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

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Chapter 2

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

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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 α-linolenic acid (α-LeA) from chloroplast membranes. After the sequential action of the plastid enzymes lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC), α-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 β-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 (COl1), 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^{COI1} 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 *jam*x*3* 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 *jam*x*3* 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 bioactive 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 yong 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

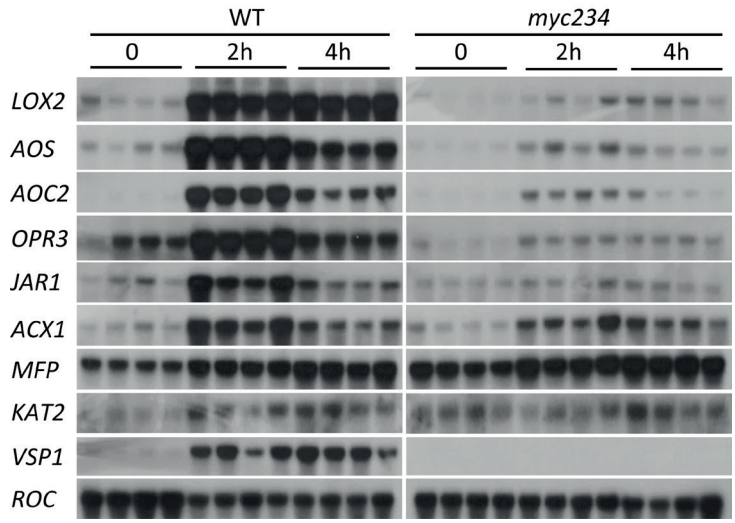

Figure 1. MYCs control the expression of JAs biosynthesis genes in response to wounding. Total RNA was isolated from 4 replicate samples of three-week-old wild-type and *myc* triple mutant Arabidopsis leaves 2 or 4 hours after wounding. The RNA gel blot was hybridized with the indicated probes. The *ROC* (*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.

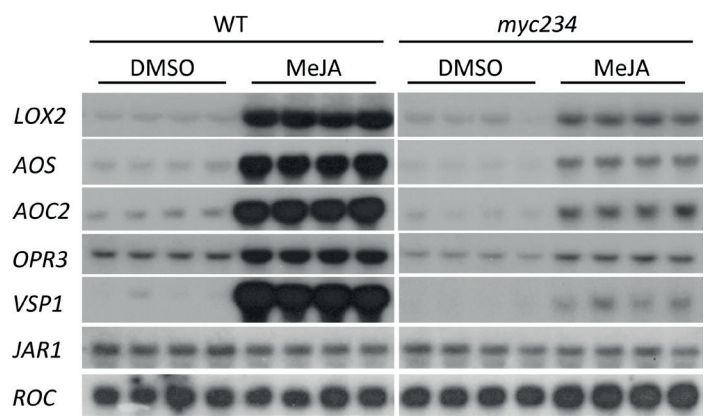

Figure 2. MYCs control the JAs-responsive expression of JAs biosynthesis genes. Total RNA was isolated from 4 replicate samples of two-week-old wild-type and *myc* triple mutant Arabidopsis seedlings treated with 50 µM MeJA or the solvent DMSO (0.05% final concentration) for 4 hours. The RNA gel blot 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 (EMSAs) 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).

