Functional analysis of ORA47, a key regulator of jasmonate biosynthesis in arabidopsis

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CHAPTER 4

Identification and analysis of ORA47-interacting proteins

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ABSTRACT

Upon herbivore or pathogen attack plants produce the jasmonate (JAs) hormones, which are perceived by the F-box protein CORONATINE-INSENSITIVE1 (COI1). This leads to degradation of JAZ repressor proteins, which sets in motion defense gene expression programmes. JAs signaling also induces all known JAs biosynthesis genes including *ALLENE OXIDE CYCLASE 2* (*AOC2*) in what is considered a positive feedback loop. The JAs-responsive AP2/ERF-domain transcription factor ORA47 controls all JAs biosynthesis genes. *AOC2* is a primary JAs-responsive gene, indicating that the activity of ORA47 is regulated by a repressor protein that is degraded in a JAs-responsive and COI1-dependent manner. The work described here aimed to identify proteins interacting with ORA47 and to determine their effect on ORA47 activity. Via yeast two-hybrid screening and pull-down assays we identified all 5 members of the Arabidopsis BTB-TAZ protein family except BT2 as ORA47 interactors. ORA47 did not directly interact with JAZ proteins, but all BT proteins except BT2 did, which is compatible with a scenario where BT proteins act as adaptors between ORA47 and JAZ. BT4 and BT5 partially repressed ORA47 activity in a transient trans-activation assay, whereas JAZ1 had no effect. Analysis of quadruple *bt* knockout plants having either a wildtype *BT2* or *BT3* gene showed no effect on basal or JA-responsive expression of *AOC2*. We conclude that BT proteins are not the hypothetical repressors or adaptor proteins that we were looking for. We hypothesize that ORA47 is regulated via interaction of its C-terminal domain with an adaptor protein that recruits certain members of the JAZ family.

INTRODUCTION

Upon insect herbivory or attack by microbial pathogens plants produce jasmonate (JAs) hormones (Pieterse et al., 2009). The bioactive amino acid conjugate jasmonoyl-isoleucine (JA-Ile) is perceived by the F-box protein CORONATINE-INSENSITIVE1 (COI1) (Thines et al., 2007; Sheard et al., 2010) which is part of an SCF complex with putative E3 ubiquitin ligase activity. This

leads to degradation of JAZ repressor proteins (Chini et al., 2007; Thines et al., 2007), which sets in motion defense gene expression programmes.

Genes involved in defense against wounding and insect herbivory are controlled by the JAs-responsive basic Helix-Loop-Helix transcription factor MYC2 (Lorenzo et al., 2004) and the related proteins MYC3 and MYC4 (Fernandez-Calvo et al., 2011; Niu et al., 2011). JAZ were shown to bind to MYC (Chini et al, 2009; Fernandez-Calvo et al., 2011). JAZ1 can repress the activity of MYC2 (Hou et al., 2010) and JAZ can bind to the corepressors 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).

JAs signaling also induces all known JAs biosynthesis genes including *ALLENE OXIDE CYCLASE 2* (*AOC2*) (Sasaki et al., 2001; Wasternack, 2007) in what is considered a positive feedback loop. The JAs-responsive AP2/ERF-domain transcription factor ORA47 controls all JAs biosynthesis genes (Chapter 2). *AOC2* is a COI1-dependent cycloheximide-independent primary JAs-responsive gene (Chapter 3; Zarei, 2006; Wang et al., 2008), indicating that the activity of ORA47 is regulated by a repressor protein that is degraded upon JAs signaling and recognition by the SCF^{COI1} complex.

The aim of the work described in this chapter was to identify proteins interacting with ORA47 and to determine their effect on ORA47 activity. Via yeast two-hybrid screening and pull-down assays we identified all 5 members of the Arabidopsis BTB-TAZ (Bric-à-brac, Tramtrack, Broad - Transcriptional Adaptor Zinc finger) protein family except BT2 as ORA47 interactors. ORA47 did not directly interact with JAZ proteins, but all BT proteins except BT2 did, which is compatible with a scenario where BT proteins act as adaptors between ORA47 and JAZ. BT4 and BT5 partially repressed ORA47 activity in a transient trans-activation assay, whereas JAZ1 had no effect. Analysis of quadruple *bt* knockout plants having either a wildtype *BT2* or *BT3* gene showed no effect on basal or JA-responsive expression of *AOC2*.

RESULTS

Determination of activation domains in ORA47 in yeast and Arabidopsis cells

For yeast two-hybrid screening it is important that the bait protein does not contain an activation domain. To determine whether ORA47 contains activation domains active in yeast, it was fused to the GAL4 DNA-binding domain (BD) and the activation of the GAL4-dependent *His3* selection gene was monitored. Expression of full-length ORA47 fused to GAL4BD in the vector pAS2.1 auto-activated the expression of the *His3* selection gene in yeast strain PJ69-4A (Fig. 1). Auto-activation was not repressed by addition of 5-50 mM 3-aminotriazole (3-AT), a competitive inhibitor of the HIS3 enzyme. Auto-activation is a frequently occurring problem with transcription factor baits and can be circumvented by removal of the activation domain. Testing a series of C-terminal deletion derivatives for auto-activation showed that derivatives extending until aa position 140 caused no auto-activation. Longer derivatives caused varying degrees of growth inhibition as well as auto-activation. An internal deletion derivative lacking aa 140-180 gave rise to normal growth and caused auto-activation with up to 50 mM 3-AT in the medium (Fig.1; ORA47Δ8). It can be concluded that the 140-160 region causes growth inhibition by deletion derivatives but not by the full-length protein or derivative ORA47Δ8. An activation domain is located in the 160-180 region which does not completely alleviate growth inhibition. Another activation domain is located in the C-terminal 15 aa (Fig. 1; ORA47Δ5).

To study whether the activation domains found in yeast also function in plant cells, the potential of deletion derivatives to activate the 600 bp *AOC2* promoter was studied in transfected Arabidopsis protoplasts. As shown in Figure 2, full-length ORA47 activated the promoter almost 6-fold. Derivative ORA47Δ3 lacking the C-terminal 55 aa was completely inactive. Addition of the 140-160 and 160-180 regions increased the activation potential in a stepwise manner to about 3-fold. Contrary to the yeast experiment, addition of the 180-195 region had no effect, indicating that although it acted as a potent activation

domain in yeast, it had no activity in Arabidopsis protoplasts. It can be concluded that the whole 140-195 C-terminal domain is necessary for the full activation potential of ORA47 in Arabidopsis protoplasts.

