Cover Page

The handle http://hdl.handle.net/1887/41032 holds various files of this Leiden University dissertation.

Author: Zhang, K.
Title: MYC transcription factors: masters in the regulation of jasmonate biosynthesis in Arabidopsis thaliana
Issue Date: 2016-07-06

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 α-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 β-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[COI1]-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-

radation 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).
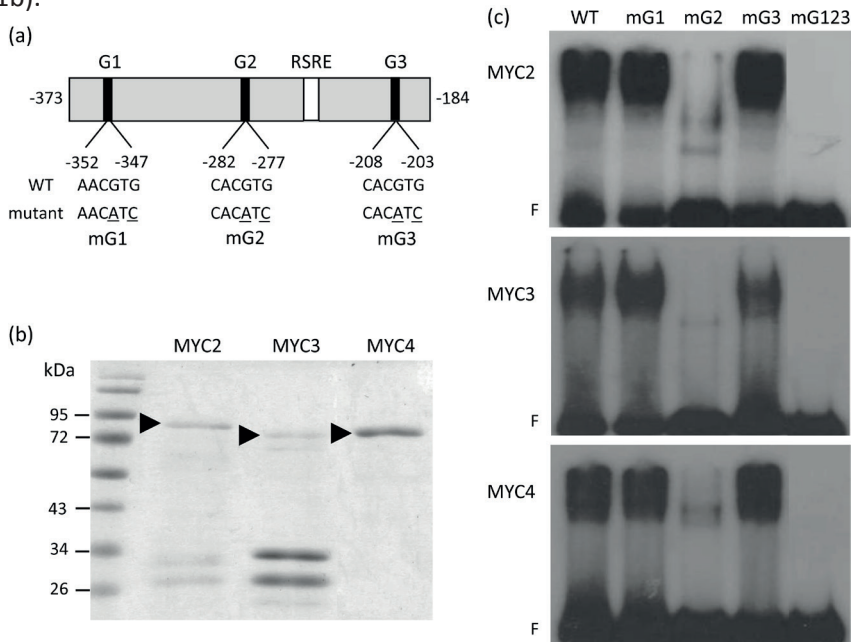
Figure 1. MYC proteins bind to one G-box sequence in the *ORA47* promoter *in vitro*. (**a**) Overview of wild-type and mutated versions of G-boxes in the *ORA47* promoter. Underlined nucleotides indicate point mutations in the G-boxes. Numbers indicate positions relative to the ATG start codon. The 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 (EMSAs) 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.
