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

Zhang, K.

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

**Kaixuan Zhang**

**张 凯 旋**

Kaixuan Zhang

MYC transcription factors:

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PhD thesis, Leiden University, The Netherlands, 2016

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

## **Proefschrift**

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**Promotor:** Prof. dr. J. Memelink

**Overige leden:** Prof. dr. B. Hause (Leibniz instituut of Plant Biochemie)

Prof. dr. C.J.M. Pieterse (Universiteit Utrecht)

Prof. dr. P.J.J. Hooykaas

Prof. dr. P.G.L. Klinkhamer

Prof. dr. H.P. Spaink

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# **Chapter1**

## **General Introduction**

Kaixuan Zhang and Johan Memelink

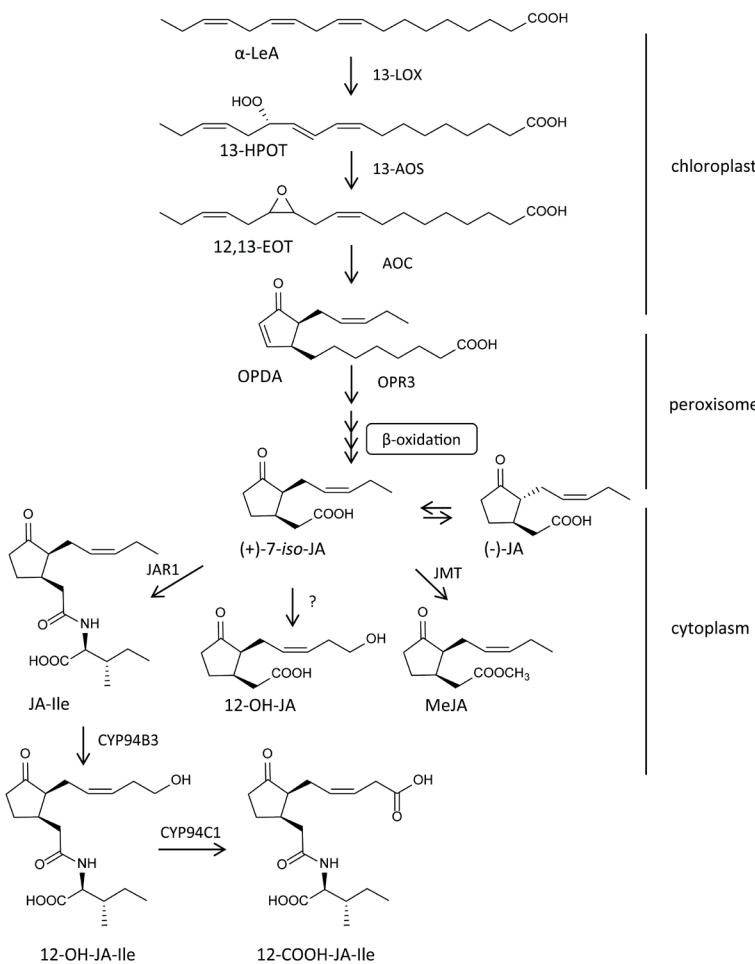
Institute of Biology, Leiden University, *Sylviusweg 72, P.O. Box 9505, 2300 RA Leiden, The Netherlands*

In nature, plants are continuously challenged by a myriad of environmental changes that comprise various biotic and abiotic stresses, such as those brought about by microbial pathogens, herbivorous insects, wounding, drought or salinity. Because plants have a sessile lifestyle, they have developed sensitive sensory systems to perceive external attacks and sophisticated strategies to mount effective responses to defend themselves against all these different types of stresses. Optimal plant fitness in the face of those threats relies on complex signal transduction networks that link damage-associated signals to appropriate changes in metabolism, growth, and development. Plant hormones, a group of structurally diverse small molecules, act as central players in the plant defensive signaling network and are key regulators of plant growth and development (Bari and Jones, 2009; Santner and Estelle, 2009). Attack or environmental stimuli result in changes in the levels of the major defense hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). Other plant hormones, including abscisic acid (ABA), gibberellins (GAs), auxin and cytokinins, are also implicated in plant defense signaling pathways (Bari and Jones, 2009; Pieterse et al., 2012).

Jasmonates (JAs), consisting of JA and its cyclic precursors and derivatives, act as important molecules in many developmental processes and in defense against environmental stresses. JAs modulate diverse processes such as vegetative growth (Staswick et al., 1992), fruit ripening (Kondo et al., 2000), trichome formation (Traw and Bergelson, 2003) and flower development (Browse, 2009). Plant responses to wounding, insect herbivory and necrotrophic pathogens are orchestrated by JAs (Yan et al., 2013). In the past decades, JAs biosynthesis, perception, signal transduction and action in *Arabidopsis* have been widely investigated and extensively reviewed.

## **JAs biosynthesis**

JAs are plant oxylipins and are synthesized via the octadecanoid pathway. Most of the enzymes of this pathway leading to JAs biosynthesis and metabolism have been identified and several reports have reviewed their structures, biochemical activities and functional regulation (Schaller, 2001; Creelman and Mulpuri, 2002; Schaller and Stintzi, 2009; Yan et al., 2013). The biosynthesis of JAs begins with the release of  $\alpha$ -linolenic acid ( $\alpha$ -LeA) from chloroplast membranes by the action of a lipid hydrolyzing enzyme. Upon  $\alpha$ -LeA liberation in the chloroplast, this fatty acid is then converted by 13-lipoxygenase (13-LOX) to 13-hydroperoxylinolenic acid (13-HPOT),



**Figure 1.** Scheme of the JAs biosynthesis pathway in *Arabidopsis thaliana*. The enzymes and the intermediates are indicated as 13-LOX for 13-lipoxygenase, 13-AOS for 13-alene oxide synthase, AOC for allene oxide cyclase, OPR3 for OPDA reductase, JAR1 for jasmonate resistant 1, JMT for JA carboxyl methyltransferase;  $\alpha$ -LeA for  $\alpha$ -linolenic acid, 13-HPOT for 13-hydroperoxylinolenic acid, 12,13-EOT for 12,13-epoxyoctadecatrienoic acid, OPDA for 12-oxo-phytodienoic acid, (+)-7-iso-JA and (-)-JA for jasmonic acid, JA-Ile for jasmonoyl-L-isoleucine, 12-OH-JA for 12-hydroxyjasmonic acid, MeJA for methyl jasmonate, 12-OH-JA-Ile for 12-hydroxy-JA-Ile and 12-COOH-JA-Ile for oxidized 12-hydroxy-JA-Ile.

which is the substrate for 13-allene oxide synthase (13-AOS). 13-AOS catalyzes the conversion of 13-HPOT to the unstable epoxide 12,13-epoxyoctadecatrienoid (12,13-EOT), providing substrate for the following enzyme allene oxide cyclase (AOC) and resulting in the formation of 12-oxo-phytodienoic acid (OPDA). OPDA is subsequently transported from chloroplasts to peroxisomes where it is reduced

by OPDA reductase (OPR) to 3-oxo-2-(2'(Z)-pentenyl)-cyclopentane-1-octanoic acid (OPC-8:0). Three rounds of  $\beta$ -oxidation reactions result in the formation of (+)-7-*iso*-JA, which readily isomerizes to the thermodynamically favored (-)-JA which is the predominant form of JA in plant tissues (Schaller et al., 2004). In addition to isomerization, JA can undergo different molecular modifications to produce a variety of derivatives in plants, among which jasmonoyl-L-isoleucine (JA-Ile) is considered as the main bioactive JAs (Fonseca et al., 2009). JA-Ile is synthesized by a JA amino acid synthetase (JAR1) that conjugates (+)-7-*iso*-JA to isoleucine (Suza and Staswick, 2008). Another well-characterized modification is the methylation to methyl-JA (MeJA) by JA carboxyl methyltransferase (JMT) (Seo et al., 2001). The cytochrome P450 enzyme CYP94B3 was found to hydroxylate bioactive JA-Ile to the inactive compound 12-hydroxy-JA-Ile (12-OH-JA-Ile) and the P450 enzyme CYP94C1 was found to act in the subsequent carboxylation step leading to the inactive compound 12-COOH-JA-Ile (Heitz et al., 2012). Together with hydroxylation of JA to 12-OH-JA (Miersch et al., 2008), hydroxylation and carboxylation of JA-Ile are assumed to be 'switch off' mechanisms in JAs signaling (Wasternack and Hause, 2013).

Perception of external stresses triggers the biosynthesis of JAs for the adaptation of plants to the changing environment. Leaf damage inflicted by mechanical wounding and herbivory are highly effective triggers for *de novo* JAs synthesis and result in rapid increases in JAs accumulation at the site of wounding (Glauser et al., 2008). The *Arabidopsis fad3fad7fad8* triple mutant, which is JAs deficient and exhibits enhanced susceptibility to insect attack (McConn et al., 1997), provides proof that *de novo* JAs synthesis is required to protect plants against attack by certain insects. The rapid production of JAs results from the sufficient occurrence of JAs biosynthesis enzymes and the release of substrates from membranes upon strong external stimuli such as wounding (Stenzel et al., 2003). Many studies have shown that most biosynthesis genes including *LOX*, *AOS*, *AOC*, *OPR3*, *JMT*, and *JAR1* are induced by JAs treatment and wounding (Sasaki et al., 2001; Schaller, 2001; Wasternack, 2007). It is widely accepted that JAs biosynthesis is regulated by a positive feedback loop. However, constitutive overexpression of *AOS* and *AOC* did not alter basal JAs levels (Laudert et al., 2000; Stenzel et al., 2003a). Surprisingly, JAs biosynthesis is not induced by endogenous JAs in tomato leaves (Miersch and Wasternack, 2000) and the accumulation of JAs is undetectable in response to JA although JAs biosynthesis genes are expressed (Scholz et al., 2015), suggesting additional post-translational regulation mechanisms. Furthermore, the abundant appearance

of LOX, AOC and AOS proteins in fully developed leaves is independent of stress-induced accumulation of the corresponding mRNAs (Stenzel et al., 2003b). Therefore, it has been suggested that substrate availability, enzyme activity and tissue specificity are important factors in JAs biosynthesis besides gene transcription (Scholz et al., 2015; Wasternack, 2007).

### JAs perception and signaling pathway

Following the production of JAs, transduction of the JAs signal occurs via interaction with a receptor that binds bioactive JA-Ile. In order to discover JAs receptor proteins, screening for *Arabidopsis* mutants insensitive to growth inhibition by MeJA or coronatine (a functional and structural analog of JA-Ile) was performed (Staswick et al., 1992; Feys et al., 1994). Exhaustive screens identified that the *coronatine insensitive1* (*coi1*) mutant was insensitive to JAs, male sterile, defective in resistance to certain insects and pathogens and failed to express JAs-related genes (Benedetti et al., 1995; McConn et al., 1997; Thomma et al., 1998). The *Arabidopsis COI1* gene was found to encode an F-box protein (Xie et al., 1998). F-box proteins were known to associate with the proteins Skp, Cullin and Rbx to form an SCF complex with E3 ubiquitin ligase activity (Bai et al., 1996). Co-immunoprecipitation experiments confirmed that COI1 associated physically with SKP1, CUL1 and Rbx1 proteins *in vivo* to assemble the SCF<sup>COI1</sup> complex (Devoto et al., 2002; Xu et al., 2002). Furthermore, additional mutants that were deficient in other components or regulators of SCF complexes also showed impaired JAs responses (Tiryaki and Staswick, 2002; Feng et al., 2003; Lorenzo and Solano, 2005), further supporting the notion that the core JAs signaling module and JAs responses depend on the actions of the SCF<sup>COI1</sup> complex. These discoveries led to the suggestion that JAs signaling involves the ubiquitination of specific proteins by the SCF<sup>COI1</sup> complex and their subsequent degradation by the 26S proteasome (Turner et al., 2002). According to this hypothesis, the substrates that COI1 recruits for ubiquitination are negative regulators of JAs responses.

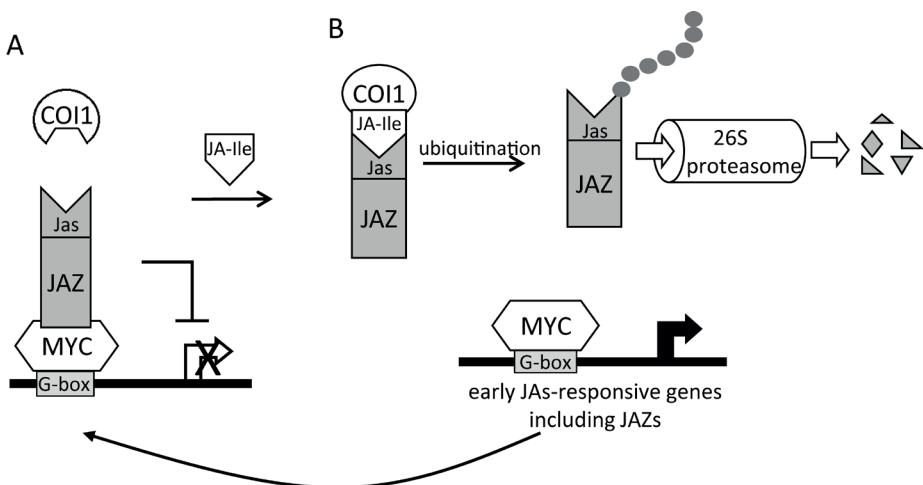
Three research groups using independent approaches simultaneously identified that a ZIM (Zinc-finger Inflorescence Meristem)-domain family protein had crucial roles in JAs responses in *Arabidopsis* (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007). The physical association between COI1 and a Jasmonate ZIM-domain (JAZ) protein, JAZ1, was detected in a yeast two-hybrid assay and in an *in vitro* pull down assay in the presence of JA-Ile (Thines et al., 2007). The degradation of JAZ3 protein in transgenic plants expressing JAZ3-GFP in wild-type background

was blocked by the proteasome-specific inhibitor MG132, implicating the 26S proteasome in JAs-mediated JAZ3 removal. Moreover, the stabilization of JAZ1-GFP, JAZ3-GFP and JAZ6-GFP in the *coi1* mutant further confirmed that JAZ proteins were direct targets of SCF<sup>COI1</sup> leading to 26S proteasome-mediated degradation (Chini et al., 2007; Thines et al., 2007). JA-Ile and coronatine, rather than OPDA, JA or MeJA, promoted the COI1-JAZ1 interaction, indicating that the JAZ proteins were the missing link of JAs signaling in *Arabidopsis* (Thines et al., 2007; Katsir et al., 2008). Different assays used for closely related COI1 and JAZ1 protein interactions in tomato, tobacco and rice established that the mechanism was not unique to *Arabidopsis* (Thines et al., 2007; Shoji et al., 2008; Sheard et al., 2010; Seo et al., 2011).

*Arabidopsis* JAZ proteins consisting of 12 members belong to the large family of proteins that share a conserved TIFY sequence within the ZIM motif (Vanholme et al., 2007). This non-DNA-binding TIFY motif mediates homo- and heteromeric interactions between most JAZs (Chung and Howe, 2009). A second defining feature of JAZs is the highly conserved Jas motif near the C terminus which is responsible for protein degradation. The dominant JAs-insensitive mutant *jai3-1* showed impaired transcriptional activation of JAs-responsive genes compared to wild-type *Arabidopsis* in addition to its phenotypic defect in response to JAs. The *jai3-1* allele encodes a mutant protein JAZ3 lacking a conserved C-terminal Jas domain and is resistant to JAs-triggered degradation (Chini et al., 2007). As for *jai3-1*, high-level expression of *JAZ1Δ3A*, a derivative with a deletion of the conserved Jas motif, blocked JAs responsiveness (Thines et al., 2007; Chung et al., 2008). Overexpression of a natural splice variant of *jasmonate-associated 1* (*JAS1*, identical to *JAZ10*) lacking part of the Jas domain resulted in reduced sensitivity to MeJA and elevated root and shoot growth upon MeJA treatment (Yan et al., 2007). Altogether, the lack of an obvious DNA-binding domain, the nuclear localization, the rapid induction by JAs and the dominant JAs-insensitive phenotype resulting from Jas motif deletion (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007; Chung et al., 2008; Chung and Howe, 2009) suggest that the hypothetical transcriptional repressor function of JAZs would be indirect by suppressing other DNA-binding transcriptional activators. Furthermore, *Arabidopsis* null mutants harboring T-DNA insertions in the *JAZ2*, *JAZ5*, *JAZ7* or *JAZ9* genes failed to exhibit JAs-related phenotypes, suggesting that these JAZ genes function redundantly (Thines et al., 2007).

The basic helix-loop-helix (bHLH) transcription factor MYC2 (also known as JIN1 or RAP-1) (de Pater et al., 1997; Lorenzo et al., 2004), was responsible for the

activation of a subset of JAs-responsive genes (Lorenzo et al, 2004). MYC2 interacted with most JAZ repressors directly (Chini et al., 2009). Similar to MYC2, the phylogenetically related transcription factors MYC3 and MYC4 interacted *in vivo* and *in vitro* with JAZ repressors (Fernández-Calvo et al., 2011; Niu et al., 2011; Goossens et al., 2015). Current models of JAs signaling indicate that, in the presence of low levels of JAs, the transcriptional activity of MYCs is repressed by JAZ proteins which recruit TOPLESS (TPL) to form a repressor complex either directly (Shyu et al., 2012) or through the adaptor protein NOVEL INTERACTOR OF JAZ (NINJA) (Pauwels et al., 2010). Elevated levels of JAs caused by external stimuli promote interaction of JAZ proteins with COI1 and subsequent degradation of JAZs by the 26S proteasome, leading to the release of MYC transcription factors and the activation of primary response genes. Interestingly, in addition to transcriptional activators, JAZ proteins also target transcriptional repressors, such as JAM1, JAM2 and JAM3, to attenuate their transcriptional function (Song et al., 2013; Fonseca et al., 2014; Sasaki-Sekimoto et al., 2014). The balance between repression and activation would lead to an appropriate expression output of JAs-responsive genes, resulting in an appropriate level of JAs responses (Song et al., 2013).



**Figure 2.** Model for the regulation of JAs-responsive gene expression by MYC and JAZ proteins. (A) In the absence of JA-Ile, JAZ proteins interact with MYCs maintaining these transcription factors inactive. (B) JA-Ile promotes the interaction between JAZs and COI1, leading to degradation of JAZs by the 26S proteasome presumably via ubiquitination. Subsequently, MYCs are liberated to activate their target genes, including JAZ genes, resulting in a negative feedback regulation. See text for other abbreviations.

## Transcription factors and JAs responses

### I) bHLH-domain Transcription factors

Transcription factors that depend on JAs signaling for activity are the key components for JAs signal transduction processes by regulating expression of downstream genes via specific binding to *cis*-acting elements in the promoters of target genes. The bHLH proteins form a superfamily in Arabidopsis with 162 bHLH-encoding genes (Bailey et al., 2003). The best characterized and most multifunctional bHLH-domain transcription factor involved in JAs signaling pathway is MYC2, which regulates a subset of JAs-responsive genes. The JAs-responsive *MYC2* gene was identified in *jin1* (*jasmonate-insensitive 1*) mutants which originated from a screening for mutants affected in JAs-induced root growth inhibition (Lorenzo et al., 2004). The *jin1/myc2* mutant showed reduced root growth inhibition in response to JAs and constitutive expression of *MYC2* conferred hypersensitivity to JAs. MYC2 displays the general characteristics of the bHLH protein family (Toledo-Ortiz et al., 2003). The bHLH domain of MYC2 is responsible for DNA-binding and the formation of homo- and/or heterodimers with other transcription factors. The G-box (CACGTG) and G-box-related hexamers are the binding sequences of MYC2 in its target promoters (de Pater et al., 1997; Dombrecht et al., 2007 ; Chini et al., 2007). In its amino-terminus, MYC2 contains a putative transcription activation domain (TAD) recruiting the Mediator complex required for transcription initiation and a JAZ interaction domain (JID) for interaction with JAZ proteins (Kazan and Manners, 2013).

