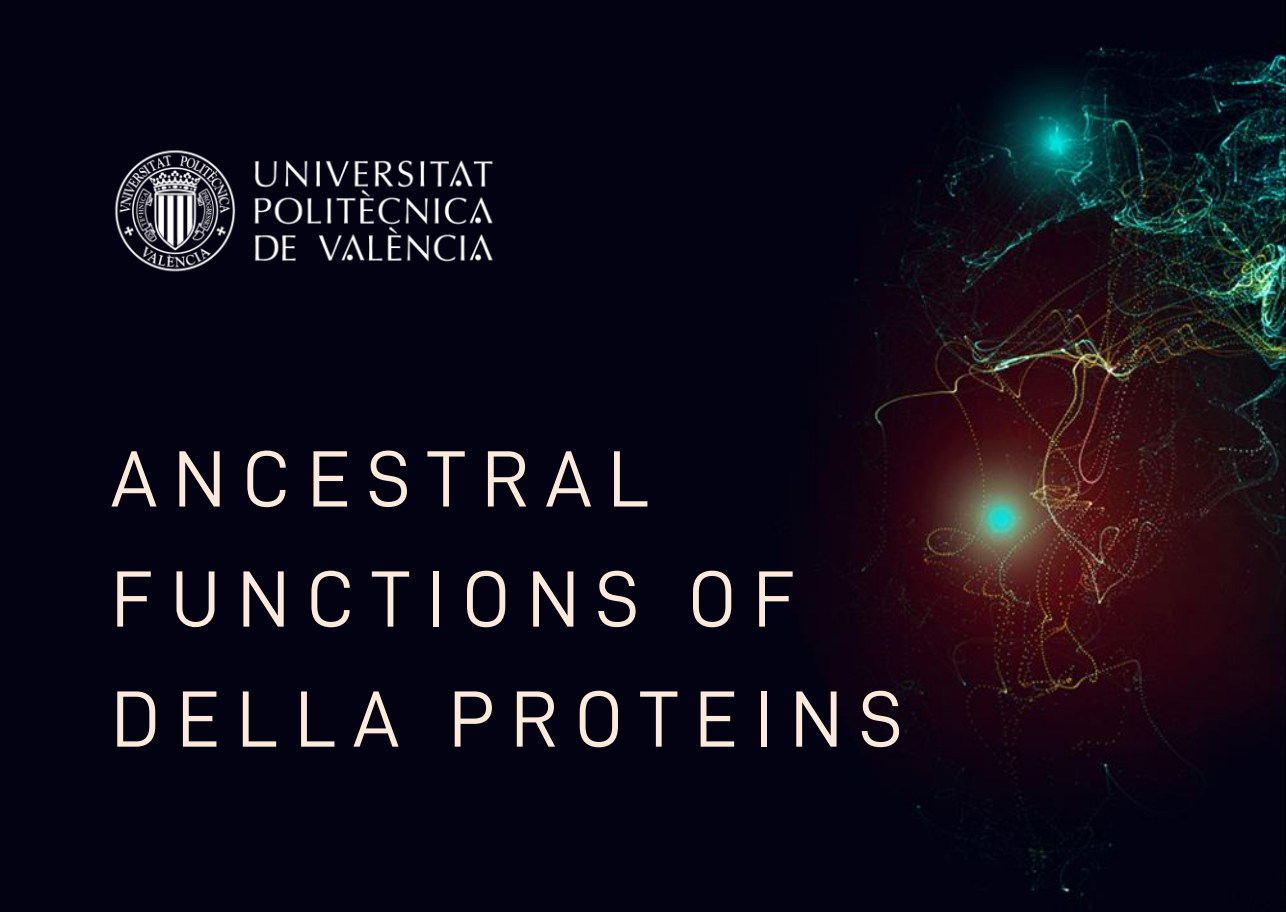

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

ANCESTRAL FUNCTIONS OF DELLA PROTEINS

PhD THESIS

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PhD in Biotechnology

ANCESTRAL FUNCTIONS OF DELLA PROTEINS

Jorge Hernández García

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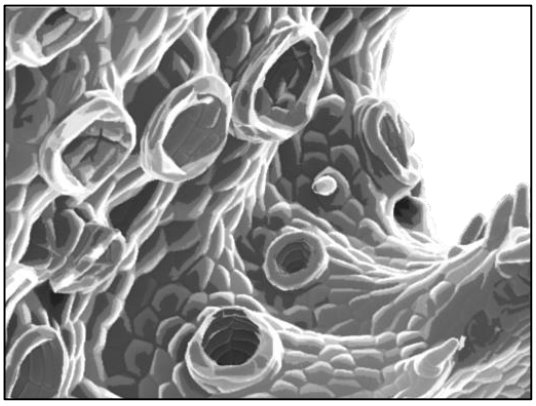
“I understood for the first time that I didn't understand what I thought I understood”

Seiji Ozawa



Marchantia polymorpha apical notch showing multiple air pores
(original: personal CryoSEM archive)

OPENING STATEMENT

Before diving into the text, I wished to inform the reader about some issues and discrepancies that could be noticed throughout the sections and chapters of this PhD, and to discuss the usage of some concepts which have "evolved" within the scientific community since the beginning of this PhD.

Formally, I have favoured the usage of UK English in all the sections. **Chapter 1** and **3** were originally written in US English (*e.g.*: signaling // signalling, analyzed // analysed, US // UK). The work in **Chapter 1** was published in *Molecular Biology and Evolution*[1], while that of **Chapter 3** is currently available as a preprint in *bioRxiv*[2]. Both works have been re-adapted here to UK English, although some US forms may inadvertently remain.

When I started this work, we (Miguel and I) still naively used evolutionary terms. An example is the use of *lower* or *higher* plants. We soon discarded the use of subjective terms and moved to the use of the more evo-friendly terms *early-diverging-* or *late-diverging -*. This can be seen in the original **Chapter 1** article[1]. However, we nowadays think such terms also tend to cause misinterpretations in phylogenetic relationships. Thus, I have got rid of this terminology using less unbiased terms. I recommend to take a look into Stuart McDaniel's 2021 viewpoint article in New Phytologist to learn more about this[3].

The figures included in **Chapter 1** are faithful to the original publication and depict phylogenetic relationships that do not reflect current views on how plant lineages diverged. Specifically, bryophytes are now widely accepted as a single clade ("Bryophyta") including the hornworts as sisters to a clade formed by mosses and liverworts ("Setaphyta"). This has not been amended in **Chapter 1**, but more accurate versions can be found together with other supplemental material online and in the Annexes section (see below).

I have created a Mendeley Data resource[4] that contains supplemental data & materials. Additionally, at the end of this dissertation, there is an **Annexes** section with data that is not considered part of the PhD thesis, but may help the reader understand some discussed points, or conclusions reached. This section is also contained in the online resource.

SUMMARY

Plants need to accommodate their growth habits to environmental conditions. For this aim, several mechanisms are used to adjust developmental responses to exogenous signals. Among them, hormonal signalling pathways participate by integrating external information with endogenous programs. One of the most relevant hormones in plant biology are gibberellins (GAs). GA signalling involves perception of the hormone by the GA receptor GID1 and subsequent degradation of the DELLA transcriptional regulators. However, only vascular plants possess a full GA perception system. Understanding the relevance of GA signalling requires elucidating how this pathway was assembled and which of the functions attributed to GAs were encoded in the ancestral DELLA proteins. Here we show by phylogenetic and biochemical analyses that DELLA proteins emerged unequivocally in a land plant common ancestor and that their recruitment into the GA-perception module relies in the presence of a conserved transactivation domain co-opted by an ancestral GID1 receptor to act as a GA-dependent degron. Moreover, this transactivation domain seems to regulate DELLA-dependent transcriptional co-activation of selected target genes by recruitment of Mediator complexes through the MED15 subunit in all land plants. Finally, we have focused on understanding the functions of DELLA proteins in bryophytes, a clade with no GA signalling. We have uncovered the role of *Marchantia polymorpha* DELLA protein as a coordinator between growth and stress responses, suggesting that this function was already present in the DELLA protein of a land plant common ancestor and has been maintained for over 450 millions of years.

RESUM

Les plantes necessiten acomodar el seu creixement a les condicions ambientals. Amb l'objectiu d'ajustar el seu desenvolupament als senyals externs, usen una sèrie de mecanismes moleculars. Un d'aquests són les rutes de senyalització hormonal, que participen en integrar la informació externa amb programes de desenvolupament propis. Una de les hormones més rellevants en la biologia vegetal són les giberel·lines (GAs). La senyalització per GAs s'inicia amb la percepció de l'hormona a través del receptor GID1, i continua per la degradació de les reguladores transcripcionals DELLA. No obstant això, només les plantes vasculars tenen un sistema complet de percepció de GAs. Entendre la rellevància de la senyalització per GAs requereix estudiar com es va assemblar la ruta i quines funcions atribuïdes a les GAs estaven ja codificades en les proteïnes DELLA ancestrals. Ací mostrem mitjançant anàlisis filogenètiques i bioquímiques que les proteïnes DELLA van emergir inequívocament en un ancestre comú de les plantes terrestres, i que el reclutament de les DELLAs al mòdul de percepció de GAs depén de la presència d'un domini de transactivació conservat que va ser co-optat pel receptor GID1 ancestral per a actuar com un degró dependent de GAs. Aquest domini de transactivació sembla regular la co-activació transcripcional de gens concrets per les DELLAs en totes les plantes terrestres mitjançant el reclutament de complexos Mediator a través de la seua subunitat MED15. Finalment, ens hem centrat en entendre les funcions de les proteïnes DELLA en briòfites, un clade sense senyalització per GAs. Hem descobert el rol de la DELLA de *Marchantia polymorpha* com a coordinadora entre les respostes de creixement i estrés, suggerint que aquesta funció estava ja codificada en proteïnes DELLA de l'ancestre comú de plantes terrestres i s'ha mantingut durant més de 450 milions d'anys.

RESUMEN

Las plantas necesitan acomodar su crecimiento a las condiciones ambientales. Con el objetivo de ajustar su desarrollo a las señales externas, usan una serie de mecanismos moleculares. Uno de estos son las rutas de señalización hormonal, que participan en integrar la información externa con programas de desarrollo propios. Una de las hormonas más relevantes en la biología vegetal son las giberelinas (GAs). La señalización por GAs se inicia con la percepción de la hormona a través del receptor GID1, y continúa por la degradación de las reguladoras transcripcionales DELLA. Sin embargo, solo las plantas vasculares tienen un sistema de percepción de GAs completo. Entender la relevancia de la señalización por GAs requiere estudiar cómo se ensambló la ruta y qué funciones atribuidas a las GAs estaban ya codificadas en las proteínas DELLA ancestrales. Aquí mostramos mediante análisis filogenéticos y bioquímicos que las proteínas DELLA emergieron inequívocamente en un ancestro común de las plantas terrestres, y que el reclutamiento de las DELLAs al módulo de percepción de GAs depende de la presencia de un dominio de transactivación conservado que fue co-optado por el receptor GID1 ancestral para actuar como un degrón dependiente de GAs. Este dominio de transactivación parece regular la co-activación transcripcional de genes concretos por las DELLAs en todas las plantas terrestres mediante el reclutamiento de complejos Mediator a través de su subunidad MED15. Por último, nos hemos centrado en entender las funciones de las proteínas DELLA en briófitas, un clado sin señalización por GAs. Hemos descubierto el rol de la DELLA de *Marchantia polymorpha* como coordinadora entre las respuestas de crecimiento y estrés, sugiriendo que dicha función estaba ya codificada en proteínas DELLA del ancestro común de plantas terrestres y se ha mantenido durante más de 450 millones de años.

ACKNOWLEDGEMENTS/AGRADECIMIENTOS

Toda persona que me conozca sabe que esta sección es impropia de mí. Por otro lado, me parecía que estaba feo no incluirla. Dicho esto, tampoco pidamos peras al olmo y esperemos una prosa inolvidable, lacrimógena y emotiva (y mucho menos miles de nombres).

Un porcentaje no pequeño de todo esto se lo debo a Miguel. Si bien es cierto que cuando llegué al lab para hacer el TFM me puse a las órdenes de David, de alguna manera que no recuerdo, Miguel y yo alcanzamos un acuerdo tácito (y no tan tácito) de que si en el 2.07 se trabajaba algún día en evolución, yo tendría que volver por aquí. Al final ocurrió, y parece que no era broma. Es difícil resumir lo que ha implicado contar con Miguel como director. Me ha enseñado más cosas de las que puedo enumerar en todos los ámbitos, desde la ciencia y la investigación, hasta aspectos más mundanos de la vida. Las horas de discusiones (muchas veces inesperadas) han ayudado a moldear esta tesis, pero también mi forma de trabajar o enfocar cada pregunta, experimento y, en parte, mi carrera. Espero que queden muchos debates y discusiones por delante (de todos modos, él sabe que, si todo va bien, probablemente le toque leerse algunas cosas de evolución de auxinas en los próximos años).

Mención especial a David, siempre prestando su expertise. Sé que parece un tío serio y rudo, pero es un cacho pan, que no os engañe. Le tengo que agradecer la oportunidad de llegar al laboratorio, y el posterior apoyo. Aunque la evolución no le motiva, a mí me lo compensó trayéndome whiskies en la boda de Esteve.

La mención de honor es para la persona que ha estado en el laboratorio día a día durante todo este proyecto: Asier. Alguna vez se me escapó alguna punta de pipeta a su cabeza, pero juro que no era bullying. Gracias a él, las largas horas de laboratorio se han hecho muy cortas y amenas. No habría podido tener mejor compañero de cabinas y proyecto. Por suerte, aunque esta etapa se haya acabado, me llevo a un gran amigo.

No puedo no mencionar a Esteve, Antonio y Noel. Hemos compartido muchas cervezas después de días enteros de trabajo, más de lo que deberíamos reconocer, pero en parte eso me ha facilitado sobrellevar esta etapa y establecer una larga amistad.

Debo mencionar a Moe: aunque está loco, sin su ayuda no habría reforzado mi entusiasmo por la investigación. A Cris Úrbez, por hacernos el día a día más llevadero y aguantar todas nuestras mierdas. La lista de miembros presentes y pasados del laboratorio es muy extensa y no voy a desglosarla, pero les agradezco innumerables horas de cafés y cervezas.

He hecho demasiadas estancias. En todas ellas he aprendido cosas que, por suerte, no siempre eran de ciencia. En Madrid, aprendí mucho de Marchantia, pero, sobre todo, que el mundo es un pañuelo. Chile me abrió las puertas al mundo, aunque de Linux y R recuerdo poco. París me forzó a darme cuenta de que el trabajo no lo es todo; aunque sigo sin tener clara cuál es la unidad de la evolución. En Wageningen descubrí que hay ambientes de

trabajo maravillosos fuera de mi burbuja; por suerte, tendré tiempo para confirmar si esto compensa sus inviernos.

Hay mucha gente que merecería aparecer aquí, tanto a nivel científico como personal, pero saben que esto se me hace más incómodo que nada, y necesitaré algunas cervezas de por medio. Desde Salamanca a Valencia, pasando por Kyoto, a cada uno de vosotros, gracias.

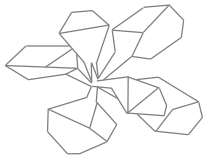
Los 'colegas' que han contribuido al contenido de esta tesis están reflejados en la portada y resumen de cada capítulo. Sin ellos esta tesis tendría muchas menos páginas.

Aunque crea que no iba a estar aquí mencionada, agradezco a Amelia el haberme encontrado y formado un clan conmigo (y con Yin). Gracias por aguantarme durante confinamiento y escritura, y siempre saber cómo animarme y darme el empujón necesario para llegar a cada meta.

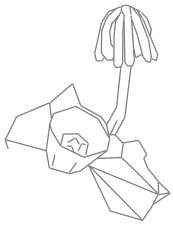
Por último, sé que peco de un 'leve' desarraigo. Sin embargo, nunca habría llegado hasta este punto sin el apoyo de mi familia. Son parte intrínseca de lo que soy y responsables de hasta dónde he llegado. Gracias por todo. Esta tesis es un poco vuestra 'culpa'.

La realización de esta Tesis Doctoral ha sido posible gracias* a una Ayuda para Contratos Predoctorales FPU (FPU15/01756), dos Ayudas para Estancias Breves FPU (EST17/00237, IPS2, París; EST18/00400, WUR, Wageningen), una ayuda EMBO Short-Term (ASTF 8239, WUR, Wageningen), y la financiación MSCA H2020 RISE para desplazamientos en el contexto del proyecto SIGNAT (RISE Action 644435, PUC, Santiago). Así mismo, el grueso del trabajo experimental incluido ha sido financiado por el proyecto HUBFUN del MINECO (BFU2016-80621-P).

*Aunque agradezco al ministerio la oportunidad de trabajar en 'lo mío', no puedo olvidar que, en este país, las condiciones salariales, derechos y regulación laboral siguen dejando mucho que desear en el ámbito de la investigación, además de implicar una más que incierta continuidad en la Academia, perpetuando hábitos y costumbres de trabajo excesivamente competitivos y tóxicos. No nos vamos: nos echan.



Preface



Plants have been part of land ecosystems for hundreds of millions of years. But the first land plant was not entirely used to this: it had to face several circumstances that their algal ancestors never thought of. Environmental conditions shaped the course of land plant evolution towards the creation of physical barriers, specialized cells, new organs, and even the recruitment of other organisms to fight for them. Many lineages of arthropods were already living there when plants arrived. Plants borrowed weapons from other organisms to fight and communicate. A plethora of new compounds helped plants thrive in this harsh environment. Better awareness of the surroundings was crucial to respond to external fluctuations. Life cycles had to adapt to their new home and neighbours. Plants had to sacrifice a great deal of their migratory potential. As a consequence, every plant biologist tagline reminds us that “as sessile organisms, plants are unable to escape from environmental changes”. While strictly not true, this made land plants really good at distinguishing between friend and foe. Indeed, plants have learnt a lot about hating each other and loving their enemies’ enemy. Millions of years of evolution have allowed plants to conquer some of the most extreme terrestrial environments. If that was not enough, plants have been dealing with humans since they appeared, and some plants have even tricked this species to nurture them. This strategy has made some plants tremendously successful. But all of this comes at a price, meaning the loss of much of its potential to understand and adapt to the environment.

We are just starting to understand some of the molecular mechanisms laying behind plant development. But how did this happen? When did this occur? How were specific mechanisms selected? Why do we care? Can we learn something studying the evolution of these mechanisms? The present document tries to address only partially some of these questions. Hopefully, some will be tangentially answered for very specific tiles in the intricate mosaic of plant molecular biology. Luckily, most of these questions and others will remain to be interrogated in the future.

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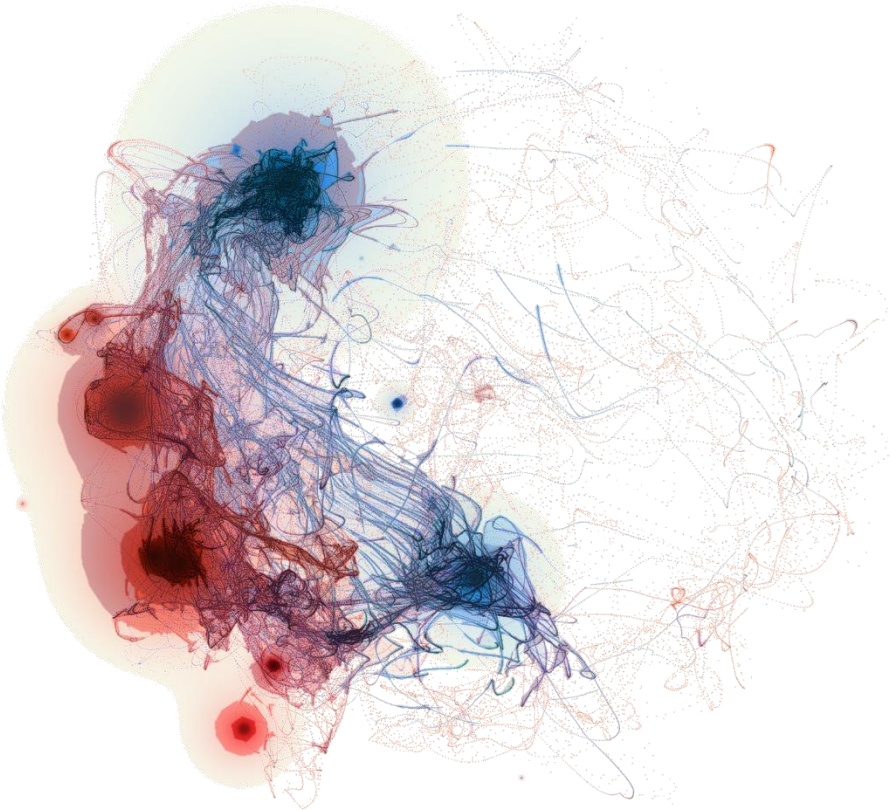
ABBREVIATIONS

3-AT, 3-aminotrizole
ARF, AUXIN RESPONSE FACTOR
ARR, ARABIDOPSIS RESPONSE REGULATOR
bHLH, basic helix-loop-helix
BiFC, Bimolecular Fluorescence Complementation
BLAST, Basic Local Alignment Search tool
BZR, BRASSINAZOLE RESISTANT
C4H, CINNAMATE 4-HYDROXYLASE
CBP, CREB-Binding Protein
ChIP, Chromatin Immunoprecipitation
CHS, CHALCONE SYNTHASE
Cit, Citrine
CKI, Cyclin-dependent Kinase Inhibitors
CPS, COPALYL DIPHOSPHATE SYNTHASE
cW, Continuous white light
CXE, Carboxyl esterase
CycB /CYCB, Cyclin B
DBD / **BD**, DNA Binding Domain
DEX, Dexamethasone
DIR, DELLA-Interacting Residue
DPBA, Diphenylborinic acid 2-aminoethyl ester
EdU, 5-ethynyl-2'-deoxyuridine
EF1, ELONGATION FACTOR1
ELF7 / PAF1, EARLY FLOWERING 7
ERF, ETHYLENE RESPONSE FACTOR
F3H, FLAVANONE 3-HYDROXYLASE
FL, Full length
FLC, FLOWERING LOCUS C
FLS, FLAVONOL SYNTHASE
FR, Far red (R, Red)
GA(**s**), gibberellin(s)
GA20ox, GIBBERELLIN 20-OXIDASE
GA2ox, GIBBERELLIN 2-OXIDASE
GA3ox, GIBBERELLIN 3-OXIDASE
GAF1 / **IDD2**, GAI ASSOCIATED FACTOR1
GAI, GIBBERELLIN A INSENSITIVE
GBR, GA-Binding Residue
GIDn, GIBBERELLIN INSENSITIVE DWARFn
GL1, GLABRA1
GLP, GID-Like Protein
GR, Glucocorticoid receptor
GRAS, GAI/RGA/SCARECROW
GUS, β-GLUCURONIDASE
HAM, HAIRY MERISTEM
HD-ZIP, Homeodomain-Leucin Zipper
HMM, Hidden Markov Model
IDD, INDETERMINATE DOMAIN
JA, JASMONIC ACID
JAZ, JASMONATE ZIM-DOMAIN
K_a, rate of nonsynonymous substitution
KAO, KAURENOIC ACID OXIDASE
KIX, Kinase-inducible domain (KID) Interacting Domain
KO, KAURENE OXIDASE (alternatively: Knock Out)
KRP, KIP-RELATED PROTEIN
KS, KAURENE SYNTHASE
K_s, rate of synonymous substitution
LAS, LATERAL SUPRESSOR
LUC, Firefly (*Photinus pyralis*) LUCIFERASE
MAPK, mitogen-activated protein kinase
MID, MED25-interacting domain
MiP, Microprotein
ML, Maximum Likelihood
MV, Methyl Viologen (Paraquat)
NAC, NAM, ATAF & CUC
PAC, Paclobutrazole
PAF1 / **ELF7**, POLYMERASE-ASSOCIATED FACTOR 1
PAL, PHENYLALANINE AMMONIA LYASE
PAT1, PHYTOCHROME A SIGNAL TRANSDUCTION1
PDB, Protein Database
PFD, PREFOLDIN
PIC, RNA polymerase II Pre-Initiation Complex
PIF, PHYTOCHROME-INTERACTING FACTORs
PIL1, PIF3-LIKE1
PKL, PICKLE
Pol II / **RNAP II**, DNA-dependent RNA polymerase II
PRE5, PACLOBUTRAZOL RESISTANCE 5
RAM, Root Apical Meristem
REN, Sea pansy (*Renilla reniformis*) LUCIFERASE
RGA, repressor of *ga1-3*
RGLn, RGA-LIKEn
RI, RNA interference
RNAP II / **Pol II**, DNA-dependent RNA polymerase II
SAM, S-Adenosyl methionine
SCF, Skp/Cullin/F-box containing complex
SCLn, SCARECROW-LIKEn
SCR, SCARECROW
SHR, SHORTROOT
SLY, SLEEPY
SMR, SIAMESE RELATED
SPL, SQUAMOSA PROMOTER-LIKE
SWI/SNF, SWITCH/SUCROSE NON-FERMENTABLE
SWI3, SWITCH3
TAD, Transcriptional Activation Domain
TES, Transcriptional End Site
TF(**s**), transcription factor(s)
TOC159, TRANSLOCASE OF CHLOROPLAST159
TR(**s**), transcriptional regulator(s)
TSS, Transcriptional Start Site
UAS, Upstream Activating Sequence
XTR7, XYLOGLUCAN ENDOTRANSGLYCOSYLASE-RELATED
Y2H, Yeast-two Hybrid
YFP, Yellow Fluorescent Protein



"Knowing how something originated often is the best clue for how it works"

Terrence Deacon

Introduction

From oceans to desserts, almost all biomes on Earth have been colonized by plants. Since their origin, their lifestyles have changed, adapting to very different habitats (Donoghue and Edwards 2014). This ability to adapt, or adaptability, has been gradually improved during evolution, probably driven by environment and habitat requirements. In this sense, adaptability could have been foreshadowed by the gain of novel molecular systems to perceive environmental signals. For example, the enhancement of light and temperature sensing systems may have been instrumental during the water to land transition (Becker and Marin 2009; Han et al. 2019). Even though these are key elements for adaptability, they represent only a fraction of the systems that allow plants to interact with their surroundings.

Changes in habitat conditions have not only to be correctly perceived, but also to be reciprocated by changes in physiology, growth and developmental patterns. For this to occur, exogenous signals have to be integrated with endogenous programs to accordingly alter plant behaviour. Hence, the capacity to coordinate these processes requires additional integrator systems. Some of these include common endogenous signalling elements such as the ubiquitous MAPK cascades, or the calcium-dependent systems, but also more specific ones as the circadian clock mechanism, or multiple hormone signalling pathways. These pathways have to be incorporated into a signalling network to generate specific output responses. An intrinsic and characteristic component of these systems are hormones, molecular signals able to regulate development and physiology. While cascades triggered by hormones have been classically seen as independent, and linear pathways, we nowadays know that all are interconnected amongst them and with other pathways.

One of these compounds are gibberellins (GAs), a group of tetracyclic diterpenoids known to influence a wide variety of processes in angiosperms, ranging from germination to flowering (Sun 2008). GA levels are tightly regulated by both environmental conditions and endogenous programs, acting as a convergence point for many signals. In turn, GAs control the stability of DELLAs, proteins that act as transcriptional regulators able to modulate the outcome of multiple transcriptional pathways. Hence, DELLA-dependent GA signalling arises as a paradigmatic example of environmental signal integration and coordination with endogenous signalling networks.

A mechanism for gibberellin perception

GAs effect on multiple aspects of flowering plant behaviour has been thoroughly studied during the last decades (Hedden and Sponsel 2015). The biosynthesis of bioactive GAs in plants, their distribution or inactivation, and the spatiotemporal regulation of these metabolic processes are particularly well understood in different angiosperms and have been exhaustively reviewed somewhere else (Yamaguchi and Kamiya 2000; Hedden and Thomas

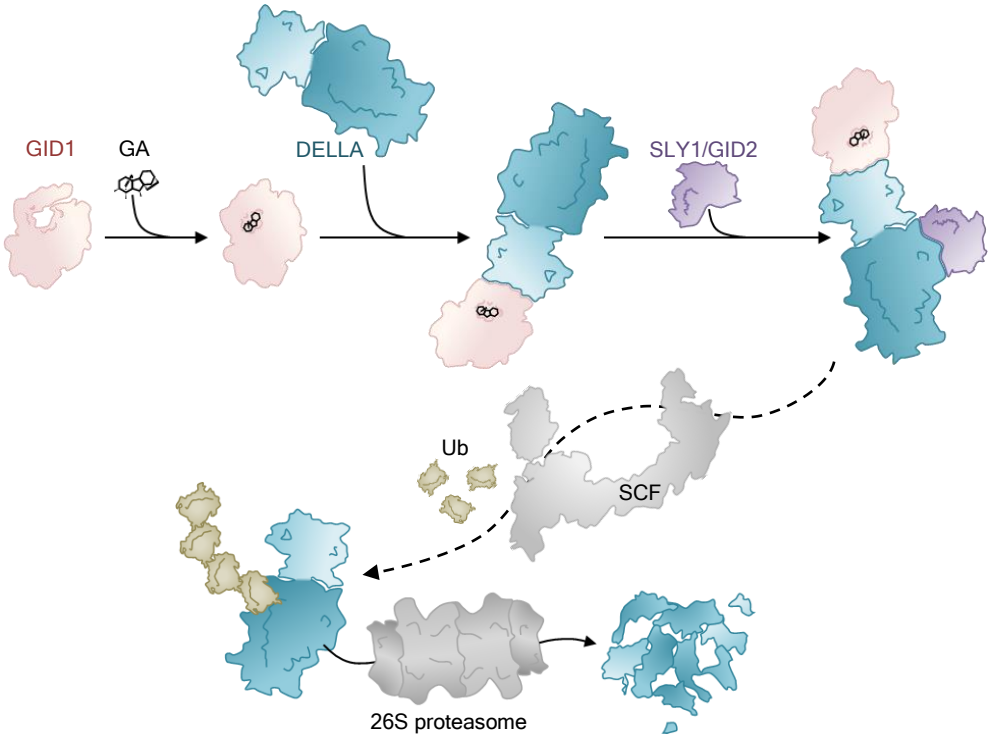


Figure 1. Gibberellin induced DELLA degradation. Model of the sequential formation of the GA-GID1-DELLA complex and SCF-mediated DELLA degradation. GID1, GIBBERELLIN INSENSITIVE1; GA, gibberellin; SLY1/GID2, SLEEPY1/ GIBBERELLIN INSENSITIVE2; SCF, Skp/Cullin/F-box containing E3 ligase complex; Ub, ubiquitin.

2012; Magome and Kamiya 2016). Contrasting with their ability to influence almost every aspect of plant physiology, the GA perception pathway is relatively simple and consists of three elements: the **GID1** receptor, the **DELLA** protein, and the **GID2** F-box protein (**Fig. 1**).

The only known GA receptors are the GIBBERELLIN INSENSITIVE DWARF1 (GID1) family of soluble proteins (Ueguchi-Tanaka et al. 2005; Griffiths et al. 2006; Nakajima et al. 2006). GID1s accommodate bioactive GAs with high affinity, suffering after this a conformational change that induces the interaction with the nuclear localized DELLA proteins (Ueguchi-Tanaka et al. 2005; Griffiths et al. 2006; Nakajima et al. 2006). Upon GID1 interaction, a rapid degradation of DELLAs takes place (Murase et al. 2008). For this to occur, the F-box proteins SLY1/GID2 (SLEEPY1/GIBBERELLIN INSENSITIVE DWARF2) are necessary (McGinnis et al. 2003; Sasaki et al. 2003). GA-GID1-DELLA complex formation promotes SLY1/GID2-dependent recruitment of the $SCF^{SLY1/GID2}$ ubiquitin E3 ligase complex, causing DELLAs to be marked for subsequent 26S proteasomal degradation (Fu et al. 2002; McGinnis et al. 2003; Sasaki et al. 2003). In the proposed model, GID1-DELLA interaction leads to a conformational change in DELLA structure, facilitating the interaction with the F-box

protein, thus promoting DELLA poly-ubiquitination and subsequent degradation of DELLA proteins.

DELLA proteins are the main players in gibberellin signalling

Most of the known GA responses in angiosperms rely on DELLA functionality. Dominant mutations in *DELLA* genes cause GA insensitivity, while loss-of-function alleles cause constitutive GA response phenotypes (Peng and Harberd 1997; Silverstone et al. 1998; Ikeda et al. 2001; Chandler et al. 2002). Hence, these proteins are considered to act as repressors of GA responses.

DELLAs are part of the GRAS (for GAI, RGA, and SCARECROW) family of proteins (Pysh et al. 1999). The homonymous family-defining domain is a globular structure resembling the α/β fold of SAM-dependent methyltransferases, and is known to stablish different types of molecular interactions (S. Li et al. 2016; Hirano et al. 2017; Hakoshima 2018). Multiple subfamilies of GRAS proteins have been shown to act in different physiological responses, and further information can be found elsewhere (Hirsch and Oldroyd 2009; Sun et al. 2012; Bolle 2016). As such, DELLAs C-terminal domain has all the canonical features of a conserved **GRAS domain** (Pysh et al. 1999; S. Li et al. 2016; Hirano et al. 2017). In addition, DELLAs are characterized by a unique N-terminal domain, the **DELLA domain**, crucial for GA-induced GID1 interaction, but dispensable for the interaction with SLY1/GID2 (Dill et al. 2001; Dill et al. 2004). The structure of this domain is mostly unknown, probably due to a high degree of intrinsic disorder (Sun et al. 2010). The exception is a small structured region harbouring GID1-interacting motifs: 1) **DELLA** are the amino acids giving name to the domain and the subfamily (Asp-Glu-Leu-Leu-Ala), and form an α-helix (αA) that greatly contributes to GID1 binding affinity (Murase et al. 2008). While there is no structural reason for it, some research groups include in the motif the next 10-12 amino acids that link αA with the next α-helix (αB) due to the 17 amino acid deletion in the *gai-1* dominant allele that includes this region (Peng et al. 1997). This motif has been suggested to undergo a conformational change from disordered to α-helix after binding to GID1 (Sun et al. 2010). 2) **LEQLE** is contained within the αB helix, and is known to directly interact with the αb helix of GID1 (Murase et al. 2008). 3) **TVHYNP** has been repeatedly shown to intervene in GID1 binding (Ueguchi-Tanaka et al.

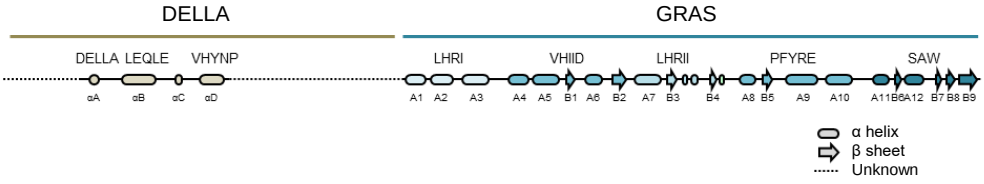


Figure 2. Structure of DELLA proteins. DELLA (AtGAI sequence) secondary structure based on GID1-interacting N-terminal region, and modelling from OsSCL7 GRAS structure (Murase et al., 2008, Li et al., 2016b). Dotted line represents structurally unknown regions.

2007; Murase et al. 2008; Hirano et al. 2010). This motif forms an irregular loop between αC and αD that is thought to be involved in binding-induced folding together with DELLA motif (Sun et al. 2010). The known structure of DELLA is summarized in **Figure 2**. Mutations in these motifs lead to stabilization of DELLA proteins due to loss of GID1 binding in response to GAs (Dill et al. 2001; Murase et al. 2008). DELLA and TVHYNP motifs have also been associated with an intrinsic ability of DELLA proteins to transactivate genes *in vivo*. These parts of the protein were shown to be important for the DELLA-dependent growth inhibition in rice, but transactivation activity has not directly been associated with this function (Ogawa et al. 2000; Hirano et al. 2012).

Most analyses on DELLA domain have been focused on its GA-regulatory function, and no other biological function has been assigned to this domain. In contrast, the GRAS domain of DELLA proteins has been subjected to extensive functional characterization. This domain mediates the interaction with SLY1/GID2 LSL domain, while some minor interactions are established simultaneously between GID1 and GRAS (Fu et al. 2004; Hirano et al. 2010). Hence, this domain is also necessary but not sufficient for the GA-dependent DELLA degradation. In short, DELLAs are destabilized by GAs. As a result, DELLAs de-repress GA transcriptional responses, but how do DELLAs accomplish this?

The manifold mechanisms of DELLA function

As other GRAS proteins, DELLAs act as transcriptional regulators (Cao et al. 2006), but have not been unequivocally shown to directly bind DNA. In turn, they regulate gene expression interacting with several transcription factors (TFs) and transcriptional regulators (TRs). To date, more than 300 interactors have been found, and more than a hundred of these have been independently associated with the regulation of specific biological processes (Marín-de la Rosa et al. 2014; Lantzouni et al. 2020). The varied nature of these interactors allows DELLAs to regulate plant physiology by different molecular mechanisms. Some of them have been extensively described, but new mechanisms are reported on and off.

Many TFs and TRs are sequestered by DELLAs. In these cases, DELLA interaction directly blocks the function of the interacting partner. Frequently, DELLAs obstruct binding of TFs to their target promoters as occurs with the first found DELLA interactors, PHYTOCHROME-INTERACTING FACTORs (PIFs) (Feng et al. 2008; de Lucas et al. 2008). Blocking also affects TRs which do not bind DNA but regulate other TFs by direct interaction, as is the case of JASMONATE ZIM-DOMAIN (JAZ) proteins that mediate jasmonic acid (JA) signalling, restricting their capacity to regulate other proteins (Hou et al. 2010; Yang et al. 2012). Negative regulation by sequestering their partners is one of the most commonly described mechanisms of DELLA function. Furthermore, DELLAs are also able to interact with subunits of different regulatory complexes to block or dampen their functions, such as with the PICKLE ATPase,

or the SWI3B subunit of the SWI/SNF family of chromatin remodelling complexes (Sarnowska et al. 2013; Zhang et al. 2014).

The ability to inhibit protein functions greatly contributed to the notion of DELLA acting as repressors. However, some TFs recruit DELLAs into chromatin contexts, where they act as co-activators. This happens with different transcriptional activators such as INDETERMINATE DOMAIN (IDD), or type B ARABIDOPSIS RESPONSE REGULATORs (ARRs) (Fukazawa et al. 2014; Yoshida et al. 2014; Marín-de la Rosa et al. 2015). While a few other TFs have been proven to act coordinately with DELLAs to activate gene transcription (Zhang et al. 2017; Tan et al. 2019), genome-wide chromatin binding analyses of DELLAs show thousands of potential target genes, suggesting that co-activation is a widespread mechanism of DELLA functioning (Marín-de la Rosa et al. 2015; Serrano-Mislata et al. 2017). In other cases, DELLAs may facilitate the function of basal transcriptional machinery proteins, such as the transcription elongation complex Paf1C, through the interaction with its PAF1 subunit (Blanco Touriñán 2020). Intriguingly, the flowering repressor FLC and DELLA cooperate at the promoters of the flowering inducing genes to repress their expression, this being the only case of a possible co-repression mechanism found for DELLA proteins so far (M. Li et al. 2016). In any case, all these mechanisms lead to a positive regulation of the partner, either by enhancing their activation, or their repressor functions. The main mechanisms of DELLA transcriptional regulation are depicted in **Figure 3**.

These mechanisms have a direct impact on gene expression regulation (Locascio et al. 2013), but DELLAs have also been linked to other cellular processes, including non-nuclear functions. For example, they regulate microtubule formation and plasma membrane trafficking through the interaction with subunits of the Prefoldin complex (Locascio et al. 2013; Salanenka et al. 2018). Additionally, DELLAs also interact with the preprotein import receptor TOC159 to

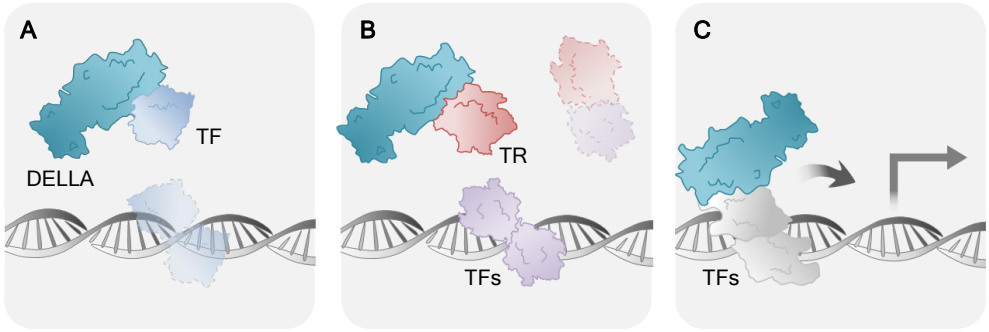


Figure 3. Main mechanisms of DELLA transcriptional regulation. (A) DELLAs interact with transcription factors (TFs) and block their ability to bind DNA, thus impeding their function in gene transcription. (B) DELLAs sequester a transcriptional regulator (TR) that regulates another TF function, releasing the later to function. (C) DELLAs are recruited to chromatin by the interaction with TFs and promote gene transcription.

inhibit chloroplast biogenesis (Shanmugabalaji et al. 2018). However, the vast majority of the functions attributed to GAs can be directly linked to the interaction of DELLAs with TFs and TRs to produce transcriptional changes.

DELLAs coordinate developmental and stress responses

DELLA function is linked to their capacity to interact with hundreds of proteins. Many of the TFs and TRs belong to a wide variety of transcriptional programs and signalling cascades, including hormone signalling pathways, and different developmental and stress-related programs (Davière and Achard 2016; Van De Velde et al. 2017). The ability of GAs to destabilize DELLA adds a layer of complexity, given that GA metabolism is tightly regulated by environmental and endogenous signals, at least in angiosperms (Yamaguchi and Kamiya 2000; Hedden and Thomas 2012). This metabolic regulation, together with the downstream function of DELLAs, allows cells to integrate external cues and their internal state with many transcriptional pathways. Due to this, DELLA proteins are proposed to act as central hubs in transcriptional networks (Thomas et al. 2016). A depiction is portrayed in **Figure 4**, and further information can be found elsewhere (Davière and Achard 2016; Vera-Sirera et al. 2016). As a consequence of their privileged position as a relay of environmental information to multiple cellular functions, DELLAs have been attributed a role in the coordination between growth and stress responses, optimizing the use of resources under limiting conditions (Claeys et al. 2014). DELLAs levels increase under different stress signals, leading to both growth arrest and the induction of defence responses (Colebrook et al. 2014). The molecular interplay between GA and JA signalling is a paradigmatic example of this function. DELLAs interact and sequester JAZ proteins, releasing the bHLH TF MYC2, thus promoting JA responses (Hou et al. 2010). In turn, JAZ impair the interaction of DELLAs with PIFs, thus releasing this growth-promoting TFs (Yang et al. 2012). In this scenario, DELLAs and JAZs correspondingly induce or repress biotic stress responses, while inhibiting or promoting plant growth.

A suitable response has critical consequences for an organism when it comes into choosing between growth or defence. As transcriptional hubs, DELLAs are integral components of these kind of decision-making circuits. However, while they indisputably act as hubs in the protein-protein interaction network due to their high connectivity, their role as signalling hubs could be subject of debate. Hubs tend to be essential pieces of regulatory networks and, as a result, null mutants on their coding genes are commonly non-viable (Vandereyken et al. 2018). This is not the case for DELLA proteins, since *della* loss-of-function mutants are known to be viable. Nonetheless, *della* mutants show pleiotropic defects in multiple biological processes, as it has been largely characterized in the *A. thaliana della* pentuple mutant (Feng et al. 2008), and even full sterility in some species when full loss-of-function mutants are checked, being the case of tomato and rice (Ikeda et al. 2001; Livne et

al. 2015). DELLAs also have structural signatures infrequent for hub proteins. Protein-protein network hubs are usually large proteins with multiple surfaces or domains. In contrast, DELLAs have a single interacting domain (i.e.: GRAS), which is relatively small (Vandereyken et al. 2018). It is unclear what properties allow DELLAs to interact with hundreds of proteins, as the relatively small GRAS domain is a highly structured and globular domain, and no common signatures can be found among all the interactors in terms of sequence, structure, or biochemistry (Lantzouni et al. 2020).

Evolutionary aspects of gibberellin signalling

Contrasting with the extensively described mechanisms of GA function in angiosperms, the evolutionary origin of this hormonal pathway has been only partially addressed. Likewise, it remains unknown how DELLAs emerged, evolved and learnt to perform these functions. Limitations on genome/transcriptome availability ranging several plant clades, and the absence of model systems for these lineages has dampened functional analyses of the

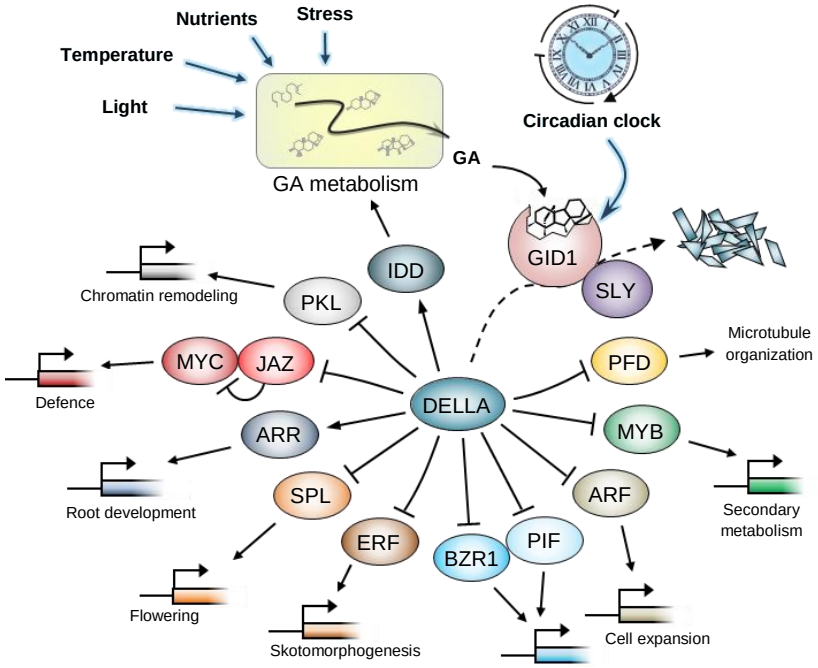


Figure 4. DELLA coordination of multiple pathways. Signals modulating GA biosynthesis and GID1 activity are represented as blue-shaded arrows. Some DELLA interactions with TFs and TRs that regulate diverse processes are indicated. Negative and positive effects of DELLA interaction are shown as T-shaped lines or arrows, respectively. Dashed line represents GID1-SLY1/GID2-regulated DELLA degradation. PFD, Prefoldin; ARF, AUXIN RESPONSIVE FACTOR; PIF, PHYTOCHROME INTERACTING FACTOR; BZR1, BRASSINAZOL RESISTANT1; ERF, ETHYLENE RESPONSIVE FACTOR; SPL, SQUAMOSA PROMOTER LIKE; ARR, ARABIDOPSIS RESPONSE REGULATORS; JAZ, JASMONATE-ZIM DOMAIN; PKL, PICKLE; IDD, INDETERMINATE DOMAIN. Adapted from Hernández-García *et al*, 2020.

conservation of different signalling pathways. Even so, some attempts to incorporate comparative studies in different plant species have been done to address the evolution of the GA signalling pathway (Hirano et al. 2007; Vandenbussche et al. 2007; Yasumura et al. 2007). The origin of the signalling pathway is commonly linked to vascular plants (*i.e.*: tracheophytes), where the main components (GAs, GID1, SLY1/GID2, and DELLA) have been found. The whole module has been shown to work in most vascular plant lineages, including lycophytes, ferns, gymnosperms, and several angiosperms (Hirano et al. 2007; Yasumura et al. 2007; Sun 2011; Tanaka et al. 2014; Du et al. 2017). What follows is a summary of knowledge of the evolutionary aspects of GA metabolism and signalling (as of the beginning of this thesis work), and the presence of elements is summarized in **Figure 5**.

Gibberellin biosynthesis evolution has escaped most evolutionary analyses, but comparisons with bacterial and fungal systems have pointed to an independent origin for the convergent synthesis of bioactive GAs on each (Hedden et al. 2001; Salazar-Cerezo et al. 2018). Intriguingly, GAs have been found in plant lineages outside vascular plants, but are derived from unidentified pathways, and have unknown functions (Mowat 1965; MacMillan 2001; Kiseleva et al. 2012). One exception is ent-2α-hydroxy-kaurenoic acid, derived from early steps of the biosynthetic pathway and act in moss development, but whose target effectors have not been found and are presumably unrelated to GA signalling elements (Miyazaki et al. 2018). The 2-oxoglutarate-dependent dioxygenase-type enzymes known to catalyse the final steps of bioactive GA synthesis in angiosperms are absent in non-vascular land plants, implicating that the canonical biosynthetic pathway exists only in tracheophytes (Hirano et al. 2007). A detailed analysis on the evolution of the biosynthetic pathway elements and its implications is included somewhere else (Hernández-García et al. 2020).

