Transcriptional Diversification and Functional Conservation between DELLA Proteins in Arabidopsis

Javier Gallego-Bartolomé,[1] Eugenio G. Minguet,†,[1] Jaime A. Marín,[2] Salomé Prat,[2] Miguel A. Blázquez,*,[1] and David Alabadí[1]

[1]Instituto de Biología Molecular y Celular de Plantas (UPV-Consejo Superior de Investigaciones Científicas), Universidad Politécnica de Valencia, Valencia, Spain

[2]Departamento de Genética Molecular de Plantas, Centro Nacional de Biotecnología (Consejo Superior de Investigaciones Científicas), Madrid, Spain

†Present address: Laboratoire Physiologie Cellulaire Végétale, UMR5168, Centre National de la Recherche Scientifique, Commissariat a l'énergie atomique, Institut National de la Recherche Agronomique, Université Joseph Fourier, Grenoble, France.

***Corresponding author:** E-mail: mblazquez@ibmcp.upv.es.

Associate editor: Neelima Sinha

Abstract

Plasticity and robustness of signaling pathways partly rely on genetic redundancy, although the precise mechanism that provides functional specificity to the different redundant elements in a given process is often unknown. In Arabidopsis, functional redundancy in gibberellin signaling has been largely attributed to the presence of five members of the DELLA family of transcriptional regulators. Here, we demonstrate that two evolutionarily and functionally divergent DELLA proteins, RGL2 and RGA, can perform exchangeable functions when they are expressed under control of the reciprocal promoter. Furthermore, both DELLA proteins display equivalent abilities to interact with PIF4 and with other bHLH transcription factors with a reported role in the control of cell growth and seed germination. Therefore, we propose that functional diversification of Arabidopsis DELLA proteins has largely relied on changes in their gene expression patterns rather than on their ability to interact with different regulatory partners, model also supported by a clustering analysis of *DELLA* transcript profiles over a range of organs and growth conditions that revealed specific patterns of expression for each of these genes.

Key words: gene duplication, redundancy, gibberellin signaling, plant development.

Introduction

Gene duplications are considered as the major source for variation and the generation of evolutionary novelties (Ohno 1970). Although the most common fate for duplicated genes is gene loss (Lynch and Conery 2000), duplicated copies are released from mutational constraints, enabling the evolution of new functions (neofunctionalization) (Ohno 1970; Taylor and Raes 2004). Alternatively, both gene duplicates can undergo simultaneous reduction of their activity thereby maintaining the total capacity of the ancestral gene (subfunctionalization) (Force et al. 1999).

In addition to providing genetic robustness against deleterious mutations through functional redundancy, gene duplications seem also to be at the core of the mechanisms that provide an unusually high degree of plasticity and robustness to plant signaling pathways (Smith 1990; Pickett and Meeks-Wagner 1995; Casal et al. 2004). For instance, it has been proposed that the multiplicity of responses triggered by auxin is governed by the optimized interaction of more than 20 Aux/IAA-ARF pairs in each cell type (Weijers and Jurgens 2004; Weijers et al. 2005). However, this phenomenon is less understood in other hormonal pathways, in which signaling is transduced by a set of structurally and phylogenetically related proteins that represent branching points in the action of a single given hormone. Such is the case for protein phosphatases type-2C in abscisic acid signaling (Rodriguez 1998), the arabidopsis response regulators response regulators in cytokinin signaling (To et al. 2004) and the Jasmonate ZIM-domain family of proteins involved in jasmonic acid signaling (Chini et al. 2007) among others.

Much work has been devoted to understand the molecular mechanisms that allow the maintenance of gene duplicates in model organisms. Nonetheless, of particular relevance is establishing the extent of the relative contribution to the functional divergence of paralogous genes of variation in the regulatory sequences versus to those in the coding region. The accumulation of polymorphisms in the coding regions of amylase (Goto et al. 2005) and fatty-acid desaturase genes (Fang et al. 2009) in Drosophila, and β-defensins in mice and humans (Maxwell et al. 2003), underscore the importance of rapid variation in the coding sequence of recently duplicated genes to generate functional divergence. However, large-scale analyses of expression divergence among duplicated genes in yeast, plants, and humans provide a less clear-cut view. In particular, it has been shown that half of the recently duplicated genes in Arabidopsis (Blanc and Wolfe 2004) or rice (Li et al. 2009) have divergent expression patterns, although no correlation is found between expression divergence and time since duplication (Haberer et al. 2004). Actually, it is likely that functional divergence between duplicated genes occurs through both mechanisms, as suggested by the analysis

© The Author 2010. Published by Oxford University Press on behalf of the Society for Molecular Biology and Evolution. All rights reserved. For permissions, please e-mail: journals.permissions@oxfordjournals.org

Research article

Downloaded from http://mbe.oxfordjournals.org/article/27/6/1247/1110303 by CSIC user on 09 September 2020

of paralogs of human transcription factors (TFs): If the DNA binding site motifs of the TF paralogs are similar, their expression has diverged, whereas two paralogs that are highly expressed in a tissue tend to have dissimilar DNA-binding site motifs (Singh and Hannenhalli 2008).

Despite all suggestive evidence based on correlations obtained through genomic analyses of duplicated genes, direct experimental evidence for the relative importance of promoter versus coding-sequence divergence is scarce. A significant exception is the demonstration that diversification of cis elements in the promoters have been essential to solve "adaptive conflicts" in the ancestor of the duplicated genes (Hittinger and Carroll 2007). For this reason, we chose to analyze the degree of conservation of the actual molecular activities of members belonging to a small family of Arabidopsis transcriptional regulators.

In Arabidopsis, functional redundancy in GA signaling has been largely attributed to the presence of five members of the DELLA family of nuclear-localized transcriptional regulators: GAI, RGA, RGL1, RGL2, and RGL3. These proteins accumulate under low GA concentrations and act as repressors of GA-activated processes, whereas a local increase in hormone concentration triggers proteasome-dependent degradation of the DELLA proteins, by the concurrent action of the GA receptor GID1 and the E3 ubiquitin ligase SCF^{SLY1} complex (Dill et al. 2004; Griffiths et al. 2006; Nakajima et al. 2006). Molecular genetic analyses have shown that each member of the DELLA family performs specific but also overlapping roles in plant development. For example, GAI and RGA are the main regulators of cell expansion in vegetative tissues, because simultaneous loss of GAI and RGA function suppresses the dwarf phenotype of GA-deficient plants to a large extent (Dill and Sun 2001; King et al. 2001). However, male fertility is primarily regulated by RGA (but not GAI), together with RGL1 and RGL2 (Cheng et al. 2004; Tyler et al. 2004). On the other hand, RGL2 has been proposed to be the main regulator of germination, because knockout mutations in *RGL2*, but not the other DELLA genes, allow germination of seeds also when GA synthesis is impaired (Lee et al. 2002; Tyler et al. 2004).

Sequence comparisons show a high degree of conservation between the five DELLA proteins, but also enough differences are observed in their N-terminal third that might justify their different activities (Hussain et al. 2005). On the other hand, it cannot be ruled out that functional specificity of the DELLA genes relies on their different expression profiles, as evidenced by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) expression analyses of different organs (Tyler et al. 2004). To distinguish between these two possibilities, we have examined the ability of RGL2 and RGA to perform exchangeable functions, by expressing *RGL2* under the control of the *RGA* promoter and vice versa.

