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The transcription factor JAM1 interacts with JAZ proteins to repress JA-responsive gene expression

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Abstract

Upon pathogen infection and herbivore attack, plants produce the signaling molecule jasmonoyl-L-isoleucine (JA-Ile), which is perceived by the COI1-JAZ receptor complex. This leads to degradation of JAZ repressor proteins via the 26S proteasome system thereby de-repressing transcription factors including MYC2, MYC3 and MYC4, basic helix-loop-helix transcription factors that play a positive regulatory role in JAs signaling, resulting in transcriptional activation of downstream target genes. Here, we describe another basic helix-loop-helix transcription factor, JA-Associated MYC2-Like1 (JAM1) as a novel target of JAZ proteins identified by yeast two hybrid screening. Bimolecular fluorescence complementation assays in Arabidopsis cell suspension protoplasts showed that JAM1 interacted with JAZ proteins in the nucleus. JAM1 repressed MYC2 target gene expression in trans-activation assays. Moreover, JAZ proteins enhance the repressive activity of JAM1. Our results indicate that JAM1 functions as a transcription repressor whereas JAZ proteins act as co-repressors to enhancing JAM1 activity.
Introduction

Jasmonates (JAs) are lipid-derived signaling molecules that regulate diverse growth and developmental processes as well as adaptive responses to biotic and abiotic stresses in plants (Pieterse et al., 2009; Hu et al., 2013). Bioactive JAs (i.e. jasmonoyl-L-isoleucine or JA-Ile) is perceived by the F-box protein CORONATINE INSENSITIVE1 (COI1), which subsequently facilitates the degradation of transcriptional repressors called JAZ via the SCFCOI1-26S proteasome pathway (Xie et al., 1998; Xu et al., 2002; Chini et al., 2007; Thines et al., 2007; Yan et al., 2009; Sheard et al., 2010). JAZ interact with and repress a variety of transcription factors (TFs) (Chini et al., 2007; Cheng et al., 2011; Fernández-Calvo et al., 2011; Niu et al., 2011; Song et al., 2011; Qi et al., 2011; Zhu et al., 2011; Hu et al., 2013; Jiang et al., 2014). The degradation of JAZ leads to release of the TF from the JAZ complex and to activation of its target genes. The basic helix-loop-helix (bHLH) TF MYC2, which regulates diverse JAs responses, was first identified as a JAZ interactor (Chini et al., 2007). More recently, two MYC2 related proteins called MYC3 and MYC4 also have been shown to be repressed by JAZ proteins (Cheng et al., 2011; Fernández-Calvo et al., 2011; Niu et al., 2011). JAZ repressors also interact with, and suppress transcriptional activities of other TFs, including the R2R3-MYB TF MYB21 and MYB24 (Song et al., 2011), and the bHLH TF GL3, EGL3 and TT8 (Qi et al., 2011), ICE1 and ICE2 (Hu et al., 2013), WRKY57 (Jiang et al., 2014), and also two ethylene (ET)-associated TFs EIN3 and EIL1 (Zhu et al., 2011), with different roles in regulating JAs-responsive gene expression.

JAM1 (JA-Associated MYC2-Like1), previously called AIB (ABA-Inducible bHLH-Type), was most recently discovered as a transcriptional repressor and negatively regulates JAs signaling (Nakata et al., 2013; Sasaki-Sekimoto et al., 2013). Compared with MYC2, JAM1 possesses transcriptional repression activity, and jam1 loss-of-function mutants show a JAs-hypersensitive phenotype and elevated JAs responsiveness. In addition, JAM1 and MYC2 competitively bind to a MYC2 target sequence, which provides a mechanism for negative regulation of JAs signaling by JAM1 through antagonizing the transcription activator MYC2 (Nakata et al., 2013; Sasaki-Sekimoto et al., 2013). Most JAZ repressors are able to interact with MYC2 in pull-down and yeast two-hybrid assays (Chini et al., 2007, 2009; Melotto et al., 2008). Similarly, most JAZ proteins also interact with MYC3 and MYC4 (Cheng et al., 2011; Fernández-Calvo et al., 2011; Niu et al., 2011).

The aim of the work described in this chapter was to determine whether JAZ proteins have an effect on JAM1 activity. Yeast two hybrid and BiFC assays showed that some of the JAZ proteins interacted with JAM1. In Arabidopsis protoplast transactivation assays, we discovered that the JAZ proteins can increase the repression activity of JAM1. Our results indicate that JAZs act as co-repressors to enhance the repressing activity of JAM1 in the JAs signaling pathway.