The GID1 receptors derive from the plant carboxylesterase (CXE) family of the α/β-hydrolase fold superfamily, but lack some of the essential residues to carry out their ancestral

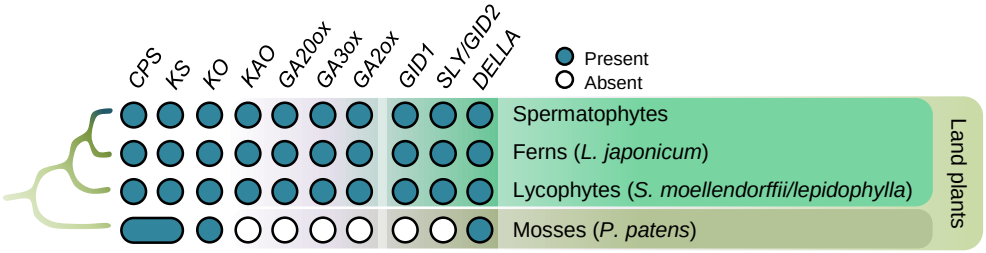


Figure 5. Evolution of GA-related biosynthesis and signalling genes. Rows represent various groups among plant lineages. Species from which the information was extracted are indicated between brackets. Circles represent the presence or absence of the genes naming each column. A tree representing evolutionary relationships is depicted at the left side. This figure represents knowledge as of late 2015. *L. japonicum*, *Lygodium japonicum*; *S. moellendorffii*/*lepidophylla*, *Selaginella moellendorffii*/*lepidophylla*; *P. patens*, *Physcomitrella patens*.

catalytic function (Ueguchi-Tanaka et al. 2005; Yoshida et al. 2018). GID1s are distinguished by their abilities to (i) bind GAs and (ii) interact with DELLAs after their so-called N-terminal lid is closed. As such, canonical GID1s with this N-terminal that recognizes DELLA motifs and lack of the CXE catalytic activity exist only in tracheophytes, while CXEs are present in the entire Archaeplastida lineage (Gazara et al. 2018; Yoshida et al. 2018). GID1 origin itself seems to coincide with that of vascular plants, and its further evolution has been proposed to follow a rather standard path of changing sensitivity towards bioactive GAs, exemplified by the ability to bind or reject different GA intermediates in their pockets. For example, GID1s from the lycophyte *Selaginella moellendorffii* have lower affinities for bioactive GAs compared to seed plant GID1, but can accommodate some forms of inactive intermediates (Hirano et al. 2007). Additional expansions and sub-functionalization of *GID1* genes have occurred within angiosperms, including the emergence of GA hypersensitivity, or the acquisition of specific expression patterns in different sub-clades (Yoshida et al. 2018).

The GRAS family, to which DELLA proteins belong, is thought to have been horizontally transferred from bacteria to plants given their resemblance to bacterial methyltransferases (Zhang et al. 2012). This event presumably involved an algal ancestor of land plants (i.e.: embryophytes), since *GRAS* genes exist in all embryophytes, but also in zygnematalean algae (Engstrom 2011; Delaux et al. 2015). On their part, DELLAs have been found exclusively in embryophytes, though those in bryophytes lack the key motifs involved in GID1 interaction (Hirano et al. 2007; Yasumura et al. 2007). DELLAs from Arabidopsis have acquired different functions mainly due to transcriptional diversification, conserving their biochemical functionality between paralogs (Gallego-Bartolomé et al. 2010). However, the extent of this conservation in a broader evolutionary scale is unclear given the lack of studies and apparently contradictory results. For instance, one work claimed that DELLAs intrinsic function is conserved from bryophytes to angiosperms based on the ability of PpDELLAs and SmDELLAs to restore growth of *A. thaliana della* mutants (Yasumura et al. 2007). At the same time, other work proposed that the function is conserved among vascular plant DELLAs but not in bryophytan DELLAs, based on their ability or not to restrain growth in rice (Hirano et al. 2007). Both these works were completed prior to the discovery of DELLA molecular function during 2008, and performed under different conditions in two very different systems (i.e.: a mutant Arabidopsis and a wild-type rice). Further analyses on DELLAs function have been done just in well-established angiosperm model systems, as Arabidopsis and rice, with only later attempts including studies on *Medicago* and tomato (Floss et al. 2013; Ye et al. 2015; Jin et al. 2016; Li et al. 2018), remaining largely unknown if DELLAs mechanism of function is conserved outside flowering plants. In fact, very little is known about direct DELLA function outside flowering plants, even under the assumption that non-angiosperm GA response is also mediated by them. For instance, GAs act as antheridiogens (i.e.: inductors of male sexual organ development) in ferns, or promote far red-induced shoot elongation in gymnosperms,

implying that DELLAs function would counteract these effects by (Tanaka et al. 2014; Li et al. 2020). Within angiosperms, their interacting promiscuity has been only validated in Arabidopsis (Marín-de la Rosa et al. 2014; Lantzouni et al. 2020), but the pleiotropic effects that GAs exert in other species would suggest for this trait to be conserved in the flowering lineage.

As occurs with the receptor, SLY1/GID2 proteins have only been found in tracheophytes (Hirano et al. 2007). They are part of the large superfamily of F-box proteins, and rely on their C-terminal motifs, namely GGF and LSL, to interact with DELLA proteins (Gomi et al. 2004). The LSL motif is conserved only in tracheophytan SLY1/GID2-type F-box proteins, but *P. patens* contains F-box proteins with putative GGF motifs (Hirano et al. 2007; Vandenbussche et al. 2007). Also, the LSL region is thought to be critical for DELLA recognition (Fu et al. 2004). SLY1/GID2 would have thus followed a two-steps path of acquiring motifs in their C-terminal domain to gain proper DELLA binding.

All things considered, it seems that late biosynthetic enzymes and the signalling components emerged, evolved, and/or were co-opted within the tracheophytan ancestral lineage. However, it is unknown how all the pieces were assembled into a hormonal module, or what underlying features allowed them to fit together. Many blanks remain to be filled in our current knowledge of DELLA evolution, both from a phylogenetic, and a functional point of view, and how it changed transcriptional networks before and after GA signalling emergence.

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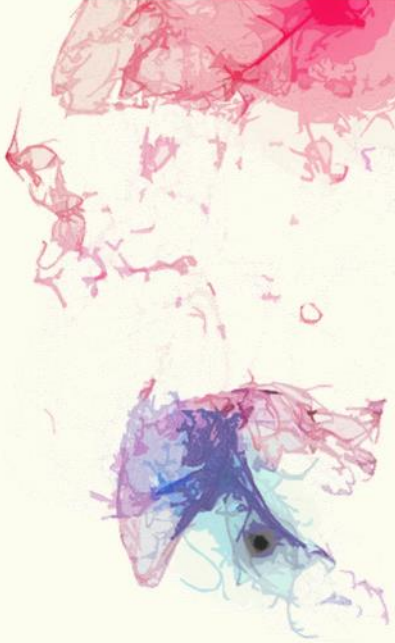
Objectives

With the advent of the genomic era, we are witnessing an incessant increase in the amount of genomic resources from non-vascular land plants and fully annotated streptophytan algae genomes. In parallel, the establishment of new genetic models from disregarded plant lineages would help us answer functional questions from a comparative point of view. We stand before a new world of possibilities to address previously unconceivable questions to understand the molecular mechanisms underlying plant evolution.

DELLAs are essential and necessary constituents of the GA signalling pathway. However, GA signalling is dispensable for DELLAs to function, given the existence of bryophytan DELLAs. It seems then reasonable to hypothesize that DELLAs operated freely when they appeared, and were recruited only later into the GA signalling. Many questions arise from this assumption: What did DELLAs do in ancestral embryophytes? Are these functions conserved? How did the recruitment into the GA signalling pathway happen? What features did DELLA need to acquire for this to occur? Altogether, we propose two specific objectives to challenge these questions:

I. **Reconstruct the evolutionary history of DELLA proteins.** We hypothesise that a deeper phylogenetic analysis of *DELLA* genes, together with the rest of the GA signalling elements will shed light into the origin – and possibly the mechanism – of the GA signalling module assembly.

II. **Analyse DELLA function in a plant without canonical gibberellin signalling**. We hypothesise that "*in situ*" studies of DELLA function in a bryophyte will help us to learn not only about possible processes regulated by DELLAs in the land plant common ancestor, but also about DELLA ancestral mechanism of function.



Chapter 1

Origin of Gibberellin-Dependent Transcriptional Regulation by Molecular Exploitation of a Transactivation Domain in DELLA Proteins

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Chapter 1

Origin of Gibberellin-Dependent Transcriptional Regulation by Molecular Exploitation of a Transactivation Domain in DELLA Proteins

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Abstract

DELLA proteins are plant-specific transcriptional regulators known to interact through their C-terminal GRAS domain with over 150 transcription factors in *Arabidopsis thaliana*. Besides, DELLAs from vascular plants can interact through the N-terminal domain with the gibberellin receptor encoded by *GID1*, through which gibberellins promote DELLA degradation. However, this regulation is absent in nonvascular land plants, which lack active gibberellins or a proper GID1 receptor. Current knowledge indicates that DELLAs are important pieces of the signalling machinery of vascular plants, especially angiosperms, but nothing is known about DELLA function during early land plant evolution or if they exist at all in charophytan algae. We have now elucidated the evolutionary origin of DELLA proteins, showing that algal GRAS proteins are monophyletic and evolved independently from those of land plants, which explains why there are no DELLAs outside land plants. *DELLA* genes have been maintained throughout land plant evolution with only two major duplication events kept among plants. Furthermore, we show that the features needed for DELLA interaction with the receptor were already present in the ancestor of all land plants and propose that these DELLA N-terminal motifs have been tightly conserved in nonvascular land plants for their function in transcriptional co-activation, which allowed subsequent exaptation for the interaction with the GID1 receptor when vascular plants developed gibberellin synthesis and the corresponding perception module.

Key words: exaptation, transcription factor, hormone signalling.

INTRODUCTION

DELLA proteins are transcriptional regulators that have been extensively characterized during the past 20 years (Vera-Sirera et al. 2015). They are involved in diverse processes ranging from seed germination to flowering, including legume nodulation, stress responses, or fern sexual reproduction (Peng and Harberd 1997; Floss et al. 2013; Tanaka et al. 2014). In fact, these proteins are responsible for the dwarf phenotype that allowed the development of new crop varieties during the Green Revolution (Peng et al. 1999). DELLAs are one of the main elements that compose the gibberellin (GA) signalling pathway in vascular plants, acting as the negative regulators of the pathway (Dill et al. 2001; Itoh et al. 2002).

As part of the GRAS family of plant-specific proteins, they present a highly conserved C-terminal domain, the GRAS domain. Initially, this domain was suggested to be distantly related to the STAT family of metazoan proteins (Richards et al. 2000). More recently, a thorough *in silico* structural analysis of the domain has evidenced a remarkable similarity to bacterial Rossman-fold SAM-dependent methyltransferases suggesting a bacterial origin of the GRAS domain (Zhang et al. 2012). Even though no chlorophytan alga presents GRAS-like genes, several charophytan species contain genes encoding GRAS proteins, pointing to an streptophytan origin of the family (Engstrom 2011; Delaux et al. 2015).

The GRAS domain of DELLA proteins is responsible for the establishment of protein-protein interactions. DELLAs cannot bind DNA, but they can interact with over 150 transcription factors and other transcriptional regulators, and modulate their functions in order to regulate gene expression (Marín-de la Rosa et al. 2014). DELLAs can either negatively affect transcription factor function, mainly through a sequestering mechanism, or positively enhance their ability to activate transcriptional activity (Locascio et al. 2013). This allows DELLAs to coordinate multiple transcriptional programs and may have been an important trait acquired during plant evolution (Briones-Moreno et al. 2017).

A second important characteristic of DELLA proteins is their GA-dependent stability. This ability relies in the N-terminal, DELLA domain. The GA receptor GIBBERELLIN INSENSITIVE1 (GID1) is able to interact directly with this N-terminal domain after GA binding (Ueguchi-Tanaka et al. 2005; Griffiths et al. 2006). Upon GA-GID1-DELLA complex formation, the SLY1/GID2 F-box protein interacts through the GRAS domain and recruits an SCF E3 ubiquitin ligase complex that marks DELLAs for degradation (Mcginnis et al. 2003; Sasaki et al. 2003; Dill 2004; Gomi et al. 2004). Three important motifs are involved in the interaction with GID1 proteins: DELLA, LEQLE, and VHYNP. Mutations in these motifs impair GID1-DELLA interaction, giving rise to GA-resistant DELLA versions (Dill et al. 2001; Murase et al. 2008).

Every land plant genome sequenced so far contains *DELLA* genes, but their characteristic features related to GA-signalling (i.e., N-terminal motifs and GA regulation, **fig. 1A**) have been reported only in vascular plants (Hirano et al. 2007; Yasumura et al. 2007). These early studies indicate that GA-dependent DELLA regulation first appeared in the vascular plants common ancestor, however the analyses were constrained by the limited availability of genomic and transcriptomic resources from only the moss *Physcomitrella patens*, and the lycophytes *Selaginella moellendorffii* and *Selaginella lepidophylla*. In these studies, neither a clear set of late GA synthesis genes nor proper GID1 receptors were detected in nonvascular plants, supporting the idea of GA-mediated regulation of DELLA proteins being vascular plant specific. In fact, no reports are available for the presence of bioactive GAs in mosses, and application of these compounds has no effect on moss growth (Hayashi et al. 2010).

Current knowledge indicates that DELLAs are important pieces of the signalling machinery of late diverging land plants, especially angiosperms, but nothing is known about DELLA function during early land plant evolution or if they exist at all in charophytan algae. The previous lack of data can now be completed with new genomic and transcriptomic sources from earlier diverging land plants and algae to understand the origin and emergence of DELLA proteins. In fact, the recently sequenced genome of the liverwort *Marchantia polymorpha* encodes a DELLA protein whose N-terminal motifs are more similar to its vascular orthologs

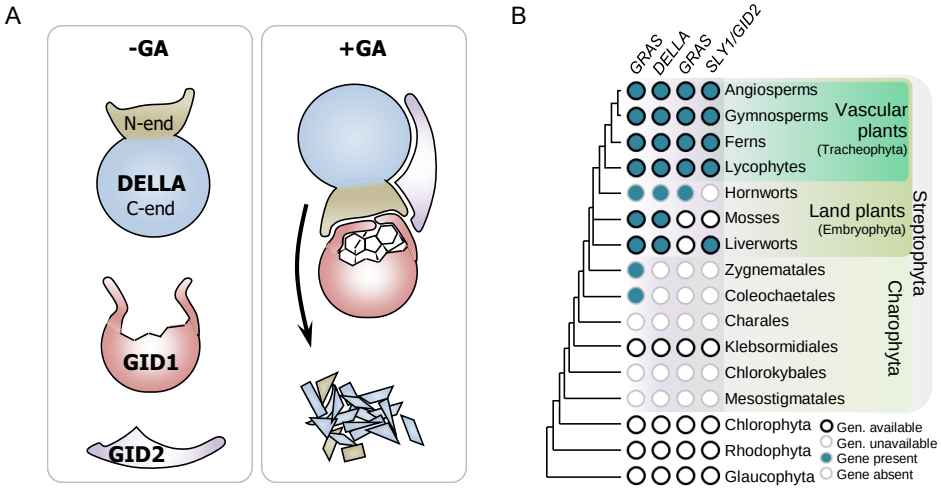

Figure 1. Gibberellin-signalling elements are present in vascular plants. (A) Gibberellin signalling in vascular plants. (B) Presence of gibberellin signalling-related sequences in different phyla. GRAS, GID1, and GID2 orthologs were retrieved from oneKP or genome databases by BlastP or TBlastX searches. GRAS proteins were validated by positive Pfam (PF03514.13) and DELLA were counted either when Pfam (PF12041.7) was positive or BlastP E-value was <1E-20 when using either AtRGA or PpDELLAa. GID1 were counted by the presence described N-lid residue presence and *α/β*-hydrolase active site conservation. SLY1/GID2 were selected by thresholding Blast results with 1E-20 based on similarity to *Selaginella moellendorffii* GID2 proteins.

than to moss sequences (Bowman et al. 2017). In the present work, we have tried to elucidate the evolutionary origin of DELLA proteins and also investigate the functionality of the N-terminal domain by analysing the conservation and diversification of specific motifs in that domain. We found that algal GRAS proteins are monophyletic and evolved independently from those of land plants, indicating that there are no DELLAs outside land plants, and propose that the ancestral role of the N-terminal domain was as a transcriptional activation module which conservation allowed the co-option for the interaction with the GID1 receptor later during land plant evolution.

RESULTS

Identification of GRAS and GA-signalling element sequences in plants

Previous studies have shown that GRAS domain sequences are present in several zygnematalean algae (Engstrom 2011; Delaux et al. 2015). We used these GRAS domains as bait to analyse available transcriptomes and genomes of all plants (i.e., Archaeplastida) in order to retrieve GRAS genes (**fig. 1B** and supplementary table 1, Supplementary Material online). After curation, we obtained GRAS sequences belonging to land plants and two groups of charophytan algae: Zygnematales and Coleochaetales. We did not find GRAS sequences in other algal groups, including the rest of charophytan groups, chlorophytes, rhodophytes, and glaucophytes. Among GRAS sequences, we detected bona fide DELLA sequence hits in all land plant extant clades. We also searched for other known GA-signalling components: the receptor GID1 and the F-box protein SLY1/GID2. Although we found similar sequences to GID1 in many clades (i.e., GID1-like proteins, or GLPs, supplementary table 2, Supplementary Material online), those present in nonvascular plants do not contain the amino-lid sequences necessary for GA perception and DELLA interaction, and resemble those found in *P. patens* (Hirano et al. 2007; Yasumura et al. 2007). Hence, we did not consider them as GID1 receptors. However, we found two hornwort sequences (an almost complete sequence for *Phaeoceros carolinianus* and a partial sequence for *Paraphymatoceros halli*) that represent good candidates for an ancestral state of GID1 receptors, pointing to a possible pretracheophytan origin of putative GID1 proteins (supplementary fig. 1, Supplementary Material online). Among nonvascular land plants, we confirmed that the presence of SLY1/GID2 orthologous sequences is not only evident in *M. polymorpha* (Bowman et al. 2017), but in all liverworts examined, and absent in other nonvascular plants (supplementary fig. 2 and supplementary table 3, Supplementary Material online). These data consolidate the presence of GRAS in charophytan algae, not only in Zygnematales but also in Coleochaetales, and are consistent with the idea of GRAS proteins appearing first in charophytes before land colonization. Besides, DELLA proteins most likely appeared in the land plant common ancestor, but the GA signalosome components can only be found simultaneously in tracheophytes (**fig. 1**). However, these views may change with further improvement of genomic

data quality and availability from Charales and/or hornworts.

Phylogenetic analysis of GRAS proteins

To elucidate the origin of the DELLA subfamily, we analysed the phylogenetic relationship between GRAS sequences in algae and land plants. For this, we used the GRAS domain of the sequences found and added previously described eubacterial GRAS sequences to use as outgroup in a phylogenetic tree (Zhang et al. 2012). Interestingly, algal and land plant sequences formed two independent and statistically supported clades (**fig. 2A**). This suggests that all land plant GRAS genes arose from a single gene present in an algal and land plant common ancestor. Further expansion and loss of GRAS subfamilies has occurred independently several times during plant evolution, and no clear correlation between the number of GRAS sequences and factors such as biological complexity seems to exist (**fig. 2B**). Among land plants, we found sequences from 12 known GRAS subfamilies in *A. thaliana* (SCL9, SHR, PAT1, SCL16/32, SCL29, SCL3, DELLA, SCL28, SCR, LAS, SCL4/7, and HAM), and sequences with no clear *A. thaliana* match in at least 2 bryophytes that resemble RAM1 sequences (**fig. 2C**). We conducted phylogenetic analysis of these GRAS domain sequences and obtained highly supported clades for these groups in all land plant lineages (**fig. 2D**). In fact, these groups greatly coincide with those recently published in the *M. polymorpha* genome (Bowman et al. 2017). Altogether, these analyses indicate that previously known GRAS subfamilies are land plant specific and appeared early in a land plant common ancestor. Consequently, we consider that only land plant genomes may contain *DELLA* genes.

Early evolution of DELLA proteins

To elucidate DELLA evolution, we generated a new phylogenetic tree adding previously undetected DELLA proteins from species belonging to different clades across land plant phylogeny (**fig. 3A**). The N-terminal domain was also excluded from this analysis because the high level of divergence in this region yielded trees that were in conflict with known taxonomic relationships (supplementary fig. 3, Supplementary Material online). We confirmed the previously reported major DELLA clades corresponding to the eudicot clades RGA and RGL (DELLA1 and DELLA2, respectively), which are fused into a single DELLA1/2 clade in non-eudicot tracheophytes, and the DELLA3 clade, also named DGLLA/SLRL, that is present in all vascular plant lineages. These three clades are found in every major clade analysed, with the sole exception of DELLA1/2 being absent in ferns. These data suggest the occurrence of two main duplication events in *DELLA* genes coinciding with the appearance of tracheophytes and the emergence of eudicots. However, multiple duplications and loses have occurred in specific groups and species, such as the lack of DELLA1 and DELLA3 in *Solanum lycopersicum*.

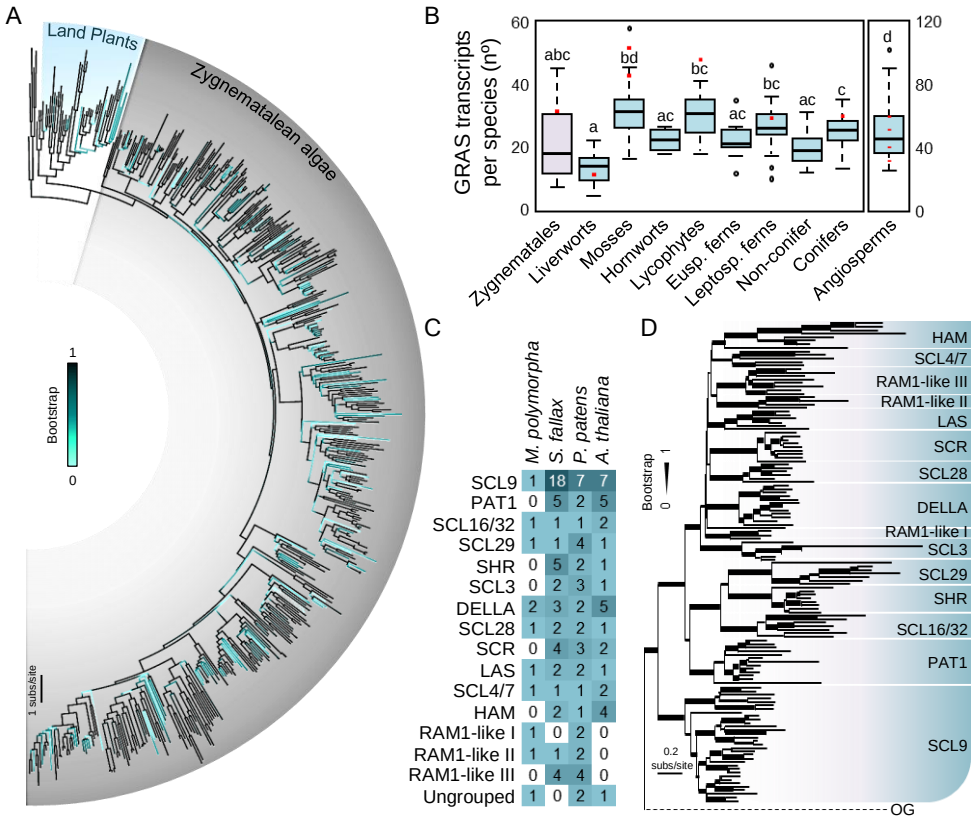

Figure 2. DELLA proteins are land plant specific. (A) Phylogenetic analysis of streptophytan GRAS proteins using GRAS domains. Support values associated with branches are maximum likelihood bootstrap values from 1,000 replicates depicted as a colour range (light turquoise to black). Black branches indicate a bootstrap of 1 (100%). Blue background denotes land plant (i.e., embryophytan) clade, grey background denotes algae sequences. (B) Number of different expressed genes found in analysed plant species belonging to different land plant clades. A number of genes found in example genomes are shown as red dots. Letters indicate significant differences between groups ($p < 0.01$, one-way ANOVA, Tukey's Honestly Significant Difference post hoc test). (C) GRAS genes per subfamily found in the non-vascular land plant genomes from *Marchantia polymorpha*, *Sphagnum fallax*, and *Physcomitrella patens*, compared with *Arabidopsis thaliana*. (D) Phylogenetic analysis of land plant GRAS proteins using GRAS domains. Support values associated with branches and displayed as bar thickness are maximum likelihood bootstrap values from 1,000 replicates.

Due to the scarce knowledge of DELLA function in non-vascular land plants, we expanded our search for DELLA sequences in liverworts, mosses, and hornworts and analysed their phylogenetic relationship (**fig. 3B**). As suggested by the previous tree, no ancient major duplications in DELLAs have been maintained in land plants prior to vasculature emergence. We also detected a type of DELLA sequences that we named DELLA-like proteins, whose phylogenetic position is unclear and most likely represent a liverwort-specific duplication with no resemblance in their N-terminal domains to those of DELLA proteins (supplementary fig.

3, Supplementary Material online). Other duplication events have also occurred independently in mosses as in the case of Funariaceae or Sphagnopsida.

DELLA domain characterization in non-vascular land plants

Since the function of DELLA proteins as GA-signalling elements requires the presence of specific motifs in their N-terminal domain and it has been proposed that there is no GA pathway in nonvascular plants (Miyazaki et al. 2018), we decided to analyse the occurrence of DELLA motifs in the N-terminal domain of DELLA proteins using automated Pfam HMM domain detection. Contrary to the highly significant scores for the presence of GRAS domains (PF03514; supplementary fig. 3, Supplementary Material online), the identification of the DELLA Pfam HMM (PF12041) resulted in strongly variable significance values (supplementary table 4, Supplementary Material online). In tracheophytes, the search was positive with independent E-values usually around 10^{-30}, with SmDELLA2 being the higher with an E-value of $1.3x10^{-11}$ (**fig. 3C**). SmDELLA2 contains highly divergent DELLA domain motifs but has been shown to be targeted by GID1 upon GA recognition (Hirano et al. 2007), setting an empirical threshold of potentially GID1-targeted DELLA domains. Among non-tracheophyte DELLAs, hornworts scored with E-values of around 10^{-19}, indicating that their DELLA domains are very similar to those found in tracheophytes. In agreement with the reported lack of DELLA canonical motifs and functionality of the DELLA domain in *P. patens* (Hirano et al. 2007; Yasumura et al. 2007), mosses show a clear trend toward DELLA Pfam loss, but their earlier diverging moss species contain relatively low E-values, reaching 10^{-18} for *Takakia lepidozioides* DELLA (**fig. 3C**).

To determine the precise motifs and residues that provide high significance value for the identity of the N-terminal domain, we aligned the corresponding regions of representative sequences from each clade (three liverworts, ten mosses, three hornworts, and three tracheophytes) (**fig. 4**). The three important motifs for the interaction with the GID1 receptor (and, therefore, for GA-signalling) in tracheophytes were differentially conserved among non-vascular plants: liverworts displayed clear DELLA and VHYNPS motifs; most mosses only contain the LEQLE motif, except *T. lepidozioides*, in which only DELLA and VHYNPS are present; and hornwort sequences contain DELLA, LEQLE, and VHYNPS motifs.

This distribution of motifs suggests that the N-terminal domain of DELLA proteins was established early in land plant ancestors and maintained during evolution. To confirm this hypothesis, we performed ancestral protein reconstruction by maximum likelihood methods, excluding late divergent moss sequences to avoid bias toward DELLA or VHYNP motif loss. To avoid the lack of consensus in bryophyte relationships and early land plant evolutionary history, we performed the analysis using different phylogenetic relationships among the three

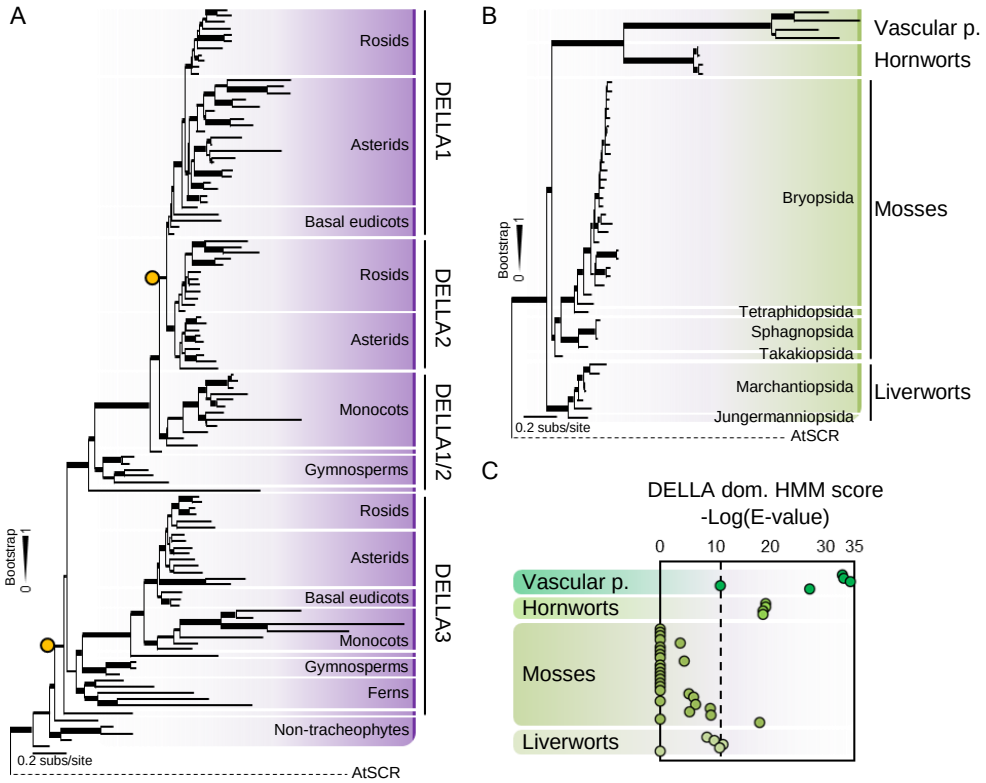

Figure 3. DELLA proteins are present in all extant land plant lineages. (A) Phylogenetic analysis of DELLA proteins using GRAS domains of all land plant lineages. Orange circles indicate inferred major duplications within DELLA subfamily. (B) Phylogenetic analysis of DELLA proteins using GRAS domains of nonvascular land plant sequences and a few land plant representatives. Support values associated with branches and displayed as bar thickness are maximum likelihood bootstrap values from 1,000 replicates. (C) Automated search for DELLA motifs in nonvascular land plants using Pfam website. Scores are represented as the negative log of the E-value retrieved from the search and represented following the phylogenetic position obtained in **figure 3B**. Absence of significant E-values for the presence of Pfam DELLA domain are plotted as 0 in the graph. Dashed line represents the value retrieved for SmDELLA2 DELLA domain, able to interact with GID1a/b with a moderately divergent DELLA domain motif.

bryophytan groups (hornworts, liverworts, and mosses), with almost identical results. The predicted ancestral sequence for the land plant common ancestor DELLA N-terminal domain harbours the canonical motifs and is strictly conserved compared with those known in tracheophytes and in hornworts (**fig. 4**). Three of the four alpha helices harboured in the N-terminal domain show an ancestral state highly conserved in late diverging DELLA proteins, coinciding with the alpha helices that form the surface interacting with GID1.

Two more pieces of evidence support that the putative function of the ancestral N-terminal domain of DELLA proteins needed to be maintained during evolution: the K_a/K_s ratio is particularly low (around 0.2) precisely in the region that interacts with GID1 in higher plants

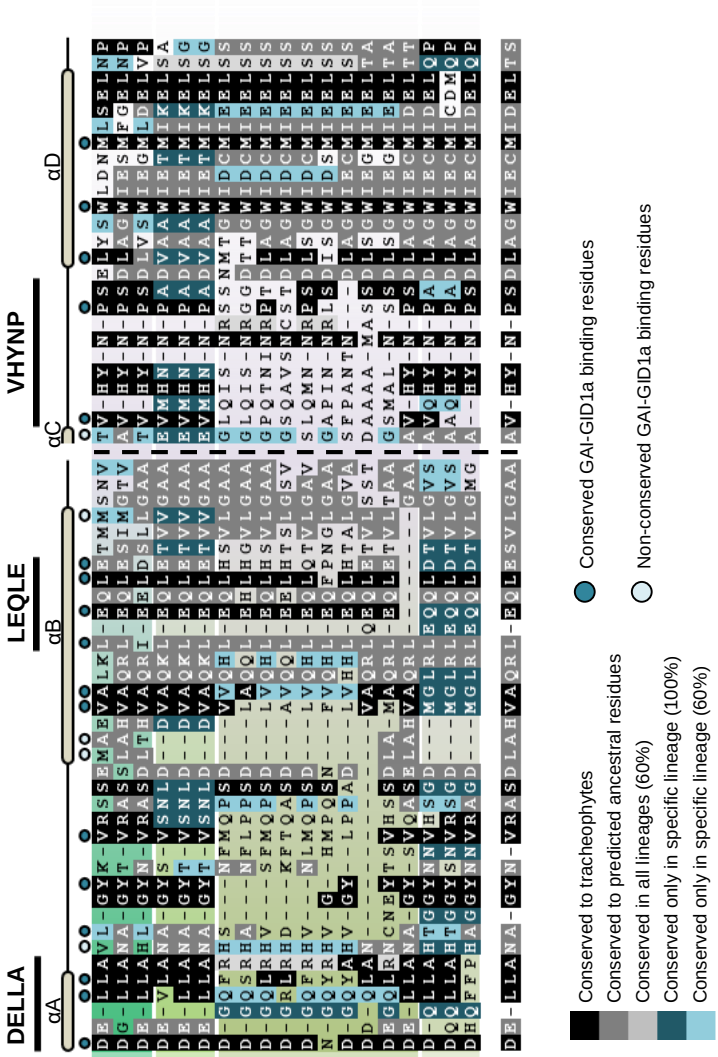

Figure 4. GID1 binding residues in DELLA proteins are highly conserved. GID1 binding residues in DELLA proteins are highly conserved. Multiple protein sequence alignment showing DELLA amino-terminal region spanning from α helix A to D. In some cases, non-conserved sequences were trimmed to avoid multiple gaps presence. Conservation percentages are based on original alignments. GAI-GID1a binding sites based on Murase et al. (2008). Ancestral DELLA sequence inferred with FastML, only the most probable residues are shown per position.

(supplementary fig. 4A and supplementary table 5, Supplementary Material online); and the characteristic intrinsic disorder of the whole N-terminal domain of DELLA proteins (Sun et al. 2010) is in fact absent in the GID1-interacting region (supplementary fig. 4B and C and supplementary table 5, Supplementary Material online).

DELLA interaction with GA-signalling components

The solid conservation of the N-terminal domain of DELLA proteins even in land plants that lack the necessary GA-signalling elements suggests that the ancestral DELLA was preadapted for subsequent interaction with the GA receptors. To gather additional experimental evidence, we first modelled the structure of putative complexes between AtGID1a and DELLAs from species of different land plant taxa based on the previously described GA_4-AtGID1a-AtGAI structure. For comparison, the DELLAs were selected from *A. thaliana* (Angiosperma), *S. moellendorffii* (Lycophyta), *Nothoceros vincentianus* (Anthocerotophyta), *P. patens* and *T. lepidozioides* (Bryophyta), and *M. polymorpha* (Marchantiophyta). The structures of the N-terminal domain of the selected DELLAs were modelled and superimposed to the structure of the DELLA-GID1 complex (Kelley et al. 2015). Using this strategy, we were able to determine if the DELLA, LEQLE, and VHYNPS motifs of the selected proteins could potentially interact with the AtGID1a protein (**fig. 5A** and supplementary fig. 5A, Supplementary Material online). As expected, the model for *A. thaliana* DELLA (AtRGA) showed a similar interaction with AtGID1a to that observed between AtGAI and AtGID1a. Despite small differences observed on the VHYNP motif of *N. vincentianus* NvDELLA (MHNNP) and the LEQLE motif of *S. moellendorffii* SmDELLA1 (IEELD), both proteins exhibit similar fold and potential interaction with AtGID1a, suggesting that lycophyte and hornwort DELLAs might indeed establish functional interactions with GA receptors. The structure of the SmDELLA1 model in complex with AtGID1 also shows similar interactions except for the LEQLE motif where the latter glutamate (E) is replaced in SmDELLA1 by an aspartate (D) (IEELD). This mutation which should decrease the interaction with GID1 K28 (**fig. 5**) does not seem important because the degradation of SmDELLA1 by GAs has already been described (Hirano et al. 2007; Yasumura et al. 2007). This result can be easily understood by the mobility of the side chain of Lysine (K) 28 which could likely interact with the aspartate residue of SmDELLA1. On the contrary, despite a similar fold, *T. lepidozioides* TIDELLA displayed critical changes in the LEQLE motif (absent in the alignment, substituted by the subsequent LGAAQ sequence in the model) of the αB helix which are predicted to prevent interaction with GID1. In addition, *M. polymorpha* and *P. patens* DELLAs present modifications not only in the αB helix but also in the DELLA and VHYNP motifs preventing interaction with AtGID1a. In summary, the models indicate that, unlike lycophytes and hornworts, moss and liverwort DELLAs should be unable to interact with AtGID1a.

To experimentally test the models, we performed yeast two-hybrid assays between the six full-length DELLAs and the Arabidopsis GID1 proteins in the presence or absence of GA_3 (**fig. 5B** and supplementary fig. 5B, Supplementary Material online). As expected, both tracheophytan DELLAs interacted with AtGID1s when GA was present. Interestingly, *N. vincentianus* DELLA was also able to interact with the receptors in a GA-dependent manner.

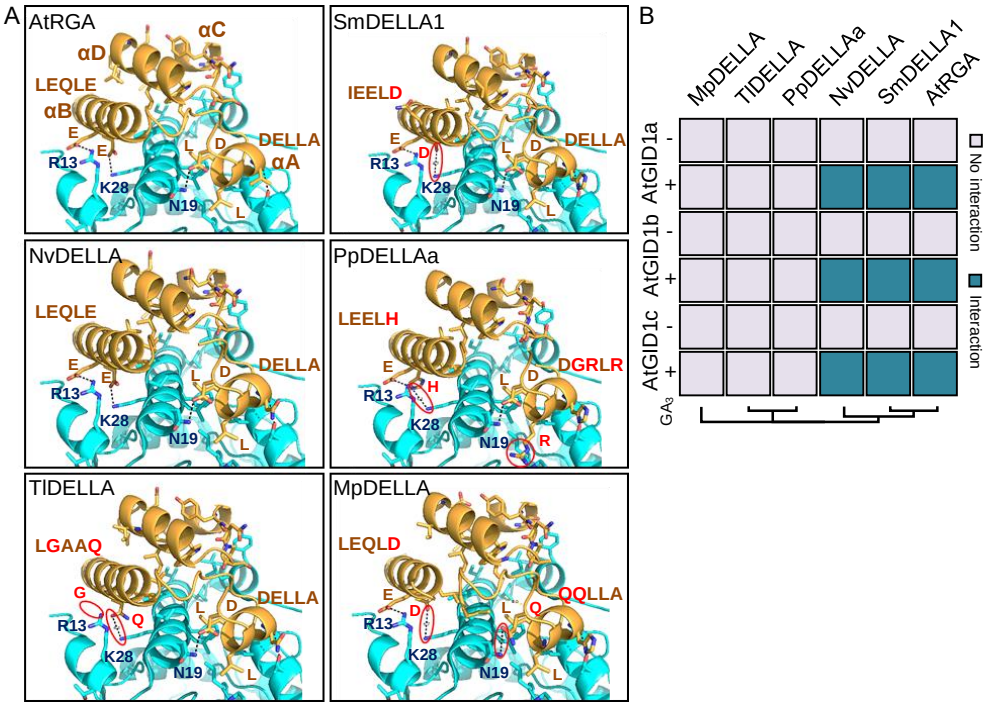

Figure 5. Some non-vascular DELLA proteins can interact with GID1 receptors in a GA-dependent manner. (A) Predicted structural model for DELLA-AtGID1a interaction using AtRGA, SmDELLA1, NvDELLA, PpDELLAa, TIDELLA, and MpDELLA. DELLA structure is shown in yellow and AtGID1a structure in light blue. AtGID1a residues involved in DELLA interaction are written in dark blue. Residues different to that of AtRGA/GAI in main motifs are presented in red. Possible residue to residue interactions affected are pointed with a red circle (B) Yeast-two-hybrid assay results between DELLA proteins and the three Arabidopsis GID1 receptors with or without GA_3. Positive interactions are accounted when yeast growth occurs in 5-mM 3-aminotriazole.

However, moss and liverwort sequences did not show interaction with the receptors either in a GA-dependent or -independent way. These results indicate that N-terminal domain conservation is necessary but not sufficient for GID1 interaction. Moreover, the ability of hornwort DELLAs to recognize the Arabidopsis GA receptors in a GA-dependent manner (**fig. 5B**), and the presence of putative GA receptor sequences in some Anthocerotophyta genomes (**fig. 1**; supplementary table 2, Supplementary Material online) suggests that the origin of GA signalling might predate the separation between hornworts and vascular plants.

An ancestral function of the DELLA N-terminal domain in transcriptional activation

The presence in N-terminal region of the ancestral DELLA protein of structured domains that were necessary for the eventual construction of a GA-signalling module begs for an additional function encoded in this region which would explain its conservation in nonvascular plants lacking GA receptors or elaborate GA biosynthesis. Interestingly, although the actual

mechanism is still unknown, Arabidopsis DELLAs have been reported to act as transcriptional coactivators in certain developmental contexts (Fukazawa et al. 2014; Yoshida et al. 2014; Marín-de la Rosa et al. 2015). In fact, when we examined the transcriptional status of loci to which AtRGA is bound (Marín -de la Rosa et al. 2015), most of the genes showed a tendency to be induced when DELLAs are active (**fig. 6A** and supplementary table 6, Supplementary Material online). It has been reported that expression of full-length rice DELLA fusions to a DNA binding domain (DBD) in yeast results in the transactivation of the corresponding reporters (Hirano et al. 2012). We have found that this transactivation capacity is conserved in the N-terminal domains of all the DELLAs tested, included those from non-vascular land plants (**fig. 6B** and supplementary fig. S6A, Supplementary Material online). Previously, it has been suggested that both DELLA and VHYNP motifs are involved in this activity (Hirano et al. 2012). However, despite of the lack of both of these motifs, the N-terminal domain of PpDELLAa is strongly capable of transcriptional activation. The most conserved region among all land plant DELLA N-terminal domains is the αD helix (**fig. 4**), and we found that deletion of this region (ΔαD) in PpDELLAa prevented the induction of reporter expression in yeast, whereas the αD helix alone was also capable of promoting transactivation in a yeast two-hybrid assay, although this activation is more robust when the whole ordered regions (N1) ranging from αA to αC are present (**fig. 6C** and supplementary fig. 6B, Supplementary Material online). To study if this also happens in planta, we confirmed these results by performing transient expression assays of a dual luciferase reporter in *Nicotiana benthamiana* (**fig. 6D** and supplementary fig. 6C, Supplementary Material online). The activity of certain transactivation domains from different origins (viral VP16, yeast GAL4 or PHO4, mammalian p53 or NFAT, etc.) has been proposed to reside in a particular nine-amino acid transactivation domain (9aaTAD) (Piskacek et al. 2007) which directly interacts with the KIX domain of general transcriptional coactivators like Mediator's MED15 subunit (Piskacek et al. 2016). Interestingly, the αD helix of the DELLA N-terminal domain displayed a high score in a 9aaTAD evaluation (supplementary fig. 7, Supplementary Material online).

DISCUSSION

The work presented here provides new clues about the origin of DELLA proteins in the common ancestor of all land plants, and a possible mechanism by which these proteins became GA-signalling elements in vascular plants, after the emergence of the GID1 GA receptor.

Previously, putative DELLA proteins had been reported in at least two nonvascular plant species: the moss *P. patens* and the liverwort *M. polymorpha* (Hirano et al. 2007; Yasumura et al. 2007; Bowman et al. 2017). Our extensive phylogenetic analyses have not only confirmed the widespread presence of clearly defined DELLA proteins in all clades of

nonvascular plants including hornworts but also add two important pieces of new information: 1) since all land plant GRAS proteins are monophyletic, the origin of DELLA proteins unequivocally coincides with the colonization of land by plants and 2) the N-terminal domain is conserved in the vast majority of DELLAs, including those in non-vascular plants. This observation contradicts the previous assumption that the recruitment of DELLAs to GA signalling was due to the appearance of GID1-interacting motifs in this N-terminal region in vascular plants (Hirano et al. 2007; Yasumura et al. 2007). This assumption was largely based on the absence of the "DELLA" and "VHYNPS" motifs in PpDELLA; but the presence of these important motifs in a basal moss species, like *T. lepidozioides*, in all the hornwort DELLA sequences analysed, and in the reconstructed ancestral DELLA protein sequence, suggest a most likely scenario in which the ancestral DELLA contained most of the motifs that would later be useful to establish the interaction with the GID1 receptor.

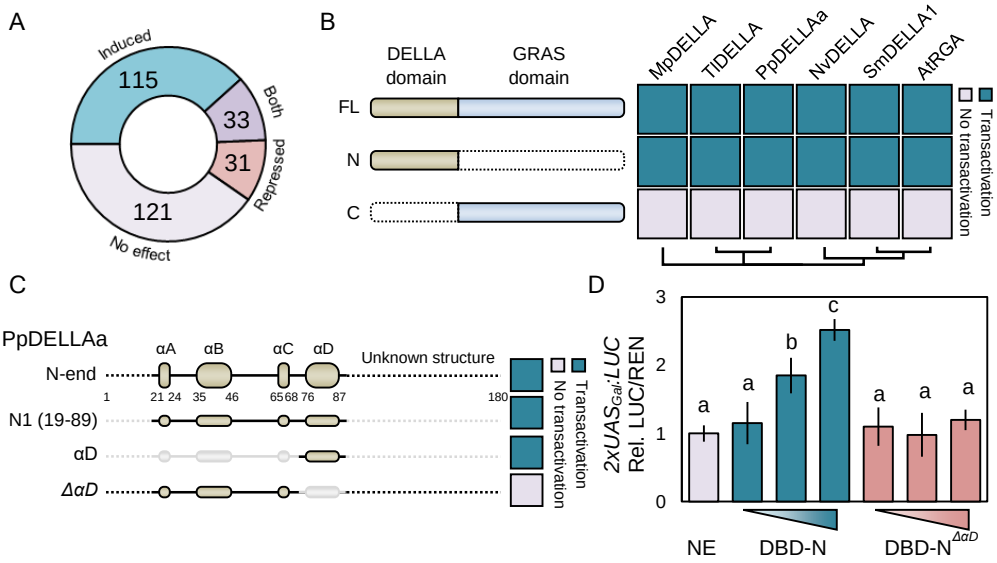

Figure 6. DELLA domain conserved region act as a transcriptional activator domain. (*A*) RGA-bound genes in ChIP-seq assays are enriched in induced versus repressed genes when compared to different transcriptomic data in DELLA induced conditions. ChIP-seq data retrieved from Marín-de la Rosa et al. 2015; Transcriptomic data obtained from several available datasets. (*B*) Yeast transactivation assay results using DELLA protein full-length coding regions (FL), or truncated versions using either the GRAS domain (C) or the DELLA domain (N). (*C*) Yeast transactivation assay results using different truncated versions of PpDELLAa DELLA domain. Transactivation is accounted when yeast growth occurs in 5 mM 3-aminotriazole. (*D*) Dual luciferase transactivation assays in *Nicotiana benthamiana* leaves using the *LUC* gene under the control of the *Gal* operon UAS promoter as the reporter, and different effector vectors fused to the GAL4 binding domain. NE, no effector; DBD-N, PpDELLAa DELLA domain fused to GAL4 DNA binding domain; DBD-N$^{\Delta\alpha D}$, PpDELLAa Δ75-88 truncated version fused to GAL4 BD. Constitutively expressed *Renilla* luciferase (*REN*) for normalization. Data shown are normalized to NE value and represent the average of three biological replicates. Error bars represent standard deviation. Letters indicate significant differences between groups ($P < 0.01$, one-way ANOVA, Tukey's HSD post hoc test).

The establishment of the GID1-DELLA interaction constitutes, as previously reasoned (Sun 2011), the key event that connects the ancestral DELLA activity with the newly emerging GA signalling. In this respect, our work contributes with the finding of GID1-like sequences in hornworts that are phylogenetically close to bona fide GID1 receptors, and the observation that the N-terminal domains of hornwort DELLAs display the intrinsic ability to interact with a vascular plant GID1 receptor in a GA-dependent manner. Therefore, at least two possible models can be contemplated: either GA signalling emerged in a common ancestor of hornworts and vascular plants, or it emerged independently in vascular plants and in hornworts, and the similar behaviour is caused by functional convergence. A third possibility would be that a putative GA-independent GID1-DELLA module in the ancestor of all land plants would have been lost in different clades, but this is highly unlikely based on recent evidence about GID1 evolution (Yoshida et al. 2018). The origin of the participation of DELLAs in GA signalling requires a more complete picture. Future work is needed to answer several key questions: 1) Do hornwort GID1-like proteins behave as GA receptors? 2) Can hornwort DELLAs interact with hornwort GID1-like proteins in a GA-dependent manner (or is any other hormone-like molecule perceived by GID1-like proteins)? 3) Is DELLA activity regulated in nonvascular plants by other GA-related compounds which are considered as GA precursors in vascular plants? Curiously, 3-hydroxy-kaurenoic acid has been proposed as a plant growth regulator in *P. patens* (Miyazaki et al. 2018), indicating a functional role for at least this metabolite in GA metabolism, but it is currently unknown if this function is conserved in other non-vascular plants.