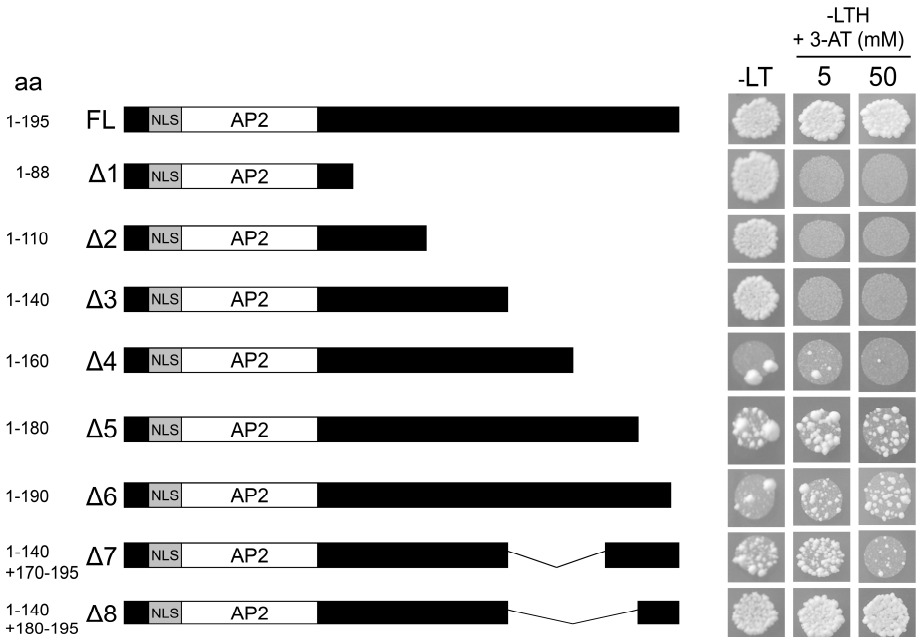

Figure 1. The C-terminal part of ORA47 contains regions that confer transcriptional activation and/or toxicity in yeast. ORA47 derivatives were fused to the GAL4 DNA-binding domain in the plasmid pAS2.1 and introduced in yeast strain pJ69-4A together with the empty vector pACT2. Cultures were spotted on minimal SD medium without Leucine and Tryptophan (-LT) to select for the plasmids and on medium additionally lacking Histidine complemented with 5 mM 3-aminotriazole (-LTH + 3-AT) to select for transcriptional activation of the *His3* gene. Growth was recorded after 7 days.

Identification of proteins that interact with ORA47

To identify proteins that interact with ORA47, yeast two-hybrid screenings were performed using derivative ORA47Δ2 extending from aa 1-110 as bait. Screening of 1.8 x 10^6 yeast transformants obtained with an Arabidopsis cDNA library generated from untreated above-ground parts of mature ecotype *Landsberg erecta* plants in the vector pACT2 resulted in 85 colonies that were able to grow on medium lacking histidine of which 74 were also able to grow

on medium lacking adenine. From 41 colonies prey plasmids were recovered. Only 14 plasmids conferred growth on selective medium after re-transformation. Eleven of these plasmids contained a cDNA encoding the small subunit of ribulose-1,5-bisphosphate carboxylase, 1 cDNA encoded the transcriptional repressor RGA1 (AT2G01570) but was not in frame with the GAL4AD, 1 cDNA encoded a protein with unknown function with AGI code AT5G50410, and 1 cDNA encoded the protein BT5 (AT4G37610). RbcS was not further analyzed because RbcS is a chloroplast protein and ORA47 is a nuclear protein, and RGA1 was discarded because it was not in frame. The analysis concentrated on AT5G50410 and BT5.

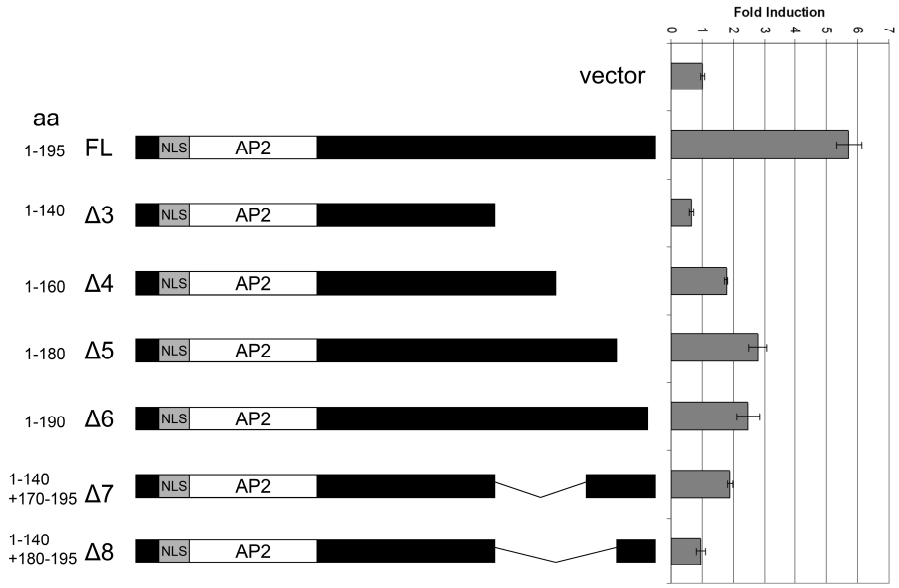

Figure 2. The C-terminal part of ORA47 contains an activation domain active in Arabidopsis protoplasts. Arabidopsis cell suspension protoplasts were co-transformed with plasmids carrying a 0.6 kb derivative of the *AOC2* promoter fused to GUS and effector plasmids without or with *ORA47* derivatives driven by the CaMV 35S promoter. GUS activities represent means ± SE of triplicate experiments and are expressed relative to the vector control.