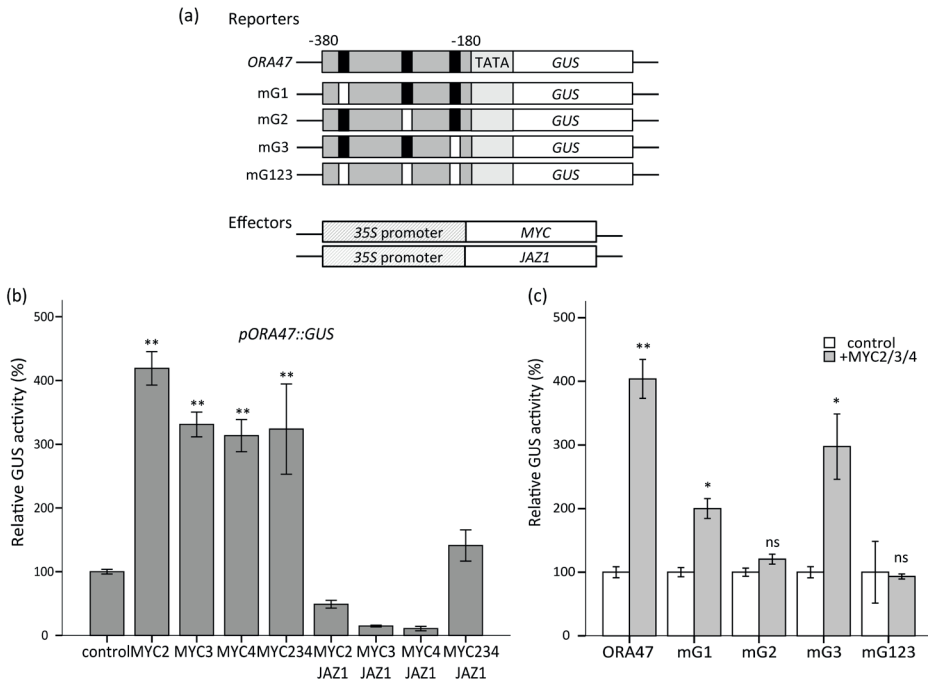
Figure 2. (**a**) Schematic representation of the constructs used for transient expression assays. The wild-type and mutated *ORA47* promoters were fused to a minimal TATA box from the CaMV 35S promoter and the *GUS* gene. Numbers indicate positions relative to the ATG start codon. (**b**) MYCs trans-activate 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 inter-action with the SCF^{COI1} complex in the presence of JA-Ile (Thines et al., 2007). Variants
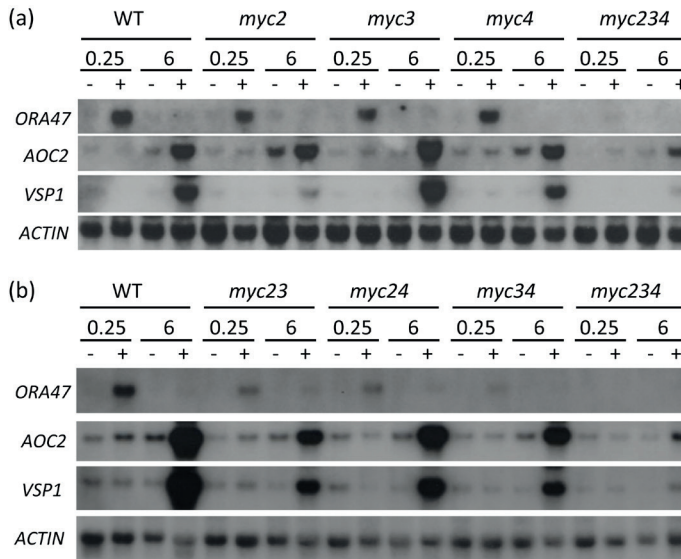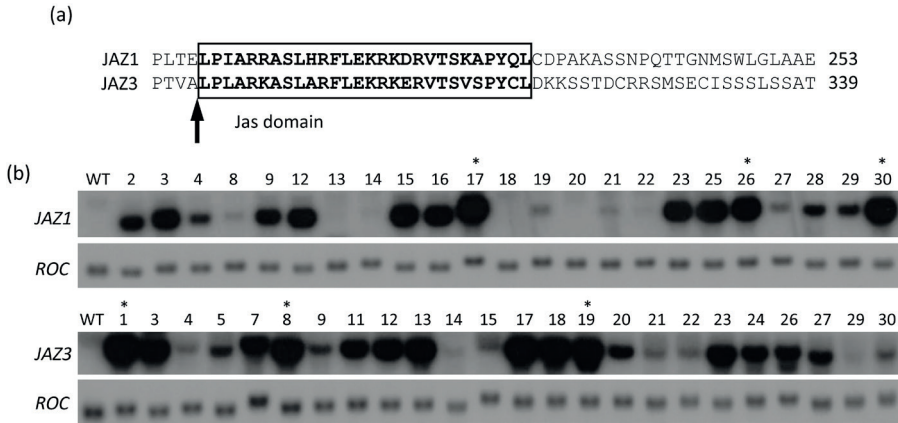
Figure 3. JA-responsive expression of *ORA47* is controlled by MYC2, MYC3 and MYC4. RNA was extracted from 2-week-old wildtype or single, double or triple mutants. Seed-lings were treated with 50 μM JA (+) or the solvent DMSO (-,0.1% v/v final concen-tration) for the number of hours indicated. The RNA 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 JAZΔC with the highest expression levels for further analysis.

