MYC transcription factors: masters in the regulation of jasmonate biosynthesis in Arabidopsis thaliana

Zhang, K.

Chapter 4

Analysis of the role of DELLA proteins in the expression of jasmonate biosynthesis genes in *Arabidopsis thaliana*

Kaixuan Zhang, Meiliang Zhou, Johan Memelink

Institute of Biology, Leiden University, Sylviusweg 72, P.O. Box 9505, 2300 RA Leiden, The Netherlands

Abstract

Jasmonates (JAs) and gibberellins (GAs) are plant hormones regulating plant growth, development and defense responses antagonistically and synergistically. JAs biosynthesis is regulated by a positive feedback loop that is transcriptionally controlled by the basic Helix-Loop-Helix (bHLH) transcription factors MYC2, MYC3, MYC4 and the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) ORA47. However, the crosstalk between JAs and GAs signaling in regulating JAs biosynthesis remains unclear. In this chapter, we describe studies to determine the involvement of DELLA proteins, repressors of the GAs signaling pathway, in the regulation of JAs biosynthesis genes. Via yeast two-hybrid screening and BiFC assays, we identified two members of the DELLA protein family, RGA and GAI, as ORA47 interactors. Transient activation assays showed that RGA and GAI slightly promoted the activity of ORA47, which was partially attenuated by co-expression of JAZ1, a repressor of the JAs signaling pathway. Furthermore, RGA and GAI significantly enhanced the activity of MYC2 on the *ORA47* promoter. The expression of *ORA47* or of the ORA47 target genes *LOX2*, *AOC2* and *OPR3*, encoding enzymes involved in JAs biosynthesis, in response to JA and/or GA3 was not affected in the quintuple *della* mutant or in transgenic plants overexpressing RGA or GAI. Thus, we were unable to establish that DELLA proteins have effects on the expression of JAs biosynthesis genes.

Introduction

Jasmonic acid (JA) and related oxylipins, collectively referred to as jasmonates (JAs), are hormones that steer a diverse set of plant development processes and that orchestrate defense responses against biotic and abiotic stresses. JAs biosynthesis and the JAs signaling pathway have been extensively investigated in Arabidopsis (Schaller and Stintzi, 2009; Gfeller et al., 2010). JA is synthesized from 12-oxo-phytodienoic acid (OPDA) and can be further enzymatically converted into numerous derivatives or conjugates, such as methyl jasmonate (MeJA), *cis*-jasmone, jasmonoyl isoleucine (JA-Ile), and jasmonoyl 1-aminocyclopropane-1-carboxylic acid (JA-ACC), some of which have well-described biological activities (Wasternack and Hause, 2013). Most of the JAs biosynthesis genes are induced by JAs treatment and wounding, implying that JAs biosynthesis is regulated by a positive feedback loop (Sasaki et al., 2001). A well-established bioactive JAs is JA-Ile, which is the only JAs that interacts with high affinity with the SCF^{COI1}-JAZ co-receptor complex thereby initiating the JAs-dependent responses (Katsir et al., 2008; Fonseca et al., 2009). JA-Ile stimulates binding of repressors of transcription factors, the JASMONATE ZIM (JAZ) proteins, to the F-box protein CORONATINE INSENSITIVE1 (COI1) of the Skp1/cullin/F-box (SCF) E3 ubiquitin ligase complex, leading to the subsequent degradation of JAZ repressors by the 26S proteasome. The degradation of JAZ repressors releases the transcription factors to regulate the expression of JAs-responsive genes and, as a consequence thereof, the onset of defense reactions (Lorenzo et al., 2004; Fernández-Calvo et al., 2011).

The bHLH transcription factor MYC2 acts as a regulatory hub within the JAs signaling pathway (Kazan and Manners, 2012). MYC2 interacts with most of the 12 JAZ repressors (Chini et al., 2009). MYC2 is a positive regulator in JAs-mediated resistance against insect pests and wounding, and in tolerance to oxidative stress (Dombrecht et al., 2007). The *myc2/jin1* mutant exhibits insensitivity to the inhibitory effect of JAs on root growth and increased resistance to necrotrophic pathogens, indicating a negative role of MYC2 in these responses (Lorenzo et al., 2004). In addition to MYC2, the transcription factors MYC3 and MYC4, which are the closest homologs of MYC2, share overlapping roles with MYC2 as well as distinct roles in JAs signaling (Cheng et al., 2011; Fernández-Calvo et al., 2011; Niu et al., 2011). In chapter 2, we discovered that MYC proteins play a crucial role in the auto-regulation of JAs biosynthesis through direct binding to the promoters of JAs biosynthesis genes. Besides MYCs, several transcription factors have been reported to be

involved in specific aspects of JAs-induced responses, such as ERFs (Fujimoto et al., 2000), ORAs (Pré, 2006), WRKYs (Schluttenhofer et al., 2014) and MYBs (Mandaokar et al., 2006) among others. The AP2/ERF-domain transcription factor ORA59 integrates the JAs/Ethylene (ET) signaling pathways and regulates resistance against necrotrophic pathogens (Pré et al., 2008). Although ORA59 is not a direct target of JAZs, the ORA59 interacting proteins ZFAR1 and ZFAR2 can recruit JAZ1 to repress the transcriptional activity of ORA59 (Zhou, 2014). Our previous studies described the function of the AP2/ERF-domain transcription factor ORA47 in JAs biosynthesis (Pré, 2006; Khurshid, 2012). Overexpression of the *ORA47* gene in Arabidopsis plants resulted in induced expression of multiple JAs biosynthesis genes and increased endogenous JAs levels. The *ORA47* gene is JAs-responsive (Pré, 2006) and its expression is controlled by MYC transcription factors (chapter 3), indicating the key roles of MYCs and ORA47 in the regulation of the auto-stimulatory loop in JAs biosynthesis.