Our results suggest that the main driving force for the conservation of the N-terminal regions of DELLA proteins has been its role in transcriptional activation, and its eventual co-option by the GID1 receptor allowed hormonal regulation of DELLA stability. Although molecular exploitation has been described as an evolutionary strategy to expand hormone receptor complexity (Bridgham et al. 2006; Baker et al. 2013), the function of the evolving receptor was equivalent to the ancestral one (i.e., interaction with the ligand). However, the origin of the GA-signalling pathway illustrates how molecular exploitation can occur upon domains with completely unrelated functions. Curiously, the coexistence of degron and transactivation motifs in the same stretches of residues has been described in several mammalian transcriptional activators (Salghetti et al. 2000, 2001). In contrast, this degron-TAD overlap has not been studied in plants but some examples can be identified, such as MYC2, in which a degron is found within the MID domain, the transactivation domain (Zhai et al. 2013). Therefore, DELLAs would have these two functions encoded in a single region, and interaction with GID1 has been reported not only to promote DELLA degradation but also to prevent transactivation by DELLAs (Hirano et al. 2012). In summary, the coincidence of transactivation and protein stability regulation in a single protein domain is a widespread property and has independently emerged several times during evolution and through different

molecular mechanisms.

MATERIALS AND METHODS

Identification of GRAS and GA-signalling element sequences in plants

GRAS homolog sequences were searched in Phytozome, OneKP, and specific databases for the charopyte *Klebsormidium flaccidum* (reassigned as *K. nitens*), red algae, and the glaucophyte *Cyanophora paradoxa* (supplementary table 7, Supplementary Material online). *A. thaliana* and *P. patens* previously identified GRAS sequences were rechecked and used as query in a BlastP initial search. In short, proteomes were examined using a BlastP local blast search using an E-value cutoff of 0.1 in most cases, further raised to 10 in red algae, chlorophytes and *C. paradoxa* in order to avoid missing highly diverging GRAS sequences. Initial results were first subjected to reciprocal Blast. Subsequently, the results were manually checked using SMART (http://smart.emblheidelberg.de/) and Pfam (http://pfam.sanger.ac.uk/) to ensure GRAS domain presence. For GID1- and GID2-related sequences, Phytozome and OneKP databases were analysed as mentioned, using *A. thaliana* and *S. moellendorffii* previously identified protein sequences as query. In this case, only reciprocal Blast was used. Manual curation of incomplete annotations was performed when needed using either transcriptomic data from the same or the closest species orthologs when available.

Phylogenetic analysis

Sequences were aligned using the MUSCLE algorithm (Edgar 2004) included in the SeaView 4.6.4 GUI (Gouy et al. 2010), with 16 iterations, default clustering methods, gap open score of -2.7, and hydrophobicity multiplier of 1.2, followed by manual curation. For phylogenetic reconstruction, C-terminal GRAS domains were used, and ambiguously aligned regions manually trimmed. In the case of DELLA phylogenetic analysis, AtSCR was included in the final alignments before tree reconstruction using MAFFT v7 method L-INS-i (Katoh and Standley 2013). ProtTest v3.4.2 (Darriba et al. 2011) was used on final multiple sequence alignments to select best-fit models of amino acid replacement using the AIC model for ranking. Maximum likelihood tree in figure 2a was produced with RAxML 8.2.3 using the LG PROTGAMMA model (Stamatakis 2014). The rest of ML trees were produced with PhyML v3.1 (Guindon et al. 2010), using the best scored model of amino acid substitution. Statistical significance was evaluated by bootstrap analysis of 1,000 replicates in all cases with the sole exception of supplemental figure 1, Supplementary Material online, which was evaluated by SH-like approximate likelihood ratio test. Phylogenetic tree graphical representations were initially generated using FigTree (version 1.4.3) software (http://tree.bio.ed.ac.uk/software/figtree/), and final cartoons edited manually.

Original sequences, raw and trimmed alignments, and trees are available at https://data.mendeley.com/datasets/bjcp6ggjk9/1.

Ancestral sequence reconstruction

The ancestral state for each codon position in the DELLA N-terminal domain was determined using MEGA7 (Kumar et al. 2015). Nucleotide coding sequences aligned following the previous result of the corresponding amino acids alignment. Ancestral sequence inference was then performed using maximum likelihood, including four different predefined tree topologies around non-vascular plants: 1) monophyletic bryophyta, 2) a moss-liverwort sister clade to other embryophytes, 3) liverwort-moss sister clade to tracheophytes, and 4) hornworts, mosses, and liverworts as successive sister lineages to tracheophytes (Puttick et al. 2018). Finally, the Tamura-Nei model of nucleotide substitution was used for ancestral state inference. Gap residues were trimmed if absent in >90% of the sequences.

Codon selection and protein disorder analysis

Analysis of selection was performed using the web-based interface Selecton v2.2 (Stern et al. 2007). M8 and M8a models of selection were used to calculate the ratio between the rates of nonsynonymous (K_a) and synonymous substitution (K_s) in previously constructed codon-based nucleotide alignments. Likelihood scores estimated by the models were evaluated by log-likelihood ratio testing with degree of freedom (df)=1, followed by Bayesian prediction of undergoing positive approach. Prediction of disorder per residue was performed with the ANCHOR web tool (https://iupred2a.elte.hu/) (Dosztányi et al. 2009). Mean predicted disorder values per residue were calculated based on the back-translated codon-based alignment using AtRGA, SmDELLA1, NvDELLA, PpDELLAa, TIDELLA, and MpDELLA. Raw data are included in supplemental table 5, Supplementary Material online.

Protein structure prediction

The N-terminal regions of all DELLA proteins were modelled with 100% confidence using AtGAI (PDB code 2ZSH) (Murase et al. 2008) as template using the PHYRE2 program (Kelley et al. 2015) and visualized with PyMOL software (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

Transactivation domain prediction

AtRGA, SmDELLA1, NvDELLA, PpDELLAa, TIDELLA, and MpDELLA protein sequences were analysed with a 9aaTAD prediction tool (http://www.med.muni.cz/9aaTAD/index.php) (Piskacek et al. 2007), using the “less stringent” pattern. Cumulative probabilities of 9aaTAD

for all the proteins were plotted versus an amino acid alignment of the DELLA domain.

Yeast-two hybrid assay

Arabidopsis GID1 was fused to the Gal4-DBD in the pGBKT7-GW vector as bait, and DELLA full-length Open Reading Frames (ORFs) from the different species were fused to the Gal4-activation domain (AD) in pGADT7-GW. DELLAs and GID1 ORFs were either amplified by polymerase chain reaction (PCR) using sequence-specific primers (supplementary table 9, Supplementary Material online) or synthesized as gBlocks (I.D.T.) and transferred to pDONR221 or pDONR207 via BP Clonase II (Invitrogen), or to pCR8 via TOPO-TA cloning (Invitrogen) to create entry vectors. Final constructs were made by recombining entry clones to GATEWAY destination vectors via LR Clonase II (Invitrogen). Direct interaction assays in yeast were performed following Clontech's small-scale yeast transformation procedure. Strain Y187 was transformed with pGADT7-derived expression vectors, whereas strain Y2HGold was transformed with pGBKT7 vectors, and selected in Synthetic Defined (SD) medium without Leu or Trp, respectively. Subsequently, diploid cells were obtained by mating and selection in SD medium lacking both Leu and Trp. Interaction tests were done in SD medium lacking Leu, Trp, and His, in the presence of different concentrations of 3-aminotriazole (Sigma-Aldrich). To assess GA-dependent interaction, the medium was supplemented (or not) with 100 μM GA_3.

Yeast transactivation assay

DELLA ORFs were obtained as described above. DELLA N-end clones and C-end clones were obtained by PCR amplification using sequence-specific primers (supplementary table 9, Supplementary Material online) and transferred to pDONR207 via BP Clonase II (Invitrogen). Entry clones were then used to create Gal4-DBD fusions in the pGBKT7-GW vector via LR Clonase II (Invitrogen), which was transformed into yeast strain Y2HGold and transformants were selected in SD medium lacking Trp. Transactivation tests were performed in SD medium without Trp and His, and increasing 3-aminotriazol concentrations as indicated.

Plant transient transactivation assay

A reporter construct containing 2xGal4 UAS followed by a 35S minimal promoter (Gendron et al. 2012) was amplified using sequence-specific primers (supplementary table 9, Supplementary Material online) and cloned upstream of the firefly luciferase gene (LUC) in pGreenII 0800-LUC (Hellens et al. 2005). The effector vectors were obtained by amplifying the GAL4 DBD-DELLA N-end fusions generated in pGBKT7 vectors as a unique PCR product with proper restriction site overhangs and ligated into pFGC5941 (http://www.ChromDB.org), between *Xho*I and *Spe*I. The GAL4 DBD control construct was obtained by excising the RGA-

N fragment from the DBD-RGA-N vector. Transient expression in *N. benthamiana* leaves was carried as previously reported (Marín-de la Rosa et al. 2015). Firefly and the control *Renilla* luciferase activities were assayed in extracts from1-cm in diameter leaf discs, using the Dual-Glo Luciferase Assay System (Promega) and quantified in a GloMax 96 Microplate Luminometer (Promega).

SUPPLEMENTARY MATERIAL

Supplementary data are available at *Molecular Biology and Evolution online* and as described at the beginning of this PhD thesis manuscript (**Opening Statement**). A phylogenetically updated version of the figures can be found in **Annexes Part 3** and Mendeley Data resource.

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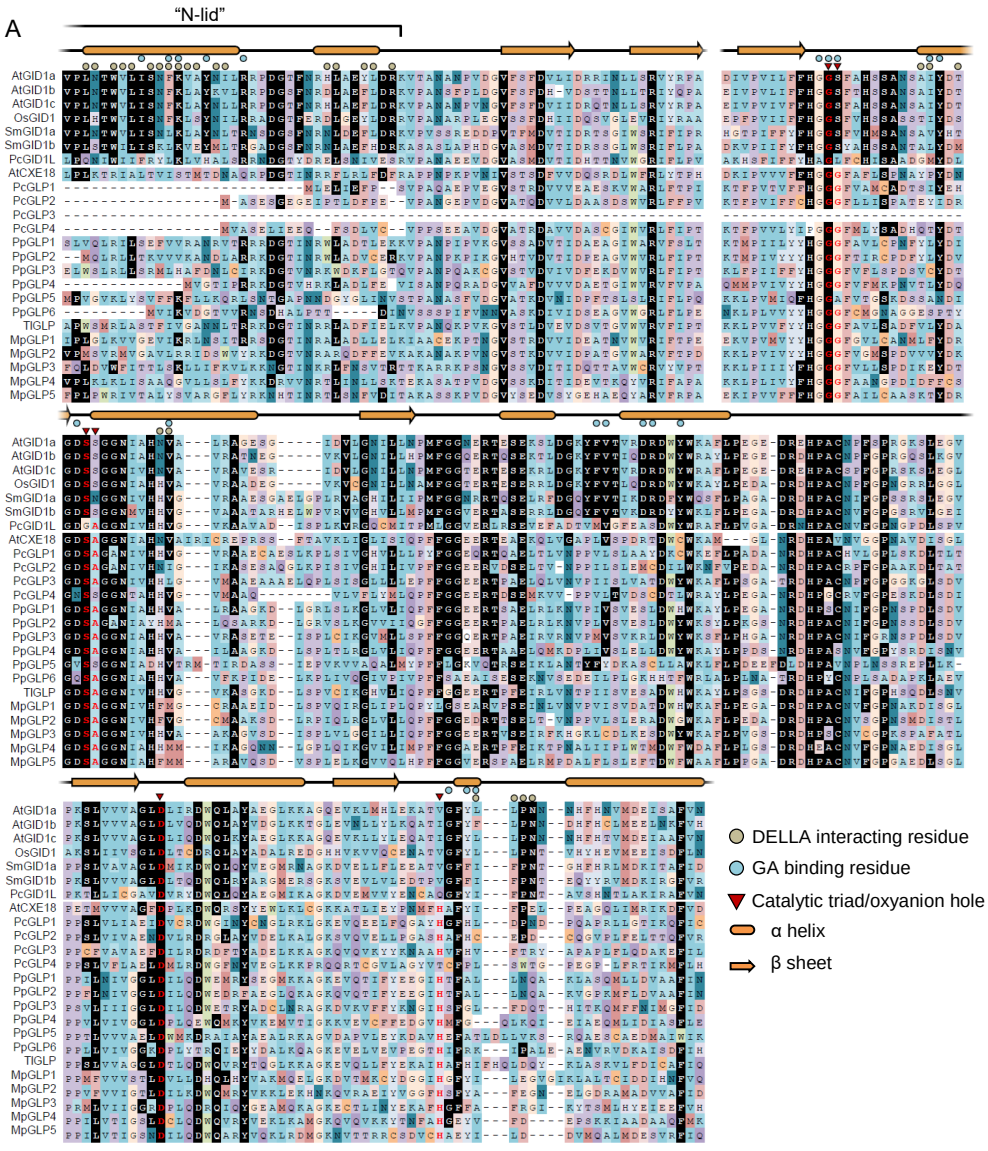
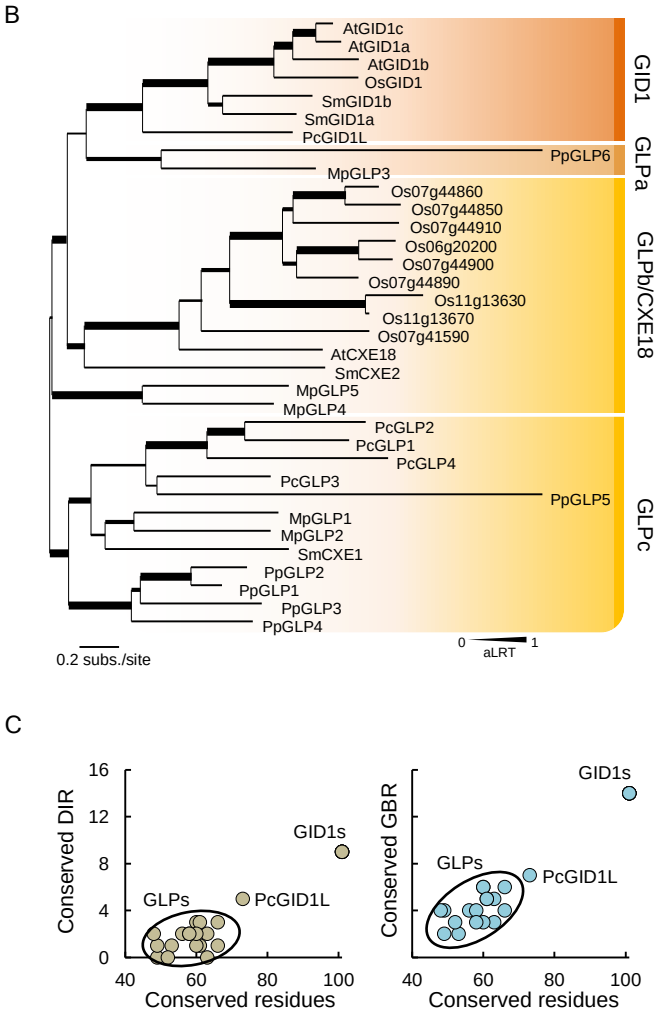
SUPPLEMENTAL FIGURE 1 – part 1



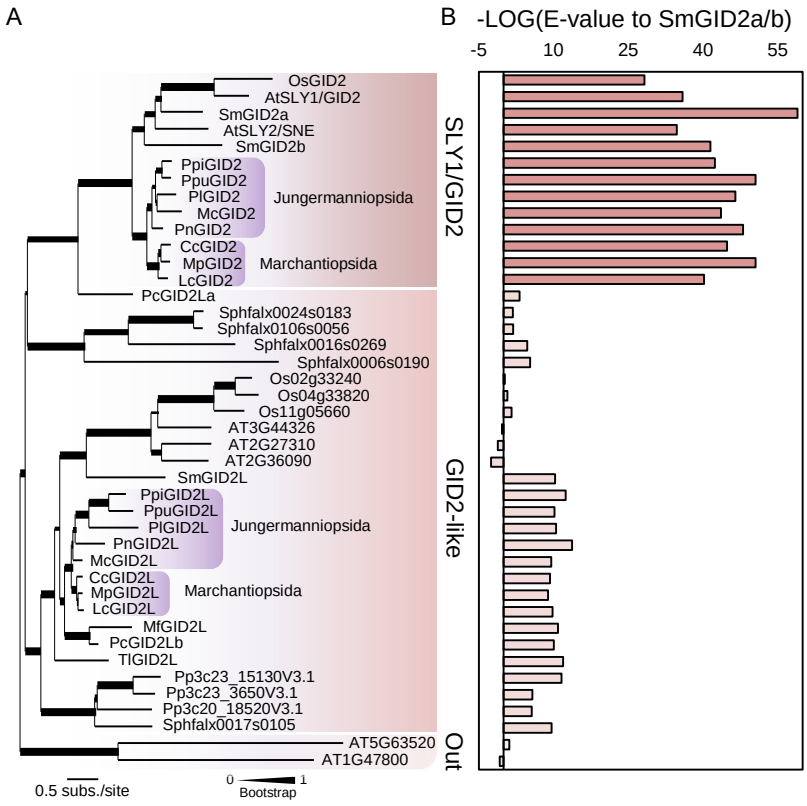
GID1 *bona fide* orthologs may be unique to vascular plants. (*A*) Multiple sequence alignment of GID1 and GID1-like proteins from several land plant species. GID1 structure and DELLA/GA binding residues based on Murase et al. 2008. Black background denotes highly conserved residues in vascular plants. Red letters indicate catalytic triad and oxyanion hole residues from the α/β hydrolase family.

SUPPLEMENTAL FIGURE 1 – part 2



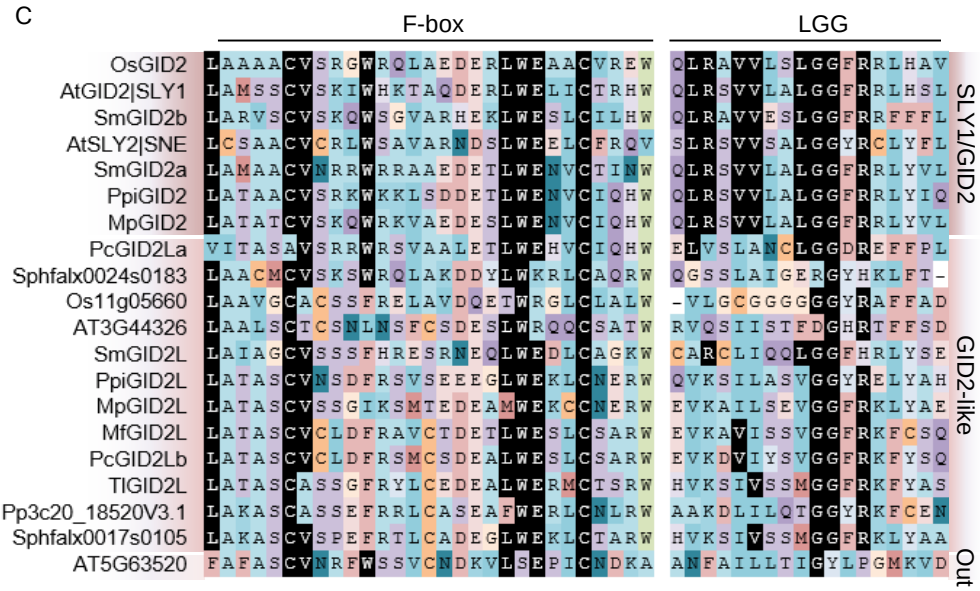
(*B*) Phylogenetic analysis of GID1 proteins. Support values associated with branches and displayed as bar thickness are SH-like approximate likelihood ratio test scores (aLRT). Unlike other non-vascular plants, the hornwort *Phaeoceros carolinianus* harbours a sequence which aligns in the same clade as bona-fide GID1 GA receptors, and contains mutations in the catalytic triad that resemble those of GA receptors (as shown in A). We have named it PcGID1L. (*C*) Comparison between the number of DELLA interacting residue (DIR) or GA binding residue (GBR) conservation and the total number of residues conserved compared to vascular plant GID1s. Only strictly conserved residues in vascular plants are counted (black background in Sup. Fig. 1A). GLPs from non-vascular land plants are encircled.

SUPPLEMENTAL FIGURE 2 – part 1



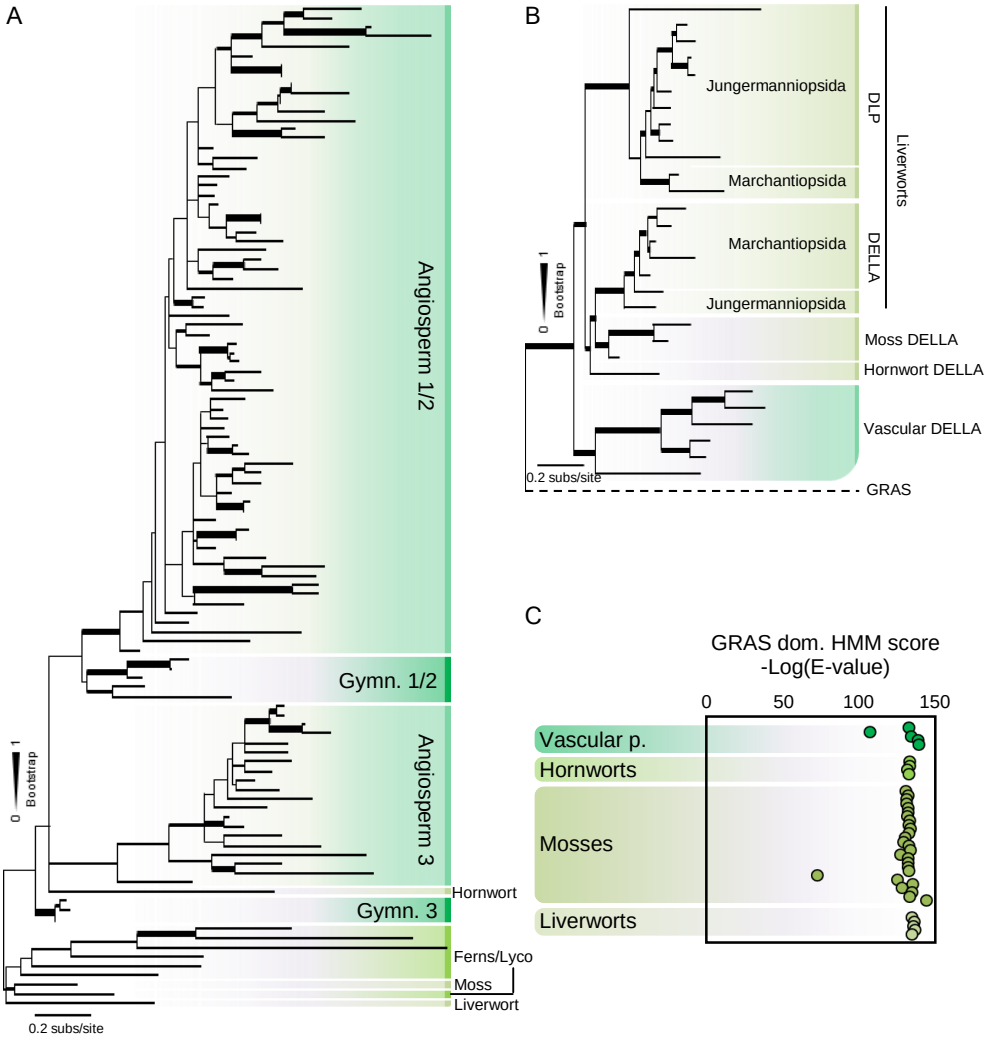
Presence of SLY1/GID2 orthologs in land plants. (*A*) Phylogenetic analysis of SLY1/GID2 and GID2-like proteins in land plant representatives. Liverwort sequences from either Jungermanniopsida and Marchantiopsida classes are pointed. Support values associated with branches and displayed as bar thickness are maximum likelihood bootstrap values from 1000 replicates. (*B*) BLASTP value obtained for SmGID2a or SmGID2b as result using SLY1/GID2 and GID2-like proteins as queries. Value shown as the -LOG of the E-value retrieved in the BLASTP search.

SUPPLEMENTAL FIGURE 2 – part 2



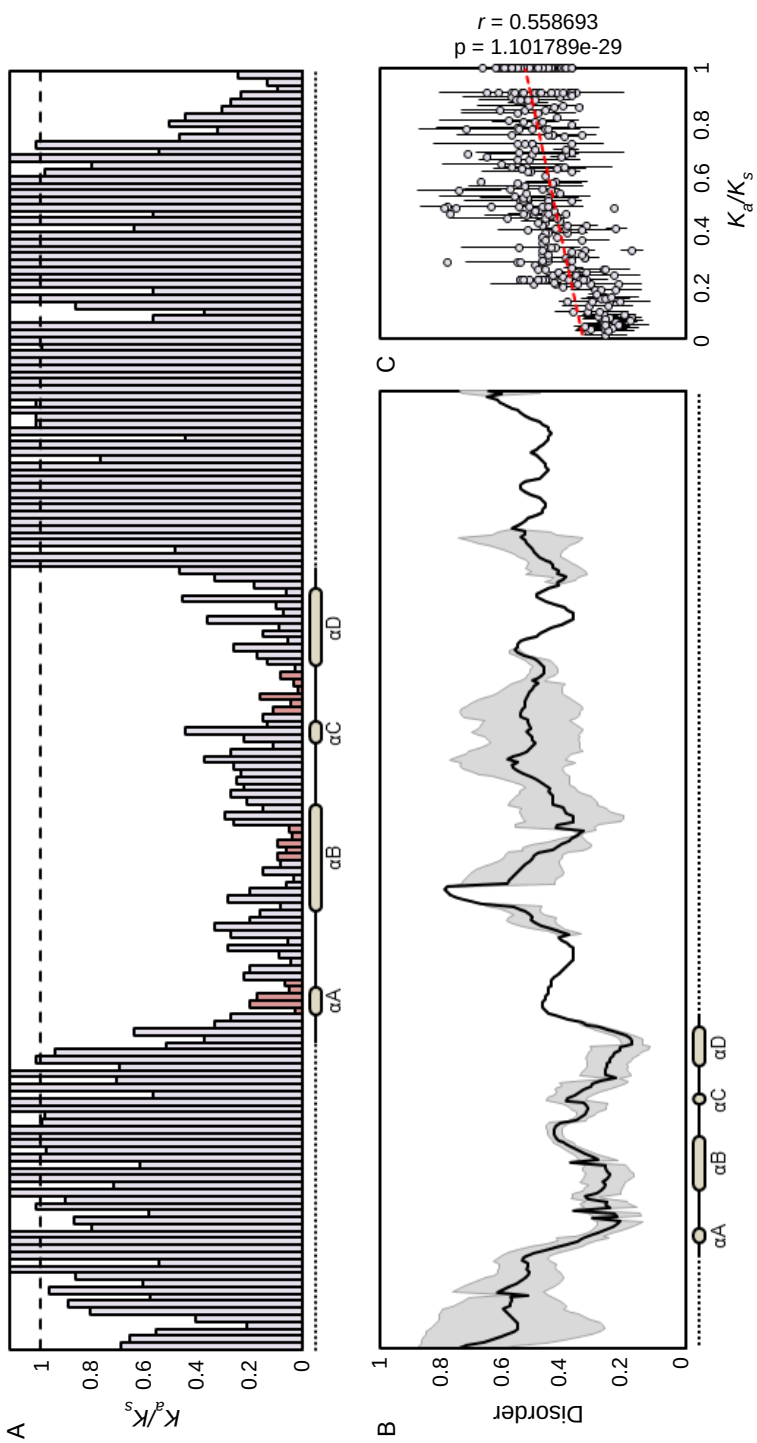
(*C*) Alignment showing highly conserved regions within the F-box domain and the LGG domain of selected SLY1/GID2 and GID2L proteins from A and B. Black colored boxes represent conserved sites in all SLY1/GID2 proteins.

SUPPLEMENTAL FIGURE 3



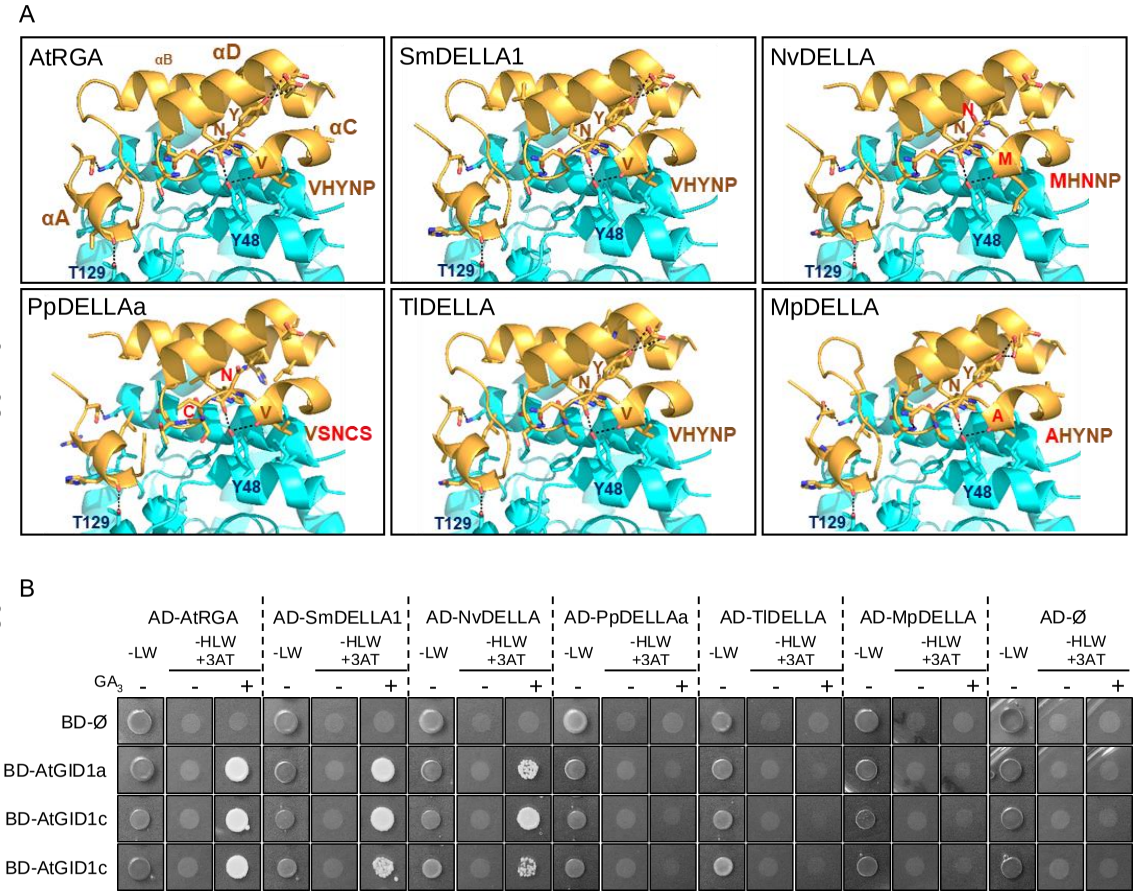
Non-vascular land plants have conserved DELLA proteins. (*A*) Phylogenetic analysis of DELLA proteins using DELLA domain. (*B*) Phylogenetic analysis of liverwort DELLA and DELLA-like proteins using GRAS domains. Support values associated with branches and displayed as bar thickness are maximum likelihood bootstrap values from 1000 replicates. (*C*) Automated analysis of GRAS domain presence in non-vascular land plants using Pfam website. Scores are represented as the -Log of the E-value retrieved from the search and represented following the phylogenetic position obtained in Fig. 3B.

SUPPLEMENTAL FIGURE 4

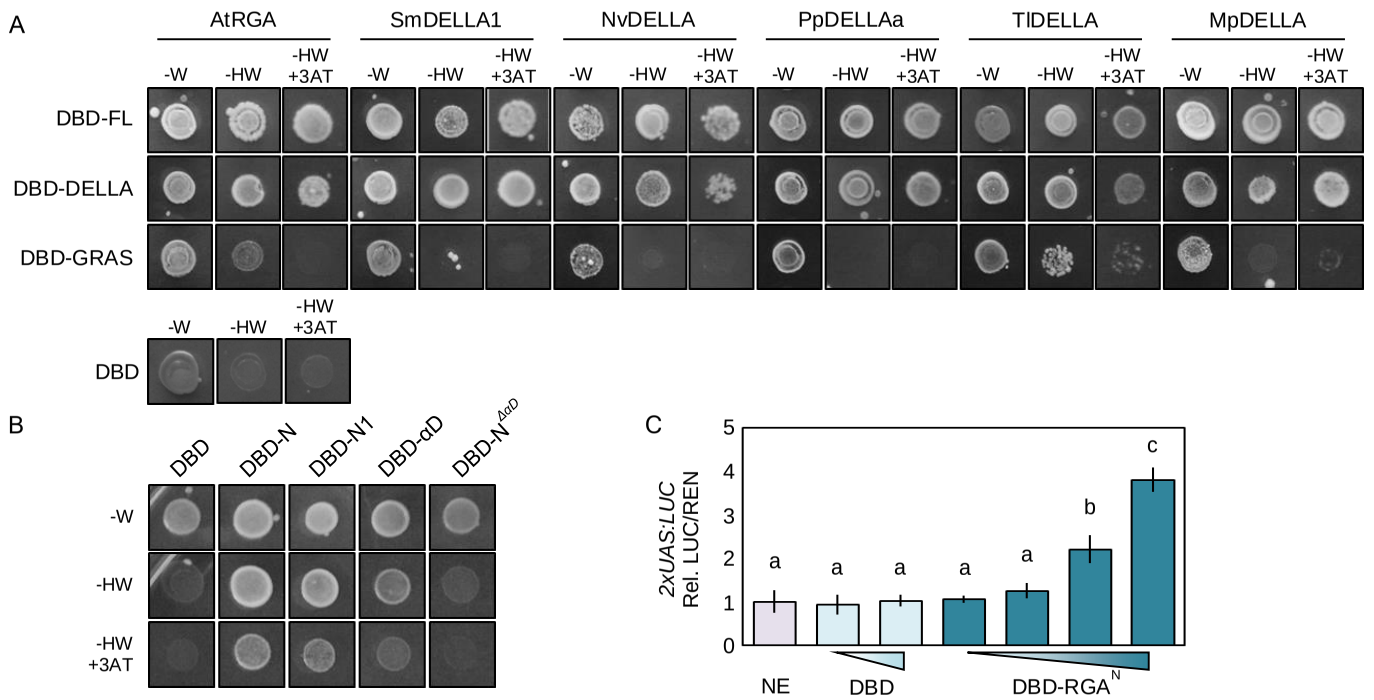


DELLA domain is structurally conserved. (*A*) Selection analysis of the DELLA N-terminal región, based on codon-aligned nucleotide sequences. Non-conserved stretches generating gaps were removed. Red bars indicate residues of DELLA, LEQLE and TVHYNP motifs in the GA-conserved degron. Dashed line indicates the theoretical threshold for positive selection (above line) or purifying selection (below). (*B*) Analysis of disorder in several DELLA domains. Disorder values are plotted in a previously made alignment. Grey forms represent standard deviation from the average (black line). Dashed line indicates threshold value for ordered (<0.5) or disordered (>0.5) residues. (*C*) Relation between disorder and selection values per residue excluding gaps. p-value indicates Pearson's product moment correlation (*r*) statistical analysis. In general, less ordered stretches were subject to stronger positive selection.

SUPPLEMENTAL FIGURE 5

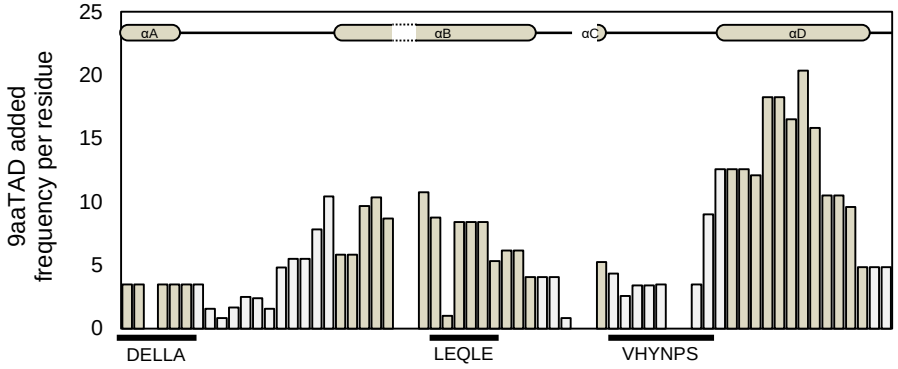


Some non-vascular DELLA proteins can interact with Arabidopsis GID1 receptors in a GA dependent manner. (*A*) Predicted structural model for DELLA-AtGID1a interaction using AtRGA, SmDELLA1, NvDELLA, PpDELLAa, TIDELLA, and MpDELLA. DELLA structure is shown in yellow and AtGID1a structure in light blue. AtGID1a residues involved in DELLA interaction are written in dark blue. Residues different to that of AtRGA/GAI in main motifs are presented in red. Possible residue to residue interactions affected are pointed with a red circle. 90° rotation compared to Fig. 5A. (*B*) Yeast-two-hybrid assay between DELLA proteins and the three Arabidopsis GID1 receptors with or without 100 µM GA_3 (L, Leucine; W, Tryptophan; H, Histidine; 3-AT,

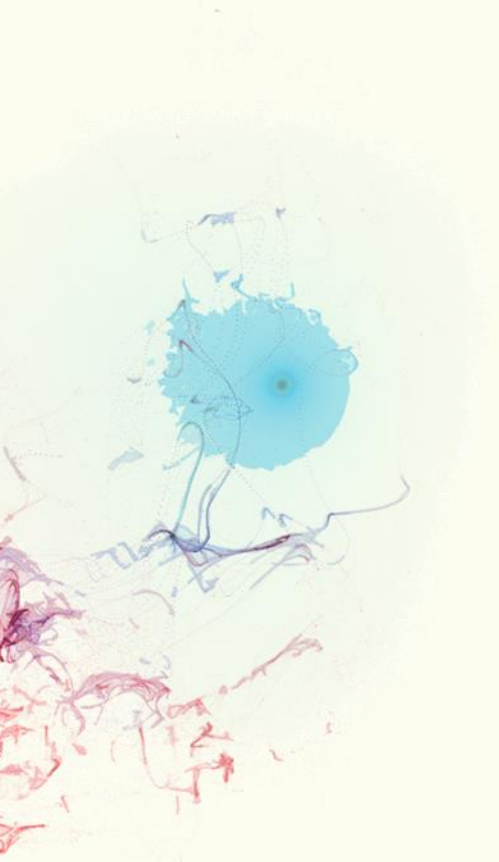


The conserved DELLA domain acts as a transcriptional activator domain. (*A*) Yeast transactivation assay using DELLA protein full-length coding regions (DBD-FL), or truncated versions using either the GRAS domain (DBD-GRAS) or the DELLA domain (DBD-DELLA). (*B*) Yeast transactivation assay using different truncated versions of PpDELLAa DELLA domain as indicated in Figure 6C. (W, Tryptophan; H, Histidine; 3-AT, 5 mM 3-aminotriazole). BD, GAL4 binding domain; AD, GAL4 activation domain. (*C*) Dual luciferase transactivation assays in *Nicotiana benthamiana* leaves using the *LUC* gene under the control of the *Gal* operon UAS promoter as the reporter, and different effector vectors fused to the GAL4 DNA binding domain. NE, no effector; DBD, GAL4 binding domain; DBD-RGA^{N}, RGA DELLA domain fused to GAL4 DNA binding domain. Constitutively expressed *Renilla* luciferase (*REN*) for normalization. Data shown are normalized to NE value and represent the average of three biological replicates. Error bars represent standard deviation. Letters indicate significant differences between groups ($P < 0.01$, one-way ANOVA, Tukey's HSD post hoc test).

SUPPLEMENTAL FIGURE 7



DELLA domain α helix D harbours a transactivation domain needed for recruitment of PolII co-activators. Transactivation prediction plotted as the cumulative probabilities of 9aaTAD presence per residue found for AtRGA, SmDELLA1, NvDELLA, PpDELLAa, TlDELLA, and MpDELLA.



Chapter 2

DELLA Proteins Recruit the Mediator Complex Subunit MED15 to Co-activate Gene Expression in Land Plants

Jorge Hernández-García, María Lozano-Quiles, Javier Forment, Miguel A. Blázquez

This chapter is far from being published. We like it anyway.

Chapter 2

DELLA Proteins Recruit the Mediator Complex Subunit MED15 to Co-activate Gene Expression in Land Plants

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Abstract

Fine tuning of adaptive responses is achieved by coordinating exogenous signals with endogenous cues. Plants harbour several systems allowing such coordination, being hormonal signalling pathways some of the most studied. DELLAs are plant-specific regulatory proteins known to act as the main effectors of the gibberellin response pathway in angiosperms, and as important hubs connecting many transcriptional programs. These proteins interact with hundreds of transcription factors and regulators, modulating their activities in multiple ways, either negatively or positively. While transcription factor sequestration by DELLA has been extensively studied as a mechanism to prevent DNA binding to the downstream targets, the mechanism by which DELLAs act as co-activators has not been explored yet. Here we have found that DELLAs are able to physically interact with the Mediator tail subunit MED15 through its conserved KIX domain, similarly to other eukaryotic transcriptional regulators. This interaction directly induces transcriptional activity, and is physiologically relevant in different gibberellin-regulated processes where transcription factors recruit DELLAs as co-activators. These include cytokinin regulation of meristem function, GAF1/IDD2-mediated gibberellin metabolism feedback, or MYB12-induced flavonol production. We suggest that DELLA-dependent recruitment of Mediator complexes to specific loci is a mechanism for DELLA-induced transcription. This mechanism could be ancestral and widespread throughout land plants, since *Marchantia polymorpha* DELLA promotes MpMYB14-dependent gene activation by recruiting MpMED15.

Key words: DELLA proteins, transactivation, Mediator, hormone signalling.

INTRODUCTION

The plant genetic toolbox responsible for the integration of environmental signals includes signalling pathways that regulate gene expression in response to signals such as light, temperature, or hormones (Casal et al. 2004). The coordination between two or more of these pathways is eventually achieved by the regulation of specific sets of genes. Examples of these convergence points between signalling cascades include the coordinated regulation of hypocotyl growth genes by light and different hormones such as auxins, brassinosteroids and gibberellins (Bai et al. 2012; Oh et al. 2014). Although transcriptional control can be exerted at different stages, a limiting step subjected to environmental regulation is transcriptional initiation, mainly characterized by the formation and activation of the RNA polymerase II (RNAP II) initiation complex (PIC). While the PIC is primed and recruited to target loci by specific transcription factors (TFs) (Näär et al. 2001), this TF-PIC bridging commonly involves the action of the Mediator complex (Soutourina 2018). The Mediator is an eukaryotic multi-modular complex that facilitates the recruitment of general TFs and the RNAP II to form the PIC (Kim et al. 1994; Lee et al. 1999). Two of the modules, the head and the middle are directly involved in RNAP II regulation, while the tail module interacts with gene-specific TFs and other regulatory proteins in order to both recruit and enhance Mediator activity. Another non-core module, CDK8, is involved in posttranslational regulation of Mediator function by direct phosphorylation of TFs, Mediator tail subunits, and RNAP II (Allen and Taatjes 2015).

Plant Mediator complexes regulate several processes in response to multiple signals (Samanta and Thakur 2015; Yang et al. 2016; Malik et al. 2017). Mediator recruitment by specific TFs has been widely documented and is widespread in many eukaryotes including plants (Allen and Taatjes 2015; Malik et al. 2017). For example, MED25-MYC2 interaction in Arabidopsis is involved in jasmonate-mediated gene activation (Çevik et al. 2012; Zhai and Li 2019). The tail subunit MED15 has caught special attention given the presence of a conserved Kinase-Inducible Domain (KID)-interacting (KIX) domain (Kim et al. 2016; Cooper and Fassler 2019). KIX domains frequently interact with transcriptional activation domains (TADs) of specific TFs such as p53, c-Myb, VP64 or Gal4 TADs (Piskacek et al. 2007; Thakur et al. 2014). Some plant TFs have been shown to interact with MED15 KIX domain in Arabidopsis but, apart from the regulation by WRINKLED1 of fatty acid biosynthesis genes (Kim et al. 2016), the relevance of most of these interactions has not been studied.

DELLAs are a plant-specific family of proteins that act as transcriptional regulators in the gibberellin (GA) signalling pathway in vascular plants. They are recognized by the gibberellin receptors GID1 after GA binding. This enables the interaction of a DELLA-GID1-GA complex with the F-box SLY/GID2, for subsequent degradation of DELLA proteins. DELLAs are part of the GRAS family of transcriptional regulators, and share with these the GRAS C-terminal

domain. This domain permits DELLA proteins to interact with hundreds of TFs and regulators, allowing the concerted regulation of multiple signalling pathway (Marín-de la Rosa et al. 2015; Lantzouni et al. 2020). They can act by direct blockage of TF DNA-binding domains (Feng et al. 2008; de Lucas et al. 2008), and also act within chromatin contexts, presumably acting as transcriptional co-activators of a set of TFs (Yoshida et al. 2014; Marín-de la Rosa et al. 2015; Tan et al. 2019). DELLA N-terminal domains - or DELLA domains - are involved in the interaction with GID1s, but are also able to directly induce gene transcription *in vivo* (Hirano et al. 2012). In fact, this activity is conserved in DELLA proteins across extant land plant lineages, suggesting an important functional role previously overlooked (see **Chapter 1**, Hernández-García et al., 2019). Here, we have studied the link between DELLA co-activator function and transactivation activity following our previous finding of a predicted TAD similar to those known to interact with KIX domains. We show that DELLA ability to co-activate gene expression by DELLA proteins involves the recruitment of Mediator complex through its MED15 subunit using the deeply conserved N-terminal TAD, and that this mechanism may represent an ancestral molecular function of DELLA, conserved in all land plants.

RESULTS

DELLA proteins recruit MED15 to promote gene expression

A nine amino acid (9aa) TAD has been predicted with high confidence score in the conserved α-helix D of N-terminal domains of all land plant DELLA proteins, evoking the well-studied presence of functional 9aaTADs in other α-helixes of TFs as mammalian p53 (**Fig. 1A**, **Fig. S1**, **Chapter 1**). These structurally conserved TADs frequently interact with KIX domains, prompting us to wonder if the mechanism of transcriptional activation by DELLA comprises the recruitment of KIX-containing proteins. Diverse KIX-containing proteins act as transcriptional activators, such as the Mediator tail subunit MED15 or the p300/CBP family of histone acetylases (Thakur et al. 2014). DELLA domains have been shown to induce transcription in plant and yeast cells (Hirano et al. 2012). Given the lack of KIX domains in the only yeast p300/CBP protein (Wang et al. 2008) and the high similarity between yeast and plant MED15 KIX domains (Dahiya et al. 2016), we tested if DELLA proteins in Arabidopsis are able to interact with MED15 proteins. We found that MED15a, the main MED15 protein in Arabidopsis, and the DELLA protein RGA are able to interact in yeast two-hybrid assays (**Fig. 1B**). This interaction involves the RGA N-terminal region (RGA^{N}) containing the predicted 9aaTAD (**Fig. 1B**) and the MED15a KIX domain ($MED15a^{KIX}$) (**Fig. S2**), whereas RGA C-terminal domain (RGA^{GRAS}) or MED15a C-terminal domain were unable to interact with any domain of their counterparts (**Fig. S2**). To further confirm this interaction, we demonstrated

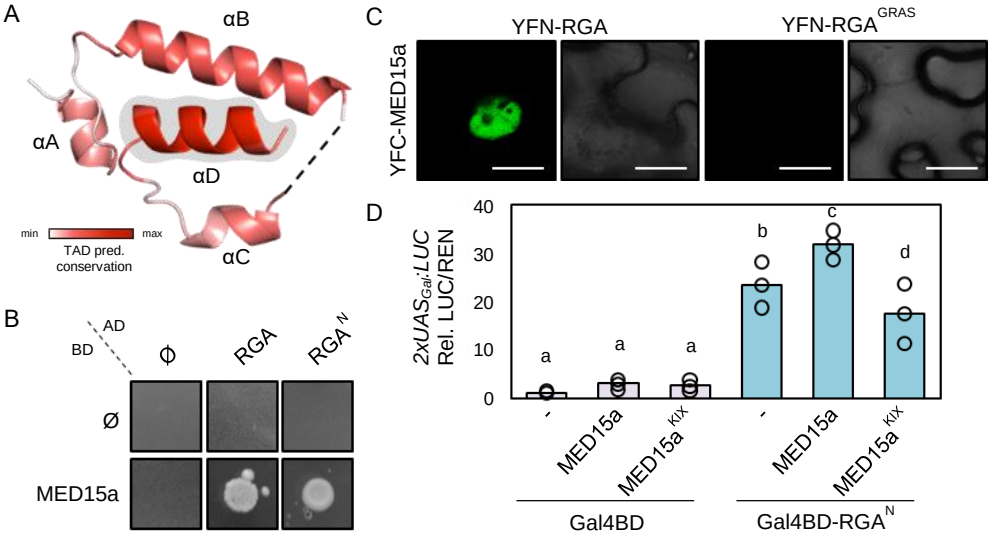


Figure 1. RGA and MED15 interaction enhances gene activation. A) Known DELLA domain structure coloured by 9aaTAD prediction in 6 land plant DELLAs from different lineages. B) Yeast two-hybrid assay using MED15a as bait, RGA and RGA DELLA domain as prey (see **Fig. S1**). C) Bimolecular fluorescence complementation assay of MED15a and RGA, using a GRAS-only version as negative control. Scale bar, 10 μm. D) Dual luciferase transactivation assay in *Nicotiana benthamiana* leaves using the *LUC* gene under the control of the *Gal* operon UAS promoter as the reporter, and either a GAL4 DNA binding domain (DBD) alone or fused to the RGA DELLA domain. MED15a full length (FL) and/or KIX domains are co-expressed with effectors.

that these proteins interact *in vivo* in BiFC assays carried out in *N. benthamiana* (**Fig. 1C**). RGA also interacted with other Arabidopsis KIX-containing MED15 subunits as MED15d and the MED15f^{KIX} domain, and other Arabidopsis DELLAs, such as GAI and RGL2, were equally able to interact with MED15 KIX domains (**Fig. S3**). These analyses confirm that the predicted DELLA 9aaTADs can physically interact with MED15-type KIX domains.