Figure 5 shows that in the JAZ1ΔC 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 JAZ3ΔC lines JA-re-sponsive expression of *ORA47*, *AOC2* and *VSP1* was not different from wild-type. As shown in Figure 4 the *JAZ3ΔC* gene was expressed in these lines at a high level similar to the *JAZ1ΔC* expression level. JAZ3ΔC 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 JAZ3ΔC 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 JAZΔC lines, demonstrating that JAZ3ΔC 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.

(a)

```
JAZ1  PLTELPIARRASLHRFLEKRKDRVTSKAPYQLCDPAKASSNPQTTGNMSWLGLAAE  253
JAZ3  PTVALPLARKASLARFLEKRKERVTSVSPYCLDKKSSTDCRRSMSECISSSLSSAT  339
```

Jas domain

(b)

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.
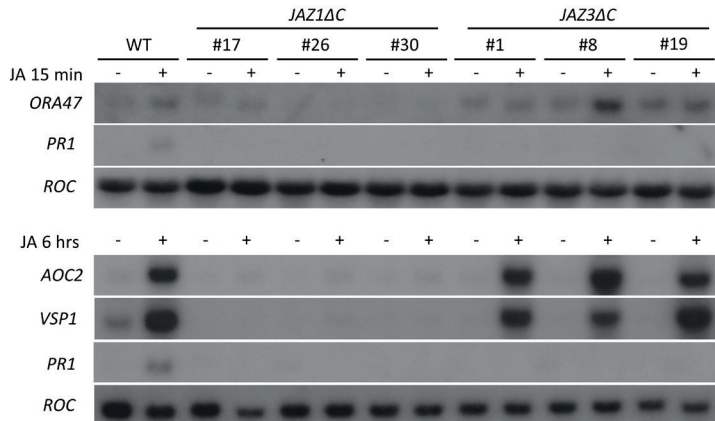
Figure 5. Overexpression of *JAZ1ΔC* represses JA-responsive expression of *ORA47* and its target gene *AOC2*. Two-week-old wildtype seedlings or seedlings from the indicated *JAZΔC* overexpression lines were treated for the indicated times with 50 μM JA (+) or the solvent DMSO (-,0.1% v/v final concentration). 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 signaling 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.
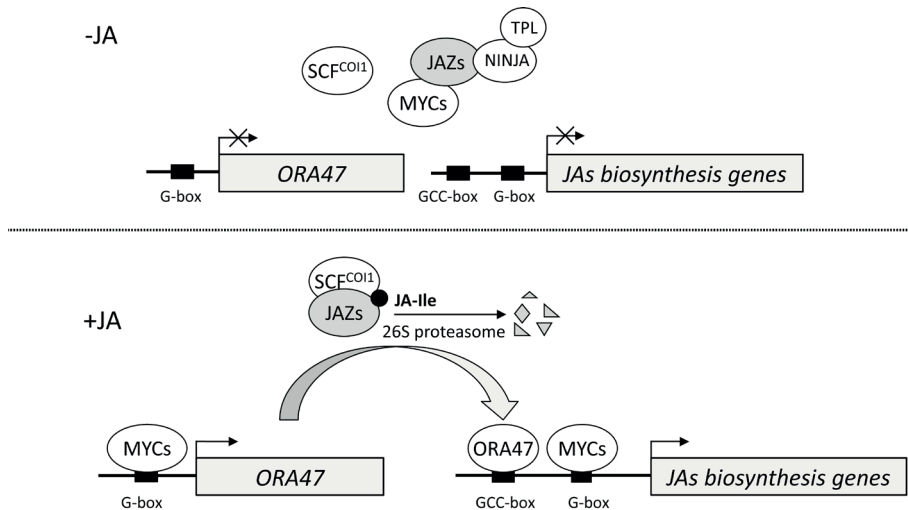
Figure 6. Model for JAs signal transduction leading to the expression of JA biosynthesis genes in Arabidopsis. In the absence of stimulus, MYC proteins are repressed by JAZ repressors, which recruit the co-repressor TOPLESS (TPL) directly or through the adaptor NOVEL INTERACTOR OF JAZ (NINJA). In the 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 µM 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 XhoI/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 SalI 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 ^{32}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 TGT 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 CTC 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 Biotagnology) 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 XhoI 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-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 µl 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.

Acknowledgements

References

Chini A, Fonseca S, Chico JM, Fernández-Calvo P, Solano R (2009) The ZIM domain mediates homo-and heteromeric interactions between Arabidopsis JAZ proteins. Plant J 59: 77-87

Chini A, Fonseca S, Fernández G, Adie B, Chico JM, Lorenzo O, Garcia-Casado G, López-Vidriero I, Lozano FM, Ponce MR, Micol JL, Solano R (2007) The JAZ family of repressors is the missing link in jasmonate signalling. Nature 448: 666-671