Results

JAM1 interacts with JAZ proteins in yeast
Most JAZ proteins were shown to interact in vitro and in yeast with MYC2 (Chini et al., 2007, 2009; Chung and Howe, 2009), and the related bHLH TFs MYC3 and MYC4 (Cheng et al., 2011; Fernández-Calvo et al., 2011; Niu et al., 2011). Since JAM1, a bHLH TF, acts as a transcriptional repressor in JAs-responsive gene expression and its transcript level was reduced in the coi1-1 mutant (Yan et al., 2007; Nakata et al., 2013; Sasaki-Sekimoto et al., 2013), we performed a yeast two-hybrid assay to test whether JAM1 protein could interact with JAZ proteins. We used MYC2 as a positive control. As shown in Figure 1, MYC2 interacted with all JAZ proteins except JAZ4 and JAZ7 consistent with previous reports (Chini et al., 2009; Fernández-Calvo et al., 2011). Interestingly, JAM1 interacted with the JAZ proteins JAZ5, JAZ6, JAZ7, JAZ8, JAZ10 and JAZ11. Yeast cells co-expressing JAM1 and JAZ were able to sustain growth at 3-AT concentrations up to 10 mM on selective medium. These interactions are considered significant since background auto-activation was undetectable. To investigate which domain of JAM1 is responsible for the interaction with JAZ proteins, we constructed a JAM1 deletion derivative which lacks the JAZ-Interaction-Domain (JID) (Fernández-Calvo et al., 2011). Consistent with a previous speculation (Fernández-Calvo et al., 2011), the JID domain of the JAM1 protein was responsible for the interactions with JAZ proteins (data not shown).

JAM1 interacts with JAZ proteins in planta

To verify the interactions between JAZ proteins and JAM1 in planta, we performed bimolecular fluorescence complementation (BiFC) assays in Arabidopsis protoplasts. The N-terminal (YN) or C-terminal (YC) fragments of the yellow fluorescent protein (nYFP or cYFP) were fused either N-terminally or C-terminally with JAZs and JAM1. The constructs were transiently co-expressed in all possible combinations of nYFP and cYFP fusion proteins in Arabidopsis suspension cell protoplasts. As shown in Figure 2, strong YFP signals were observed in the nucleus of Arabidopsis protoplasts upon co-expression of JAZ5-cYFP and nYFP-JAM1, JAZ6-cYFP and nYFP-JAM1, JAZ7-cYFP and nYFP-JAM1, cYFP-JAM1 and nYFP-JAZ8, JAZ10-cYFP and nYFP-JAM1, JAZ11-cYFP and nYFP-JAM1, respectively. A similar result was observed upon co-expression of nYFP-MYC2 with JAZ5-cYFP as a positive control. No or only background YFP fluorescence was detected in negative controls (nYFP-JAM1 co-expressed with cYFP or nYFP co-expressed with JAZ-cYFP) (data not shown). These results indicated that the JAM1 protein interacts with JAZ proteins in planta in the nucleus.

JAM1 repressor activity is enhanced by JAZ proteins

In order to elucidate the functional significance of the interaction between JAZ and JAM1, trans-activation assays were performed. MYC2 trans-activated the JAs-responsive element (also called ‘D’) of the CrORCA3 gene in the protoplast assay as previously reported (Montiel et al., 2011). Since JAM1 and MYC2 have similar DNA sequence-specific binding preferences (Nakata et al., 2013), we used the 4D-GUS gene as a reporter. Effector constructs for JAM1, JAZ, or MYC2 were expressed under the
control of the CaMV 35S promoter. Arabidopsis cell suspension protoplasts were transiently co-transformed with a 4D-GUS reporter and effectors. As a positive control, the MYC2 protein activated the expression of 4D-GUS about four-fold, whereas co-expression of JAZ5 with MYC2 largely abolished MYC2-activated 4D-GUS expression (Figure 3). In contrast, JAM1 functioned as a transcription repressor. Co-expression of any of the 6 tested JAZ proteins resulted in even lower reporter gene expression, indicating that JAZ act as co-repressors to enhance JAM1 repression activity. These results suggest that JAM1 acts as a transcriptional repressor, and that JAZ proteins interact with JAM1 to enhance its repression activity.