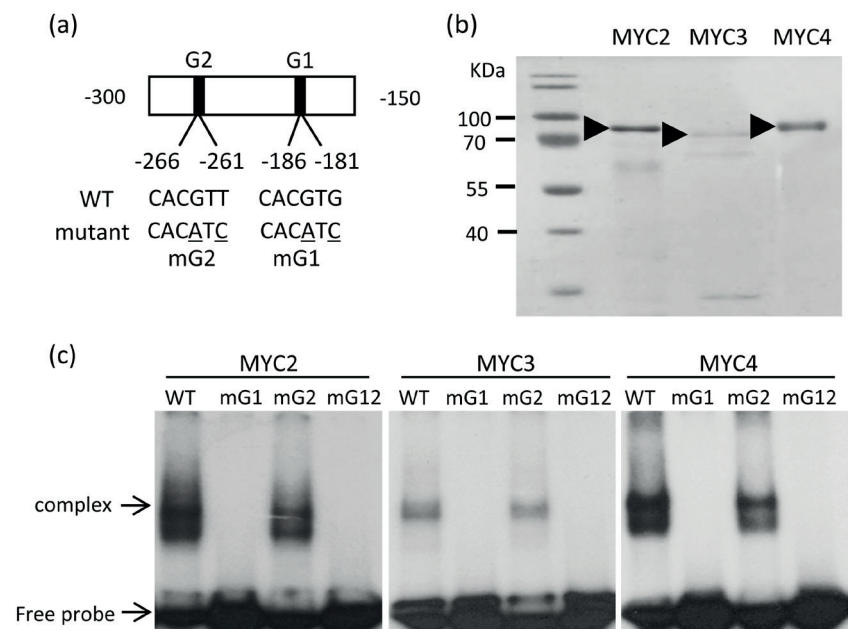

Figure 3. MYC proteins bind to one G-box sequence in the AOC2 promoter *in vitro*. (**a**) Schematic diagram of wild-type and mutated versions of G-boxes in the *AOC2* promoter. Underlined nucleotides indicate point mutations in the G-boxes. Numbers indicate positions relative to the ATG start codon. (**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 β-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

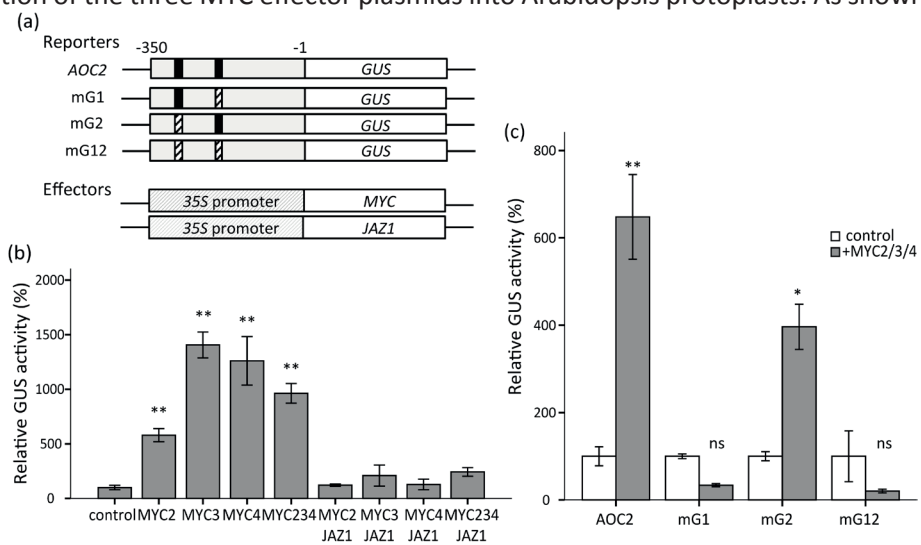

Figure 4. MYC proteins trans-activate the *AOC2* promoter via the G-box sequence *in vivo*. (**a**) Schematic representation of the constructs used for transient expression assays. Numbers indicate positions relative to the ATG start codon. (**b**) MYCs trans-activate the *AOC2* promoter in Arabidopsis protoplasts. Arabidopsis cell suspension protoplasts were co-transformed with plasmids carrying *pAOC2::GUS* (2 μg) and effector plasmids containing *MYCs* (2 μg) alone or in combination with *JAZ1* (2 μg), 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 ±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.