To assess if this interaction mediates transcriptional activation by DELLAs, we used a previously established Gal4-based dual luciferase system (Gendron et al. 2012). As previously demonstrated in **Chapter 1**, a Gal4 binding domain fusion to RGA^{N} was sufficient to induce luciferase activity in plants. The addition of MED15a further increased the ability of DELLA to activate transcription, while adding the MED15a^{KIX} domain alone reduced the ability of DELLA to promote luciferase activity, probably due to competition with plant endogenous MED15 (**Fig. 1D**). Altogether, these data suggest that DELLA could recruit Mediator complexes through the interaction with MED15 to induce transcriptional activation *in vivo* and support a direct role of DELLA proteins as transcriptional co-activators.

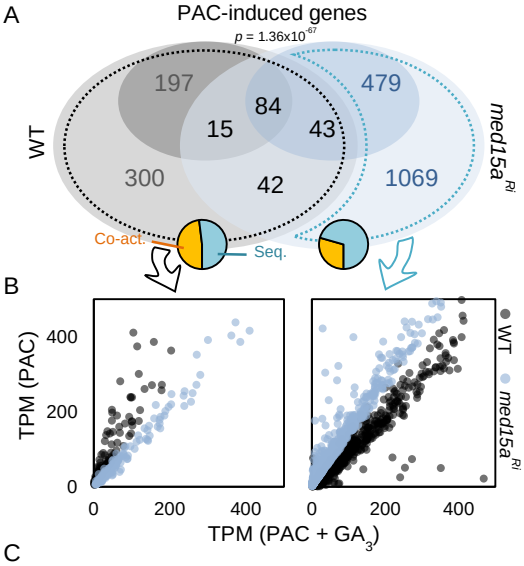
MED15 is necessary for the activation of a subset of DELLA-dependent transcriptional responses

To check if GA signalling interferes with MED15 function, we analysed the expression of *MED15* genes, and found no deregulation in the transcriptomes of the pentuple At*della* mutant or in PAC-treated wild-type plants (**Fig. S4, Table S1**). Likewise, we discarded intrinsic transcriptomic defects in GA signalling genes due to lack of proper MED15 function among *med15a*^{Ri} deregulated genes (**Table S2**).

Next, to evaluate the involvement of MED15 in DELLA-regulated transcription, we analysed the DELLA-dependent transcriptome in a previously reported RNAi line targeting *MED15a* (termed here *med15a*^{Ri}, Kim et al., 2016). For this, we compared the transcriptomic profile of wild-type and *med15a*^{Ri} seedlings with high vs low DELLA levels (i.e., treated with the GA synthesis inhibitor paclobutrazol (PAC) or with PAC + GA_3, respectively). To identify possible direct targets of DELLA-MED15 activity, we searched for genes differentially induced in the wild type under high DELLA levels, which were not induced (or induced at significant lower levels) in the *med15a*^{Ri} line. For this, we first selected genes with at least 1 TPM in both genotypes, and that were differentially expressed in PAC vs PAC+GA_3 (p-adj<0.01).

We found a total of 681 genes induced by DELLA accumulation in wild-type plants and 1737 in the *med15a*^{Ri} line (**Table S3**). Up to 44% of the genes (296) show a fold change of two or more in the wild-type, whereas only 34% of the genes are above this threshold in *med15a*^{Ri} plants (606), which could be attributed to a reduced ability of DELLAs to promote transcriptional activation in the presence of a defective Mediator complex (**Fig. 2A, B**). Up to 1548 (89%) of the genes up-regulated in the *med15a*^{Ri} line were not induced in the wild type, suggesting that impaired Mediator activity increases the sensitivity towards DELLA accumulation, for which we do not have a mechanistic explanation. More importantly, we detected 497 genes which were significantly up-regulated by DELLAs only in the presence of MED15 (**Fig. 2A, B**). Moreover, a large portion of the 184 genes upregulated by DELLAs in both genotypes also displayed statistically significant defects in the extent of induction under impaired MED15 activity (**Fig. S5A-D**). In fact, this pattern was absent in the subset of genes up-regulated only in *med15a*^{Ri} (**Fig. 2B**). This prompted us to treat the list of up-regulated genes in wild-type as the putative DELLA-MED15 target candidates' list.

DELLAs are unable to directly bind DNA, instead they are brought to chromatin by the interaction with different DNA-binding TFs of different families (Vera-Sirera et al. 2016). Therefore, we looked for TFs involved in the transcriptional regulation of the putative DELLA-MED15 targets using the EAT-UpTFv0.1 tool to find enriched TF binding sites in their upstream regulatory sequences (Shim and Seo 2020). We found that the binding sites of 52 TFs were significantly enriched in the regulatory sequences of the putative DELLA-MED15



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Families	#	TF-BS in DELLA ChIPs
bZIP	20	Yes
ERF	13	No
HD-ZIP	8	Yes
IDD (C2H2)	5	Yes
NAC	4	No
BEH	4	No
MYB	3	Yes
bHLH	1	Yes

Figure 2. MED15 is involved in DELLA-mediated transcriptional activation. A) Venn's diagram showing the overlap of curated genes (showing at least 1 TPM in WT and *med15a*^{Ri}, padj <0.01) up-regulated in PAC-treated plants (compared to PAC+GA_3-treated plants). p-value shown indicates the statistical significance of the overlap. Orange-blue circles represent predicted DELLA mechanistic effect on EAT-Up-derived TFs found to be enriched in each subset. Orange, co-activation; blue, sequestering. B) TPM comparison of the genes up-regulated in WT (grey dot line in A), or exclusively in *med15a*^{Ri} (blue dot line in A). Zoomed out axes are shown in **Fig. S4**. C) TF numbers per family and the presence of TF binding sites in DELLA ChIP-seq experiments (Marín-de la Rosa et al., 2015). Colours represent the predicted DELLA mechanistic effect on that TF family. Expression analyses carried out by whole RNA sequencing of 7-days-old seedlings treated for 3 days either with 1 µM PAC, or 1 µM PAC + 100 µM GA_3.

targets, all belonging to 8 TF families which are known to interact with DELLAs (**Table S4**). Moreover, there is experimental evidence that DELLAs act as co-activators of key representative TFs of 4 of these families, such as the statistically significant enrichment of these *cis* elements also among the DELLA-binding regions identified by ChIP-seq (Marín-de la Rosa et al. 2015; Serrano-Mislata et al. 2017). In contrast, a similar analysis with the set of genes upregulated in the *med15a*^{Ri} line yielded an enrichment for only 17 TFs. These belong to 7 different TF families, of which 2 have never been associated with DELLA activity. Besides, only less than a third of these TFs are predicted to act in concert with DELLAs to activate gene transcription, and most of them are regulated by DELLAs through sequestration (**Fig. 2A**).

Among the GO terms enriched within the putative DELLA-MED15 targets, we found "response to GA stimulus" (GO:0009739), together with other GA-mediated processes as "response to oxidative stress" (GO:0006979), and "secondary metabolism" (GO:0019748) (**Fig. S6**, **Table S5**). DELLA co-activation partners as IDDs are known to be involved in GA response as part of a negative feedback loop, while secondary metabolism such as flavonol biosynthesis is regulated by MYB12-dependent co-activation. Conversely, the genes up-regulated only in the *med15a*^{Ri} line have a varied list of terms in wich some are associated

with GA responses (but not the term itself), as "growth" (GO:0040007), or "response to cold" (GO:0009409), but are not linked to co-activation in any way. Overall, it is reasonable to propose that MED15 could be required for the regulation of a subset of previously known GA transcriptional responses.

MED15 is required for DELLA regulation of biological processes as a co-activator

Our transcriptomic analysis indicates that MED15 is required for the up-regulation of a large portion of DELLA target genes, and also points to some of the DELLA-interacting TFs that might trigger transcription at those loci to regulate certain GA-related processes. We decided to take advantage of this information to validate this transcriptional regulation at the functional level. The predicted co-activators that could regulate the putative DELLA-MED15 targets include three of the four well-demonstrated TF families that require DELLA as co-activators: the INDETERMINATE DOMAIN (IDD) proteins of the C2H2 family (Fukazawa et al. 2014; Yoshida and Ueguchi-Tanaka 2014), MYB proteins (Y. Zhang et al. 2017; Tan et al. 2019), and bZIP TFs (Lim et al. 2013). Intriguingly, we did not find enrichment of type-B ARR TFs using EAT-UpTF or other TF enrichment analysis tools as that in PlantRegMap (Tian et al. 2019), representing a likely technical issue with enrichment analyses in type-B ARR BS. Nevertheless, we found 63 robust BS for ARR10 among the promoters of the 681 DELLA-MED15 targets ($p < 0.0001$), comparable to the 72 BS we could recover using the same approach on the promoters of 804 documented ARR10 targets (**Table S6**, Zubo et al., 2017). Moreover, these DELLA-MED15 targets significantly overlapped with the ARR10 targets, contrarily to the genes induced by DELLAs exclusively in the *med15a*^{Ri} line (**Fig. S7**). Therefore, we tested the involvement of MED15 in biological processes known to be regulated by DELLA co-activation.

DELLAs have been shown to regulate developmental programs in apical meristems acting as co-activators of type-B ARRs (Marín-de la Rosa et al. 2015). For instance, accumulation of DELLA proteins induces cotyledon opening during skotomorphogenic development (Alabadí et al. 2004), but lack of ARR1, ARR10 and ARR12 proteins reverts this effect (Marín-de la Rosa et al. 2015). In agreement with a requirement for MED15 in DELLA co-activation, the *med15a*^{Ri} displayed a defective response to PAC-induced cotyledon opening (**Fig. 3A-B**). Similarly, type-B ARR-DELLA interaction restricts root apical meristem (RAM) size, but the *med15a*^{Ri} line was impaired in DELLA-mediated reduction of RAM size (**Fig. S8A**), phenocopying *arr1;12* mutants (Moubayidin et al. 2010). These results indicate that the participation of MED15 is relevant for the regulation of developmental processes mediated by DELLA's enhancement of ARR transcriptional activity. One of the key processes regulated by

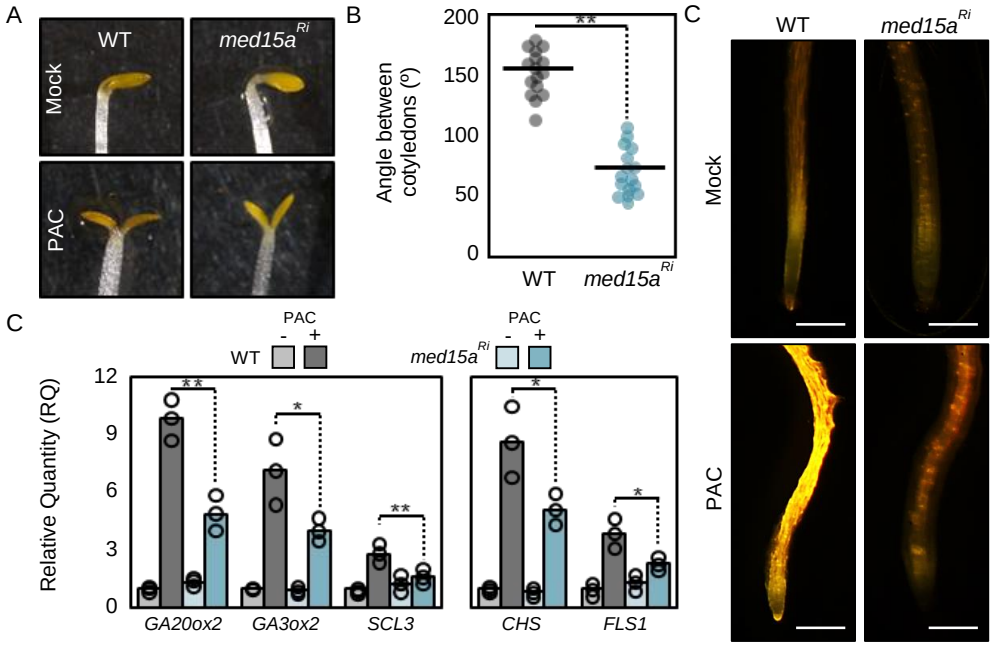

Figure 3. MED15 is needed for DELLA-dependent co-activation responses. A) 5 days-old WT and *med15a*^{Ri} seedlings grown in darkness with or without 1 µM PAC. Scale bar, 1 mm. B) Cotyledon opening in 5 days-old WT and *med15a*^{Ri} seedlings grown in darkness with or without 1 µM PAC. Measurements in mock plants not shown (mean = 0; SD = 0). C) right, RT-qPCR analysis of GA and IDD-responsive genes in 5 days-old WT and *med15a*^{Ri} seedlings grown in darkness with or without 1 µM PAC, and left, GA and MYB12-responsive genes in 7 days-old WT and *med15a*^{Ri} seedlings grown in continuous light with or without 1 µM PAC. D) DPBA staining of 8 days-old WT and *med15a*^{Ri} seedlings root tips grown in long day conditions (16L:8D), and treated for four days with or without 1 µM PAC. Scale bar, 100 µm. B shows experimental data of one representative experiment of three with at least 15 plants per genotype and treatment. C data are medians (bar) of 3 biological replicates, referred against mock. Biological replicate means are depicted as empty circles. *PDF2.1* was used to normalize data.

DELLA-IDD interaction is the negative feedback of regulation of GA biosynthesis involving up-regulation of GA20- and GA3-oxidases in response a reduction in GA levels (Fukazawa et al. 2014). As expected, PAC treatment promoted the expression of *GA20ox2* and *GA3ox1* genes, but it was significantly reduced in the *med15a*^{Ri} line (**Fig. 3C**), reproducing *gaf1/idd2* mutants effect in breaking the feedback regulation (Fukazawa et al. 2014). *SCL3*, a direct target of IDD1 and other IDD proteins together with DELLAs (Yoshida et al., 2014), displayed a milder but similar trend. Finally, flavonol production has been reported to respond to DELLAs through their interaction with MYB12, acting as co-activators (Tan et al. 2019) . As suggested by our previous analysis, PAC-dependent transcriptional activation of the *FLS1* and *F3H* flavonoid biosynthesis genes, and flavonoid accumulation in roots were impaired in *med15a*^{Ri} (**Fig. 3C-D)**.

These experiments show that TFs belonging to different families, all relying on DELLA co-activation to exert some of their physiological roles require MED15 to achieve full regulatory capacity. This idea predicts that the participation of MED15 in processes regulated by DELLA-TF interaction through a sequestration mechanism should be minor. The bHLH PIF TFs promote hypocotyl elongation during skotomorphogenesis, and DELLAs counteract this effect by impeding PIF binding to their target DNA (Alabadí et al. 2007; Feng et al. 2008; de Lucas et al. 2008). As predicted, the reduction of hypocotyl elongation in the presence of PAC was equally effective in wild-type and *med15a*^{Ri} seedlings (**Fig. S8B**). In agreement, we found no change in the expression levels on the PIF-repressed genes *PIL1* and *XTR7,* or in the induced gene *PRE5,* one of the final effectors of hypocotyl elongation (**Fig. S9**).

To promote gene transcription, the Mediator complex facilitates the recruitment of RNAP II to specific genes by forming and activating the PIC at their transcriptional start sites (TSS, Allen and Taatjes, 2015). We reasoned that DELLA accumulation should have a positive effect in RNAP II binding to these spots and examined previously reported RNAP II ChIP-seq data of plants treated with GA_3 or in mock conditions. In such studies, a generalized loss of RNAPII occupancy upon GA treatments was reported, including TSS, transcriptional end sites (TES), and to a lower extent, gene bodies, agreeing with a role of DELLAs in regulating the recruitment of the transcriptional elongation complex Paf1c (Blanco-Touriñán, 2020). Contrary to Mediator, Arabidopsis Paf1c complex is involved in transcriptional elongation rather than initiation (Antosz et al. 2017), suggesting that total RNAP II patterns of occupancy with GA perturbations would be difficult to interpret if both transcriptional steps are partially controlled by DELLA proteins (i.e.: by Mediator and Paf1c regulation). Surprisingly, looking at genes specifically regulated by DELLA co-activation (as *SCL3* and *GA20ox2*), we did not perceive a clear decrease in the overall RNAP II occupancy under reduced DELLA protein levels (**Fig. S10**). Instead, we observed a clear reduction in peaks neighbouring the TSS. Consistently, the PIF-regulated *XTR7* gene showed a clear overall decrease in RNAP II occupancy throughout the gene body and TES in the absence of DELLAs, but not in the peaks at the TSS. These observations support a mechanism involving DELLA-mediated recruitment of Mediator to specific loci thus promoting TF-DELLA co-activation of target genes.

DELLA recruitment of MED15 is a conserved mechanism in land plants

The high level of conservation of the 9aaTAD in the N-terminal domain of DELLA proteins suggests that the involvement of Mediator in DELLA transactivation capacity observed in Arabidopsis is common to all embryophytes (**Chapter 1**). To functionally test this idea in evolutionary distant plants, we decided to analyse DELLA function as a co-activator in the liverwort *Marchantia polymorpha*. *MED15* orthologs in *M. polymorpha* have not been identified, so we performed a preliminary search using BLASTP with known MED15 proteins

using an in-house made database composed of several plant proteomes (See Materials & Methods). MED15 identity was confirmed by the presence of MED15-type KIX domains (Pfam family KIX_2, PF16987). After protein sequence alignment, we constructed a maximum likelihood phylogenetic tree including several species spanning land plant phylogeny using exclusively the KIX domains due to the high divergence in the rest of the protein. We confirmed the presence of *bona fide* orthologs in all the species clustering together with Arabidopsis MED15a (**Fig. 4A, Fig. S11A**). The amino acid composition of MED15^{KIX} domains is well conserved in all plant species, including two of the hits we found in *M. polymorpha*, Mp8g02180 and Mp8g01860 (**Fig. S11B**). Though we retrieved other candidates, only these two proteins grouped with the rest of MED15 proteins and, as expected, forming a clade with other bryophytes. A deeper analysis showed that Mp8g01860 is a small protein encompassing only a KIX domain, and shared a 90% identity with the Mp8g02180 KIX domain, and also up to 90% of their DNA sequences. Other expressed gene, *Mp8g01900*, also shared a strikingly high degree of sequence with a different region of *Mp8g02180* (**Fig. S12A**). This suggests that *Mp8g01860* and *Mp8g01900* are derived from a partial duplication of *Mp8g02180* and neither of them encode a full-length MED15 protein. Interestingly, both genes are expressed and properly spliced (**Fig. S12B**). We concluded that Mp8g02180 is the only gene encoding a full-length MED15 protein in *M. polymorpha*, named hereafter as Mp*MED15*.

Subsequently, we examined if MpMED15 interacts with the single DELLA protein in *M. polymorpha* species, MpDELLA. Contrarily to AtMED15a, MpMED15 alone was able to induce *HIS3* transcription in yeast (**Fig. S13A**), suggesting that MpMED15 can stimulate basal transcription on its own in yeasts. Since the MpMED15^{KIX} domain alone did not cause transactivation, it allowed us to confirm the positive interaction between MpDELLA and MpMED15^{KIX}, (**Fig. 4B**, **Fig. S13B**). For this interaction to occur the DELLA N-end domain (MpDELLA^{N}) was sufficient, as in the case of Arabidopsis DELLAs. However, unlike with Arabidopsis DELLAs, the MpDELLA GRAS domain alone was also able to interact with MpMED15^{KIX}. It is unclear if this interaction is relevant, since the GRAS domain does not trigger gene activation in yeast on its own (**Chapter 1**). We also examined this interaction by BiFC (**Fig. S13C**), confirming it occurs in plant cell nuclei. This suggests that a deeply conserved interaction between MpMED15 and MpDELLA exists, and the transactivation capacity of the MpDELLA N-terminal domain is also conserved, as confirmed by Gal4-based dual luciferase assay (**Fig. 4C**, **Fig. S14A**). In this assay, the addition of MpMED15 consistently enhanced the ability of MpDELLA^{N} to induce LUC activity, similarly to the results obtained in Arabidopsis and supporting a conserved mechanism of MED15 recruitment by DELLAs to induce gene transcription in land plants.

To confirm that this MpDELLA-MpMED15 interaction is relevant in the context of a *M. polymorpha* promoter, we decided to identify a potential target gene assuming its conservation

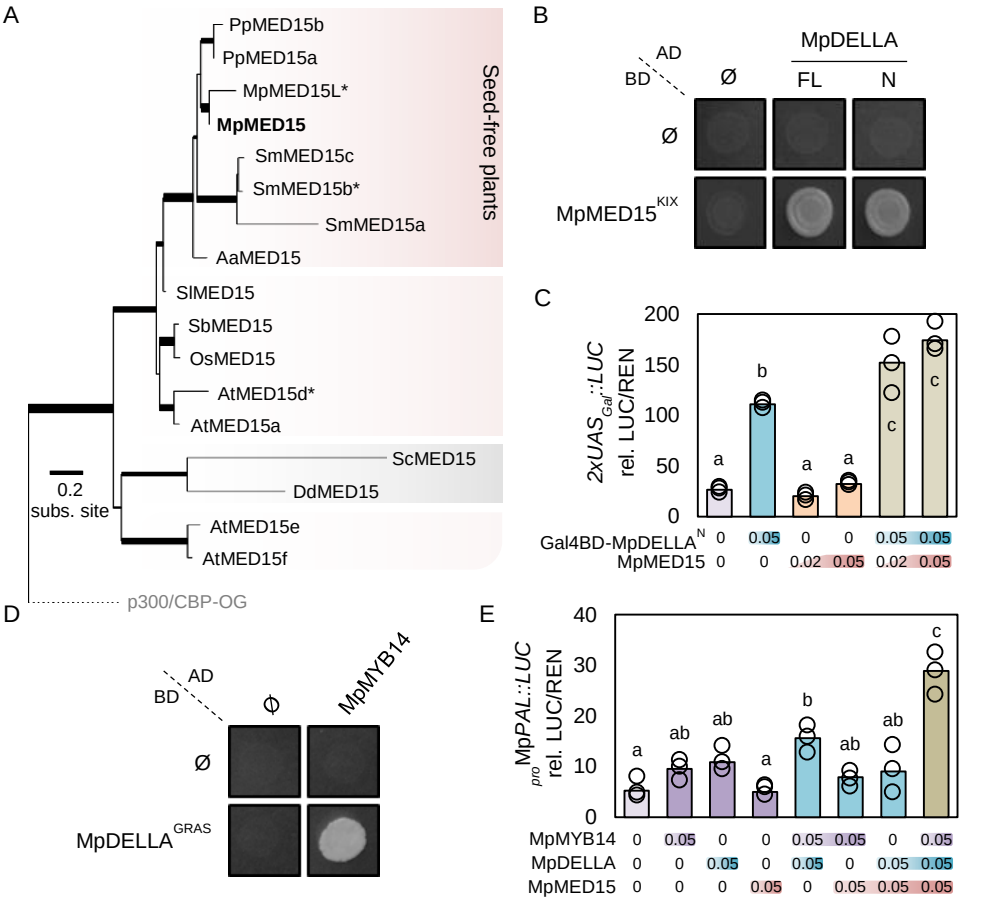

Figure 4. *Marchantia polymorpha* MpDELLA acts as a co-activator by recruiting MED15. A) Phylogenetic analysis of MED15 proteins using KIX domains. Outgroup (OG) is composed of non-MED15 KIX domains. Grey shaded proteins are non-plant eukaryotes (Dd, *Dictyostelium discoideum*; Sc, *Saccharomyces cerevisiae*). Support values associated with branches and displayed as bar thickness are maximum likelihood bootstrap values from 1,000 replicates. Branch length represent distance in substitutions per site. Asterisks denote proteins without one or more MED15 canonical domains. B) Yeast two-hybrid assay using MpMED15 KIX domain as bait, and MpDELLA full length (FL) or N-terminal domain (N) as preys. C) Dual luciferase transactivation assay in *Nicotiana benthamiana* leaves using the *LUC* gene under the control of the *Gal* operon UAS promoter as the reporter, and a GAL4 DNA binding domain (DBD) fused to the MpDELLA N-terminal domain, and MpMED15 co-expressed as effectors. D) Yeast two-hybrid assay using MpDELLA GRAS domain as bait, and MpMYB14 as prey. E) Dual luciferase transactivation assay in *N. benthamiana* leaves using the *LUC* gene under the control of Mp*PAL* (*Mp1g05190*) promoter as the reporter, and HA-FLAG-MpMYB14, YFP-MpDELLA, and cMyc-MpMED15 co-expressed as effectors. Effector level in C and E is indicated below each bar as the agroinfiltrated OD_{600}.

with the known targets in Arabidopsis. According to the results shown above, induction by DELLA of flavonoid biosynthesis genes in Arabidopsis roots occurs through physical interaction with MYB12 and is facilitated by Mediator (**Fig. 3C, D**). Three additional pieces of

evidence support the hypothetical conservation of this particular regulatory mechanism in *M. polymorpha*: (1) we have observed that flavonoid biosynthesis is induced by MpDELLA (**Chapter 3**); (2) an ortholog of *MYB12* in *M. polymorpha* (Mp*MYB14*) has been shown to act on flavonoid biosynthesis by promoting Mp*PAL* (*Mp1g05190*) transcription among other genes (Kubo et al. 2018; Carella et al. 2019); (3) in a parallel effort in our lab, a screening for MpDELLA TF interactors had identified MpMYB02, which is the closest paralog of MpMYB14. For these reasons, we hypothesized that MpMYB14-MpDELLA would act concertedly to promote Mp*PAL* transcription by recruiting MpMED15. We first confirmed that these proteins interact using the GRAS domain of MpDELLA by yeast two-hybrid (**Fig. 4D**, **Fig. S15A**), and that this interaction occurs predominantly in plant cell nuclei as seen by BiFC assays (**Fig. S15B**). In agreement, MpMYB14 could activate Mp*PAL* transcription using a $_{pro}$Mp*PAL::LUC* reporter in a dual luciferase assay (**Fig. S14B**), while adding MpDELLA as a co-effector steadily increased MpMYB14 effect on LUC activity (**Fig. S14C, D**). This indicates that MpDELLA cooperates with MpMYB14 as a co-activator of its target genes, confirming that MpDELLA function as a TF co-activator.

Finally, to check whether MED15 recruitment is required for this co-activation, we added MpMED15 as a third effector to the transactivation assays. MpMED15 alone did not trigger obvious changes in LUC activity, while its presence together with MpMYB14, MpDELLA enhanced the ability of MpMYB14 to promote transcription (**Fig. 4E**). Altogether, these results support the idea that the recruitment of Mediator by DELLA is a conserved mechanism of transcriptional co-activation in land plants.

DISCUSSION

Here, we suggest a molecular mechanism of DELLA functioning as co-activators based on Mediator recruitment to enhance transcription in specific loci. We show that DELLA transactivation through Mediator is essential to modulate a subset of GA-regulated processes. This function is attributable to a eukaryotic-type 9aaTAD-KIX mechanism of interaction, and constitutes an ancestral mechanism of DELLA function.

Our findings point to a simple mechanism for DELLA function as co-activators by the interaction with the Mediator subunit MED15. However, DELLAs have been linked to the regulation of transcription through other basal transcription machinery complexes. It has been previously shown that the SWI3 or PKL subunits of Swi/Snf-related chromatin remodelling complexes are both modulated by DELLA proteins (Sarnowska et al. 2013; Zhang et al. 2014). This interaction seems to prevent SWI3 or PKL from directly binding chromatin, thus inhibiting the function of these proteins, which can be either activation or repression depending on the chromatin context (Han et al. 2015). It is likely that other mechanisms of transcriptional regulation by DELLAs may remain to be uncovered. For example, our assumption that DELLA

9aaTAD shall be interacting with MED15-type KIX and not CBP histone acetyltransferase (HAT)-type KIX is inherently biased by the assumption that gene transactivation in yeast should follow the same mechanism that occurs in plants, that do have KIX-containing HATs (Thakur et al. 2013). Therefore, it is plausible that DELLAs N-terminal domains directly recruit plant HATs to activate transcription. Other TFs as SREBP1a have been shown to recruit CBP and MED15 in different situations (Zhao and Yang 2012), and it would not be surprising for DELLA to also have HAT-recruiting activity. Furthermore, DELLAs have been proposed to act in the regulation of transcriptional elongation by the modulation of Paf1c complex activity by still unknown mechanisms (Blanco-Touriñán 2020). Interestingly, this function likely relies on ELF7/PAF1 protein interaction through the GRAS domain, so the N-terminal would remain free to act as a TAD. The apparently multifaceted ability of DELLAs to control transcription could be a consequence of their interaction with hundreds of TFs and TRs, which would have eventually facilitated the emergence of multiple context-specific regulatory mechanisms.

Many TFs are able to facilitate PIC formation and even initial recruitment. Hence, the existence of several intermediary bridges connecting specific TFs to RNAP II activation suggests that they provide regulatory benefits. The recruitment of Mediator is presumed to act as a fine-tuning of transcriptional regulation, not only promoting transcriptional initiation, but ensuring continuity and quantitative modulation of activated transcription (Malik and Roeder 2010; Borggrefe and Yue 2011). In this sense, DELLAs could add an environmentally-controlled layer of regulation, acting between TFs and Mediator to rapidly adjust transcription in response to multiple converging signals. For example, type-B ARRs activate gene expression, but need to interact with specific partners (Zhang et al. 2017), suggesting that the use of co-activation bridges or scaffolds is a common requirement for certain TFs. Other TFs do not need bridging, such as MYB12, known to directly promote transcription probably through the interaction with MED15a KIX domain (Mehrtens et al. 2005; Stracke et al. 2017; Kumar et al. 2018). The MYB12 TAD region is also involved in the direct interaction with DELLA GRAS domains to co-activate target genes (Tan et al. 2019). This interaction seems to enhance binding affinity of MYB12 to its DNA targets. In this case, DELLA GRAS domain could sterically impede MYB12 TAD functionality, but DELLA domain would then act as the TAD of the heterodimer. Alternatively, if GRAS does not obstruct MYB12 TAD function, DELLA could be instead adding its own N-terminal TAD to strengthen transcriptional activation.

A recurrent observation in our *in silico* analysis is the widespread presence of KIX-domain-only genes derived from full-length *MED15* genes. Such is the case of the Arabidopsis *MED15d* (AT1G15790), a two-KIX domain containing gene, but also *SmMED15b* (421987) or Mp*MED15L* (*Mp8g01860*), which all seem to have independently emerged by gene duplication and fragmentation in a lineage-specific manner (**Fig. 4A**). Does the occurrence of these shorter versions have any functional meaning? Interestingly, single domain-proteins

frequently act as microproteins (MiP), small mono-domain proteins that have a negative effect on their related complexes activity by direct protein-protein competition with full length multi-domain proteins (Eguen et al. 2015). Although we do not have definitive proof, several observations lead in that direction: (1) KIX-domain-only genes are expressed and spliced forming KIX-containing ORF at least in *A. thaliana* and *M. polymorpha* (**Fig. S7**, **Fig. S12B**); (2) interaction between the shorter versions of MED15 proteins and DELLA still occurs, at least for MED15d (**Fig. S3A**); and (3) a KIX-only deletion of MED15a interferes with DELLA-dependent transactivation (**Fig. 1D**). A GA-dependent transcriptional regulation could also be interpreted as a KIX/MiP feedback regulation, since *MED15d* appears to be slightly upregulated in response to an increase in DELLA levels. If MED15d competes with MED15a in vivo, it could constitute a titration mechanism to modulate the intensity of the co-activation. Moreover, it could be used as a biotechnological strategy to selectively inhibit DELLA activity in specific processes, tissues or organs where full loss of MED15 may be deleterious.

Another mechanism that could be directly associated with DELLA-dependent MED15 recruitment is GID1 inhibition of DELLA transactivation (Hirano et al. 2012). This mechanism seems to be masked by the predominant GID1 function as a proteolytic regulator of DELLAs (Ariizumi et al. 2008). The DELLA region responsible for GID1 recognition overlaps with the TAD described here (**Chapter 1**). Competition between GID1 and MED15 for DELLA interaction would explain previous observations, and would allow for a rapid and transient GA-dependent shut-down of DELLA function as co-activator, and could act before a slower non-reversible GA-dependent proteolysis of DELLA occurred. For example, GID1 was suggested to obstruct GAF1/IDD2 direct interaction with DELLA (Fukazawa et al. 2015). On top of that, this could be reminiscent of an evolutionary intermediate step in the GID1-DELLA relationship, predating DELLA degradation.

Overall, our results show a DELLA mechanism of co-activation that follows a eukaryotic KIX-dependent recruitment of the MED15 subunit to enable transcriptional initiation. We have previously discussed the widespread phenomenon of degron-TAD overlap (see **Chapter 1**). The data presented here could lie beneath the molecular basis for DELLA degron-TAD coincidence. While more evidence might be needed to definitely confirm this, we propose that the transcriptional activation mechanism by Med recruitment is an ancient function of DELLA and may underlie the co-option of DELLA TAD for the establishment of the GA-GID1-DELLA module.

MATERIALS & METHODS

Transactivation prediction and conservation

DELLA domains and p53 proteins of plants and metazoan species were selected based on a similar evolutionary distance among the groups (earlier diverging grouping bifurcating > 500 mya) (**Table S7**). The sequences were aligned with MAFFT 7.0, using the L-INS-I method (Katoh and Standley 2013), followed by manual curation. Transactivation domain prediction was conducted as previously described using the 9aaTAD prediction tool (http://www.med.muni.cz/9aaTAD/index.php) using the "less stringent" pattern (Piskacek et al. 2007). 9aaTAD conservation indices was calculated for each residue based on the alignment and setting as reference sequence the crystalized form of each protein (*Arabidopsis thaliana* partial GAI DELLA domain structure, extracted from PDB:2ZSH; *Homo sapiens* p53 TAD structure, PDB:2l14). Final score represents the cumulative probability of 9aaTAD presence in all the sequences at each residue. Colouring was done with ProtSkin (Ritter et al. 2004), for subsequent mapping on PDB structures using PyMOL software (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

Yeast-two hybrid assays

For bait Gal4-DNA Binding Domain (BD) fusions, MED15 related sequences (full length, KIX domain and C-terminal domain), and a truncated version of MpDELLA based on previously described DELLA deletions were introduced in the pGBKT7-GW vector. DELLA full-length, N-terminal and C-terminal sequences from Arabidopsis and Marchantia, and MpMYB14 were fused to the Gal4-Activation Domain (AD) in pGADT7-GW (Rossignol et al. 2007). Entry clones and sources are listed in **Table S8**. New entry vectors were obtained by transferring PCR-amplified CDSs to pDONR207 via BP Clonase II (Invitrogen). Newly obtained vectors and sequence-specific primers used for amplification are listed in **Table S9**. Final constructs were made by recombining entry clones with Gateway destination vectors via LR Clonase II (Invitrogen). Direct interaction assays in yeast were performed following Clontech's small-scale yeast transformation procedure. Strain Y187 was transformed with pGADT7 derived expression vectors, while strain Y2HGold was transformed with pGBKT7 vectors, and selected in SD medium without Leu or Trp, respectively. Subsequently, diploid cells were obtained by mating and selection in SD medium lacking both Leu and Trp. Interaction tests were done in SD medium lacking Leu, Trp and His, in the presence of different concentrations of 3-aminotriazol (3-AT) (Sigma-Aldrich).

Bimolecular fluorescent complementation (BiFC) assays

For BiFC, DELLA-related entries and MED15 entries were recombined with pMDC43-YFN

and pMDC43-YFC (Belda-Palazón et al. 2012), respectively. *Agrobacterium tumefaciens* GV3101 containing binary plasmids were used to infiltrate 4-week-old *Nicotiana benthamiana* leaves. Three days after infiltration, leaves were analyzed with a Zeiss LSM 780 confocal microscope. Reconstituted YFP signal was detected with emission filters set to 503-517 nm. Nuclei presence in abaxial epidermal cells was verified by transmitted light.

Dual luciferase transactivation assay

For Gal4 based assays, a previously reported *2xUAS$_{Gal}$:LUC* based on pGreenII 0800 LUC was used. Gal4DBD-RGA^{N} effector plasmids have been described. MED15 related, full length MpDELLA, and MpMYB14 constructs were introduced into pEarleyGate203, pEarleyGate104 and pEarleyGate201 destination vectors (Earley et al. 2006) via LR Clonase II (Invitrogen) from their entry vectors, respectively. The Gal4BD-MpDELLA^{N} effector vector was obtained by amplifying the GAL4 BD-MpDELLA N-end fusion previously generated in pGBKT7 vectors as a unique PCR product with proper restriction site overhangs and ligated into pFGC5941 (http://www.ChromDB.org), between *Xho*I and *Spe*I. $_{pro}$Mp*PAL::LUC* was generated amplifying with sequence-specific primers a 2296 pb upstream region of the Mp*PAL* gene *Mp1g05190* from *M. polymorpha* Tak-1 genomic DNA, and cloned upstream of the firefly luciferase gene (LUC) between an *Spe*I and *Nco*I in pGreenII 0800-LUC (Hellens et al. 2005). Transient expression in *Nicotiana benthamiana* leaves was carried as previously reported (Marín-de la Rosa et al. 2015). Firefly and the control *Renilla* luciferase activities were assayed in extracts from 1-cm in diameter leaf discs, using the Dual-Glo Luciferase Assay System (Promega) and quantified in a GloMax 96 Microplate Luminometer (Promega). Statistical differences were analysed by one-way ANOVA followed by Tukey's HSD post hoc test. Letters denote differences between groups with $p<0.01$, unless specified.

Plant material and growth conditions

The transgenic *med15a^{Ri}* line in the Col-0 background has been described before (Kim et al. 2016). All seeds were surface sterilized and sown on half-strength MS (Duchefa) plates containing 0.8% agar pH 5.7. Seedlings were grown at a constant temperature of 22 ºC under long day conditions (16 h light 60-70 µmol $m^{-2}s^{-1}$:8 h darkness) unless specified. For hypocotyl growth and cotyledon opening assessment during skotomorphogenic development, seedlings were germinated in light for 6-8 hours and transferred to darkness with or without 1 µM PAC for 5 days before evaluation. For RAM growth analysis, seedlings were grown during 5 days in vertical plates under standard conditions, and transferred to identical plates with or without 10 µM PAC for 16 hours before confocal analysis. Statistical analyses of biological samples were performed by t-test analyses between two groups, or one-way ANOVA followed by Tukey's HSD post hoc test in multiple group comparisons. For t-tests, one asterisk indicates $p<0.01$, and two $p<0.001$. Letters denote differences between groups after ANOVA analyses

($p<0.01$).

Microscopy & histochemical analysis

Diphenylborinic acid 2-aminoethyl ester (DPBA) staining was used to visualize flavonoids as previously described (Peer et al. 2001). Whole seedlings were stained for 15 minutes at 0.25% (w/v) DPBA and 0.1% (v/v) Triton X-100. Epifluorescence microscopy of stained flavonoids in roots was performed on a Leica DMS1000 dissecting microscope using a GFP filter for detection of DPBA fluorescence. Root meristems were imaged with an LSM 5 Pascal Zeiss Confocal microscope with a water-immersion objective lens (C-Apochromat 40X/1.2; Zeiss) using propidium iodide to stain cell walls. Meristem size was measured as the number of cortex cells.

RNA isolation, cDNA synthesis, and qRT-PCR analysis

Total RNA was isolated using NucleoSpinTM RNA Plant Kit (Macherey-Nagel) according to the manufacturer's instructions. cDNA was prepared from 1 µg of total RNA with NZY First-Strand cDNA Synthesis Kit (NZYTech). Quantitative real-time PCR was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems) with SYBR premix ExTaq (Tli RNaseH Plus) Rox Plus (Takara Bio Inc). All relative expression levels were calculated following Hellemans et al. (2008), and *PDF2.1* (AT1G13320) was used as the reference gene (Czechowski et al. 2005). Primers are listed in **Table S9**.

RNA sequencing and data analysis

For whole RNA sequencing, seedlings were grown under long day conditions as described during 4 days and then transferred to either 1 µM PAC or 1 µM PAC + 100 µM GA_3 for 3 days prior to RNA isolation. Total RNA was isolated as described above and sent to BGI Europe to perform quality control, library construction and sequencing on a DNBSEQ platform (BGI). The obtained 100 bp paired-end reads were analyzed with FastQC (v 0.11.9) using parameters by default to assess quality, and adaptor sequences removed with Cutadapt (with parameters '--minimum-length=20 --max-n=0.1 --quality-cutoff=30,30') (Martin 2011), and then mapped to the TAIR10 *A. thaliana* reference genome with HISAT2 (Kim et al. 2019). htseq-count was used for read count (parameters: '--format=bam --order=name --stranded=no') (Anders et al. 2015), and TPMs calculated as a proxy to absolute levels of gene expression. Genes with 1 TPM or more 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), Only differentially expressed genes (DEGs) whose adjusted p values (padjust) were under 0.01 were used for metaanalyses. DEGs between PAC-treated and PAC+GA-treated seedlings can be found in **Table S3**.

Enriched biological process terms were calculated using the GO Enrichment tool available at AgriGO using the Fisher's test and the Yekutieli adjustment method (**Table S5**). For the graphic display, only Biological Process terms with p-adj values lower than 0.01 were considered and merged manually to discard duplicity in highly related terms. R code for ggplot2-based plotting can be found in our Mendeley Data resource.

TF binding-site and TF enrichment prediction

TF binding-site enrichment among selected genes was calculated using the EAT-UpTFv0.1 (Shim and Seo 2020). The hypergeometric statistical model with a Bonferroni post hoc test (alpha level 0.01) was applied. Inspection of type-B ARR enrichment in previous datasets was performed using the TF enrichment tool available at PlantRegMap (Tian et al. 2019). Targeted type-B ARR binding site prediction on the 1000 bp upstream of the genes in a subset was performed using FIMO (MEME Suite) with the ARR10 power weight matrix (Grant et al. 2011).

Sequence identification and phylogenetic analysis

To identify MED15 sequences, we first built a custom BLAST database web interface based on Sequenceserver (Priyam et al. 2019) with different available plant proteomes annotations: Arabidopsis thaliana Araport11 (Cheng et al. 2017) was downloaded from https://www.arabidopsis.org/, *Marchantia polymorpha* v5.1 (Montgomery et al. 2020) was downloaded from https://marchantia.info/, *Anthoceros agrestis* Bonn (Li et al. 2020) was downloaded from https://www.hornworts.uzh.ch/en.html, tomato iTAG4.0 (Hosmani et al. 2019) was downloaded from https://solgenomics.net/, *Oryza sativa* v7, *Sorghum bicolor* v3.1.1, *Physcomitrella patens* v3.3, *Selaginella moellendorffii* v1.0, *Micromonas* sp. RCC299 v3.0, and *Chlamydomonas reindhardtii* v5.5 were downloaded from https://phytozome-next.jgi.doe.gov/ (Goodstein et al. 2012). The in-house database was questioned by BlastP with the protein sequence of MED15a (AT1G15780) as query using an E-value cutoff of 0.1 or 10 in the case of the chlorophytes *Micromonas* and *Chlamydomonas*. The obtained sequences were checked for presence of KIX domains using Pfam (http://pfam.sanger.ac.uk/search). We included in our sequence list the previously characterized Arabidopsis p300/CBP protein (AT1G16705) to use as an outgroup, and the identified MED15 subunits from the yeast *S. cerevisiae* (Gal11, YOL051W), and the amoeba *Dictyostelium discoideum* (DDB_G0293914).

MED15 sequences were aligned with MAFFT 7.0, using the L-INS-I method (Katoh and Standley 2013), followed by manual curation. For phylogenetic reconstruction, KIX domains were used, and ambiguously aligned regions manually trimmed. ProtTest v3.4.2 (Darriba et al. 2011) was used on final multiple sequence alignment to select best-fit model of amino acid replacement using the AIC model for ranking. Maximum likelihood tree was produced with

PhyML v3.1 (Guindon et al. 2010), using the best scored model of amino acid substitution. Statistical significance was evaluated by bootstrap analysis of 1000 replicates. Phylogenetic tree graphical representation was initially generated using FigTree (version 1.4.3) software (http://tree.bio.ed.ac.uk/software/figtree/), and final cartoons edited manually.

Yeast transactivation assay

MpMED15 full length ORF entry was obtained as described above, and then used to create a Gal4-BD fusion in the pGBKT7-GW vector via LR Clonase II (Invitrogen), and then transformed into yeast strain Y2HGold and transformants were selected in SD medium lacking Trp. Transactivation tests were performed in SD medium without Trp and His, and increasing 3-aminotriazol concentrations as indicated.

SUPPLEMENTAL INFORMATION

Supplemental information is available as described at the beginning of this PhD thesis manuscript (**Opening Statement**). Additionally, supplemental figures can be found at the end of this chapter.

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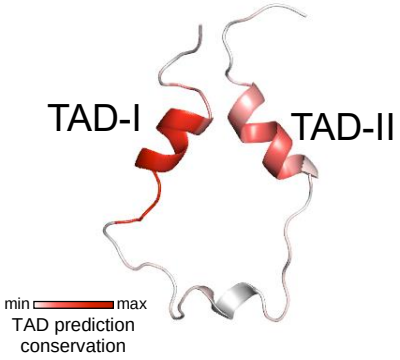
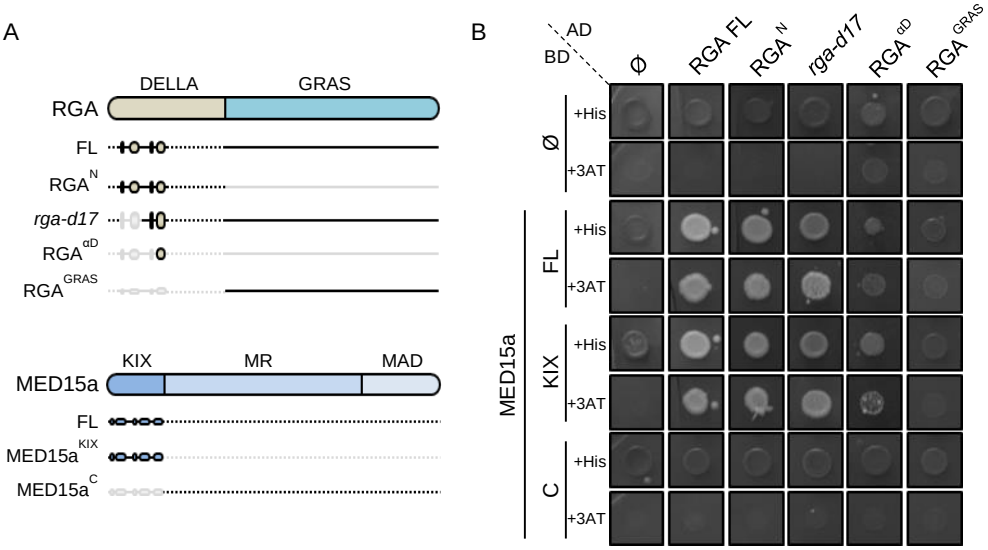
SUPPLEMENTAL FIGURE 1



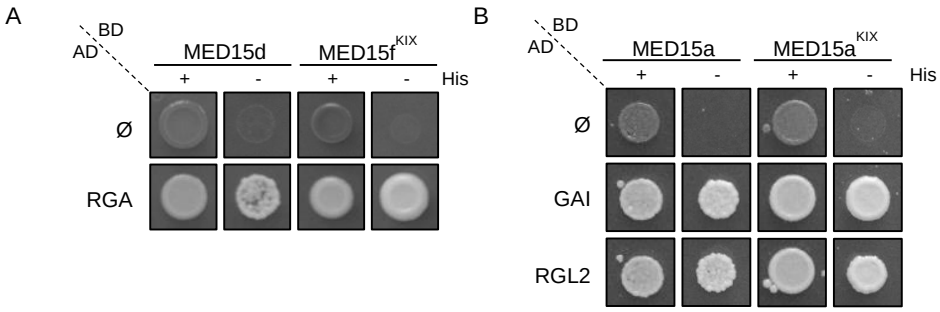
Mammalian p53 structure involved in ARC105 recruitment. Protein structure pf the p53 transcriptional activation domains involved in the human Med15 subunit (ARC105) recruitment. Coloured gradient represents 9aaTAD prediction merged from 6 different metazoan lineages (**Table S7**). Both TAD-I and II are known to independently interact with KIX domains.