**Figure 1.** JAM1 interacts with JAZ proteins in yeast. Yeast cells expressing JAM1 or MYC2 fused to the GAL4 activation domain (AD) and JAZ fused to the GAL4 DNA-binding domain (BD-) were spotted on minimal SD medium without Leucine and Tryptophan (-2) to select for the plasmids and on medium additionally lacking Histidine with 10 mM 3-aminotriazole (-3) to select for transcriptional activation of the His3 gene. Growth was monitored after 7 days. Yeast cells transformed with the empty plasmids pAS2.1 and pACT2, expressing GAL4 BD and AD, respectively, were used as controls.
Figure 2. JAM1 interacts with JAZ proteins in planta. YFP fluorescence and merged fluorescence/bright field images of Arabidopsis cell suspension protoplasts co-transformed with constructs encoding the indicated fusion proteins with YFP at the C-terminus (YC) or the N-terminus (YN). Scale bar = 10 \( \mu \)m.

Figure 3. JAZ proteins enhance JAM1 repression activity. Arabidopsis cell suspension protoplasts were co-transformed with plasmids carrying 4D::GUS (2 \( \mu \)g) and overexpression vectors containing 35S::JAM1 (2 \( \mu \)g) or MYC2 (2 \( \mu \)g) and JAZ (6 \( \mu \)g) as indicated. Protein concentrations were used to correct for differences in protein extraction efficiencies. Values represent means \( \pm \) SE of triplicate experiments.
Figure 4. Model of JAM and MYC activity regulated by JAZ proteins. Upon perception of JA-Ile, SCF\(^{COI1}\) bind JAZs which are subsequently degraded via the 26S proteasome. Released MYC activate expression of their target genes. JAM negatively regulate the targets of MYC. JAM transcriptional repressor activity is enhanced by interacting JAZ proteins.

Discussion

*JAM1* encodes a MYC2-like bHLH TF and was previously reported to be a positive regulator of ABA signaling (Li et al., 2007). As the *JAM1* transcript level was reduced in the *aos* mutant and was induced by wounding (Yan et al., 2007), we explored the hypothesis that JAM1 is involved in JAs signaling. Very recently, JAM1 was shown to act as a transcriptional repressor and to negatively regulate JAs signaling (Nakata et al., 2013). Transgenic plants over-expressing *JAM1* showed substantial reduction of JA responses, whereas *jam1* loss-of-function mutants showed enhanced JAs responsiveness (Nakata et al., 2013). As MYC2 and JAM1 protein share significant amino acid identities (83.9% identity) around the bHLH domain, it is tempting to speculate that JAM1 and MYC2 have similar DNA binding affinities. The results of electrophoretic mobility shift assays (EMSAs) showed that JAM1 can bind MYC2 target sequences, including the G-box (CACGTG), TG-box (CACGTT), and MYC2 binding sequence (MBS) in *rd22* (CACATG) (Abe et al., 1997; Nakata et al., 2013). Therefore, it is likely that JAM1 and MYC2 compete for binding to G-box related sequences, which would explain their antagonistic regulation of downstream genes. Previous reports showed that MYC2 can activate the JAs-responsive element (also called ‘D’) of the *CrORCA3* gene in the Arabidopsis protoplast assay (Montiel et al., 2011). Our results also show that MYC2 activated the expression of 4D-GUS, whereas JAM1 repressed 4D-GUS expression. These results indicate that the JAM1 protein negatively regulates JAs responsive gene expression. In addition, three bHLHs (bHLH003/At4g16430, bHLH13/At1g01260 and bHLH14/At4g00870), which are closely related to JAM1 belong to the family of bHLH subgroup IIId TFs, and were shown to have a similar activity as JAM1 (Sasaki-Sekimoto et al., 2013; Song et al., 2013).
The JAZ proteins negatively regulate JAs mediated responses via interaction with TFs such as MYC2 (Chini et al., 2009; Chung and Howe, 2009), and the related bHLH subgroup IIIe TFs MYC3 and MYC4 (Cheng et al., 2011; Fernández-Calvo et al., 2011; Niu et al., 2011). Here, we identified the bHLH subgroup IIId TF JAM1 as a new target of JAZ proteins (Figure 1). However, our results contradict a recent publication showing that JAM1/bHLH17 interacts with all JAZ proteins except JAZ7 and JAZ12 (Song et al., 2013). Our results showed that JAM1 interacts with six JAZ proteins, i.e. JAZ5, JAZ6, JAZ7, JAZ8, JAZ10 and JAZ11. It is possible that the contradictory observations are caused by differences in yeast two hybrid assays, as we evaluated the positive interactions on selective medium containing 10 mM 3-AT which suppresses auto-activation. To examine if the JAZ proteins contribute to modulation of JAM1 transcriptional activity, we further performed Arabidopsis cell suspension protoplasts assay. The results reveal that JAZ proteins act as co-repressors to enhance JAM1 repression activity. However, Song et al. (2013) reported that JAZ proteins function as negative regulators to inhibit JAM1 repression activity, which is based on theoretical considerations an unlikely mechanism. Their conclusions were based on experiments with JAZ1, which did not interact with JAM1 in our yeast two-hybrid assays, and no other JAZ proteins were tested.