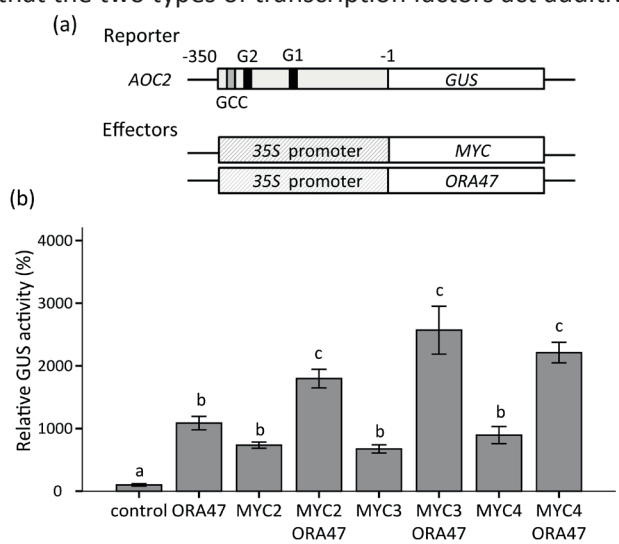

Figure 5. The *AOC2* promoter is additively trans-activated by MYCs and ORA47. (**a**) Schematic representation of the constructs used for transient expression assays. Numbers indicate positions relative to the ATG start codon. (**b**) MYCs and ORA47 trans-activate the *AOC2* promoter additively. Arabidopsis cell suspension protoplasts were co-transformed with plasmids carrying *pAOC2::GUS* (2 µg) and effector plasmids containing *ORA47* (2 µg) and/or *MYCs* (2 µg), as indicated. Protein concentrations were used to correct for differences in protein extraction efficiencies. letters show statistically significant differences between values according to a post hoc Tukey HSD test (ANOVA, $P<0.05$). Values represent means ±SE of triplicate experiments and are expressed relative to the vector control.

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.

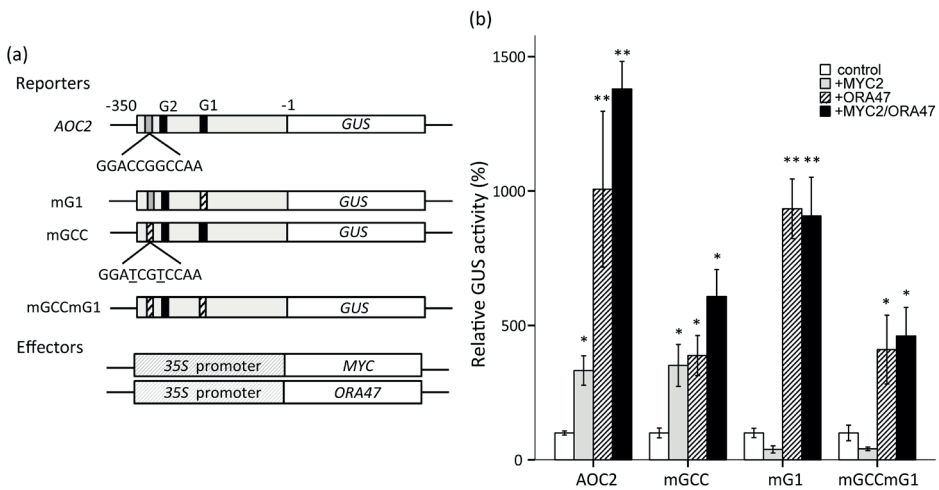

Figure 6. Effects of GCC-box and G-box mutations on trans-activation of the *AOC2* promoter by MYC2 and/or ORA47. (**a**) Schematic representation of the constructs used for transient expression assays. Underlined nucleotides indicate point mutations in the GCC-box. Numbers indicate positions relative 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 µg) and effector plasmids containing *ORA47* (2 µg) and/or *MYC2* (2 µg), 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 ±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.