In Arabidopsis, MYC2 differentially modulates JAs-dependent responses through direct regulation of its target genes and physical interaction with other transcription factors. As a positive regulator in response to wounding, MYC2 activates the expression of *LOX2* and *TAT1* genes by directly binding to their promoters (Hou et al., 2010). MYC2 positively regulated JAs-mediated resistance to insect pests, such as *Helicoverpa armigera*, and flavonoid biosynthesis possibly via modulating the expression of positive transcriptional regulators in Arabidopsis (Dombrecht et al., 2007). In addition, most JAZ genes were constitutively expressed in plants overexpressing *MYC2* and JAs-responsive expression of most JAZ genes was reduced in a loss-of-function *myc2* mutant, indicating that MYC2 fine-tunes the JAs signaling pathway by regulating the expression of JAZ repressors (Chini et al., 2007). Further studies have demonstrated that MYC2 directly targeted the G-boxes in the promoters of at least three JAZ genes, *JAZ1* (Pauwels and Goossens, 2008), *JAZ2*

(Niu et al., 2011) and *JAZ3* (Chini et al., 2007). In contrast, the genes involved in defenses against pathogens are repressed by MYC2 consistent with the increased resistance to necrotrophic pathogens of the *jin1/myc2* mutant (Lorenzo et al., 2004). The negative effect of MYC2 is due to the MYC2-mediated direct suppression of two genes, encoding ETHYLENE RESPONSE FACTOR 1 (ERF1) and AP2/ERF-domain protein ORA59 (Dombrecht et al., 2007; Zhai et al., 2013), which regulate defense against pathogens. More recently, it has been reported that MYC2 physically interacted with ETHYLENE INSENSITIVE3 (EIN3) to inhibit its DNA binding activity and simultaneously induced the expression of the gene encoding the F-box protein *EIN3 BINDING F-BOX PROTEIN1 (EBF1)* to promote EIN3 degradation during apical hook development (Song et al., 2014; Zhang et al., 2014).

The two *Arabidopsis* bHLH-domain proteins MYC3 and MYC4 share high sequence similarity with MYC2, suggesting they probably have similar biological function. Indeed, MYC3 and MYC4 interact *in vitro* and *in vivo* with JAZ repressors and also form homo-dimers or hetero-dimers with each other or MYC2. They are nuclear proteins and have similar DNA binding specificity as MYC2. Analysis of single, double and triple mutants revealed that MYC2, MYC3 and MYC4 regulate both overlapping and distinct functions (Fernández-Calvo et al., 2011; Niu et al., 2011). Moreover, some other bHLH transcription factors including TT8, GL3 and EGL3, essential components of WD-repeat/bHLH/MYB transcriptional complexes mediating diverse plant responses, such as anthocyanin biosynthesis and trichome initiation, are targets of JAZ repressors (Qi et al., 2011). More recently, the bHLH transcription factors JAM1, JAM2 and JAM3 were found to act as transcriptional repressors and to negatively regulate JAs responses by forming protein-protein interactions with JAZs (Sasaki-Sekimoto et al., 2013; Fonseca et al., 2014). The mechanism for the negative regulation of JAs signaling by JAMs may also be based on the competitive binding to the target sequences of MYC2 (Nakata et al., 2013; Song et al., 2013).

## II) AP2/ERF-domain transcription factors

The AP2/ERF superfamily is defined by the AP2/ERF domain, which consists of approximately 60 amino acids and is involved in recognizing a GCC (AGCCGCC) motif for DNA binding (Mizoi et al., 2012). In *Catharanthus roseus*, Octadecanoid-derivative Responsive *Catharanthus* AP2-domain (ORCA) proteins, ORCA2 and ORCA3, belong to the AP2/ERF-domain family and the expression of the encoding genes was rapidly induced by MeJA (Menke et al., 1999; van der Fits and Memelink, 2001).

ORCA2 and ORCA3 trans-activated the promoter of the monoterpenoid indole alkaloid (MIA) biosynthesis gene *Strictosidine synthase* (*STR*) through binding to a JAs- and elicitor-responsive element (JERE) containing a GCC motif (Menke et al., 1999; van der Fits and Memelink, 2001). Overexpression of *ORCA3* in transgenic *C. roseus* suspension cells resulted in enhanced expression of several genes encoding enzymes involved in primary and secondary metabolism, including *STR* and, consequently, in increased accumulation of MIA (van der Fits and Memelink, 2000). This demonstrates that the JAs-induced expression of *STR* gene is controlled by the JAs-responsive AP2/ERF-domain transcription factors ORCA2 and ORCA3, providing the first evidence for a link between members of the AP2/ERF-domain family and JAs signaling.

In *Arabidopsis*, the AP2/ERF transcription factor family comprises 145 members (Sakuma et al., 2002). Based on the observation that *ORCA* genes were induced by JAs, 14 *Octadecanoid-Responsive Arabidopsis AP2/ERF-domain* (*ORA*) genes were identified as being rapidly induced by JAs in a *COI1*-dependent manner (Atallah, 2005). The expression of *ORA59* was induced by JAs or ethylene (ET) and was synergistically induced by both hormones (Pré et al., 2008). Genome-wide microarray analysis showed that overexpression of *ORA59* activated the expression of several JA- and ET-responsive defense genes, including the defense gene *PLANT DEFENSIN 1.2* (*PDF1.2*). Plants overexpressing *ORA59* were more resistant to infection by the necrotrophic fungus *Botrytis cinerea*. The expression of the AP2/ERF-domain transcription factor *ERF1*, a close parologue of *ORA59*, was similar to *ORA59* and dependent on JAs and/or ET signals (Lorenzo et al., 2003). Constitutive overexpression of *ERF1* rescued the defense response defect of *coi1* and *ein2* mutants and induced the expression of several genes responsive to both ET and JAs, including *PDF1.2* and *basic chitinase* (*ChiB*) (Lorenzo et al., 2003). In terms of these similarities in gene expression patterns and in target gene sets, as well as their phylogenetic relationship in the AP2/ERF-domain family, *ORA59* and *ERF1* hypothetically have redundant functions in JAs and ET signal transduction. However, the essential role of *ORA59* as an integrator of JAs and ET signals to regulate defense genes was demonstrated with *ORA59*-silenced plants generated by the RNAi approach (Pré et al., 2008). In response to JAs and/or ET, or after infection with the necrotrophic fungi *B. cinerea* or *Alternaria brassicicola*, the expression of defense-related genes such as *PDF1.2*, *hevein-like* (*HEL*) and *ChiB* was blocked in *ORA59*-silenced plants. As expected from the dramatic reduction of defense gene expression, the silenced plants exhibited

more susceptibility to *B. cinerea* infection, further supporting the crucial role of ORA59 in the integration of JAs and ET signals. The activity of ORA59 in defense against necrotrophic pathogens was repressed by JAZ1 via the ZFAR1 and ZFAR2 adaptor proteins (Körbes, 2010; Zhou, 2014).

The AP2/ERF-domain transcription factor ORA47 functions in the regulation of the JAs biosynthesis pathway (Pré, 2006; Chen et al., 2016). Overexpression of the *ORA47* gene conferred obvious JAs-related phenotypes, such as inhibition of growth and anthocyanin production, and induced expression of most JAs biosynthesis genes. JAs measurements in plants overexpressing *ORA47* showed an increase in the amounts of the JA precursor OPDA, JA, the bioactive JA-Ile and 12-hydroxy-JA (Khurshid, 2012). Probably, as a consequence of JAs accumulation, several JAs-responsive defense genes including *VSP1* were upregulated in *ORA47*-overexpressing plants. ORA47 activated the expression of the *AOC2* gene, encoding an enzyme of JAs biosynthesis, via specifically binding to a GCC-like motif in the *AOC2* promoter (Zarei, 2007; Chen et al., 2016). These findings indicate that ORA47 act as an important regulator in the positive JAs-responsive feedback loop by controlling the expression of JAs biosynthesis genes.

AtERF3 and AtERF4 act as repressors and downregulated not only the transcription levels of their target genes but also interfered with the activity of other activating transcription factors (Fujimoto et al., 2000). The expression of *AtERF4* gene was induced by JAs, ET, ABA, wounding or infection with *Fusarium oxysporum* (Fujimoto et al., 2000; McGrath et al., 2005; Yang et al., 2005; Pré, 2006). In untreated *AtERF4*-overexpressing plants, the basic transcript levels of defense genes *PDF1.2* and *ChiB* remained at a similar level as in wild-type plants. A dramatic increase in the *PDF1.2* basal transcript level was found in *erf4-1* mutant plants compared to wild-type plants, whereas *ChiB* transcript level remained unchanged (McGrath et al., 2005). Nevertheless, in response to JAs or ethylene treatment, expression of a subset of defense-related genes, including *PDF1*, was significantly repressed and enhanced in *AtERF4* overexpressing plants and *AtERF4*-silenced plants, respectively (McGrath et al., 2005; Pré, 2006). It is therefore suggested that AtERF4 is able to repress the expression of the same genes that are positively regulated by ORA59 and ERF1 in response to JAs and/or ET. AtERF3 and AtERF4 differ from the JAs-responsive AP2/ERF transcriptional activators by the presence of an ERF-associated amphiphilic repression (EAR) motif in the C-terminal part of the proteins. This motif has been shown confer the repression ability to AtERF3 and AtERF4 because muta-

tion within this motif eliminated the capacity for repression (Ohta et al., 2001). The co-repressor TOPLESS (TPL) was shown to interact directly with the EAR motifs of NINJA and JAZ8 proteins to repress the transcriptional activation of JAs-responsive genes (Pauwels et al., 2010; Shyu et al., 2012). Thus, AtERF3 and AtERF4 probably repress the expression of JAs-responsive genes through both directly binding to the GCC-box in the promoters and the association with the co-repressor TPL.

### **III) R2R3-MYB transcription factors**

MYB transcription factors are widely distributed in plants, and can be classified into four groups, 1R, 2R, 3R and 4R, based on the number of adjacent repeats in the DNA-binding domain. The DNA binding domain consists of one to four imperfect repeats (R1, R2, R3 and R4), where each repeat is about 50-53 amino acids long and consists of three  $\alpha$ -helices, with the second and third helices forming a helix-turn-helix structure which can bind the major groove of DNA. The *Arabidopsis* R2R3-MYB transcription factors MYB21 and MYB24 were identified as key regulators of stamen and pollen maturation processes triggered by JAs (Mandaokar et al., 2006). The *myb21-1* knockout mutant exhibited shorter anther filaments, delayed anther dehiscence and strongly reduced male fertility, which could not be rescued by exogenous JAs. Although the *myb24* mutant was phenotypically wild-type, the *myb21myb24* double mutant exacerbated all three phenotypic aberrations of the *myb21* mutant. The expression of the *MYB21* and *MYB24* genes was induced by JAs (Mandaokar et al., 2006). MYB108, also encoded by a JAs-responsive gene, shared overlapping functions with MYB24 and acted downstream of MYB21 in a transcriptional cascade that mediates stamen and pollen maturation in response to JAs (Mandaokar, 2009). The direct interaction between a select set of JAZ repressors (JAZ1, JAZ8 and JAZ11) and MYB21 and MYB24 revealed a mechanism in which JAs triggers COI1-dependent JAZ degradation to control MYB21 and MYB24 in stamen development (Song et al., 2011). Overexpression of the R2R3-MYB transcription factor *MYB75* restored anthocyanin accumulation and trichome initiation in the *coi1* mutant. JAZ proteins directly interacted with the MYB factors (MYB75 and GL1) of WD-repeat/bHLH/MYB complexes, attenuated their transcriptional function, and subsequently repressed anthocyanin biosynthesis and trichome initiation (Qi et al., 2011).

### **JAs signal interaction with SA, GAs and Ethylene**

Upon changing environmental conditions, JAs and SA are recognized as major defense hormones of plant immunity. However, the hormones ET, GAs, auxin and ABA function as modulators of the plant immune signaling network as well (Pieterse et al., 2012). This so-called hormone crosstalk enables plants to rapidly adapt to external attack and to utilize their limited resources in a cost-efficient manner (Walters and Heil, 2007).

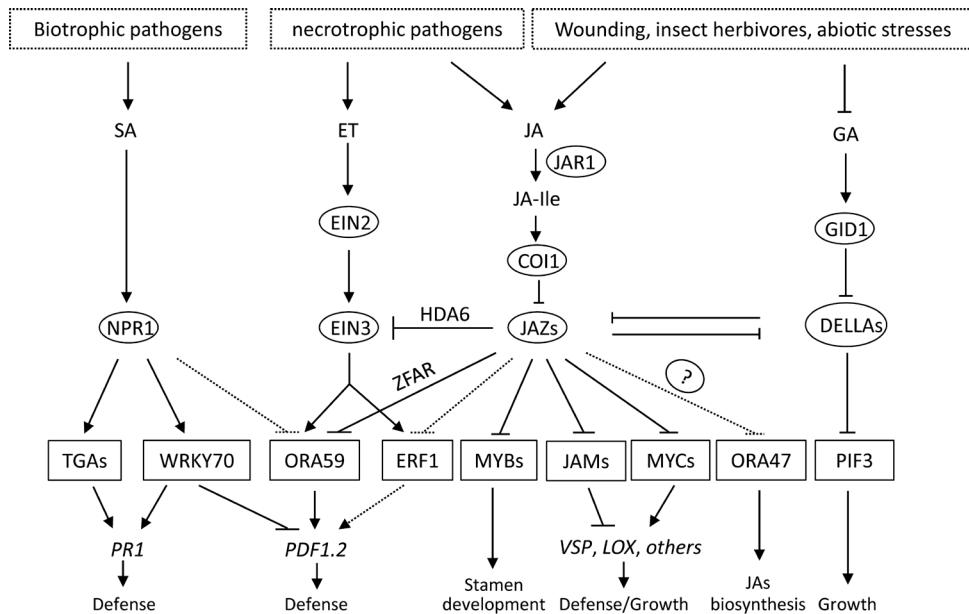
### JAs-SA crosstalk

Generally speaking, SA functions as a plant hormone required for innate immunity against biotrophic pathogens, while JAs are effective in the activation of defense against necrotrophic pathogens (Glazebrook, 2005). When plants are infected by biotrophic pathogens, the accumulation of high levels of SA triggers the localization of NON-EXPRESSOR OF PR GENE1 (NPR1) from the cytoplasm to the nucleus. In the nucleus, NPR1 binds to TGA (TGACG motif binding) transcription factors, initiating the expression of a set of defense-related genes including the *PR* (*Pathogenesis-Related*) genes. Many reports described the antagonistic interplay between SA and JAs. Arabidopsis unable to accumulate SA showed enhanced expression of JAs-responsive genes *PDF1.2*, *LOX2* and *VSP* in response to infection by *Pseudomonas syringae* pv *tomato* DC3000. Analysis of the Arabidopsis mutant *npr1*, which was impaired in SA signal transduction, revealed that the regulatory protein NPR1 was required in the SA-mediated suppression of JAs-regulated genes (Spoel et al., 2003). SA suppressed JAs signaling downstream of the action of the SCF<sup>COI1</sup>-JAZ complex by targeting JAs-responsive GCC promoter motifs through the reduction of accumulation of ORA59 protein (van der Does et al., 2013). Treatment with exogenous JAs inhibited the expression of several SA-dependent *PR* genes, suggesting that JAs also suppressed SA signaling (Niki et al., 1998). Arabidopsis *coi1* and *mpk4* mutants, which were impaired in JAs-responsive gene expression, exhibited increased SA levels, constitutive expression of *PR1* and enhanced resistance against *P. syringae* (Petersen et al., 2000; Kloek et al., 2001). However, treatment of Arabidopsis with low concentrations of JAs and SA resulted in a synergistic effect on the JAs- and SA-responsive genes *PDF1.2* and *PR1*, respectively, implying that the outcome of JAs-SA interaction is dose-dependent (Mur et al., 2006). The transcription factor WRKY70 acts as a node of convergence for integration of JAs-SA signaling. Expression of *WRKY70* was activated by SA but suppressed by JAs. Overexpression of *WRKY70* in Arabidopsis up-regulated *PR* genes and down-regulated *PDF1.2* gene, leading to

enhanced resistance to biotrophic pathogens and enhanced susceptibility to necrotrophic pathogens (Li et al., 2004). Recently, the JAs-induced R2R3-MYB transcription factor AtMYB44 was reported to modulate the antagonistic JAs-SA interaction via directly activating expression of *WRKY70* through a conserved sequence in the *WRKY70* promoter (Shim et al., 2013).

### **JAs-GAs crosstalk**

GAs play essential roles in controlling plant growth and development by regulating the degradation of growth-repressing DELLA proteins (Sun, 2010). Like JAs signaling, binding of GAs to the receptor GA INSENSITIVE DWARF1 (GID1) promotes the SCF<sup>SLY1/GID1</sup>-DELLA interaction, presumably resulting in ubiquitination and leading to degradation of DELLA repressors and the subsequent de-repression of different transcription factors, such as the positive regulators PHYTOCHROME-INTERACTING FACTORs (PIFs) involved in GAs-mediated plant growth promotion. The quadruple-DELLA mutant was partially insensitive to gene induction by JAs and, moreover, the GAs-deficient mutant *ga1* showed upregulated expression of JAs-responsive genes, suggestive of a positive role of DELLA proteins in JAs signaling (Navarro et al., 2008; Hou et al., 2010). A ‘relief of repression’ model was proposed for the JAs-GAs interaction, in which DELLAs competed for binding to JAZ1 with the key transcriptional activator of JAs signaling, MYC2, and, thus, liberated MYC2 to regulate its target genes (Hou et al., 2010). Consistent with this model, induction of primary JAs-responsive genes, including *LOX2*, *TAT1* and *VSP2*, was enhanced in transgenic plants overexpressing *RGL3*, encoding a DELLA protein, and reduced in the *rgl3-5* mutant (Wild et al., 2012). On the other hand, JAZ9 inhibited the interaction of the DELLA protein RGA with PIF3, indicating that JAs prioritized defense over growth by interfering with the GAs signaling cascade through the COI1-JAZ-DELLA-PIF signaling module (Yang et al., 2012). In addition, DELLAs positively regulated JAs/ET-mediated resistance to the necrotrophic fungus *B. cinerea* via preventing inhibitory JAZ1-ZFAR interaction with ORA59 and enhancing the activity of ORA59 on its target *PDF1.2* promoter (Zhou, 2014). Interestingly, JAs and GAs can also act synergistically to promote stamen and trichome development. GAs were found to promote JAs biosynthesis through DELLAs to control the expression of *MYB21*, *MYB24*, and *MYB57*, which in turn promoted stamen filament growth (Cheng et al., 2009). GAs and JAs induced degradation of DELLAs and JAZ proteins to coordinately activate the WD-repeat/bHLH/MYB complex and synergistically and mutually dependently



**Figure 3.** A model for the roles of transcription factors in the stress-responsive network involving JAs, ET, GAs and SA signaling. Attack by wounding, insects and necrotrophic pathogens induces the synthesis of JA, which can be converted to the biologically active JA-Ile by JAR1. Perception of JA-Ile by its receptor COI1 triggers the degradation of JAZ repressors, leading to the release of downstream transcription factors, such as ORA59, ORA47, MYB21/24, MYC2/3/4 and JAM1/2/3, and the regulation of JAs-responsive genes. R2R3-MYB transcription factors MYB21 and MYB24 are positive regulators of stamen development. The bHLH-type transcription factors MYC2/3/4 and JAM1/2/3 antagonistically regulate various JAs-related responses. The AP2/ERF-domain transcription factor ORA47 functions in the regulation of JAs biosynthesis and is hypothesized to be repressed by JAZ family members via an adaptor protein (indicated by the dashed lines). Infection with necrotrophic pathogens simultaneously induces ET together with JAs, resulting in the synergistic induction of *ORA59* and *ERF1* genes depending on EIN3 and COI1. *ORA59* repressed by the JAZ1-ZFAR complex is the key regulator of JAs/ET-responsive defense gene *PDF1.2*, whereas the role of *ERF1* in gene regulation remains unclear and awaits analysis of a knockout mutant (indicated by the dashed lines). JAZ repressors interact with EIN3 and repress the transcriptional activity of EIN3 via recruiting HDA6. GAs regulate plant growth and development through the degradation of DELLA repressors, which is mediated by the SCF<sup>SLY1/GID1</sup> complex and the 26S proteasome. The interaction between JAZ and DELLA proteins mediates JAs-GAs crosstalk, providing the molecular switch for the balance between plant growth and defense. SA signaling is generally triggered by biotrophic pathogens, in which nuclear NPR1 binds TGA transcription factors to initiate SA-associated responses. SA suppresses JAs signaling by targeting JAs-responsive GCC promoter motifs through the reduction of accumulation of *ORA59* protein. On the other hand, SA induces the expression of *WRKY70* in an NPR1-dependent manner to repress the expression of *PDF1.2*.

induced trichome initiation (Qi et al., 2014).