SUPPLEMENTAL FIGURE 2



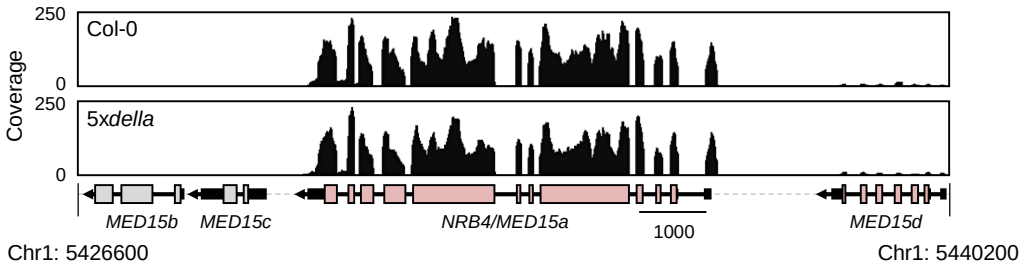
RGA-MED15a yeast-two hybrid deletion analysis. A) Illustration of RGA and MED15a deletions. MR, Middle Region; MAD, Mediator Activation/Association Domain. B) Yeast two-hybrid assay using MED15a deletions as bait (Gal4BD), and RGA deletions as prey (Gal4AD).

SUPPLEMENTAL FIGURE 3



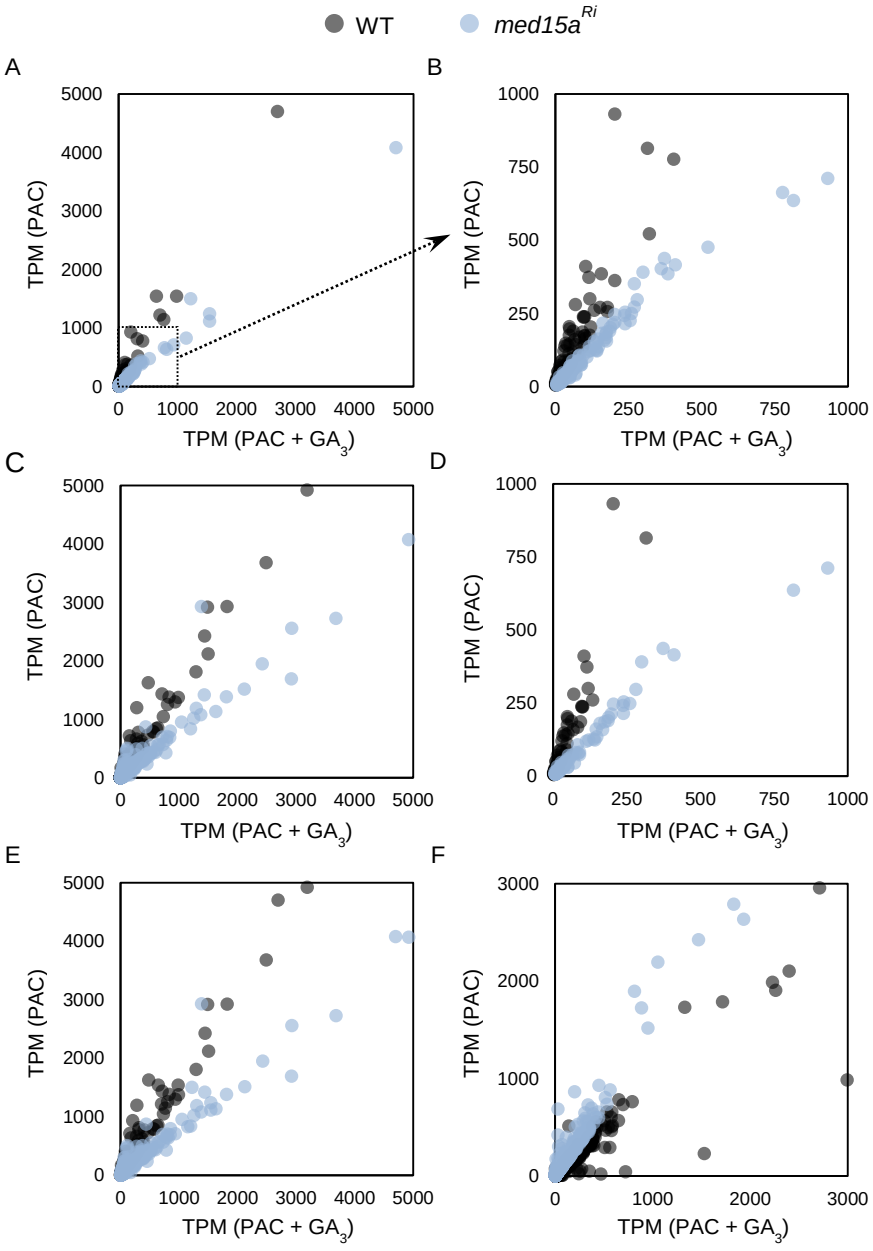
Yeast-two hybrid of DELLA-MED15 paralogs in Arabidopsis. Yeast two-hybrid assays using A) MED15e and MED15f double KIX domain as baits (Gal4BD), and RGA full length as prey (Gal4AD), and B) MED15a as bait, and GAI and RGL2 full length versions as preys. MED15d protein is composed only of two tandem KIX domains, while MED15f is a highly divergent full length MED15 subunit, encoded by a low expression gene, probably under a pseudogenization process, similarly to its closest paralog, the pseudogene *MED15e* (see Fig. 4). GAI and RGL2 are each a close and a distant paralog of RGA, respectively.

SUPPLEMENTAL FIGURE 4



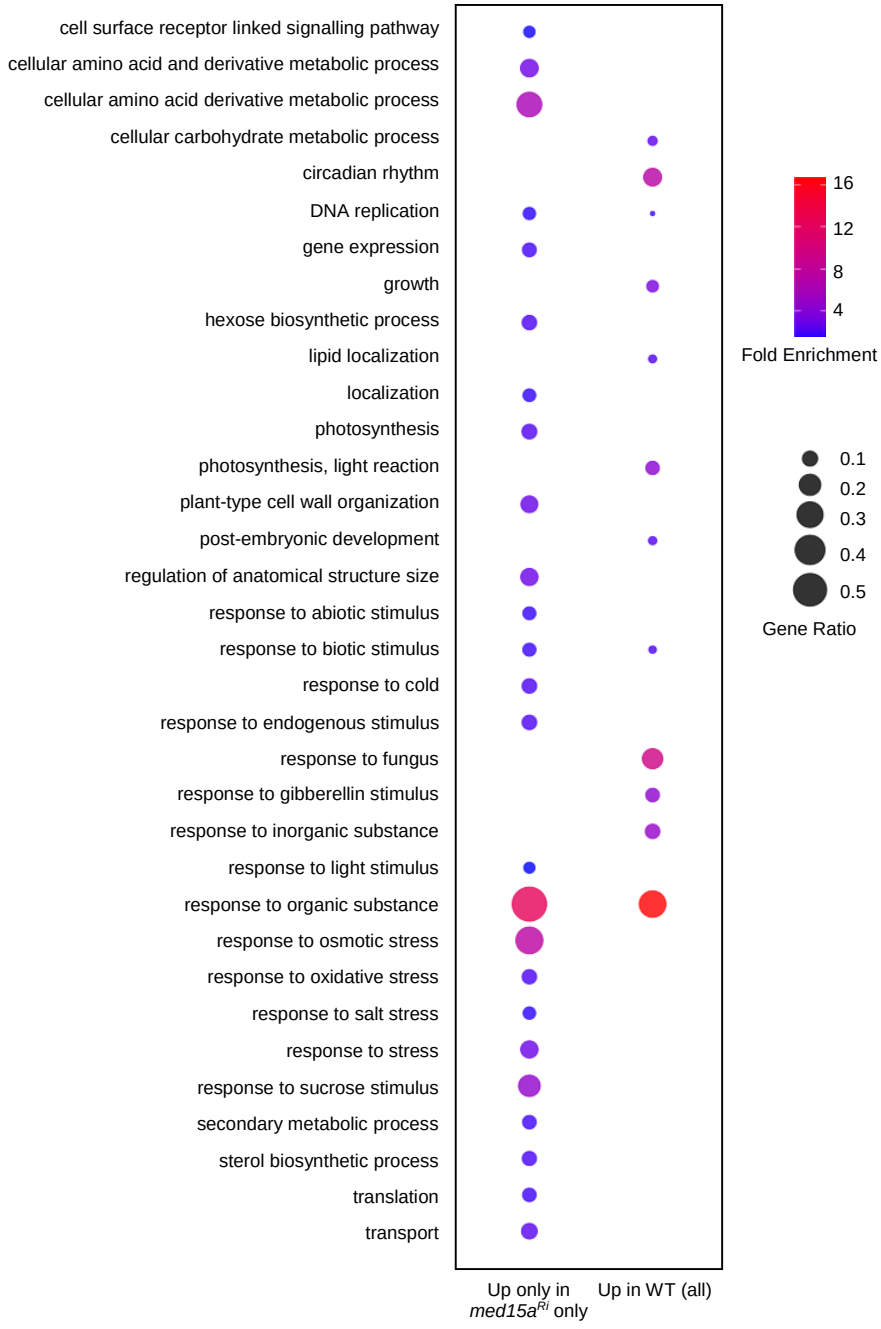
RNA-seq coverage of *MED15* gene cluster in Arabidopsis in the 5x*della* mutant. RNA-seq coverage of minus (-) strand at the *MED15* cluster in the *Arabidopsis thaliana* chromosome 1 in 7-day-old seedlings of Col-0 wild-type and pentuple *della* mutant. Grey-shaded exons represent lack of reads. *MED15b* and *MED15c* are pseudogenes. Data extracted from Briones-Moreno 2020.

SUPPLEMENTAL FIGURE 5



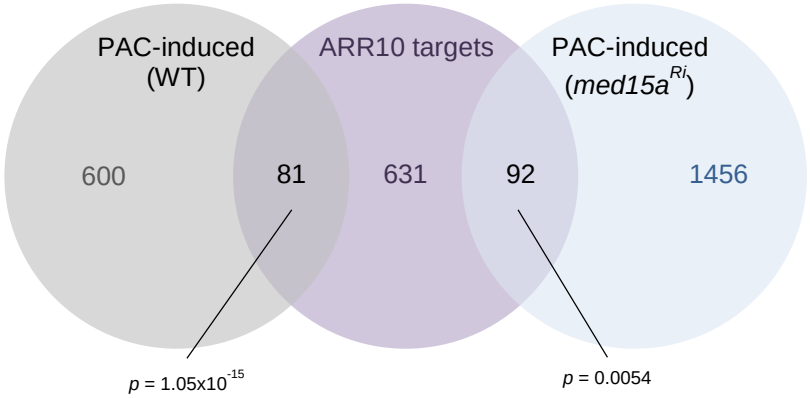
RNA-seq TPM comparison scatter plots. A, B) Scatter plot showing TPM counts for each treatment of A) the 184 genes in the intersection between those up-regulated by PAC compared to PAC+GA_3 treatment in wild-type (WT, gray) and *amiR*^{Med15} line (blue), and B) zoom-in of the same subset to 1000 TPM axes. C) Scatter plot of TPM count in the genes up-regulated only in the wild-type (497) D) Scatter plot of TPM of the 84 genes included in B with a fold change greater than 1 in PAC-treated plants compared to PAC+GA_3 in wild-type and *amiR*^{Med15} line intersection. E, F) Full zoomed-out scatter plot of TPM count represented in Fig. 2B plots.

SUPPLEMENTAL FIGURE 7



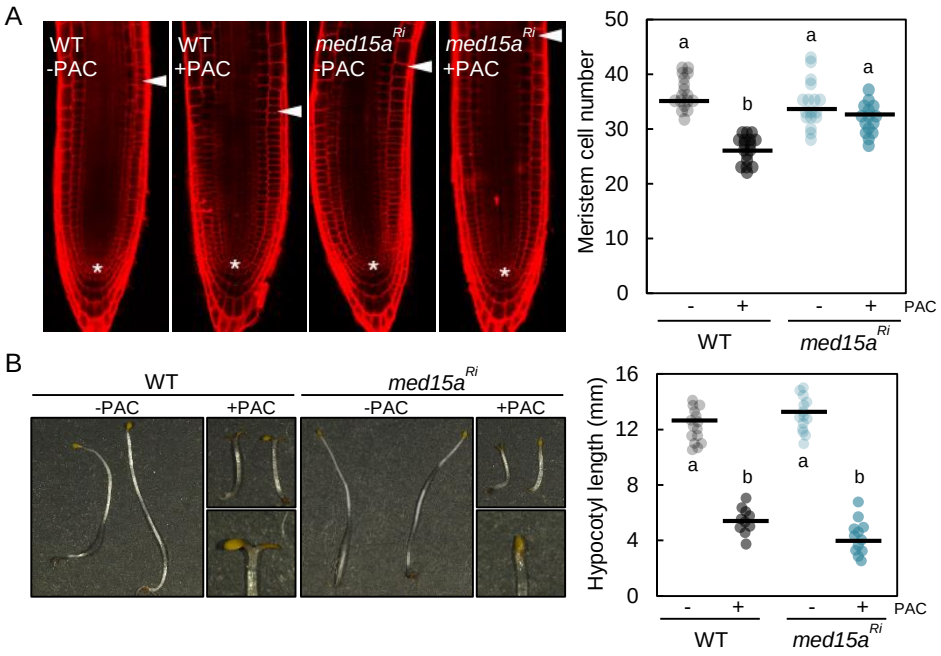
Biological processes GO term enrichment in DELLA-MED15 targets. See table S5. GO enrichment only in Biological Processes found for the list of genes up-regulated by PAC in the MED15a RNAi line, or all the genes up-regulated by PAC in the wild-type, which is accounted as the list of putative DELLA-MED15 targets. GO enrichment was first calculated based on AgriGO. Code for enrichment plot can be found in the Mendeley Data resource.

SUPPLEMENTAL FIGURE 8



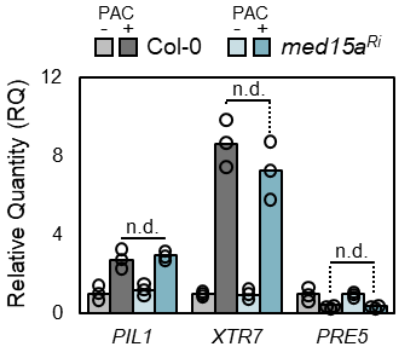
Venn's diagram of ARR10 targets compared to PAC induced genes. Overlap between genes predicted to be direct targets of ARR10 (Zubo et al, 2017) and genes up-regulated by DELLA accumulation (PAC-induced) in both genotypes. Statistical significance between datasets calculated by Fisher's exact test of the intersection.

SUPPLEMENTAL FIGURE 9



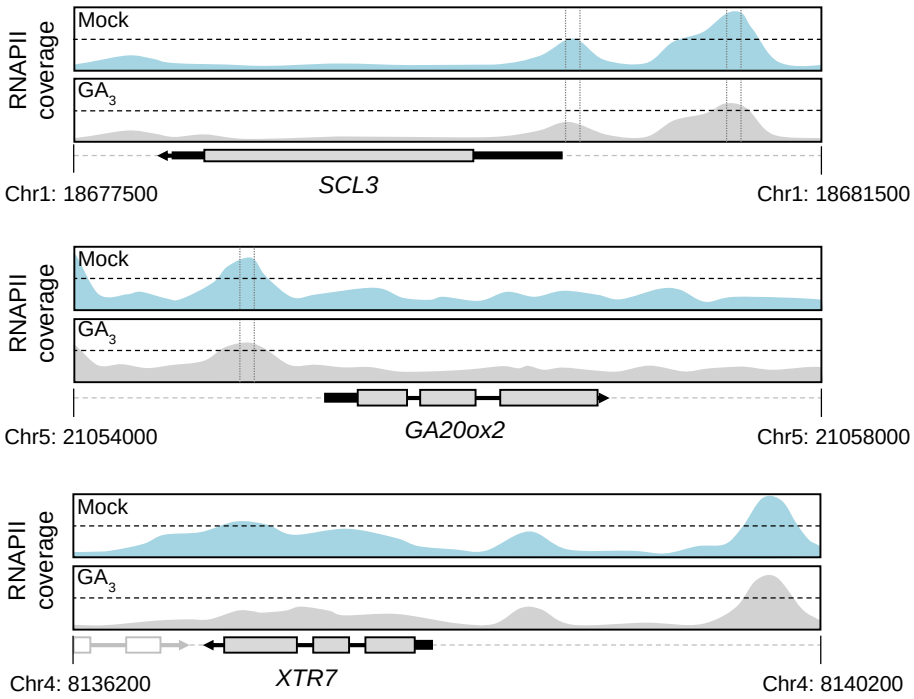
MED15 involvement in DELLA co-activation responses. A) Meristem size measured as cortex cell number in 5 days-old *med15a*^{Ri} seedlings grown with or without 10 µM PAC for 16 hours. Data in B represents two merged replicates with at least 10 plants per genotype and treatment. B) Hypocotyl length in 5 days-old wild-type and *med15a*^{Ri} seedlings grown in darkness with or without 1 µM PAC. Close-up pictures emphasizing the cotyledon open phenotype shown in Fig. 2A and B. Experimental data of three independent merged replicates with at least 15 plants per genotype and treatment.

SUPPLEMENTAL FIGURE 9



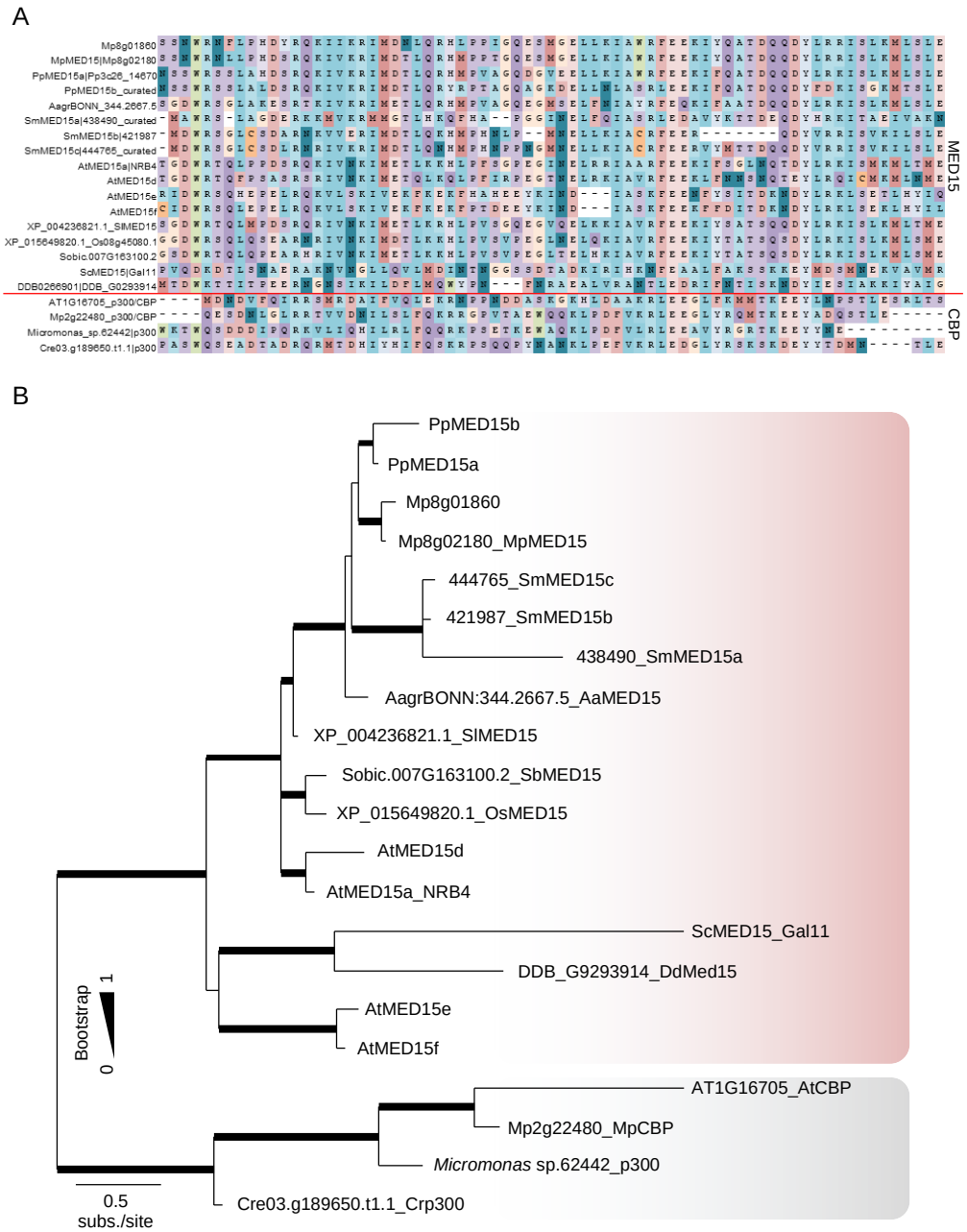
Gene expression analysis of DELLA-PIF targets in *med15a^{Ri}*. A) RT-qPCR analysis of GAs and PIFs responsive genes in 5 days-old Col-0 and *med15a^{Ri}* seedlings grown in darkness with or without 1 µM PAC. Data are medians (bar) of 3 biological replicates, referred against mock and 2 technical repeats (means per replicate shown as empty circles), referred against mock. *PDF2.1* was used to normalize data.

SUPPLEMENTAL FIGURE 10



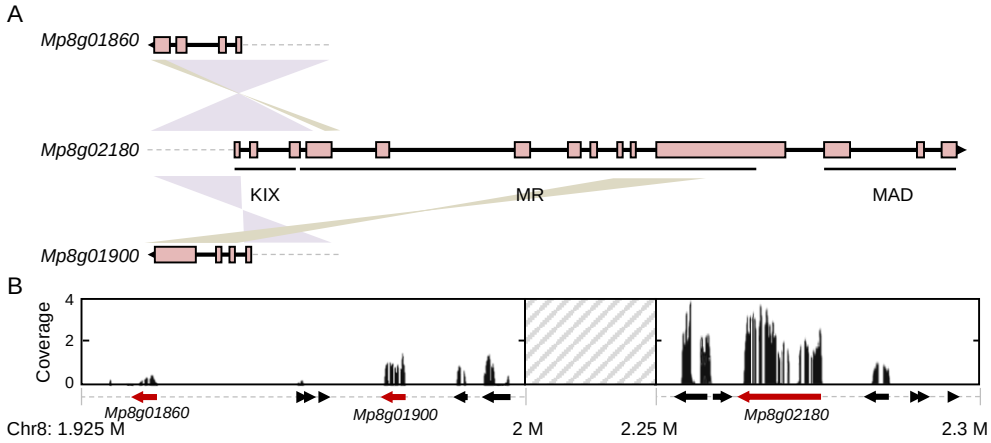
RNAP II ChIP-seq coverage of selected genes with or without GAs. RNAP II ChIP-seq coverage of the DELLA-induced genes *SCL3* (AT1G50420), *GA20ox2* (AT5G51810), and *XTR7* (AT4G14130) in 7-day-old seedlings grown on half strength MS plates supplemented with 100 µM GA_3 or not (mock). Coverage height are equaled to mock condition. Genome view spans 4 kilobases between the indicated positions. Grey boxes represent exons. Flanking gene upstream of *XTR7* appears shaded (AT4G14120). Data extracted from Blanco-Touriñán 2020.

SUPPLEMENTAL FIGURE 11



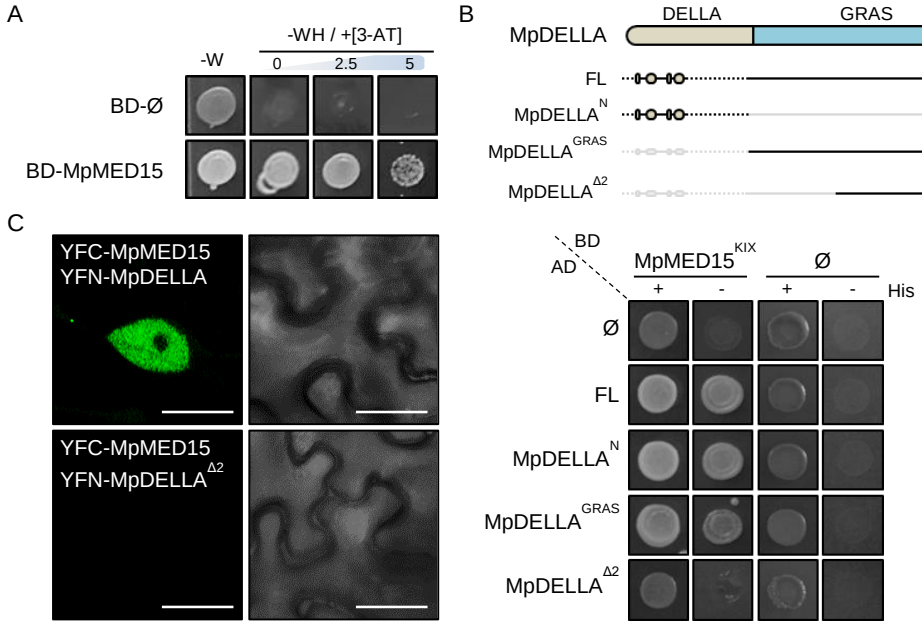
MED15 KIX-domains alignment and phylogeny tree. A) MAFFT alignment of KIX domains from BLASTP-selected proteins. B) Phylogenetic analysis of MED15 proteins using KIX domains as depicted in **Fig. 4**. Grey shaded clade is composed of CBP KIX domains and represents the outgroup (OG). Support values associated with branches and displayed as bar thickness are maximum likelihood bootstrap values from 1,000 replicates. Branch length represent distance in substitutions per site.

SUPPLEMENTAL FIGURE 12



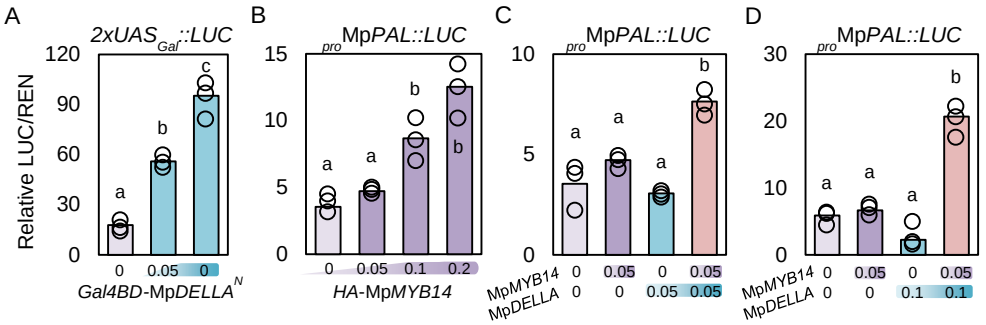
MED15* genes fragments found in *M. polymorpha. A) *MED15* genes *Mp8g01860* and *Mp8g1900* sequence coincidence with *Mp8g02180* gene. MR, Middle Region; MAD, Mediator Activation/Association Domain. B) RNA-seq coverage of minus (-) strand of *Marchantia polymorpha* Tak-1 wild-type chromosome 8. Red arrows represent *MED15*-related genes, while black arrows are unrelated genes. Data extracted from the *marchantia.info* database.

SUPPLEMENTAL FIGURE 13



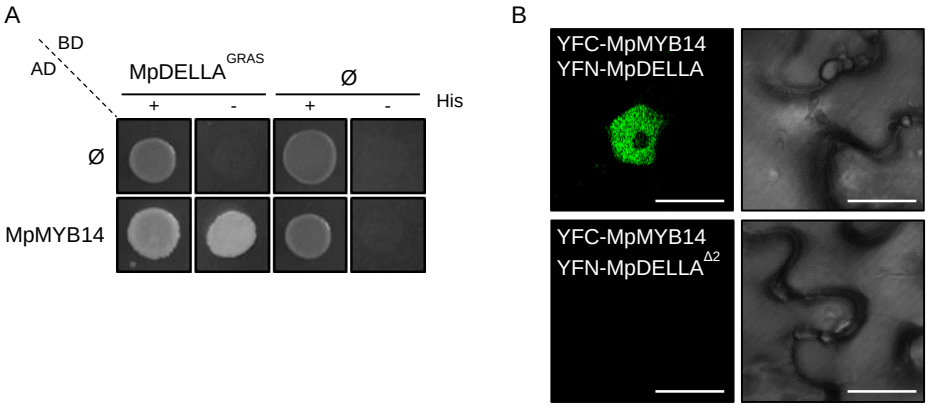
MpMED15-MpDELLA interaction analysis. A) Yeast one-hybrid assay using MpMED15 full length fused to a Gal4 DNA binding domain in His auxotrophic strain with a *UAS_{Gal}::HIS3 reporter*. B) Up, illustration of MpDELLA deletions. Down, yeast two-hybrid assay using $MpMED15^{KIX}$ as bait (Gal4BD), and MpDELLA deletions as prey (Gal4AD). C) Bimolecular fluorescence complementation assay of MpMED15 and MpDELLA, using a MpDELLA deletion version as negative control. Scale bar, 10 µm.

SUPPLEMENTAL FIGURE 14

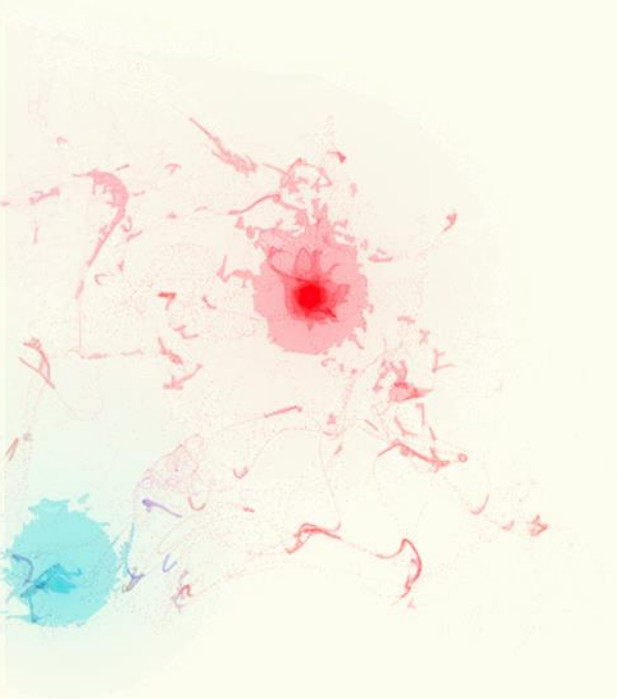


MpDELLA-related dual luciferase assays. Dual luciferase transactivation assay in *Nicotiana benthamiana* leaves. A) Dual luciferase using the *LUC* gene under the control of the *Gal* operon UAS promoter as the reporter (*2xUAS$_{Gal}$::LUC*) and the GAL4 DNA binding domain (BD) fused to the MpDELLA DELLA domain (MpDELLA^{N}) as effector. B-D) Dual luciferase using the *LUC* gene under the control of the Mp*PAL* gene promoter, and the HA-fused MpMYB14 as effector alone (B), or together with YFP-fused MpDELLA (C & D). Effectors were infiltrated with a preparation of *A. tumefaciens* with the optical densities (O.D.) indicated below the bar plots. Data represent means (bar) of three biological replicates. Circles are median values of three technical replicates in a biological replicate. Letters indicate statistically different groups calculated by one-way ANOVA with a Tukey HSD post-hoc analysis ($p < 0.01$).

SUPPLEMENTAL FIGURE 15



MpDELLA-MpMYB14 interaction analysis. A) Yeast one-hybrid assay using B) Bimolecular fluorescence complementation assay of MpMYB14 and MpDELLA, using a MpDELLA deletion version as negative control. Scale bar, 10 µm.



Chapter 3

Coordination Between Growth and Stress Responses by DELLA in the Liverwort *Marchantia polymorpha*

Jorge Hernández-García, Rui Sun, Antonio Serrano-Mislata, Keisuke Inoue, Carlos Vargas-Chávez, David Esteve-Bruna, Vicent Arbona, Shohei Yamaoka, Ryuichi Nishihama, Takayuki Kohchi, Miguel A. Blázquez

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Chapter 3

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Abstract

Plant survival depends on the optimal use of resources under variable environmental conditions. Among the mechanisms that mediate the balance between growth, differentiation, and stress responses, the regulation of transcriptional activity by DELLA proteins stands out. In angiosperms, DELLA accumulation promotes defence against biotic and abiotic stress and represses cell division and expansion, while the loss of DELLA function is associated with increased plant size and sensitivity towards stress (Thomas et al. 2016). Given that DELLA protein stability is dependent on gibberellin (GA) levels (Sun 2011), and GA metabolism is influenced by the environment (Hedden and Thomas 2012), this pathway is proposed to relay environmental information to the transcriptional programs that regulate growth and stress responses in angiosperms (Claeys et al. 2014; Davière and Achard 2016). However, *DELLA* genes have been identified only in vascular plants (Hirano et al. 2007; Yasumura et al. 2007; Hernández-García et al. 2019; Blázquez et al. 2020; Hernández-García et al. 2021). Thus, it is not clear whether these regulatory functions of DELLA predated or emerged with typical GA signalling. Here we show that, as in vascular plants, the only DELLA in the liverwort *Marchantia polymorpha* also participates in the regulation of growth and key developmental processes, and promotes oxidative stress tolerance. Moreover, part of these effects is likely caused by the conserved physical interaction with the MpPIF transcription factor. Therefore, we suggest that the role in the coordination of growth and stress responses was already encoded in the DELLA protein of the common ancestor of land plants, and the importance of this function is underscored by its conservation over the past 450 M years.

Key words: gibberellin, *Marchantia polymorpha*, plant growth, oxidative stress, plant hormone, plant evolution.

RESULTS AND DISCUSSION

MpDELLA accumulation affects cell division

The genome of *M. polymorpha* encodes a single Mp*DELLA* gene (Mp5g20660; Bowman et al. 2017; Hernández-García et al. 2019). Attempts to generate Mp*della* loss-of-function mutants with several sgRNAs yielded only mutations that did not significantly alter the protein sequence (*i.e.*, the locus was editable, but hypomorphic alleles were not recovered) (Figure S1A). Thus, to investigate its biological function, we generated transgenic plants overexpressing Mp*DELLA* either under the control of the CaMV *35S* promoter, or the *M. polymorpha ELONGATION FACTOR1α* (Mp*EF1α*) promoter. In all cases, Mp*DELLA* constitutive overexpressors displayed smaller thallus sizes than the wild type, which was already evident in two-week-old plants (**Figures 1A**, **1C**, and **S1A**). To test MpDELLA function within its native expression range, plants were also transformed with additional copies of the gene including its own promoter, coding sequence and a translationally fused β-glucuronidase reporter (*g*Mp*DELLA-GUS*). These lines were moderately high in MpDELLA expression (**Figure S1B**) but also showed significantly smaller thallus (**Figures 1A** and **1C**). Introducing additional copies, native-promoter driven translational fusion with the β-glucuronidase (GUS) reporter (*g*Mp*DELLA-GUS*) had a moderate, but similar effect (**Figures 1A**, **1C**, and **S1A**). As members of the GRAS family transcriptional regulators, DELLA proteins have been shown to function in the nuclei of angiosperms (Silverstone et al. 2001; Itoh et al. 2002). Nuclear localization of MpDELLA was also observed for MpDELLA-Cit fusion proteins (**Figure S1D**). Following such observation, we constructed the inducible Mp*DELLA-GR* lines, which constitutively expressed Mp*DELLA* fused with the rat glucocorticoid receptor. Dexamethasone (DEX)-induced growth impairment was observed in a dose-dependent manner (**Figure S1E** and **S1F**). These results support the hypothesis that DELLA accumulation inhibits vegetative growth in *M. polymorpha* and is similar to size alterations observed in several flowering plant species (Peng et al. 1999; Dill et al. 2001; Ikeda et al. 2001; Martí et al. 2007).

In Arabidopsis, one of the mechanisms proposed for controlling plant size is the DELLA-dependent reduction of cell proliferation rate (Achard et al. 2009; Davière et al. 2014; Serrano-Mislata et al. 2017). To investigate if cell division is affected in Mp*DELLA* overexpressors, we labelled S-phase cells with 5-ethynyl-2'-deoxyuridine (EdU) and observed the nuclear signals around the apical region. All overexpressor lines showed significant reduction in the total number of EdU-positive nuclei, which were distributed in a smaller area compared with wild-type plants (**Figures 1B**, **1D-E**, and **S1G-I**). For further confirmation of the cell-cycle regulation by Mp*DELLA*, we introduced a G2-M phase reporter (*pro*Mp*CYCB;1:Dbox-GUS*) into the

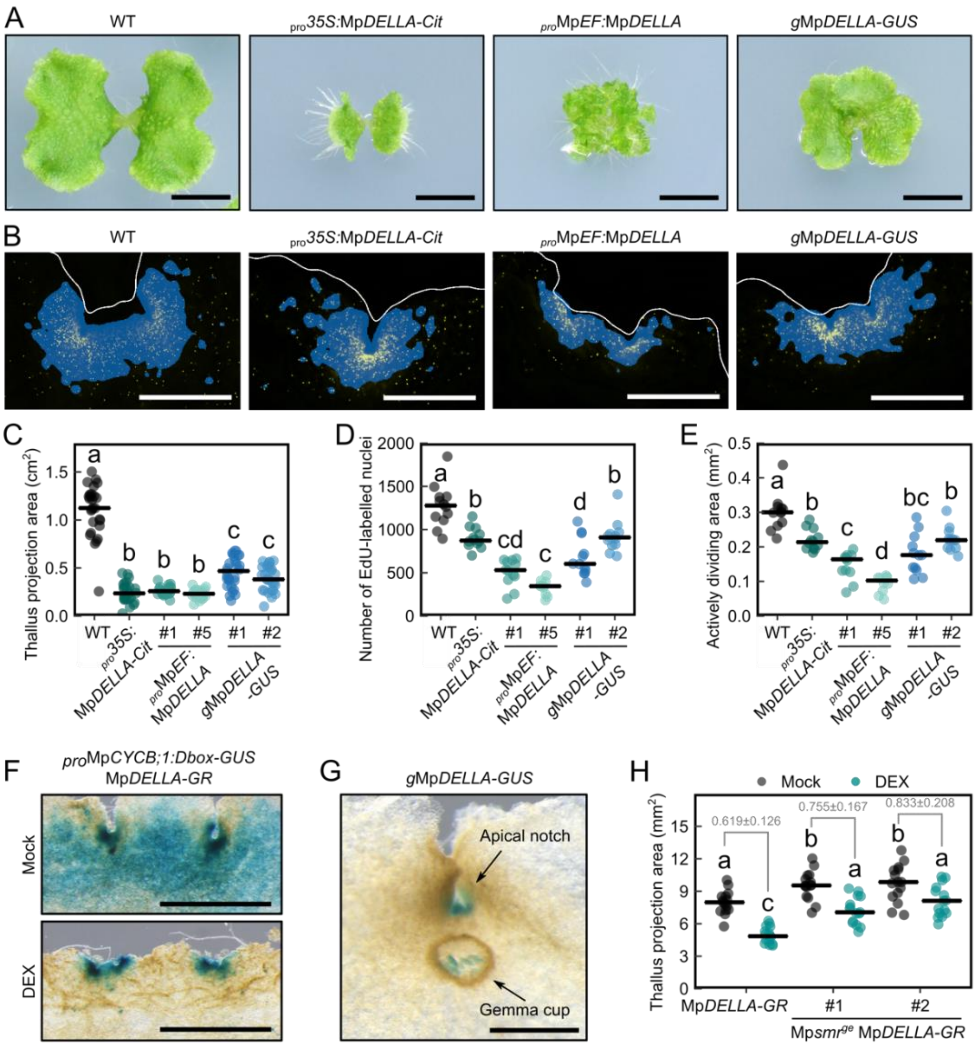

Figure 1. Mp*DELLA* overexpression inhibits plant growth via cell division. See also Figure S1. (A) Morphology of 14-day-old gemmallings in Mp*DELLA* overexpression lines. Scale bar, 5 mm. (B) Apical notches of 7-day-old gemmallings labelled with EdU (yellow signals). Plant boundaries are marked with white lines, and blue color indicates the area occupied by dividing cells (See STAR methods for definition). Scale bar, 500 μm. (C) Measurement of plant sizes in 14-day-old gemmallings. n=26 for *pro35S*:Mp*DELLA-Cit*; n=27 for others. (D and E) Number of EdU-labelled nuclei (D) and area of actively dividing regions (E) in the apical notches of 7-day-old gemmalings. n=10 for *pro*Mp*EF*:Mp*DELLA* #5; n=12 for others. (F) Images of 9-day-old Mp*DELLA-GR* Mp*CYCB;1-GUS* plants stained for GUS activity after mock or 1 μM DEX treatment for 3 days. Scale bar, 1 mm. (G) A representative image of 21-day-old g*MpDELLA-GUS* plant stained for GUS activity. Scale bar, 500 μm. (H) Plant sizes of 7-day-old Mp*smr*^{ge} Mp*DELLA-GR* gemmallings after mock or 1 μM DEX treatment for 5 days. Ratio of plant sizes (±propagated SE) for each pair is shown in grey. n=15. All plants were grown under continuous white light except long-day conditions in H. In C, D, E, H, dots represent individual plants, and the horizontal lines represent mean values. Statistical groups are determined by Tukey's Post-Hoc test ($p<0.05$) following one-way ANOVA.

Mp*DELLA-GR* background. After a two-day treatment with 1 µM DEX, the spatial range of GUS signals was notably restricted compared to the mock group (**Figure 1F**), suggesting a reduction in active cell divisions.

Histochemical analysis of transcriptional and translational GUS reporters showed that MpDELLA is broadly expressed in the thallus, but natively expressed MpDELLA protein preferentially accumulated in the apical notch region, where cell division actively occurs (**Figures 1G** and **S1J**). This result is comparable with the observations in Arabidopsis showing that DELLA proteins are expressed in the shoot and root apical meristems (Ubeda-Tomás et al. 2009; Shani et al. 2013; Serrano-Mislata et al. 2017). Therefore, Mp*DELLA* may also restrict growth by inhibiting cell proliferation in the meristematic regions of *M. polymorpha*.

Cyclin-dependent kinase inhibitors (CKIs) have been shown to participate in the DELLA-mediated decrease of cell proliferation in Arabidopsis (Achard et al. 2009; Serrano-Mislata et al. 2017). The *M. polymorpha* genome contains two CKI genes, Mp*SMR* (Mp1g14080) and Mp*KRP* (Mp3g00300), which belong to the plant-specific SIAMESE (SIM) protein family and the conserved Kip-related proteins (KRP), respectively (Bowman et al. 2017). To test genetically if MpDELLA acts through MpSMR to control thallus size, we introduced Mp*SMR* loss-of-function mutations into a Mp*DELLA-GR* line using the CRISPR/Cas9 system (Sugano et al. 2018) (**Figure S1K**) and examined growth in the absence and the presence of DEX. Gemmallings carrying the Mp*smr* alleles were moderately larger than the wild type in mock conditions. More importantly, the growth inhibition caused by activation of Mp*DELLA-GR* was attenuated in the Mp*smr*^{ge} mutants (**Figure 1G**), supporting the functional relevance of cell division in Mp*DELLA*-mediated growth restriction. Mp*smr* alleles did not fully abolish the response to DEX induction, indicating that Mp*DELLA* might also suppress cell proliferation through additional pathways. Taken together, these results suggest that the regulation of plant size through the interference with cell division is a shared DELLA function in land plants.

MpDELLA regulates development through physical interaction with MpPIF

Distribution of the *g*Mp*DELLA-GUS* signal was also detectable inside gemmae cups, preferentially in developing gemmae (**Figures 1H** and **S1J**). Interestingly, both constitutive and induced Mp*DELLA* overexpression exhibited a loss of gemma dormancy, revealed by early gemma germination inside the gemma cups (**Figures 2A** and **2B**). This effect on gemma dormancy resembles the capacity to germinate in the dark of Mp*pif*^{ko}, which is a loss-of-function mutant of Mp*PHYTOCHROME INTERACTING FACTOR* (Mp*PIF,* Mp3g17350; Inoue et al. 2016). Indeed, we observed a similar loss of dormancy in gemma cups of Mp*pif*^{ko} (**Figures 2C** and **S2A**). Reciprocally, DEX induction was found to promote gemma germination in darkness in the Mp*DELLA-GR* lines (**Figure S2B**). In addition, Mp*DELLA* overexpressors,

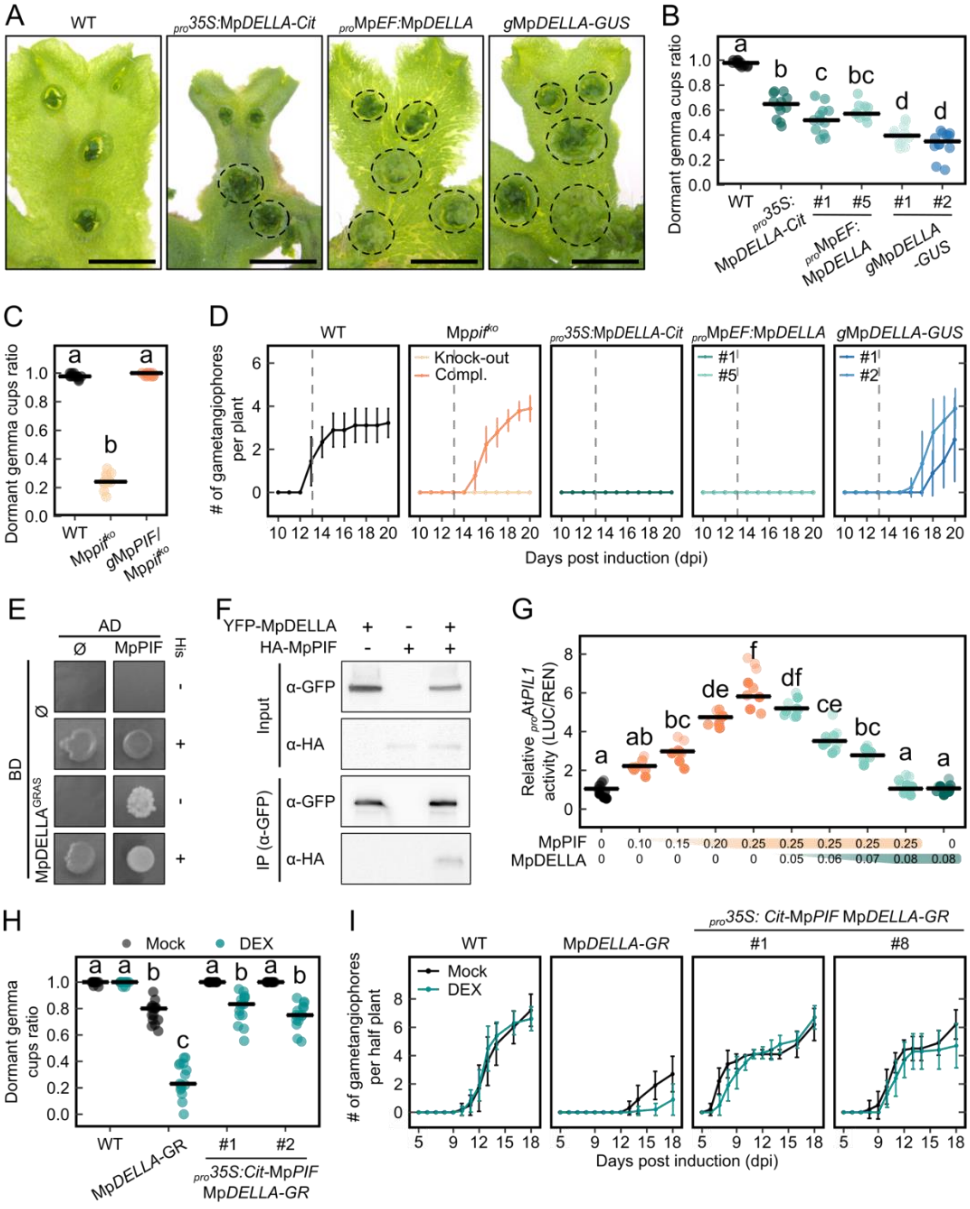

Figure 2. Functional interaction between MpDELLA and MpPIF. See also Figure S2. (A) Gemma dormancy in 28-day-old plants showing premature gemma germination inside the cups of Mp*DELLA* overexpression lines. Dashed circles indicate non-dormant gemma cups. Scale bar, 5 mm. (B and C) Proportion of dormant gemma cups in 28-day-old MpDELLA overexpressors (B) or Mp*pif*^{ko} mutants (C). n=12. (D) Progress of gametangiophore formation in Mp*pif*^{ko} mutants and Mp*DELLA* overexpression lines, after induction with far-red light. n=9. (E) Physical interaction between MpDELLA and MpPIF shown by yeast two-hybrid assay. BD and AD denote the fusions to the GAL4 DNA binding domain or the activation domain, respectively. (F) Physical interaction between YFP-MpDELLA and HA-MpPIF shown by co-immunoprecipitation after agroinfiltration in *N. benthamiana* leaves. (continues in next page)

including *g*Mp*DELLA-GUS*, displayed a significant delay in the induction of sexual reproduction (**Figure 2D**), which has also been observed in Mp*pif*^{ko} (Inoue et al. 2019). These similarities indicate a possible functional connection between Mp*DELLA* and Mp*PIF*, which has been previously reported in Arabidopsis for the regulation of apical hook formation and other developmental processes (Feng et al. 2008; de Lucas et al. 2008; Gallego-Bartolomé et al. 2011).

