# Regulation of ORA59, a key modulator of disease resistance in Arabidopsis

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# CHAPTER 3

## Jasmonic acid induces stabilization and nuclear localization of ORA59, an AP2/ERF-domain transcription factor essential for defense responses in Arabidopsis

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**Abstract**

Plant defense against pathogens and herbivores depends on the action of several endogenously produced hormones, including jasmonic acid (JA) and ethylene. In certain defense responses, JA and ethylene signaling pathways synergize to activate a specific set of defense genes. The AP2-domain transcription factor ORA59 acts as the integrator of the JA and ethylene signaling pathways. How JA and ethylene affect the activity of ORA59 is not known. The aim of the studies reported here was to determine whether JA has an activating effect on ORA59 at the protein level. The results show that JA caused stabilization as well as nuclear localization of ORA59. Interestingly, nuclear localization of ORA59 did not require a functional COI1 protein. We postulate that there is a jasmonate receptor distinct from COI1, an F-box protein that targets ORA59 for degradation, and a repressor protein that sequesters ORA59 in the cytoplasm.

**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 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 (Turner et al., 2002). Several components of the JA signal transduction pathway have been characterized. The JA-insensitive *coi1-1* mutant is affected in a gene encoding an F-box protein that forms part of a putative E3 ubiquitin ligase complex of the SCF type (Xie et al., 1998; Xu et al., 2002; Devoto et al., 2002). COI1 interacts with members of a family of repressor proteins called JAZ (Thines et al., 2007; Chini et al., 2007). Several members of this family were shown to also interact with the JA-responsive transcription factor AtMYC2 (Chini et al., 2007; Chung et al., 2009). In response to biologically active jasmonates the repressor proteins are rapidly degraded (Thines et al., 2007; Chini et al., 2007), which is proposed to lead to derepression of AtMYC2 activity resulting in expression of a set of genes involved in defense against wounding and herbivory.

Another set of genes distinct from those regulated by AtMYC2 is synergistically induced by JA in combination with the stress hormone ethylene (Penninckx et al., 1996; Lorenzo et al., 2003, 2004). The transcription factors ORA59 and ERF1 have been suggested to act as integrators of JA and ethylene signaling pathways in Arabidopsis to control this gene subset (Lorenzo et al., 2003, Pré et al., 2008). Overexpression of *ORA59* as well as *ERF1* activates the expression of several defense-related genes including *plant defensin1.2* (*PDF1.2*; Lorenzo et al., 2003; Pré et al., 2008) and confers resistance to the necrotrophic fungus *Botrytis cinerea* (Berrocal-Lobo et al., 2002; Pré et al., 2008). Analysis of plants in which *ORA59* expression is knocked out by RNAi shows that the JA- and ethylene-responsive expression of defense genes including *PDF1.2* is not controlled by ERF1 as previously reported (Lorenzo et al., 2003), but instead by the related transcription factor ORA59 (Pré et al., 2008). Expression of *ORA59* (Atallah, 2005; Pré et al., 2008) and the subset of genes controlled by ORA59 including *PDF1.2* is also dependent on COI1 (Lorenzo et al., 2003; Pré et al., 2008). However ORA59 is not known to interact with members of the JAZ family of repressors.

The aim of the studies reported here was to determine whether JA has an activating effect on ORA59 at the protein level. The results show that JA caused stabilization as well as nuclear localization of ORA59. Interestingly, nuclear localization of ORA59 did not require a functional COI1 protein.

## Results

### *PDF1.2* is not an immediate-early JA-responsive gene

As a first step, we wanted to get some indication that JA induces *PDF1.2* gene expression by activating the transcription factor ORA59, for example via covalent modifications or protein-protein interactions. Therefore we determined whether *PDF1.2* is a primary JA-responsive gene. Primary response genes generally do not require de novo protein synthesis, because the signal activates pre-existing regulatory proteins including transcription factors active in the signal transduction pathway (Pauw and Memelink, 2005). Fourteen-days old seedlings were treated with JA alone or in combination with the ethylene releasing compound ethephon in the absence or presence of the protein synthesis inhibitor cycloheximide (CHX). As shown in Figure 1, *PDF1.2* expression was induced by JA alone and superinduced by JA and ethephon consistent with previous reports (Penninckx et al., 1998; this thesis, Chapter 2). CHX completely abolished this response, indicating that the expression of *PDF1.2* in response to JA and ethephon requires de novo protein expression. *PDF1.2* is therefore not an immediate-early response gene. *ORA59* on the other hand is an immediate-early response gene, since its expression in response to JA or JA/ethephon treatment was not negatively affected by CHX. In fact CHX alone induced *ORA59* mRNA accumulation, and in combination with JA or JA/ethephon superinduction of mRNA accumulation was observed. (Super)-induction by CHX is commonly observed with immediate-early response genes in mammalian cells (Edwards and Mahadevan, 1992), and is usually attributed to decreased mRNA degradation.