### **JAs-ET crosstalk**

ET is another important plant hormone in many aspects of the plant life cycle and in response to environmental stimuli. ET is perceived by a family of five membrane-localized receptors in Arabidopsis, which activate the downstream negative regulator Raf-like kinase CTR1 (CONSTITUTIVE TRIPLE RESPONSE 1) through physical interaction in the absence of ET. Binding of ET inactivates the receptors, resulting in inactivation of CTR1, which allows EIN2 to positively regulate the stability of the transcription factors EIN3, EIN3-like 1 (EIL1) and EIL2 in the nucleus to activate ET-responsive genes (Wang et al., 2002). An Arabidopsis microarray experiment showed that nearly half of the ET induced genes were also induced by JAs treatment, indicating that JAs and ET coordinately regulated many defense-related genes (Schenk et al., 2000). As mentioned above, JAs and ET synergistically induced the expression of *ORA59*, *ERF1* and *PDF1.2* in response to necrotrophic pathogens. Nevertheless, in a JAs or ET insensitive mutant (*coi1* or *ein2*, respectively), JAs and ET alone or in combination failed to induce the expression of these defense genes (Lorenzo et al., 2003; Pré et al., 2008). Crosstalk between JAs and ET was reported to be mediated through the direct interaction of JAZ proteins (JAZ1, JAZ3 and JAZ9) with two positive transcription factors for ET responses, EIN3 and EIL1, inhibiting the activity of these regulators by recruitment of the co-repressor HDA6 (HISTONE DEACETYLASE6) (Zhu et al., 2011). Therefore, ET-mediated EIN3/EIL1 stabilization and JAs-mediated EIN3/EIL1 release from JAZ repression resulted in the synergistic activation of *ERF1*, *ORA59* and their downstream target genes such as *PDF1.2*, providing a plausible explanation for the synergy in many JAs/ET-regulated responses (Zhu et al., 2011).

### **Conclusions**

Knowledge about the biosynthesis, signaling and regulation mechanisms of JAs has substantially increased in the last two decades. JAs biosynthesis showed self-activation, in which the products positively regulate the expression of genes encoding enzymes involved in JAs biosynthesis. Currently, the JA conjugate JA-Ile has been exclusively identified to act as the bioactive ligand to promote JAs signaling by enhancing the interaction of JAZ with the  $SCF^{COI1}$  complex. In the presence of low levels of JAs, JAZ proteins repress the expression of JAs-responsive genes via interaction with transcription activators such as MYC2, MYC3 and MYC4. In response to envi-

ronmental stimuli, the bioactive JA-Ile is quickly synthesized and perceived by its receptor COI1 to form the SCF<sup>COI1</sup>-JA-Ile-JAZ complex, resulting presumably in the ubiquitination and in the degradation of JAZ repressors and the de-repression of primary response genes. JAs do not exert the function independently but cooperatively with SA, GAs and ET signals. The positive and negative regulatory components of hormone pathways are potential targets to modify hormone crosstalk during disease and defense. Several important mediators of hormone crosstalk are transcription factors or repressors, implying that crosstalk is predominantly executed in the nucleus downstream of signal transduction, at the level of gene transcription.

## Outline of this thesis

JAs are crucial plant signaling molecules that regulate defense responses against wounding, insects and necrotrophic pathogens. Changing environmental conditions triggers quick biosynthesis, perception and transduction of JAs signals. Several transcription factors have been identified that appear to be involved in the expression of JAs-responsive genes, including ORA59, ERF1, ORA47 and MYCs. Transcriptional regulation of JAs biosynthesis in a positive feedback loop has been repeatedly described, however the regulatory mechanism behind this positive feedback loop remains to be elucidated. The studies described in this thesis focused on the functional analysis of the bHLH-domain transcription factors MYC2, MYC3 and MYC4 in the auto-regulatory loop in JAs biosynthesis in *Arabidopsis*.

**Chapter 2** describes the roles of MYC proteins in the regulation of JAs biosynthesis genes. Induction of a large number of genes involved in JAs biosynthesis in response to wounding or MeJA treatment was obviously attenuated in the *myc234* triple mutant compared to wild-type *Arabidopsis*. MYC proteins bound to a G-box (CACGTG) sequence in the *AOC2* promoter *in vitro* and activated the expression of *AOC2* through direct binding to this G-box sequence *in vivo*. In addition, MYCs and ORA47 additively controlled the activity of promoters of the JAs biosynthesis genes *LOX2*, *AOS*, *AOC2* and *OPR3*. These results indicated that MYCs act as key positive regulators of the auto-stimulatory loop in JAs biosynthesis.

**Chapter 3** describes the roles of MYC proteins in the JAs-responsive expression of *ORA47*. The results showed that MYC proteins interacted with a single G-box in the *ORA47* promoter *in vitro*. Transient assays revealed that this G-box sequence was essential for MYC-mediated activation of the *ORA47* promoter and another G-box and a G-box-like sequence contributed to a higher expression level

of the *ORA47* promoter. Triple knockout of the *MYC* genes or overexpression of a stable *JAZ1* derivative abolished JAs-responsive *ORA47* expression, demonstrating the crucial role of the *MYC-JAZ* module in the regulation of *ORA47* expression.

**Chapter 4** describes the effect of DELLA proteins on the regulation of JAs biosynthesis genes in *Arabidopsis*. All five DELLA proteins have been reported to interact with *MYC2* directly and to interfere with the *MYC2*-mediated JAs signaling output. Via yeast two-hybrid screening two members of the *Arabidopsis* DELLA protein family, *RGA* and *GAI*, were identified as *ORA47* interactors. *RGA* and *GAI* slightly promoted the activity of *ORA47* in the activation of the *AOC2* promoter, which was partially attenuated by co-expression of *JAZ1*. *RGA* and *GAI* significantly enhanced the activation of the *ORA47* promoter through interaction with *MYC2*. The expression of *LOX2*, *OPR3* and *AOC2* was not affected in the quintuple *della* mutant or in plants constitutively overexpressing *RGA* or *GAI*.

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# Chapter 2

## MYC transcription factors regulate jasmonate biosynthesis genes in *Arabidopsis thaliana*

Kaixuan Zhang and Johan Memelink

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

## Abstract

Jasmonates (JAs), comprising jasmonic acid (JA) and its cyclic precursors and conjugates, are plant specific hormones that regulate diverse plant developmental processes as well as defense responses against biotic and abiotic stresses. Pathogen or herbivore attack or wounding induce the biosynthesis of JAs, including the bioactive amino acid conjugate JA-Ile. Perception of JA-Ile by its receptor CORONATINE INSENSITIVE1 (COI1) triggers the degradation of JASMONATE ZIM DOMAIN (JAZ) repressors and the subsequent release of basic-helix-loop-helix-type MYC transcription factors, resulting in the activation of JAs-responsive genes. We report here that the expression of most genes encoding enzymes involved in JAs biosynthesis was MeJA- and wound-induced in a *MYC*-dependent manner. *In vitro* assays showed that MYC proteins directly bound to one of the two G-box sequences present in the promoter of the *AOC2* gene, encoding an enzyme in JAs biosynthesis. Furthermore, transient activation assays in protoplasts demonstrated that MYCs activate the promoters of a set of JAs biosynthesis genes additively with ORA47, an AP2/ERF-domain activator of JAs biosynthesis. These results indicate that MYCs act as key positive regulators of the auto-stimulatory loop in JAs biosynthesis.

## Introduction

Plants are exposed to a wide variety of stresses, including attack by pathogens, herbivory, and wounding. Plants can recognize stress signals and rapidly mount appropriate defense responses. Recognition of stress signals leads to accumulation of endogenous signaling molecules including the plant hormone jasmonic acid (JA) and its cyclic precursors and derivatives, collectively called jasmonates (JAs). JAs play major roles in the activation of defense responses against herbivorous insects, necrotrophic pathogens and wounding (Glazebrook, 2005; Glauser et al., 2008; Howe and Jander, 2008). Thus, the defense response involving JAs is a two-step process. First, perception of the external stress induces endogenous JAs biosynthesis. Then, JAs perception leads to the expression of a large number of defense-related genes (Turner et al., 2002).

Biosynthesis of JAs originates from the release of  $\alpha$ -linolenic acid ( $\alpha$ -LeA) from chloroplast membranes. After the sequential action of the plastid enzymes lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC),  $\alpha$ -LeA is converted to 12-oxo-phytodienoic acid (OPDA), the cyclic precursor of JA. OPDA is transported to peroxisomes and reduced by OPDA reductase (OPR3) followed by three rounds of  $\beta$ -oxidation to (+)-7-*iso*-JA, which can spontaneously epimerize into the more stable (-)-JA. As the last step JA is conjugated to the amino acid isoleucine by a JA amido synthetase (JAR1) to form the bioactive jasmonoyl-L-isoleucine (JA-Ile) (Schaller et al., 2004; Staswick and Tiryaki, 2004; Schaller and Stintzi, 2009). JA-Ile is perceived by the receptor CORONATINE INSENSITIVE1 (COI1), which is an F-box protein which is part of a Skp1-Cul1-F-box protein (SCF) complex with presumed E3 ubiquitin ligase activity. Binding of JA-Ile recruits JASMONATE ZIM-DOMAIN (JAZ) repressors to the SCF<sup>COI1</sup> complex, presumably resulting in ubiquitination and leading to subsequent degradation of JAZ proteins. The degradation of JAZ repressors liberates transcription factors to regulate the expression of various JAs-responsive genes (Gfeller et al., 2010).

The basic-helix-loop-helix (bHLH) transcription factor MYC2 has been reported as a regulatory hub in many aspects of the JAs signaling pathway in *Arabidopsis*. At low JAs levels, the transcriptional activity of MYC2 is repressed by interaction with JAZ proteins which recruit the repressor TOPLESS (TPL) directly or through the adaptor protein NOVEL INTERACTOR OF JAZ (NINJA) to form a repressor complex. Increase of cellular JAs levels caused by diverse stresses triggers the degradation of JAZ proteins and the release of MYC2 for JAs-dependent responses (Chini et

al., 2007; Dombrecht et al., 2007; Pauwels et al., 2010). The bHLH domain of MYC2 protein is responsible for DNA-binding and the formation of homo- and/or heterodimers with related bHLH proteins called MYC3 and MYC4. Previous studies indicated that MYC2 can bind to the G-box (CACGTG) sequence and G-box-related hexamers (de Pater et al., 1997; Toledo-Ortiz et al., 2003; Yadav et al., 2005; Dombrecht et al., 2007; Chini et al., 2007). MYC3 and MYC4, phylogenetically closely related to MYC2, have similar DNA binding affinity as MYC2 and act additively with MYC2 in the activation of JAs responses (Fernández-Calvo et al., 2011; Niu et al., 2011). Recently, the bHLH transcription factors JAM1, JAM2 and JAM3 were identified as transcriptional repressors that negatively regulate JAs responses via interaction with JAZ repressors (Nakata and Ohme-Takagi, 2013; Sasaki-Sekimoto et al., 2013; Fonseca et al., 2014; Sasaki-Sekimoto et al., 2014). The mechanism for the negative regulation of JAs signaling by JAMs is proposed to be also based on the competitive binding to the target sequences of MYCs (Nakata et al., 2013; Song et al., 2013).

The levels of JAs in plants vary as a function of tissue type, developmental stage and in response to different environmental stimuli. High levels of JAs were found in flowers, pericarp tissues of developing fruits, and in response to wounding (Creelman and Mullet, 1997). Several studies have shown that most genes encoding enzymes of JAs biosynthesis are induced by JAs (Sasaki et al., 2001; Wasternack, 2007) and wounding (Schaller, 2001), implying that JAs biosynthesis is regulated by a positive feedback loop. The phenomenon of self-activation of JAs biosynthesis has already been extensively investigated and reviewed in recent years, but the regulatory mechanism behind this positive feedback loop has not been elucidated yet. The expression levels of JAs biosynthesis genes were reduced in the *myc2* mutant and enhanced in the *jamx3* triple mutant compared with the wild type (Shin et al., 2012; Sasaki-Sekimoto et al., 2013; Zhai et al., 2013). Furthermore, the accumulation of JAs induced by wounding in the *jamx3* triple mutant was significantly higher than in wild type (Sasaki-Sekimoto et al., 2013). These results revealed that JAMs and MYC2 antagonistically regulate the JAs biosynthesis pathway. Overexpression of the AP2/ERF-domain transcription factor ORA47 led to elevated expression of a whole suite of JAs biosynthesis genes and increased levels of JAs, indicating that ORA47 controlled the positive feedback regulatory system (Kurshid, 2012; Pré, 2006). Both ORA47 and MYC2 were able to trans-activate the promoter of the *LOX3* gene, encoding an enzyme involved in JAs biosynthesis (Pauwels et al., 2008). However, as the key regulators of JAs signaling cascade, the direct involvement of MYC proteins

in the regulation of JAs biosynthesis has not been established yet.

The work described in this chapter is aimed at unraveling the function of MYC2, MYC3 and MYC4 in the auto-regulatory loop in JAs biosynthesis. We discovered that the expression of genes involved in JAs biosynthesis was attenuated in the *myc234* triple mutant after treatment with MeJA or wounding. Moreover, we found that MYC proteins bind to one of two G-box sequences in the *AOC2* promoter *in vitro* and that this G-box is essential for MYC-mediated activation of the *AOC2* promoter *in vivo*. In addition, MYCs and ORA47 can additively activate the promoters of the JAs biosynthesis genes *LOX2*, *AOS*, *AOC2* and *OPR3*.

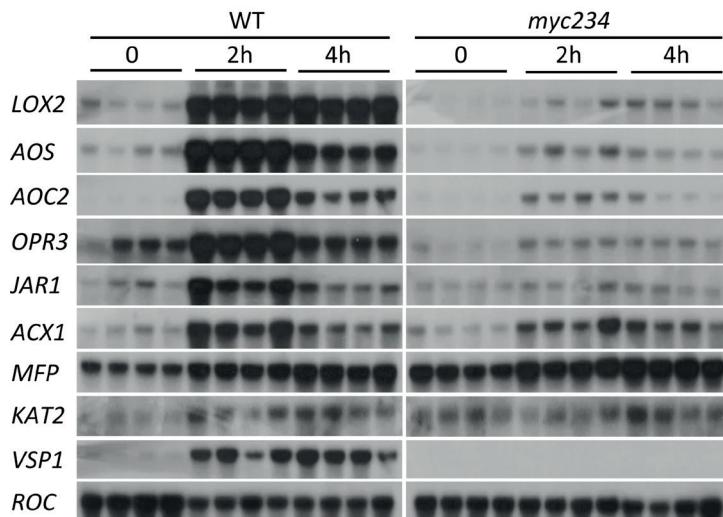
## Results

### Expression of JAs biosynthesis genes requires MYC2, MYC3 and MYC4

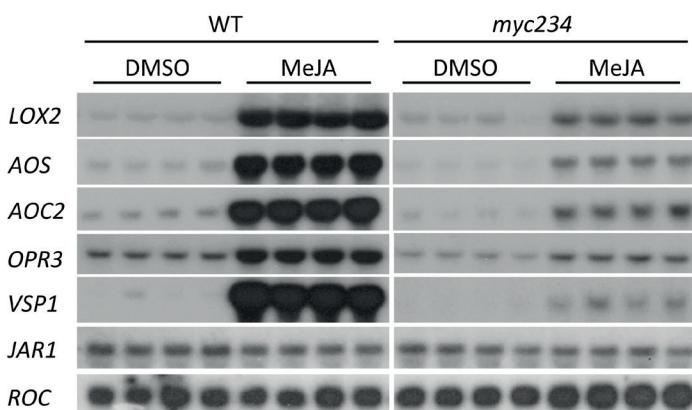
To determine whether MYC proteins transcriptionally control JAs biosynthesis, we examined the expression of the JAs biosynthesis genes in wild type *Arabidopsis* and in *myc234* triple mutants in response to leaf wounding or MeJA treatment. RNA gel blots revealed that expression of the *LOX2*, *AOS*, *AOC2*, *OPR3*, *JAR1*, *ACX1*, *MFP* and *KAT2* genes, encoding enzymes involved in the synthesis of bio-active JA-Ile, was strongly induced in three-week-old wild type *Arabidopsis* after 2 and 4 hours treatment by leaf wounding. In *myc234* triple mutants, wound-induced expression of most tested genes was severely attenuated, whereas the expression of *ACX1*, *MFP* and *KAT2* showed no difference compared with wild type (Fig. 1). In addition to JAs biosynthesis genes, wound-induced expression of the defense gene, *VSP1*, a JAs-responsive gene controlled by MYCs, was undetectable in *myc234* mutants. This demonstrates that wound-induced expression of *LOX2*, *AOS*, *AOC2*, *OPR3* and *JAR1* genes is largely MYC dependent in young *Arabidopsis* leaves. Figure 2 shows that MeJA induced *LOX2*, *AOS*, *AOC2* and *OPR3* gene expression in 3 weeks old wild type consistent with their expression in response to leaf wounding, except for the *JAR1* gene which was not upregulated by MeJA treatment although it was wound-responsive. In the *myc234* triple mutant seedlings the MeJA-responsive expression of the JAs biosynthesis genes was strongly reduced.

### MYC proteins bind to a G-box sequence in the *AOC2* promoter *in vitro*

Several reports have confirmed that MYC proteins binds to the G-box and G-box related hexameric sequences (Kazan and Manners, 2013). The *AOC2* promoter contains one G-box (CACGTG) and one G-box-like sequence (CACGTT) (Fig. 3a). As a first

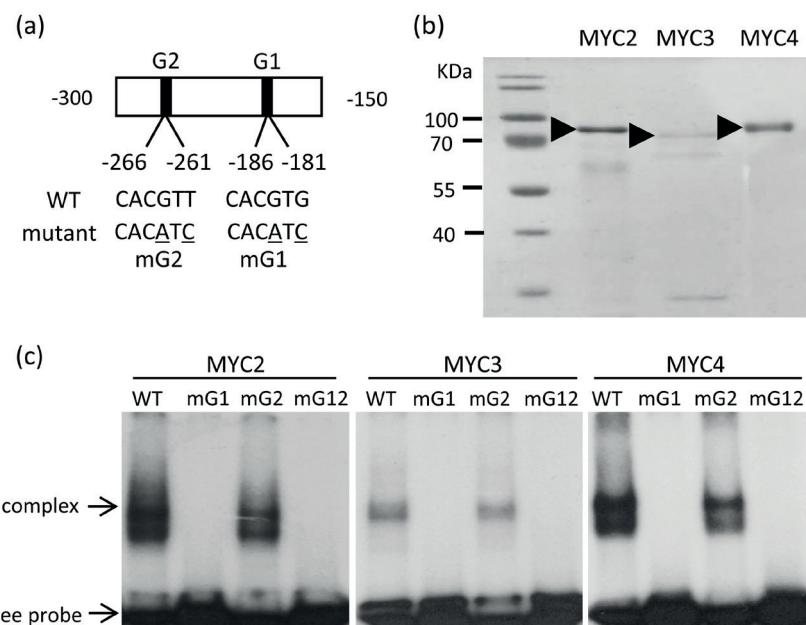


**Figure 1.** MYCs control the expression of JAs biosynthesis genes in response to wounding. Total RNA was isolated from 4 replicate samples of three-week-old wild-type and *myc* triple mutant *Arabidopsis* leaves 2 or 4 hours after wounding. The RNA gel blot was hybridized with the indicated probes. The *ROC* (*Rotamase cyp*) probe was used to verify RNA loading. The two panels for each probe were on the same blot and exposed to film for the same time allowing direct comparison of expression levels.



**Figure 2.** MYCs control the JAs-responsive expression of JAs biosynthesis genes. Total RNA was isolated from 4 replicate samples of two-week-old wild-type and *myc* triple mutant *Arabidopsis* seedlings treated with 50  $\mu$ M MeJA or the solvent DMSO (0.05% final concentration) for 4 hours. The RNA gel blot was hybridized with the indicated probes. The *ROC* (*Rotamase cyp*) probe was used to verify RNA loading. The two panels for each probe were on the same blot and exposed to film for the same time allowing direct comparison of expression levels.