In Arabidopsis, DELLA proteins interact physically with the PIF transcription factors and prevent their binding to downstream targets (Feng et al. 2008; de Lucas et al. 2008). It is likely that this mechanism is also conserved in *M. polymorpha*, since we observed that MpDELLA interacts physically with MpPIF in a yeast two-hybrid assay and *in vivo* by co-immunoprecipitation, and also by Bimolecular Fluorescence Complementation assays (**Figures 2E**, **2F** and **S2C**). Further MpPIF deletion analyses suggested that the GRAS domain of the MpDELLA protein specifically interacts with the bHLH domain of MpPIF (**Figures S2D** and **S2E**), paralleling results seen in Arabidopsis (de Lucas et al. 2008). The inhibitory effect of the interaction was verified by dual-luciferase transactivation assays in tobacco. In a dose-dependent manner, MpDELLA inhibited the MpPIF-activation of the At*PIL1* promoter (**Figure 2G**), a known direct target for PIFs in Arabidopsis (Zhang et al. 2013).

To assess the biological relevance of the interaction between MpDELLA and MpPIF, we tested if an increase in the dosage of MpPIF would suppress the phenotypical defects caused by high MpDELLA levels. Indeed, the reduction of gemma dormancy in gemma cups caused by MpDELLA induction was notably attenuated in $_{pro}$*35S*:*Cit*-Mp*PIF* Mp*DELLA-GR* plants (**Figures 2H** and **S1B-C**). Similarly, gemma germination in darkness and the delay in gametangiophore formation of Mp*DELLA-GR* plants were significantly suppressed in the presence of higher Mp*PIF* levels, both in the absence and presence of DEX treatments (**Figures 2I** and **S2F**). No rescue of plant growth by elevated Mp*PIF* levels was observed in the double overexpression lines (**Figure S2G**). Given the normal vegetative growth of Mp*pif*^{ko} (Inoue et al. 2016), the cell-cycle-repressing function of MpDELLA does not appear to be mediated by MpPIF. These results suggest that DELLA/PIF-mediated modulation of

Figure 2. (continued) (G) Transient expression assay of the At*PIL1:LUC* reporter in *N. benthamiana* leaves after agroinfiltration with different levels of Mp*PIF* and Mp*DELLA* (shown below the graph as infiltrated OD_{600}). n=9 in total. (H) Quantification of gemma cup dormancy in 30-day-old $_{pro}$*35S*:Mp*PIF-Cit* Mp*DELLA-GR* plants, after treatment with mock or 1 µM DEX for 20 days. (I) Progress of gametangiophore formation in $_{pro}$*35S*:Mp*PIF-Cit* Mp*DELLA-GR* plants, induced with far-red light and treated with mock or 1 nM DEX. n=10. In A, B and C, plants were grown on ½ Gamborg's B5 plates with 1% sucrose under cW. In B, C, and H, dots represent individual plants. In G dots represent biological replicates from three independently performed experiments. All horizontal lines represent total mean values. Statistical groups are determined by Tukey's Post-Hoc test ($p<0.05$) following ANOVA analysis. In D and I, error bars represent standard deviation.

developmental programs could be a conserved mechanism that was already present in the common ancestor of land plants.

MpDELLA promotes flavonoid accumulation and oxidative stress tolerance

To investigate the downstream targets of the MpDELLA-MpPIF module, we analysed the transcriptomic changes in $_{pro}$*35S*:Mp*DELLA-Cit* line and the Mp*pif*^{ko} mutant. As MpPIF proteins are stabilized by far-red light (Inoue et al. 2016), Mp*pif*^{ko} plants were evaluated at 0, 1, or 4 hours after far-red light irradiation (see STAR Methods). Mp*DELLA* overexpression caused the upregulation of 1483 genes and the downregulation of 560 genes (**Figure 3A** and Data S1). The analysis of differential gene expression in the Mp*pif*^{ko} mutant yielded a total of 339 and 333 genes, up- and down-regulated by at least at one time point, respectively (Data S1). As expected, the most abundant set of Mp*PIF*-regulated genes was obtained after the 4-hour

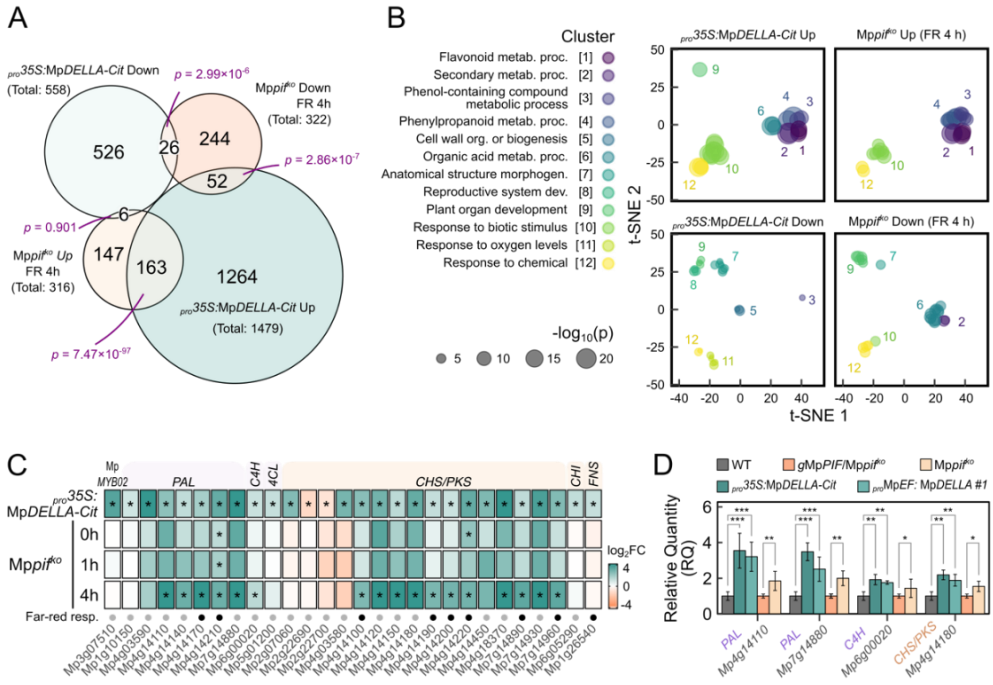

Figure 3. Genome-wide co-regulation of gene expression by MpPIF and MpDELLA. See also Figure S3 and Data S1. (A) Venn diagram showing genes differentially expressed in $_{pro}$*35S*:Mp*DELLA-Cit* and the Mp*pif*^{ko} mutant (after 4 hours of far-red light irradiation). P-values were calculated by Fisher's exact tests. (B) Two-dimensional t-SNE plot visualizing GO categories over-represented in the sets of genes differentially expressed in $_{pro}$*35S*:Mp*DELLA-Cit* and in Mp*pif*^{ko}. (C) Heatmap showing gene expression changes of the flavonoid biosynthesis pathway. Asterisks indicate genes considered as significantly changed (|log2FC|>1; adjusted p<0.01). Black dots in the bottom row indicate genes significantly changed in response to FR irradiation at any time point in either WT or Mp*pif*^{ko}. (D) Expression level of selected flavonoid biosynthesis genes determined by RT-qPCR. Error bars represent standard deviation. n=3. *, p<0.05; **, p<0.01 by Student's t-test.

far-red treatment (**Figure S3A**), so we used this set for further analyses. More than half of the upregulated genes in Mp*pif*^{ko} were also upregulated in $_{pro}$*35S*:Mp*DELLA*, and there was a statistically significant overlap also among genes downregulated in both genotypes (**Figure 3A**), indicating a strong correlation between Mp*DELLA* overexpression, and loss of Mp*PIF* functions. As a confirmation, three differentially expressed genes were tested by qPCR, and they all showed expression changes consistent with the RNA-seq (**Figure S3B**).

Gene ontology enrichment analysis highlighted the regulation of stress response and secondary metabolism processes in both $_{pro}$*35S*:Mp*DELLA-Cit* and Mp*pif*^{ko} up-regulated datasets, especially with the enrichment of terms involving phenylpropanoid and flavonoid biosynthesis (**Figure 3B** and Data S1). In particular, many genes encoding PHENYLALANINE AMMONIA LYASE (PAL), CINNAMATE 4-HYDROXYLASE (C4H) or CHALCONE SYNTHASE (CHS) were indeed upregulated, as also confirmed by qPCR (**Figures 3C** and **3D**). In the case of Mp*pif*^{ko}, the observed net upregulation of flavonoid biosynthesis genes was mainly due to far-red-induced downregulation in the wild type (**Figure S3C**), suggesting a suppressive role for MpPIF.

Staining with diphenylborinic acid 2-aminoethyl ester confirmed the increased accumulation of flavonoid compounds caused by Mp*DELLA* overexpression or Mp*PIF* loss-of-function (**Figures 4A** and **S4A**). Furthermore, the Mp*DELLA*-induced increase of flavonoid signals was less evident when Mp*PIF* is also overexpressed in Mp*DELLA-GR* (**Figure S4B**). Quantitative analysis of flavonoids showed increases in luteolin 7'-O-glucuronide, and 4',7-dihydroxyflavan-3-ol in Mp*DELLA* overexpressors and Mp*pif*^{ko} plants (**Figure S4C** and Table S1). Similar to other plants, increased flavonoid biosynthesis is shown as a protective response against UV-B induced oxidative stress in *M. polymorpha* (Clayton et al. 2018). The ability to enhance the production of these antioxidant compounds suggests a general function for MpDELLA in stress response, which might be fulfilled in coordination with its inhibition of MpPIF.

To test if MpDELLA levels influence the response to oxidative stress, we examined the tolerance of plants overexpressing Mp*DELLA* to methylviologen (MV), an inducer of oxidative stress (Babbs et al. 1989). Six-day-old gemmallings were transferred to plates containing MV for 10 days, after which they were allowed to recover. All Mp*DELLA* overexpressing plants, including those with the native Mp*DELLA* promoter, showed a significantly higher survival rate compared to wild-type plants (**Figure 4B**). These results suggest that the increased production of flavonoids caused by higher Mp*DELLA* levels could be responsible for the protection against oxidative stress. This hypothesis is further supported by the observation that Mp*pif*^{ko} mutants also displayed enhanced resistance to MV (**Figure 4B**), and that the Mp*DELLA*-dependent tolerance was attenuated by Mp*PIF* overexpression (**Figure S4D**).

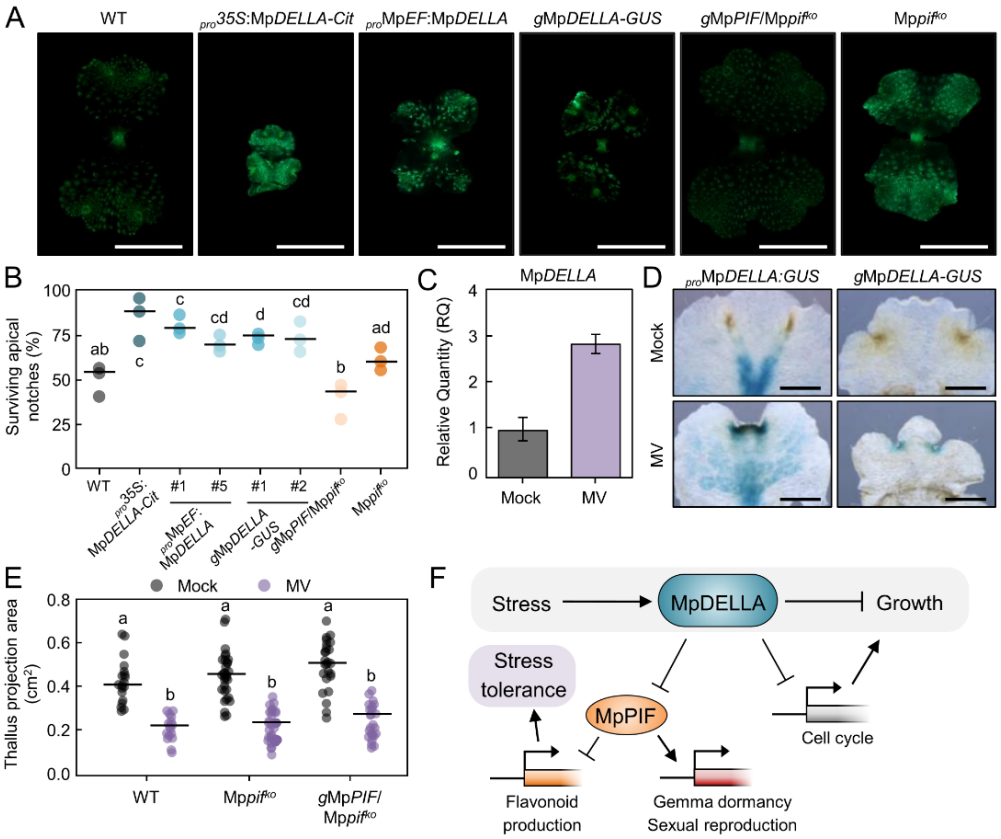

Figure 4. Involvement of Mp*DELLA* in the response to oxidative stress. See also Figure S4 and Table S1.
(A) Images of 14-day-old gemmalings, stained with diphenylboric acid 2-aminoethyl ester to show general flavonoid content. Scale bar, 5 mm. (B) Percentage of surviving apical notches after a 10-day treatment with 100 µM MV in different transgenic lines. Dots represent independent experiments (n=3). (C) Relative expression level of Mp*DELLA* determined by RT-qPCR in 14-day-old gemmallings grown in mock or 10 µM MV-supplemented media. Error bars represent standard error; n=3. (D) GUS-stained Mp*DELLA* reporter lines showing the increased signals in the apical notches of 13-day-old plants after a 6-day treatment with mock or 10 µM MV. (E) Size of 14-day-old gemmallings grown on mock or 0.5 µM MV-supplemented medium. n=19 (WT Mock), 16 (WT MV), 36 (Mp*pif*^{ko} Mock), 28 (Mp*pif*^{ko} MV), 25 (*g*Mp*PIF*/Mp*pif*^{ko} Mock), 24 (*g*Mp*PIF*/Mp*pif*^{ko} MV). (F) Model for the regulation of growth, development and stress responses by Mp*DELLA*. Under stress, MpDELLA would accumulate in apical notches protecting them through the MpPIF-dependent production of antioxidant compounds, and suppressing growth by inhibiting cell divisions. The interaction with MpPIF also causes alterations in developmental processes, such as gemma dormancy or gametangiophore formation. All plants were grown under long-day conditions. In B, E, dots represent biological replicates, and the horizontal lines represent mean values. Statistical groups were determined by Tukey's Post-Hoc test ($p<0.05$) following ANOVA analysis.

In Arabidopsis, the role of DELLAs in the coordination between growth and stress responses is visualized by GA reduction and accumulation of DELLA in response to certain types of stress, coupled to increased tolerance and a variable degree of growth impairment

(Achard et al. 2008). Although *M. polymorpha* does not possess a GID1-like GA receptor that might modulate MpDELLA protein stability, we found that exposure of 10-day-old gemmallings to MV provoked an increase in Mp*DELLA* gene expression (**Figure 4C** and **4D**). Such MpDELLA accumulation was concomitant with marked growth arrests and reduced cell division (**Figures 4E** and **S4E**). Interestingly, Mp*pif*^{ko} mutants were as large as wild-type plants both in the absence and in the presence of MV (**Figure 4E**), confirming that the control of *M. polymorpha* thallus size is largely independent of Mp*PIF*.

In summary, we have shown that MpDELLA can modulate cell division, developmental responses and tolerance to oxidative stress in *M. polymorpha* through molecular mechanisms that are shared with angiosperms (**Figure 4F**). That our results reflect the function of the endogenous MpDELLA protein is supported by the following observations: (i) mild overexpression from the native promoter caused similar phenotypic effects as constitutive and ectopic overexpression; (ii) local induction of MpDELLA-GR in apical notches (where endogenous MpDELLA accumulates) caused growth impairment (**Figure S4F-H**); and (iii) the effect of MpDELLA accumulation on growth is dose-dependent. The involvement of MpDELLA in growth control is in contrast with the previous proposal that this function emerged with vascular plants, based on the phenotype of *P. patens della* mutants (Yasumura et al. 2007). However, this might reflect a specific functional loss in mosses, given that PpDELLAa still retains the capacity to impair growth in particular contexts, as through heterologous expression in Arabidopsis (Yasumura et al. 2007). Thus, the functional conservation between angiosperm and bryophyte DELLAs implies that the role in the optimization of growth and the responses to disadvantageous environments was already encoded in the ancestral land plant DELLA protein, and the canonical GA signaling might have simply hijacked these functions when the pathway emerged in vascular plants.

STAR Methods

RESOURCE AVAILABILITY

Data and code availability

Raw RNA sequencing datasets generated during this study were deposited to the Short Read Archive at the National Center for Biotechnology Information (NCBI) or the Sequence Read Archive at DNA Data Bank of Japan (DDBJ) under Bioprojects PRJNA695248 and PRJDB11176. The modified ITCN plugin for ImageJ is available at https://github.com/PMB-KU/CountNuclei. R scripts used for processing EdU data, Blast2GO annotation and RNA-seq analysis were deposited to https://github.com/dorrenasun/MpDELLA.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Plant materials and growth conditions

Marchantia polymorpha accession Takaragaike-1 (Tak-1; male) (Ishizaki et al. 2008) was used in this study as the wild-type (WT). Female lines Mp*pif*^{ko} and *g*Mp*PIF*/Mp*pif*^{ko} were previously described as *pif*^{KO} #1 and $_{pro}$*PIF:PIF*/*pif*^{KO} #1, respectively(Inoue et al. 2016). *M. polymorpha* plants were cultured on half-strength Gamborg's B5 (Gamborg et al. 1968) medium with 1% agar at 21-22ºC. Light conditions are specified in each figure; generally, long day (LD) conditions refer to cycles with 16 hours of light (90-100 µmol m^{-2} s^{-1}) and 8 hours of darkness, while continuous white light (cW) was supplemented at the intensity of 50-60 µmol m^{-2} s^{-1}.

METHOD DETAILS

Cloning and generation of transgenic *M. polymorpha* plants

Various Gateway-compatible entry vectors related to Mp*DELLA* were generated. The full length CDS, GRAS domain (amino acids 173 to 560), promoter (4.3kb upstream of ATG), and genomic (promoter and CDS) regions were amplified from genomic DNA by PCR using Phusion High-Fidelity Polymerase (Thermo Fisher Scientific) with attB-containing primers, and introduced into pDONR221 (Thermo Fisher Scientific) vector using Gateway BP Clonase II Enzyme mix (Thermo Fisher Scientific) to generate pENTR221-MpDELLA, -MpDELLA^{GRAS}, -$_{pro}$MpDELLA and -gMpDELLA, respectively. The CDS region was also amplified with KOD FX Neo DNA polymerase (Toyobo Life Science) and directionally cloned into pENTR/D-TOPO (Thermo Fisher Scientific) to create pENTR-MpDELLA. For pENTR1A-$_{pro}$MpDELLA-short, a slightly shorter promoter region was amplified with PrimeSTAR GXL DNA Polymerase (TaKaRa Bio) and inserted between the *Sal*I and *Not*I sites of pENTR1A (Thermo Fisher Scientific) with T4 DNA ligase (TaKaRa Bio). pENTR1A-gMpDELLA-short was further created by seamless integration of the CDS fragment with the In-Fusion Cloning kit (TaKaRa Bio). Finally, both constructs were extended at the 5' end by In-Fusion insertion to create pENTR1A-$_{pro}$MpDELLA and pENTR1A-gMpDELLA, matching with the lengths of pENTR221 counterparts. To create the vectors for Mp*DELLA* overexpression, pENTR221-MpDELLA and pENTR211-gMpDELLA were recombined with pMpGWB106 and pMpGWB107 (Ishizaki et al. 2015) using Gateway LR Clonase II Enzyme mix (Thermo Fisher Scientific) to generate pMpGWB106-MpDELLA and pMpGWB107-gMpDELLA, respectively. pENTR-MpDELLA was recombined with pMpGWB310 and pMpGWB313 for the generation of pMpGWB310-MpDELLA and pMpGWB313-MpDELLA, while pENTR1A-$_{pro}$MpDELLA and pENTR1A-gMpDELLA were recombined with pMpGWB304 to generate pMpGWB304-$_{pro}$MpDELLA and pMpGWB304-gMpDELLA. All these binary vectors were introduced into Tak-1 plants.

To monitor the cell division activity, the promoter (3.8 kb upstream of ATG) and coding sequence of the first 116 amino acids (including the destruction box) of Mp*CYCB;1* (*Mp5g10030*) was amplified with KOD -Plus- Ver.2 (Toyobo Life Science) and ligated into the the *Sal*I and *Eco*RV sites of pENTR1A (Thermo Fisher Scientific) with Ligation high Ver.2 (Toyobo Life Science). The resulting plasmid was recombined with pMpGWB104 and then transformed into the *M. polymorpha* transgenic line Mp*DELLA-GR* #5.

CRISPR/Cas9-based genome editing of Mp*DELLA* and Mp*SMR* (*Mp1g14080*) was performed as previously described (Sugano et al. 2014). For Mp*DELLA*, various guide RNAs were designed in the coding sequence and the 5'-untranslated region. For Mp*SMR*, the guide RNA was designed upstream of the CDKI domain with Benchling (Anon 2021). Double stranded DNA corresponding to the guide RNA protospacers were generated by annealing complementary oligonucleotides and inserted into *Bsa*I-digested pMpGE_En03 (Sugano et al. 2014) by ligation using DNA T4 ligase (Promega), and then transferred to the binary vector pMpGE010 (Sugano et al. 2014) using Gateway LR Clonase II Enzyme mix (Thermo Fisher Scientific). *M. polymorpha* transformation was carried out in Tak-1 for Mp*DELLA*, or the transgenic line Mp*DELLA-GR* #5 for Mp*SMR*. The targeted loci were examined by sequencing from crude G1 DNA samples and confirmed in G2 plants.

For the construction of Mp*PIF*-Mp*DELLA* double overexpressors, the Mp*PIF* (*Mp3g17350*) coding region containing the stop codon was amplified from cDNA, cloned into pENTR/D-TOPO (Thermo Fisher Scientific), then recombined with pMpGWB105 using Gateway LR Clonase II Enzyme mix (Thermo Fisher Scientific). The resulting construct was transformed into the *M. polymorpha* transgenic line Mp*DELLA-GR* #5.

All the *M. polymorpha* transgenic lines are listed in the Key Resources Table. Transformants were obtained by agrobacterium-mediated transformation from regenerating thalli, using *Agrobacterium tumefaciens* strains GV3101 (pMP90 C58) or GV2260 (Kubota et al. 2013).

Plant growth and EdU analysis

For the measurement of plant sizes, images of the whole culturing plates were taken vertically above with a digital camera (Canon EOS Kiss X7i). The thallus projection areas were analysed with ImageJ 1.52a (Schneider et al. 2012) by thresholding the images with the default algorithm on the blue colour channel and batch-measured with the function "Analyse Particles".

For the detection of S-phase cells, constitutive- and native-promoter MpDELLA overexpressors were grown from gemmae for seven days under cW. Mp*DELLA-GR* lines were

grown from gemmae under cW for five days, then transferred onto the plates containing mock solvent or 1 µM dexamethasone (DEX) and cultured for two days. All the plants were labelled with 20 µM 5-ethynyl-2'-deoxyuridine (EdU) in liquid half-strength Gamborg's B5 medium under cW for 2 h. Then they were fixed with 3.7% formaldehyde for 1 h, washed for 5 min twice with phosphate buffer saline (PBS), and permeabilised in 0.5% Triton X-100 in PBS for 20 min. After two 5-min washes with 3% bovine serum albumin (BSA) in PBS, samples were incubated with the reaction mixture from Click-iT EdU Imaging Kit with Alexa Fluor 488 (Invitrogen) in the dark for 1 h. After staining, samples were protected from light, washed twice with 3% BSA in PBS and soaked in ClearSee solution (Kurihara et al. 2015) for 3-7 days. After that, the samples were mounted to slides with 50% glycerol and observed with Keyence BZ-X700 all-in-one fluorescence microscope.

Z-stacks of fluorescence images were taken in 2-µm steps with the YFP filter (Keyence 49003-UF1-BLA, excitation at 490-510 nm, detection range 520-550 nm) and merged together with the BZ-X Analyzer software (1.3.1.1). EdU-labelled nuclei were marked and counted with a modified version of the ITCN plugin (Kuo and Byun) in ImageJ 1.52a (Schneider et al. 2012). Spatial coordinates for the nuclei were exported and processed with R scripts (R-Core-Team 2020) to calculate density maps using the *spatstat* package (Baddeley and Turner 2005). Actively dividing area was measured as with nucleus densities higher than 0.001 μm^{-2}. See Key Resources Table for the depository of plugins and scripts used.

Microscopy & histochemical analysis

For GUS activity assay, plants were vacuum-infiltrated with GUS staining solution (50 mM sodium phosphate buffer pH 7.2, 0.5 mM potassium-ferrocyanide, 0.5 mM potassium-ferricyanide, 10 mM EDTA, 0.01% Triton X-100 and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) for 15 min, and then incubated at 37 ºC overnight (>16 hours). Samples were de-stained with 70% ethanol and imaged under an Olympus SZX16 stereoscope. To prepare agar sections, stained samples were embedded in 6% agar and sectioned into 100-µm slices with LinearSlicer PRO 7 (DOSAKA EM, Kyoto, Japan), then imaged with Keyence BZ-X700 microscope in the bright-field.

Confocal laser scanning microscopy on *g*Mp*DELLA-Cit* gemma was performed using a Leica TCS SP8 equipped with HyD detectors. A white light laser was used to visualize Citrine (excitation 509 nm). Diphenylborinic acid 2-aminoethyl ester (DPBA) staining was used to visualize flavonoids as previously described (Peer et al. 2001). Whole thalli were stained for 15 minutes at 0.25% (w/v) DPBA and 0.1% (v/v) Triton X-100. Epifluorescence microscopy of stained flavonoids in gemmallings was performed on a Leica DMS1000 dissecting microscope using a GFP filter for detection of DPBA fluorescence.

Scoring of gemma cup dormancy

To score the dormancy of gemma cups, constitutive- and native-promoter Mp*DELLA* overexpressors, as well as Mp*pif*^{ko} plants were grown from gemmae on half-strength Gamborg's B5 plates with 1% sucrose under cW for 28 days. Mp*DELLA-GR* and $_{pro}$*35S:Cit*-Mp*PIF* Mp*DELLA-GR* lines were grown on sugar-free half-strength Gamborg's B5 plates under cW for 10 days, then transferred onto plates containing mock solvent or 1 μM DEX and cultured for another 20 days before evaluation. Gemma cups with observable gemmae were observed carefully under stereoscopes, marked on photos taken with a digital camera and then counted. If a gemma with rhizoid and/or growth expansion was observed in a certain gemma cup, it is considered as non-dormant. Representative plants were also photographed with Leica M205C stereo microscopes to show the dormancy of gemma cups in different transgenic lines.

Gemma germination assay

Gemma germination assays were carried out following the previous publication (Inoue et al. 2016). In each experiment, fifty gemmae of each group were planted onto half-strength Gamborg's B5 plates containing 1% sucrose under green light, then treated with far-red light (30 μmol photons m^{-2} s^{-1}) for 15 minutes. For Mp*DELLA-GR* related experiments, 1 μM DEX or the mock solvent was supplemented in the agar plates. After one day of imbibition in the dark, gemmae were irradiated with nothing or a pulse of red light (4500 μmol photons m^{-2}) and then cultured for another six days in the dark. Photos of each gemmae were taken using Leica M205C or Olympus SZX16, then evaluated for germination based on growth expansion and/or the development of rhizoids.

Gametangiophore formation observation

To monitor the progress of gametangiophore formation in transgenic lines shown in Figure 2B, plants were grown from gemmae on half-strength Gamborg's B5 plates with 1% sucrose under continuous white light supplemented with far-red light (~20 μmol photons m^{-2} s^{-1}, cW+FR). Individual plants were examined and counted for visible gametangiophores each day under stereoscopes. For the experiment in Figure 2I, gemmae of inducible lines or the wild-type control were grown on DEX-free half-strength Gamborg's B5 plates with 1% sucrose under cW for 10 days, then half pieces of thallus were transferred onto the plates containing 1 nM DEX or mock solvent and cultured under cW+FR. Gametangiophore formation progresses were observed for half plants similarly as described above.

Yeast-two hybrid assays

For yeast two-hybrid analyses, Mp*PIF* full length CDS and CDS fragments were amplified from cDNA and introduced into pCR8 using the pCR8/GW/TOPO TA Cloning Kit (Thermo Fisher Scientific) to generate pCR8-MpPIF and -MpPIFdel1-4. Then they were recombined with pGADT7-GW (Rossignol et al. 2007) using Gateway LR Clonase II Enzyme mix (Thermo Fisher Scientific) to produce Gal4-activation domain (AD) fusion proteins. To avoid the previously shown N-terminal transactivation of MpDELLA (Hernández-García et al. 2019), only the GRAS domain (pENTR221-MpDELLA^{GRAS}) was introduced into pGBKT7-GW(Rossignol et al. 2007) to fuse with the GAL4 DNA-binding domain. Yeast transformation was performed by lithium acetate/single-stranded carrier DNA/polyethylene glycol method as previously described. Y187 and Y2HGold yeast strains were transformed with pGADT7 and pGBKT7-derived expression vectors and selected with Synthetic Defined (SD) medium lacking leucine (-Leu) or tryptophan (-Trp), respectively. Subsequently, haploid yeasts were mated to obtain diploid cells by selection in SD/-Leu-Trp medium. Protein interactions were assayed by the nutritional requirement on histidine (His). SD/-Leu-Trp plates were used as growth control, and SD/-Leu-Trp-His plates supplemented with 5 mM 3-amino-1,2,4-triazole (3-AT, Sigma-Aldrich) was used for interaction evaluation. Spotting assays were performed using cultures with optical density = 1 at a wavelength of 600 nm (OD_{600} = 1) as initial concentration in sequential drop dilutions, and plated with a pin multi-blot replicator. Photos of the same-fold dilutions were taken 3 days after plating.

Co-immunoprecipitation (Co-IP) and Bimolecular Fluorescence Complementation (BiFC) assays

Co-IP vectors were obtained by introducing Mp*DELLA* CDS (pENTR221-Mp*DELLA*) into pEarleyGate104 and MpPIF fragments (pCR8-Mp*PIF* and -Mp*PIFdel3*) into pEarleyGate201 (Earley et al. 2006). For BiFC, pENTR211-Mp*DELLA* and pCR8-Mp*PIF* were recombined with pMDC43-YFN and pMDC43-YFC (Belda-Palazón et al. 2012), respectively. *Agrobacterium tumefaciens* GV3101 containing binary plasmids for Co-IP and BIFC were used to infiltrate 4-week-old *Nicotiana benthamiana* leaves.

For Co-IP, leaves were re-infiltrated with a solution of 25 μM MG-132 8 hours before collection 3 days after *A. tumefaciens* infiltration, grinded in liquid nitrogen and homogenized in 1 ml extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Triton, 2 mM PMSF, and 1x protease inhibitor cocktail [Roche]). Proteins were quantified using the Bradford assay. 50 μg of total proteins were stored as input. One milligram of total proteins was incubated for 2 h at 4ºC with anti-GFP-coated paramagnetic beads and loaded onto μColumns (Miltenyi). Wash and elution from beads was performed according to manufacturer's instructions.

Samples were analysed by Western-blot after running two 12% SDS-PAGE gels in parallel. One gel was loaded with 25 µg of input, and 10% of eluted proteins; following wet transfer, the PVDF membrane was incubated with an anti-GFP antibody (JL8, 1:5000). The second gel was loaded with 25 µg of input, and 90% of eluted proteins and, after transfer, the membrane incubated with an anti-HA-HRP antibody (3F10, 1:5000). Chemiluminiscence was detected with SuperSignal West Femto substrates (Thermo-Fisher Scientific) and imaged with a LAS-3000 imager (Fujifilm).

For BiFC, leaves were analysed with a Zeiss LSM 780 confocal microscope. Reconstituted YFP signal was detected with emission filters set to 503-517 nm. Nuclei presence in abaxial epidermal cells was verified by transmitted light.

Dual luciferase transactivation assay

Mp*DELLA* and Mp*PIF*-expresssing vectors used for Co-IP (pEarleyGate104-MpDELLA and pEarleyGate201-MpPIF) were used as effector plasmids. A previously available construct with the *Arabidopsis thaliana PIL1* promoter controlling the firefly luciferase gene expression, and a constitutively expressed *Renilla* luciferase gene was used as reporter plasmid(Zhang et al. 2013). The promoter consists of 1.8 kb upstream of the gene ATG codon, including three consecutive G-boxes known to be bound *in vivo* by PIF3. Transient expression in *N. benthamiana* leaves was carried by agroinfiltration as previously reported (Marín-de la Rosa et al. 2015). The amount of infiltrated bacteria was set by OD_{600} measurement of *A. tumefaciens* liquid cultures. Combinations of pre-set reporter-carrying bacteria (OD600 = 0.1) and varying amounts of effector-carrying bacteria were mixed and co-infiltrated together. All the mixes were co-infiltrated alongside *p19* vector-carrying bacteria at a OD_{600} = 0.01. Firefly and the control *Renilla* luciferase activities were assayed in extracts from 1-cm in diameter leaf discs, using the Dual-Glo Luciferase Assay System (Promega) and quantified in a GloMax 96 Microplate Luminometer (Promega). Three leaf disc extracts were quantified per sample in each experiment and repeated for three times. Final quantifications represent means of ratios between firefly luciferase and *Renilla* luciferase read-outs in three independent experiments.

RNA isolation, cDNA synthesis, and qRT-PCR analysis

To examine the expression levels of Mp*DELLA* and Mp*PIF* in different transgenic lines, 14-day-old plants grown under cW were homogenized in liquid nitrogen. Total RNA was isolated with TRIzol reagent (Thermo Fisher Scientific) following the manufacturer's instructions. After checking the concentration and quality of RNA using a NanoDrop 2000 spectrophotometer (Thermo Scientific), up to 3 µg of total RNA was digested with RQ1 RNase-Free DNase (Promega) and reverse-transcribed using ReverTra Ace (Toyobo Life Science). Quantitative real-time PCR (qPCR) was performed in a CFX96 real-time PCR detection system (Bio-Rad)

using TaKaRa Ex Taq (TaKaRa Bio) and SYBR Green I Nucleic Acid Gel Stain (Lonza).

For other qPCR experiments, total RNA was extracted with a RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was prepared from 1 µg of total RNA with PrimeScript 1st Strand cDNA Synthesis Kit (Takara Bio Inc). PCR was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems) with SYBR premix ExTaq (Tli RNaseH Plus) Rox Plus (Takara Bio Inc).

All relative expression levels were calculated following Hellemans et al. (2008), and Mp*EF1α* (Mp*ELONGATION FACTOR 1α, Mp3g23400*) was used as the reference gene. Primers are listed in Table S2.

RNA sequencing and data analysis

For the Mp*DELLA* overexpression data set, WT and $_{pro}$*35S:*Mp*DELLA-Cit* plants were grown from gemmae on half-strength Gamborg's B5 plates containing 1% sucrose under LD conditions for 30 days. Then whole plants for two biological replicates were collected for total RNA extraction total RNA with a RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA concentration and integrity [RNA integrity number (RIN)] were measured with an RNA nanochip (Bioanalyzer, Agilent Technologies 2100). Library preparation with TruSeq RNA Sample Prep Kit v.2 (Illumina) and sequencing of 75-nt single-end reads on Illumina NextSeq 550 were carried out at the Genomics Service of the University of Valencia.

For the Mp*pif*^{ko} dataset, Tak-1 and Mp*pif*^{ko} were grown from gemmae on half-strength Gamborg's B5 plates containing 1% sucrose under continuous red-light conditions (50 µmol photons m^{-2} s^{-1}) for 7 days, then irradiated with far-red light (50 µmol photons m^{-2} s^{-1}). Whole plant materials for three biological replicates were collected each at 0, 1, and 4 h after the irradiation. Total RNAs were extracted using RNeasy Plant Mini Kit (Qiagen) and purified with the RNeasy MinElute Cleanup Kit (Qiagen). RNA concentration and qualities were examined by Qubit Assays (Thermo Fisher Scientific) and the Agilent 2100 Bioanalyzer. Libraries were prepared using a TruSeq RNA Sample Prep Kit v.2 (Illumina), quantified by KAPA Library Quantification Kit (Kapa Biosystems), and sequenced of 126-nt single-end reads on Illumina HiSeq 1500 at National Institute for Basic Biology (Okazaki, Japan).

For data processing, reads from both sources were mapped to the *M. polymorpha* reference genome and quantified using Salmon 1.3.0 (Patro et al. 2017). Reads from male lines were mapped to the MpTak1 v5.1 genome (Montgomery et al. 2020), while reads from female Mp*pif*^{ko} plants were mapped to autosome sequences from MpTak1 v5.1 plus the known U-chromosome scaffolds from genome ver 3.1 (Bowman et al. 2017). Differential gene

expressions between sample pairs were analysed with the R package DESeq2 (Love et al. 2014), in which both autosome and V chromosome genes were considered for the Mp*DELLA* overexpression set, while only autosome genes were compared between Tak-1 and Mp*pif*^{ko}. Genes with a minimum fold change of 2 and adjusted p-value smaller than 0.01 were considered as significantly changed in expression. Compared with the wild type, $_{pro}$*35S:*Mp*DELLA-Cit* led to the significant up- and down-regulation of 4 and 2 V-chromosome genes, respectively. The total number of MpTak1 v5.1 autosome genes was used for checking if Mp*DELLA*-regulated genes were enriched in differentially expressed genes caused by Mp*pif*^{ko} using Fisher's exact test. UpSet plots were created using the R package ComplexHeatmap (Gu et al. 2016).

Fuzzy Gene Ontology (GO)(Gene Ontology Consortium 2021) annotations for the v5.1 (plus ver 3.1 U-chromosome) genes were generated using the Blast2GO algorithm (Götz et al. 2008) written in R scripts. Briefly, all *M. polymorpha* reference proteins were blasted (Camacho et al. 2009) against a database containing all UniProtKB (The Uniprot Consortium 2019) entries with non-IEA (inferred from electronic annotation) GO annotations, plus all Swiss-Prot entries (release 2020_05) with an e-value threshold of 0.001. Then the GO annotations from top 25 blast hits for each target protein were scored and concatenated based on their similarity and the GO hierarchy (release 2020-06-01). Annotations with scores higher than the user-defined thresholds (40 for cellular component, 55 for biological process, 50 for molecular function) were transferred to *M. polymorpha* proteins. GO enrichment analysis was conducted with biological process terms with the classic fisher's test from the *topGO* package (Alexa and Rahnenfuhrer 2020). R packages *GO.db* (Carlson 2019), *Rtsne* (Krijthe 2015), *GOSemSim* (Yu et al. 2010; Yu 2020), *rrvgo* (Sayols 2020) and *AnnotationForge* (Carlson and Pagès 2020) were used for the clustering and visualization of top-enriched GO terms. Depositories for the raw sequence datasets, GO annotations and R scripts are listed in the Key Resources Table.

Non-targeted flavonoid-related metabolite profiling

Various Analysis of secondary metabolites in freeze-dried Marchantia samples was carried out following a non-targeted approach as previously described (Zandalinas et al. 2017). Briefly, samples (c.a. 10 mg) were extracted in 80% aqueous MeOH containing biochanin A at 1 mg L^{-1} (Sigma-Aldrich, Madrid, Spain) as internal standard by ultrasonication for 10 min. Crude extracts were centrifuged and clean supernatants recovered and filtered through PTFE 0.2 µm syringe filters directly into dark chromatography vials. Extracts were injected into a UPLC system (10 µL) (Acquity SDS, Waters Corp. Ltd. USA) and separations carried out on a C18 column (Luna Omega Polar, C18, 1.6 µm, 100 × 2.1 mm, Phenomenex, CA, USA) using acetonitrile and ultrapure water, both supplemented with formic acid at a

concentration of 0.1% (v/v), as solvents at a flow rate of 0.3 mL min^{-1}. A gradient elution program starting from 5% to 95% acetonitrile in 17 min followed by a 3 min re-equilibration period was employed. Compounds were detected by mass spectrometry using a hybrid quadrupole time-of-flight mass spectrometer (QTOF-MS, Micromass Ltd., UK) coupled to the UPLC system through an electrospray source. Samples were analysed in both positive and negative electrospray modes within 50-1000 Da mass range using two simultaneous acquisition modes: 1) low CID energy for profiling purposes and 2) high CID energy for MS/MS of selected compounds, this was achieved by setting an energy ramp from 5-60 eV. During measurements cone and capillary voltages were set at 30 V and 3.5 kV, respectively; source and block temperatures were kept at 120ºC. Desolvation gas (N_2) was kept at 350 ºC at a flow rate of 800 L h^{-1}. Nebulization gas was also N_2 at a flow rate of 60 L h^{-1}. In the collision cell, pure Ar was used as the collision gas. Exact mass measurements were achieved by monitoring the reference compound lockmass leucine-enkephalin ($[M+H]^+$ 556.2771 and $[M-H]^-$ 554.2514, respectively); therefore, the resulting mass chromatograms were acquired in centroid mode.

Processing of mass chromatograms was performed with xcms (Smith et al. 2006) after conversion to mzXML with MSConvert (Chambers et al. 2012) using default settings. Chromatographic peak detection was performed using the matchedFilter algorithm, applying the following parameter settings: snr = 3, fwhm = 15 s, step = 0.01 D, mzdiff = 0.1 D, and profmethod = bin. Retention time correction was achieved in three iterations applying the parameter settings minfrac = 1, bw = 30 s, mzwid = 0.05D, span = 1, and missing = extra = 1 for the first iteration; minfrac = 1, bw = 10 s, mzwid = 0.05 D, span = 0.6, and missing = extra = 0 for the second iteration; and minfrac = 1, bw = 5 s, mzwid = 0.05 D, span = 0.5, and missing = extra = 0 for the third iteration. After final peak grouping (minfrac = 1, bw = 5 s) and filling in of missing features using the fillPeaks routine of the xcms package, a data matrix consisting of feature × sample was obtained. When available, identification of metabolites was achieved by comparison of mass spectra and retention time with those of authentic standards or alternatively were tentatively annotated by matching experimental mass spectra in public databases (metlin, Massbank or HMDB). Known and tentative flavonoid-related compounds were chosen for comparison. Before statistical analyses, raw peak area values were normalized to internal standard area and sample weight. Pairwise comparisons were carried out using a two-tailed Student's t-test comparing two groups of samples of identical variance.

Analysis of survival after oxidative stress

For survival measurement, 10 gemmae per genotype and experiment were grown on top of Whatman filter papers discs (Thermo Fisher) on half-strength Gamborg's B5 1% agar medium for 6 days, and then transferred to half-strength Gamborg's B5 1% agar medium

supplemented with 100 µM methylviologen (MV) to produce severe oxidative stress for 10 days. Gemmallings were transferred back to half-strength Gamborg's B5 1% agar medium for recovery. Survival was counted when independent apical regions resumed growth and represented as the percentage of growth-resuming apical regions of the total number at the beginning of the stress treatment.

In Mp*DELLA-GR* related assays, the same procedure was followed, but mock and 1 µM dexamethasone (DEX) were included for DELLA activity induction during the 10 days of oxidative stress treatment. In addition, DEX or mock (ethanol) were added in water solution 24 hours before stress treatment.

SUPPLEMENTAL INFORMATION

Data S1. RNA-seq expression profiles, GO annotations and top-enriched GO terms. Related to Figure 3.
Table S1. Differentially accumulated flavonoid-related metabolites. Related to Figure S4C.
Table S2. List of oligonucleotides, related to the STAR Methods.
Supplementary Figures 1-4.

Supplemental information is available as described at the beginning of this PhD thesis manuscript (**Opening Statement**). Additionally, supplemental figures can be found at the end of this chapter.

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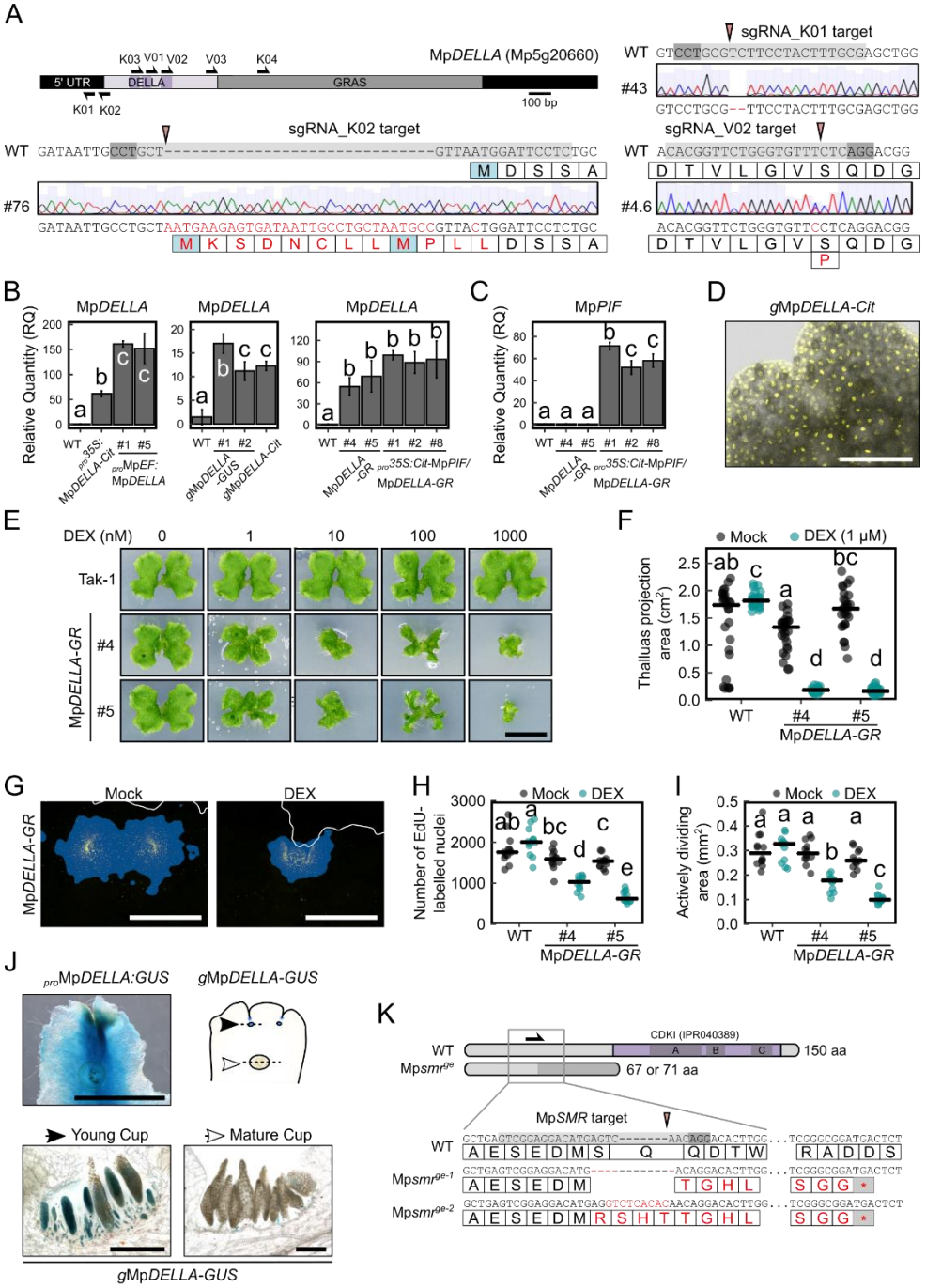
SUPPLEMENTAL FIGURE 1



SUPPLEMENTAL FIGURE 1

Figure S1. Related to Figure 1.

(A) CRISPR-Cas9 genome editing attempts to obtain an Mp*della* mutant. Positions of short guide RNA (sgRNA) targets designed independently in Kyoto (K) or Valencia (V) were indicated in the illustration, and chromatograms showed sequencing data for three mutations obtained. #76 from sgRNA_K02 changed the sequences around the start codon but caused no frameshifts. sgRNA_V02 generated the 1-bp substitution in #4.3 in a chimeric manner and was eventually taken over by the wild-type tissue. The 2-bp deletion with sgRNA_K01 was successful but it did not change the coding sequence.

(B) Relative expression level of Mp*DELLA* determined by RT-qPCR in 14-day-old gemmallings of multiple transgenic lines.

(C) Relative expression level of Mp*PIF* determined by RT-qPCR in 14-day-old gemmallings in $_{pro}$*35S*:Mp*PIF-Cit* Mp*DELLA-GR* lines

(D) Microscopic image of the apical notch of *g*Mp*DELLA-Cit* gemma showing MpDELLA-Cit nuclear localization. Scale bar, 200 µm.

(E) Morphology of Mp*DELLA-GR* plants, grown from gemmae for 14 days with mock or 1 µM DEX. Scale bar, 5 mm.

(F) Measurement of plant sizes in Mp*DELLA-GR* gemmalings, grown from gemmae with mock or 1 µM DEX for 14 days. n=27.

(G) Apical notches of 7-day-old Mp*DELLA-GR* gemmalings, treated with mock or 1 µM DEX for two days and labelled with EdU (yellow signals). Plant boundaries are marked with white lines, and blue color indicates the area occupied by dividing cells. Scale bar, 500 µm.