JAs signaling exhibits cross-talk with signaling pathways of other phytohormones including gibberellins (GAs), salicylic acid (SA), ethylene (ET), abscisic acid (ABA), auxin and cytokinins. GAs play essential roles in promotion of plant growth and development, including root growth (Ubeda-Tomás et al., 2008), stem elongation (Achard et al., 2009), leaf expansion (Olszewski et al., 2002), seed germination (Piskurewicz et al., 2008), flower development (Cheng et al., 2004) and trichome initiation (Gan et al., 2007), as well as responses to changing environmental conditions (Colebrook et al., 2014). The GAs signaling pathway, like JAs signaling, uses the ubiquitin–proteasome pathway to control gene expression through protein degradation. When bioactive GAs is perceived by the receptor GID1 (GA INSENSITIVE DWARF1), DELLA repressors are recruited to an F-box protein (SLY1 in Arabidopsis and GID2 in rice) of the E3 ubiquitin ligase complex $SCF^{SLY1/GID2}$ (McGinnis et al., 2003), leading to rapid degradation of DELLAs via the proteasome pathway and to de-repression of the transcriptional activity of transcription factors (Itoh et al., 2003; Sun, 2010).

In Arabidopsis, DELLAs are encoded by a family of five genes, i.e. *GIBBERELLIC ACID INSENSITIVE* (*GAI*), *REPRESSOR OF ga1-3* (*RGA*), and three *RGA-LIKE* genes (*RGL1*, *RGL2*, and *RGL3*) (Peng et al., 1997; Silverstone et al., 1998; Lee et al., 2002). DELLA proteins have been shown to regulate gene expression by association with various transcription factors, such as PHYTOCHROME-INTERACTING FACTORs (PIFs), BOTRYTIS SUSCEPTIBLE1 INTERACTOR (BOI), JAZs and MYC2 (Feng et al., 2008; Hou

et al., 2010; Hong et al., 2012; Park et al., 2013). DELLA proteins interact with the bHLH-type transcription factors PIF3 and PIF4 and prevent them from binding to their target gene promoters and regulating gene expression, and therefore abrogate plant growth (de Lucas et al., 2008; Feng et al., 2008). The RING domain protein BOI together with DELLA proteins are targeted to the promoters of a subset of GAs-responsive genes and repress their expression (Park et al., 2013). By interacting with DELLA proteins, MYC2 integrates both GAs and JAs signals into the induction of sesquiterpene production by flowers (Hong et al., 2012). DELLAs are involved in cross-talk between JAs and GAs signaling among others by preventing the inhibitory JAZ1 interaction with the key transcriptional activator of JAs responses, MYC2, and, thus, enhance the ability of MYC2 to regulate its target defense genes (Hou et al., 2010). Similar to DELLAs, which compete with MYC2 to bind JAZs, JAZ9 promotes plant growth by inhibiting RGA interaction with PIF3 (Yang et al., 2012). As outlined above, JAZs and DELLAs function antagonistically to regulate the conflict between defense and growth. In addition, DELLAs function as positive regulators of ORA59 to modulate JA/ET mediated resistance to the necrotrophic fungus *Botrytis cinerea* (Zhou, 2014). Interestingly, JAs and GAs can also act synergistically to promote stamen and trichome development. GA3 has been found to promote JAs biosynthesis through DELLAs to control the expression of *MYB21*, *MYB24*, and *MYB57*, which in turn promote stamen filament growth (Cheng et al., 2009). It was proposed that GAs and JAs induce degradation of DELLAs and JAZ proteins to coordinately activate the WD-repeat/bHLH/MYB complex and synergistically and mutually dependently induce trichome initiation (Qi et al., 2014). Although these observations indicate the existence of crosstalk between GAs and JAs signaling in pathogen resistance and plant development, the detailed molecular mechanisms by which DELLAs modulate JAs biosynthesis still remain elusive.

The aim of the work in this chapter is to determine whether DELLA proteins affect JAs biosynthesis in Arabidopsis. Via yeast two-hybrid screening and assays we identified 2 members of the Arabidopsis DELLA protein family, RGA and GAI, as ORA47 interactors. ORA47 did not directly interact with JAZs, but RGA and GAI did (Hou et al., 2010), which is compatible with a scenario where DELLAs act as adaptors to promote binding between ORA47 and JAZs. RGA and GAI slightly promoted ORA47 activity in a transient trans-activation assay, whereas co-expression of JAZ1 and DELLAs had a slight negative effect on the activity of ORA47. The expression of the JAs biosynthesis genes *LOX2*, *AOC2* and *OPR3* was not affected in the quintuple

della mutant or in transgenic plants constitutively overexpressing RGA or GAI. All five DELLAs have been reported to interact with MYC2 directly, and GA and JA jointly regulate the biosynthesis of sesquiterpenes through a DELLA-MYC2 interaction (Hong et al., 2012). Here we report that RGA and GAI significantly promoted the activation of the *ORA47* promoter by MYC2 in Arabidopsis protoplasts. The JAs-responsive expression of the *ORA47* gene was not affected in the quintuple *della* mutant or by constitutive overexpression of RGA and GAI. Thus, we were unable to establish that DELLA proteins have effects on the expression of JAs biosynthesis genes.

Results

Identification of DELLAs that interact with ORA47

To identify proteins that interact with ORA47, yeast two-hybrid screenings were performed using derivative ORA47Δ3 extending from aa 1-140 as bait. Screening of 5.2 x10^5 yeast transformants obtained with an Arabidopsis cDNA library generated from mature ecotype *Landsberg erecta* plants in the vector pACT2 resulted in 120 colonies that were able to grow on medium lacking histidine. Recovered prey plasmids were re-transformed and seven plasmids conferred growth on selective medium. All seven cDNA sequences were in frame with the GAL4 activation domain and encoded the protein GAI.