### ORA59 accumulates in the nucleus in response to JA

The previous result is not in favour of a mechanism where ORA59 activity is affected at the post-translational level by JA. In fact, the CHX experiments indicate that JA switches on *PDF1.2* expression by inducing *ORA59* gene expression resulting in an increase in ORA59 protein abundance. However such a scenario does not exclude that JA also affects ORA59 activity at the protein level. Of all possible changes in transcription factor activity we decided to study nuclear localization and protein stability since these are two prominent mechanisms whereby transcription factor activity is regulated (Vom Endt et al., 2002). Nuclear localization was studied by expressing ORA59 fused N-terminally or C-terminally to green fluorescent protein (GFP) in Arabidopsis cell suspension protoplasts and observing localization by confocal laser scanning microscopy.

The first remarkable observation was that N- (Figure 2a) and C-terminal (data not shown) ORA59-GFP fusions showed a similar localization as GFP alone in untreated protoplasts in both the cytoplasm as the nucleus. In contrast, several other AP2-domain transcription factors tested (data not shown) including ORA37 (Figure 2a) were nuclear localized. ERF1, which is closely

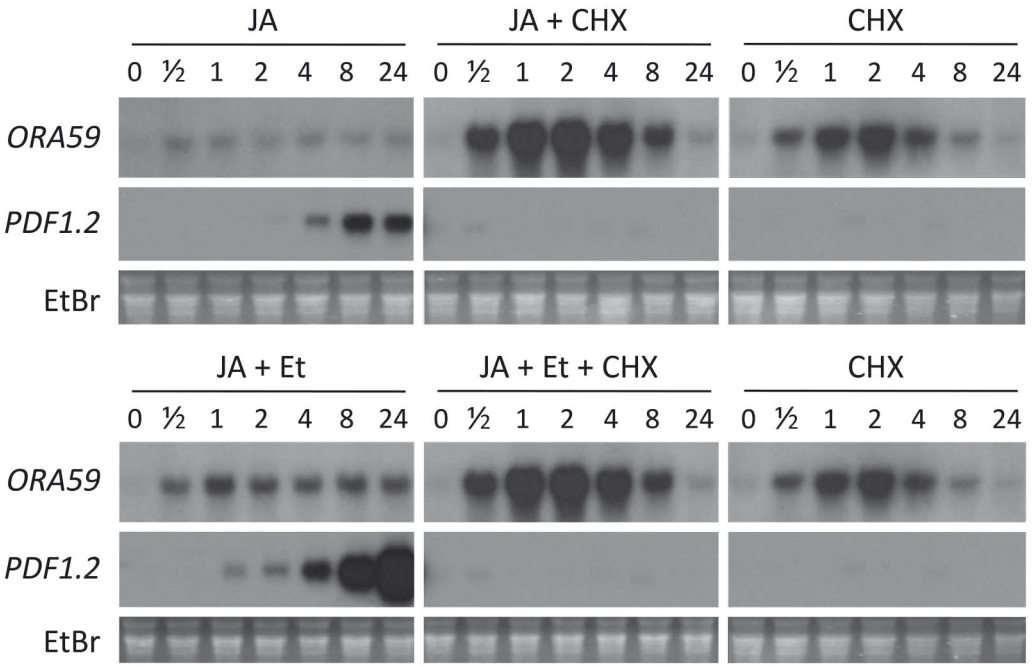


**Figure 1.** PDF1.2 is not an immediate-early JA-responsive gene. Fourteen-days old seedlings were treated with JA alone or in combination with the ethylene-releasing agent ethephon (Et) in the presence or absence of cycloheximide (CHX) for number of hrs as indicated. All panels hybridized with the same probe were on the same blot and exposed to film for the same time, therefore signal intensities can be directly compared. The ethidium bromide (EtBr) stained gel is shown as a control or RNA loading.

related to ORA59 and can also switch on *PDF1.2* expression when overexpressed (Lorenzo et al., 2003; Pré et al., 2008), was also constitutively localized in the nucleus (Figure 2a).