ORA47 activity is not affected by AT5G50410

As a first step, interaction between ORA47 and AT5G50410 was tested using the in vitro pull-down technique. Radiolabelled AT5G50410 protein translated in

vitro was mixed with resin-bound recombinant Strep/His-tagged ORA47 or MYC2 proteins produced in *E. coli*. As shown in Figure 3, AT5G50410 was pulled down by ORA47-containing resin but not by empty resin. AT5G50410 also was pulled down by MYC2-coated beads, but much less efficiently.

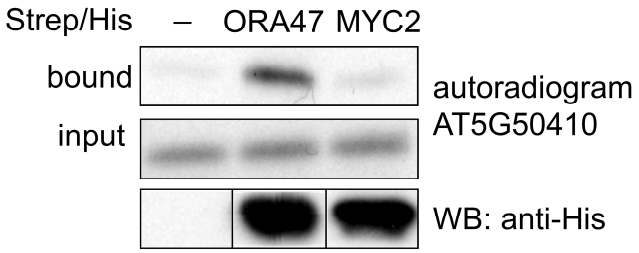

Figure 3. ORA47 interacts with AT5G50410 protein in vitro. Pull-down experiments were carried out with in vitro synthesized radiolabeled AT5G50410 protein and resin-bound recombinant Strep/His-tagged ORA47 or MYC2. Shown are autoradiograms of the input and pull-down reactions and western blots of the pull-down reactions with anti-His antibodies. The samples were analyzed on the same western blot allowing direct comparison of signals.

Next we tested whether AT5G50410 had an effect on the activation activity of ORA47. As a first step we determined the dose-response relationship for trans-activation of gene expression via the 600 bp *AOC2* promoter by ORA47. Arabidopsis cell suspension protoplasts were co-transformed with plasmids carrying the *AOC2* promoter fused to GUS and varying amounts of effector vectors without or with *ORA47* driven by the CaMV 35S promoter. As shown in Figure 4, 100 ng of ORA47 effector plasmid activated the *AOC2* promoter 4-fold. Fold activation increased with an increasing amount of effector plasmid to 7-fold with 3 µg plasmid. Based on the titration series we tested ORA47 activity with an equal amount of AT5G50410 effector plasmid and with a 5-fold mass excess. Figure 5 shows that AT5G50410 did not significantly affect ORA47 activity.

ORA47 interacts with BT proteins

Next we investigated the BT5 interactor. *BT5* belongs to a small family of 5 *BT* genes in Arabidopsis. The encoded proteins are characterized by an N-terminal

BTB (Bric-à-brac, Tramtrack, Broad) or POZ (POx virus Zinc finger) domain, a TAZ (Transcriptional Adaptor Zinc finger) domain and a C-terminal CaMBD (CalModulin Binding) Domain. The BTB/POZ and TAZ domains mediate protein-protein interactions, and the CaMBD mediates calmodulin binding in a Ca^{2+}-dependent manner. The interaction of all 5 BT proteins with ORA47 was tested in yeast. As shown in Figure 6 ORA47 interacted with BT1, BT3, BT4 and BT5. The interaction was weak since yeast growth was inhibited by addition of 5 mM 3-AT in the medium. Only BT5 showed stronger interaction allowing growth, consistent with its recovery in the screening procedure.

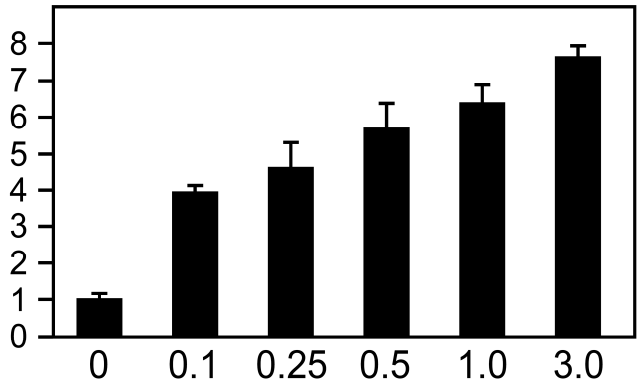

Figure 4. Dose-response relationship for trans-activation of gene expression via the *AOC2* promoter by ORA47. Arabidopsis cell suspension protoplasts were co-transformed with plasmids carrying a 0.6 kb derivative of the *AOC2* promoter fused to GUS and varying microgram amounts as indicated of overexpression vectors without or with *ORA47* driven by the CaMV 35S promoter. Total effector plasmid amounts were equalized in all reactions by addition of empty overexpression vector. GUS activities represent means ± SE of triplicate experiments and are expressed relative to the zero ORA47 effector control set at 1.

To confirm the interaction we used the in vitro pull-down technique. Radiolabelled BT4 and BT5 proteins translated in vitro were mixed with resin-bound recombinant Strep/His-tagged ORA47, MYC2 or ERF4 proteins produced in *E. coli*. As shown in Figure 7, BT4 and BT5 were pulled down by ORA47-containing resin but not by empty resin or by MYC2- or ERF4-coated resin.

ORA47 does not interact with JAZ proteins

The observation that *AOC2* is a COI1-dependent, cycloheximide-independent primary JA-responsive gene (Chapter 3; Wang et al., 2008; Zarei, 2006) indicates that pre-existing ORA47 is activated via COI1-mediated protein degradation. The most straightforward model is that a protein repressing ORA47 activity is degraded analogous to the MYC2-JAZ model. Besides interacting with MYC2, JAZ also interact with MYC3 and MYC4 and several other transcription factors belonging to different classes (Pauwels and Goossens, 2011). JAZ were not isolated in the two-hybrid screen with ORA47. However a deletion derivative was used for screening leaving open the possibility that JAZ interact with full-length ORA47. We tested this option in a yeast two-hybrid assay using MYC2 as a positive control. As shown in Figure 8 MYC2 interacted with all JAZ proteins except JAZ4 and JAZ7 consistent with previous reports (Chini et al., 2009; Fernandez-Calvo et al., 2011). On the other hand, none of the JAZ proteins interacted with ORA47.