(H) Number of EdU-labelled nuclei in the apical notches of 7-day-old Mp*DELLA-GR* gemmalings, treated with mock or 1 µM DEX for two days. n=12.

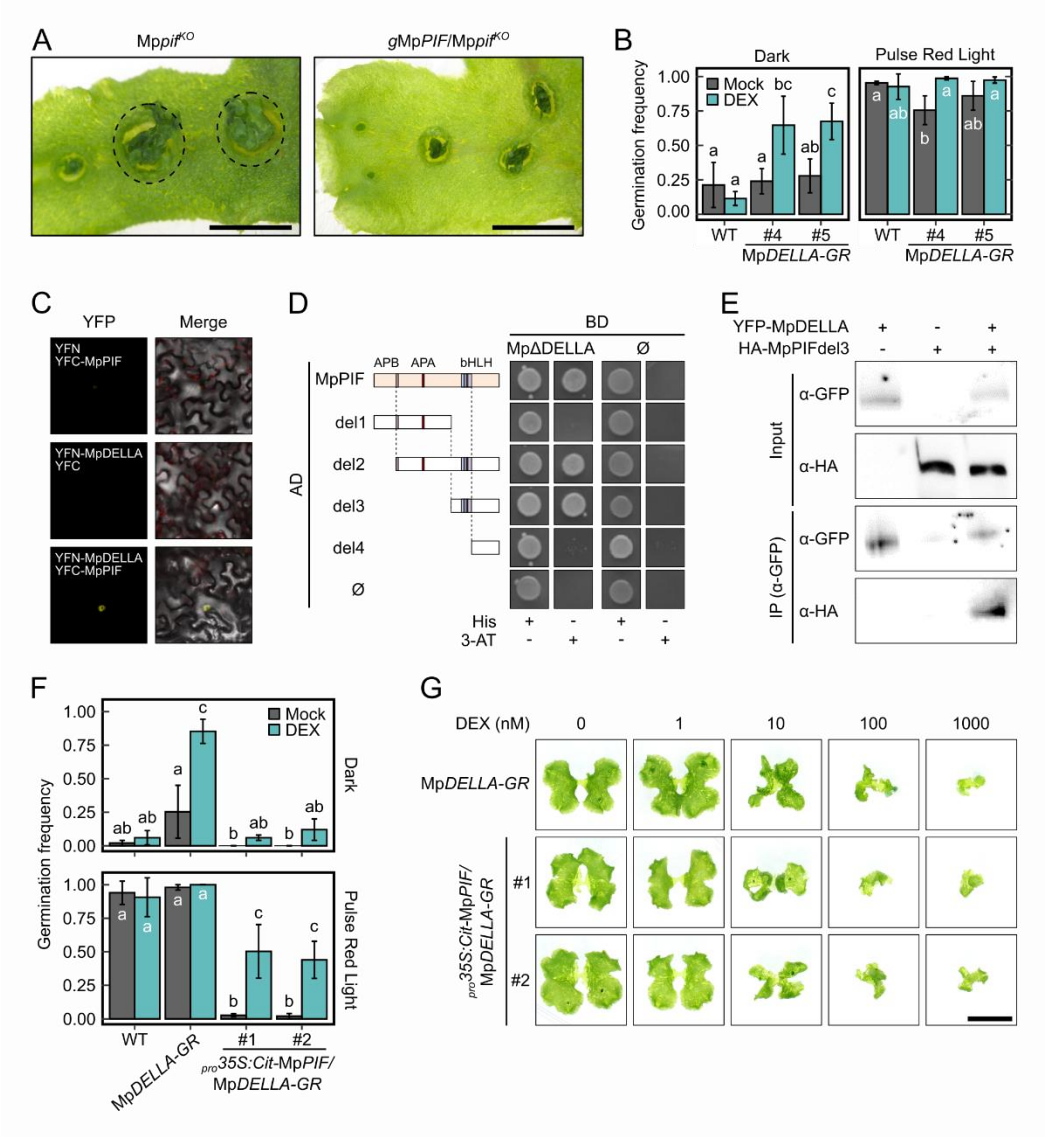
(I) Quantification of actively dividing area in the apical notches of 7-day-old Mp*DELLA-GR* gemmalings, treated with mock or 1 µM DEX for two days. n=12.

(J) GUS-stained thallus of 21-day-old Mp*DELLA* reporter lines, showing the range of promoter activity across the thallus ($_{pro}$Mp*DELLA:GUS*, Scale bar, 5 mm) or MpDELLA protein accumulation in developing (black arrow) and mature (white arrow) gemma cups (agar sectionings of *g*Mp*DELLA-GUS*. Scale bars, 200 µm).

(K) Genotype information for the Mp*smr*^{ge} Mp*DELLA-GR* lines. Predicted protein products from wild-type and genome-edited Mp*SMR* locus were illustrated (purple boxes: CDKI functional domains; dark-grey shade: frameshifts caused by genome editing). Sequences of wild-type and both CRISPR/Cas9-derived alleles were shown in alignments.

In A, B, error bars represent standard error of three biological replicates. In E, F, G, dots represent individual plants and horizontal lines represent mean values. Statistical groups were determined by Tukey's Post-Hoc test ($p<0.05$) following one-way ANOVA.

SUPPLEMENTAL FIGURE 2



SUPPLEMENTAL FIGURE 2

Figure S2. Related to Figure 2.

(A) Images of 28-day-old plants showing premature gemmae germination inside the cups of Mp*pif*^{ko} and the complemented line (*g*Mp*PIF*/Mp*pif*^{ko}). Dashed circles indicate non-dormant gemma cups. Scale bar, 5 mm.

(B) Germination frequencies of the wild-type and Mp*DELLA-GR* gemmae under different light conditions. Gemmae were imbibed and treated without (Dark) or with a pulse of red light (4500 µmol photons m^{-2}) followed by incubation in the dark. Dark grey and turquoise columns represent gemmae supplemented with mock or 1 µM DEX, respectively.

(C) Bimolecular fluorescence complementation assays showing MpDELLA-MpPIF interaction in *N. benthamiana* abaxial leaves.

(D) Physical interaction between MpDELLA GRAS domain and MpPIF bHLH domain shown by yeast two-hybrid assays of MpPIF deletion fragments. Although there is no conserved APB domain in MpPIF, its theoretical position was marked by *ψ*APB and used for fragmentation. MpPIF amino acids present on each fragment are 1-472 (del1), 132-760 (del2), 473-760 (del3) and 588-760 (del4). Histidine (His) supplemented media used as growth control. 5 mM 3-amino-1,2,4-triazole (3-AT) was added to His-depleted medium.

(E) Physical interaction between YFP-MpDELLA and HA-MpPIFdel3 (aa 473-760) shown by co-immunoprecipitation after agroinfiltration in *N. benthamiana* leaves.

(F) Germination frequencies of Mp*DELLA-GR* gemmae with additional expression of Mp*PIF* ($_{pro}$*35S:Cit*-Mp*PIF*) under different light conditions as indicated in (B). Dark grey and blue columns represent gemmae supplemented with mock or with 1 µM DEX, respectively.

(G) Morphology of 14-day-old Mp*DELLA-GR* and $_{pro}$*35S:Cit*-Mp*PIF*/Mp*DELLA-GR* plants, grown with different concentrations of DEX. Scale bar, 1 cm.

In B, F, error bars represent standard deviation from three independent experiments (n = 50 per experiment). Statistical groups were determined by Tukey's Post-Hoc test ($p<0.05$) following ANOVA analysis.

SUPPLEMENTAL FIGURE 3

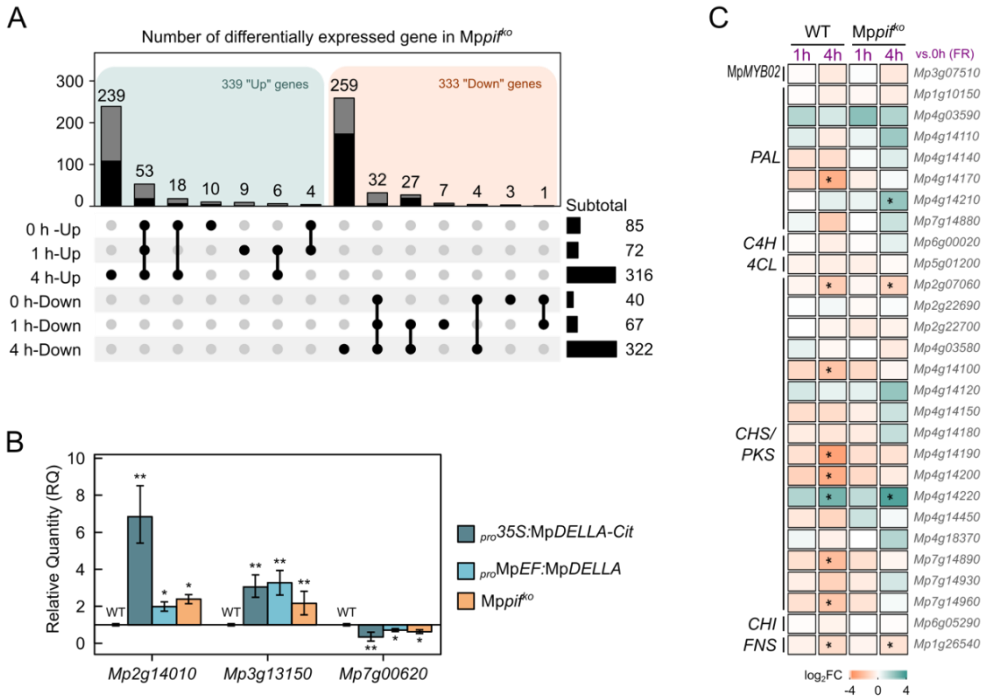


Figure S3. Related to Figure 3. (A) UpSet plot showing differentially expressed genes between Mp*pif*^{ko} and the wild-type at different time points after far-red light (FR) irradiation. Black proportions in the top column plot represent genes ever changed significantly (with |log2FC|>1 and adjusted p<0.01 calculated by DESeq2) in response to far-red treatment in either genotypes. (B) Relative expression level of selected genes by RT-qPCR, as a verification for the RNA-seqs. Error bars represent standard deviation from three biological replicates. Asterisks indicate statistically significant differences with respect to the wild type (*, p<0.05; **, p<0.01, after a Student's t-test). (A) Fold changes in expression (log2 scale) of genes related to flavonoid biosynthesis, compared to time point 0 in both genotypes. Asterisks indicate genes considered as significantly changed (with |log2FC|>1 and adjusted p<0.01 calculated by DESeq2).

SUPPLEMENTAL FIGURE 4

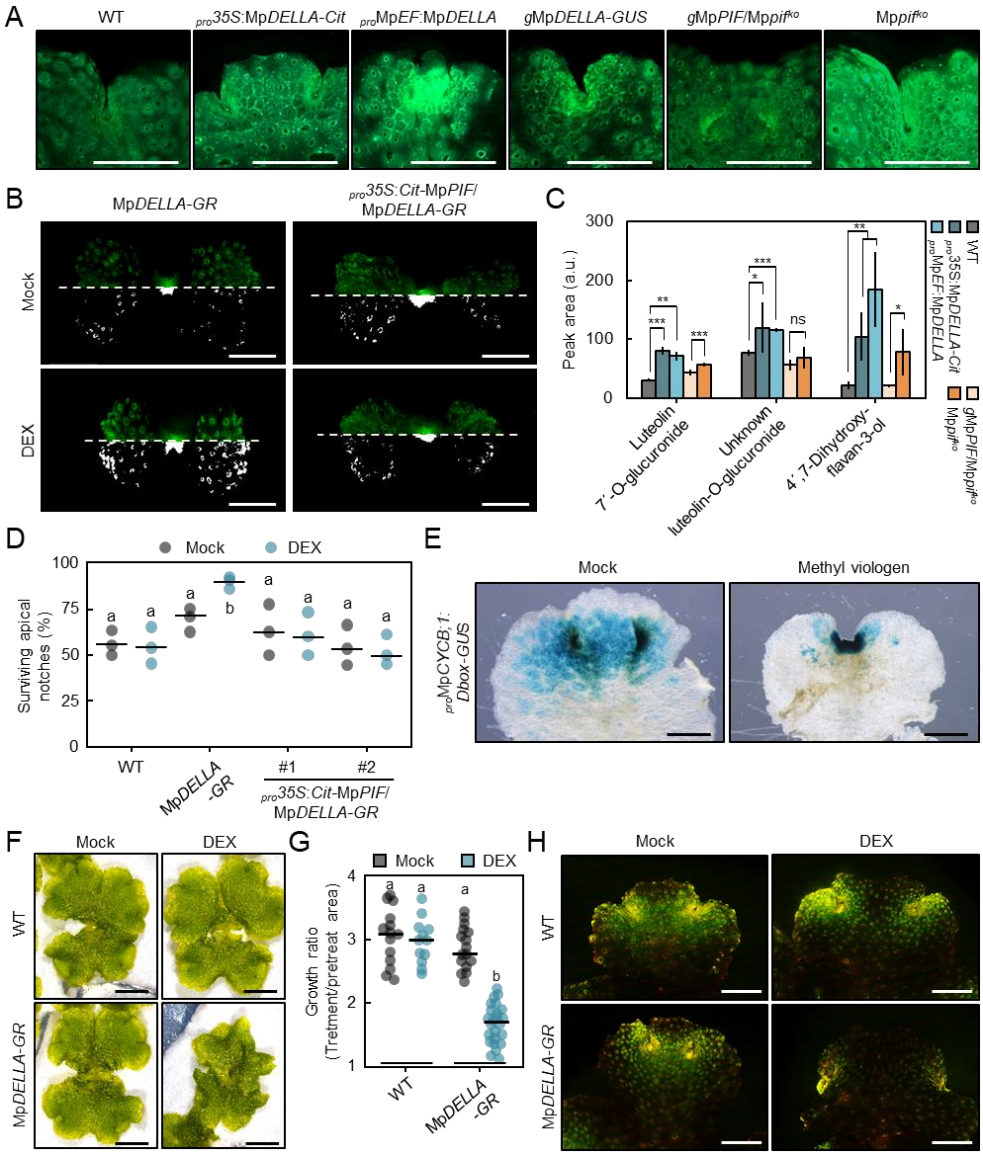


Figure S4 captions can be found in next page (124)

Figure S4. Related to Figure 4.

(A) Images of apical region of Figure 4A gemmalings, stained by DPBA. More intense fluorescent signals denote higher general flavonoid content. Scale bar, 1 mm.

(B) Images showing DPBA staining of 9-day-old Mp*DELLA-GR* and $_{pro}$*35S:Cit*-Mp*PIF* Mp*DELLA-GR* gemmalings, grown with or without 1 µM DEX for 3 days. Upper halves, original picture taken with GFP filter; Lower halves, pseudo-color intensity binary maps (threshold at 30%) to facilitate the comparison of the differences in fluorescence signal between images. Scale bar, 2 mm.

(C) Differentially accumulated flavonoid-related compounds in different genotypes as found by untargeted metabolomics analyses. Original data with individually detected ions and parameters of detection can be found in Table S1.

(D) Percentage of surviving apical notches after a 10-day treatment with 100 µM methylviologen (MV). Mp*DELLA-GR* induction with 1 µM DEX started one day earlier before MV application and was further maintained throughout the stress treatment.

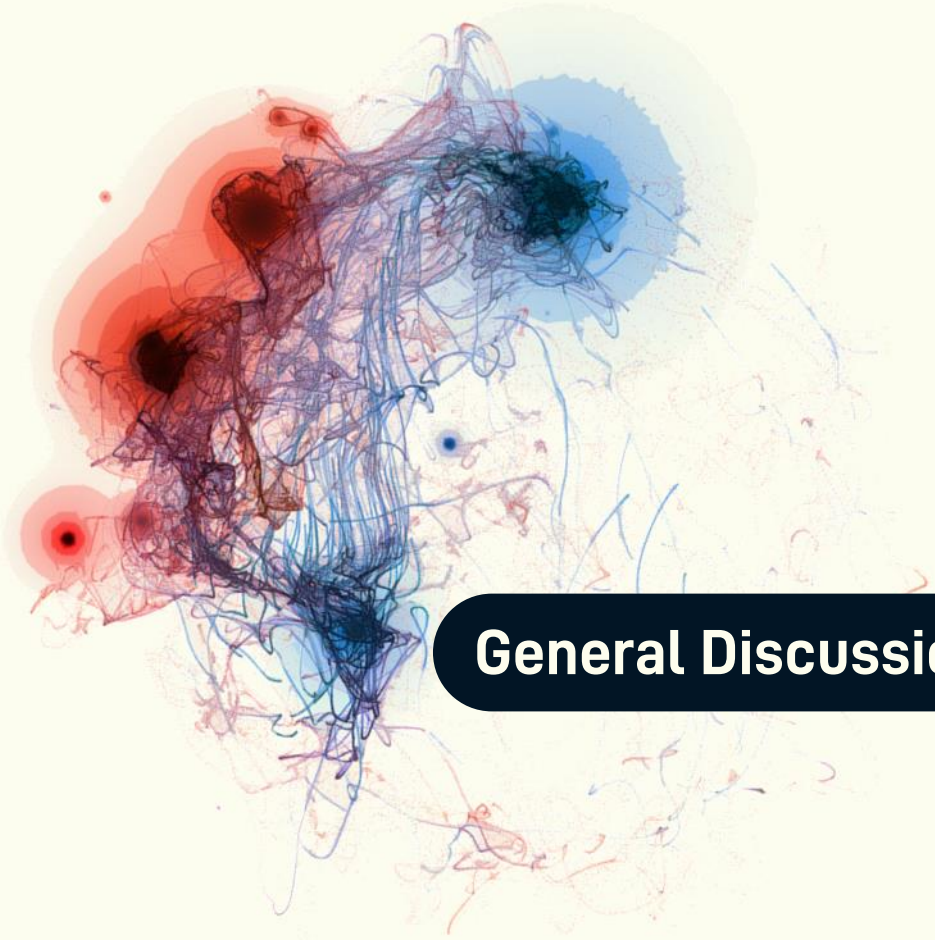
(E) Images of 13-day-old Mp*CYCB;1-GUS* Mp*DELLA-GR* plants stained for GUS activity after mock or 10 µM MV treatment for 6 days. Scale bar, 1 mm.

(F) Morphology of 14 days-old wild-type and Mp*DELLA-GR* plants grown for three days after local application of 20 µM DEX at apical notches. Scale bar, 4 mm.

(G) Quantification of the growth ratio in F accounted as the thallus projection area three days after DEX treatment versus thallus projection area the day of application.

(H) Images of DBPA staining showing the effect of apical notch local DEX application in plants identical to those in F. Scale bar, 2 mm.

In C, fold changes and p-values from Student's t-tests are calculated with quantifications from four samples per genotype with the sole exception of Tak-1 (three samples). In D, E dots represent biological replicates and horizontal lines median values from three independent experiments, in G, dots represent individual measurements in two independent experiments, statistical support is provided by one-way ANOVA analysis, and groups determined by Tukey's Post-Hoc test ($p<0.01$).



General Discussion

The 21st century has witnessed remarkable advancements in our understanding of GA function, from the discovery of the GID1 receptors, to the control of gene expression. However, the elucidation of the so-called canonical GA signalling pathway has left many fundamental questions still unanswered. Some of these have not been interrogated due to technical limitations, while others have been incompletely addressed or their answers are limited. The work shown here tries to harness some of these questions, and particularly, re-assess some of them from an evolutionary point of view. DELLA proteins have been shown to act as central coordinators of multiple transcriptional programs, a function achieved through an enormous array of interactions with multiple transcriptional regulators and factors. Their intrinsic connection to GA signalling seems to be irreducible, mainly due to being the regulatory proteins performing most if not all GA-dependent transcriptional responses in angiosperms. Yet, evolution of DELLAs was only addressed at the early 2000s. Consequently, GA (and DELLAs) signalling emergence and evolution was solved too hastily with a reduced set of genomic data. We have taken advantage of the new evo-devo and 'comparative molecular biology' waves, and the availability of hundreds of new transcriptomes and genomes from land plants and algae, to more accurately study the evolutionary origin of DELLA and their functions. We have significantly updated our knowledge on the origin of DELLAs, and generated new evidence regarding when they appeared, how they functioned, and even how they became part of the GA signalling.

Something old, something new, something borrowed

DELLA emergence paved the way to a new hormonal pathway

During more than a decade, GA signalling has been directly associated with tracheophytes. This assumption is based in a pair of studies published in 2007 (Hirano et al. 2007; Yasumura et al. 2007), just until after the GID1 receptor was found and characterized. Both works use a single bryophyte, the moss *Physcomitrium* (*Physcomitrella*) *patens*, whose genome sequence was to be published soon after (Rensing et al. 2008), and two different lycophytes from the *Selaginella* genus: *S. moellendorffii*, and *S. kraussiana*. Both pieces of work unquestionably found that GA perception and signalling only occur in the lycophytes, thought to be one of the earliest lineages to branch out within the tracheophytes, while any form of canonical GA signalling could be discarded in the moss. The amount of evidence supporting the lack of GA signalling in mosses has accumulated during the last years: a dedicated GA synthesis pathway (or at the very least the late-pathway enzymes) has not been found, the closest GID1 orthologs lack the residues known to be characteristic for both GA perception and DELLA binding in agreement with the results found in the experiments trying to assess these functions, and the two DELLA proteins found in *P. patens* lack the essential motifs for the GID1 interaction, including DELLA and the VHYNP motifs. In contrast, lycophytes contain all the

necessary orthologs, residues and enzymes to assemble a full GA signalling pathway, and they do. This has been also shown to be the case for all the vascular plant lineages as ferns and gymnosperms (Tanaka et al. 2014; Du et al. 2017).

The absence of a proper GA cascade in *P. patens* has been widely extrapolated to all bryophytes. However, a single species does not define a clade. This is especially true for the bryophytes, composed of three lineages that diverged more than 400 million years ago from each other (*i.e.*: mosses, liverworts, and hornworts; Puttick et al. 2018). To date, no other functional analysis has been done in any other bryophytan species, though lack of "canonical" bioactive GAs in the moss *P. patens* has been repeatedly confirmed, and a different set of molecules has been found to derive from the GA early pathway (Miyazaki et al. 2018). The new array of genomic resources has contributed greatly to confirm that mosses lack proper orthologs of the cytosolic enzymes devoted to GA synthesis This can be extended to both liverworts and hornworts, suggesting that synthesis of GAs is absent in all bryophytes, or that, if it exists, it occurs either through a non-devoted set of enzymes or by enzymes evolved independently from those of vascular plants (see **Annexes Part 1**, Hernández-García et al. 2020). Apart from metabolism, GA signalling assumptions were biased due to the availability of a single moss genomic resource. Our systematic search for orthologs of the cascade components in all plant clades has showed that GA signalling has followed a more intricate trajectory (**Fig. 1**). DELLA proteins themselves, the main regulators of virtually all GA-dependent transcriptional responses, are present in every land plant analysed, including the three clades of bryophytes. While this does not refute the conclusions reached by Yasumura et al. and Hirano et al., the presence of *bona fide* DELLA signature motifs in bryophytan DELLAs, previously associated to vascular plant DELLAs, is difficult to reconcile with their proposed scenario of DELLA acquiring the ability to interact with GID1 in a tracheophytan

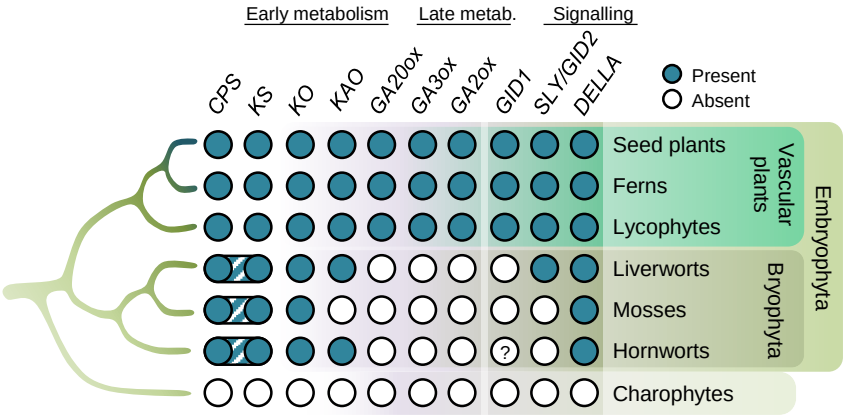


Figure 1. Evolution of GA-related biosynthesis and signalling genes. Rows represent various groups among plant lineages. Circles represent the presence or absence of the genes naming each column. A tree representing evolutionary relationships is depicted at the left side. This figure represents knowledge as of 2021, see **Introduction, Fig. 5** for comparison to knowledge at the beginning of this PhD.

ancestor and not before. Just by looking at protein sequences, it was clear that the loss of these motifs in mosses is a derived trait of this bryophytan subclade (**Chapter 1, Fig. 3**). In the absence of a GA/GID1 perception module, this loss has no foreseen functional consequences. DELLAs are not involved in major growth or developmental processes in *P. patens* (Yasumura et al. 2007), contrarily to what is known in angiosperms, or what we have seen in the liverwort *M. polymorpha* (**Chapter 3**). Thus, it is plausible for moss DELLAs to have suffered some kind of selection constraint leading to acquire a lineage-specific role, with the DELLA domain as the main "target" of this selection. This idea of a domain-specific selection is reinforced by data shown in a parallel PhD project developed in our lab, where all the GRAS domains of all DELLA proteins manifested a conserved and intrinsic promiscuity, something that does not happen with the GRAS domains of other members of the GRAS family (Briones-Moreno 2020).

The deep conservation of the alpha helix adjacent to the VHYNP motif suggests it is functionality preserved in all DELLAs. While the DELLA domain was known to harbour a sequence capable of transactivation (Hirano et al. 2012), no physiological role was attributed to it. By a classical molecular biology approach, we have found that this TAD is relevant for DELLA co-activation responses, and it uses MED15 subunit in order to initiate transcription in specific loci. Interestingly, the region containing the TAD at least partially, overlaps with the GID1-binding region. GID1 binds DELLA-VHYNP motifs upon GA binding, triggering DELLA ubiquitination and finally de-stabilization, thus this region can be considered the GA-dependent degron. The coexistence of degron and TAD in the same region has been documented in several unrelated yeast and mammalian TFs, such as GCN4, Myc, or p53 (Salghetti 2000; Salghetti et al. 2001). There is no clear explanation for this overlap, but one possibility is that quickly destroying TADs confers an advantage through the reprogramming of transcriptional responses. No matter how this convergence has originated, the overlap also occurs in plant proteins (see **Annexes Part 2, Fig. 1**), as we have found for DELLA proteins. Interestingly, we can predict this in other plant TFs such as MYC2, WUS, or PIFs. MYC2 contains within its MID domain both the proteolytic-target region and the TAD activity (Zhai et al. 2013). WUS, whose degron is mapped to the acid and W-box domains (Snipes et al. 2018), both in charge of direct transcriptional activation. In the case of PIFs, the APB (Active phyB-Binding) domain could be regarded as the light-dependent degron, and coincidently shows transactivation activity (Leivar and Monte 2014). The degron-TAD coincidence in different very unrelated proteins from distant eukaryotes suggests that it must be an important and recurrent tool to adjust and tune transcriptional regulation. In the case of DELLA proteins, this co-occurrence may underlie the very birth of the GA signalling pathway. DELLAs in plants lacking GA signalling (*i.e.*: bryophytes) harbour a demonstrated transactivation capacity (See **Chapter 1, Fig. 6** and Hirano et al. 2012), which we have shown to be linked to TF-DELLA co-activation of target genes (**Chapter 2, Fig. 4**). The presence of the TAD within a structurally conserved

region and with a high level of intrinsic order may have thus permitted GID1 exaptation, allowing for GA-dependent titration of DELLA function in vascular plants. The process of co-option of a previous TAD to a degron thus would create a hormone signalling pathway. However, there are blanks left to fill in order to understand what steps followed the whole pathway to assemble as we know it in vascular plants.

If it ain't broke, don't fix it...or adjust it somehow

DELLAs regulate processes using conserved and non-conserved regulatory modules

One of the most relevant roles attributed to DELLAs in Arabidopsis, and angiosperms in general, is the coordination between growth and environmental conditions (Claeys et al. 2014). In **Chapter 3**, we have unravelled the role of *M. polymorpha* DELLA as a coordinator of both growth and stress responses, suggesting that this function could have been maintained for over 450 millions of years. A previous *in silico* analysis suggested that regulation of stress responses by DELLAs could be ancestral, deduced from the conservation of the potential DELLA transcriptional networks' properties between *P. patens* and angiosperms (Briones-Moreno et al. 2017). Our transcriptomic approach in *M. polymorpha* supported this possibility, indicating that biotic and oxidative stress responses were positively modulated by MpDELLA (**Chapter 3, Fig. 3**). Strikingly, GO analysis has never rendered "growth" as a conserved process regulated by DELLA, either in our transcriptomic analysis or in the networks' predictions (Briones-Moreno et al. 2017; Briones-Moreno 2020). This could probably be due to differences in terms of how growth is achieved in the different lineages, including transcriptional programs leading to the differentiation of cell-types and tissues, but also due to technical difficulties related to poorly annotated gene functions in plants other than *A. thaliana*.

Interestingly, while coordinating growth and stress can be ascribed to both AtDELLAs and MpDELLA, it seems that the pathways involved may vary (**Fig. 2**). DELLAs regulation of a process can be accomplished in two species by different sets of interactors and transcriptional networks. For example, MpDELLA prevents growth mainly through cell cycle inhibition in the apical notches of the thallus (**Chapter 3, Fig. 1 & Fig. S1**). While the mechanism behind this cell division inhibition is unknown, we did not find obvious signs of transcriptional control of cell cycle-related genes. Contrarily, Arabidopsis DELLAs regulate stem growth by direct transcriptional regulation of cell cycle genes (Davière et al. 2014; Serrano-Mislata et al. 2017). This indicates that DELLAs repress cell divisions in *M. polymorpha* and *A. thaliana*, but the transcriptional network in charge of this must be different between each species.

In other cases, DELLA-TF interactions are highly conserved, but these interactions are involved in the regulation of different downstream processes. For example, AtDELLAs are known to restrain hypocotyl growth by sequestering PIFs, thus inhibiting cell expansion (Feng et al. 2008; de Lucas et al. 2008). Conversely, MpDELLA interaction with MpPIF does not

regulate growth. Instead, MpDELLA-MpPIF interaction regulates processes as gametangiophore formation or gemma dormancy, none of which is governed by cell expansion (Inoue et al. 2017; Kato et al. 2020). Hence, the involvement of DELLAs in species-specific pathways/processes does not depend on the molecular activity of DELLAs, but on the different downstream targets of PIFs. This observation matches the findings in a transcriptomic/interactomic approach to study DELLA evolution that indicate that DELLAs functional innovation during evolution can be mainly attributed to the evolution of their interacting partners and their cis-regulatory networks (Briones-Moreno 2020).

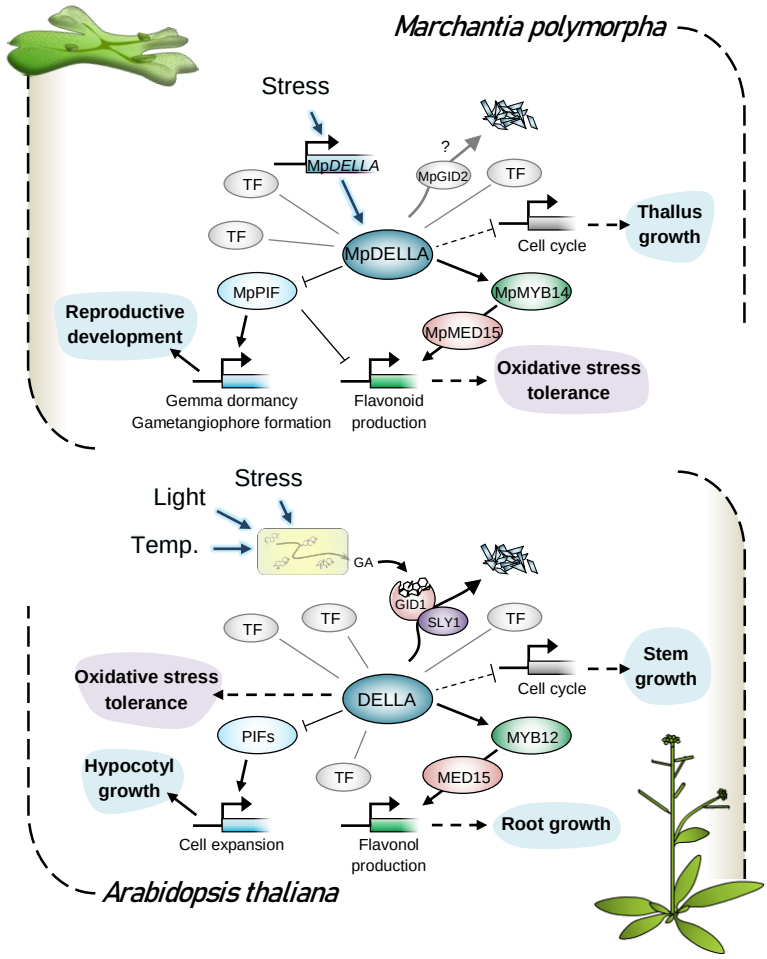


Figure 2. Comparison of DELLA regulation & regulatory networks in *A. thaliana* and *M. polymorpha*. Selected DELLA regulation mechanisms (transcriptional, posttranslational) and DELLA-TF interactions comparing knowledge gathered during in this PhD about *M. polymorpha* DELLA function with the related pathways and processes regulated by DELLA in *A. thaliana*. Arrows indicate positive regulations. Dashed lines indicate indirect regulation or mechanisms yet to be uncover. Grey shaded lines and elements are unknown (as for MpGID2) or unrelated interactions to the presented processes.

A third case involves the conservation of both the underlying mechanism and the regulated processes. DELLA-dependent activation of phenylpropanoid and flavonoid biosynthesis in *M. polymorpha* and *A. thaliana* involves a MYB-based co-activation mechanism. In comparison, this response has not been linked to oxidative stress tolerance in *A. thaliana*, but to a developmental trait (*i.e.*: root growth). However, two observations suggest that this role is conserved: it is triggered by nutrient deficiency, an environmental stress (Tan et al. 2019); and GAs and DELLAs have been repeatedly associated to oxidative stress tolerance in *A. thaliana* (Achard et al. 2008; Xie et al. 2016). Hence, it is possible that MYB12-induced flavonol accumulation is also part of the stress tolerance response deployed by DELLAs. It is reasonable to propose that the activation mechanism leading to flavonoid accumulation, and the subsequent antioxidant production could be conserved between these two species.

It is nearly impossible to know all the functions that DELLAs accomplished when they first appeared, but our comparative analysis suggests that a set of processes related to growth and stress responses were probably in the pack. Furthermore, it seems certain that their molecular function as transcriptional hubs has been conserved in all land plants, since DELLAs operate as such in bryophytes and tracheophytes (Briones-Moreno 2020).

The chicken or the egg, or both

The steps leading to the formation of the GA signalling pathway remain unsolved

A missing piece of information is when and how SLY1/GID2 was recruited into the pathway. Its intriguing presence in liverworts, but not in mosses and hornworts could be credited to either loss of the gene in these two lineages, or to a rarer event of horizontal gene transfer (HGT) from vascular plants. Gene loss is the simplest and more common process, though it would have needed to occur twice due to the phylogenetic relationship among the three bryophytan clades, with mosses and liverworts as sisters, forming the Setaphyta clade (Puttick et al. 2018; Sousa et al. 2019). Furthermore, HGT between multicellular eukaryotes, and specially between Viridiplantae species, is rather uncommon compared to gene loss (Tang et al. 2018). Furthermore, most documented HGT events among land plants took place between closely related species or during parasite-host interactions (Wickell and Li 2020), and a single report shows a bryophyte-to-tracheophyte HGT (Li et al. 2014). Therefore, the most likely scenario involves a double gene loss, implying the emergence of SLY1/GID2 in the last land plant common ancestor (LLCA), as proposed for DELLA proteins. The coincidence of SLY1/GID2 and DELLA in a LLCA rise the possibility for them to have had a functional relationship already at this point, necessarily independent of GAs and GID1 assuming their absence in this LLCA. SLY1/GID2 F-box triggers DELLA ubiquitination after GID1-GA binding and SCF ligase recruitment. Nevertheless, it has been shown that SLY1 is able to interact with the GRAS domain of RGA *in vitro* and in yeasts with no need of GID1 nor GAs (Dill et al. 2004).

This is in line with our own data, where we found SLY1 interaction with not only RGA, but also *Takakia lepidozioides* (moss) and *Marchantia polymorpha* (liverworts) DELLAs (see **Annexes**, **Additional Data Fig. 2a**). The relevance of the GID1-independent interaction of SLY1 and AtDELLAs remains unsolved, although it has been proposed that GAs are not necessary for the SLY1-dependent DELLA ubiquitination and destruction, but significantly stimulates this process (Ariizumi et al. 2011). It is therefore reasonable to propose that SLY1/GID2s establish a basal DELLA destabilization, and hence, this could serve as the main trigger of DELLA protein turnover in non-GA containing species. Indeed, MpGID2 interacts with MpDELLA, and 26S proteasome inhibition stabilizes MpDELLA proteins to some degree in vivo (**Annexes Part 2**, **Fig. 2b-c**), supporting this scenario. The regulation of DELLA stability by SLY1/GID2 can represent an ancestral form of DELLA posttranslational regulation already present in a LLCA, which was exploited by the GA-GID1 perception module at some point. One possible (and parsimonious) scenario would be DELLAs appearance predating that of SLY1/GID2. This requires the adaptation of SLY1/GID2 from a previous F-box protein to acquire relatively few motifs to recognize the DELLA GRAS domain. On the other hand, SLY1/GID2 predating DELLA could represent a more challenging scenario, since SLY1/GID2 proteins have been only linked to DELLAs regulation, however, this possibility cannot be unequivocally ruled out. Given that DELLAs probably acted as promiscuous proteins in the LLCA, coordinating multiple process in a context dependent manner (**Chapter 3** and Briones-Moreno 2020), it is reasonable to think that they were affected by both transcriptional and posttranslational regulatory mechanisms to adjust final protein levels. In this line, it has been suggested that the regulation of DELLA stability by the E3 ligase encoded by *COP1* observed in Arabidopsis could have originated early during land plant evolution (Blanco-Touriñán 2020; Blanco-Touriñán et al. 2020). Later, the appearance of the more flexible and likely tuneable mechanism of GA signalling could have taken control of DELLA levels regulation.

Another puzzling piece in the evolutionary history of GA signalling is the GID1 receptor. GID1 is undoubtedly absent *sensu stricto* in liverworts and mosses, as previously published (Hirano et al. 2007; Yasumura et al. 2007) and confirmed by us (**Chapter 1**) and other groups (Yoshida et al. 2018). Intriguingly, we detected a partial GID1-like ortholog in the hornworts *Phaeoceros carolinianus* and *Paraphymatoceros halli*, opening the door to additional complexity in the evolution of the GA pathway. These sequences perfectly fit within the GID1 clade, as sisters to all tracheophytan GID1s. The wild origin of these samples (One Thousand Plant Transcriptomes Initiative 2019) suggested that contamination could occasionally occur. In addition, the sequencing of three different *Anthoceros* species indicated that no *GID1* sequences were present in their genomes (Li et al. 2020; Zhang et al. 2020). In short, the presence of GID1 or some GID1-like in hornworts -and thus in bryophytes- was initially discarded. However, if the existence of bona fide GID1 hornwort sequences is confirmed, the most likely scenario for the origin of the GA signalling pathway would be the following: GID1s

appeared in a tracheophytan common ancestor that most probably contained tracheophytan/hornwort-like DELLA domains, encountering an already functional DELLA protein capable of strong co-activation in a chromatin context. In this context, GID1 learned to recognize the TAD neighbouring motifs DELLA/VHYNP, producing two outcomes: a) GID1 binding to this region blocked TAD availability to transcriptional regulators, inhibiting gene activation (i.e.: Mediator); b) this interaction facilitated SLY1/GID2-DELLA interaction, and thus DELLA proteolytic degradation. While we do not know if both processes emerged at the same time or were gradually acquired, the first process does not require any kind of conformational change within DELLA structure, since GID1 can directly compete with MED15 physically. However, GID1 and SLY1 directly interact (Ariizumi et al. 2011), indicating that, if this was the case for the ancestral GID1 and SLY1/GID2, this could have occurred as soon as GID1 learned to interact with DELLAs as well.

A final intriguing question is how GID1 became GA receptors from their CXE ancestors. The family seems to have a broad range of substrates (Ileperuma et al. 2007), indicating that GA-related compounds may have been used as substrates by proto-GID1s. However, it is also plausible for proto-GID1 to have first learned to interact with DELLAs independently of GA binding, something that occurs in some GID1 proteins, at least with lower but decent affinities (Yoshida et al. 2018). For a more detailed discussion on the possible evolutionary pathway followed by GID1 see **Annexes 1** (Hernández-García et al. 2020). In any case, it seems feasible that the appearance of a chemically-dependent inhibitor (GID1) of an already promiscuous protein (DELLA) could have laid the foundations of a powerful and flexible transcriptional regulatory module (GA signalling).

Don't count your chickens before they hatch

Phaeoceros carolinianus may contain a functional GID1 protein

The presence of a possible GID1 protein in hornworts had never been previously considered given the unavailability of a proper genomic source, fostered by the absence of similar sequences in the recently sequenced hornwort genomes. Yet, we have obtained *Phaeoceros carolinianus* cultures and retrieved a PcGID1 full sequence. Moreover, we found that PcGID1 interacts with PcDELLA in a GA-dependent manner, much like tracheophytes GID1s (**Annexes Part 2**, **Fig. 3**). It indicates that GID1 could have been present in a LLCA. Ockham's Razor principle supports a single origin and multiple losses, although other possibilities as HGT or convergence cannot be categorically ruled out. If the LLCA already harboured a functional GID1, then the full GA signalling system could have been already present before tracheophytes and bryophytes divergence, pushing back the origin of the canonical signalling pathway to the LLCA. Under such circumstances, a devoted late GA biosynthetic pathway could represent the only GA-related basic feature specifically evolved in tracheophytes. There

are several blank spots to fill to understand the emergence of the GA signalling pathway. As such, we still need to dig into the origin of GID1, especially regarding the recently found PcGID1; to understand if GID2-DELLA interaction has a functional role in liverworts; and also to re-asses the roles of GAs in non-seed tracheophytes as ferns and lycophytes, were DELLA functions and GAs have been overlooked. GA metabolism origin in tracheophytes remains unclear, and we would need to study the possibility of a different biosynthetic pathway of GA-like compounds in bryophytes, especially in hornworts as *P. carolinianus*. The presence or not of GAs would suggest that PcGID1-PcDELLA can interact 1) independently of any chemical signal, 2) in response to other endogenous substances unrelated to GAs, or 3) in response to exogenous GA-related molecules, maybe derived from fungi or bacteria where devoted GA metabolic pathways are widespread.

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Conclusions

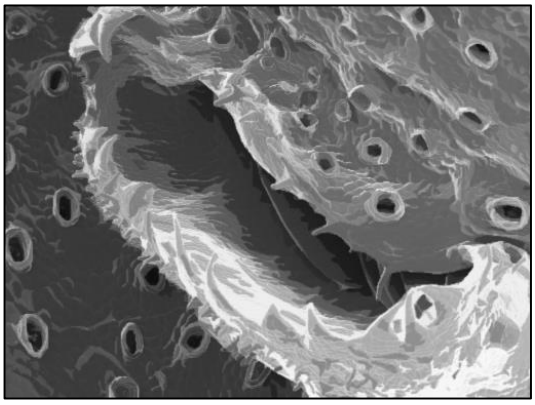
The findings here described have allowed us to establish a new, more complete **model for the origin and evolution of the GA signalling pathway**. Our work on DELLA proteins has helped uncover their most likely **ancestral roles** and **mechanism of action**, which were conserved after becoming an essential constituent of the GA signalling pathway. The conclusions extracted from these pages can be summarised in:

1. DELLA proteins emerged in an embryophyte common ancestor. Their capacity to modulate TF activity by sequestration and co-activation is ancestral.

2. The incorporation of DELLA proteins to the GA signalling module took place by molecular exploitation of a pre-existing transactivation domain to act as a GA-regulated degron.

3. DELLA function as co-activators relies on Mediator, which is recruited through physical interaction between DELLA's N-terminal domain and MED15.

4. The ability to coordinate growth and stress responses by DELLA proteins predates the emergence of the GA signalling pathway.



Marchantia polymorpha developing gemma cup
(original: personal CryoSEM archive)

Annexes

Part 1. Hernández-García et al. 2021. *Semin Cell Dev Biol* 109:46-54. doi: 10.1016/j.semcdb.2020.04.009.

Part 2. Additional figures for General Discussion

Part 3. Updated Chapter 1 Figures

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Origin and evolution of gibberellin signaling and metabolism in plants

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ABSTRACT

Gibberellins modulate multiple aspects of plant behavior. The molecular mechanism by which these hormones are perceived and how this information is translated into transcriptional changes has been elucidated in vascular plants: gibberellins are perceived by the nuclear receptor GID1, which then interacts with the DELLA nuclear proteins and promote their degradation, resulting in the modification of the activity of transcription factors with which DELLAs interact physically. However, several important questions are still pending: how does a single molecule perform such a vast array of functions along plant development? What property do gibberellins add to plant behavior? A closer look at gibberellin action from an evolutionary perspective can help answer these questions. DELLA proteins are conserved in all land plants, and predate the emergence of a full gibberellin metabolic pathway and the GID1 receptor in the ancestor of vascular plants. The origin of gibberellin signaling is linked to the exaptation by GID1 of the N-terminal domain in DELLA, which already acted as a transcriptional coactivator domain in the ancestral DELLA proteins. At least the ability to control plant growth seems to be encoded already in the ancestral DELLA protein too, suggesting that gibberellins' functional diversity is the direct consequence of DELLA protein activity. Finally, comparative network analysis suggests that gibberellin signaling increases the coordination of transcriptional responses, providing a theoretical framework for the role of gibberellins in plant adaptation at the evolutionary scale, which further needs experimental testing.

1. Introduction

All living beings evolve to survive in changing environments, to thrive despite scarce nutrients, predators and diseases. It has been especially difficult for plants to overcome hostile conditions, as they are sessile organisms and once they settle down in a certain location, they can only acclimate. To that end, they make use of intricate and sophisticated signaling networks to respond to the environment [1]. An intrinsic component of these networks are phytohormones, molecular signals that modulate developmental processes and physiological responses, often following external stimuli. In this respect, gibberellins (GAs) are particularly important for adaptation because their metabolism depends on external conditions, and their functions are widespread along the plant's life cycle [2]. Apart from being widely known for promoting plant growth via cell expansion and division [3–5], GAs regulate numerous developmental processes through the whole plant life cycle like seed germination [6,7], photomorphogenesis [8], floral transition [9,10], male fertility [11] and fruit set [12,13]. They are also instrumental in the response to different environmental stimuli such as gravity [14,15], light [16], or temperature [17]. Moreover, GA levels affect the defense against abiotic stress caused by reactive oxygen species [18], salt [19], or cold [20], and biotic stress caused by pathogens [21]. Although most of these functions are common to angiosperms, monocots and gymnosperms, some others are specific to certain plant clades. For instance, they have been shown to control sex determination in ferns [22], or nodulation and arbuscular mycorrhizal associations [23]. In this review, we address the evolutionary history of GAs, from their biosynthesis to the components of their signaling pathway, to understand the design principles supporting their pervasive role in plant life.

1.1. Introduction to GA metabolism

As diterpenoids, GAs are derived from the isoprenoid biosynthetic pathway (Fig. 1). In the majority of land plants, GAs are formed from the methylerythritol 4-phosphate (MEP) pathway, a plastid-specific pathway for isoprenoid production [24–26]. The first step occurs in proplastids using *trans*-geranylgeranyl diphosphate (GGPP) to produce

Abbreviations: 2OH-KA, *ent*-2α-hydroxy-kaurenoic acid; 3OH-KA, *ent*-3β-hydroxy-kaurenoic acid; CPS, *ent*-copalyl diphosphate synthase; ER, endoplasmic reticulum; GGPP, *trans*-geranylgeranyl diphosphate; KAO, *ent*-kaurenoic acid oxidase; KO, *ent*-kaurene oxidase; KS, *ent*-kaurene synthase; pGID2, pre-GID1-recruited GID2; PP, proplastid

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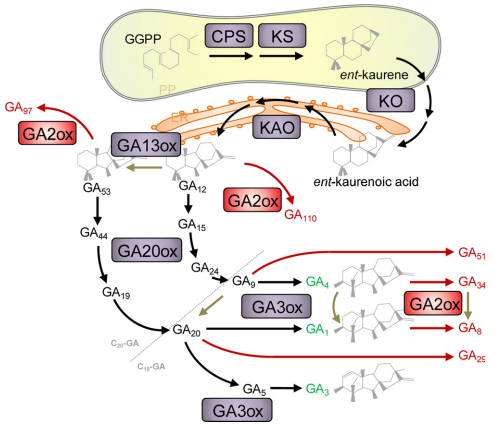

Fig. 1. GA biosynthesis in seed plants. Enzymatic flow depicted as arrows from substrates to products. Enzymes catalyzing each step are shown near each arrow or arrows group. Enzymes channeling biosynthesis towards bioactive GAs are enclosed in dark purple boxes, and their reactions shown as black arrows. Catabolic enzymes appear in red boxes and their reactions as red arrows. Unknown reactions or steps catalyzed by unknown enzymes are shown as brown arrows. Bioactive and inactive GAs are shown in green and red, respectively. Grey dotted line separates the phases of the cytosolic pathway (i.e.: C_{20} and C_{19} backbone GAs).

ent-kaurene [27]. In seed plants, two monofunctional diterpene cyclases (DTC), *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS), act in consecutive cyclizations to produce *ent*-copalyl diphosphate (*ent*-CPP) and then *ent*-kaurene from GGPP. Then, oxidation by a plastid membrane-bound P450 dioxygenase (*ent*-kaurene oxidase [KO]) and an endoplasmic reticulum-bound P450 dioxygenase (*ent*-kaurenoic acid oxidase [KAO]), act sequentially to yield GA_{12}. These oxidations convert the *ent*-kaurene ring into the *ent*-gibberellane ring common to all GAs. The subsequent steps occur in the cytosol to form bioactive GAs by the action of 2-oxoglutarate-dependent dioxygenases (2-OGD), namely GA20ox and GA3ox. These enzymes convert the C_{20} gibberellic backbone into C_{19} GAs by succeeding oxidations. A third type of 2-OGD, GA13ox, can act on the cytosolic pathway creating the alternative 13-hydroxylated-GA pathway [28], although this reaction can also be performed by CYPs in certain species Some P450 dioxygenases can also act as GA13ox either early or late in the pathway to convert 13-H GAs into 13−OH GAs [29,30].