The second remarkable observation was that when protoplasts transformed with either GFP-ORA59 (Figure 2a) or ORA59-GFP (Figure 2b) expression plasmids were treated with JA for 4 hrs, an increase in the proportion of cells showing nuclear localization of the GFP fusion protein was observed. A low frequency of nuclear localization was always observed in untreated or DMSO-treated protoplasts. Nuclear re-localization was a relatively slow process with most of the fusion protein in the nucleus after 1-2 hrs (Figure 2b).

To show unequivocally that the GFP-ORA59 fusion protein accumulated inside the nucleus and not outside around the nuclear membrane, the plasmid was co-transformed with a plasmid carrying a fusion between *Discosoma sp.* red fluorescent protein (DsRFP) and the nuclear tobacco (*Nicotiana tabacum*) protein NtKIS1a (Jasinski et al., 2002). As shown in Figure 2c NtKIS1a-DsRFP and ORA59-GFP showed complete overlap in nuclear localization when co-expressed in protoplasts that show nuclear localization of ORA59-GFP in response to JA.

To demonstrate that the observed relocalization of ORA59 reflects a property of a functional

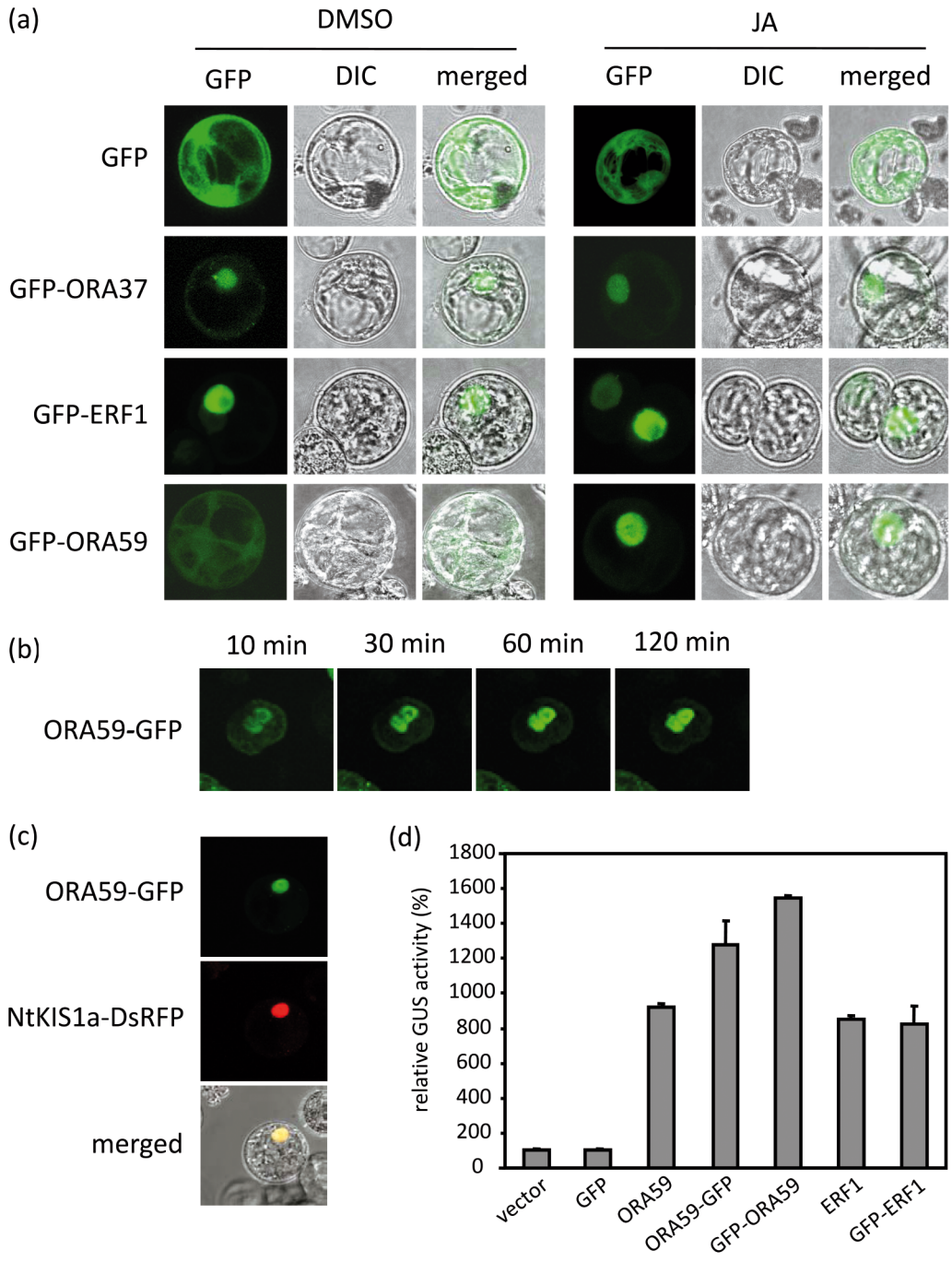


**Figure 2.** ORA59 accumulates in the nucleus of Arabidopsis protoplasts in response to JA. **(a)** GFP, GFP-ORA37, GFP-ERF1, GFP-ORA59 or ORA59-GFP constructs were transformed to Arabidopsis cell suspension protoplasts and examined by confocal laser scanning microscopy after treatment with 50 µM JA or 0.1% (v/v) DMSO as indicated for 4 hrs.

transcription factor, we determined the ability of GFP fusion proteins to trans-activate the *PDF1.2* promoter in Arabidopsis cell suspension protoplasts. As shown in Figure 2d, the fusion proteins of ORA59 and ERF1 with GFP trans-activated the *PDF1.2* promoter to a similar level as the unfused ORA59 and ERF1 proteins. These results show that the GFP fusion proteins were functionally active as transcription factors.