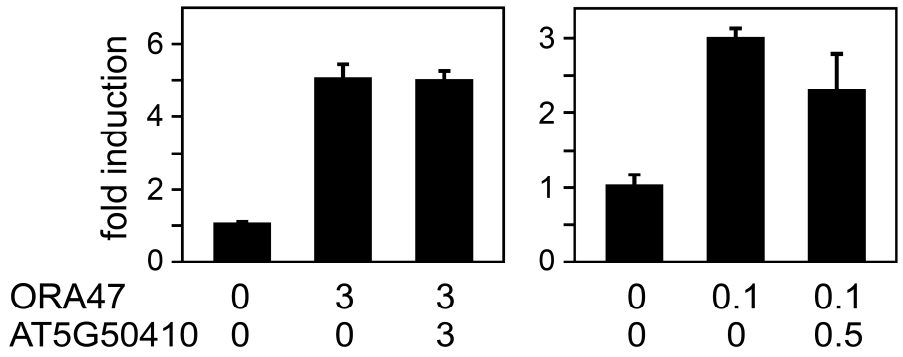

Figure 5. ORA47 activity is not affected by AT5G50410. Arabidopsis cell suspension protoplasts were transiently co-transformed with a *GUS* reporter gene driven by a 0.6 kb derivative of the *AOC2* promoter, and combinations of effector plasmids carrying *ORA47* or AT5G50410 in microgram amounts as indicated. GUS activities represent means ± SE of triplicate experiments and are expressed relative to the zero ORA47/AT5G50410 effector control set at 1.

BT proteins interact with JAZ in yeast

Another option that would still implicate JAZ proteins in regulation of ORA47 activity would be indirect binding via an adaptor protein. BT proteins are ideal candidates for such an adaptor role due to the presence of two protein-protein interaction domains. Therefore we tested interaction between the BT proteins and the JAZ family in a two-hybrid assay in yeast. As shown in Figure 9B, except for BT2, all BT proteins interacted with 2 or more JAZ proteins.

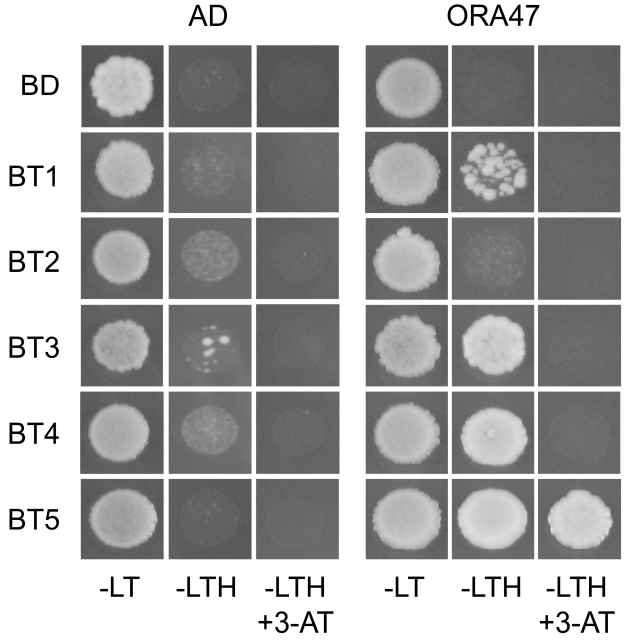

Figure 6. ORA47 interacts with BT proteins in yeast. Yeast cells expressing deletion derivative ORA47Δ2 fused to GAL4BD and BT fused to GAL4AD were spotted on minimal SD medium without Leucine and Tryptophan (-LT) to select for the plasmids and on medium additionally lacking Histidine without (-LTH) or with 5 mM 3-aminotriazole (-LTH + 3-AT) 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 the binding domain (BD) and activation domain (AD) of GAL4, respectively, were used as controls.

Interaction in most cases was weak since yeast growth was inhibited by addition of 5 mM 3-AT in the medium (Fig. 9C). Only yeast cells expressing BT4

and JAZ1 or JAZ8 were able to grow under these conditions. Interestingly, these same JAZ proteins were the only ones showing interaction with BT5 on selection medium without 3-AT (Fig. 9B). In summary, the in vitro pull-down and yeast interaction results are compatible with a scenario where BT4 and BT5 serve as adaptors to connect JAZ1 or JAZ8 to ORA47.

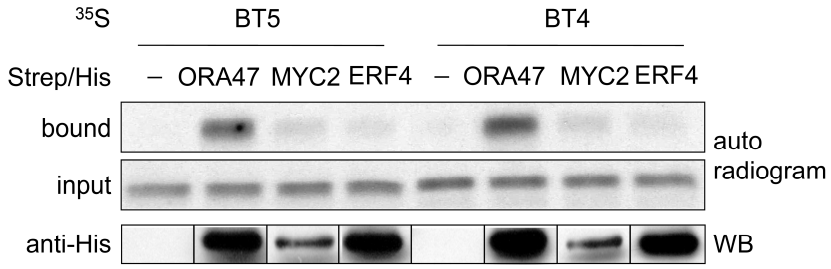

Figure 7. ORA47 interacts with BT4 and BT5 in vitro. Pull-down experiments were carried out with in vitro synthesized radiolabeled BT4 and BT5 proteins and resin-bound recombinant Strep/His-tagged ORA47, MYC2 or ERF4. Shown are autoradiograms of the input and pull-down reactions and western blots of the pull-down reactions with anti-His antibodies. The samples were analyzed on the same western blot allowing direct comparison of signals.

BT4 and BT5 proteins repress ORA47 activity

We tested whether BT4, BT5, JAZ1 or combinations thereof had an effect on the activation activity of ORA47. Arabidopsis cell suspension protoplasts were transiently co-transformed with a *GUS* reporter gene driven by a tetramer of the GCC box from the *AOC2* promoter, and combinations of effector plasmids carrying *ORA47*, BT4, BT5 and *JAZ1*. As shown in Figure 10, JAZ1 had no effect on the activity of ORA47. On the other hand, BT4, BT5 or both combined reduced ORA47 activity about 2-fold. Co-expression of JAZ1 with the BT proteins did not have an additional negative effect on ORA47 activity.