Chung HS, Howe GA (2009) A critical role for the TIFY motif in repression of jasmonate signaling by a stabilized splice variant of the JASMONATE ZIM-domain protein JAZ10 in *Arabidopsis*. Plant Cell 21: 131-145

de Pater S, Pham K, Memelink J, Kijne J (1997) RAP-1 is an Arabidopsis MYC-like R protein homologue, that binds to G-box sequence motifs. Plant Mol Biol 34: 169-174

Devoto A, Nieto-Rostro M, Xie D, Ellis C, Harmston R, Patrick E, Davis J, Sherratt L, Coleman M, Turner JG (2002) COI1 links jasmonate signalling and fertility to the SCF ubiquitin–ligase complex in *Arabidopsis*. Plant J 32: 457-466

Dombrecht B, Xue GP, Sprague SJ, Kirkegaard JA, Ross JJ, Reid JB, Fitt GP, Sewelam N, Schenk PM, Manners JM (2007) MYC2 differentially modulates diverse jasmonate-dependent functions in *Arabidopsis*. Plant Cell 19: 2225-2245

Fernández-Calvo P, Chini A, Fernández-Barbero G, Chico JM, Gimenez-Ibanez S, Geerinck J, Eeckhout D, Schweizer F, Godoy M, Franco-Zorrilla JM, Pauwels L, Witters E, Puga MI, Paz-Ares J, Goossens A, Reymond P, Jaeger GD, Solano R (2011) The Arabidopsis bHLH transcription factors MYC3 and MYC4 are targets of JAZ repressors and act additively with MYC2 in the activation of jasmonate responses. Plant Cell 23: 701-715

Figueroa P, Browse J (2012) The Arabidopsis JAZ2 promoter contains a G-box and thymidine-rich module that are necessary and sufficient for jasmonate-dependent activation by MYC transcription factors and repression by JAZ proteins. Plant Cell Physiol 53: 330-343

Figueroa P, Browse J (2015) Male sterility in Arabidopsis induced by overexpression

of a MYC5-SRDX chimeric repressor. Plant J 81: 849-860

Fonseca S, Chico JM, Solano R (2009) The jasmonate pathway: the ligand, the receptor and the core signalling module. Curr Opin Plant Biol 12: 539-547

Godoy M, Franco-Zorrilla JM, Pérez-Pérez J, Oliveros JC, Lorenzo Ó, Solano R (2011) Improved protein-binding microarrays for the identification of DNA-binding specificities of transcription factors. Plant J 66: 700-711

Heim MA, Jakoby M, Werber M, Martin C, Weisshaar B, Bailey PC (2003) The basic helix–loop–helix transcription factor family in plants: a genome-wide study of protein structure and functional diversity. Mol Biol Evol 20: 735-747

Hou X, Lee LYC, Xia K, Yan Y, Yu H (2010) DELLAs modulate jasmonate signaling via competitive binding to JAZs. Dev Cell 19: 884-894

Khurshid M (2012) Functional analysis of ORA47, a key regulator of jasmonate biosynthesis in Arabidopsis. PhD thesis. Leiden University, Leiden, The Netherlands

Lorenzo O, Chico JM, Sánchez-Serrano JJ, Solano R (2004) *JASMONATE-INSENSITIVE1* encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. Plant Cell 16: 1938-1950

Memelink J, Swords KM, Staehelin LA, Hoge JHC (1994) Southern, Northern and Western blot analysis. Plant Mol Biol manual. F1: 1-23

Montiel G, Zarei A, Körbes AP, Memelink J (2011) The jasmonate-responsive element from the ORCA3 promoter from *Catharanthus roseus* is active in Arabidopsis and is controlled by the transcription factor AtMYC2. Plant Cell Physiol 52: 578-587

Niu Y, Figueroa P, Browse J (2011) Characterization of JAZ-interacting bHLH transcription factors that regulate jasmonate responses in *Arabidopsis*. J Exp Bot 62: 2143-2154

Pasquali G, Ouwerkerk PB, Memelink J (1994) Versatile transformation vectors to assay the promoter activity of DNA elements in plants. Gene 149: 373-374

Pauwels L, Barbero GF, Geerinck J, Tilleman S, Grunewald W, Pérez AC, Chico JM, Bossche RV, Sewell J, Gil E, García-Casado G, Witters E, Inzé D, Long JA, Jaeger GD, Solano R, Goossens A (2010) NINJA connects the co-repressor TOPLESS to jasmonate signalling. Nature 464: 788-791