Materials and Methods

Plant Lines and Growth Conditions

Arabidopsis thaliana mutant plants (*ga1-3*, *ga1-3 gai-t6*, *ga1-3 gai-t6 rga-24*, and *rgl2-1*) were all in the L*er* ecotype. For germination tests, L*er* and *rgl2-1* seeds were surface sterilized and sown on sterile Whatman filter papers placed in plates of half-strength MS medium (Duchefa) with 0.8% w/v agar and 1% w/v sucrose and stratified at 4 °C for 7 days in darkness. *ga1*, *ga1 gai-t6* and *ga1 gai-t6 rga-24* seeds were sterilized and imbibed at 4 °C for 7 days in water containing 20 μM GA_3. Before transfer to MS plates, they were extensively rinsed with sterile water to remove any remaining GA_3.

Germination took place under continuous white fluorescent light (90–100 μmol m^{-2} s^{-1}) at 23 °C in a Percival growth chamber E-30B. Soil-grown plants were kept at 23 °C in cabinets with a 16-h photoperiod.

Construction of Vectors and Generation of Transgenic Lines

For promoter-swapping experiments, 2-kb promoter regions of *RGA* and *RGL2*, which included the 5′ untranslated region, were polymerase chain reaction–amplified from genomic DNA of wild-type L*er* plants using the pairs of oligonucleotide primers:

pRGA-F-XbaI (TCTAGATATAACCTCATCCATCTATAG) and pRGA-R-XbaI (TCTAGATTACAAGATCTGATGGAG) for p*RGA* and pRGL2-F-XbaI (TCTAG ATCAGGATGCGAGGTTAAGAATGG) and pRGL2-R-*Hin*dIII (AAGCTTTTACTT TACTTCATGGGT) for p*RGL2*.

The PCR products were subcloned into pCR2.1 (Invitrogen) and transferred into the multiple cloning site (MCS) of the Gateway binary vector pSBright (Bensmihen et al. 2004) by *Xba*I digestion in case of pRGA to generate pSBright-pRGA and *Xba*I/*Hin*dIII for pRGL2 to generate pSBright-pRGL2. The *RGA* and *RGL2* cDNAs were obtained from the REgulatory Gene Initiative in Arabidopsis consortium as pDONR201 (Invitrogen) clones. Both cDNAs were subcloned into pSBright-pRGA or -pRGL2 using LR clonase (Invitrogen).

The constructs were introduced into the *Agrobacterium tumefaciens* strain C58 by electroporation, and these were then used to transform the *Arabidopsis ga1 gai-t6 rga-24* and *rgl2-1* mutants by the floral dip method (Clough and Bent 1998). Transgenic seedlings in the T_1 and T_2 generations were selected based on their resistance to glufosinate. Transgenic lines with a 3:1 (resistant:sensitive) segregation ratio were selected, and at least 19 homozygous lines were identified in the T_3 generation for each construct. Data from two representative lines per construct are shown in this work.

Phylogenetic Analysis

Iterative search by TBlastN was done on the NCBI public database (http://www.ncbi.nlm.nih.gov) with the *A. thaliana* DELLA amino acid sequences as baits, and representative full-length sequences were selected. Species-specific databases were also consulted to determine the number of DELLA genes present in each genome. Alignments of protein sequences were done with ClustalX (Thompson et al. 1997) and only the informative part of the alignments were used for subsequent analyses. A phylogenetic tree was

Downloaded from https://academic.oup.com/mbe/article/27/6/1247/1110303 by CSIC user on 09 September 2020

obtained with the PhyML software v2.4.4 (Guindon and Gascuel 2003) using the Jones, Taylor, and Thorton amino acid substitution model as indicated upon comparison of the different models with Prottest (Abascal et al. 2005). The consensus tree was supported by Bootstrap analysis (n = 1,000). Visualization and manipulation of trees were made with TreeView (Page 1996).

Real-Time Quantitative RT-PCR

Total RNA from imbibed seeds was extracted using a modified RNAeasy Mini kit (Qiagen) protocol, where the ground tissue was previously incubated with 600 µl of RNeasy Lysis buffer-Polyvinylpyrrolidone (RLT-PVP) buffer (540RLT buffer from RNAeasy Mini Kit + 60 µl PVP40 10% + 6 µl β-mercaptoethanol) followed by a 30-s centrifugation, recovery of the supernatant, and application to the lilac column. For adult plant tissues, total RNA extraction was carried out as described previously (Frigerio et al. 2006). cDNA synthesis and quantitative PCR, as well as primer sequences for amplification of GA metabolism and EF1-α genes, have been described previously (Frigerio et al. 2006).

The primers used for the quantitative PCR analysis of mRNA levels of *GFP*, *RGA*, *RGL2*, *GAI*, *ATHB16*, and *AtMYB34* were, respectively, GFPqRT-F (TCATATGAAGCGGCACGACTT) and GFPqRT-R (GATGGTCCTCTCCTGCAC GTA); RGAqRT-F (ACTTCGACGGGTACGCAGAT) and RGAqRT-R (TGTCGT CACCGTCGTTCC); RGL2qRT-F (GACGGCGCGTAGAGTTCAC) and RGL2qRT-R (TGCATCCCTTGATTAAGCCC); GAIqRT-F (GCTTATGCAGGCTCTTGCG) and GAIqRT-R (AACCGGAAAACAGGAGGACC); ATHB-16qRT-F (GCGCCGTTCTT AACGACGAAACAA) and ATHB-16qRT-R (TAAGAAACTCCCGCCAGTAACCGT); MYB34qRT-F (TTAACCGCGTCGCAAGCAAATACG) and MYB34qRT-R (TTGAGC AATGTGGAGGTCGGAGAA).

Protein Extraction and Western Blot

Total proteins were extracted by homogenizing seedlings in one volume of cold extraction buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 10% glycerol, 1 mM phenylmethanesulfonyl fluoride, and 1× complete protease inhibitor cocktail [Roche]). Extracts were centrifuged at 13,000 × g for 10 min at 4 °C. Protein concentration in the supernatants was quantified by the Bradford assay (Bradford 1976). Aliquots (40 µg) of denatured total proteins were separated in Precise 8% Tris–N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid–sodium dodecyl sulfate gels (Pierce) and transferred onto polyvinylidene difluoride membrane (Bio-Rad). RGA–green fluorescent protein (GFP) fusion was detected using the monoclonal anti-GFP antibody (clone JL-8) from Clontech.

Yeast Two-Hybrid Assay

For two-hybrid experiments, truncated forms of RGA and RGL2, lacking the DELLA domain, were PCR amplified from a first-strand cDNA of L*er* seedlings using the following combinations of oligonucleotide primers:

RGA1-F (CACCCTGGTTGACTCGCAAGAGAACG) and RGA1-R (GTCAAAC TCAGTACGCCGCCG) for RGA; and RGL2-F (CACCCTCGTTGACTCTCAGGAG ACCG) and RGL2-R (GCCGCGACTCAGGCGAGTTTCC) for RGL2.

For PCR amplification of the complete coding regions for the PIF4, PIL2, PIL5, and SPT bHLH factors, the combinations of primers used were PIF4-F (CACCATGGAACACCAAGGTTGGAG) and PIF4-R (GGCTCACCAACCT AGTGGTCC) for PIF4; PIL2-F (CACCATGATGTTCTTACCAACCG) and PIL2-R (CAGGGAGAATTCCTTCATCTG) for PIL2; PIL5-F (CACCATGCATCATTTTGTC CCTG) and PIL5-R (GTTAACCTGTTGTGTGGTTTC) for PIL5; and SPT-F (CACCATGATATCACAGAGAGAAGAAAG) and SPT-R (GGACACTGTTCAAGT AATTCG) for SPT.

The PCR products were subcloned into the pENTR/D vector using the pENTR/D-TOPO cloning kit (Invitrogen) and mobilized by LR clonase (Invitrogen) into the pGBKT7 and pGADT7 Gateway vectors (kindly provided by Marta Boter) generated by inserting the Gateway *ccd*B cassette into the MCS *Nde*I –*Xho*I/*Sal*I sites of these vectors.