**ORA59 nuclear accumulation is also observed in *C. roseus* cells**

To study whether this nuclear relocalization is a peculiarity of Arabidopsis protoplasts or a more general phenomenon, the localization of the GFP-ORA59 fusion was compared to GFP fusions with ORA37 or ERF1 in bombarded *Catharanthus roseus* suspension cells with and without treatment with methyl-jasmonic acid (MeJA). As in Arabidopsis protoplasts, the GFP-ORA59 fusion protein showed a similar localization as GFP alone in *C. roseus* cells, and the ORA37 and ERF1 fusion proteins were constitutively nuclear (Figure 3). Treatment with MeJA for 2 hrs resulted in nuclear localization of the GFP-ORA59 fusion protein in the transformed cells (Figure 3). This shows that nuclear localization of ORA59 in response to jasmonates occurs both in protoplasts and in cells via a mechanism that is conserved across plant species.

**JA-responsive ORA59 nuclear localization is independent of COI1**

We examined the role of COI1, an important component of JA signal transduction, in the JA-induced nuclear accumulation of ORA59. The ORA59-GFP construct was introduced in wild-type leaf protoplasts and protoplasts derived from the mutant *coi1-1*. In *coi1-1* leaf protoplasts, the ORA59-GFP fusion protein relocalized to the nucleus in response to JA in a similar percentage of the cells as in wild-type protoplasts (Figures 4a and 4b), indicating that JA-induced nuclear accumulation of ORA59 did not require the COI1 protein.