Quadruple *bt* mutants have wildtype levels of JA-responsive *AOC2* expression

To test whether BT proteins affect the activity of ORA47 and hence the expression of its target genes in plants, quadruple *bt* knockout plants were

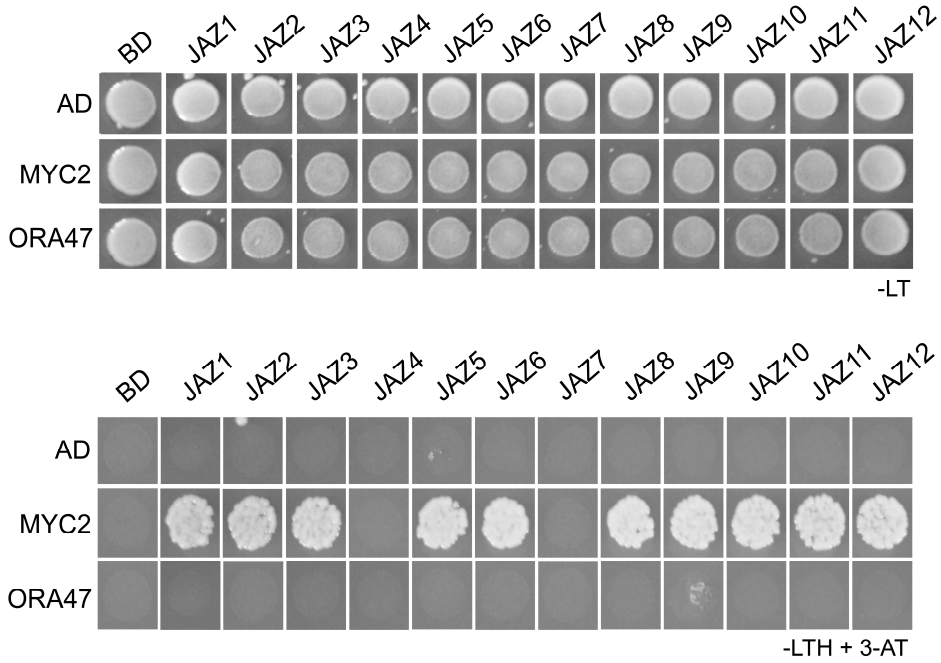

Figure 8. ORA47 does not interact with JAZ repressors in yeast. Yeast cells expressing ORA47 or MYC2 fused to GAL4AD and JAZ fused to GAL4BD were spotted on minimal SD medium without Leucine and Tryptophan (-LT) to select for the plasmids and on medium additionally lacking Histidine complemented with 5 mM 3-aminotriazole (-LTH + 3-AT) 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 the binding domain (BD) and activation domain (AD) of GAL4, respectively, were used as controls.

analyzed for JA-responsive *AOC2* expression. The mutant *bt* genes contain T-DNA or transposon insertions and do not give rise to the normal full-length transcripts (Robert et al., 2009). Since a quintuple *bt* mutant is not viable (Robert et al., 2009), we analyzed quadruple mutants which either have a wildtype *BT2* or a wildtype *BT3* gene. BT2 showed no interaction with ORA47 or JAZ proteins in yeast two-hybrid assays (Figs. 6 and 9B). Wildtype and mutant seedlings were treated for various times with 5 or 50 µM JA or the solvent DMSO and the expression of the *AOC2* gene was analyzed. From Figure 11

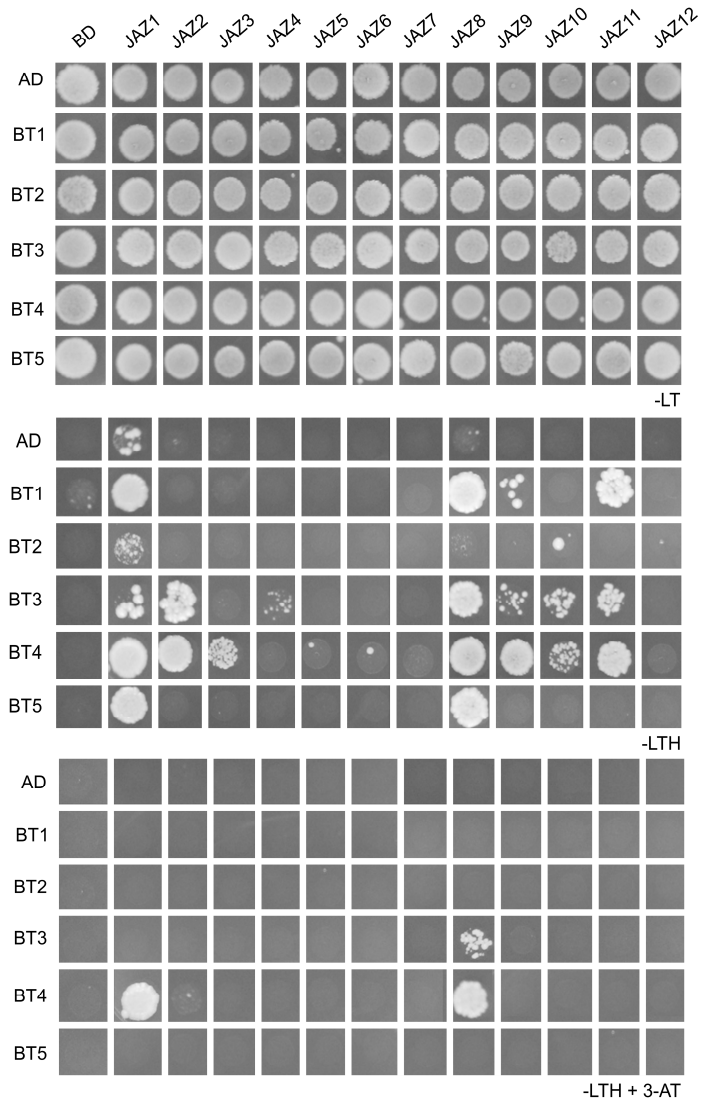

Figure 9. BT proteins interact with JAZ repressors in yeast. Yeast cells expressing BT proteins fused to GAL4AD and JAZ fused to GAL4BD were spotted on minimal SD medium without Leucine and Tryptophan (-LT) to select for the plasmids and on medium additionally lacking Histidine without (-LTH) or with 5 mM 3-aminotriazole (-LTH + 3-AT) to select for transcriptional activation of the His gene. Growth was recorded after 5 days. Yeast cells transformed with the empty plasmids pAS2.1 and pACT2, expressing the binding domain (BD) and activation domain (AD) of GAL4, respectively, were used as controls.

showing the result of a northern blot hybridization experiment it can be concluded that quadruple *bt* knockout did not change the basal expression level of the *AOC2* gene. Although a slight variation was observed between plant lines after JA treatment, there was also not a consistent effect of quadruple *bt* knockout on JA-responsive *AOC2* expression.