Constructs were transformed into the AH109 yeast strain (*MAT*a *ura3-52 his3-200 trp1-901 leu2-3,112 gal4Δ gal80Δ LYS2::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3 GAL2*$_{UAS}$*- GAL2*$_{TATA}$*-ADE2 URA3::MEL1*$_{UAS}$*-MEL1*$_{TATA}$*-lacZ*), using the lithium acetate/polyethilene glycol method, and yeast cells containing the different DELLA-BD and bHLH-AD fusion combinations were selected on SD-Leu-Trp and SD-Leu-Trp-His-Ade plates. Each construct was also transformed with the pGBKT7 or pGADT7 empty vectors to test for autoactivation activity. Protein extracts were obtained from the transformed yeast cells and western probed with anti-HA (Roche) and anti-GAL4BD (Santa Cruz) antibodies to ensure proper expression of the protein fusions.

Confocal Microscopy

Seedlings were rinsed for 2 min with 10 µg/ml propidium iodide (PI), then 5 min with water. Fresh stained seedlings were mounted on slides only with water. Images were taken using a Leica TCS SL confocal laser microscope (Leica, http://www.leica.com) with excitation at 488 nm. For GFP detection, channel 1 was configured between 500 and 540 nm, and for PI detection, channel 2 was configured between 590 and 660 nm.

Results and Discussion

Phylogenetic Relationships of DELLA Proteins in Angiosperms

Examination of public gene sequence databases and the available full genome sequences of several plant species indicates that, unlike in monocots, genes encoding DELLA proteins in dicots are frequently duplicated. In some cases, such as the *Brassicaceae*, the genomes contain up to five DELLA genes. To investigate the origin and the possible evolutionary history of these duplications, we assembled full-length sequences of DELLA proteins from different dicots and monocots, and analyzed their phylogenetic relationships. As shown in figure 1A, two large clades (I and II)

Downloaded from https://academic.oup.com/mbe/article/27/6/1247/1110303 by CSIC user on 09 September 2020

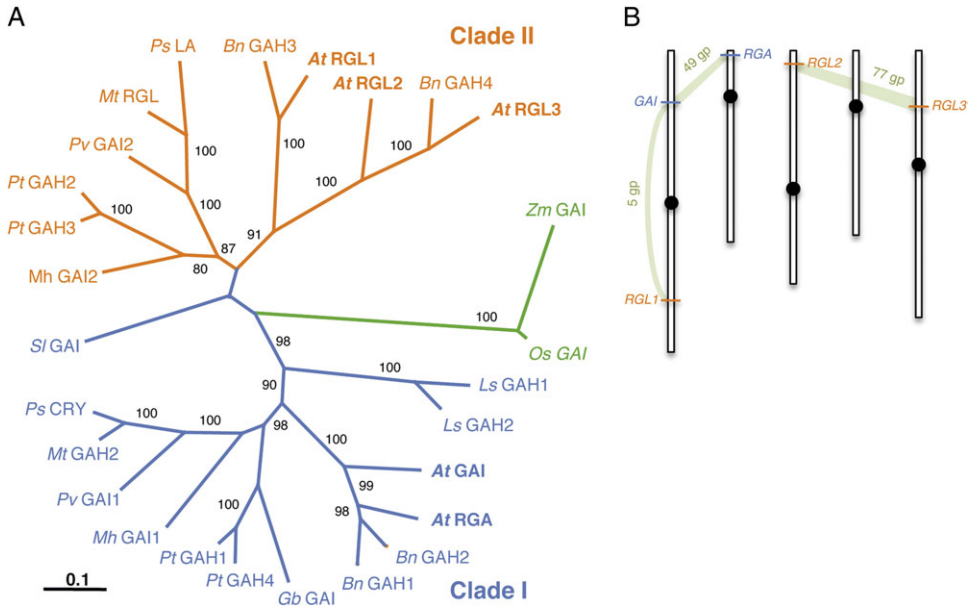

Fig. 1. Repeated duplication of DELLA genes in dicots. (*A*) Phylogenetic maximum likelihood tree of DELLA proteins displaying two large clades (blue and orange). See Material and Methods for details on sequence analysis. Arabidosis proteins are in bold. Numbers represent percentage bootstrap value ($n = 1{,}000$). Monocot sequences are depicted in green. (*B*) Syntenic regions in Arabidopsis chromosomes that include DELLA genes. *At*, *Arabidopsis thaliana*; *Bn*, *Brassica napus*; *Gb*, *Gossypium barbadense*; *Ls*, *Lactuca sativa*; *Mh*, *Malus hupehensis*; *Mt*, *Medicago truncatula*; *Os*, *Oryza sativa*; *Ps*, *Pisum sativum*; *Pt*, *Populus trichocarpa*; *Pv*, *Phaseolus vulgaris*; *Sl*, *Solanum lycopersicum*; and *Zm*, *Zea mays*. GAH stands for GAI Homolog. *gp* is the number of gene pairs syntenically conserved between the indicated regions.

of DELLA proteins can be found in dicots. However, although species in the Rosids genera such as *Populus*, *Pisum*, *Medicago*, and the *Brassicaceae* possess members in both subfamilies, species in the Asterids like *Solanum lycopersicum* and *Latuca sativa* posses either only one DELLA protein or two paralogs, respectively, that always group in clade I. In these cases, the absence of DELLA proteins associated with clade II could be a consequence of the lack of sequence information for these species, although a loss-of-function mutant identified in *S. lycopersicum* in the single reported *DELLA* gene displays a phenotype that covers all the functions attributed to GAs in this organism (Marti et al. 2007; Bassel et al. 2008; Jasinski et al. 2008). Hence, it is likely that it indeed represents the only functional *DELLA* gene in tomato.

The presence of two clades of DELLA proteins suggests that the diversification of this family was initiated by a duplication of a single ancestor within the Rosids. After this event, subsequent independent duplications would have given rise to the variety of DELLA proteins present in different species. In fact, the observation of syntenic regions in the Arabidopsis genome that include the five DELLA genes (fig. 1*B*) reveals a possible mechanism for the multiplication of these genes in the *Brassicaceae*, involving the rearrangement of large chromosomal fragments.

Expression of Chimeric Versions of DELLA Genes

According to genetic analysis, repeated duplication of DELLA proteins in Arabidopsis has been accompanied by certain degree of functional diversification, given that mutants in the different DELLA genes are affected only in a subset of responses regulated by GAs. To determine if this diversification has been caused by changes in the patterns of expression of these paralogs, or in the molecular activity of the different DELLA proteins, we decided to construct chimeric versions of two representative DELLA genes, one from each clade, under the control of their own and the reciprocal promoters (fig. 2*A*). *RGA* and *RGL2* were chosen because single loss-of-function mutants in each of these genes render a visible phenotype under certain conditions, a prerequisite to score the functionality of each chimera. For instance, seeds of the *rgl2-1* mutant are able to germinate in the presence of paclobutrazol (PAC) (Lee et al. 2002), and mutation of *RGA* in a *ga1-3 gai-t6* background rescues the dwarf phenotype caused by the lack of GA synthesis in this background (Dill and Sun 2001).