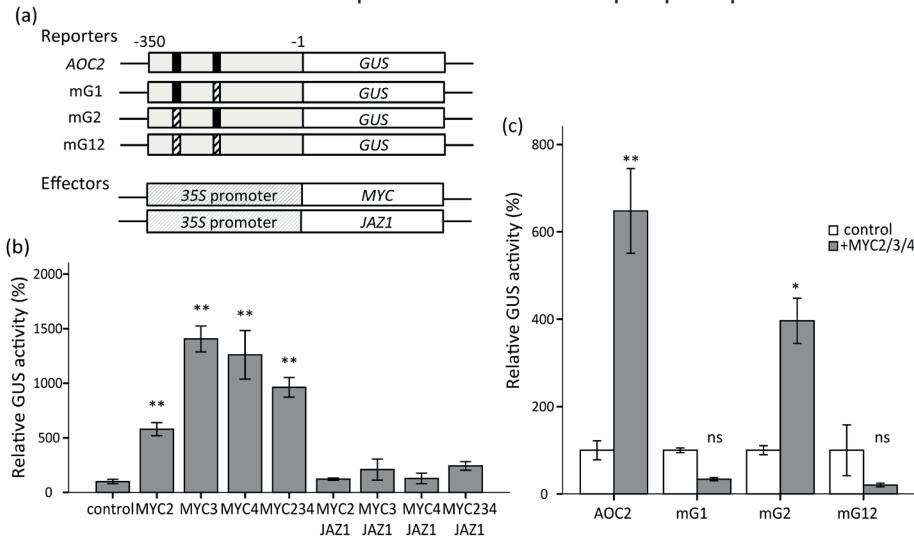
experiment to test whether the expression of the *AOC2* gene is regulated by MYC2, MYC3 and MYC4, binding of recombinant MYC proteins to the *AOC2* promoter *in vitro* was tested. Mutations were introduced in both sequences (Fig. 3a) and single and double mutated versions of the *AOC2* promoter were generated. Recombinant MYC2, MYC3 and MYC4 proteins with a C-terminal His-tag were produced in *Escherichia coli* and Coomassie brilliant blue staining showed the presence of bands of the expected sizes, as well as smaller bands presumably representing degradation products (Fig. 3b). Electrophoretic mobility shift assays (EMSA) with the recombinant MYC proteins showed that MYC proteins were able to interact *in vitro* with the wild-type *AOC2* promoter (Fig. 3c). In EMSAs with mutant versions, mutation of G-box G1 or of both G-boxes abolished *in vitro* binding of MYC proteins, whereas mutation of G-box G2 had no effect on the binding, indicating that G1 was the only binding site within the tested promoter fragment (Fig. 3c).



**Figure 3.** MYC proteins bind to one G-box sequence in the *AOC2* promoter *in vitro*. **(a)** Schematic diagram of wild-type and mutated versions of G-boxes in the *AOC2* promoter. Underlined nucleotides indicate point mutations in the G-boxes. Numbers indicate positions relative to the ATG start codon. **(b)** Analysis of recombinant MYC proteins. MYC2, MYC3 and MYC4 were purified by His tag affinity chromatography. Sizes of relevant marker (M) bands are indicated in kD. The arrowheads indicate the full-length proteins. **(c)** Electrophoretic mobility shift assays. Radio-labeled wild-type and mutated fragments of *AOC2* promoter as indicated in (a) were used as probes in *in vitro* binding.

## MYC proteins trans-activate the *AOC2* promoter via the G-box sequence *in vivo*

Next we performed transient activation assays to determine whether MYC proteins activate the promoter of the *AOC2* gene via direct binding to the G-box sequence *in vivo*. Arabidopsis protoplasts were co-transformed with a plasmid carrying the  $\beta$ -glucuronidase (*GUS*) reporter gene fused to 350 bp of the *AOC2* promoter and effector plasmids carrying the *MYC* and *JAZ1* open reading frames (ORF) under the control of the Cauliflower Mosaic Virus (CaMV) 35S promoter (Fig. 4a). *MYC2*, *MYC3*, *MYC4* and the combination of *MYCs* strongly trans-activated the *AOC2* promoter in Arabidopsis protoplasts and *JAZ1* had a significant negative effect on the activity of *MYC* proteins (Fig. 4b). In addition, plasmids carrying the *GUS* reporter gene fused with mutated versions of the *AOC2* promoter were co-transformed with the combination of the three *MYC* effector plasmids into Arabidopsis protoplasts. As shown in

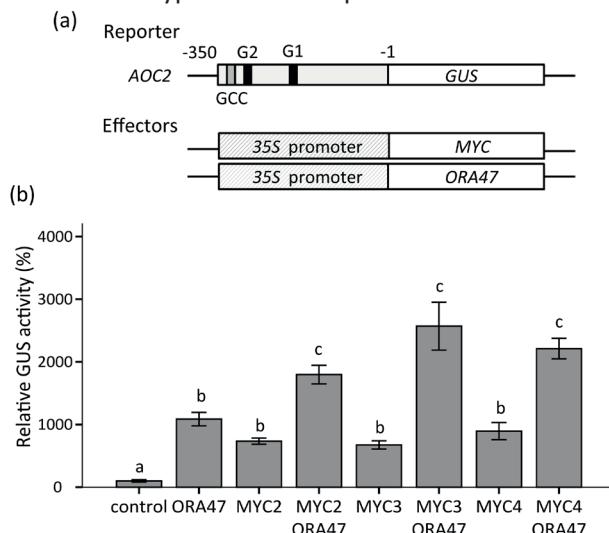


**Figure 4. MYC proteins trans-activate the *AOC2* promoter via the G-box sequence *in vivo*.** (a) Schematic representation of the constructs used for transient expression assays. Numbers indicate positions relative to the ATG start codon. (b) MYCs trans-activate the *AOC2* promoter in Arabidopsis protoplasts. Arabidopsis cell suspension protoplasts were co-transformed with plasmids carrying *pAOC2::GUS* (2  $\mu$ g) and effector plasmids containing *MYCs* (2  $\mu$ g) alone or in combination with *JAZ1* (2  $\mu$ g), as indicated. (c) One G-box motif in *AOC2* promoter is required for the activation by MYCs. Arabidopsis cell suspension protoplasts were co-transformed with plasmids carrying wild-type or mutated versions of *pAOC2::GUS* and effector plasmids with or without *MYCs*. Protein concentrations were used to correct for differences in protein extraction efficiencies. Asterisks show statistically significant differences according to a post hoc Tukey HSD test (ANOVA). \*\* $P<0.01$ , \* $P<0.05$  and ns (not significant different). Values represent means  $\pm$ SE of triplicate experiments and are expressed relative to the vector control.

figure 4c, the wild-type promoter and the mG2 mutant were significantly trans-activated by MYCs, whereas the mG1 or the mG12 mutant promoters did not respond to MYCs. Thus there is a perfect correlation between the effects of mutations on *in vitro* binding of MYCs to the G-box sequences and the ability of MYCs to trans-activate promoter derivatives *in vivo*. These results indicate that MYC proteins trans-activated the *AOC2* promoter *in vivo* via direct binding to the G-box sequence G1.

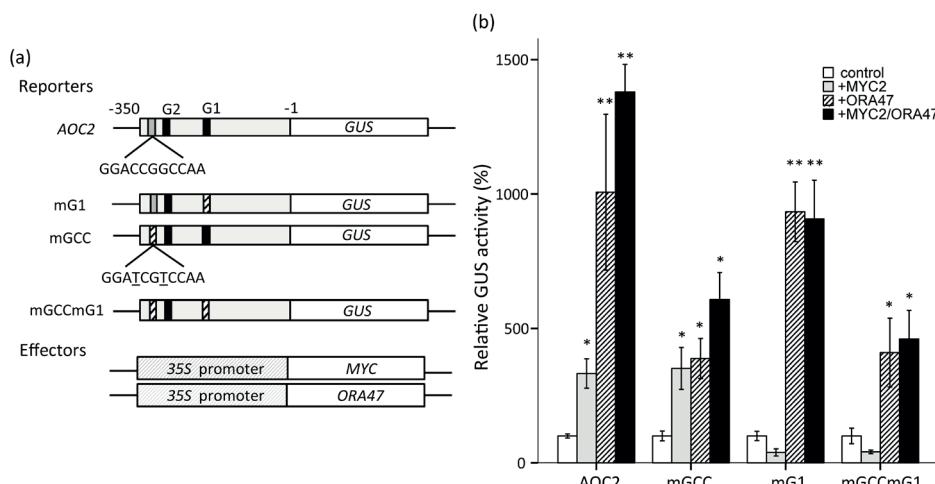
### MYCs and ORA47 trans-activate the promoter of JAs biosynthesis genes additively

Our previous studies indicated that the AP2/ERF-domain transcription factor ORA47 activated the *AOC2* promoter (-350 to -1) via binding to a GCC-box (ACCGGCC) (Fig. 5a; Zarei, 2007). Therefore we examined the transactivation effects of MYCs and ORA47 individually or in combination. The 350 bp wild-type *AOC2* promoter was activated 11-fold by ORA47, 7.3-fold by MYC2, 6.7-fold by MYC3, 9-fold by MYC4 and 18-, 25- and 22-fold by simultaneous expression of ORA47 and MYC effectors (Fig. 5b), indicating that the two types of transcription factors act additively.



**Figure 5.** The *AOC2* promoter is additively trans-activated by MYCs and ORA47. (a) Schematic representation of the constructs used for transient expression assays. Numbers indicate positions relative to the ATG start codon. (b) MYCs and ORA47 trans-activate the *AOC2* promoter additively. *Arabidopsis* cell suspension protoplasts were co-transformed with plasmids carrying *pAOC2::GUS* (2  $\mu$ g) and effector plasmids containing *ORA47* (2  $\mu$ g) and/or *MYCs* (2  $\mu$ g), 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  $\pm$ SE of triplicate experiments and are expressed relative to the vector control.

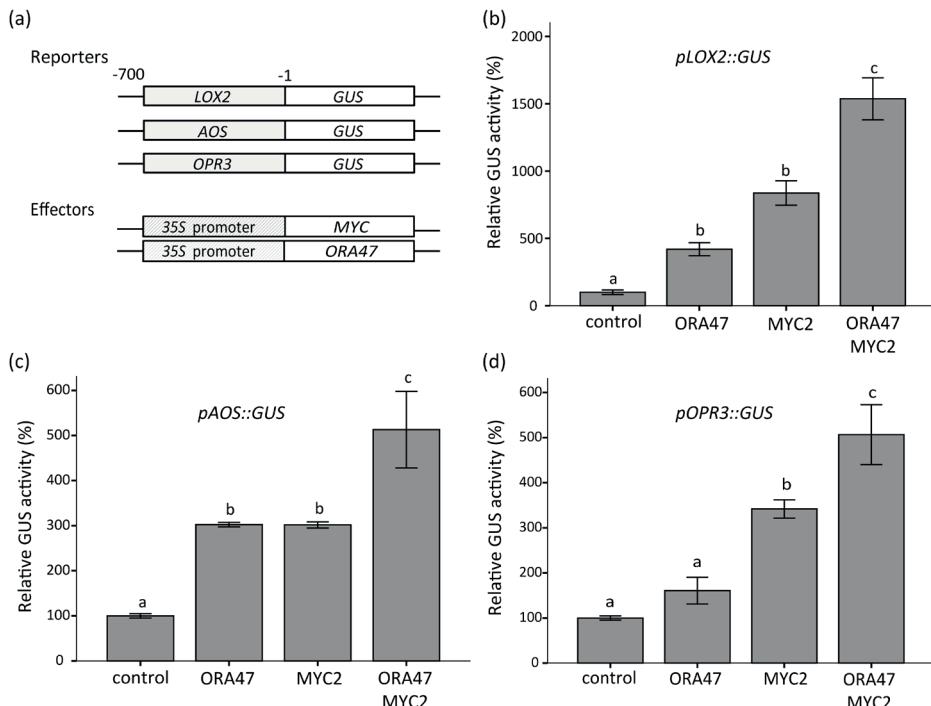
Figure 6 shows that mutation of the GCC-box (mGCC) had a strong negative effect on the ORA47-induced activation of the *AOC2* promoter, but had no effect on the MYC2-induced activation. Co-expression of MYC2 and ORA47 additively activated the mGCC promoter but to a lower level compared with the wild-type promoter. Conversely, MYC2 was ineffective in activating the *AOC2* promoter in which the G-box1 was mutated (mG1), alone or in combination with ORA47, whereas ORA47 activated the mG1 promoter to the same level as the wild-type promoter. When both the GCC-box and G-box1 were mutated, there was a much lower activation level by ORA47 or ORA47 and MYC2 combined and no activation by MYC2 alone.



**Figure 6.** Effects of GCC-box and G-box mutations on trans-activation of the *AOC2* promoter by MYC2 and/or ORA47. **(a)** Schematic representation of the constructs used for transient expression assays. Underlined nucleotides indicate point mutations in the GCC-box. Numbers indicate positions relative to the ATG start codon. **(b)** Trans-activation of *pAOC2::GUS* and its derivatives, which carried mutations in the GCC-box (mGCC), G-box1 (mG1) or both (mGCCmG1), by MYC2 and/or ORA47. *Arabidopsis* cell suspension protoplasts were co-transformed with plasmids carrying *pAOC2::GUS* (2  $\mu$ g) and effector plasmids containing *ORA47* (2  $\mu$ g) and/or *MYC2* (2  $\mu$ g), as indicated. Protein concentrations were used to correct for differences in protein extraction efficiencies. Asterisks show statistically significant differences according to a post hoc Tukey HSD test (ANOVA, \*\*P<0.01, \*P<0.05). Values represent means  $\pm$ SE of triplicate experiments and are expressed relative to the vector control.

Next we extended our analysis to the promoters of other JAs biosynthesis genes whose JAs-responsive expression was MYC-dependent. As shown in figure 7a, putative MYC2 binding sites, G-box or G-box-like sequences, were identified in the promoters of *LOX2*, *AOS* and *OPR3*. Plasmids carrying the *GUS* reporter gene fused

with 700 bp promoter fragments of *LOX2*, *AOS* and *OPR3* were co-transformed with MYC2 and/or ORA47 effector plasmids in *Arabidopsis* protoplasts. These transient transactivation assays gave the results that the *LOX2*, *AOS* and *OPR3* promoters were activated 8.4-, 3- and 3.4-fold respectively by overexpression of MYC2, 4.2-, 3- and 1.6-fold by ORA47 and 15.4-, 5.2- and 5.1-fold by both effectors (Fig. 7b, c, d), indicating that MYC2 and ORA47 act additively on these promoters, as in the case of the *AOC2* promoter.



**Figure 7.** MYCs and ORA47 trans-activate the promoter of JAs biosynthesis genes additively. **(a)** Schematic representation of the constructs used for transient expression assays. Numbers indicate positions relative to the ATG start codon. MYC2 and ORA47 additively trans-activate the **(b)** *LOX2*, **(c)** *AOS* and **(d)** *OPR3* promoters. *Arabidopsis* cell suspension protoplasts were co-transformed with plasmids carrying *pAOC2::GUS* (2  $\mu$ g) and effector plasmids containing *ORA47* (2  $\mu$ g) and/or *MYC2* (2  $\mu$ g), 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  $\pm$ SE of triplicate experiments and are expressed relative to the vector control.

## Discussion

Jasmonates regulate specific plant developmental processes as well as diverse re-

sponses to external biotic or abiotic stress stimuli. The biosynthesis of JAs is controlled by a positive feedback loop, however little is known about the regulatory mechanisms controlling JAs biosynthesis. The results described here showed that loss-of-function of three bHLH MYC transcription factors resulted in dramatically reduced expression of genes encoding JAs biosynthesis enzymes, including *LOX2*, *AOS*, *AOC2*, *OPR3* and *JAR1*, in response to wounding (Fig. 1). In the *myc234* triple mutant gene expression was also decreased in response to MeJA treatment, except for *JAR1* (Fig. 2), suggesting that *JAR1* expression is MYC-dependent but not induced by MeJA. This is consistent with the results that *JAR1* transcript levels increased dramatically in wounded tissue after about 1 h (Suza and Staswick, 2008) but showed no increase up to 24 h after treatment with MeJA (Staswick and Tiryaki, 2004). Additionally, expression of some genes from  $\beta$ -oxidation steps, including *ACX1*, *MFP* and *KAT2*, were tested as well. Previous studies reported that mechanical damage triggered the expression of the *ACX1* and *KAT2* genes and that the *acx1* or *ped1/kat2* mutation resulted in lower accumulation of JAs in wounded tissues (Castillo et al., 2004; Afitlhile et al., 2005; Schilmiller et al., 2007). Only *ACX1* and *KAT5* transcripts accumulated in a dose-dependent manner by treatment with JA (Castillo et al., 2004). Wounding of the *aim1/mfp2* mutant, disrupted in fatty acid  $\beta$ -oxidation, resulted in a reduced JAs level and in decreased expression of JAs-responsive genes compared to wild-type Arabidopsis (Delker et al., 2007). In wild type Arabidopsis, the expression of *ACX1*, *MFP* and *KAT2* was induced by wounding. However in the *myc234* triple mutant the wound-induced expression of *ACX1* was slightly attenuated, *MFP* showed no differences and *KAT2* did not exhibit obvious induction, which indicates that MYCs control the  $\beta$ -oxidation genes to a lesser degree.

Further evidence obtained by EMSAs demonstrated that MYC transcription factors directly bound to one of two G-box sequences in the promoter of the JAs biosynthesis gene *AOC2*. MYC2, MYC3 and MYC4 show the strongest binding affinity for the G-box (CACGTG) palindromic hexamer, and display slightly different affinities for certain G-box variants (Fernández-Calvo et al., 2011). In the promoter of the *AOC2* gene, one G-box and one G-box-like (CACGTT) sequence were identified. The DNA binding of MYC proteins was abolished by mutating the G-box but not by mutating the G-box-like sequence (Fig. 3). Mutation of the G-box also abolished the activation of the *AOC2* promoter by MYCs in Arabidopsis protoplasts, whereas mutation of the G-box-like sequence had a minor effect on trans-activation (Fig. 4). These results indicate that the G-box is the major functional binding site for MYCs in

*vivo* and that the G-box-like sequence has a minor quantitative contribution to the activation of *AOC2* promoter activity by MYCs.

Our previous studies revealed that the AP2/ERF-domain transcription factor ORA47 appears to act as the regulator of the positive feedback loop in JAs biosynthesis. Overexpression of the *ORA47* gene in *Arabidopsis* resulted in induced expression of multiple JAs biosynthesis genes and in elevated endogenous JAs levels (Kurshid, 2012; Pré, 2006). The *AOC2* gene contains both canonical G-box and GCC-box sequences in its proximal promoter region, and its expression was trans-activated by overexpression of MYCs or ORA47 in *Arabidopsis* protoplasts and the effects of these effectors were additive (Fig. 5). Mutation of the G-box and/or GCC-box dramatically reduced the activation level of the *AOC2* promoter by MYC2 and/or ORA47 (Fig. 6). MYC2 and ORA47 additively trans-activated the promoters of the JAs biosynthesis genes *LOX2*, *AOS* and *OPR3* (Fig. 7). We conclude that MYCs and ORA47 act as key regulators of JAs biosynthesis genes via binding to their cognate *cis*-elements in the promoters.

In *Arabidopsis*, MYCs differentially modulate JAs-dependent gene expression through direct binding to their target promoters and through physical interaction with other transcription factors. In the regulation of glucosinolate (GS) biosynthesis, MYC2 was shown to bind directly to the promoters of more than half of the GS biosynthesis genes *in vivo*, and MYC2, MYC3 and MYC4 interact directly with GS-related MYBs, which positively co-regulate the expression of GS biosynthesis genes (Schweizer et al., 2013). DELLAAs, the GAs (Gibberellic acids) signaling repressors, directly interact with MYC2 in regulating sesquiterpene synthase gene expression (Hong et al., 2012) and interfere with the MYC2-JAZ1 interaction via competitive binding to JAZs (Hou et al., 2010). More recently, it has been reported that MYC2 physically interacts with ETHYLENE INSENSITIVE3 (EIN3) and attenuated the transcriptional activity of EIN3 during apical hook development (Song et al., 2014; Zhang et al., 2014). We did not find interaction between MYC2 and ORA47 using the yeast two-hybrid assay (data not shown), but it is possible that MYC2-ORA47 interaction is facilitated by other proteins. For instance, MED25 physically associates with MYC2 and exerts a positive effect on the MYC2-regulated gene transcription (Chen et al., 2012).