GAI is one of the five DELLA proteins in Arabidopsis. DELLAs are repressors of the GAs signal transduction pathway, which have been shown to interact with JAZ1 and to prevent inhibitory JAZ1 interaction with the transcription factor MYC2 (Hou et al., 2010). To test whether all five DELLAs interact with ORA47, yeast two-hybrid assays were performed. The results showed that GAI and RGA interacted with ORA47, whereas no interaction was detected between RGL1, RGL2, RGL3 and ORA47 (Fig. 1a). DELLA proteins contain conserved DELLA and leucine heptad repeats (LZ) protein-interaction motifs in the N-terminal part (Fig. 1b)(Itoh et al., 2003). To investigate the functional domains mediating the interaction between ORA47 and DELLAs, we tested the interaction of two RGA deletion derivatives (RGAΔN1 and RGAΔN2) with ORA47. Co-expression of RGAΔN1, RGAΔN2 or RGA fused to AD with BD-ORA47 in yeast cells indicated that the LZ1 domain in RGA is required for the interaction between ORA47 and RGA (Fig. 1c). Previous studies showed that the LZ1 domain also contributes to the interaction of RGA with JAZ1 (Hou et al., 2010).

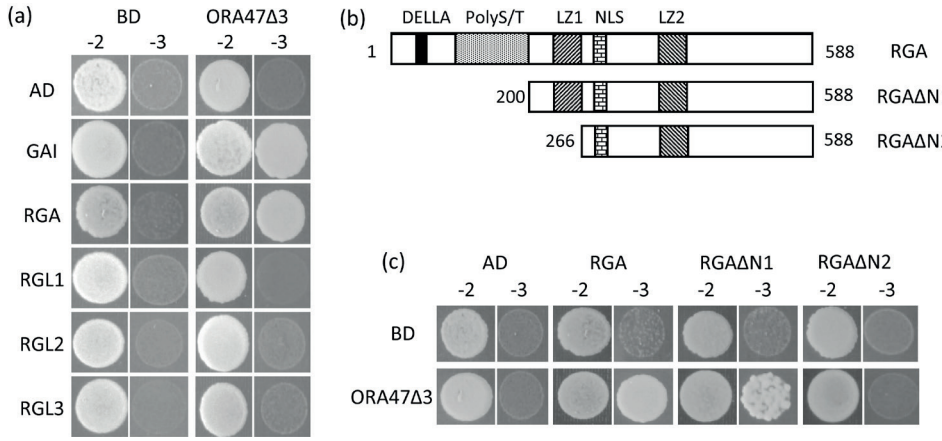

Figure 1. DELLA proteins interact with ORA47 in yeast. (**a**) GAI and RGA interact with ORA47. (**b**) Schematic representation of RGA derivatives. Poly S/T, polymeric Ser and Thr; LZ, Leu zipper; NLS, nuclear localization signal. Numbers indicates the amino acid position. (**c**) The LZ1 domain of RGA is necessary for interaction with ORA47. Yeast cells expressing ORA47 derivative Δ3 (1-140 aa) fused to the GAL4 DNA-binding domain (BD) in plasmid pAS2.1 and DELLA proteins and RGA derivatives fused to the GAL4 activation domain (AD) in plasmid pACT2 were spotted on minimal SD medium without Leucine and Tryptophan (-2) to select for the plasmids and on medium additionally lacking Histidine complemented with 5 mM 3-aminotriazole (-3) to select for transcriptional activation of the *His3* gene. Growth was recorded after 5 days. Yeast cells transformed with the empty plasmids pAS2.1 and pACT2, expressing GAL4BD and AD, respectively, were used as control.

ORA47 interacts with GAI and RGA *in planta*

To confirm the interaction of ORA47 with DELLAs *in planta*, the Bimolecular Fluorescence Complementation (BiFC) assay was performed. The N-terminal or C-terminal fragments of the yellow fluorescent protein (nYFP or cYFP) were fused either N-terminally (YN) or C-terminally (YC) with ORA47, GAI and RGA. The constructs were transiently co-expressed in all possible combinations of YN and YC fusion proteins in Arabidopsis suspension cell protoplasts. Reconstitution of a fluorescing YFP chromophore occurred only upon co-expression of certain combinations of fusion proteins. Co-transformation of YN-ORA47 and GAI-YC, or ORA47-YC and YN-RGA, resulted in YFP fluorescence in the nucleus of Arabidopsis protoplasts cells (Fig. 2), whereas cells transfected with single plasmids and any combination of empty YFP vectors produced no or only background YFP fluorescence (data not shown). These results demonstrated that ORA47 can interact with GAI and RGA in the nucleus of plant cells.

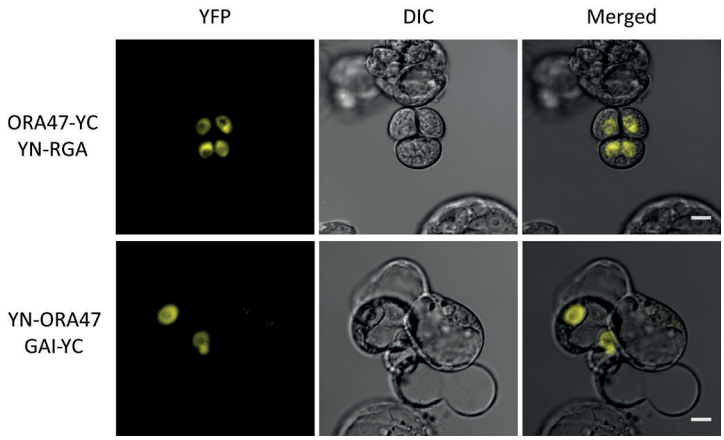

Figure 2. ORA47 interacts with RGA and GAI in the nucleus. YFP fluorescence, bright field (DIC) and merged images of Arabidopsis cell suspension protoplasts co-transformed with constructs encoding the indicated fusion proteins with nYFP or cYFP at the C-terminus (YC) or the N-terminus (YN). Scale bar = 10 μm.