Confocal microscopic images are shown at the left (GFP), the corresponding differential interference contrast (DIC) images are in the middle and the merged images are at the right. **(b)** Time-lapse confocal laser scanning microscopy of ORA59-GFP in individual protoplasts. Projections of series of confocal optical sections are shown at each time point. Protoplasts were treated with 50 µM of JA. **(c)** Confocal laser scanning microscopic images of Arabidopsis protoplasts transformed simultaneously with NtKIS1a-DsRed and ORA59-GFP expression plasmids taken after treatment with 50 µM JA or 0.1% DMSO control for 1 hr. **(d)** GFP-transcription factor fusions are functional in transcriptional activation assays in Arabidopsis protoplasts. Arabidopsis protoplasts were co-transformed with the reporter construct *GUS* gene driven by a 277 bp *PDF1.2* promoter and one of the effector plasmids and the reference plasmid. The effector constructs consisted of an expression vector carrying the *CaMV 35S* promoter without or with the *ORA59* or *ERF1* cDNAs alone or fused to GFP. The *Renilla* luciferase (*LUC*) gene fused to the *CaMV 35S* promoter served as a reference gene to correct for differences in transformation and protein extraction efficiencies. Bars represent average GUS/LUC ratios from triplicate experiments ± SE expressed relative to the vector control set at 100%.

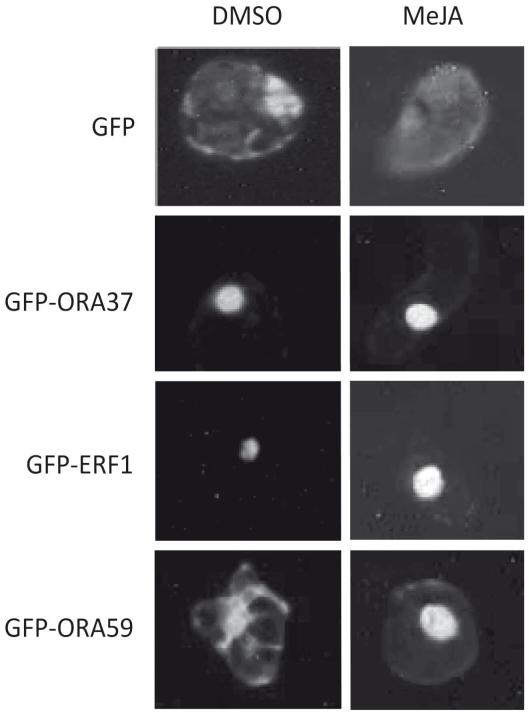


**Figure 3.** ORA59 accumulates in the nucleus of C. roseus cells in response to MeJA. GFP, GFP-ORA37, GFP-ERF1 and GFP-ORA59 expression plasmids were transformed to cell suspension cells of *C. roseus* and examined by confocal laser scanning microscopy after treatment with 100 µM methyl-jasmonic acid (MeJA) or 0.1% DMSO for 2 hrs.

**Mapping the ORA59 domain responsible for JA-responsive nuclear localization**

We attempted to map the domain responsible for JA-responsive nuclear localization by analyzing nuclear/cytosolic distribution of a range of deletion derivatives. Domains with distinct features derived from *in silico* analysis of the ORA59 protein are shown in Figure 5a. The full-length ORA59 protein with GFP fused at its N-terminal end localized to the nucleus in about 45% of JA-treated protoplasts, whereas nuclear localization was observed in about 20% of untreated protoplasts (Figure 5b). ORA59 contains a putative bipartite nuclear localization signal (NLS) flanking the C-terminal end of the AP2 DNA-binding domain. Derivatives containing amino acids 48-139 or 81-139, both lacking the NLS, showed a dramatic reduction in nuclear localization, while derivatives 48-180 and 81-180, lacking both Ser-rich domains were virtually constitutively nuclear localized. Derivative 1-180 showed a qualitatively similar pattern of nuclear localization as full-length ORA59 but with 2-fold enhanced nuclear localization in cells without JA treatment (around 40% of the cells). Derivatives 48-244 and 81-244 did not show nuclear accumulation after JA treatment.

The conclusion from these experiments is that there is not a single domain responsible for cytoplasmic retention, since this function is present both in the N-terminal and the C-terminal part of the protein (compare for example derivatives 1-180 and 81-244). In contrast there is a single domain responsible for JA-responsive nuclear localization since this function is only