The research described in this chapter provides novel insight in the roles of JAZ and JAM1 in the JAs signaling pathway. JAM1, a novel target of JAZ proteins, functions as a negative regulator in the MYC2/3/4-regulated pathway to attenuate JAs responses (Figure 4). The complex mechanism of JAs signal transduction mediated by the transcription repressors JAM and the transcription activators MYC may provide an important strategy for plant survival in their fluctuating environment.

Materials and Methods

Yeast two hybrid assays

Full-length MYC2, JAM1 and JAM1 deletion derivative JAM1ΔJID cloned in pACT2 (acc. No. U29899) were co-transformed with empty pAS2.1 (acc. No. U30497), and pAS2.1-JAZ to yeast strain PJ69-4A (James et al., 1996). JAM1 (At2g46510) was PCR amplified with the primer set 5’-TGC CAT GGA GAT GAA TAT GAG TGA TTT AGG TTG-3’ and 5’-CCC TCG AGT TAT ATA TCA CCA GAG ACC TG-3’, digested with NcoI and XhoI and cloned in pACT2. JAM1ΔJID cloned in pACT2 with the following primer set: 5’-TGC CAT GGA GAT GAA TAT GAG TGA TTT AGG TTG-3’ and 5’-CGG GAT CCG TCG ACG AAT GTC GAG TTC TAT GGA ATG TTC -3’, and JAM1ΔJID fragment 1 (JAM1ΔJID-F1), 5’-GAA GAT CTT TCT TAG CTT CCA TGT ATT TCT TC-3’ and 5’-CCC TCG AGT TAT ATA TCA CCA GAG ACC TG-3’ for JAM1ΔJID fragment 2 (JAM1ΔJID-F2), JAM1ΔJID-F1 fragment digested with NcoI and BamHI and cloned in pACT2 digested with NcoI and BamHI, the JAM1ΔJID-F2 fragment was digested with BglII and XhoI and cloned in pACT2- JAM1ΔJID-F1 digested with BamHI and XhoI. MYC2 was digested from pAS2.1 with NcoI, and cloned in pACT2. JAZ cloned in pAS2.1 were amplified with the primers 5’-CGG GAT CCG TCG ACG AAT GTC GAG TTC TAT GGA ATG TTC-3’ and 5’-
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CGG GAT CCC GTC GAC 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 GCA ACT GAG CCA AGC T-3' for JAZ2 (At1g74950), 5'-CGG GAT CCG TCG GTA TGG AGA GAG ATT TTC TCG G-3' and 5'-CGG TCG ACG TTT TAG GTT GTA GTA ACT TGA A G-3' for JAZ3 (At3g17860), 5'-CGG GAT CCG TCG GTA TGG AGA TTT TTC GAC GTA CTA TAT TAG AGC T-3' and 5'-CGG GAT CCC GTC GAC TAT TAG AGA TTA AGC T-3' for JAZ5 (At1g17380), 5'-CGG GAT CCG TCG ACG AAT GTC GTC GAG CAA TGA AAA TGC and 5'-CGG GAT CCC GTC GAC TAT TTC AGC TGC TAA ACC G-3' for JAZ9 (At1g70700), 5'-CGC GTC GAC GTA TGG AGA GAG ATT TTC TCG G-3' and 5'-CGG TCG ACG TTT TAG GTT GTA GTA ACT TGA A G-3' for JAZ10 (At5g13220), 5'-GAA GAT CTC TCG AGG AAT GAA GCT ACA GCA AAA TG TTT G-3' and 5'-GAA GAT CTC TCG AGG AAT GAA GCT ACA GCA AAA TG TTT G-3' for JAZ8 (At1g30135), 5'-CGG GAT CCG TCG ACG AAT GGA AAG AGA GTA TTT TCT GGG T-3' and 5'-CGG GAT CCG TCG ACG AAT GGA AAG AGA GTA TTT TCT GGG T-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 AGG AAT GAA GCT ACA GCA AAA TTG TG-3' for JAZ20 (At1g72450), 5'-CGG GAT CCG GTC GTA TCA TCA TCA TCA AAA ACT G-3' and 5'-CGG GAT CCG GTC GTA TCA TCA TCA TCA AAA ACT G-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 AGG AAT GAA GCT ACA GCA AAA TTG TG-3' for JAZ8 (At1g30135), 5'-CGG GAT CCG TCG ACG AAT GGA AAG AGA GTA TTT TCT GGG T-3' and 5'-CGG GAT CCG TCG ACG AAT GGA AAG AGA GTA TTT TCT GGG T-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 AGG AAT GAA GCT ACA GCA AAA TTG TG-3' for JAZ20 (At1g72450), 5'-CGG GAT CCG GTC GTA TCA TCA TCA TCA AAA ACT G-3' and 5'-CGG GAT CCG GTC GTA TCA TCA TCA TCA AAA ACT G-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 AGG AAT GAA GCT ACA GCA AAA TTG TG-3' for JAZ20 (At1g72450).