Effects of DELLAs on the activity of ORA47

To test whether DELLAs had an effect on the activation activity of ORA47, Arabidopsis cell suspension protoplasts were transiently co-transformed with a plasmid carrying the GUS reporter gene driven by the *AOC2* promoter, and combinations of effector plasmids carrying *ORA47*, *RGA*, *GAI*, *RGL1* or *JAZ1* under the control of the CaMV *35S* promoter (Fig 3a). As shown in Figure 3b, expression of ORA47 strongly activated the *AOC2* promoter and co-expression of JAZ1 had no effect. GAI and RGA slightly and not significantly enhanced the activity of ORA47 and this effect was repressed by co-transformation with JAZ1. RGL1, not interacting with ORA47 in yeast, had no significant effects on the activity of ORA47 and JAZ1 either (Fig. 3c). Based on the observation that DELLAs modulate the JAs signaling pathway via competition with MYC2 for binding to JAZ1 (Hou et al., 2010), we next tested whether DELLAs affect activation of the *AOC2* promoter by MYC2. All five DELLAs had no effect on the activity of MYC2 (Fig. 3d and e), and co-expression of RGA did not affect the repression of MYC2 activity by JAZ1 (Fig. 3e).

DELLAs promote the activation of the *ORA47* promoter by MYC2 in Arabidopsis protoplasts

In chapter 3, we discovered that MYCs controlled the expression of *ORA47*. Previous studies reported that DELLAs differentially modulate the JAs signaling pathway through direct binding to JAZ1 or MYC2 (Hou et al., 2010; Hong et al., 2012).

Although DELLAs did not affect the ability of MYC2 to activate the *AOC2* promoter, it cannot be ruled out that DELLAs might have an effect on the activation of the *ORA47* promoter. The *GUS* reporter gene controlled by a 200 bp fragment of the *ORA47* promoter was co-transformed with combinations of effector plasmids carrying *MYC2*, *RGA*, *GAI*, *RGL1*, *RGL2* and *RGL3* in Arabidopsis protoplasts (Fig 4a). We found that co-transformation of RGA and GAI significantly enhanced the ability of MYC2 to activate the *ORA47* promoter (Fig. 4b). Figure 4c shows that

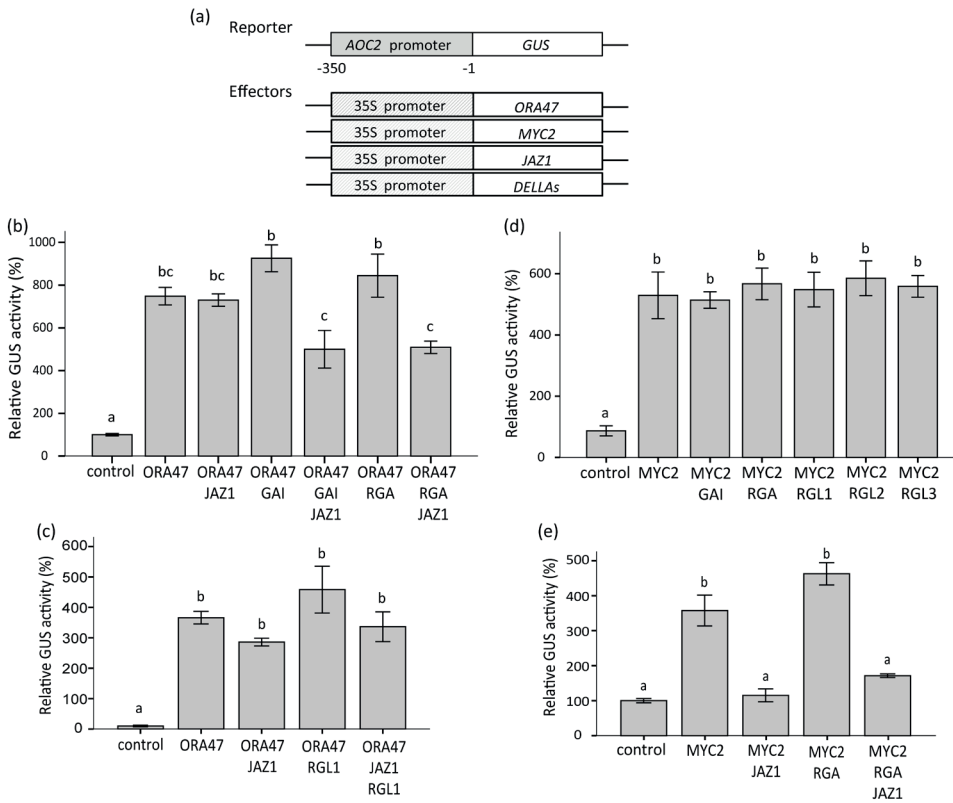

Figure 3. Effects of DELLAs on trans-activation of the *AOC2* promoter by ORA47 or MYC2. (**a**) Schematic representation of the constructs used for transient expression assays. Numbers indicate positions relative to the ATG start codon. (**b**)(**c**) Effect of DELLAs on the activity of ORA47. (**d**)(**e**) Effect of DELLAs on the activity of MYC2. Arabidopsis cell suspension protoplasts were co-transformed with plasmids carrying *pAOC2::GUS* (2 µg) and effector plasmids containing *ORA47* (2 µg) or *MYC2* (2 µg) and *DELLAs* (2 µg in (d), 6 µg in (e)) and/or *JAZ1* (2 µg), as indicated. Protein concentrations were used to correct for differences in protein extraction efficiencies. letters show statistically significant differences between values according to a post hoc Tukey HSD test (ANOVA, $P<0.05$). Values represent means ±SE of triplicate experiments and are expressed relative to the vector control.