The transcriptional regulation of JAs biosynthesis in *Arabidopsis* shows similarities to the regulation of nicotine biosynthesis in *Nicotiana* species. MYC homologs of *N. benthamiana* were shown to function as positive regulators of nicotine

biosynthesis via binding G-box elements in the *PMT* promoter (Todd et al., 2010). In *N. tabacum*, the *NIC2*-locus AP2/ERF-domain transcription factor ERF189 and NtMYC2 additively regulated JAs-induced nicotine biosynthesis and NtMYC2 was required for the expression of *ERF189* (Shoji et al., 2010; Shoji and Hashimoto, 2011). In *Catharanthus roseus*, CrMYC2 directly activated JAs-responsive expression of the *ORCA3* gene, encoding an AP2/ERF transcription factor closely related to ORA47 which acts in the regulation of alkaloid biosynthesis genes (Zhang et al., 2011). As described in the next chapter, the JAs-responsive expression of *ORA47* is controlled by MYC transcription factors in Arabidopsis.

Based on the results in this study and on studies by others, we propose a model for JAs biosynthesis gene expression mediated by bHLH and AP2/ERF-domain transcription factors in a cooperative manner in Arabidopsis (Fig. 8). In the absence of stimulus, the expression of JAs biosynthesis genes is suppressed through the action of JAZ repressors, which recruit the co-repressor TOPLESS (TPL) directly or via the adaptor protein NINJA (NOVEL INTERACTOR OF JAZ) to repress the activity of MYC proteins. On the other hand, our previous studies indicate that the activity of ORA47 is also regulated by members of the JAZ family presumably via an adaptor protein. In response to stress, bioactive JA-Ile is rapidly synthesized and perceived by its receptor COI1, leading to the degradation of JAZ repressors. Subsequently MYC proteins and ORA47 are released from repression to activate the expression of JAs biosynthesis genes additively through directly binding to the G-box and GCC-box sequences present in the promoters.

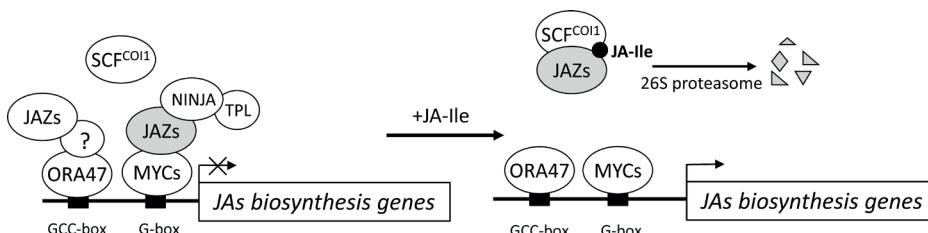


Figure 8. Model of the regulation of JAs biosynthesis genes. In the absence of stimulus, the activity of ORA47 and MYC proteins is repressed by JAZ repressors, which recruit the co-repressor TOPLESS (TPL) directly or through the adaptor NOVEL INTERACTOR OF JAZ (NINJA). Perception of bioactive JA-Ile by its receptor COI1 leads to degradation of JAZ repressors by the 26S proteasome, causing the liberation of transcriptional activators. Subsequently, MYCs and ORA47 additively activate the expression of JAs biosynthesis genes through the G-box and GCC-box sequences in their promoters.

## Materials and Methods

### Plant material, growth conditions and chemical treatments

*Arabidopsis thaliana* wild-type and *myc234* triple mutant plants are in the genetic background of ecotype Columbia (Col 0) (Fernández-Calvo et al., 2011). Following stratification for 3 days at 4°C, surface-sterilized seeds were first incubated at 21°C in a growth chamber (16 h light/8 h dark, 2500 lux) for 10 days on plates containing MA medium with 0.6% agar (Masson and Paszkowski, 1992). For MeJA treatments, 20 to 25 seedlings were transferred to 50 ml polypropylene tubes (Sarstedt, Nümbrecht, Germany) containing 10 ml liquid MA medium and incubated on a shaker at 120 rpm for 4 additional days before treatment. Seedlings were treated for different time periods with 50 µM MeJA (Sigma-Aldrich, St. Louis, MO) dissolved in dimethylsulfoxide (DMSO; 0.05% final concentration). As controls, seedlings were treated with 0.05% DMSO. For the wounding assay, plants were grown for 3 weeks under 16 h light/8 h dark conditions on MA medium with 0.6% agar, and then six rosette leaves per plant were wounded by crushing across the midrib with a hemostat. Sixty damaged leaves from ten damaged plants at the indicated time points after wounding and sixty undamaged leaves from ten undamaged plants were harvested for each RNA sample.

### RNA extraction and Northern blot analysis

Total RNA was extracted from frozen ground 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 <sup>32</sup>P-labeled by random priming with the DecaLable DNA labeling kit (Thermo Fisher Scientific). (Pre-) hybridization of blots, hybridization of probes and subsequent washings were performed as described (Memelink et al., 1994) with minor modifications. Blots were exposed on X-ray films (Fuji, Tokyo, Japan). For probe preparation, DNA fragments were PCR amplified using the following primer sets: 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 GCT TCT ATT TCA ACC CC-3' and 5'-CTA AAA GCT AGC

TTT CCT TAA CG-3' for *Allene oxide synthase* (*AOS*, At5g42650); 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'-ATG TTG GAG AAG GTT GAA AC-3' and 5'-TCA AAA CGC TGT GCT GAA G-3' for *Jasmonate amido synthetase* (*JAR1*, At2g46370); 5'-CGG GAA GGA TCG TGA TGG A-3' and 5'-CCA ACC TTC TCG ATG GCC T-3' for *Rotamase cyp* (*ROC*, At4g38740).

### **Isolation of recombinant MYC proteins**

Plasmid pASK-IBA45 (IBA Biotechnology, Gottingen, Germany) containing MYC2 was described before (Montiel et al., 2011). MYC3 (At5g46760) was amplified with primer set 5'-CGA GCT CGA TGA ACG GCA CAA CAT CAT C-3' and 5'-CCC ATG GAT TAG TTT TCT CC GAC TTT CGT C-3', digested with SacI/Ncol and cloned in pASK-IBA45plus. MYC4 (At4g17880) was amplified with the primer set 5'-GGA ATT CGA TGT CTC CGA CGA ATG TTC AAG-3' and 5'-CCC ATG GAT GGA CAT TCT CCA ACT TTC TC-3', digested with EcoRI/Ncol and cloned in pASK-IBA45plus. Double Strep/His-tagged MYC proteins were expressed in *E. coli* strain BL21 (DE3) pLysS and purified by Ni-NTA agarose (Qiagen) chromatography.

### **Electrophoretic mobility shift assays**

The wild-type and mutated fragments of the *AOC2* promoter were amplified from the construct pAOC2-GusSH with the primers 5'-GGA TCC CAA CTT AAA TCC AAG ACC-3' and 5'-GTC GAC TGG ATG AGT GAT GAA TGG-3' and cloned in pJET1.2 (Thermo Fisher Scientific). Fragments were isolated with BamHI/Sall and labelled by filling in the overhangs with the Klenow fragment of DNA polymerase I and [ $\alpha$ -<sup>32</sup>P] dCTP. DNA binding reactions contained 0.1 ng of end-labelled DNA fragment, 500 ng of poly(dAdT)-poly(dAdT), binding buffer (25 mM HEPES-KOH pH 7.2, 100 mM KCl, 0.1 mM EDTA, 10% v/v glycerol) and protein extract in a 10  $\mu$ l volume, and were incubated for 30 min at room temperature before loading on 5% (w/v) acrylamide/bisacrylamide (37:1)-0.5 $\times$ Tris-Borate-EDTA gels under tension. After electrophoresis at 100 V for 1 hour, gels were dried on Whatman DE81 paper and exposed to Fuji X-ray films.

### **Arabidopsis protoplast transient expression assays**

A 350 bp *AOC2* promoter fragment was PCR-amplified from *Arabidopsis* genomic DNA with the primer set 5'-TCT AGA GAT TCA TTA CAT TTA GAA G -3' and 5'-GTC

GAC TGA TAA AAA TAA AAT AAA AAG -3', digested with XbaI and Sall and cloned in plasmid pGusSH (Pasquali et al., 1994). Mutations were generated according to the QuickChange Site-Directed Mutagenesis protocol (Stratagene) using the primers 5'-GT AAT TTA CG CAC ATC CTA CTT CAT CAA TC -3' and 5'- GA TTG ATG AAG TAG GAT GTG CG TAA ATT AC-3' for G-box (mG1) and 5'- CAA TGC TTA GAT CAC ATC CCG ACC ATG GAA AC-3' and 5'- GT TTC CAT GGT CGG GAT GTG ATC TAA GCA TTG -3' for G-box-like (mG2). The *MYC2* (*At1g32640*) gene was excised from the *Rap-1* cDNA in pBluescript SK (GenBank acc. No. X99548;(de Pater et al., 1997) with XmaI and cloned in pRT101 (Töpfer et al., 1987). The *MYC3* (*At5g46760*) gene was PCR amplified from a cDNA library using the primer set 5'-CCT CGA GAA TGA ACG GCA CAA CAT CAT C-3' and 5'-CGG ATC CTC AAT AGT TTT CTC CGA CTT TC-3', digested with Xhol/BamHI and cloned in pRT101. The *MYC4* (*At4g17880*) gene was amplified with the primer set 5'-GAT CGA ATT CAT GTC TCC GAC GAA TGT TCA AG-3' and 5'- CAG TGG ATC CTC ATG GAC ATT CTC CAA CTT -3', digested with EcoRI/BamHI and cloned in pRT101. The *ORA47* (*At1g74930*) 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. 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 Sall and cloned in pRT101. 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 *MYCs*, *ORA47* or *JAZ1* fused to the *CaMV 35S* promoter were carried out with a ratio of 2:2:2 ( $\mu$ g *GUS*:*MYCs*:*ORA47* or *GUS*:*MYCs*:*JAZ1*). 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.

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# Chapter 3

## **MYC transcription factors control the jasmonate-responsive expression of the *ORA47* gene encoding a regulator of jasmonate biosynthesis in *Arabidopsis thaliana***

Muhammad Khurshid\*, Kaixuan Zhang\*, Johan Memelink

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

\*These authors contributed equally to this work

## Abstract

Upon herbivore or pathogen attack plants produce the jasmonate hormones (JAs). The bioactive JAs triggers degradation of JAZ repressor proteins thereby activating transcription factors including MYC2, MYC3 and MYC4, which sets in motion defense gene expression programs. JAs signaling also induces all known JAs biosynthesis genes in what is considered a positive feedback loop. Overexpression of the AP2/ERF-domain transcription factor ORA47 leads to elevated expression of all JAs biosynthesis genes and to elevated levels of JAs, indicating that ORA47 controls the positive feedback loop. ORA47 is itself encoded by a JAs-responsive gene. The aim of the work described in this chapter was to identify the promoter element(s) and the transcription factor(s) responsible for JAs-responsive expression of the *ORA47* gene. We explored the hypothesis that *ORA47* is regulated by the functionally redundant JAs-responsive transcription factors MYC2, MYC3 and MYC4. Our results show that the MYC proteins can trans-activate the *ORA47* promoter via binding to one of the three G-box sequences present in the promoter. Triple knockout of the *MYC* genes or overexpression of a stable JAZ1 derivative abolished JAs-responsive *ORA47* expression, demonstrating the crucial role of the MYC-JAZ module in regulation of *ORA47* expression.

## Introduction

Plant fitness and survival is dependent on the ability to mount fast and highly adapted responses to diverse environmental stress conditions including microbial pathogen attack and insect herbivory. Perception of stress signals results in the production of one or more of the secondary signaling molecules jasmonates (JAs), ethylene (ET) and salicylic acid (SA).

JAs are a group of related lipid-derived signaling molecules including the namesake compound jasmonic acid (JA) which are involved in defense against wounding, herbivores and necrotrophic pathogens (Pieterse et al., 2009). In response to damage or pathogen attack JAs are synthesized via conversion of  $\alpha$ -linolenic acid to 12-oxo-phytodienoic acid (OPDA) by the sequential action of the plastid enzymes lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC). OPDA is reduced by OPDA reductase (OPR3), followed by three rounds of  $\beta$ -oxidation to yield JA (Wasternack, 2007). Subsequently JA is conjugated to the amino acid isoleucine by JA amido synthetase (JAR1) yielding the biologically active jasmonoyl-isoleucine (JA-Ile) (Fonseca et al., 2009). This signaling molecule stimulates the interaction between the receptor COI1 (CORONATINE INSENSITIVE1) and members of a family of repressor proteins called JAZ (JASMONATE ZIM-DOMAIN) (Thines et al., 2007; Sheard et al., 2010). COI1 is an F-box protein that forms part of a Skp1-Cul1-F-box protein (SCF) complex with putative E3 ubiquitin ligase activity (Devoto et al., 2002; Xu et al., 2002). Several members of the JAZ family were shown to interact with the JAs-responsive basic Helix-Loop-Helix (bHLH) transcription factor MYC2 (Chini et al., 2007; Chini et al., 2009; Chung and Howe, 2009) and the related proteins MYC3 and MYC4 (Fernández-Calvo et al., 2011; Niu et al., 2011). JAZ1 can repress the activity of MYC2 (Hou et al., 2010) and JAZs can bind to the co-repressors TOPLESS (TPL) and TPL-related proteins either directly (Shyu et al., 2012) or via the adaptor protein NOVEL INTERACTOR OF JAZ (NINJA) (Pauwels et al., 2010). More recently a variety of transcription factors were shown to interact with members of the JAZ family (Pauwels and Goossens, 2011). In response to JA-Ile JAZ proteins are rapidly degraded by the 26S proteasome presumably via SCF<sup>COI1</sup>-mediated ubiquitination (Chini et al., 2007; Thines et al., 2007), which leads to de-repression of interacting transcription factors resulting in expression of the corresponding sets of target genes. Wounding and herbivory activate JAs signaling leading to the expression of a set of defense genes controlled by MYC2, MYC3 and MYC4 (Lorenzo et al., 2004; Fernández-Calvo et al., 2011).

The expression of all JAs biosynthesis genes, including *LOX2*, *AOS*, *AOC* and *OPR3*, is induced by wounding or treatment with exogenous JA or MeJA (Sasaki et al., 2001; Sasaki-Sekimoto et al., 2005; Wasternack, 2007; Pauwels and Goossens, 2008) in what is considered to be a positive feedback loop. Overexpression of the AP2/ERF-domain transcription factor *ORA47* leads to elevated expression levels of all JAs biosynthesis genes (Pré, 2006; Khurshid, 2012), indicating that *ORA47* controls the positive feedback loop. *ORA47* is itself encoded by a JAs-responsive gene (Pauwels and Goossens, 2008) and shows a very fast (30 min) and transient response which is *COI1*-dependent (Wang et al., 2008).

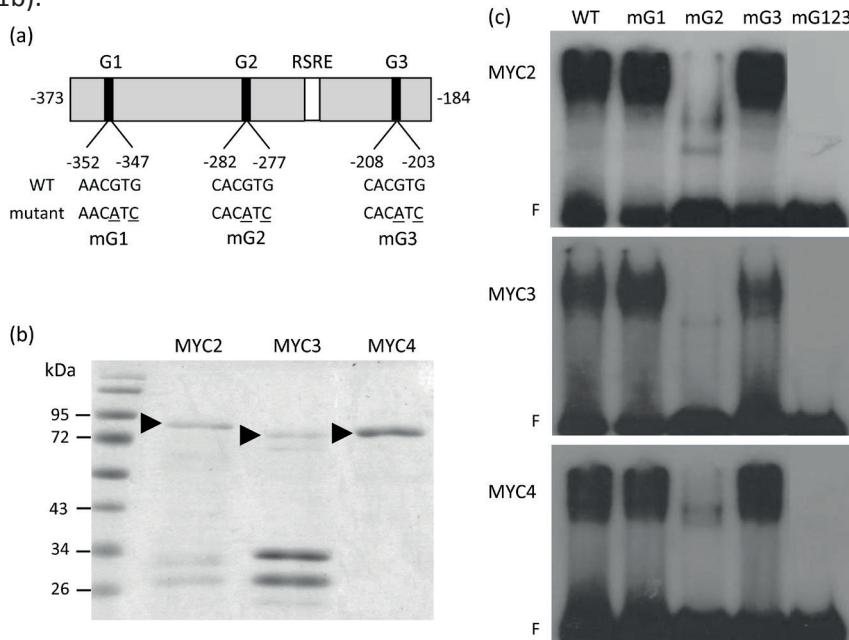
The aim of the work described in this chapter was to identify the transcription factor(s) responsible for JAs-responsive expression of the *ORA47* gene. Based on the facts that *ORA47* is a very fast JAs-responsive gene and that several reports link its expression to *MYC2* expression (Pauwels et al., 2008; Wang et al., 2008), we explored the hypothesis that *ORA47* is regulated by *MYC2*, *MYC3* and *MYC4*. We discovered that the *MYC* proteins can bind to a single G-box in the *ORA47* promoter *in vitro*. Transient assays revealed that this G-box sequence was essential for *MYC*-mediated activation of the *ORA47* promoter and that the other G-box and a G-box-like sequence contributed to a higher expression level of the *ORA47* promoter *in vivo*. Triple knockout of the *MYC* genes or overexpression of a stable *JAZ1* derivative abolished JA-responsive *ORA47* expression, demonstrating the crucial role of the *MYC*-*JAZ* module in the regulation of *ORA47* expression.

## Results

### **MYC proteins bind to the *ORA47* promoter *in vitro***

As a first step to test the hypothesis that JAs-responsive *ORA47* expression is regulated by *MYC2*, *MYC3* and *MYC4*, we tested binding of recombinant *MYC* proteins to the *ORA47* promoter *in vitro*. The 1.4 kb *ORA47* promoter contains two G-boxes (CACGTG) and one G-box-like sequence (AACGTG). These sequences have been described as high affinity binding sites for *MYC* proteins (Chini et al., 2007; Dombrecht et al., 2007; Godoy et al., 2011). Mutations were introduced in each of the three sequences (Fig. 1a) and single and triple mutant versions of the *ORA47* promoter fragment were generated. Analysis of recombinant *MYC2* and *MYC3* proteins produced in *Escherichia coli* and purified by His tag affinity chromatography by denaturing gel electrophoresis and Coomassie Brilliant Blue staining showed the presence of bands of the expected sizes, as well as smaller bands presumably representing deg-

radiation products (Fig. 1b). Further purification of MYC4 by Strep tag affinity chromatography resulted in a preparation showing a single band of the expected size (Fig. 1b).



**Figure 1.** MYC proteins bind to one G-box sequence in the *ORA47* promoter *in vitro*. (a) Overview of wild-type and mutated versions of G-boxes in the *ORA47* promoter. Underlined nucleotides indicate point mutations in the G-boxes. Numbers indicate positions relative to the ATG start codon. The Rapid Stress Response Element (RSRE) located at positions -234 to -229 (Walley et al., 2007) is also indicated. (b) Analysis of recombinant MYC proteins. The proteins were separated by 10% SDS-PAGE and stained with Coomassie Brilliant Blue. Sizes of relevant marker bands are indicated in kD. MYC2 and MYC3 were purified by His tag affinity chromatography while MYC4 was purified by sequential His and Strep tag affinity chromatography. The arrowheads indicate the full-length proteins. (c) Electrophoretic mobility shift assays. Radio-labeled wild-type and mutated versions of the *ORA47* promoter as indicated in (a) were used as probes in *in vitro* binding. F indicates free probes.

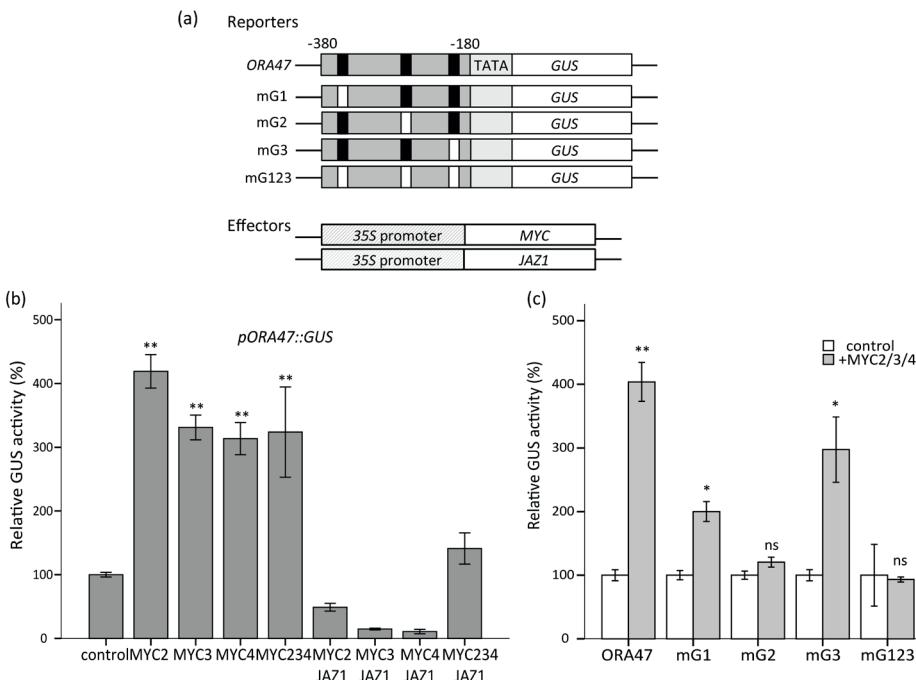
Electrophoretic mobility shift assays (EMSA) with the radio-labeled wild-type and mutant versions of a 200 bp *ORA47* promoter fragment and the MYC protein preparations showed identical patterns of binding (Fig. 1c). In EMSAs with versions with a single mutated G-box, only mutation of sequence G2 had a strong negative effect on binding of the MYC proteins, indicating that it was the main binding site. Whereas the single mG2 mutant showed a low level of residual binding especially with MYC2 and MYC4, the triple mutant version showed no binding at all to the MYC proteins.