Therefore, a 2-kb fragment of the *RGL2* promoter was used to drive the expression of the *RGL2* (R2R2) or *RGA* (R2RA) coding sequences fused to *GFP* as a visualization marker, and the constructs were introduced into the *rgl2-1* mutant. Nineteen and 21 independent transformants were isolated, respectively. Given that *RGL2* is expressed predominantly in seeds (Lee et al. 2002; Tyler et al. 2004), the expression of the corresponding transgenes was measured by RT-qPCR in the seeds of the transformants and, based on their expression level, two homozygous lines from each class were selected for further analyses: one representative of the lower-expressing lines and one for the higher-expressing lines (fig. 2*B*). None of the transgenes displayed significant expression in tissues in which *RGL2* is not expressed (data not shown). Similarly, a 2-kb fragment of the *RGA* promoter was fused to the *RGA* (RARA) or *RGL2*

Downloaded from https://academic.oup.com/mbe/article/27/6/1247/1110303 by CSIC user on 09 September 2020

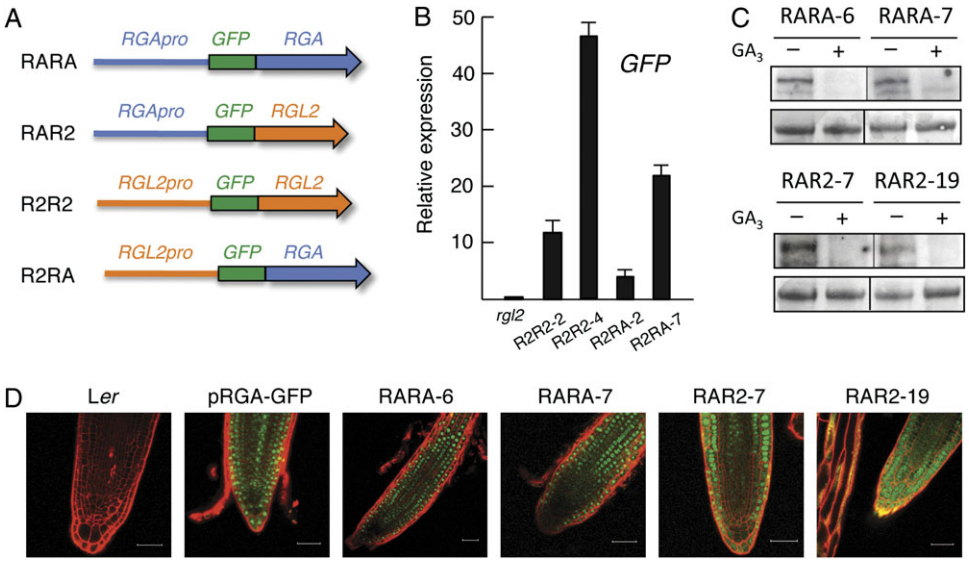

Fig. 2. Transgenic lines expressing *RGA* and *RGL2* under the control of their own and reciprocal promoters. (*A*) Scheme of the constructs used in this study. (*B*) Expression *of RGL2pro::GFP:DELLA* transgenes, determined by RT-qPCR analysis of *GFP* in *rgl2* mutants. Error bars represent standard deviation (SD) of three replicates. (*C*) Production of functional DELLA protein in transgenic lines expressing *RGApro::GFP:DELLA* in *ga1 gai-t6 rga-24* mutants, determined by western blot in seedlings with mock and 50-μM GA_3 treatments. (*D*) Nuclear localization of DELLA proteins in the roots of transgenic plants described in (*C*). Line pRGA-GFP is used as control (Dill et al. 2001). Size bar is 40 μm.

(RAR2) coding sequences and *GFP*, and the resulting constructs were introduced into the triple knockout line *ga1-3 gai-t6 rga-24*. Twenty-two and 25 independent transformants were isolated. The functionality of the constructs was tested through the detection of the GFP–DELLA proteins in extracts of 7-day-old seedlings and, as expected, their stability was severely reduced after incubation of the seedlings with 50 μM GA_3 for 3 h (fig. 2*C*). Furthermore, the transgenic DELLA fusion proteins displayed nuclear localization (fig. 2*D*), and two homozygous lines of each class were selected for further analyses.

Functional Substitution of RGA by RGL2

Loss of *GA1*, encoding *ent*-copalyldiphosphate synthase (Sun and Kamiya 1994), blocks the early steps in GA biosynthesis and causes severe dwarfism due to the accumulation of DELLA proteins, which affect the size of the shoot, the leaves, the hypocotyls, and other organs (Silverstone et al. 2001). This defect cannot be rescued by a mutation of *GAI*, due to redundancy with *RGA*, but simultaneous knockout mutations of both DELLA genes restores growth almost to the size of a wild type (Dill and Sun 2001; King et al. 2001). On the other hand, the endogenous *RGL2* gene does not have a role in cell expansion, because mutations in this gene do not restore growth (Lee et al. 2002; Tyler et al. 2004), in agreement with the very low expression of *RGL2* in these tissues. However, our analysis of the RAR2 lines indicates that the RGL2 protein is capable of exerting RGA function when expressed under control of the *RGA* promoter. As shown in figure 3*A*, expression of RGA under the 2-kb *RGA* promoter could complement the loss of RGA function, to different extent depending on the line examined. Even more interestingly, expression of *RGL2* could also complement the lack of *RGA* function to an equivalent extent, when expressed under the *RGA* promoter. Such complementation ability was observed not only in leaves and shoots (fig. 3*A* and *B*) but also in etiolated hypocotyls (results not shown). Actually, the degree of stem dwarfism rescue was dependent on the age of the plant but not on the transgenic DELLA used (fig. 3*B*), an effect that might be caused by the lack of additional regulatory sequences lying beyond the 2-kb promoter fragment used in this study.

RGA is also involved in feedback regulation of GA metabolism genes in tissues in which *RGL2* is not normally expressed, such as hypocotyls, leaves, and shoots (Silverstone et al. 1998; Dill and Sun 2001; Frigerio et al. 2006). *RGL2*, when expressed under the control of the *RGA* promoter, was able to restore feedback control of the *GA20ox1*, *GA20ox2*, and *GA2ox8* genes, which was lost in the *ga1-3 gai-t6 rga-24* mutant (fig. 3*C*). Therefore, the RGL2 protein seems to display the biochemical activity characteristic of RGA function, functional divergence of these genes being caused, at least in this case, by changes in their spatial pattern of expression. To confirm this hypothesis, we performed the reciprocal analysis by testing the ability of RGA to substitute RGL2.

Functional Substitution of RGL2 by RGA

A major function of RGL2 in seed physiology is revealed by the observation that GA-deficient seeds are able to germinate only if RGL2 activity is suppressed but not when any of the other four *DELLA* genes are mutated (Lee et al. 2002; Cao et al. 2005). This is particularly evident for instance in the *rgl2-1* allele, whose seeds germinate in the presence of 20 or even 120 μM PAC, whereas these inhibitor

Downloaded from https://academic.oup.com/mbe/article/27/6/1247/1110303 by CSIC user on 09 September 2020