MYC2 activity with or without JAZ1 repression was increased by co-expression of RGA, although this effect was not statistically significant in the case of co-expression with JAZ1.

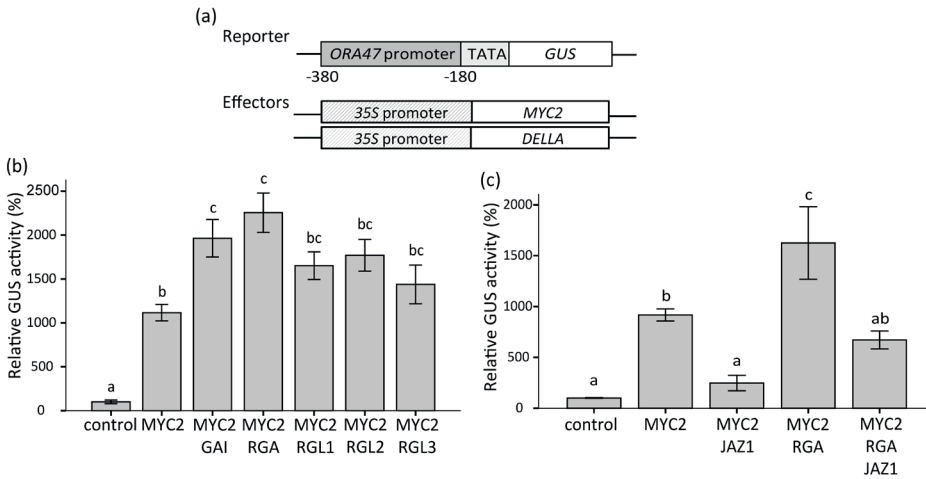

Figure 4. DELLAs enhanced the activation of the *ORA47* promoter by MYC2. (**a**) Schematic representation of the constructs used for transient expression assays. The *ORA47* promoter fragment was fused to a minimal TATA box from the CaMV 35S promoter and the *GUS* gene. Numbers indicate positions relative to the ATG start codon in the *ORA47* gene context. (**b**)(**c**) DELLAs modulated the activation of the *ORA47* promoter by MYC2. Arabidopsis cell suspension protoplasts were co-transformed with plasmids carrying *pORA47::GUS* (2 µg) and effector plasmids containing *MYC2* (2 µg) and/or *DELLAs* (2 µg in (b), 6 µg in (c)) and/or *JAZ1* (2 µg), as indicated. Protein concentrations were used to correct for differences in protein extraction efficiencies. letters show statistically significant differences between values according to a post hoc Tukey HSD test (ANOVA, $P<0.05$). Values represent means ±SE of triplicate experiments.

Expression of *ORA47* or JAs biosynthesis genes is not modulated by DELLAs

The previous experiments in protoplasts indicated that DELLAs had a minor effect on the activity of ORA47 (Fig. 3b) and promoted the activation of the *ORA47* promoter by MYC2 (Fig. 4). We examined the expression of the *ORA47* gene and of its target genes *AOC2*, *LOX2* and *OPR3* in the quintuple *della* mutant and in RGA or GAI overexpressing plants by RNA gel blot analysis. As shown in Figure 5, the JA-responsive expression levels of *ORA47*, *AOC2, LOX2* and *OPR3* were not affected by co-treatment with GA3 in wildtype plants, in the quintuple *della* mutant or in plants constitutively overexpressing RGA or GAI.

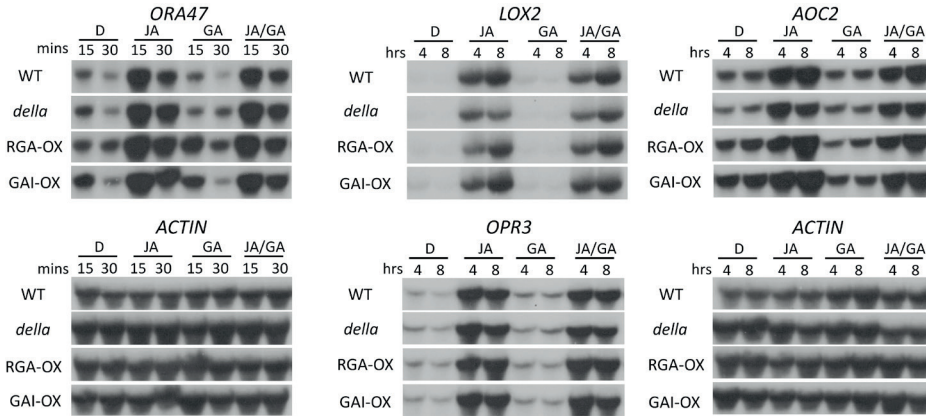

Figure 5. Expression of *ORA47* and JAs biosynthesis genes in Arabidopsis wild-type plants (Ler), quintuple *della* mutants and RGA or GAI overexpressing plants. RNA gel blot analyses of two-weeks-old Arabidopsis seedlings treated for indicated times with the solvents DMSO (D), 50 µM JA, 100 µM GA3 alone or in combination. The *ACTIN* probe was used to verify RNA loading. All four panels for each gene are from the same blot allowing direct comparison of band intensities.