DISCUSSION

Based on the hypothesis that ORA47 activity is regulated by a repressor protein that is degraded upon JA signaling via recognition by the SCF^{COI1} complex, the aim of the work described in this chapter was to identify proteins interacting with ORA47 by yeast two-hybrid screening and to determine their effect on ORA47 activity.

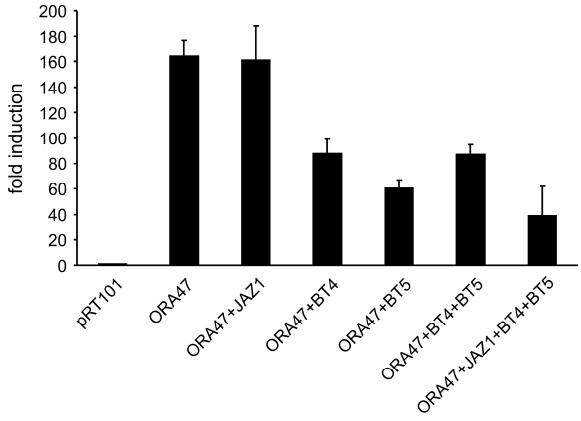

Figure 10. BT4 and BT5 proteins repress ORA47 activity. Arabidopsis cell suspension protoplasts were transiently co-transformed with a *GUS* reporter gene driven by a tetramer of the GCC box from the *AOC2* promoter, and combinations of effector plasmids carrying *ORA47*, BT4, BT5 and *JAZ1* as indicated. Total effector plasmid amounts were equalized in all reactions by addition of empty overexpression vector. GUS activities represent means ± SE of triplicate experiments and are expressed relative to the vector control.

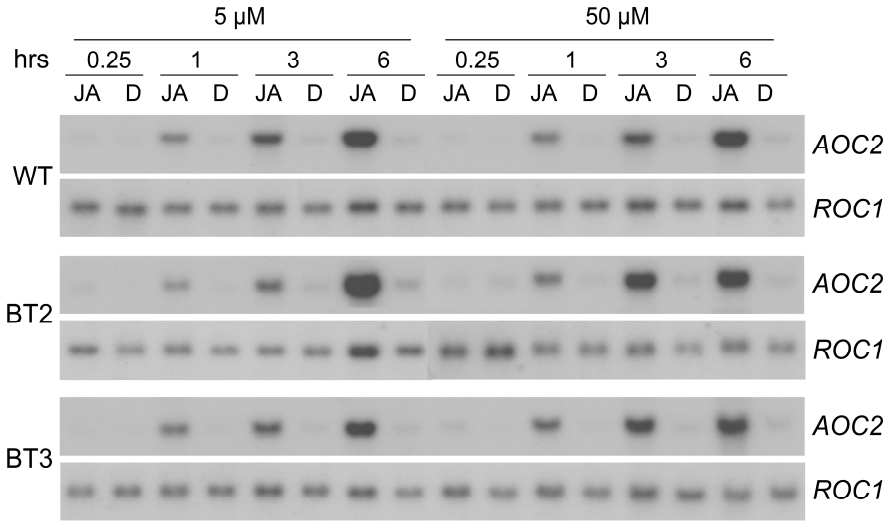

Figure 11. Quadruple *bt* mutants have wildtype levels of JA-responsive *AOC2* expression. RNA was extracted from 14-days-old wild-type or mutant Arabidopsis seedlings treated with 5 or 50 µM jasmonic acid (JA), or with the solvent DMSO (D), for the number of hours indicated. The RNA gel blot was hybridized with an *AOC2* probe. The *ROC1* probe was used to verify RNA loading. All panels for each probe were on the same blot and exposed to film for the same time allowing direct comparison of expression levels.

Screening was performed with an ORA47 derivative lacking the C-terminal half due to the fact that this region contained several domains that activated transcription in yeast. Also in plant cells the C-terminal region was required for the activity of ORA47. Screening resulted only in 2 clones that were considered worthwhile to pursue. One encoded the protein AT5G50410 with unknown function and the other encoded the BTB-TAZ protein BT5. Interaction was confirmed by in vitro pull-down experiments. AT5G50410 did not affect the activity of ORA47 in a transient trans-activation assay in Arabidopsis protoplasts. All 5 members of the BT protein family except BT2 interacted with ORA47 in yeast. BT5 showed the strongest interaction. ORA47 did not directly interact with JAZ proteins, but all BT proteins except BT2 did, which is compatible with a scenario where BT proteins act as adaptors between ORA47 and JAZ. BT4 and BT5 partially repressed ORA47 activity in a transient trans-

activation assay, whereas JAZ1 had no effect. The combination of BT4, BT5 and JAZ1 had the same partially repressing effect as the BT4 and BT5 combination. Analysis of quadruple *bt* knockout plants having either a wildtype *BT2* or *BT3* gene showed no effect on basal or JA-responsive expression of the ORA47 target gene *AOC2*.

We conclude that although BT proteins interact specifically with ORA47 and have a negative effect on its activity, they are not the hypothetical repressor or adaptor proteins that we were looking for. It is possible that the hypothetical repressor or adaptor interacts with the C-terminal domain that is missing in the ORA47 derivative used as a bait for screening. In MYC proteins for example, the relative orientation of the DNA-binding domain and the activation domain is reversed, and JAZ were shown to bind to an N-terminal region close to the putative activation domain (Fernandez-Calvo et al., 2011). Although experimental evidence is missing, the current model is that the MYC-JAZ complex while bound to its target promoters recruits the co-repressor TPL and TPL-like proteins either directly (Shyu et al., 2012) or via the adaptor protein NINJA (Pauwels et al., 2010). Therefore, it seems worthwhile to perform the search for ORA47 interactors with another method allowing the use of full-length ORA47. One such method is the TAP-tag method which allows the determination of the composition of native complexes formed in planta (Pauwels et al., 2010; Fernandez-Calvo et al., 2011).