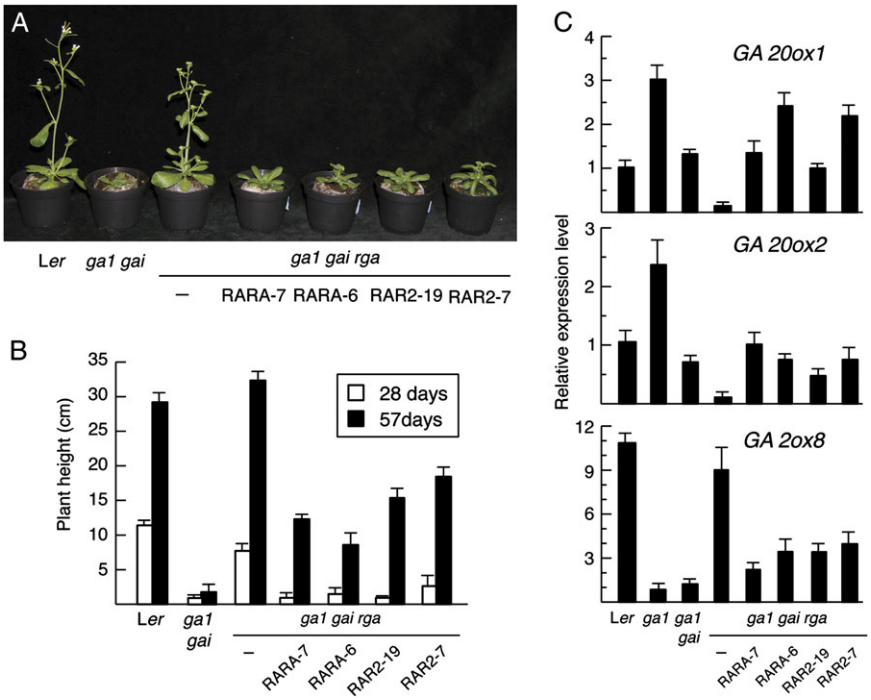

Fig. 3. Complementation by *RGL2* of *rga* loss-of-function mutants. (*A*) Photograph of 4-week-old plants grown under long days. (*B*) Plant height at different ages of control plants and plants transformed with *RGApro::GFP:DELLA*, showing that *RGA* and *RGL2* are capable of inhibiting shoot elongation to a similar extent when expressed under the *RGA* promoter. Error bars represent SD ($n > 15$). (*C*) Expression of GA metabolism genes subject to regulation by RGA in shoots, measured by RT-qPCR, showing that RGA and RGL2 complement the loss of RGA function. Error bars represent SD of three replicates. Only two representative homozygous transgenic lines are shown for each construct, although equivalent results were observed for over 14 lines.

concentrations reduce germination efficiency of wild-type seeds to values below 5% (fig. 4*A*). As expected, expression of *RGL2* under the control of its 2-kb promoter region caused a decrease in germination of *rgl2-1* in the presence of PAC, although it never reached the values of the wild type (fig. 4*A*). More importantly, expression of *RGA* under the control of the *RGL2* promoter (R2RA lines) also complemented the *rgl2-1* to an equivalent extent when compared with RGL2, indicating that RGA and RGL2 proteins can perform equivalent functions during seed germination, provided that they are both expressed under the *RGL2* promoter. This conclusion was confirmed at the molecular level by measuring the expression level of *ATHB-16* and *MYB34* in germinating seeds, by RT-qPCR. These genes are normally upregulated during germination and have been proposed to be repressed by RGL2 in imbibed seeds (Ogawa et al. 2003; Cao et al. 2006). As shown on figure 4*B*, both marker genes were induced in *rgl2-1* mutant seeds during germination in GA-limiting conditions, compared with seeds of the wild type and the transgenic lines expressing RGL2 and RGA under the control of the *RGL2* promoter.

The observation that the RGA protein can perform the function of RGL2 in seeds seems to be in conflict with the fact that RGA is also expressed in seeds, but suppression of this gene does not allow germination of GA-deficient seeds (Tyler et al. 2004; Cao et al. 2005). Where does the specificity of RGL2 function reside in wild-type seeds? One possibility is that expression of *RGA* is restricted to cell types in the seed that are not relevant for germination. This is unlikely because RGA and GAI have indeed been found to contribute to control seed germination under normal conditions (Cao et al. 2005). Another possibility is that the *RGA* and *RGL2* promoters are differentially regulated under GA deficiency, as suggested by the observation that the RGL2 protein is more abundant than RGA in seeds incubated with PAC (Piskurewicz et al. 2008). To explore this option, we analyzed the expression of *GAI*, *RGA*, and *RGL2* during seed imbibition and germination, in the GA-deficient *ga1* mutant, and in its corresponding parental wild type. Interestingly, although *RGA* and *RGL2* expression levels were equivalent in the wild type during germination (when seeds are exposed to light), *RGL2* expression was highly predominant in GA-deficient seeds (fig. 5*A*). Besides, elements mediating GA-regulation of the *RGL2* promoter would lie- within the 2-kb fragment used in the R2R2 and R2RA lines, as confirmed by RT-qPCR analysis of the GFP fusion transcript in germinating seeds of the transgenic lines in mock and GA-deficient (20 μM PAC) conditions (fig. 5*B*).

Conservation of DELLA Protein Interactions

The results presented here indicate that a major driving force in the subfunctionalization of DELLA proteins may in fact rely on different expression patterns arisen after duplication, hence pointing to variations in the promoters of

Downloaded from https://academic.oup.com/mbe/article/27/6/1247/1110303 by CSIC user on 09 September 2020

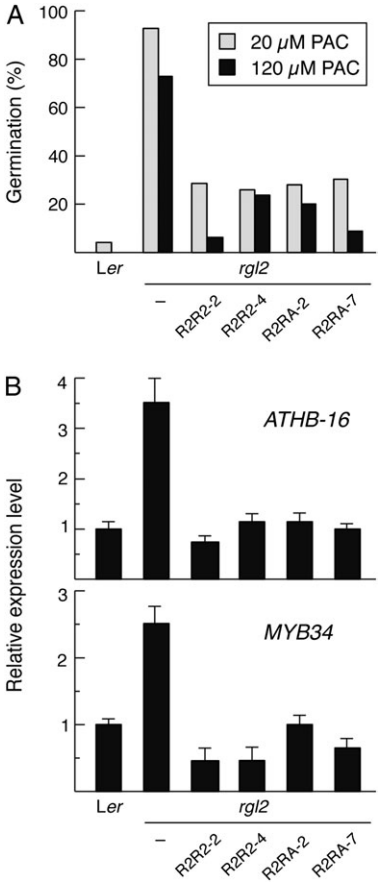

FIG. 4. Complementation by *RGA* of *rgl2* loss-of-function mutants. (*A*) Germination of wild type and *rgl2* mutant seeds transformed with *RGL2pro*::*GFP*:*DELLA* under increasing concentrations of PAC. All lines tested germinated with almost 100% efficiency in the absence of PAC. $n > 200$ seeds. (*B*) Expression of germination marker genes in seeds imbibed for 11 days in the presence of 20-μM PAC, 6 days after exposure to light. Error bars represent SD of three replicates. Only two representative homozygous transgenic lines are shown for each construct, although equivalent results were observed for over 12 lines.

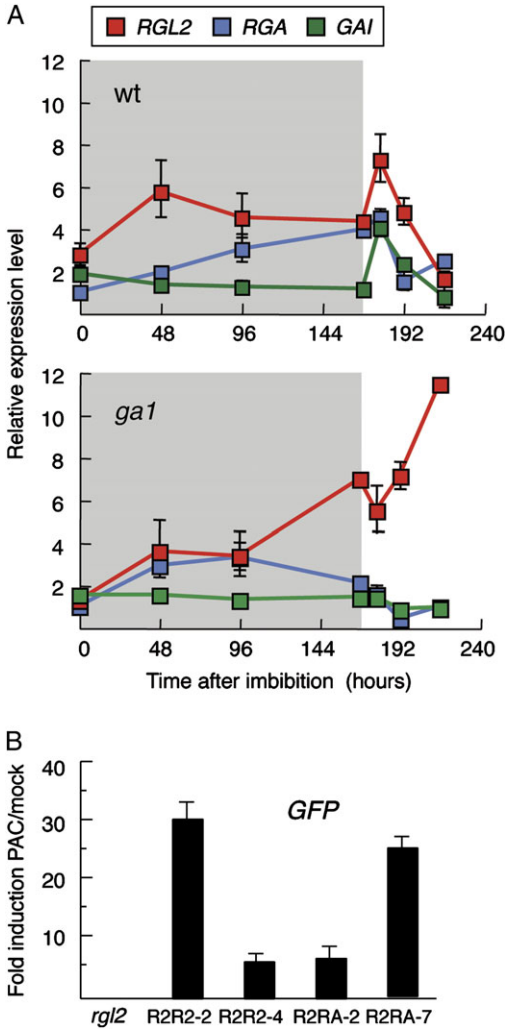

FIG. 5. Expression of *DELLA* genes during seed imbibition and germination. (*A*) Time course of the expression of *GAI*, *RGA*, and *RGL2* during imbibition of wild-type and *ga1* mutant seeds in darkness (shaded area in the graphs) and after exposure to light, determined by RT-qPCR. (*B*) Expression of *RGL2pro*::*GFP*:*DELLA* transgenes, determined by RT-qPCR analysis of *GFP* in *rgl2* mutants 48 h after exposure of imbibed seeds to light. In this experiment, seeds were incubated with mock or 20-μM PAC solutions. Error bars represent SD of three biological replicates.