Interaction assays were performed by co-transformation of bait and prey plasmids into yeast strain PJ69-4A according to a yeast transformation protocol modified from Gietz et al. (1992), and plated on minimal synthetic defined (SD)-glucose medium, supplemented with Met/Ura/His and lacking Leu and Trp (-LW) 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-LW and 10 µL of 10 and 100-fold dilutions were spotted on selective solid SD medium lacking Leu, Trp and His (LWH) supplemented with increasing 3-AT concentrations ranging from 0 to 50 mM. Yeast cells were allowed to grow for 7 days at 30 °C.

Arabidopsis protoplast transformation and microscopic analysis

Primer sets used for BiFC cloning were: 5'-GAT CGT CGA CAA TGA ATA TGA GTG ATT TAG GTT GTA A G-3' and 5'-GCA AGC GGC GGC GTC GTA ATT TCA CCA ACC GAG ACC 'TG TGT GAA C-3' for JAM1 cloning with SalI and NotI in pRTL2-EEYN (728) and -HAYC (738); 5'-GAT CGT CGA CAA TGA ATA TGA GTG ATT TAG GTT G-3' and 5'-CAG TAG ATC TTT ATT TCA TAT ACC CAG AGA CCT C-3' for JAM1 cloning with SalI and BglII in pRTL2-YNEE (736) and -YCHA (735); 5'-GAT CGT CGA CAA TGT CGT CGA GCA ATG AAA ATG C-3' and 5'-GCA AGC GGC GGC GTC GTA ATT TCA CCA ACC GAG ACC 'TG TGT GAA C-3' for JAM1 cloning with SalI and NotI in pRTL2-EEYN and -HAYC; 5'-CGG GAC TAG TAT TTC GTC GTA GAG CCA AAA TGC-3' and 5'-CGG GAT CCC GTC GAC TAT TTC AGC TGC TAA ACC G-3' for JAZ1 (At1g72450), 5'-CGG GAT CCG TCG ACG AAT GTC GTC GAG CAA TGA AAA TGC and 5'-CGG GAT CCC GTC GAC TAT TTC AGC TGC TAA ACC G-3' for JAZ9 (At1g70700).
TTC G-3’ for JAZ5 cloning with SpeI and BamHI in pRTL2-YNEE and -YCHA; 5’-GAT CGT CGA CAA TGT CAA CGG GAC AAG CGC CG-3’ and 5’-GCA AGC GGC CGC GTA AGC TTG AGT CAA AGG TTT TTG-3’ for JAZ6 cloning with SalI and NorI in pRTL2-EEYN and -HAYC; 5’-GAT CGT CGA CAA TGT CAA CGG GAC AAG CGC CG-3’ and 5’-CAG TAG ATC TCT AAA GCT GTA TAA CAA GGT TTT TG-3’ for JAZ6 cloning with SalI and BglII in pRTL2-YNEE and -YCHA; 5’-GAT CGT CGA CAA TGT CAA CGG GAC AAG CGC CG-3’ and 5’-GCA AGC GGC CGC GTC TCA TCA TCA TCA TCA TCA TCA AAA ACT GCG-3’ and 5’-GCA AGC GGC CGC GTC TCA TCA TCA TCA TCA TCA AAA ACT GCG-3’ and 5’-GCA AGC GGC CGC GTC TCA TCA TCA TCA TCA TCA AAA ACT GCG-3’ and 5’-GCA AGC GGC CGC GTC TCA TCA TCA TCA TCA TCA AAA ACT GCG-3’ and 5’-GCA AGC GGC CGC GTC TCA TCA TCA TCA TCA TCA AAA ACT GCG-3’ and 5’-GCA AGC GGC CGC GTC TCA TCA TCA TCA TCA TCA AAA ACT GCG-3’ and 5’-GCA AGC GGC CGC GTC TCA TCA TCA TCA TCA TCA AAA ACT GCG-3’ and 5’-GCA AGC GGC CGC GTC TCA TCA TCA TCA TCA TCA AAA ACT GCG-3’ and 5’-GCA AGC GGC CGC GTC TCA TCA TCA TCA TCA TCA AAA ACT GCG-3’ and 5’-GCA AGC GGC CGC GTC TCA TCA TCA TCA TCA TCA AAA ACT GCG-3’ and 5’-GCA AGC GGC CGC GTC TCA TCA TCA TCA TCA TCA AAA ACT GCG-3’ and 5’-GCA AGC GGC CGC GTC TCA TCA TCA TCA TCA TCA AAA ACT GCG-3’ and 5’-GCA AGC GGC CGC GTC TCA TCA TCA TCA TCA TCA AAA ACT GCG-3’ and 5’-GCA AGC GGC CGC GTC TCA TCA TCA TCA TCA TCA AAA ACT GCG-3’ and 5’-GCA AGC GGC CGC GTC TCA TCA TCA TCA TCA TCA AAA ACT GCG-3’ and 5’-GCA AGC GGC CGC GTC TCA TCA TCA TCA TCA TCA AAA ACT GCG-3’ and 5’-GCA AGC GGC CGC GTC TCA TCA TCA TCA TCA TCA AAA ACT GCG-3’ and 5’-GCA AGC GGC CGC GTC TCA TCA TCA TCA TCA TCA AAA ACT GCG-3’. 