Discussion

JAs play important roles in numerous plant defense responses including those against insect herbivores, necrotrophic pathogens and wounding. Upon external attack plants produce JAs, including bioactive JA-Ile, which is perceived by the receptor F-box protein COI1. The SCF^{COI1} complex with putative E3 ubiquitin ligase activity triggers degradation of JAZ repressors leading to the release of transcription factors controlling JAs-responsive genes (Chico et al., 2008; Staswick, 2008). JAs signaling also induces most JAs biosynthesis genes in a positive feedback loop (Turner et al., 2002). The JAs-responsive bHLH transcription factors MYC2, MYC3 and MYC4 and the AP2/ERF-domain transcription factor ORA47 control the expression of all known JAs biosynthesis genes (chapter 2; Pré, 2006; Kurshid, 2012). MYC proteins are major JAs-responsive regulators and direct targets of JAZ repressors in a myriad of defense responses (Lorenzo et al., 2004; Memelink, 2009; Fernández-Calvo et al., 2011; Kazan and Manners, 2013). ORA47 did not interact with JAZ repressors in a yeast two-hybrid assay (Khurshid, 2012). The AP2/ERF-domain transcription factor ORA59 integrates the JAs and ET signaling pathways. ORA59 interacted with JAZ1 via the ZFAR adaptors resulting in the suppression of its activity in the absence of JAs (Zhou, 2014). Based on these observations, we proposed two alternative hypotheses, (1) that the activity of ORA47 is regulated by a novel repressor protein

that is degraded upon JAs signaling dependent on the SCF^{COI1} complex or (2) by an adaptor protein recruiting certain members of the JAZ repressor family.

We used yeast two-hybrid screening and assays to identify proteins interacting with ORA47. Two members of the DELLA protein family, RGA and GAI, were found to directly interact with ORA47. In transient assays in Arabidopsis protoplasts measuring ORA47 transcriptional activity, RGA and GAI slightly enhanced the activity of ORA47, while JAZ1 had no effect. Co-expression of DELLAs and JAZ1 partially repressed the activity of ORA47. However, these repressors had a minor effect on the activity of ORA47, suggesting that they are not the hypothetical repressor and/or adaptor proteins. Since the yeast two-hybrid screening was performed with an ORA47 derivative lacking the C-terminal region, which is responsible for auto-activation in yeast and is required for the activity of ORA47 in Arabidopsis protoplasts (Khurshid, 2012), it is possible that the hypothetical repressors or adaptors interact with the C-terminal domain.

The JAs-induced expression of the *LOX2*, *AOC2* and *OPR3* genes, encoding JAs biosynthesis enzymes, was not affected in the quintuple *della* mutant or in transgenic plants overexpressing RGA or GAI, indicating that DELLAs do not modulate the expression of JAs biosynthesis genes. DELLAs have been reported to prevent inhibitory JAZ1 interaction with MYC2 via competitively binding to JAZ1, and thereby to enhance the ability of MYC2 to transcriptionally upregulate its target genes (Hou et al., 2010). In a more recent study direct association of DELLAs with MYC2 was reported and was suggested to have a negative effect on MYC2 activity in sesquiterpene biosynthesis (Hong et al., 2012). In contrast to these reports, activation of the *AOC2* promoter by MYC2 was not negatively or positively affected by any DELLA protein and co-expression of RGA did not attenuate the repression of MYC2 activity by JAZ1. Thus we found no evidence for modulation of MYC2 activity by DELLA proteins through direct binding to MYC2 or by interfering with MYC2-JAZ1 interaction.

Our previous studies demonstrated that MYCs controlled the expression of *ORA47*, therefore it is possible that DELLA proteins regulate *ORA47* expression via MYCs. RGA and GAI significantly promoted the trans-activation of the *ORA47* promoter by MYC2 in Arabidopsis protoplasts (Fig. 4b), possible by competitive binding to endogenous JAZ proteins. The repressive effect of exogenous JAZ1 on MYC2 activity was reduced by co-expression of RGA, although this effect was not statistically significant (Fig. 4c). In RGA or GAI overexpressing plants or in the quintuple *della* mutant the expression of the *ORA47* gene was not visibly affected (Fig 5). Recently,

the bHLH transcription factors JAM1, 2 and 3 were reported as negative regulators of JAs responses (Nakata and Ohme-Takagi, 2013; Sasaki-Sekimoto et al., 2013; Song et al., 2013). The DNA-binding specificity of JAMs is similar to that of MYC2, suggesting that they might regulate similar or overlapping sets of genes (Nakata et al., 2013; Fonseca et al., 2014). The expression of *JAM* genes is JAs-induced and partially MYC2-dependent (Sasaki-Sekimoto et al., 2013; Fonseca et al., 2014). If DELLAs affect the expression of *JAMs*, this in turn will affect the expression of *ORA47*. JAMs physically interact with some JAZs, leading to the fact that JAZs inhibit the function of JAMs as transcriptional repressors (Song et al., 2013; Sasaki-Sekimoto et al., 2014). According to the 'relief of repression' model, DELLAs might break the interaction between JAZs and JAMs, releasing JAMs to suppress their target genes, including *ORA47*.

The signaling pathway for the important growth regulators GAs shows crosstalk with the JAs signaling pathway to modulate plant responses to diverse environmental conditions. Several studies reported that DELLAs contribute to gene expression in response to JAs signaling. For example, constitutive overexpression of *RGL3* enhanced the expression of *VSP2*, *TAT1*, and *LOX2*, whereas the *rgl3-5* mutant exhibited reduced induction levels of these genes (Wild et al., 2012). Also JAs biosynthesis genes were reported to show a different expression pattern in GA-related mutants. For example, the expression of *LOX1* was down-regulated in the *ga1-3* mutant and at wild-type level in the quintuple *della* mutant, whereas the *AOC2* gene was up-regulated in this mutant (Cheng et al., 2009). Although we found interaction of DELLAs with ORA47 and effects of DELLAs on MYC2 activity in transient assays, we did not measure major changes in the expression of JAs biosynthesis genes in DELLA overexpressing plants or in the quintuple *della* mutant.