the *DELLA* genes as the main cause for the differential roles of DELLAs in plant development. Common tools for promoter analysis did not allow the identification of specific sequences that explain the divergent behavior of the *DELLA* promoters. However, cluster analysis of the expression of the five Arabidopsis *DELLA* genes using the over 100 conditions microarray data available through Genevestigator (Zimmermann et al. 2004) revealed a topology that faithfully reproduces the phylogenetic structure of the DELLA group (fig. 6; see also fig. 1A), suggesting an intimate link between the evolution of DELLA function and *DELLA* gene expression patterns. This view is coherent with the observation that at least one-fifth of the alterations responsible for phenotypic evolution in multicellular organisms reside in regulatory regions (Stern and Orgogozo 2008). On the other hand, the study of the expression patterns of duplicate genes using microarrays has established a link between expression divergence and coding-sequence divergence in animals (Makova and Li 2003; Conant and Wagner 2004; Li et al. 2005). Because the correlation between these two processes is less clear in Arabidopsis (Blanc and Wolfe 2004), and there is experimental evidence that recently duplicated proteins in Arabidopsis undergo rapid changes in protein activity (Tominaga et al. 2007), we decided to investigate the extent of divergence in DELLA protein activity that could have arisen as a result of expression changes.

Although biochemical activity of DELLA proteins is not yet fully established, at least two features have been defined that are intimately linked to the protein sequences: 1) They have been proposed to regulate gene expression through protein–protein interactions with TFs of the bHLH family (de Lucas et al. 2008; Feng et al. 2008); and 2) they interact physically with the GID1 GA receptors (Nakajima et al. 2006; Ueguchi-Tanaka et al. 2007; Murase et al. 2008). To evaluate the degree of conservation of the biochemical

Downloaded from https://academic.oup.com/mbe/article/27/6/1247/1110303 by CSIC user on 09 September 2020

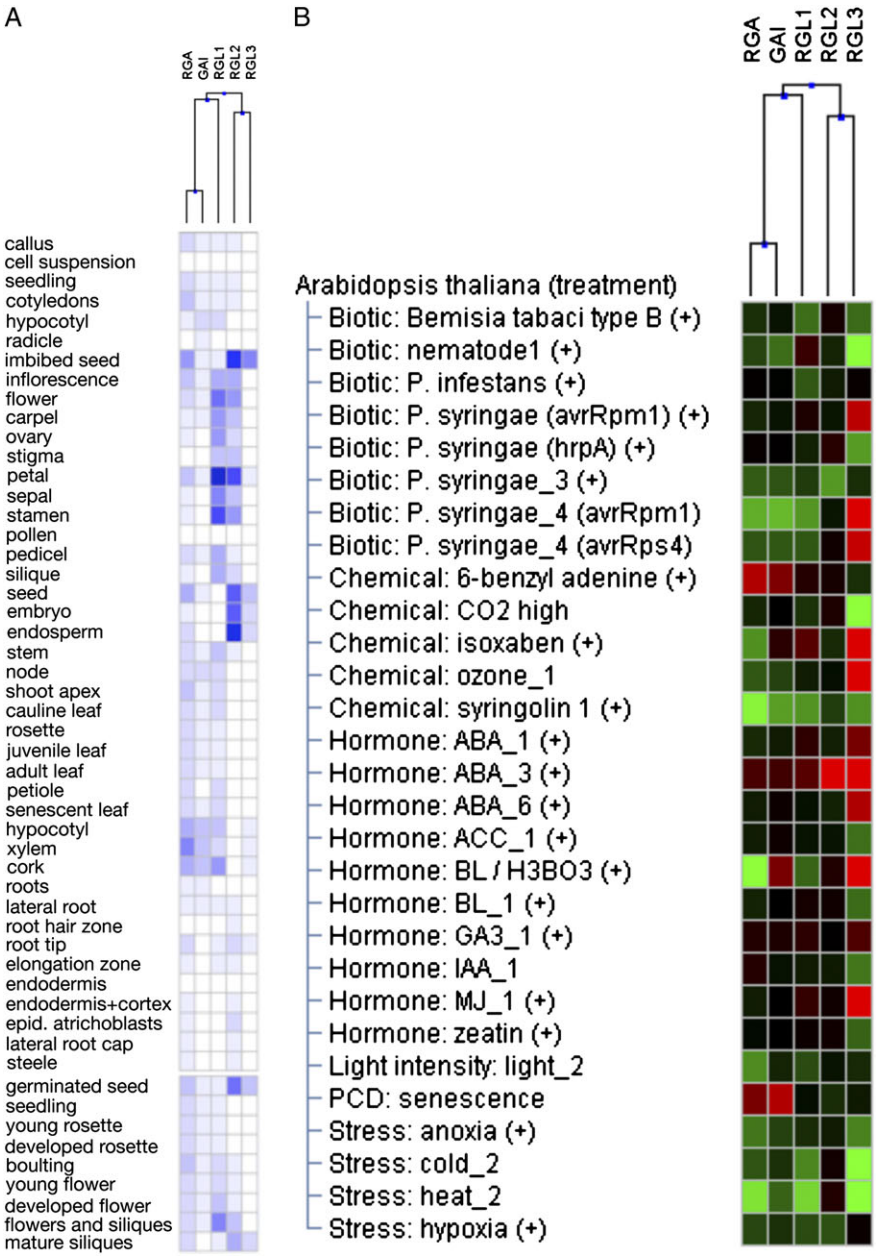

Fig. 6. Cluster analysis of *DELLA* gene expression. (*A*) Expression patterns of *DELLA* genes during plant development and across tissues. (*B*) Expression of *DELLA* genes in response to biotic and abiotic stress treatments. Multiple microarray data were analyzed with the tools provided by Genevestigator (Zimmermann et al. 2004) (http://www.genevestigator.ethz.ch), and the dendrogram displaying the relationships between *DELLA* gene expression patterns was constructed using all the experiments in the database. On the left panel, the intensity of the blue color is proportional to the level of expression, whereas on the right panel, green and red colors indicate lower and higher levels, respectively, compared with the control situation in each treatment.

properties of these proteins, we examined the ability of RGA and RGL2 to interact with PIF4 and also with other bHLH TFs of subfamily 15, with a reported role in seed germination control (Penfield et al. 2005; Oh et al. 2006, 2009; Kim et al. 2008). As seen previously, RGA displayed strong interaction with PIF4, and a similar interaction ability was also observed for RGL2 (fig. 7). PIF4–DELLA interaction has been reported to involve the bHLH DNA recognition domain, which is highly conserved among members of subfamily 15. Thus, it is possible that this interaction is not restricted to PIF3/PIF4 but involves other members of this gene family. In fact, we observed a strong interaction between RGA and RGL2 and the bHLH proteins PIL5, PIL2, and SPATULA (SPT), suggesting that DELLAs may interact with all members of this TF subfamily.