PCR-amplified inserts were digested with the restriction enzymes mentioned above and cloned in the mentioned pRTL2 derivatives digested with the corresponding enzymes (Bracha-Drori et al., 2004). Co-transformation 10 µg each of plasmids carrying N-terminal YFP fused protein (728 and 736) and C-terminal YFP fused protein (738 and 735) were introduced by PEG-mediated transfection as previously described into Arabidopsis protoplasts (Schirawski et al., 2000). Images of transfected protoplasts were acquired with a Leica DM IRBE confocal laser scanning microscope equipped with an Argon laser line of 488 nm (excitation) and a band pass emission filter of 500-550 nm.

**Arabidopsis protoplast transactivation assays**

The JAM1 open reading frame (ORF) was PCR amplified from cDNA of JA-treated seedlings with the primer set 5’-CGC TCG AGA TGA ATA TGA GTG ATT TAG GTT G-3’ and 5’-GCG GTA CCT TAT ATA TCA CCA GAG ACC TG-3’, digested with XhoI and KpnI and cloned into pRT101 (Topfer et al., 1987). MYC2 in pRT101 was described previously (Montiel et al., 2011). Tetramerized ORCA3 promoter fragment D in reporter plasmid GusSH-47 (4D-GUS) was described previously (Vom Endt et al., 2007). JAZ5, JAZ6 and JAZ10 were digested from pGEMT-Easy with BamHI and cloned in pRT101 digested with BamHI. JAZ7 was
digested from pGEMT-Easy with SalI and cloned in pRT101 digested with XhoI. JAZ8 was digested from pGEMT-Easy with XhoI and cloned in pRT101 digested with XhoI. JAZ11 was digested from pGEMT-Easy with EcoRI and cloned in pRT101 digested with EcoRI. 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 4D-GUS and effector plasmids carrying JAM1, MYC2, or JAZ5, or JAZ6, or JAZ7, or JAZ8, or JAZ10, or JAZ11 fused to the CaMV 35S promoter were carried out. To study a possible effect of JAZ interaction with the regulators, a ratio of 2:2:6 (µg GUS: MYC2 or JAM1: JAZ: effector plasmid) was chosen. As controls, co-transformations of 4D-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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