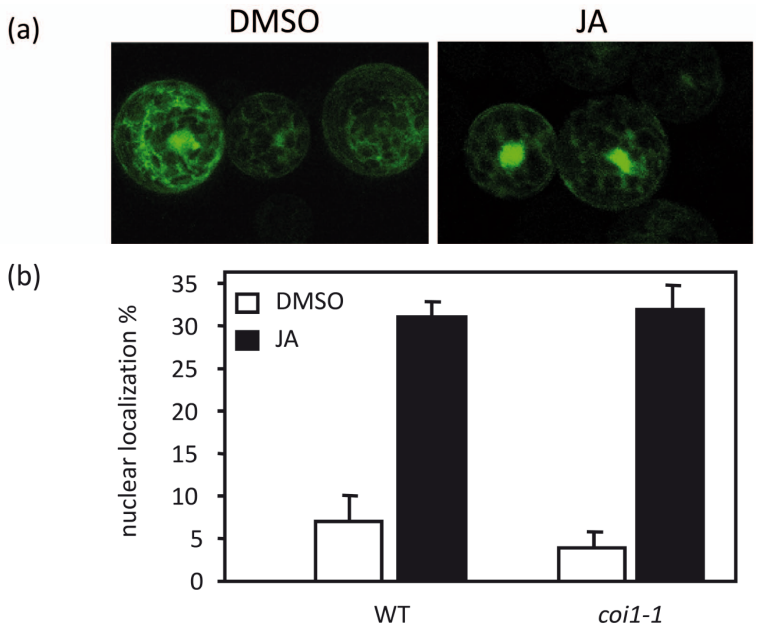


**Figure 4.** JA-responsive ORA59 nuclear localization is independent of COI1. **(a)** The ORA59-GFP expression plasmid was transformed to *coi1-1* leaf protoplasts and cells were examined by confocal laser scanning microscopy after treatment with 50 µM JA or 0.1 % DMSO for 2 hrs. **(b)** Nuclear localization of ORA59-GFP in wild-type and *coi1-1* leaf protoplasts expressed as the percentage of cells showing nuclear localization relative to the total number of GFP-expressing cells analyzed by confocal laser scanning microscopy. For each data point at least 100 GFP-expressing protoplasts were analyzed. The experiment was repeated twice with similar results.

present in full-length protein and in derivative 1-180. Based on the studied truncated proteins, both Ser-rich domains emerge as candidates for cytoplasmic retention, while the first Ser-rich domain could be involved in nuclear relocalization.

**Inhibition of nuclear export leads to nuclear localization of ORA59**

Many nuclear proteins shuttle between the cytoplasm and the nucleus via interaction with nuclear import and export receptors which interact with NLS and nuclear export signals (NES), respectively. The presence of a putative NLS and a putative NES suggested that ORA59 might also shuttle between the cytoplasm and the nucleus. To test this hypothesis we used leptomycin B (LMB), which is a specific inhibitor of the major nuclear export receptor CRM-1. Treatment of protoplasts transformed with ORA59-GFP with LMB for 3 hrs increased the proportion of cells showing nuclear localization to 100% (Figure 6a). Re-compartmentalization was clearly visible after 30 min of incubation in the presence of LMB and was nearly complete after 50 min (Figure