Materials and Methods

Yeast two-hybrid assays

The *ORA47* (*At1g74930*) deletion derivative 1-140 cloned in pAS2.1 (acc. No. U30497) was used as bait for yeast two-hybrid screening. Using the Stratagene cDNA synthesis kit amplified cDNA libraries representing $2x10^6$ primary transformants were prepared from an equal mixture of RNA from stems, leaves, roots and flowers of mature ecotype *Landsberg erecta* plants in the vector λACTII (Memelink, 1997). The λACTII library was converted in a pACT2 (acc. No. U29899) plasmid library via Cre-lox excision in *E.coli* strain BNN132. Co-transformation of bait and

cDNA library at a ratio of 1:1 was performed into yeast strain PJ64-4A according to a modified yeast transformation protocol (Gietz et al., 1992). Transformants were plated on minimal synthetic defined (SD)-glucose medium containing 5 mM 3-AT and lacking Trp, Leu and His (-LWH). Full-length *RGA* (*At2g01570*) and deletion derivatives were PCR amplified with the primer sets 5'-GCC ATG GAA GAG CTC ATG AAG AGA GAT CAT CAC CAA TTC-3' and 5'-GGA TCC TCT AGA TCA GTA CGC CGC CGT CGA GAG TTT C-3' for *RGA*, 5'-GCC ATG GAA GAG CTC ACG GCG GCG GGT GAG TCA ACT CGT TC-3' and 5'-GGA TCC TCT AGA TCA GTA CGC CGC CGT CGA GAG TTT C-3' for *RGAΔN1* (598-1764), 5'-GCC ATG GAA GAG CTC GCC GAA GCT TTA GCG CGG CGG ATC TAC C-3' and 5'-GGA TCC TCT AGA TCA GTA CGC CGC CGT CGA GAG TTT C-3' for *RGAΔN2* (796-1764), digested with NcoI and BamHI and cloned in pACT2 digested with NcoI and BamHI. The *GAI* gene (*At1g14920*) was PCR amplified with the primer set 5'-GCC ATG GAA GAG CTC ATG AAG AGA GAT CAT CAT CAT CAT C-3' and 5'-GGA TCC TCT AGA CTA ATT GGT GGA GAG TTT CC-3', digested with NcoI and BamHI and cloned in pACT2 digested with NcoI and BamHI. The *RGL1* gene (*At1g66350*) was PCR amplified with the primer set 5'-CGG GAT CCG AAT GAA GAG AGA GCA CAA CCA C-3' and 5'-CGA GCT CTT ATT CCA CAC GAT TGA TTC-3', digested with BamHI and SacI and cloned in pACT2 digested with BamHI and SacI. The *RGL2* gene (*At3g03450*) was PCR amplified with the primer set 5'-GCC ATG GAG ATG AAG AGA GGA TAC GGA GAA AC-3' and 5'-TCC CCC GGG TCA GGC GAG TTT CCA CGC CG-3', digested with NcoI and SmaI and cloned in pACT2 digested with NcoI and SmaI. The *RGL3* gene (*At5g17490*) was PCR amplified with the primer set 5'-TCC CCC GGG GAT GAA ACG AAG CCA TCA AGA AAC-3' and 5'-CGA GCT CCT ACC GCC GCA ACT CCG CCG C-3', digested with SmaI and SacI and cloned in pACT2 digested with SmaI and SacI. Interaction assays were performed by co-transformation of bait and prey plasmids into yeast strain PJ64-4A and plated on SD-LT medium. As control, empty pAS2.1 and pACT2 were used. Transformants were allowed to grow for 4-5 days. Subsequently, cells were grown for 16 hours in liquid SD-LT and 10 µl of 10 and 100-fold dilutions were spotted on solid SD-LTH supplemented with increasing 3-AT concentrations ranging from 0 to 50 mM. Yeast cells were allowed to grow for up to 7 days at 30 °C.

Plant material, growth conditions and chemical treatments

Arabidopsis thaliana wild-type, quintuple homozygous mutant (*gai*/*rga*/*rgl1*/*rgl2*/*rgl3*) (N16298) plants, RGA (N16291) and GAI (N16293) overexpressing plants are

in the genetic background of ecotype *Landsberg erecta* (*Ler*) (Feng et al., 2008). Seeds were surface-sterilized in a closed container with chlorine gas for three hours. Surface-sterilized seeds were grown on solid MA medium (Masson and Paszkowski, 1992). Following stratification for 3 days at 4°C, seeds were first incubated at 21°C in a growth chamber (16 h light/8 h dark, 2500 lux) for 10 days. For RGA and GAI overexpressing lines, seedlings were grown on solid MA medium supplemented with 40 mg/L gentamicin. Twenty to 25 seedlings were transferred to 50 ml polypropylene tubes (Sarstedt, Nümbrecht, Germany) containing 10 ml liquid MA medium without antibiotic and incubated on a shaker at 120 rpm for 4 additional days before treatment. Seedlings were treated for different time periods with 50 µM JA (Sigma-Aldrich, St. Louis, MO) dissolved in dimethylsulfoxide (DMSO; 0.05% final concentration), 100 µM GA3 dissolved in DMSO, or a combination of JA and GA3. As controls, seedlings were treated with 0.05% DMSO.