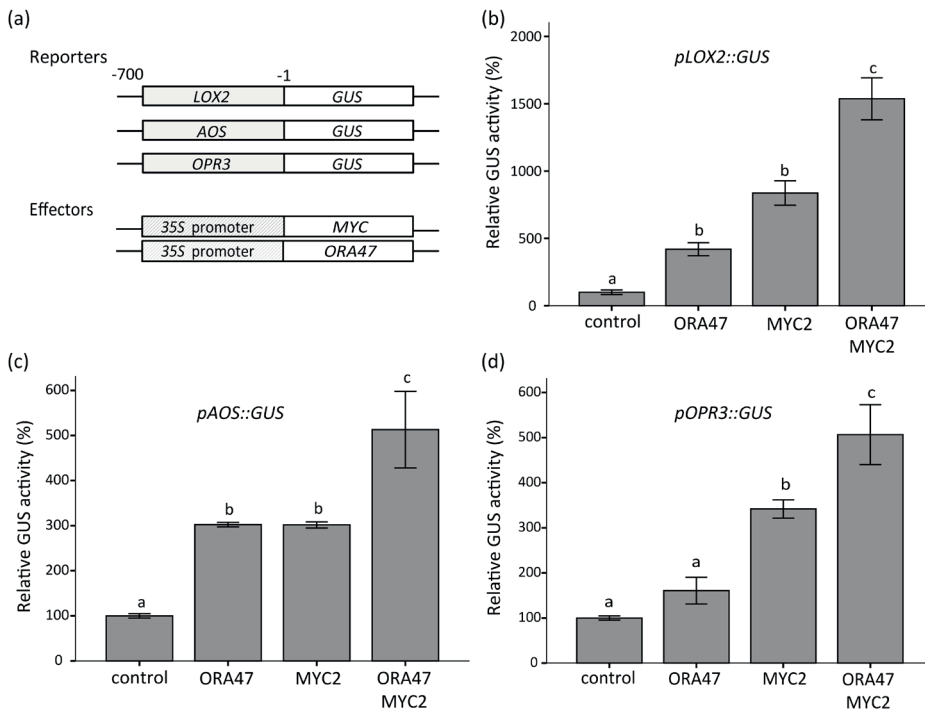

Figure 7. MYCs and ORA47 trans-activate the promoter of JAs biosynthesis genes additively. (**a**) Schematic representation of the constructs used for transient expression assays. Numbers indicate positions relative to the ATG start codon. MYC2 and ORA47 additively trans-activate the (**b**) *LOX2*, (**c**) *AOS* and (**d**) *OPR3* promoters. Arabidopsis cell suspension protoplasts were co-transformed with plasmids carrying *pAOC2::GUS* (2 µg) and effector plasmids containing *ORA47* (2 µg) and/or *MYC2* (2 µg), as indicated. Protein concentrations were used to correct for differences in protein extraction efficiencies. letters show statistically significant differences between values according to a post hoc Tukey HSD test (ANOVA, $P<0.05$). Values represent means ±SE of triplicate experiments and are expressed relative to the vector control.

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 β-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 β-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 β-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). DELLAs, 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.

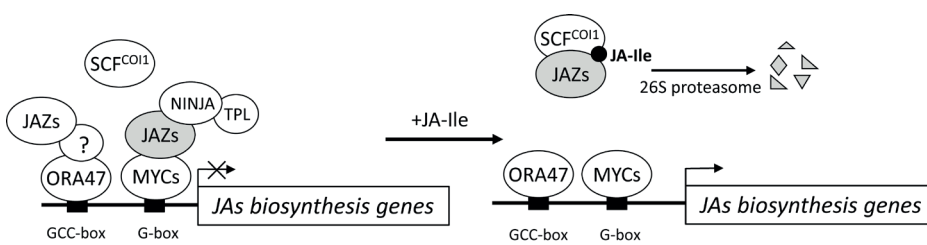

Figure 8. Model of the regulation of JAs biosynthesis genes. In the absence of stimulus, the activity of ORA47 and MYC proteins is repressed by JAZ repressors, which recruit the co-repressor TOPLESS (TPL) directly or through the adaptor NOVEL INTERACTOR OF JAZ (NINJA). Perception of bioactive JA-Ile 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 ^{32}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/NcoI 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/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) chromatography.

Electrophoretic mobility shift assays

The wild-type and mutated fragments of the *AOC2* promoter were amplified from the construct p*AOC2*-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/SalI and labelled by filling in the overhangs with the Klenow fragment of DNA polymerase I and [α-^{32}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 µl volume, and were incubated for 30 min at room temperature before loading on 5% (w/v) acrylamide/bisacrylamide (37:1)-0.5×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 SalI 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 XhoI/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 SalI 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 (µg 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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