Pauwels L, Goossens A (2008) Fine-tuning of early events in the jasmonate response. Plant Signal Behav 3: 846-847

Pauwels L, Goossens A (2011) The JAZ proteins: a crucial interface in the jasmonate signaling cascade. Plant Cell 23: 3089-3100

Pauwels L, Morreel K, De Witte E, Lammertyn F, Van Montagu M, Boerjan W, Inzé

D, Goossens A (2008) Mapping methyl jasmonate-mediated transcriptional reprogramming of metabolism and cell cycle progression in cultured Arabidopsis cells. Proc Natl Acad Sci USA 105: 1380-1385

Pieterse CM, Leon-Reyes A, Van der Ent S, Van Wees SC (2009) Networking by small-molecule hormones in plant immunity. Nat Chem Biol 5: 308-316

Pré M (2006) ORA EST: Functional analysis of jasmonate-responsive AP2/ERF-doamin transcription factors in *Arabidopsis thaliana*. PhD thesis. Leiden University, Leiden, The Netherlands

Qi T, Huang H, Song S, Xie D (2015) Regulation of jasmonate-mediated stamen development and seed production by a bHLH-MYB complex in Arabidopsis. Plant Cell 27: 1620-1633

Sasaki-Sekimoto Y, Taki N, Obayashi T, Aono M, Matsumoto F, Sakurai N, Suzuki H, Hirai MY, Noji M, Saito K (2005) Coordinated activation of metabolic pathways for antioxidants and defence compounds by jasmonates and their roles in stress tolerance in Arabidopsis. Plant J 44: 653-668

Sasaki Y, Asamizu E, Shibata D, Nakamura Y, Kaneko T, Awai K, Amagai M, Kuwata C, Tsugane T, Masuda T (2001) Monitoring of methyl jasmonate-responsive genes in Arabidopsis by cDNA macroarray: self-activation of jasmonic acid biosynthesis and crosstalk with other phytohormone signaling pathways. DNA Res 8: 153-161

Schirawski J, Planchais S, Haenni AL (2000) An improved protocol for the preparation of protoplasts from an established *Arabidopsis thaliana* cell suspension culture and infection with RNA of turnip yellow mosaic tymovirus: a simple and reliable method. J Virol Methods 86: 85-94

Sheard LB, Tan X, Mao H, Withers J, Ben-Nissan G, Hinds TR, Kobayashi Y, Hsu FF, Sharon M, Browse J (2010) Jasmonate perception by inositol-phosphate-potentiated COI1-JAZ co-receptor. Nature 468: 400-405

Shyu C, Figueroa P, DePew CL, Cooke TF, Sheard LB, Moreno JE, Katsir L, Zheng N, Howe GA (2012) JAZ8 lacks a canonical degron and has an EAR motif that mediates transcriptional repression of jasmonate responses in *Arabidopsis*. Plant Cell 24: 536-550

Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, Browse J (2007) JAZ repressor proteins are targets of the SCF[COI1] complex during jasmonate signalling. Nature 448: 661-665

Töpfer R, Matzeit V, Gronenborn B, Schell J, Steinbiss HH (1987) A set of plant expression vectors for transcriptional and translational fusions. Nucleic Acids Res 15: 5890

van der Fits L, Memelink J (1997) Comparison of the activities of CaMV 35S and

FMV 34S promoter derivatives in *Catharanthus roseus* cells transiently and stably transformed by particle bombardment. Plant Mol Biol 33: 943-946

Walley JW, Coughlan S, Hudson ME, Covington MF, Kaspi R, Banu G, Harmer SL, Dehesh K (2007) Mechanical stress induces biotic and abiotic stress responses via a novel *cis*-element. PLoS Genet 3: 1800-1812

Wang Z, Cao G, Wang X, Miao J, Liu X, Chen Z, Qu LJ, Gu H (2008) Identification and characterization of COI1-dependent transcription factor genes involved in JA-mediated response to wounding in Arabidopsis plants. Plant Cell Rep 27: 125-135

Wasternack C (2007) Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. Annals of botany 100: 681-697

Xu L, Liu F, Lechner E, Genschik P, Crosby WL, Ma H, Peng W, Huang D, Xie D (2002) The SCF[COI1] ubiquitin-ligase complexes are required for jasmonate response in Arabidopsis. Plant Cell 14: 1919-1935