Arabidopsis protoplast transient expression assays

A 350 bp *AOC2* (*At3g25770*) promoter fragment was PCR-amplified on Arabidopsis genomic DNA with the primer set 5'-GCT CTA GAG ATT CAT TAC ATT TAG AAG-3' and 5'-TGG TCG ACT GAT AAA AAT AAA ATA AAA AG-3', digested with XbaI and SalI and cloned in plasmid pGusSH (Pasquali et al., 1994). A 200 bp *ORA47* (*At1g74930*) promoter fragment was PCR-amplified with the primers 5'- GGA TCC AAG TCG CGA CGA AAA TCT C-3' and 5'- CTG CAG GCT GAC TGG CGC GTG AAG-3', digested with BamHI and PstI and cloned in plasmid pGusSH47 (Pasquali et al., 1994). The *ORA47* open reading frame (ORF) was amplified using the primer set 5'-GAA GAT CTC ATA TGG TGA AGC AAG CGA TGA AG-3' and 5'-GAA GAT CTT CAA AAA TCC CAA AGA ATC AAA G-3', digested with BglII and cloned into BamHI digested pRT101 (Töpfer et al., 1987). The *JAZ1* (*At1g19180*) ORF was PCR-amplified using the primer set 5'-CGG GAT CCG TCG ACG AAT GTC GAG TTC TAT GGA ATG TTC-3' and 5'- CGG GAT CCC GTC GAC TCA TAT TTC AGC TGC TAA ACC G-3', digested with SalI and cloned in pRT101. The *GAI* (*At1g14920*) and *RGA* (*At2g01570*) ORF DNA fragments were PCR amplified with the primer sets 5'- GAG CTC ATG AAG AGA GAT CAT CAT CAT C-3' and 5'- TCT AGA CTA ATT GGT GGA GAG AGT TTC CAA G-3' for *GAI*; 5'- GAG CTC ATG AAG AGA GAT CAT CAC CAA TTC-3' and 5'- TCT AGA TCA GTA CGC CGC CGT CGA GAG TTT C-3' for *RGA*, digested with SacI and XbaI and cloned in pRT101 digested with SacI and XbaI. Protoplasts were isolated from Arabidopsis cell suspension ecotype Col-0 and plasmid DNA was introduced by polyethylene glycol (PEG)-mediated transfection

as previously described (Schirawski et al., 2000). Co-transformation with plasmids carrying *AOC2*-promoter-*GUS* and effector plasmids carrying *ORA47*, *RGA*, *RGAΔN*, *GAI* or *JAZ1* fused to the *CaMV 35S* promoter were carried out. To study a possible effect of DELLA interaction with transcription factors, a ratio of 2:2:2:2 or 2:2:6:2 (µg GUS: ORA47 or MYC2: DELLA: JAZ1) was chosen. As controls, co-transformations of *AOC2*-promoter-*GUS* with the empty pRT101 expression vector were used. Protoplasts were incubated at 25°C for at least 16 hrs prior to harvesting by centrifugation and immediately frozen in liquid nitrogen. GUS activity assays were performed as described (van der Fits and Memelink, 1997). GUS activities from triplicate transformations were normalized against total protein content to correct for differences in protein extraction efficiencies.

Arabidopsis protoplast transformation and microscopic analysis

Primer sets used for BiFC cloning were: 5'-GA TCG TCG ACA ATG AAG AGA GAT CAT CAC CAA TTC-3' and 5'-CG GGA TCC TCA GTA CGC CGC CGT CGA GAG TTT C-3' for *RGA* cloning with SalI and BamHI in pRTL2-YNEE (736); 5'-CCG GAC TAG TAT GAA GAG AGA TCA TCA TCA TCA TC-3' and 5'-CGG GAT CCC TAA TTG GTG GAG AGT TTC CAA G-3' for *GAI* cloning with SpeI and BamHI in pRTL2-YCHA (735); 5'-GA TCG TCG ACA ATG GTG AAG CAA GCG ATG AAG-3' and 5'-CGG TCA GCT CAA AAA TCC CAA AGA ATC AAA G-3' for *ORA47* cloning with SalI and SpeI in pRTL2-YCHA (735) and pRTL2-YNEE (736); PCR-amplified inserts were digested with the restriction enzymes mentioned above and cloned in the mentioned pRTL2 derivatives digested with the corresponding enzymes (Bracha-Drori et al., 2004). Plasmids were co-transformed by PEG-mediated transfection as previously described into Arabidopsis protoplasts (Schirawski et al., 2000). Images of transfected protoplasts were acquired with a Leica DM IRBE confocal laser scanning microscope equipped with an Argon laser line of 488 nm (excitation) and a band pass emission filter of 500-550 nm.

RNA extraction and Northern blot analyses

Total RNA was extracted from frozen tissue by phenol/chloroform extraction followed by overnight precipitation with 2 M lithium chloride, washed with 70% ethanol, and resuspended in water. For RNA-blot analysis, 10 µg RNA samples were subjected to electrophoresis on 1.5% agarose/1% formaldehyde gels, and blotted to GeneScreen nylon membranes (Perkin-Elmer Life Sciences, Boston, MA). Probes were ^{32}P-labeled by random priming. (Pre-) hybridization of blots, hybridization of

probes and subsequent washings were performed as described (Memelink et al., 1994) with minor modifications. Blots were exposed to X-ray films (Fuji, Tokyo, Japan). DNA fragments used as probes were PCR amplified from Arabidopsis genomic DNA. The following primer sets were used: 5'-ATG GCT CT TCA GCA GTG TC-3' and 5'-TTA GTT GGT ATA GTT ACT TAT AAC-3' for *Allene oxide cyclase2* (*AOC2*, *At3g25770*); 5'-CGG GAT CCG TGC GGA ACA TAG GCC ACG G-3' and 5'-CGG GAT CCG GAA CAC CCA TTC CGG TAA C-3' for *Lipoxygenase2* (*LOX2, At3g45140*); 5'-ATG ACG GCG GCA CAA GGG AAC-3' and 5'-TCA GAG GCG GGA AGA AGG AG-3' for *OPDA reductase3* (*OPR3*, *At2g06050*); 5'-CTG TGC CAA TCT ACG AGG GTT-3' and 5'-GGA AAC CTC AAA GAC CAG CTC-3' for *ACTIN* (*At3g18780*). For ORA47, the fragment was excised with XbaI and EcoRI from pRT101.

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