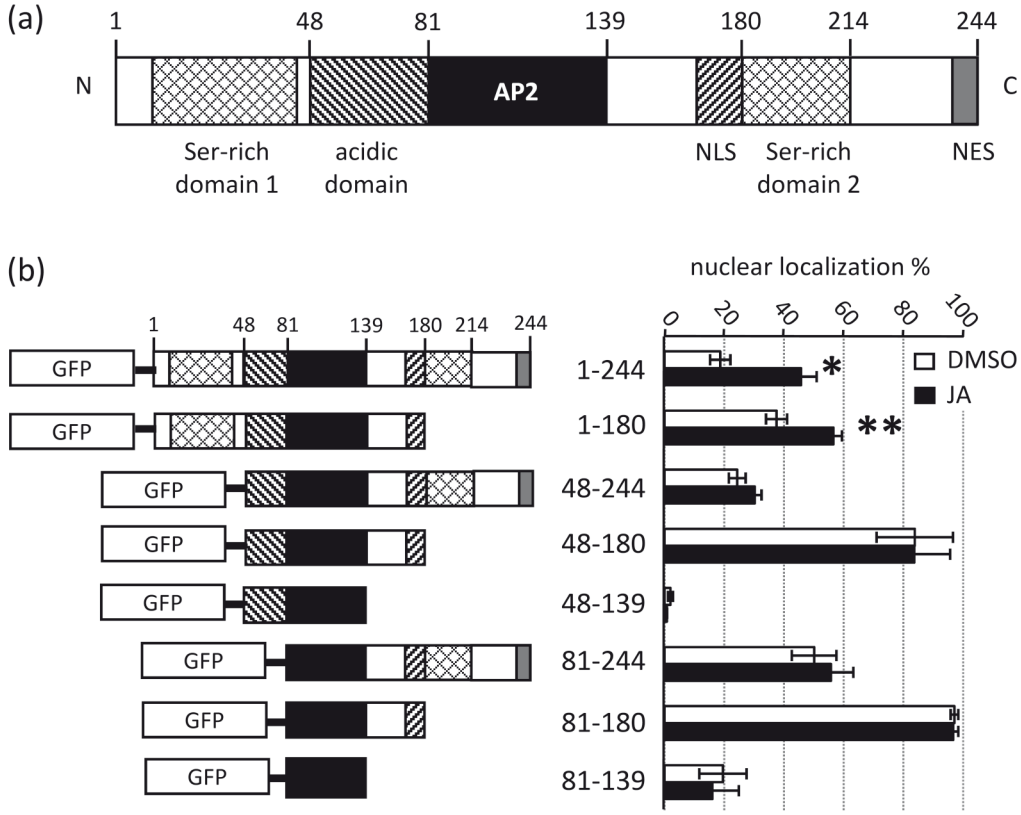


**Figure 5.** Mapping the ORA59 domain responsible for JA-responsive nuclear localization. **(a)** Schematic overview of domains in the ORA59 protein, which contains two Serine-rich domains (Ser-rich), an acidic domain, the AP2 domain, a putative bipartite nuclear localization signal (NLS) and a putative nuclear export signal (NES). **(b)** Sixteen hrs after transformation Arabidopsis cell suspension protoplasts transformed with the indicated ORA59 deletion derivatives fused to GFP were treated with 0.1% DMSO or 50 µM JA. Values represent cells with nuclear localization as a percentage of the total number of GFP-expressing cells analysed by confocal laser scanning microscopy. For each data point at least 150 GFP-expressing protoplasts were analyzed. Bars represent average from triplicate experiments ± SE. Asterisks indicate significant differences between DMSO and JA treatments (T-test; *= 0.005; **= 0.014).

6b). This indicates that ORA59 shuttles between cytoplasm and nucleus at a slow rate.

**ORA59 localization is determined by an active NLS and NES**

Truncated proteins as used in the experiments in Figure 5 can exhibit conformational changes and thereby create experimental artefacts. To extend the observations of ORA59 localization

shown in Figures 5 and 6, GFP-fused ORA59 derivatives with point mutations in the NLS or specific deletions of the NLS or NES were studied. Since ORA59 contains a putative bipartite NLS, mutations were created in the first (m1), in the second (m2) or in both (m3) NLS parts (Figure 7a). Observation of Arabidopsis protoplasts expressing the mutant proteins showed that mutations in the first and second NLS parts dramatically reduced nuclear localization, whereas the double mutation resulted in exclusive cytosolic localization (Figures 7b and 7c). Deletion of the NLS (m4) had the same effect as the double mutation (Figures 7c). An interesting observation is that mutation m1 and also the double mutation m3 resulted in GFP signal mainly detected in the cytoplasm and often no signal at all in the nucleus, as shown in protoplasts co-transformed with a plasmid carrying a fusion between the nuclear tobacco protein NtKIS1a with the red fluorescent protein (DsRFP; Figure 7a - merged picture) (Jasinski et al., 2002). Mutation of the second NLS resulted in an even distribution of GFP signal in nucleus and cytoplasm, with a small proportion of cells with only nuclear signal. In agreement with the results obtained with the nuclear export inhibitor LMB (Figure 6), deletion of the NES (m5) prevented nuclear export and resulted in a high proportion of cells with nuclear localization of the GFP fusion protein (Figure 7b and 7c). The combination of double mutated NLS and lack of NES (m6) resulted in exclusive cytosolic distribution of the GFP fusion protein. These results confirm that indeed ORA59 shuttles between cytoplasm and nucleus due to the activity of a bipartite NLS and a NES.