### **MYC transcription factors trans-activate the *ORA47* promoter via one G-box sequence *in vivo***

Next, the ability of MYCs to trans-activate the *ORA47* promoter via interaction with the G-boxes was tested. *Arabidopsis* protoplasts were co-transformed with combinations of a plasmid carrying the *GUS* reporter gene controlled by wild-type or mutated version of a 200 bp *ORA47* promoter fragment and an overexpression plasmid that was empty or carried *MYC2*, *MYC3*, *MYC4* or *MYC2/3/4* and/or *JAZ1* controlled by the *CaMV 35S* promoter (Fig. 2a). As shown in Fig. 2b and 2c, MYC proteins were able to trans-activate the wild-type *ORA47* promoter sequence significantly and co-expression of *JAZ1* repressed the activation by MYCs. Reporter constructs carrying the *mG1* and *mG3* mutations were trans-activated 2- and 3-fold by a combination of *MYC2/3/4*. However, the mutants *mG2* and *mG123* did not confer transactivation by *MYC2/3/4*. These results indicated that MYC proteins trans-activated the *ORA47* gene *in vivo* via direct binding to a single G-box sequence in the promoter.

### **JAs-responsive expression of *ORA47* is controlled by *MYC2*, *MYC3* and *MYC4***

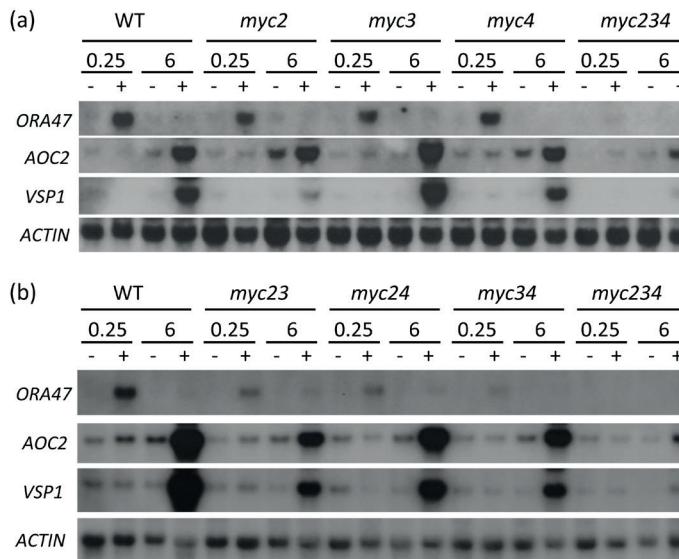
To obtain more evidence for regulation of *ORA47* expression by MYC transcription factors, we determined the expression of *ORA47* and one of its target genes, *AOC2*, in *myc* mutants. The *myc2/jin1-2* mutant has a point mutation introducing a stop codon early in the gene, and therefore lacks most of the *MYC2* protein including the DNA-binding domain (Lorenzo et al., 2004). The *myc3* and *myc4* mutants carry a T-DNA insertion in the genes and do not express the corresponding full-length transcripts (Niu et al., 2011). Plant lines were treated for 15 min and 6 hrs with JA to be able to observe induction of *ORA47* and *AOC2*, respectively. In addition we determined the expression of *VSP1*, which is probably a direct target gene of MYCs. As shown in Figure 3a, mutation of a single *MYC* gene had no effect on JA-responsive gene expression, except for the *myc2* mutant which had a lower level of *VSP1* expression. Simultaneous mutation of two *MYC* genes resulted in a lower expression of JA-responsive genes (Fig. 3b). The observed effect was stronger for *ORA47* than for its target gene *AOC2*. Simultaneous mutation of all three *MYC* genes strongly reduced JA-responsive gene expression (Figs. 3a and b). However still some induction of *ORA47* (clearly visible in Fig. 3a) and *AOC2* and *VSP1* occurred, indicating that although the three MYC proteins are the main regulators there must be additional transcription factors controlling the residual JAs-responsive expression.



**Figure 2. (a)** Schematic representation of the constructs used for transient expression assays. The wild-type and mutated *ORA47* promoters were fused to a minimal TATA box from the CaMV 35S promoter and the *GUS* gene. Numbers indicate positions relative to the ATG start codon. **(b)** MYCs trans-activate the *ORA47* promoter *in vivo*. Arabidopsis cell suspension protoplasts were co-transformed with plasmids carrying *pORA47::GUS* (2 µg) and effector plasmids containing *MYCs* (2 µg) alone or in combination with *JAZ1* (2 µg), as indicated. **(c)** MYCs trans-activate *ORA47* promoter via binding to one G-box site. Arabidopsis cell suspension protoplasts were co-transformed with plasmids carrying wild-type or mutated versions of *pORA47::GUS* and effector plasmids with or without *MYCs*. Protein concentrations were used to correct for differences in protein extraction efficiencies. Asterisks show statistically significant differences according to a post hoc Tukey HSD test (ANOVA). \*\*P<0.01, \*P<0.05 and ns (not significantly different). Values represent means ± SE of triplicate experiments and are expressed relative to the vector control.

## Overexpression of *JAZ1ΔC* represses JAs-responsive expression of *ORA47*

If MYC transcription factors are controlling JAs-responsive expression of *ORA47*, the current widely accepted model of JAs signaling predicts that *ORA47* expression should also be regulated by JAZ proteins. To test whether this is correct, we made transgenic plants overexpressing *JAZ1* or *JAZ3* variants lacking the C-terminal part which contains the conserved Jas domain. The Jas domain acts as a degron by interaction with the SCF<sup>CO1</sup> complex in the presence of JA-Ile (Thines et al., 2007). Variants



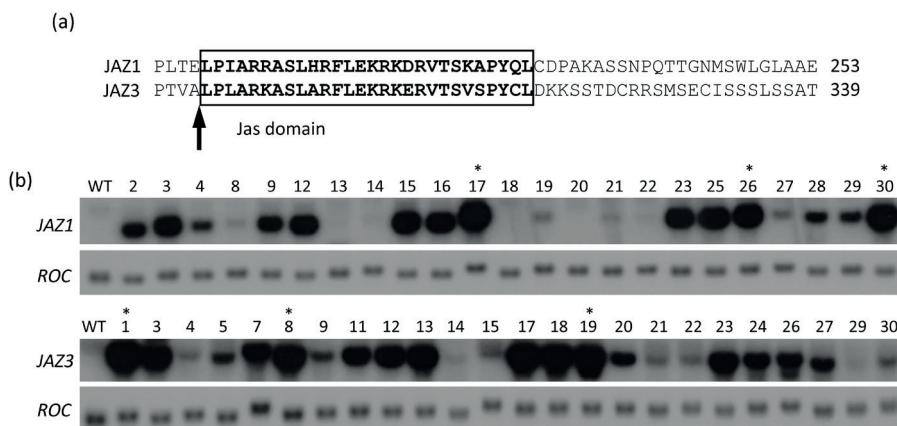
**Figure 3.** JA-responsive expression of *ORA47* is controlled by MYC2, MYC3 and MYC4. RNA was extracted from 2-week-old wildtype or single, double or triple mutants. Seedlings were treated with 50  $\mu$ M JA (+) or the solvent DMSO (-, 0.1% v/v final concentration) for the number of hours indicated. The RNA gel blot was hybridized with the indicated probes. The *ACTIN* probe was used to verify RNA loading.

of JAZ1 (Thines et al., 2007) and JAZ3 (Chini et al., 2007) lacking the Jas domain are stable and repress JAs responses. Based on expression analysis (Fig. 4), we selected three lines for each JAZ $\Delta$ C with the highest expression levels for further analysis.

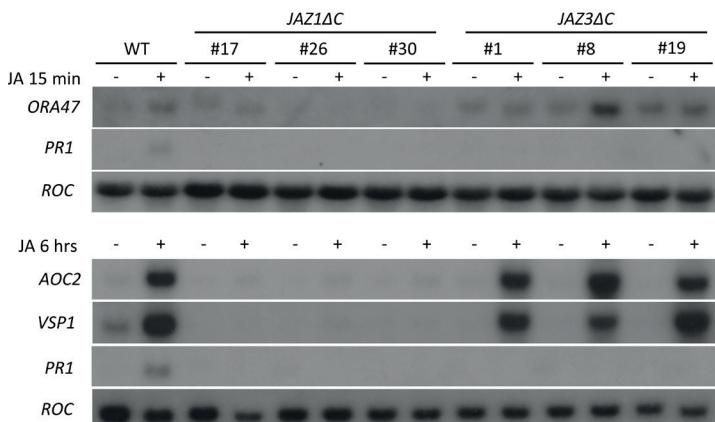
Figure 5 shows that in the JAZ1 $\Delta$ C lines the JA-responsive expression of *ORA47* and its target gene *AOC2* was strongly reduced. Also the expression of the putative MYC target gene *VSP1* was strongly reduced. In the JAZ3 $\Delta$ C lines JA-responsive expression of *ORA47*, *AOC2* and *VSP1* was not different from wild-type. As shown in Figure 4 the JAZ3 $\Delta$ C gene was expressed in these lines at a high level similar to the JAZ1 $\Delta$ C expression level. JAZ3 $\Delta$ C corresponds to the mutant *jai3-1* described in Chini et al. (2007). Consistent with our results in that report *VSP1* did not emerge as a repressed gene in a micro-array analysis of the *jaz3/jai3-1* mutant line. To get an indication that JAZ3 $\Delta$ C is functional, we determined the expression of a *PR1* gene (*At2g14610*) which was among the strongest repressed genes in the micro-array analysis. As shown in Figure 5 the relatively weak induction of the *PR1* gene in response to JA was completely repressed in all JAZ $\Delta$ C lines, demonstrating that JAZ3 $\Delta$ C is functional.

In conclusion, consistent with the widely accepted MYC-JAZ model, JAs-re-

sponsive *ORA47* expression is positively regulated by MYC transcription factors and repressed by a stable variant of JAZ1.



**Figure 4.** Selection of *JAZ1ΔC* and *JAZ3ΔC* overexpression lines. (a) Amino acid sequences of the C-terminal ends of JAZ1 and JAZ3. The conserved Jas domain is shown in a box. The arrow indicates the C-terminal end of the JAZΔC derivatives. (b) Equal amounts of RNA from 2-week-old seedlings of independent *JAZΔC* overexpression T2 lines were hybridized with the indicated *JAZ* probes. The lines with the highest expression indicated with asterisks were selected for further analysis. The *ROC* (*Rotamase* *cyp*) probe was used to verify RNA loading.



**Figure 5.** Overexpression of *JAZ1ΔC* represses JA-responsive expression of *ORA47* and its target gene *AOC2*. Two-week-old wildtype seedlings or seedlings from the indicated *JAZΔC* overexpression lines were treated for the indicated times with 50  $\mu$ M JA (+) or the solvent DMSO (-, 0.1% v/v final concentration). The RNA gel blots were hybridized with the indicated probes. The *ROC* (*Rotamase* *cyp*) probe was used to verify RNA loading.

## Discussion

JAs are major signaling molecules for plant defense against wounding, necrotrophic pathogens and insect herbivores. The AP2/ERF-domain transcription factor *ORA47* acts as a key regulator in the JAs positive feedback loop. *ORA47* is itself encoded by a JAs-responsive gene, however knowledge about the molecular details of control of this JAs responsiveness is limited. In this study, we have shown that the JAs-responsive expression of *ORA47* is controlled by the central MYC-JAZ module as depicted in figure 6. The genetic evidence shows that functional MYC2, MYC3, MYC4 and JAZ1 proteins are required for JAs-responsive expression of the *ORA47* gene. Binding of the MYC proteins to a G-box in the *ORA47* promoter and their ability to trans-activate the promoter in protoplasts indicated that *ORA47* is a direct target gene of the MYCs. In chapter 2, we found that MYC2, MYC3 and MYC4 regulated the expression of JAs biosynthesis genes through directly binding to their promoters. The JAs-responsive induction of *ORA47* gene is transient and occurs much earlier than that of JAs biosynthesis genes. Therefore, in response to external attack, MYC proteins quickly up-regulate *ORA47* gene and subsequently activate JAs biosynthesis genes additively with *ORA47* in the positive feedback loop controlling JAs biosynthesis (Fig. 6).

The *ORA47* gene was previously identified as a wounding-responsive gene (Walley et al., 2007). These authors reported a Rapid Stress Response Element (RSRE) present in promoters of rapid wound-responsive genes, which also occurs in the *ORA47* promoter between G-boxes G2 and G3 (Fig. 1a). A tetramer of the RSRE conferred a transcriptional response to wounding and a variety of other stress signals, but it was not reported whether it responds to JAs or any other defense hormone, or whether its response depends on COI1 or any other receptor or signalling protein. Based on our results it is unlikely that the RSRE contributes to JAs-responsive expression since this depends on the three redundant MYC transcription factors. These proteins bind mainly to G-box G2 in the *ORA47* promoter and a triple G-box mutant promoter showed no MYC binding at all demonstrating that MYCs do not bind to the RSRE.

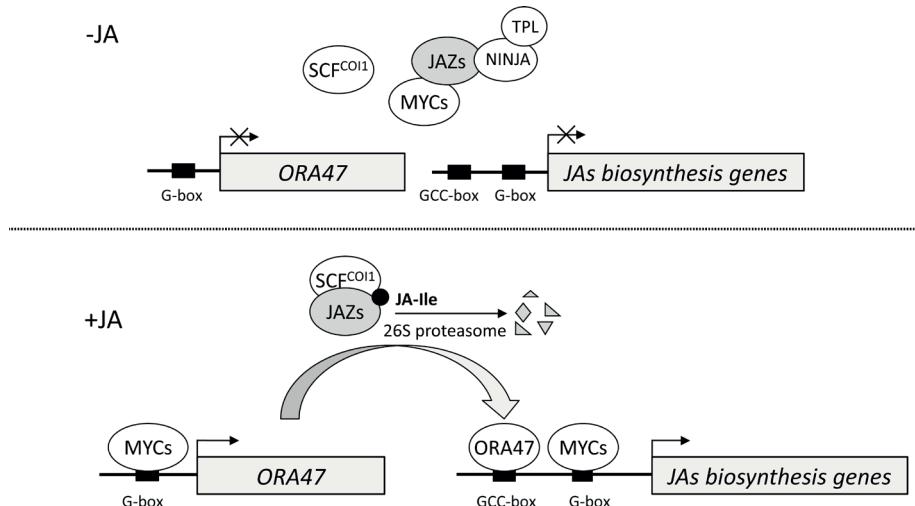
The three G-box or G-box-like sequences in the *ORA47* promoter all perfectly fit the sequence requirements for high-affinity MYC binding sites (Chini et al., 2007; Dombrecht et al., 2007; Godoy et al., 2011). Surprisingly, only G-box 2 turned out to be a high-affinity *in vitro* binding site for MYC proteins. This indicates that besides the G-box flanking nucleotides contribute to MYC binding. The *JAZ2* promoter

contains 2 G-boxes and 2 G-box-like elements, but only one of them is involved in JAs- and MYC-responsive expression (Figueroa, 2012). These authors show evidence indicating that 4 thymidine nucleotides flanking the G-box at the 3' side are essential for JAs-responsive activity. However in that study no EMSAs were performed, therefore it remains unclear whether G-box activity correlates with MYC binding. G-box 2 in the *ORA47* promoter is flanked at the 3' side by an adenine followed by three thymidine nucleotides. Further experiments are needed to establish whether these nucleotides contribute to binding of MYCs to G-box 2 and to JAs-responsive activity of the *ORA47* promoter. Transient activation assay performed in *Arabidopsis* protoplasts with mutated G-box versions of the *ORA47* promoter revealed that G-box G2 is essential for MYC-mediated activation of the *ORA47* promoter. The lower levels of *ORA47* activation caused by mutation of G1 or G3 indicate that both G1 and G3 sequences contribute to a higher activation level of the *ORA47* promoter *in vivo* despite their low contribution to binding of MYC proteins *in vitro*.

The three MYC proteins were redundant in regulating JAs-responsive gene expression. Single and double mutants showed only slightly lower expression levels than the wild-type, whereas in the triple mutant gene expression was strongly reduced consistent with a previous report (Fernández-Calvo et al., 2011). However, the triple mutant still showed readily detectable expression of *AOC2* and *VSP1* in response to JA treatment, indicating that additional transcription factors are involved in JAs-responsive gene expression. A candidate is MYC5 (bHLH28) which belongs to the same subgroup IIIe of the *Arabidopsis* bHLH family as MY2, MYC3 and MYC4 (Heim et al., 2003). MYC5 was ruled out as a JAs-responsive transcription factor because interaction with tested JAZ in yeast two-hybrid assays or in *in vitro* pull-down assays was not detected (Niu et al., 2011) and it was not captured in TAP tagging screens for *in vivo* complexes using JAZ3 or JAZ5 as baits (Fernández-Calvo et al., 2011). However, more recently it was reported that MYC5 interacts with certain JAZ repressors to function redundantly with MYC2, MYC3 and MYC4 in the regulation of stamen development and seed production (Qi et al., 2015). Figueroa and Browse (2015) found that MYC5 trans-activated the JAZ2 promoter via binding to the G-box element and that the transcriptional activity of MYC5 was inhibited by a stable JAZ1 derivative. Therefore it is likely that MYC5 is also involved in the regulation of JAs biosynthesis through directly controlling the expression of *ORA47* and JAs biosynthesis genes.

Overexpression of a stable JAZ1 derivative abolished JA-responsive gene

expression. Overexpression of a similar C-terminal deletion derivative of JAZ3 had no effect on JA-responsive *AOC2* or *VSP1* expression, whereas it did abolish JAs-responsive *PR1* gene expression. Apparently JAZ1 and JAZ3 are not functionally equivalent. It also indicates that different transcription factor-JAZ complexes operate at different JAs-responsive promoters. Apparently JAZ3 does not assemble in repressive complexes on the *VSP1* promoter.



**Figure 6.** Model for JAs signal transduction leading to the expression of JA biosynthesis genes in *Arabidopsis*. In the absence of stimulus, MYC proteins are repressed by JAZ repressors, which recruit the co-repressor TOPLESS (TPL) directly or through the adaptor NOVEL INTERACTOR OF JAZ (NINJA). In the presence of JA-Ile, interaction between COI1, an F-box protein forming part of SCF complex, and JAZ repressors is enhanced leading to the degradation of JAZs. MYC2, MYC3 and MYC4 quickly activate transcription of the gene encoding the AP2/ERF-domain transcription factor ORA47. Subsequently, MYCs and ORA47 recognize G-box and GCC-box sequences in the promoters of JAs biosynthesis genes and additively activate their expression.

## Materials and Methods

### Growth conditions and treatments

*Arabidopsis thaliana* ecotype Columbia (Col-0) is the genetic background for all wild type, mutant and transgenic plants. The *myc2/jin1-2* mutant (Lorenzo et al., 2004) and *myc3* (GK445B11) and *myc4* (GK491E10) and double and triple mutants (Fernández-Calvo et al., 2011) have been described before. Following stratification for 3 days at 4°C, the surface-sterilized seeds were germinated for 10 days at 21°C in a growth chamber (16 h light/8 h dark, 2500 lux at 70% humidity) on plates contain-

ing MA medium (Masson and Paszkowski, 1992) with 0.6% agar supplemented with 20 mg/L hygromycin for selection of transgenic plants. Batches of 15-20 seedlings were transferred to 50 ml polypropylene tubes (Sarstedt) containing 10 ml liquid MA medium without antibiotic and the tubes were incubated on a shaker at 120 rpm for 4 additional days before treatments. Seedlings were treated with 50  $\mu$ M JA (Sigma-Aldrich, St. Louis, MO) dissolved in dimethyl sulfoxide (DMSO; 0.1% v/v final concentration). As control, seedlings were treated with 0.1% DMSO.