Until now, the only proteins known to be degraded in a COI1-dependent manner upon JA signaling are the JAZ proteins (Chini et al., 2007; Thines et al., 2007). However, COI1 has been reported to interact with histone deacetylase 6 (HDA6) in yeast and in planta (Devoto et al., 2002), although JA-responsive HDA6 degradation was not established. In auxin signaling, which has many similarities to JA signaling, the transcriptional repressors Aux/IAA are the only known targets for SCF^{TIR1}-mediated degradation (Chapman and Estelle, 2009). Assuming that COI1 control of ORA47 activity proceeds via JAZ degradation, the most plausible model is that the C-terminal part of ORA47 interacts with certain members of the JAZ family via an adaptor protein.

MATERIALS AND METHODS

Plant material, growth conditions and treatments

Arabidopsis thaliana wild-type plants and the mutants *bt1BT2bt3bt4bt5* and *bt1bt2BT3bt4bt5* are in the genetic background of ecotype Col-0. Surface-sterilized seeds were grown for 10 days at 21°C in a growth chamber (16 h light/8 h dark photoperiod at 200 µE m^{-2} s^{-1} at 70% relative humidity) on solid MA medium (Masson and Paszkowski, 1992). Fifteen to 20 seedlings per sample were transferred to 50 mL polypropylene tubes (Sarstedt, Nümbrecht, Germany) containing 10 mL MA medium and incubated on a shaker at 120 rpm for 4 additional days before treatment. Seedlings were treated with 5 or 50 µM JA (Sigma-Aldrich, St. Louis, MO). As control, seedlings were treated with the solvent DMSO (0.1%).

Yeast two-hybrid screening

Full-length *ORA47* and deletion derivatives cloned in pAS2.1 (acc. No. U30497) were co-transformed with empty pACT2 (acc. No. U29899) to yeast strain PJ64-4A (James et al., 1996). For auto-activation assays, transformants were plated on minimal synthetic defined (SD)-glucose medium supplemented with Ade/Met/Ura/His and lacking Leu and Trp (-LT). Ability to activate transcription in yeast was evaluated by monitoring growth after 7 days on selective SD medium lacking Leu, Trp and His (-LTH) supplemented with increasing 3-AT concentrations ranging from 0 to 50 mM. *ORA47* deletion derivative 1-110 cloned in pAS2.1 was used as bait for the screening. Using the Stratagene cDNA synthesis kit amplified cDNA libraries representing $2x10^6$ primary transformants were prepared from an equal mixture of RNAs from stems, leaves and flowers of mature ecotype *Landsberg erecta* plants in the vector λACTII. The λACTII library was converted in a pACT2 (Clontech) 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 yeast transformation protocol modified from Gietz et al. (1992). Transformed cells were plated on SD medium containing 5 mM 3-AT and

lacking Trp, Leu and His. BTs were amplified with the primers 5′-GGA TCC GTA TGG CTA TAA CCG CCA CTC A-3′ and 5′- GGA TCC CTA TAT AAT TCG ACC GAC CA-3′ for *BT1* (*At5g63160*), 5′- GGA TCC GTA TGG AAG CTG TTC TTG TCG C-3′ and 5′- GGA TCC TTA AAC CCC TTG TGC TTG TT-3′ for *BT2* (*At3g48360*), 5′- GGA TCC GTA TGT CTA GTA GTA CCA AGA AC-3′ and 5′- GGA TCC CTA TAT CAA ACC AGA AGA AC-3′ for *BT3* (*At1g05690*), 5′-GAG GAT CCC AAT GCA GGG AAG AGA AGA TAA GC-3′ and 5′-GAG GAT CCC ATT AAC AGT TTG TCA CCG GTA-3′ for *BT4* (*At5g67480*) and 5′-GAG GAT CCC AAT GGA GAA CAT GGA CGA TTT CT-3′ and 5′-GAG GAT CCC ATC ATA AAG TAA CAT CAA TTG CT-3′ for *BT5* (*At4g37610*). JAZs were amplified with the primers 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′ for *JAZ1* (*At1g19180*), 5′-CGG GAT CCG TCG ACG AAT GTC GAG TTT TTC TGC CGA GT-3′ and 5′-CGG GAT CCC GTC GAC TTA CCG TGA ACT GAG CCA AGC T-3′ for *JAZ2* (*At1g74950*), 5′-CGC GTC GAC GTA TGG AGA GAG ATT TTC TCG G-3′ and 5′-CGG TCG ACG TTT TAG GTT GCA GAG CTG AGA G-3′ for *JAZ3* (*At3g17860*), 5′-CGC GTC GAC GTA TGG ATT GGT CAT TCT CAA G-3′ and 5′-CGG TCG ACG TTT TAG TGC AGA TGA TGA GCT G-3′ for *JAZ4* (*At1g48500*), 5′-CGG GAT CCG TCG ACG AAT GTC GTC GAG CAA TGA AAA TGC and 5′-CGG GAT CCC GTC GAC CTA TAG CCT TAG ATC GAG ATC T-3′ for *JAZ5* (*At1g17380*), 5′-CGG GAT CCG TCG ACG AAT GTC AAC GGG ACA AGC G-3′ and 5′-CGG GAT CCC GTC GAC CTA AAG CTT GAG TTC AAG GTT-3′ for *JAZ6* (*At1g72480*), 5′-CGC GTC GAC GTA TGA TCA TCA TCA TCA AAA ACT G-3′ and 5′-CGG TCG ACG TTC TAT TCG GTA ACG GTG GTA A-3′ for *JAZ7* (*At2g34600*), 5′-GAA GAT CTC TCG AGG AAT GAA GCT ACA GCA AAA TTG TG-3′ and 5′-GAA GAT CTC TCG AGC ATT ATC GTC GTG AAT GGT ACG-3′ for *JAZ8* (*At1g30135*), 5′-CGG GAT CCG TCG ACG AAT GGA AAG AGA TTT TCT GGG T-3′ and 5′-CGG GAT CCC GTC GAC TCA TAA GCC TCT CTT TGC G-3′ for *JAZ9* (*At1g70700*), 5′-CGG GAT CCG TCG ACG AAT GTC GAA AGC TAC CAT AGA ACT-3′ and 5′-CGG GAT CCC GTC GAC TTA GGC CGA TGT CGG ATA GT-3′ for *JAZ10* (*At5g1322*), CGC GTC GAC GTA TGG CTG AGG