Bioactive GAs can be inactivated by a fourth type of 2-OGD, GA2ox. Different GA2ox can act in earlier steps of GA biosynthesis to deplete the substrates for bioactive GA, but GA2ox acting on C_{19}-GA skeletons are the main GA inactivation pathway [31]. Some modifications, such as methylation, are thought to reversibly inactivate GAs [32]. Inactivation mechanisms and its regulation have been reviewed elsewhere [33].

In angiosperms, GA biosynthesis is tightly regulated by both endogenous and environmental factors. Most of this regulation is thought to be exerted at the transcriptional level, while no post-translational regulatory mechanisms of enzymatic activity are known [2,34]. Genes responsible for several steps of GA biosynthesis are developmentally regulated, while environmental signals and endogenous feedback responses generally target the 2-OGD genes. For example, in Arabidopsis, complex patterns of regulation have been reported for the different *GA20ox* and *GA3ox* genes, with some of them induced as a response to decreasing GA levels [35] and repressed under stress conditions [36].

1.2. Introduction to GA signaling

GA-dependent transcriptional regulation is supported by a relatively simple signaling pathway, similar to that of other plant hormones [37], consisting on the degradation of a transcriptional regulator triggered by the recognition of the active GA molecules by a receptor (Fig. 2). The GA receptor, encoded by GIBBERELLIN INSENSITIVE DWARF1 (GID1), is a soluble protein found in the nucleus and the cytoplasm, formed by a C-terminal domain with a GA-binding pocket, and a N-terminal extension (N-ex), which has a flexible structure [38–41]. When bioactive GAs bind the C-terminal pocket, an allosteric change is induced, the N-ex folds over the C-terminus to cover the pocket like a lid, and a new surface is exposed, able to interact with the transcriptional regulator

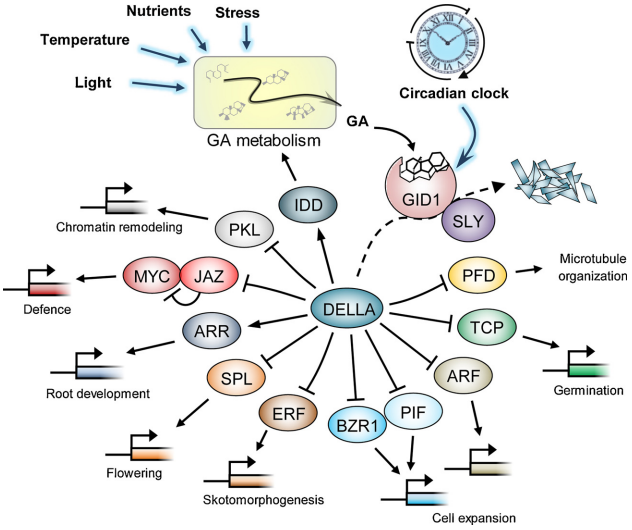

Fig. 2. GA signaling in seed plants. GA metabolism (Fig. 1) is summarized as a yellow square. Environmental signals modulating GA biosynthesis and GID1 activity are represented as blue-shaded arrows. Some representative interactions of DELLA with transcription factors and transcriptional regulators that regulate diverse processes are shown. Negative and positive effects of DELLA interaction are shown as T-shaped lines or arrows, respectively. Dashed line represents GID1-SLY1/GID2 regulated DELLA degradation by the 26S proteasome. PFD, Prefoldin; TCP, TEOSINTE BRANCHED 1/CYCLOIDEA/PCF1; ARF, AUXIN RESPONSIVE FACTOR; PIF, PHYTOCHROME INTERACTING FACTOR; BZR1, BRASSINAZOL RESISTANT1; ERF, ETHYLENE RESPONSIVE FACTOR; SPL, SQUAMOSA PROMOTER LIKE; ARR, ARABIDOPSIS RESPONSE REGULATORS; JAZ, JASMONATE-ZIM DOMAIN; PKL, PICKLE; IDD, INDETERMINATE DOMAIN.

encoded by the *DELLA* genes [42,43]. DELLAs are soluble nuclear proteins that belong to the GRAS family of transcriptional regulators [44]. The DELLA subfamily is characterized by having a C-terminal domain common to other GRAS proteins, and an N-terminal domain that contains the conserved motifs DELLA (for the amino acid sequence Asp-Glu-Leu-Leu-Ala), LEQLE and TVHYNP, which can be recognized by GID1 [42,43,45]. This physical interaction between GA-GID1 and DELLA promotes the interaction with a particular F-box protein (SLY1 in Arabidopsis and GID2 in rice [46–48]), the recruitment of an SCF ubiquitin E3 ligase complex, and the subsequent degradation by the 26S proteasome [49].

Although GA levels are the main source of information for the regulation of DELLA stability, additional environmentally-modulated mechanisms have been described that provide fine tuning. For instance, *GID1* gene expression is under the control of the circadian clock [50]. The oscillation of GID1 abundance thus gates the response to GAs, promoting degradation of DELLA proteins around the end of the night, and contributing to rhythmic growth. DELLA stability seems to be regulated as well by certain post-translational modifications (PTMs). Several studies indicate that DELLA phosphorylation confers resistance to proteolysis, while dephosphorylation facilitates their degradation [51–53]. Similarly, SUMOylation of DELLAs enhances their stability through a GA-independent interaction between sumoylated DELLA and GID1 [54]. Two other PTMs affect DELLA activity instead of its accumulation: O-GlcNAcylation by SECRET AGENT (SEC) and O-fucosylation by SPINDLY (SPY) respectively inhibit and enhance the ability of DELLAs to interact with specific TFs [55,56].

The best documented output of the control of DELLA levels is the dramatic alteration in the plant transcriptome in response to GAs (reviewed in [57]). This is a direct consequence of the activity of DELLA proteins as transcriptional regulators that interact with dozens of TFs [58,59]. From a mechanistic point of view, these DELLA-TF interactions can be catalogued in three groups: (i) those that cause the sequestration of the TF, impairing its binding to the target promoters [16,60–62]; (ii) those that recruit DELLA to the target promoters, in which DELLA acts as a transcriptional coactivator [35,63–65]; and (iii) the interactions with other non DNA-binding transcriptional regulators that indirectly affect TF activity [66,67]. Moreover, these physical interactions provide an explanation for long-standing questions, like the physiological interactions between GA signaling and other signaling pathways including light [16,60] or hormones [58,62,63,67], or the multitude of processes controlled by GAs, such as seed germination [61,65], cell expansion [68], flowering [69,70], the establishment of root-microbe interactions [71–73], or the control of iron homeostasis [74], among others.

Besides TFs, DELLAs can also interact in the nucleus with other regulators involved in different aspects of cell physiology. For instance, DELLAs interact with components of the chromatin remodelling machinery, such as PICKLE [75] or SWI3C [76]; or retain the chaperonin Prefoldin in the nucleus, to eventually impair tubulin folding and cell elongation [77].

In summary, the current view of GA and DELLA function is that they relay environmental information to multiple transcriptional circuits to promote adaptation through the optimization and coordination of plant responses [78]. Environmental signals would be integrated by GA metabolism, while the GA-GID1 perception module would control DELLA levels through their N-terminal domain, and the C-terminal GRAS domain would interact with (and modulate) TFs and possibly other nuclear regulators. However, this view is challenged by the fact that bioactive GAs, or bona-fide GID1 receptors are not present in all plant lineages (Fig. 3), therefore raising the question of how GA metabolism and signaling emerged and evolved.

2. Evolution of gibberellin metabolism

As explained in the previous section, GA biosynthesis can be divided into three phases: (i) the cyclization of GGPP to *ent*-copalyl diphosphate (*ent*-CPP) and ent-kaurene by the consecutive action of CPS and KS; (ii) the oxidation by cytochrome P450 (CYP) mono-oxygenases to yield *ent*-KA and GA_{12}; and (iii) further oxidation by a family of 2-OGDs to form the bioactive GAs. While the first two phases are largely conserved in all land plants, the cytosolic oxidations catalyzed by 2-OGDs are specific to tracheophytes (Fig. 4).

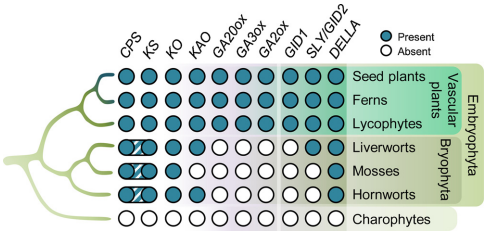

Fig. 3. Evolution of GA-related biosynthesis and signaling genes. Rows represent various groups among green plant lineages. Circles represent the presence or absence of the genes named in each column. A tree representing accepted evolutionary relationships among plant lineages is depicted at the left side. The charophytan algae are presented here as a single branch (charophytes) rather than a multiple-branched polyphyletic group for simplification. The rest of the branches represent monophyletic groups according to recent findings.

In fact, CPS and KS probably originated from bacterial DTC precursors via horizontal gene transfer to an ancestor of land-plants, since green algae do not contain genes encoding similar proteins (Fig. 3) [79]. In mosses, endogenous *ent*-kaurene produced by a bifunctional CPS/KS is linked to different developmental responses [80,81]. In seed plants, the enzymatic activities involved in these steps, are performed by independent but closely related enzymes, CPS and KS, which probably emerged after duplication and subfunctionalization of a moss-like bifunctional CPS/KS [82]. However, other plant specific compounds are known to stem from GGPP by DTC-dependent cyclization, as an indication of the promiscuity of this enzyme class, specially the bifunctional CPS/KS (Fig. 4) [83–85]. It has been proposed that KS consecutively acquired its specificity both to recognize ent-CPP and to form ent-kaurene during land plant evolution [86]. The lycopod *Selaginella moellendorffii* KS has broad substrate specificity for different CPP stereoisomers, while angiosperm KS enzymes are highly specific for *ent*-CPP recognition. The intrinsic ability of CPS/KS DTCs to vary largely between substrates and products has been proposed to support a scenario in which hormonal metabolism would be exploited to create novel secondary metabolic pathways [87]. While CPS and KS tend to be single copy genes in angiosperms and there are no bifunctional DTCs, CPS/KS genes in non-vascular land plants exist in higher copy numbers [88,89]. This suggests that plants outside the angiosperm lineage possess an enriched diterpenoid metabolism probably due to the existence of multiple copies of flexible DTCs. However, the function of ancestral GGPP-derived diterpenoids is unknown. Broad substrate specificity and promiscuity are features of extant DTCs that could be present in ancestral DTCs, and it is tempting to speculate that the acquisition of CPS/KS-like cyclase activity by neo-functionalization of terpene synthases in the ancestral land plant could have provided of new compounds, either with a direct defensive function (e.g.: phytoalexins) or as signaling effectors (e.g.: ent-kaurene derivatives) to cope with land environment.

The plastidial and/or endoplasmic envelope-located KO and KAO share a common origin from an ancestral P450 but are part of different CYP families: CYP701A and CYP88A, respectively [90]. Both enzymes perform several sequential oxidations, to first form *ent*-kaurenoic acid, and then GA_{12}, with intermediates not being accumulated [91]. This may have constrained the possible branching of the pathway to these two final products of each enzyme. P450-dependent KO activity is likely

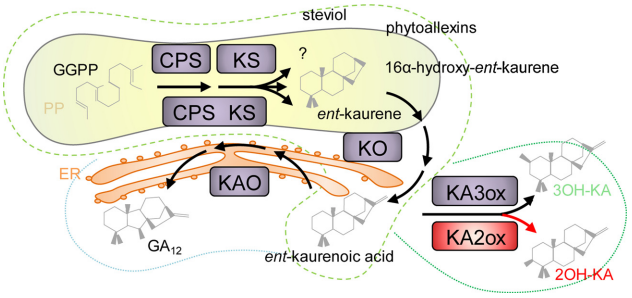

Fig. 4. GA biosynthesis in bryophytes. Enzymatic flow depicted as arrows from substrates to products. Enzymes catalyzing each step are shown enclosed in dark purple boxes near each arrow or arrows group. CPS/KS activities can be performed by mono- and bifunctional DTCs. Different products derived from DTC activities initiating multiple pathways are presented. Light green dashed line contains deeply conserved Embryophyta steps of the pathway. Light blue dotted line (lower left) includes a second oxidation step present in liverworts, hornworts, and vascular plants, while dark green dotted line (lower right) represents a moss specific pathway based on *ent*-kaurenoic acid hydroxylations. Green and red compounds represent biologically active and inactive molecules in mosses, respectively. An unidentified KA3ox appears in a dark-purple box guiding the production of 3OH-KA. The antagonistic enzyme (KA2ox) is represented in a red-shaded box and its reaction depicted as a red arrow toward the inactive product.

to be present in all extant land plant lineages, since closely related sequences have been unambiguously predicted, and at least one has been linked to *ent*-kaurenoic acid production [92]. Their absence in sequenced algae suggests that KO originated in the common ancestor of land plants, but its original activity remains unknown. Some studies have found that AtKO is able to recognize and produce diverse (but closely related) molecules, suggesting that the ancestral KO could have recognized multiple substrates.

Genes encoding KAO are present in both bryophytes and vascular plants suggesting they originated in the ancestor of land plants (Fig. 3) [93]. However, they are missing in extant mosses [81,94], pointing to an early loss of KAO in that lineage. While substrate specificity has not been studied in KAOs, early analyses showed multiple unexpected intermediate metabolites being produced by pea KAO [95]. As in bacteria, KAO activity can also be performed by some plant 2-OGD enzymes [96].

The cytosolic steps of GA biosynthesis and inactivation are performed by 2-OGD enzymes. While this family of proteins exists in many organisms, plant *GA20ox*, *GA3ox*, and *GA2ox* genes share a common ancestor. These genes are widely present in vascular plants (Fig. 3), but *bona fide* orthologous genes have not been found in early-diverging land plants, in agreement with the absence of canonical bioactive GAs in bryophytes [88,89,97,98]. Recently, a new GAox-related 2-OGD has been found in mosses, KA2ox, which oxidizes *ent*-kaurenoic acid into the inactive *ent*-2α-hydroxy-kaurenoic acid (2OH-KA), thereby decreasing the alternative oxidation of *ent*-kaurenoic acid into the active 3OH-KA during caulonemal development [97]. Other GAox-related 2-OGD are predicted to exist not only in the moss *Physcomitrella patens,* but also in vascular plants such as *S. moellendorffii,* suggesting that other GA oxidation-related activities exist in bryophytes and vascular plants apart from the canonical ones [99]. In the case of mosses, this may reflect an independent evolution derived from the lack of KAO enzymes, and possibly the acquisition of an increased substrate specificity for *ent*-kaurenoic acid. As in the case of many enzymes, some of these 2-OGD have evolved towards different specificities and catalytic efficiencies. Such is the case of the fern *Lygodium japonicum*, that harbours a GA3ox enzyme with increased specificity towards GA_9 when compared with most seed plants' GA3ox [22].

Other plant lineages, bacteria, and fungi have been found to produce GAs, but through biosynthesis pathways which appear to have evolved independently to that of land plants [100–102]. For instance, while fungi and non-vascular land plants possess a single bifunctional CPS/KS enzyme, GA-producing bacteria such as *Bradyrhizobium japonicum* harbour two independent monofunctional enzymes [103]. Both KO and KAO activities in fungi and bacteria are performed by CYP enzymes, but they are unrelated to their plant counterparts [104,105]. Similarly, GA20ox and GA3ox enzymes are not found either in bacteria nor fungi. These steps are also accomplished 2-OGD enzymes in bacteria, while fungi are able to produce GAs using a different set of CYPs after KAO [101]. Besides, other enzymatic differences between homologous steps exist between these organisms. This is the case of *Gibberella fujikuroi* KAO, that produces GA_{14} from *ent*-kaurenoic acid instead of GA_{12} [104]. On the other hand, the algal groups Chlorophyta, Charophyta, and Phaeophyceae, have been reported to produce bioactive GAs in more than 30 species [106–108], while none has been found in Rhodophyta species [109]. Given that Chlorophyta and Charophyta lack clear *CPS* and *KS* orthologs [79], and Phaeophyceae algae are distantly related to Archaeplastid lineages [110], the most plausible explanation is that so far unidentified pathways for GA biosynthesis may have evolved independently in multiple lineages, in an extreme case of convergent evolution between kingdoms and domains.

One could speculate that GAs may possess chemical features that make them prone to act as physiological regulators. The vast number of known gibberellin-related structures may represent the opportunity for new regulatory molecules to emerge. By-products of other hormonal metabolism pathways have evolved to act as signaling molecules, such as phaseic acid [111]. Jasmonic acid has also been proposed to have evolved as a by-product of fatty acid catabolism followed by co-evolution between ligand and receptor [112]. In the case of GAs, 2OH-KA may have acquired a function as a regulatory molecule in *P. patens* after its emergence as an inactive product of *ent*-kaurenoic acid [97].

3. Evolution of gibberellin signaling

3.1. Origin and diversification of DELLA proteins

Genome sequencing efforts have shown that *DELLA* genes are found both in vascular and non-vascular land plants (Fig. 3). For example, a single *DELLA* gene is present in the genome of the liverwort *Marchantia polymorpha* [89] or the hornworts *Anthoceros agrestis* [113] and *A. angustus* [114], and two paralogous genes are found in the mosses *P. patens* [115] and *Sphagnum fallax* [94]. Thorough examination of available transcriptomes [116] has confirmed the presence of DELLAs in all extant lineages of land plants but not in algae [117], pointing to an origin of DELLAs in the ancestor of all land plants. This model is supported by a more general analysis of GRAS gene evolution, which shows that algal GRAS gene families are monophyletic, while the expansion of GRAS families in land plants (including the DELLA subgroup) has occurred independently [117].

From a phylogenetic perspective, DELLAs in early-diverging land plants belong to an ancestral type ("DELLA1/2/3"), which later duplicated in the ancestor of vascular plants ("DELLA1/2" and "DELLA3"), and once again in eudicots ("DELLA1", "DELLA2" and "DELLA3") [117]. The actual number of DELLA paralogs in different species reflects the particular history of genome losses and gains during evolution. For instance, only one *DELLA* gene (*PROCERA*) is found in

Solanum lycopersicum [118,119] due to loss of DELLA1 and DELLA3; one in *O. sativa* [120] due to the loss of the DELLA domain in DELLA3; or five in Brassicaceae [121–124] as the result of loss of DELLA3 and duplications of DELLA1 and DELLA2 [117]. Although mutant analyses in species with multiple *DELLA* genes are scarce, distinct functions have been assigned to different paralogous DELLAs. This is the case of the Arabidopsis *RGL2* gene, with a major role in the control of seed germination, and *RGA* in the regulation of stem elongation. However, subfunctionalization of at least these paralogs seems to have occurred through diversification at the level of *DELLA* gene promoters, rather than in DELLA activity [125].

Given that DELLA activity resides in the capacity to interact with TFs, the study of the evolution of DELLA activity requires the comparative analysis of the DELLA interactome in different species. Although no systematic study has been reported yet, functional studies in different plant species indicate that there is a degree of conservation in DELLA-TF interactions. For example, interaction of DELLA with NAC TFs regulates cellulose biosynthesis in rice and Arabidopsis [126], and interaction with JAZ regulators modulates the response to jasmonic acid also in both species [127,128]. On the other hand, although certain physical interactions are conserved, they may not regulate the same processes. This is the case of DELLA interaction with NF-Y, which regulates nodulation in *Medicago truncatula* [71] and flowering time in Arabidopsis [129]. Despite the lack of information about DELLA activity other than in angiosperms, more indirect studies also suggest that a large part of DELLA activity is conserved across land plants. Heterologous expression of the *S. moellendorffii DELLA1* gene in rice caused dwarfism, and treatment of *S. moellendorffii* plants with the GA biosynthesis inhibitor uniconazole impaired growth to some extent [99], suggesting that at least the function of DELLAs on growth is probably conserved across vascular plants. Similarly, growth was reduced by the expression of the *P. patens DELLAa* in an Arabidopsis mutant lacking the two major DELLAs [115] but not in rice [99], indicating that the control of plant growth may have been already encoded in the ancestral DELLA protein, with additional species-specific fine tuning.

The fact that *DELLA* genes are present in all land plant genomes examined, while active GAs and GID1 are only typical of vascular plants, indicates that DELLAs predate the GA perception module, and their recruitment was the origin of GA signaling. According to our knowledge of the regulation of DELLA stability by GA/GID1 (see above), the emergence of this regulatory mechanism required the establishment of GA-dependent physical interaction between GID1 and the N-terminal domain of DELLA in the ancestor of all vascular plants. However, the conservation of motifs and residues important for the interaction [39,42] is also extensive to DELLAs in non-vascular plants. This indicates that the N-terminal domain in the ancestral DELLA was already configured for the interaction with GID1, and that this domain must have an additional function that justifies its conservation in extant non-vascular plants lacking GID1. Three observations suggest that such function may be the activity as a transcriptional coactivator: (i) Arabidopsis and rice DELLAs display intrinsic transactivation capacity in heterologous systems, like yeast or rice callus cells [16,130]; (ii) Arabidopsis DELLAs enhance the transactivation capacity of certain TFs with which they interact [35,63–65]; and (iii) the transactivation capacity resides in the N-terminal domain, based on deletion analyses in Arabidopsis and rice [130], and it is conserved also in non-vascular plants [117]. Thus, the establishment of GA signaling probably involved the exaptation by the newly-emerged GA receptor of a pre-existing transactivation domain in the N-terminal domain of DELLA proteins (Fig. 5). The observation that the sole interaction with GID1 blocks DELLA's transactivation capacity [130] suggests that ubiquitination-dependent regulation of DELLA stability may have occurred in a second evolutionary step as a refinement of this primary mode of GA-dependent control of DELLA activity (Fig. 5).

In the light of the stepwise construction of GA signaling along the plant lineage, with DELLAs predating the appearance of GAs and GID1,

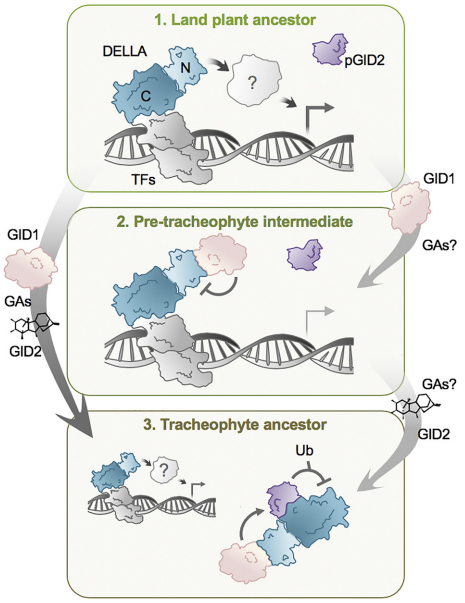

Fig. 5. Possible paths of GA signaling evolution. 1. Land plant ancestor containing DELLA able to transactivate gene expression through an unknown co-activator recruited with the N-terminal domain (N). Some transcriptional factors (TFs) would be in charge of recruiting DELLA to chromatin. GID2 would not be part of a GA-based DELLA regulation. 2. A possible intermediate state in pre-tracheophytan ancestors. True GID1s would recognize DELLA and impede transactivation, either in a GA-independent way or after GA binding. 3. The full GA-signalosome is fully assembled in vascular plants. GID1 would recognize DELLA proteins after GA binding and facilitate GID2 interaction to recruit a SCF complex for DELLA degradation through the 26S proteasome. The single-step path (left) suggests the sudden assembly of the whole signalosome from 1 to 3, skipping scenario 2. The two-steps path (right) suggests an intermediate state between 1 and 3 with no –or low– levels of DELLA stability regulation by GID1.

a relevant question is what properties have DELLAs and GAs provided to cellular homeostasis. An *in silico* approach based on comparative expression network analysis between species with key differences within DELLA signaling has shown that the presence of this pathway correlates with a marked increase in the coordination of transcriptional responses [131]. In this study, gene coexpression networks were built from a chlorophyte without GA/GID1 or DELLAs (*Chlamydomonas reinhardtii*), a moss that harbours *DELLA* genes but no GA/GID1 perception module (*P. patens*), and two angiosperms with a full GA pathway. Then, subnetworks were extracted with the sets of putative DELLA-dependent transcriptional targets, and the characteristics of these subnetworks were compared across species. Several parameters indicative of network interconnectivity increased in species with *DELLA* genes, specially in those capable of GA perception [131], suggesting a role of DELLAs and GAs in the coordination of transcriptional responses. However, further experimental studies are needed to assess this property.

3.2. Evolution of the gibberellin perception module

The GA perception module (i.e.: GA-GID1) has only been shown to be functional in vascular plants [99,115]. Phylogenetic and structural analyses have confirmed that GID1 receptors are part of the

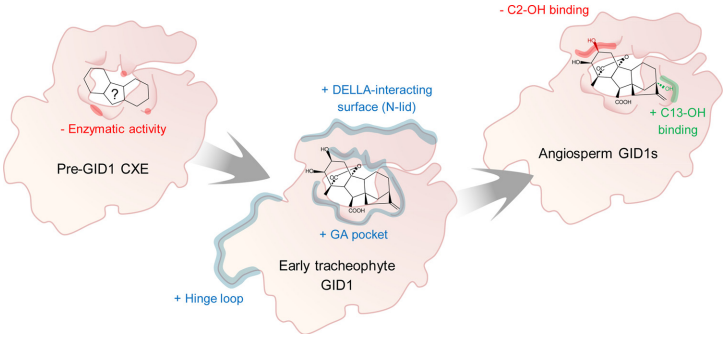

Fig. 6. GID1 receptor evolution in vascular plants. The structure of GID1a has been adapted from Murase et al. (2008). The three features associated with the transformation of a CXE into a GA receptor (N-end lid, hinge and the GA pocket) are highlighted.

carboxylesterase family (CXE) [40,42,132], belonging to the α/β-hydrolase superfamily [133]. The two features that distinguish GID1s are: (i) the ability to recognize GAs in their catalytic pocket, and (ii) the ability to interact with DELLAs after their pocket lid is closed [39]. In this sense, canonical GID1 with a characteristic N-terminal that recognizes DELLA motifs and lack of the CXE catalytic triad exist only in vascular plants, while CXEs are present in all Archaeplastida. However, the availability of new genomic and transcriptomic data from early-diverging land plants and algae has not uncovered yet the presence of a CXE/GID1 representing a possible proto-GID1 with intermediate characteristics. No information exists about how GAs became ligands of proto-GID1s, or whether their binding occurred in a catalytically active CXE. But it is plausible that the ancestral GID1 was a CXE which lost its catalytic activity and adapted its substrate pocket to accommodate GA-related compounds (Fig. 6).

Nevertheless, some studies have defined that vascular GID1s radiated into several clades [134], and further evolved in terms of sensitivity towards specific GAs or to abolish the recognition of others [135]. Examples of adaptation in terms of sensitivities include GID1-1 from the fern *L. japonicum*, which copes with very low concentrations of its substrate in prothalli to induce antheridial formation, and thus presents between one to two orders of magnitude higher affinity for GA_4 than seed plant GID1s [22]. In turn, *S. moellendorffii* contains GID1s with lower affinities for GAs than seed plant GID1s [99], and they are able to bind also the inactive intermediates GA_9 and GA_{34}. Coinciding with the predicted absence of the 13-hydroxylation alternative pathway in non-seed vascular plants, they cannot accommodate C13−OH bioactive GAs (Fig. 6) [135]. In eudicots, sub-functionalization after duplication of a single copy GID1 gave rise to two clades: GID1ac and GID1b [134,135]. These clades differ not only in terms of expression patterns that are well documented in soybean, *Medicago* or Arabidopsis [134,136,137], but in their sensitivities to different amounts of GAs [135]. Most of these GID1s are able to interact in a GA-dependent manner with DELLA proteins. Interestingly, some vascular GID1s are able to bind DELLA in the absence of GAs due to a semi-closed set up of their lids [138,139]. Mutations in a hinge loop present in OsGID1 suggest that this hinge evolved to allow lid closure upon GA binding, but intermediate states are possible. GID1b clade resembles this structural state, making it hypersensitive to GA [139]. This opens the possibility to a first evolutionary step in which recognition of DELLA by GID1 was independent of GAs, followed by GA recognition to enable accurate regulation of DELLA-GID1 binding (Fig. 5).

How the functional consequence of DELLA-GID1 interaction emerged is also unclear. A key element in GA signaling is the F-box SLY1/GID2 responsible for proteasome-dependent DELLA degradation, but studies with Arabidopsis *sly1* and rice *gid2* mutants suggest that GID1 can inhibit the transactivation capacity encoded in the N-terminal domain of DELLA proteins [130] and possibly in its GRAS domain [140], in a proteasome-independent way [141,142]. Therefore, GID1 could have originally evolved to inhibit DELLA function before incorporating GID2 to the GA-GID1-DELLA complex. Considering that genes encoding SLY1/GID2 have been unequivocally found in liverworts [89,117], an alternative evolutionary model would be that a land plant ancestral SLY1/GID2 could have been involved in the regulation of DELLA stability prior to the emergence of the GA-GID1 module.

4. Gibberellin function: lessons from evolution

The past 25 years have witnessed tremendous advances in our knowledge of GA action in plants. The identification of the receptor, the elucidation of the signal transduction mechanism through DELLA degradation, and the control of gene expression through the interaction between DELLAs and transcriptional regulators have solved many intriguing questions, and several strategies have been proposed to harness GA metabolism and signaling, and generate crop improvement. At a more physiological level, two important questions remain, to which current and future approaches from an evolutionary perspective can contribute.

GAs can perform many functions depending on the organ, growth phase, developmental stage, or environmental conditions. Where does this functional diversity reside? Unlike other hormones, like auxin, GA signaling relies on a relatively small set of elements, which in some plant species consists of a single gene for the receptor, and a single DELLA gene [37]. In Arabidopsis, it is well established that DELLAs interact with dozens of TFs, providing a mechanistic framework to understand diversification of DELLA activity. What current models for the origin and evolution of GA signaling propose is that this promiscuity was hijacked by GAs when the GID1 receptor emerged in the ancestor of vascular plants [117]. Therefore, GA functional diversity is a direct consequence of a pre-existing property of DELLA proteins. Future research should explain how DELLAs became transcriptional hubs during evolution, whereas future biotechnological applications should then be targeted at generating DELLA alleles with different interaction abilities.

It has also been proposed that GAs regulate the balance between growth and defence responses [78], based on observations using loss- or gain-of-function mutants of GA activity (e.g., metabolism and signaling), or with pharmacological treatments that enhance or decrease GA activity. However, studies are missing that demonstrate the participation of GAs in the short-term adaptive response of individual plants, or in the long-term adaptation of plant populations to different ecological niches. What *in silico* evolutionary models show is that GAs seem to be linked to an increased ability to coordinate transcriptional

programs [131], suggesting that this hormone pathway may have a potential impact in adaptation at least in an evolutionary scale. It will be interesting to obtain experimental evidence that supports, or not, an ancestral role of DELLAs or GAs in this transcriptional coordination.

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PART 2 – ADDITIONAL FIGURES FOR GENERAL DISCUSSION

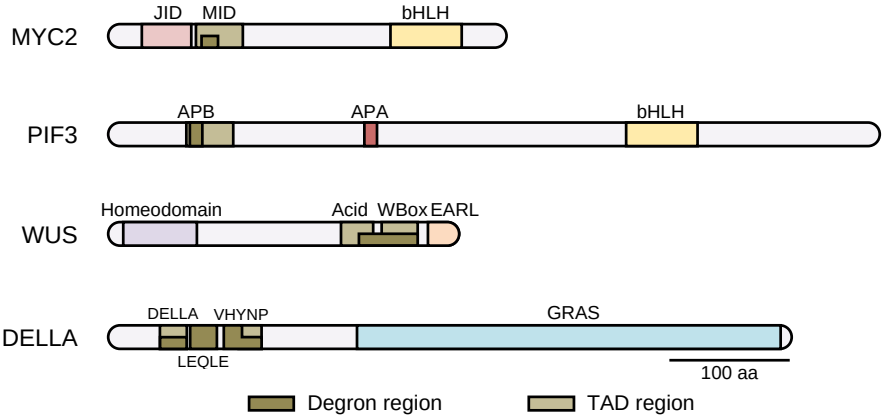


Figure 1. Physical overlap of degron and TAD regions in *Arabidopsis thaliana* transcription factors. Most of these have been found independently, and none have been functionally linked.

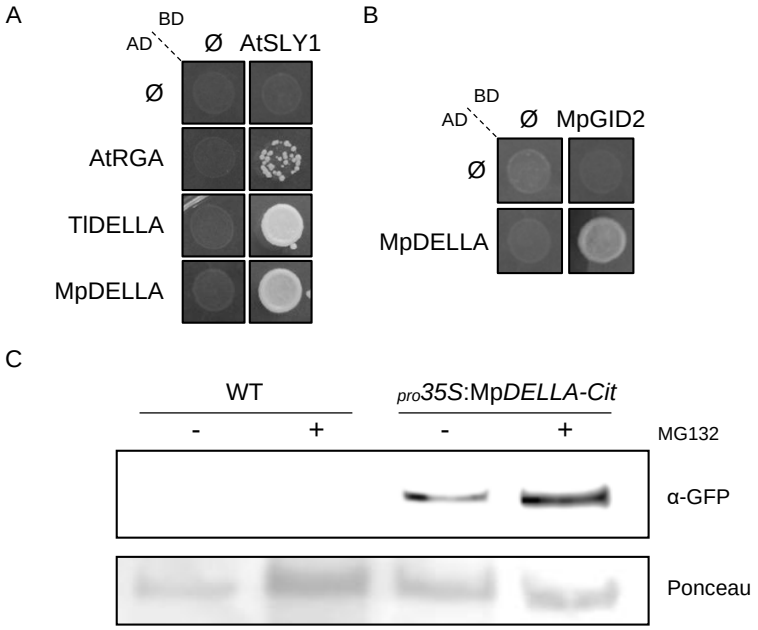


Figure 2. A) Yeast two-hybrid assay using Arabidopsis SLY1 as bait and different DELLAs as preys. AtRGA, *Arabidopsis thaliana* DELLA; TIDELLA, *Takakia lepidozioides* DELLA; MpDELLA, *Marchantia polymorpha* DELLA. B) Yeast two-hybrid assay of MpGID2 (SLY1 ortholog in *M. polymorpha*) as bait and MpDELLA as prey. C) Western-blot of MpDELLA-Cit protein stability with or without the 26S proteasome inhibitor MG132. Citrine-tagged Mp*DELLA* overexpression lines were grown in half-strength Gamborg's B5 axenic medium for 10 days, and treated during 3 hours with or without MG132 (100 µM) before collection, protein extraction, and western-blot analysis.

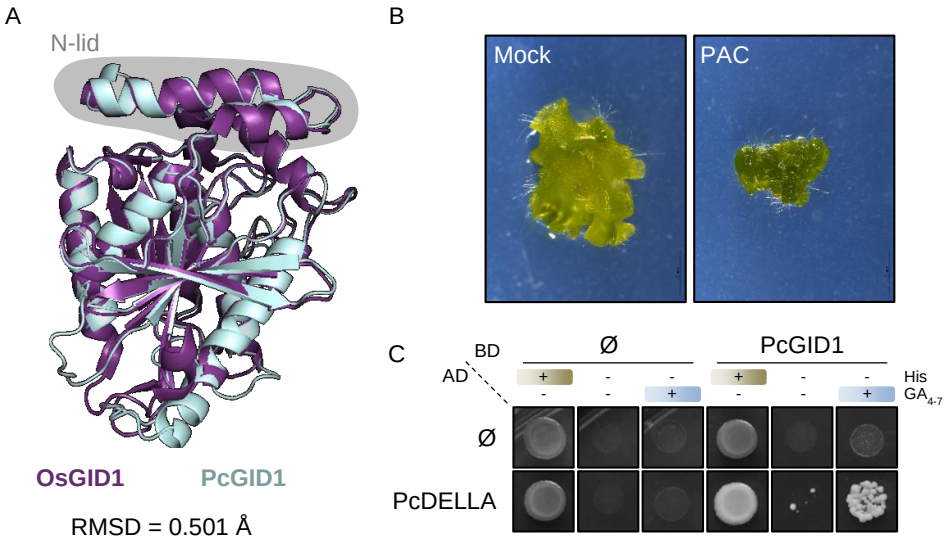


Figure 3. A) Predicted structure of PcGID1 superimposed on OsGID1 known structure. RMSD calculated with the whole protein. Grey shaded region delimits the so-called N-lid present in the PcGID1 structure (contrary to most CXE proteins). B) *Phaeoceros carolinianus* thallus explants grown in BCD+$CaCl_2$ medium for 30 days with or without paclobutrazol 1 μM. Note that *Phaeoceros* is usually grown from thallus cuttings. C) Yeast two-hybrid assay of PcGID1-PcDELLA GA-dependent interaction. Full-length PcGID1 is used as bait, while PcDELLA N-terminal domain is used as prey. Histidine is added as positive growth control.

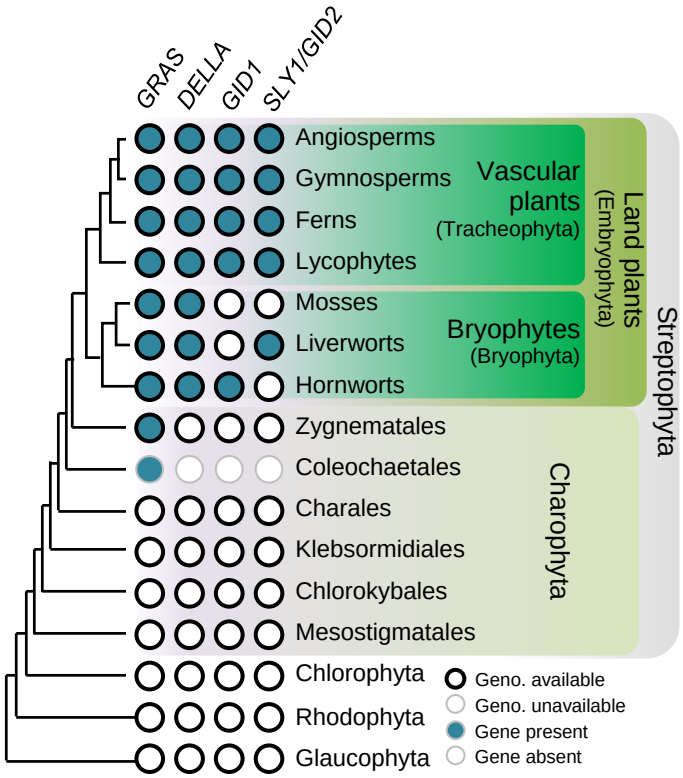


Figure 1B. Gibberellin signalling elements are present in vascular plants. Presence of gibberellin-signalling related sequences in different phyla. GRAS, GID1 and GID2 orthologs were retrieved from oneKP or genome databases by BLASTP or TBLASTX searches.

Compared to **Figure 1B** in page **29** (Originally published version from 2019 based on 2018 data). Main changes arise from 1) newly published genomes, especially in charophytan lineages, 2) newly found phylogenetic relationships among bryophytes, which are now widely accepted as a monophyletic tree.

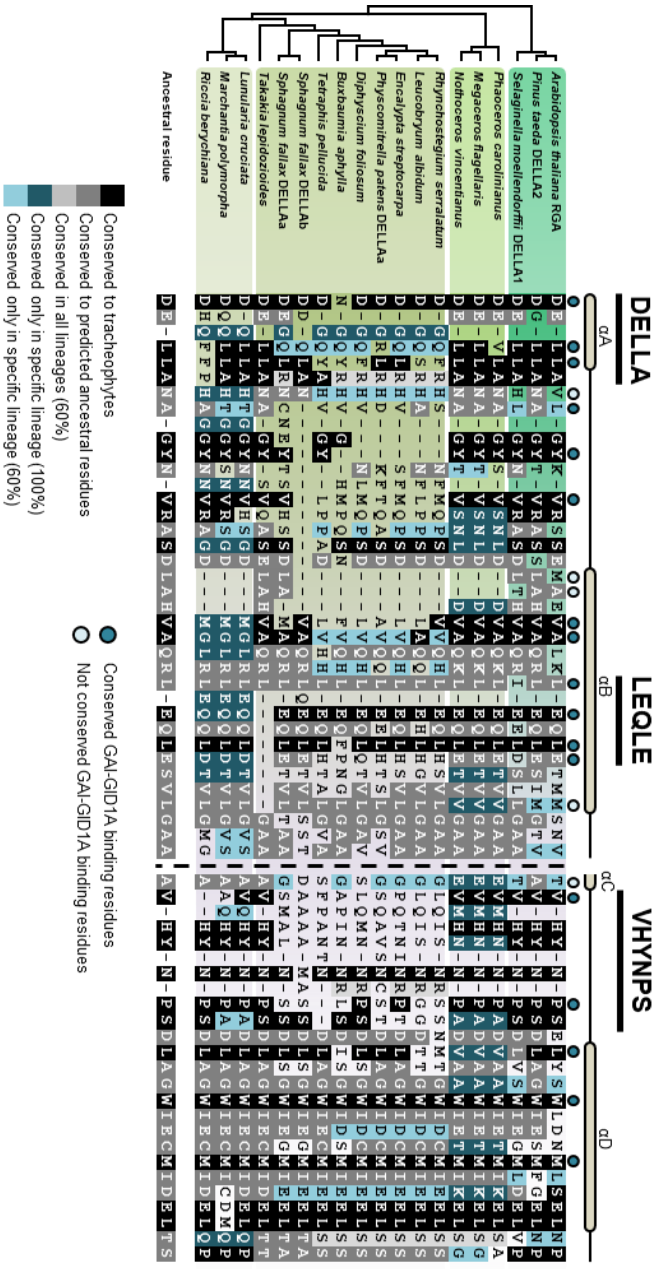


Figure 4. GID1 binding residues in DELLA proteins are highly conserved. Multiple protein sequence alignment showing DELLA amino-terminal region spanning from α helix A to D. In some cases, non-conserved sequences were trimmed to avoid multiple gaps presence. Conservation percentages are based on original alignments. GAI-GID1A binding sites based on Murase et al. 2008. Ancestral DELLA sequence inferred with FastML, only the most probable residues are shown per position.

Compared to **Figure 4** in page **35.** The only change is the species tree based on the monophyly of bryophytes.

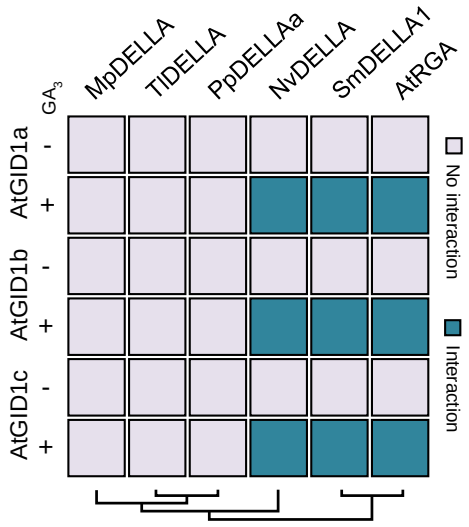


Figure 5B. Some non-vascular DELLA proteins can interact with GID1 receptors in a GA dependent manner. Yeast-two-hybrid assay results between DELLA proteins and the three Arabidopsis GID1 receptors with or without GA_3. Positive interactions are accounted when yeast growth occurs in -His SD media supplemented with 5 mM 3-aminotriazol.

Compared to **Figure 5B** in page **37**. The only change is the species tree based on the monophyly of bryophytes.

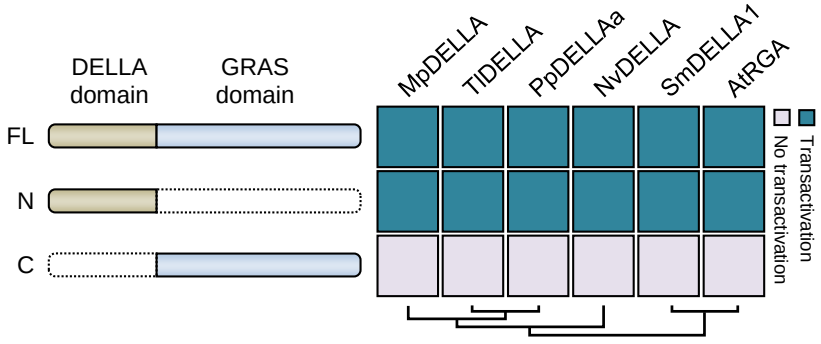
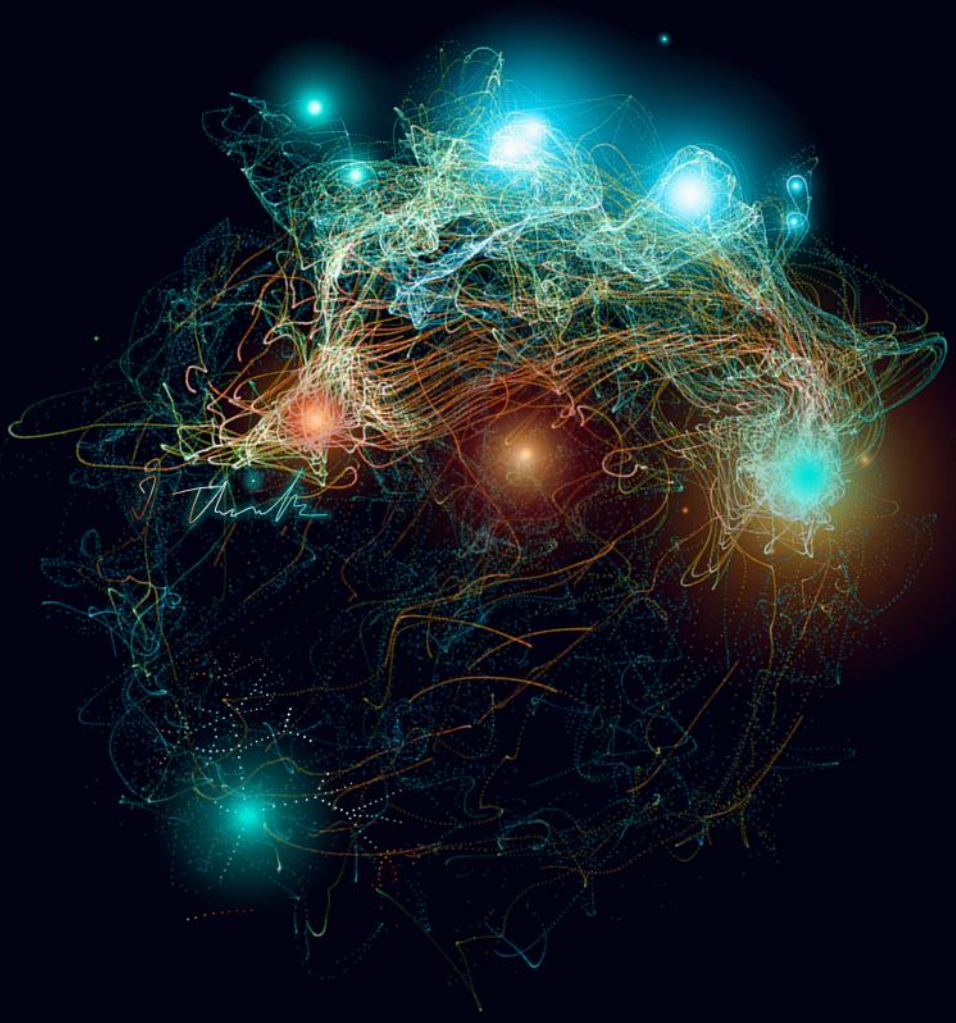


Figure 6B. DELLA domain conserved region act as a transcriptional activator domain. Yeast transactivation assay results using DELLA protein full-length coding regions (FL), or truncated versions using either the GRAS domain (C) or the DELLA domain (N). Transactivation is accounted when yeast growth occurs in 5 mM 3-aminotriazol.

Compared to **Figure 6B** in page **39**. The only change is the species tree based on the monophyly of bryophytes.

"When radium was discovered no one knew that it would prove useful in hospitals. The work was one of pure science. And this is a proof that scientific work must not be considered from the point of view of the direct usefulness of it. It must be done for itself, for the beauty of science"

Marie Salomea Sklodowska Curie



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