### Binary constructs and plant transformation

The *JAZ1ΔC* and *JAZ3ΔC* sequences were amplified by PCR using the primer sets 5'-CGG GAT CCG TCG ACG AAT GTC GAG TTC TAT GGA ATG TTC-3' and 5'-GGG ATC CGT CGA CTC AAA GTT CTG TCA ATG GTG TTG G-3' for *JAZ1ΔC* and 5'-CGG AAT TCA CCA TGG AGA GAG ATT TTC TCG GG-3' and 5'-CCG CTC GAG CTA CAC GTT GGA GCC ATT ACA TTG-3' for *JAZ3ΔC*, respectively. *JAZ1ΔC* and *JAZ3ΔC* were cloned as BamHI and EcoRI fragments respectively into pRT101 (Töpfer et al., 1987). The 35S expression cassettes were excised with PstI and cloned in pCAMBIA1300. The binary vectors were introduced into *Agrobacterium tumefaciens* strain EHA105 (Hood et al., 1993). Arabidopsis plants were transformed using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on MA medium containing 100 mg/L timentin and 20 mg/L hygromycin.

### Transient expression assay

A 200 bp *ORA47* promoter fragment was amplified with the primer set 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 pGusSH-47 (Pasquali et al., 1994). Mutations were generated according to the QuickChange Site-Directed Mutagenesis protocol (Stratagene) using the primers 5'-CTC AGT ATT TAA AAC AAA CAT CCC TAA ACA AAT AGA G-3' (MG1), 5'-GAG AGT TGA ATT AAA TCA CAT CGA AAA CAA GGA ACA CG-3' (MG2) or 5'-CTC AAT ACA ATC CGC CAC ATC TCC CAT CTT CAC GCG CCA G-3' (mG3) and their respective reverse complementary primers. The *MYC2* (At1g32640) gene was excised from the *Rap-1* cDNA in pBluescript SK (GenBank acc. No. X99548; (de Pater et al., 1997)) with XmaI and cloned in pRT101. The *MYC3* (At5g46760) gene was PCR amplified from a cDNA library using the primer set 5'-CCT CGA GAA TGA ACG GCA CAA CAT CAT C-3' and 5'- CGG ATC CTC AAT AGT TTT CTC CGA CTT TC-3', digested with Xhol/BamHI and cloned in pRT101. The *MYC4*

(At4g17880) gene was PCR amplified from a cDNA library using the primer set 5'-GAT CGA ATT CAT GTC TCC GAC GAA TGT TCA AG-3' and 5'-CAG TGG ATC CTC ATG GAC ATT CTC CAA CTT-3', digested with EcoRI/BamHI and cloned in pRT101. 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 Sall and cloned in pRT101. Protoplasts prepared from *Arabidopsis* cell suspension ecotype Col-0 were co-transformed with plasmids carrying the *ORA47* promoter-*GUS* fusion and effector plasmids carrying *MYC* and/or *JAZ1* fused to the CaMV 35S promoter. As a control, co-transformation of *ORA47* promoter-*GUS* with the empty pRT101 expression vector was carried out. Protoplasts were transformed using polyethylene glycol (PEG)-mediated transfection as described previously (Schirawski et al., 2000) with the constructs in a ratio of 2:2:2:2 (μg *GUS*:*MYC2*:*MYC3*:*MYC4*:*JAZ1*). The protoplasts were harvested 18 hrs after transformation and were frozen in liquid nitrogen. GUS activity assays were performed as described (van der Fits and Memelink, 1997). GUS activities were related to protein concentrations to correct for differences protein extraction efficiencies.

### **RNA extraction and Northern blot analyses**

Total RNA was extracted from frozen ground tissue by phenol/chloroform extraction followed by overnight precipitation with 2 M lithium chloride and two washes with 70% v/v ethanol, and resuspended in water. Ten μg RNA samples were subjected to electrophoresis on 1.5% w/v agarose/1% v/v formaldehyde gels and blotted onto Genescreen nylon membranes (Perkin-Elmer Life Sciences, Boston, MA). Probes were <sup>32</sup>P-labeled by random priming. (Pre-) hybridization and subsequent washings of blots were performed as described (Memelink et al., 1994) with minor modifications. The following sets of primers were used: 5'-GAA GAT CTC AAT GGA AGA AGA ATC GGG TTT AGT A-3' and 5'-GAA GAT CTC ATC AAA AAT CCC AAA GAA TCA-3' for *ORA47* (At1g74930), 5'-GTC GAC TTC ATG AAA TTA AAA TGT TTC TC-3' and 5'-GTC GAC CCA AAA GAT TAC AAA GAC TTT TC-3' for *AOC2* (At3g25770), 5'-CGG GAT CCA TGA AAA TCC TCT CAC TTT-3' and 5'-CCC TCG AGT TAA GAA GGT ACG TAG TAG G-3' for *VSP1* (At5g24780), 5'-CGG GAT CCG TCG ACG AAT GTC GAG TTC TAT GGA ATG TTC-3' and 5'-GGG ATC CGT CGA CTC AAA GTT CTG TCA ATG GTG TTG G-3' for *JAZ1* (At1g19180), 5'-CGG AAT TCA CCA TGG AGA GAG ATT TTC TCG GG-3' and 5'-CCG CTC GAG CTA CAC GTT GGA GCC ATT ACA TTG-3' for *JAZ3* (At3g17860), 5'-ATG AAT TTT

ACT GGC TAT TCT CG-3' and 5'-TCA GTA TGG CTT CTC GTT C-3' for *PR1* (*At2g14610*), 5'-CTG TGC CAA TCT ACG AGG GTT-3' and 5'-GGA AAC CTC AAA GAC CAG CTC-3' for *ACTIN* (*At1g18780*) and 5'-CGG GAA GGA TCG TGA TGG A-3' and 5'-CCA ACC TTC TCG ATG GC C T-3' for *ROC* (*At4g38740*).

### **Isolation of recombinant MYC proteins and EMSAs**

Plasmid pASK-IBA45 (IBA Biotechnology, Göttingen, Germany) containing *MYC2* was described before (Montiel et al., 2011). *MYC3* (*At5g46760*) was amplified with primer set 5'-CGA GCT CGA TGA ACG GCA CAA CAT CAT C-3' and 5'- CCC ATG GAT TAG TTT TCT CC GAC TTT CGT C-3', digested with *SacI*/*NcoI* and cloned in pASK-IBA45plus. *MYC4* (*At4g17880*) was amplified with the primers 5'- GGA ATT CGA TGT CTC CGA CGA ATG TTC AAG-3' and 5'- CCC ATG GAT GGA CAT TCT CCA ACT TTC TC-3', digested with *EcoRI*/*NcoI* and cloned in pASK-IBA45plus. Double Strep/His-tagged MYC proteins were expressed in *E. coli* strain BL21 (DE3) pLysS and purified by Ni-NTA agarose (Qiagen, <http://www.qiagen.com>) chromatography followed in the case of *MYC4* by Strep-Tactin sepharose (IBA Biotechnology) chromatography. The wild-type *ORA47* promoter fragment was amplified with the primers 5'- GAT CCT CGA GAA AAT CTC AGT ATT TAA AAC A-3' and 5'- CAG TCT CGA GTG GCG CGT GAA GAT GGG A-3' and cloned in pJET1.2 (Fermentas). Mutations were generated according to the QuickChange Site-Directed Mutagenesis protocol (Stratagene) using the primers 5'-CTC AGT ATT TAA AAC AAA CAT CCC TAA ACA AAT AGA G-3' (MG1), 5'- GAG AGT TGA ATT AAA TCA CAT CGA AAA CAA GGA ACA CG-3' (MG2) or 5'- CTC AAT ACA ATC CGC CAC ATC TCC CAT CTT CAC GCG CCA G-3' (mG3) and their respective reverse complementary primers. Fragments were isolated with *XbaI* and labelled by filling in the overhangs with the Klenow fragment of DNA polymerase I and [ $\alpha$ -<sup>32</sup>P]dCTP. DNA-binding reactions contained 0.1 ng of end-labeled DNA fragment, 500 ng of poly(dAdT)-poly(dAdT), binding buffer (25 mM HEPES-KOH pH 7.2, 100 mM KCl, 0.1 mM EDTA, 10% v/v glycerol) and protein extract in a 10  $\mu$ l volume. Following the addition of protein extract, reactions were incubated for 30 min at room temperature before loading on a 5% w/v acrylamide/bisacrylamide (37:1) gel in half-strength Tris-Borate-EDTA buffer under tension. After electrophoresis at 125 V for 30 min, the gel was dried on Whatman DE81 paper and autoradiographed.

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# 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*-jasnone, 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 JA is JA-Ile, which is the only JA that interacts with high affinity with the SCF<sup>COI1</sup>-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 (Schluttonhofer et al., 2014) and MYBs (Mandakar 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 are 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<sup>SLY1/GID2</sup> (McGinnis et al., 2003), leading to rapid degradation of DELLA proteins via the proteasome pathway and to de-repression of the transcriptional activity of transcription factors (Itoh et al., 2003; Sun, 2010).

In *Arabidopsis*, DELLA proteins are encoded by a family of five genes, i.e. *GIBBEREL-LIC ACID INSENSITIVE* (*GA1*), *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

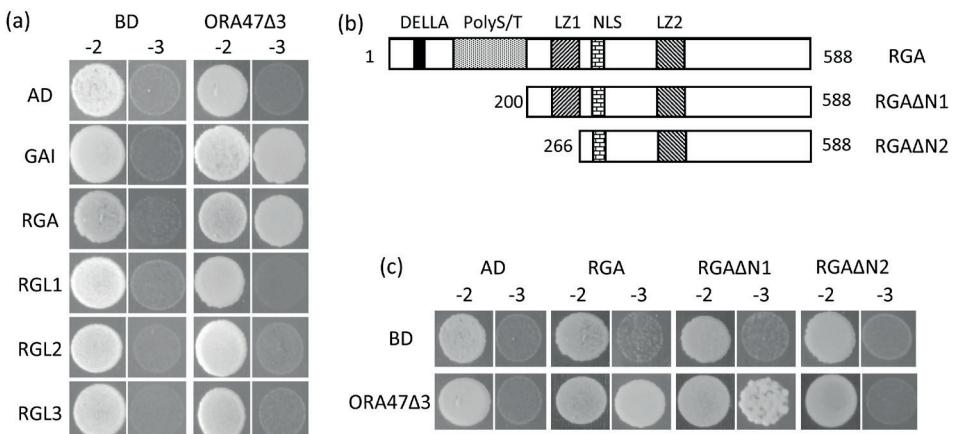
*della* mutant or in transgenic plants constitutively overexpressing RGA or GAI. All five DELLA proteins have been reported to interact with MYC2 directly, and GA and JA jointly regulate the biosynthesis of sesquiterpenes through a DELLA-MYC2 interaction (Hong et al., 2012). Here we report that RGA and 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 DELLA proteins 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  $5.2 \times 10^5$  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.

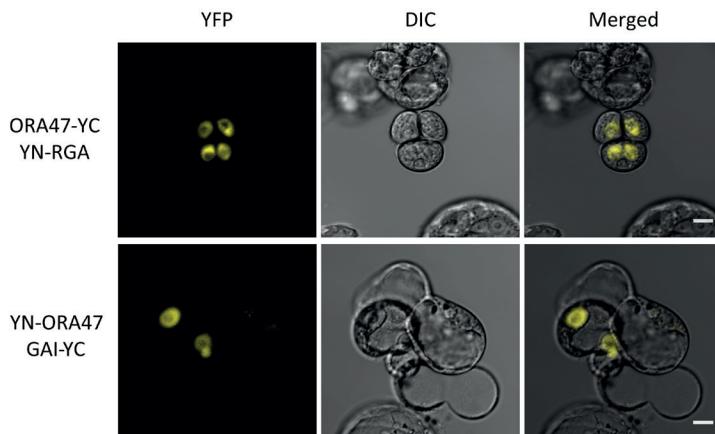
GAI is one of the five DELLA proteins in *Arabidopsis*. DELLA proteins are repressors of the GAs signal transduction pathway, which have been shown to interact with JAZ1 and to prevent inhibitory JAZ1 interaction with the transcription factor MYC2 (Hou et al., 2010). To test whether all five DELLA proteins 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 DELLA proteins, 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 DELLA 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  $\mu$ m.

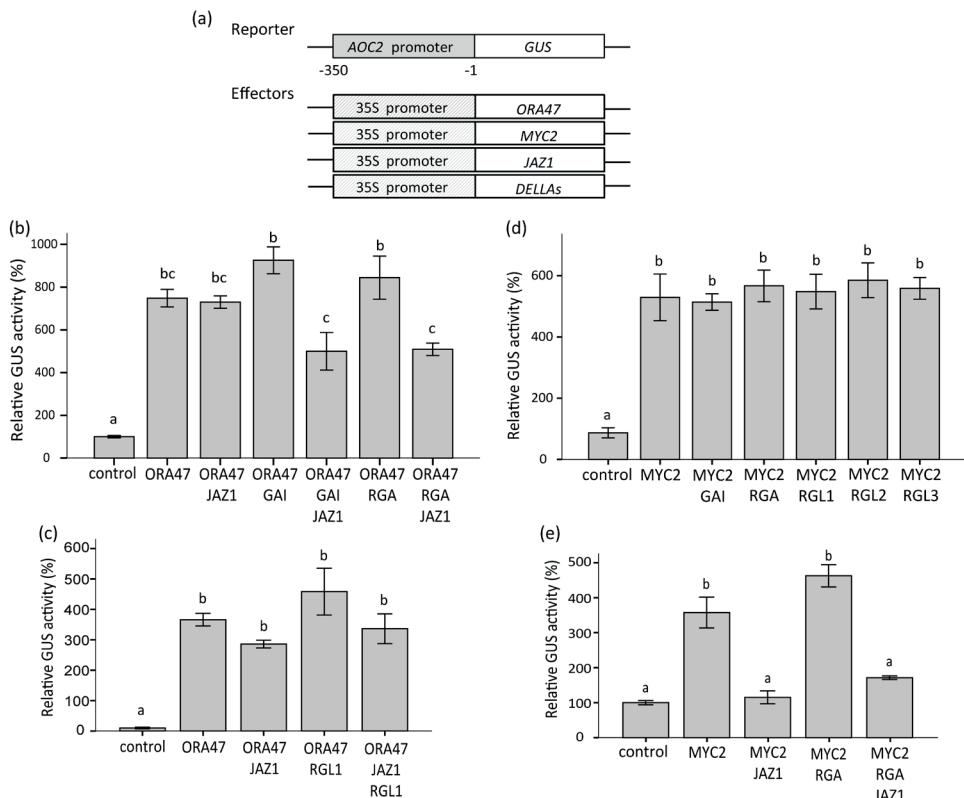
### Effects of DELLA on the activity of ORA47

To test whether DELLA 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 DELLA modulate the JAs signaling pathway via competition with MYC2 for binding to JAZ1 (Hou et al., 2010), we next tested whether DELLA affect activation of the *AOC2* promoter by MYC2. All five DELLA 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).

### DELLA promotes the activation of the *ORA47* promoter by MYC2 in *Arabidopsis* protoplasts

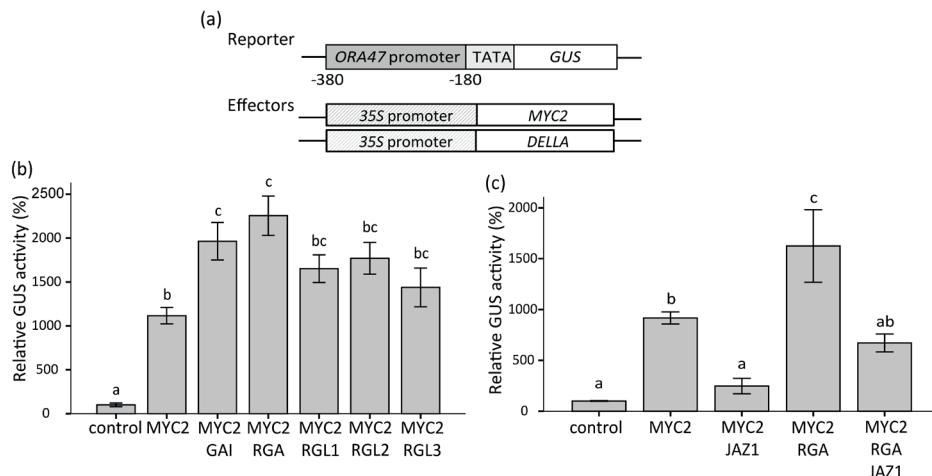
In chapter 3, we discovered that MYCs controlled the expression of *ORA47*. Previous studies reported that DELLA differentially modulate the JAs signaling pathway through direct binding to JAZ1 or MYC2 (Hou et al., 2010; Hong et al., 2012).

Although DELLA proteins did not affect the ability of MYC2 to activate the *AOC2* promoter, it cannot be ruled out that DELLA proteins 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 DELLA proteins 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 DELLA proteins on the activity of *ORA47*. **(d)(e)** Effect of DELLA proteins on the activity of *MYC2*. *Arabidopsis* cell suspension protoplasts were co-transformed with plasmids carrying *pAOC2::GUS* (2  $\mu$ g) and effector plasmids containing *ORA47* (2  $\mu$ g) or *MYC2* (2  $\mu$ g) and *DELLAs* (2  $\mu$ g in (d), 6  $\mu$ g in (e)) and/or *JAZ1* (2  $\mu$ g), 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  $\pm$ SE of triplicate experiments and are expressed relative to the vector control.

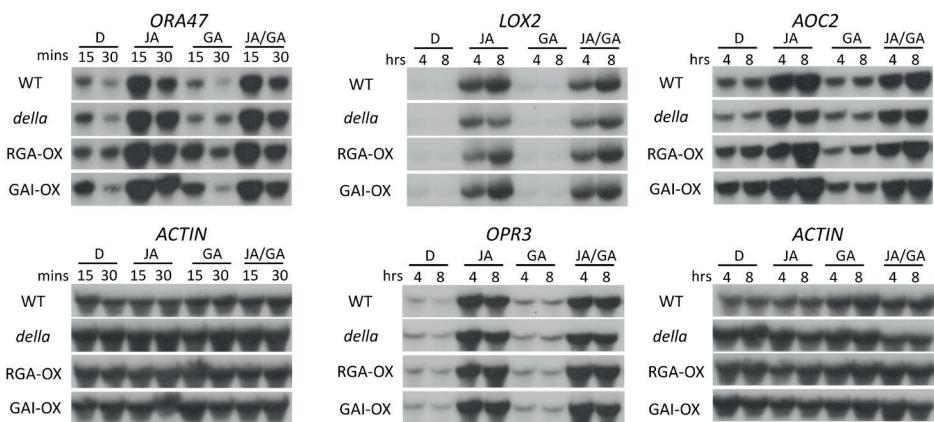
MYC2 activity with or without JAZ1 repression was increased by co-expression of RGA, although this effect was not statistically significant in the case of co-expression with JAZ1.



**Figure 4.** DELLA proteins 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)** DELLA proteins 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 DELLA proteins (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 DELLA proteins

The previous experiments in protoplasts indicated that DELLA proteins 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  $\mu$ M JA, 100  $\mu$ M 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<sup>COI1</sup> 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  $SCF^{COI}$  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 DELLA 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 DELLA do not modulate the expression of JAs biosynthesis genes. DELLA 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 DELLA 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 DELLAAs 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, DELLAAs 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 DELLAAs 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 DELLAAs with *ORA47* and effects of DELLAAs 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  $2 \times 10^6$  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  $\lambda$ ACTII (Memelink, 1997). The  $\lambda$ ACTII 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 Ncol and BamHI and cloned in pACT2 digested with Ncol 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 Ncol and BamHI and cloned in pACT2 digested with Ncol 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 Ncol and SmaI and cloned in pACT2 digested with Ncol 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 *Xba*I and *Sall* 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 *Bam*HI and *Pst*I 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 *Bgl*II and cloned into *Bam*HI 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 *Sall* 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 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 *Sac*I and *Xba*I and cloned in pRT101 digested with *Sac*I and *Xba*I. 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 Sall 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 Spel 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 Sall and Spel 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 <sup>32</sup>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.