DELLA proteins have been also described to display differential abilities to interact with the three GID1 receptors in Arabidopsis, based on yeast two-hybrid analyses (Nakajima et al. 2006). Taking into account the reported differential expression pattern of the three Arabidopsis GID1 receptors and the five DELLA genes, it seems likely that GA signaling is governed by combinatorial tissue- and stage-specific

Downloaded from https://academic.oup.com/mbe/article/27/6/1247/1110303 by CSIC user on 09 September 2020

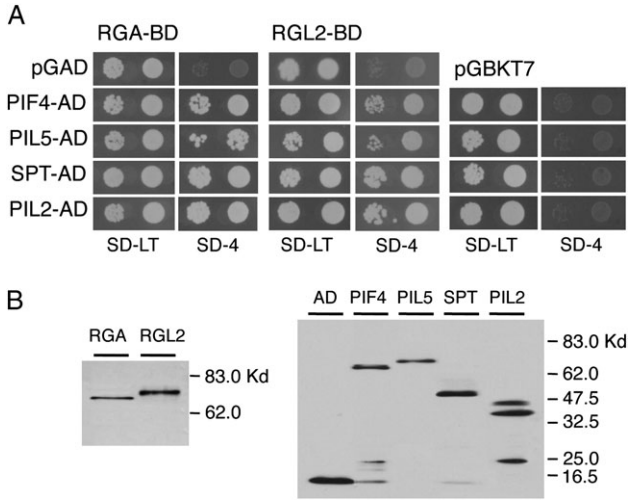

FIG. 7. Conserved interaction between DELLA proteins and bHLH TFs. (*A*) Growth of the yeast cells transformed with the DELLA-GAL4BD and bHLH-GAL4AD constructs on SD-Leu-Trp (SD-LT) and SD-Leu-Trp-His-Ade (SD-4) plates. (*B*) Western blot detection of the GAL4BD and GAL4AD fusion proteins using anti-GAL4BD and anti-HA antibodies.

expression patterns of DELLA proteins and most likely also by subsequent diversification of their interactors.

Therefore, the experimental evidence presented here demonstrates the existence of at least two levels of regulation that have contributed with different impact to the evolutionary fixation of diversity in GA signaling: first, and more critically, the subfunctionalization of *DELLA* gene expression patterns (either spatially or in terms of regulatory responses), and then a more recent optimization of protein interactions between DELLAs and different sets of TFs (and possibly other proteins) within the cellular context defined by the new expression domain for each DELLA protein. It remains to be assessed if the multiplication of *DELLA* genes in Rosids, and especially in the *Brassicaceae*, represents an improvement in the plasticity of the responses mediated by GAs.

Acknowledgments

We deeply appreciate the help of Marta Trénor and Laura García-Cárcel in the initial stages of this work. We also thank Tai-ping Sun (Duke University) and the Arabidpsis Biological Resource Center for seeds, Marta Boter for the pGBKT7 and pGADT7 Gateway vectors, Santiago Elena (IBMCP, CSIC-UPV) for useful comments on the manuscript, and François Parcy (IRTSV, CNRS-CEA) for fruitful discussions and hosting MAB. Work in the authors' laboratories is funded by grants BIO2007-60923 and BIO2005-07284 from the Spanish Ministry of Science and Innovation. J.G.B. is the recipient of a CSIC I3P Fellowship and J.A.M. is the recipient of a Fellowship from the Fundación "la Caixa."

References

Abascal F, Zardoya R, Posada D. 2005. ProtTest: selection of best-fit models of protein evolution. *Bioinformatics* 21:2104–2105.

Bassel GW, Mullen RT, Bewley JD. 2008. Procera is a putative DELLA mutant in tomato (*Solanum lycopersicum*): effects on the seed and vegetative plant. *J Exp Bot*. 59:585–593.

Bensmihen S, To A, Lambert G, Kroj T, Giraudat J, Parcy F. 2004. Analysis of an activated ABI5 allele using a new selection method for transgenic *Arabidopsis* seeds. *FEBS Lett*. 561: 127–131.

Blanc G, Wolfe KH. 2004. Functional divergence of duplicated genes formed by polyploidy during *Arabidopsis* evolution. *Plant Cell*. 16:1679–1691.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 72:248–254.

Cao D, Cheng H, Wu W, Soo HM, Peng J. 2006. Gibberellin mobilizes distinct DELLA-dependent transcriptomes to regulate seed germination and floral development in *Arabidopsis*. *Plant Physiol*. 142:509–525.

Cao D, Hussain A, Cheng H, Peng J. 2005. Loss of function of four DELLA genes leads to light- and gibberellin-independent seed germination in *Arabidopsis*. *Planta* 223:105–113.

Casal JJ, Fankhauser C, Coupland G, Blázquez MA. 2004. Signalling for developmental plasticity. *Trends Plant Sci*. 9:309–314.

Cheng H, Qin L, Lee S, Fu X, Richards DE, Cao D, Luo D, Harberd NP, Peng J. 2004. Gibberellin regulates *Arabidopsis* floral development via suppression of DELLA protein function. *Development* 131:1055–1064.

Chini A, Fonseca S, Fernandez G, et al. (12 co-authors). 2007. The JAZ family of repressors is the missing link in jasmonate signalling. *Nature* 448:666–671.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. *Plant J*. 16:735–743.

Conant GC, Wagner A. 2004. Duplicate genes and robustness to transient gene knock-downs in *Caenorhabditis elegans*. *Proc Biol Sci*. 271:89–96.

de Lucas M, Davière JM, Rodríguez-Falcón M, Pontin M, Iglesias-Pedraz JM, Lorrain S, Fankhauser C, Blázquez MA, Titarenko E, Prat S. 2008. A molecular framework for light and gibberellin control of cell elongation. *Nature* 451:480–484.

Dill A, Jung HS, Sun TP. 2001. The DELLA motif is essential for gibberellin-induced degradation of RGA. *Proc Natl Acad Sci U S A*. 98:14162–14167.

Dill A, Sun T. 2001. Synergistic derepression of gibberellin signaling by removing RGA and GAI function in *Arabidopsis thaliana*. *Genetics* 159:777–785.

Dill A, Thomas SG, Hu J, Steber CM, Sun TP. 2004. The *Arabidopsis* F-box protein SLEEPY1 targets gibberellin signaling repressors for gibberellin-induced degradation. *Plant Cell*. 16:1392–1405.

Fang S, Ting CT, Lee CR, Chu KH, Wang CC, Tsaur SC. 2009. Molecular evolution and functional diversification of fatty acid desaturases after recurrent gene duplication in *Drosophila*. *Mol Biol Evol*. 26:1447–1456.

Feng S, Martinez C, Gusmaroli G, et al. (14 co-authors). 2008. Coordinated regulation of *Arabidopsis thaliana* development by light and gibberellins. *Nature* 451:475–479.

Force A, Lynch M, Pickett FB, Amores A, Yan YL, Postlethwait J. 1999. Preservation of duplicate genes by complementary, degenerative mutations. *Genetics* 151:1531–1545.

Frigerio M, Alabadí D, Pérez-Gómez J, García-Cárcel L, Phillips AL, Hedden P, Blázquez MA. 2006. Transcriptional regulation of gibberellin metabolism genes by auxin signaling in *Arabidopsis*. *Plant Physiol*. 142:553–563.

Goto H, Szmidt AE, Yamazaki T, Inomata N. 2005. Effect of nucleotide polymorphism in cis-regulatory and coding regions on amylase activity and fitness in *Drosophila melanogaster*. *Heredity* 95:369–376.