TAA ACG GAG A-3′ and 5′-CGG TCG ACG TTT CAT GTC ACA ATG GGG CTG G-3′ for *JAZ11* (*At3g43440*) and 5′-CGG GAT CCG TCG ACG AAT GAC TAA GGT GAA AGA TGA GC-3′ and 5′-CGG GAT CCC GTC GAC CTA AGC AGT TGG AAA TTC CTC-3′ for *JAZ12* (*At5g20900*). Interaction assays were performed by co-transformation of bait and prey plasmids into yeast strain PJ64-4A and plating 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 incubated for 16 hours in liquid SD-LT and 5 µl of 100-fold dilutions were spotted on solid SD-LTH supplemented with 5 mM 3-AT. Yeast cells were allowed to grow for 7 days at 30°C.

In vitro pull-down assays

ERF4 (*At3g15210*) was amplified with the primers 5′-CGG AAT TCA ATG GCC AAG ATG GGC TTG AAA CCC-3′ and 5′-CGG TCG ACC CTT GGG CCT GTT CCG ATG GAG-3′ and cloned in EcoRI/SalI digested pASK-IBA45 (IBA Biotagnology, Göttingen, Germany). MYC2 in pASK-IBA45 (Montiel et al., 2011) and ORA47 in pASK-IBA45plus (Zarei et al., 2011) were described previously. *BT4* (*At5g67480*) was amplified with the primers 5′-GAG GAT CCC AAT GCA GGG AAG AGA AGA TAA GC-3′ and 5′-GAG GAT CCC ATT AAC AGT TTG TCA CCG GTA-3′ and *BT5* (*At4g37610*) was amplified with the primers 5′-GAG GAT CCC AAT GGA GAA CAT GGA CGA TTT CT-3′ and 5′-GAG GAT CCC ATC ATA AAG TAA CAT CAA TTG CT-3′. PCR fragments were digested with BamHI and cloned in pBluescript II SK+ plasmid. In vitro synthesis of full-length BT4 and BT5 proteins was achieved using a pBluescript II SK+ plasmid template with the TNT Coupled Wheat Germ Extract System (Promega) in the presence of [^{35}S]methionine. Recombinant Strep/His-tagged proteins were expressed in *Escherichia coli* BL21 (DE3) pLysS cells. Crude protein extracts containing about 5 µg of Strep/His-tagged protein were mixed with 100 µL of 50% Strep-Tactin resin slurry, incubated for 1 hr at 4 ^{o}C with mixing and washed twice. For each pull-down experiment 10-15 µl of in vitro translated protein reaction in 500 µl of incubation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 0.2% Nonidet P40, 1 mM

phenylmethylsulphonyl fluoride, protease inhibitor cocktail (Roche)) was added. After 60 min rotation at room temperature, the samples were washed 6 times. The washed resin was resuspended in 30 µl of SDS-PAGE loading buffer. Following boiling for 5 min, 20 µl of the samples were subjected to 10% SDS-PAGE. The gel was incubated in Amplify reagent (Amersham), dried and exposed to X-ray film (Fuji, Tokyo, Japan). For western blot analysis 5 µl of the samples were subjected to 10% SDS-PAGE and transferred to Protran nitrocellulose (Schleicher&Schuell) by semidry blotting. Detection was done by incubation with Penta-His HRP antibody conjugate (Qiagen 1:20000), following blocking with Penta-His HRP blocking agent, washing and incubation in 6 ml luminol solution (250 µM sodium luminol (Sigma), 0.1 M Tris-HCl pH 8.6, 0.01% H_2O_2) mixed with 60 µl enhancer solution (67 µM p-hydroxy coumaric acid (Sigma) in DMSO) to visualize the proteins by enhanced chemiluminescence detection using X-ray films.

Plasmid construction and protoplast assays

A 600 bp *AOC2* promoter fragment was amplified on Arabidopsis genomic DNA with the primer set 5'-GCT CTA GAA TAA AAA TCA GTG TTC TAT CC-3' and 5'-TGG TCG ACT GAT AAA AAT AAA ATA AAA AG-3', digested with XbaI and SalI and cloned in plasmid GusSH (Pasquali et al., 1994). The *ORA47* (At1g74930) open reading frame (ORF) was PCR-amplified using the primer set 5'-GAA GAT CTC ATA TGG TGA AGC AAG CGA TGA AG-3' and 5'-GAA GAT CTT CAA AAA TCC CAA AGA ATC AAA G-3', digested with BglII and cloned into BamHI digested pRT101 (Töpfer et al., 1987). The *BT4* (*At5g67480*) ORF was PCR-amplified using the primer set 5'-GAG GAT CCC AAT GCA GGG AAG AGA AGA TAA GC-3' and 5'-GAG GAT CCC ATT AAC AGT TTG TCA CCG GTA-3', digested with BamHI and cloned in pRT101. The *BT5* (*At4g37610*) ORF was PCR-amplified using the primer set 5'-GAG GAT CCC AAT GGA GAA CAT GGA CGA TTT CT-3' and 5'-GAG GAT CCC ATC ATA AAG TAA CAT CAA TTG CT-3', digested with 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 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* fused to the CaMV 35S promoter were carried out. To study a possible effect of BT interaction with the transcription factors, a ratio of 2:2:2 or 2:6:2 (µg GUS::effector plasmid) 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.

RNA extraction and Northern blot analyses

Total RNA was extracted from pulverized 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). All probes were ^{32}P-labeled by random priming. Pre-hybridization of blots, hybridization of probes and subsequent washings were performed as described (Memelink et al., 1994) with minor modifications. Blots were exposed on X-ray films. DNA fragments used as probes were PCR amplified from Arabidopsis genomic DNA. The following primer sets were used: 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)* and 5,-CGG GAA GGA TCG TGA TGG A-3' and 5'- CCA ACC TTC TCG ATG GCC T-3' for *ROC1 (At4g38740).*

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