### Acknowledgements

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# **Summary**

Jasmonates (JAs), consisting of jasmonic acid (JA) and several of its cyclic precursors and derivatives, are signaling molecules involved in the regulation of a number of processes in plants, including certain developmental processes, senescence and responses to biotic and abiotic attack (Wasternack, 2007). JAs are oxylipins which are synthesized via the octadecanoid pathway. External stresses trigger the biosynthesis of JAs, which then switch on gene expression leading to the adaptation of plants to the changed environmental conditions. The major bioactive JAs is the amino acid conjugate jasmonoyl-isoleucine (JA-Ile) (Fonseca et al., 2009). Perception of JA-Ile by the F-box protein CORONATINE-INSENSITIVE1 (COI1), which is part of a Skp-Cullin-F-box protein (SCF) complex with putative E3 ubiquitin ligase activity leads to the degradation of JAZ repressor proteins, the release of JAZ-targeted transcription factors from repression and the subsequent activation of JAs-responsive genes (Gfeller et al., 2010).

Most of the enzymes leading to JAs biosynthesis and metabolism have been identified and the corresponding genes are known (Yan et al., 2013). The expression of most biosynthesis genes including *LOX*, *AOS*, *AOC*, *OPR3*, *JMT*, and *JAR1* is induced by JAs treatment and wounding (Wasternack, 2007), implying that JAs biosynthesis is regulated by a positive feedback loop. The transcriptional regulation of JAs biosynthesis genes by this feedback loop is widely accepted but its regulatory mechanisms remain to be elucidated. Our previous studies showed that overexpression of the AP2/ERF-domain transcription factor ORA47 resulted in elevated expression of JAs biosynthesis genes and increased levels of JAs. However, the expression of JAs biosynthesis genes was not altered by downregulation of the expression level of *ORA47*, suggesting that *ORA47* regulates the positive feedback loop together with (an) unidentified transcription factor(s) (Pré, 2006; Khurshid, 2012). The current knowledge about the JAs biosynthesis pathway, the JAs signal transduction pathway and about JAs-related transcription factors is reviewed in **Chapter 1**.

It has been reported that the transcription factors MYC2 and ORA47 were able to activate the *LOX3* promoter in a transient assay in tobacco protoplasts (Pauwels et al., 2008). The bHLH-domain transcription factor MYC2 and the closely related proteins MYC3 and MYC4 are key regulators of JAs responses in *Arabidopsis* (Lorenzo et al., 2004; Fernández-Calvo et al., 2011). However, little is known about the roles of MYC2, MYC3 and MYC4 in the regulation of JAs biosynthesis. Therefore, the aim of the studies described in this thesis was to study the roles of MYC proteins in the auto-regulatory loop in JAs biosynthesis in *Arabidopsis thaliana*.

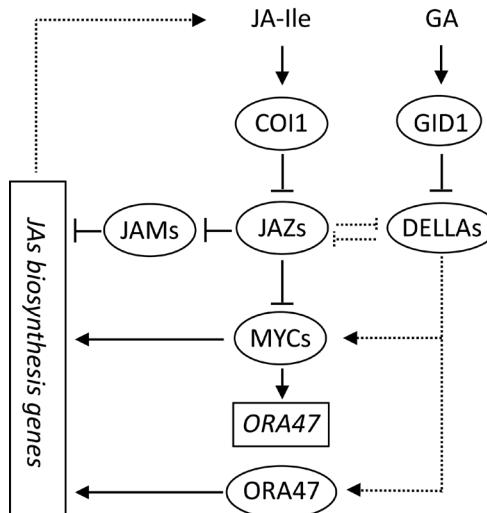
**Chapter 2** describes the roles of MYC2, MYC3 and MYC4 in the expression of JAs biosynthesis genes. In *myc234* triple mutants the expression of a large number of genes encoding enzymes involved in JAs biosynthesis was dramatically reduced after treatment with MeJA or after wounding compared to wild-type plants. EMSAs showed that MYC proteins were able to bind *in vitro* to only one of the two G-boxes that are present in the *AOC2* promoter. This G-box was essential and sufficient for MYC-mediated activation of the *AOC2* promoter in *Arabidopsis* protoplasts. There was a perfect correlation between the *in vitro* binding of MYCs to the G-box sequences and the ability of MYCs to trans-activate *AOC2* promoter derivatives with mutated G-boxes *in vivo*. Furthermore, transient trans-activation assays showed that MYCs and ORA47 additively activated the promoters of the JAs biosynthesis genes *LOX2*, *AOS*, *AOC2* and *OPR3*. These results indicate that MYC2, MYC3 and MYC4 act together with ORA47 as key positive regulators of the auto-stimulatory loop in JAs biosynthesis.

**Chapter 3** describes the roles of MYC2, MYC3 and MYC4 in the JAs-responsive expression of *ORA47*. As a positive regulator of JAs biosynthesis, ORA47 is itself encoded by a JAs-responsive gene. Based on literature data, the hypothesis that *ORA47* is regulated by the functionally redundant transcription factors MYC2, MYC3 and MYC4 was explored. The *ORA47* promoter contains 3 G-box and G-box-like motifs. The results showed that the MYC proteins could bind to only one of those G-box motifs *in vitro*. Transient trans-activation assays revealed that this G-box sequence was essential for MYC-mediated activation of the *ORA47* promoter and that the other G-box and a G-box-like sequence contributed to a higher expression level of the *ORA47* promoter *in vivo*. Triple knockout of the *MYC* genes or overexpression of a stable JAZ1 derivative abolished JAs-responsive *ORA47* expression, indicating the crucial role of the MYC-JAZ module in the regulation of *ORA47* expression.

JAs exert their function in crosstalk with gibberellins (GAs) signaling to regulate plant development and defense. **Chapter 4** aimed to study the effect of DELLA proteins, repressor proteins acting in the GAs signaling pathway, on the regulation of JAs biosynthesis in *Arabidopsis*. All five DELLA have been reported to interact with MYC2 directly and to interfere with the MYC2-mediated JAs signaling output. Y2H and BiFC assays show that two members of the *Arabidopsis* DELLA protein family, RGA and GAI, interact with ORA47. In transient activation assays, RGA and GAI slightly promoted the activity of ORA47 in the activation of the *AOC2* promoter, which was partially attenuated by addition of JAZ1. The same two DELLA proteins,

GAI and RGA, significantly enhanced MYC2-mediated activation of the *ORA47* promoter. The expression of the JAs biosynthesis genes *LOX2*, *AOC2* and *OPR3* in response to JA, GA3 or both combined was not changed in the quintuple *della* mutant or in transgenic plants constitutively overexpressing RGA or GAI compared to wild-type. Thus, although members of the DELLA repressor family interact with the regulators MYC2, MYC3, MYC4 and ORA47, we did not find evidence that DELLA proteins modulate the expression of JAs biosynthesis genes in *Arabidopsis*.

In short, the studies described in this thesis show that MYC2, MYC3 and MYC4 positively regulate JAs biosynthesis genes directly and by controlling the expression of the *ORA47* gene. GAs may be involved in the regulation of JAs biosynthesis genes via the interaction of DELLA proteins with MYCs and ORA47. A model summarizing the main results presented in this thesis is depicted in Figure 1.



**Figure 1.** Model for the transcriptional regulation of the positive feedback loop in JAs biosynthesis in *Arabidopsis*. See text for details.

The rapid production of JA-Ile in response to changing environmental conditions triggers the degradation of JAZ repressors, resulting in the release of MYC transcription factors to activate their target genes including *ORA47*. MYCs and ORA47 respectively bind to G-box and GCC-box sequences in target gene promoters and additively activate the expression of JAs biosynthesis genes, which in turn promote JAs accumulation. Combining two distinct *cis*-elements, which are targeted by two distinct transcription factors, in single promoters may enhance the specificity and responsiveness of this positive feedback loop. Members of the DELLA repressor

family, which were identified as crucial components of the GAs signaling pathway, can interact with MYCs and ORA47. They may thus promote the expression of JAs biosynthesis genes. It was reported by others that JAZ proteins interact not only with the crucial MYC activator proteins but also with the JAM repressors, which are hypothesized to compete for binding to G-box sequences with MYCs. JAMs are therefore indicated in the model as negative regulators of JAs biosynthesis genes. This model highlights that plants have in place a complex regulatory system that should be able to fine-tune the auto-regulatory loop of JAs biosynthesis to ensure adequate JAs responses.

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# **Samenvatting**

Jasmonaten (JAs), een groep chemische verbindingen die jasmonzuur (JA) en haar cyclische precursors en afgeleiden omvatten, zijn signaalmoleculen die betrokken zijn bij de regulatie van een aantal processen in planten, met inbegrip van bepaalde ontwikkelingsprocessen, veroudering en reacties op biotische en abiotische stress (Wasternack, 2007). JAs zijn oxylipines die via de octadecanoid route worden gemaakt. Externe stress signalen leiden tot de biosynthese van JAs, die vervolgens de expressie van bepaalde genen aanzetten hetgeen leidt tot de aanpassing van de plant aan de gewijzigde omstandigheden. De belangrijkste bioactieve JAs is het aminozuur conjugaat jasmonoyl-isoleucine (JA-Ile) (Fonseca et al., 2009). Percepcie van JA-Ile door het F-box-eiwit CORONATINE-INSENSITIVE1 (COI1), dat deel uitmaakt van een Skp-Cullin-F-box-eiwit (SCF) complex met vermeende E3 ubiquitine ligase activiteit, leidt tot de afbraak van JAZ repressor eiwitten. De transcriptiefactoren die door JAZ onderdrukt worden komen daardoor vrij en schakelen JAs-responsieve genen aan (Gfeller et al., 2010).

De meeste van de enzymen betrokken bij JAs biosynthese en metabolisme zijn geïdentificeerd en de overeenkomstige genen zijn bekend (Yan et al., 2013). De expressie van de meeste biosynthese genen waaronder *LOX*, *AOS*, *AOC*, *OPR3*, *JMT* en *JAR1* wordt aangeschakeld door JAs of door verwonding, een stressomstandigheid die leidt tot JAs biosynthese (Wasternack, 2007). Dit impliceert dat JAs biosynthese wordt geregeld door een positief terugkoppelingsmechanisme. Dit model van transcriptionele regulatie van JAs biosynthese genen door terugkoppeling is algemeen aanvaard, maar de betrokken regulatie mechanismen zijn nog niet bekend. Erdere studies in onze onderzoeksgrondtoonden aan dat verhoogde expressie van de AP2/ERF-domein transcriptiefactor ORA47 resulteerde in verhoogde expressie van JAs biosynthese genen en verhoogde JAs niveaus. Verlaging van de expressie van ORA47 leidde echter niet tot een verandering van het expressieniveau van de JAs biosynthesegenen, hetgeen suggereert dat ORA47 de positieve terugkoppeling reguleert samen met (een) niet-geïdentificeerde transcriptiefactor (en) (Pré, 2006; Khurshid, 2012). De huidige kennis over de JAs biosynthese route, de JAs signaal-transductie route en over JAs-gerelateerde transcriptiefactoren wordt besproken in

### **Hoofdstuk 1.**

De basische helix-loop-helix (bHLH) -domein transcriptiefactor MYC2 en de nauw verwante eiwitten MYC3 en MYC4 zijn belangrijke regulatoren van JAs reacties in *Arabidopsis thaliana* (Lorenzo et al, 2004; Fernández-Calvo et al, 2011). De MYC eiwitten worden gereguleerd worden door de JAZ repressors. Andere onderzoekers

hebben eerder gerapporteerd dat MYC2 en ORA47 de *LOX3* promoter konden anschakelen in een trans-activatie test in tabaksprotoplasten (Pauwels et al., 2008). Er is echter weinig bekend over de rol van MYC2, MYC3 en MYC4 in de regulatie van JAs biosynthese. Daarom was het doel van de in dit proefschrift beschreven studies om te bepalen wat de rol van MYC eiwitten is in het terugkoppelingsmechanisme dat JAs biosynthese in *Arabidopsis* reguleert.

**Hoofdstuk 2** beschrijft de rollen van MYC2, MYC3 en MYC4 in de expressie van JAs biosynthese genen. In de drievoudige *myc234* mutant was na behandeling met methyl-jasmonaat (MeJA) of na verwonding de expressie van de JAs biosynthese genen een stuk lager dan in wildtype planten. Uit “electrophoretic mobility shift assays” (EMSA's) bleek dat MYC eiwitten in vitro konden binden aan slechts één van de twee G-boxen die in de *AOC2* promoter voorkomen. Deze G-box was essentieel en voldoende voor de activering van de *AOC2* promoter door MYC transcriptiefactor- en in *Arabidopsis* protoplasten. Er was een perfecte correlatie tussen de in vitro binding van MYC eiwitten aan de wildtype G-box en gemuteerde sequenties en het vermogen van de MYC eiwitten om *AOC2* promoter afgeleiden met de wildtype en gemuteerde G-boxen in vivo te trans-activeren. Bovendien toonden de trans-activatie testen aan dat de MYC eiwitten en ORA47 gezamenlijk op een additieve wijze de promoters van de JAs biosynthese genen *LOX2*, *AOS*, *AOC2* en *OPR3* konden activeren. Deze resultaten geven aan dat MYC2, MYC3 en MYC4 samen met ORA47 optreden als belangrijke positieve regulatoren in het terugkoppelingsmechanisme dat JAs biosynthese reguleert.

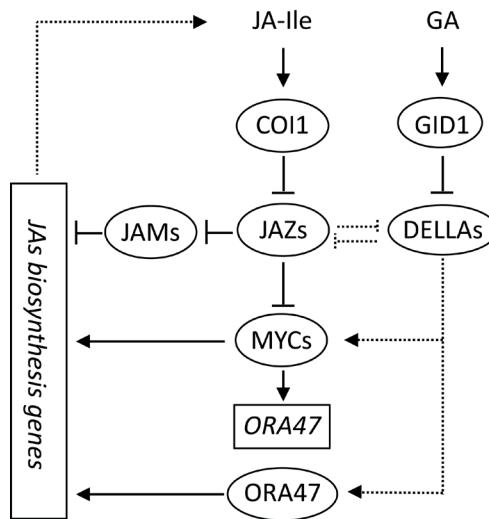
**Hoofdstuk 3** beschrijft de rol van MYC2, MYC3 en MYC4 in de JAs-responsieve expressie van het *ORA47* gen. ORA47 is een positieve regulator van JAs biosynthese, en wordt zelf gecodeerd door een JAs-responsief gen. Op basis van literatuurgegevens werd de hypothese verkend dat ORA47 wordt gereguleerd door de functioneel redundant transcriptiefactoren MYC2, MYC3 en MYC4. De *ORA47* promoter bevat twee G-boxen en een G-box-achtig motief. De resultaten toonden aan dat de MYC eiwitten aan slechts één van deze G-box motieven in vitro konden binden. Uit trans-activatie testen bleek dat deze G-box sequentie essentieel is voor het anschakelen van de *ORA47* promoter door MYC eiwitten en dat de andere G-box en de G-box-achtige sequentie bijgedragen aan een hoger expressieniveau van de *ORA47* promoter in vivo. Drievoudige mutatie van de *MYC* genen of overexpressie van een stabiele JAZ1 afgeleide onderdrukte de JAs-responsieve *ORA47* expressie volledig, wat aangeeft dat de MYC-JAZ module een cruciale rol speelt in de regulatie

van *ORA47* expressie.

De regulatie van de ontwikkeling en verdediging door JAs wordt beïnvloed door gibberellines (GAs), een ander belangrijk plantenhormoon. Studies in **Hoofdstuk 4** hadden tot doel om het effect van DELLA eiwitten, repressor eiwitten met een belangrijke rol in de GAs signaleringsroute, op de regulatie van JAs biosynthese in *Arabidopsis* te bestuderen. Andere onderzoekers hebben eerder gerapporteerd dat alle vijf leden van de DELLA eiwitfamilie in *Arabidopsis* kunnen binden aan MYC2 en daardoor interfereren met de respons die door MYC2 wordt teweeg gebracht. Uit gist twee-hybride (Y2H) testen en uit bi-moleculaire fluorescentie complementatie (BiFC) testen bleek dat twee van de DELLA eiwitten, namelijk RGA en GAI, kunnen binden aan *ORA47*. In trans-activatie testen hadden RGA en GAI een licht positief effect op het aanschakelen van de *AOC2* promoter door *ORA47*, wat weer gedeeltelijk werd afgezwakt door gelijktijdige toevoeging van de JAZ1 repressor. Dezelfde twee DELLA eiwitten, GAI en RGA, hadden een duidelijk positief effect op het aanschakelen van de *ORA47* promotor door MYC2. De expressie van de JAs biosynthese genen *LOX2*, *AOC2* en *OPR3* na toediening van JA, GA3 of beide gecombineerd was niet veranderd in de vijfvoudige *DELLA* mutant of in transgene planten met constitutief verhoogde expressie van RGA of GAI vergeleken met wildtype planten. Dus hoewel deze leden van de DELLA repressor familie interactie vertonen met de transcriptiefactoren MYC2, MYC3, MYC4 en *ORA47*, hebben we niet kunnen vast stellen dat deze DELLA eiwitten de expressie van JAs biosynthese genen in *Arabidopsis* beïnvloeden.

De in dit proefschrift beschreven studies laten zien dat MYC2, MYC3 en MYC4 de JAs biosynthese genen positief reguleren zowel rechtstreeks als indirect door het reguleren van de expressie van het *ORA47* gen. GAs zijn mogelijk betrokken bij de regulatie van de JAs biosynthese genen via de interactie van DELLA eiwitten met de MYC eiwitten en met *ORA47*. Figuur 1 toont een model waarin de belangrijkste resultaten in dit proefschrift zijn weergegeven. De snelle productie van JA-Ile in reactie op ongunstige omgevingsomstandigheden leidt tot de afbraak van JAZ repressoren, wat resulteert in het vrijkomen van MYC transcriptiefactoren die hun doelwitgenen met inbegrip van *ORA47* aan schakelen. De MYC eiwitten en *ORA47* binden respectievelijk aan de G-box en aan de GCC-box in de promoters van doelwitgenen en reguleren additief de expressie van JAs biosynthese genen, hetgeen leidt tot verhoogde JAs niveaus. Het combineren van twee verschillende cis-elementen, die het doelwit zijn van twee verschillende transcriptiefactoren, in pro-

motors heeft mogelijk tot doel om de specificiteit en gevoeligheid van het positieve terugkoppelingsmechanisme te verbeteren. Leden van de DELLA repressor familie, cruciale onderdelen van de GAs signaaltransductieroute, kunnen binden aan MYC eiwitten en aan ORA47. Zo zouden zij de expressie van JAs biosynthese genen kunnen bevorderen. Er werd eerder gerapporteerd door andere onderzoekers dat de JAZ repressors niet alleen binden aan de MYC activator eiwitten maar ook interactie vertonen met de JAM repressor eiwitten. Dit zijn ook eiwitten met een bHLH domein voor binding aan DNA die worden verondersteld te concurreren met MYC eiwitten voor binding aan G-box-sequenties. JAM eiwitten zijn daarom in het model weergegeven als negatieve regulatoren van JAs biosynthese genen. Dit model benadrukt dat planten een complex regelmechanisme bezitten dat ze in staat stelt de productie van JAs heel gevoelig af te stemmen voor het vereiste niveau van verdediging tegen ongunstige omstandigheden.



**Figuur 1.** Model voor de transcriptionele regulatie van de positieve terugkoppelingsmechanisme in JAs biosynthese in *Arabidopsis*.



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Kaixuan Zhang

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# Curriculum Vitae

Kaixuan Zhang was born on the 16<sup>th</sup> of November 1986 in Yantai, Shandong Province, China. After she finished her high school studies in Yantai No.1 middle school, she started her bachelor studies at Northeast Forestry University in Harbin in September 2004 and obtained the Bachelor degree in Life Science in July 2008. She continued her master studies in Tree Genetic Improvement and Biotechnology at Northeast Forestry University. In July 2011, she obtained the Master's degree with the thesis 'Cloning and functional analysis of *PnsGA20ox1* gene in *Populus simonii* × *P. nigra*' under the supervision of Prof. Dr. Zhigang Wei. In October 2011, she started her PhD research under the supervision of Prof. Dr. J. Memelink in the Plant Cell Physiology group, Institute of Biology, Leiden University, The Netherlands, with financial support from the China Scholarship Council. Her work about the transcriptional regulation of jasmonate biosynthesis in *Arabidopsis* is described in this thesis.