Downloaded from https://academic.oup.com/mbe/article/27/6/1247/1110303 by CSIC user on 09 September 2020

Griffiths J, Murase K, Rieu I, et al. (11 co-authors). 2006. Genetic characterization and functional analysis of the GID1 gibberellin receptors in *Arabidopsis*. *Plant Cell*. 18:3399–3414.
Guindon S, Gascuel O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol*. 52:696–704.
Haberer G, Hindemitt T, Meyers BC, Mayer KF. 2004. Transcriptional similarities, dissimilarities, and conservation of cis-elements in duplicated genes of *Arabidopsis*. *Plant Physiol*. 136:3009–3022.
Hittinger CT, Carroll SB. 2007. Gene duplication and the adaptive evolution of a classic genetic switch. *Nature* 449:677–681.
Hussain A, Cao D, Cheng H, Wen Z, Peng J. 2005. Identification of the conserved serine/threonine residues important for gibberellin-sensitivity of Arabidopsis RGL2 protein. *Plant J*. 44:88–99.
Jasinski S, Tattersall A, Piazza P, Hay A, Martinez-Garcia JF, Schmitz G, Theres K, McCormick S, Tsiantis M. 2008. PROCERA encodes a DELLA protein that mediates control of dissected leaf form in tomato. *Plant J*. 56:603–612.
Kim DH, Yamaguchi S, Lim S, Oh E, Park J, Hanada A, Kamiya Y, Choi G. 2008. SOMNUS, a CCCH-type zinc finger protein in *Arabidopsis*, negatively regulates light-dependent seed germination downstream of PIL5. *Plant Cell*. 20:1260–1277.
King KE, Moritz T, Harberd NP. 2001. Gibberellins are not required for normal stem growth in *Arabidopsis thaliana* in the absence of GAI and RGA. *Genetics* 159:767–776.
Lee S, Cheng H, King KE, Wang W, He Y, Hussain A, Lo J, Harberd NP, Peng J. 2002. Gibberellin regulates *Arabidopsis* seed germination via RGL2, a GAI/RGA-like gene whose expression is up-regulated following imbibition. *Genes Dev*. 16:646–658.
Li WH, Yang J, Gu X. 2005. Expression divergence between duplicate genes. *Trends Genet*. 21:602–607.
Li Z, Zhang H, Ge S, Gu X, Gao G, Luo J. 2009. Expression pattern divergence of duplicated genes in rice. *BMC Bioinformatics*. 10(Suppl 6):S8.
Lynch M, Conery JS. 2000. The evolutionary fate and consequences of duplicate genes. *Science* 290:1151–1155.
Makova KD, Li WH. 2003. Divergence in the spatial pattern of gene expression between human duplicate genes. *Genome Res*. 13:1638–1645.
Marti C, Orzaez D, Ellul P, Moreno V, Carbonell J, Granell A. 2007. Silencing of DELLA induces facultative parthenocarpy in tomato fruits. *Plant J*. 52:865–876.
Maxwell AI, Morrison GM, Dorin JR. 2003. Rapid sequence divergence in mammalian beta-defensins by adaptive evolution. *Mol Immunol*. 40:413–421.
Murase K, Hirano Y, Sun TP, Hakoshima T. 2008. Gibberellin-induced DELLA recognition by the gibberellin receptor GID1. *Nature* 456:459–463.
Nakajima M, Shimada A, Takashi Y, et al. (13 co-authors). 2006. Identification and characterization of *Arabidopsis gibberellin* receptors. *Plant J*. 46:880–889.
Ogawa M, Hanada A, Yamauchi Y, Kuwahara A, Kamiya Y, Yamaguchi S. 2003. Gibberellin biosynthesis and response during *Arabidopsis* seed germination. *Plant Cell*. 15:1591–1604.
Oh E, Kang H, Yamaguchi S, Park J, Lee D, Kamiya Y, Choi G. 2009. Genome-wide analysis of genes targeted by phytochrome interacting factor 3-like5 during seed germination in *Arabidopsis*. *Plant Cell*. 21:403–419.
Oh E, Yamaguchi S, Kamiya Y, Bae G, Chung WI, Choi G. 2006. Light activates the degradation of PIL5 protein to promote seed germination through gibberellin in *Arabidopsis*. *Plant J*. 47:124–139.
Ohno S. 1970. Evolution by gene duplication. New York: Springer.
Page RD. 1996. TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci*. 12:357–358.
Penfield S, Josse EM, Kannangara R, Gilday AD, Halliday KJ, Graham IA. 2005. Cold and light control seed germination through the bHLH transcription factor SPATULA. *Curr Biol*. 15:1998–2006.
Pickett FB, Meeks-Wagner D. 1995. Seing double: appreciating genetic redundancy. *Plant Cell*. 7:1347–1356.
Piskurewicz U, Jikumaru Y, Kinoshita N, Nambara E, Kamiya Y, Lopez-Molina L. 2008. The gibberellic acid signaling repressor RGL2 inhibits *Arabidopsis* seed germination by stimulating abscisic acid synthesis and ABI5 activity. *Plant Cell*. 20: 2729–2745.
Rodriguez PL. 1998. Protein phosphatase 2C (PP2C) function in higher plants. *Plant Mol Biol*. 38:919–927.
Silverstone AL, Ciampaglio CN, Sun T. 1998. The *Arabidopsis* RGA gene encodes a transcriptional regulator repressing the gibberellin signal transduction pathway. *Plant Cell*. 10:155–169.
Silverstone AL, Jung HS, Dill A, Kawaide H, Kamiya Y, Sun TP. 2001. Repressing a repressor: gibberellin-induced rapid reduction of the RGA protein in *Arabidopsis*. *Plant Cell*. 13:1555–1566.
Singh LN, Hannenhalli S. 2008. Functional diversification of paralogous transcription factors via divergence in DNA binding site motif and in expression. *PLoS ONE*. 3:e2345.
Smith H. 1990. Signal perception, differential expression within multigene families and the molecular basis for phenotypic plasticity. *Plant Cell Environ*. 13:585–594.
Stern DL, Orgogozo V. 2008. The loci of evolution: how predictable is genetic evolution? *Evolution* 62:2155–2177.
Sun TP, Kamiya Y. 1994. The *Arabidopsis* GA1 locus encodes the cyclase ent-kaurene synthetase A of gibberellin biosynthesis. *Plant Cell*. 6:1509–1518.
Taylor JS, Raes J. 2004. Duplication and divergence: the evolution of new genes and old ideas. *Annu Rev Genet*. 38:615–643.
Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*. 25:4876–4882.
To JP, Haberer G, Ferreira FJ, Deruere J, Mason MG, Schaller GE, Alonso JM, Ecker JR, Kieber JJ. 2004. Type-A *Arabidopsis* response regulators are partially redundant negative regulators of cytokinin signaling. *Plant Cell*. 16:658–671.
Tominaga R, Iwata M, Okada K, Wada T. 2007. Functional analysis of the epidermal-specific MYB genes CAPRICE and WEREWOLF in *Arabidopsis*. *Plant Cell*. 19:2264–2277.
Tyler L, Thomas SG, Hu J, Dill A, Alonso JM, Ecker JR, Sun TP. 2004. Della proteins and gibberellin-regulated seed germination and floral development in *Arabidopsis*. *Plant Physiol*. 135: 1008–1019.
Ueguchi-Tanaka M, Nakajima M, Katoh E, et al. (11 co-authors). 2007. Molecular interactions of a soluble gibberellin receptor, GID1, with a rice DELLA protein, SLR1, and gibberellin. *Plant Cell*. 19:2140–2155.
Weijers D, Benkova E, Jager KE, Schlereth A, Hamann T, Kientz M, Wilmoth JC, Reed JW, Jurgens G. 2005. Developmental specificity of auxin response by pairs of ARF and Aux/IAA transcriptional regulators. *Embo J*. 24:1874–1885.
Weijers D, Jurgens G. 2004. Funneling auxin action: specificity in signal transduction. *Curr Opin Plant Biol*. 7:687–693.
Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W. 2004. GENEVESTIGATOR. *Arabidopsis* microarray database and analysis toolbox. *Plant Physiol*. 136:2621–2632.

Downloaded from https://academic.oup.com/mbe/article/27/6/1247/1110303 by CSIC user on 09 September 2020