**ORA59 activation potential is determined by nuclear localization signals**

Transcription factors work in the cell nucleus. Therefore, the fact that ORA59 shuttles between nucleus and cytoplasm prompted us to investigate whether ORA59 transcriptional activity was dependent on nuclear localization signals. ORA59 mutant proteins depicted in Figure 7a fused to GFP were tested for the ability to trans-activate the *PDF1.2* promoter in protoplasts. As shown in Figure 8a, ORA59 derivatives able to accumulate in the nucleus (m2 and m5) trans-activated the *PDF1.2* promoter. Surprisingly, the deletion derivative lacking the NES (m5) which showed increased nuclear accumulation activated the *PDF1.2* promoter to a 2-fold lower level than wild-type ORA59 protein. Trans-activation assays with unfused ORA59 derivatives gave similar results but with a less strong reduction of activation by m5 (Figure 8b). In conclusion, a functional NLS is necessary for nuclear localization and trans-activation, which is not surprising. What is surprising however is that removal of the NES resulted in a higher percentage of nuclear localization but in significantly lower trans-activation.

**JA stabilizes ORA59 protein**

A prominent mechanism of regulating the activity of a protein in a cell is by regulating its abundance. Especially ubiquitin/proteasome-mediated degradation emerged in the past

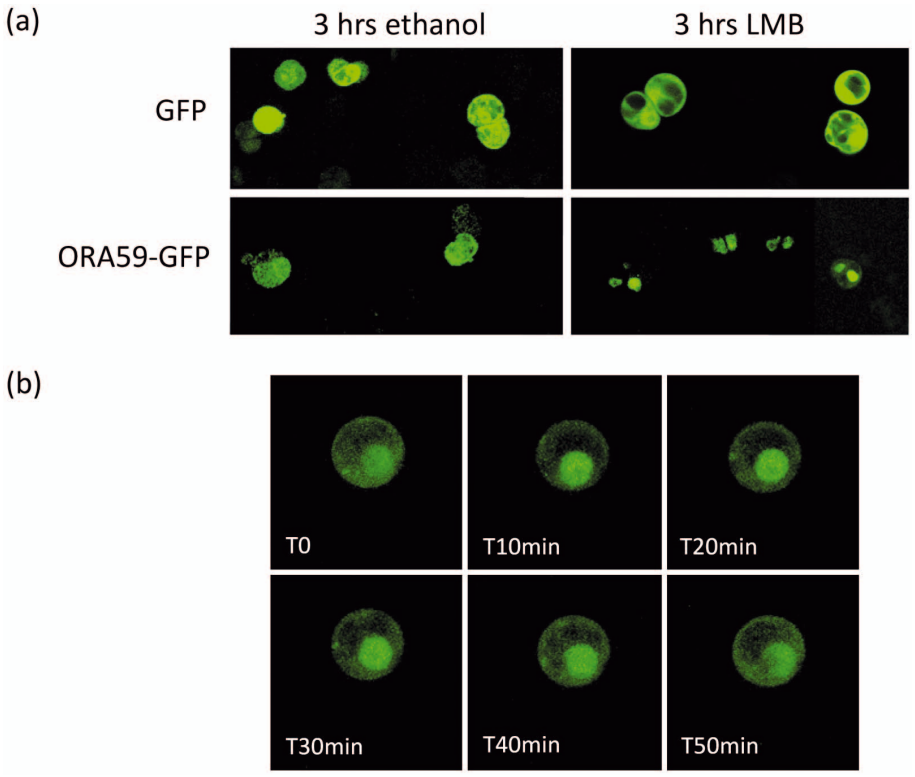


**Figure 6.** Inhibition of nuclear export leads to nuclear localization of ORA59. **(a)** Confocal laser scanning microscopy images of Arabidopsis cell suspension protoplasts expressing ORA59-GFP or GFP. Sixteen hrs after transformation with expression plasmids protoplasts were treated for 3 hrs with 2 µM of the nuclear export inhibitor leptomycin B (LMB) or with the solvent ethanol at a final concentration of 0.5% (v/v). **(b)** Time-lapse confocal laser scanning microscopy of an individual protoplast expressing ORA59-GFP after treatment with 2 µM LMB.

two decades as a predominant mechanism for regulating the activity of proteins including transcription factors (Bach and Ostendorff, 2003). Therefore we asked the question whether JA affected the level of ORA59 protein post-translationally. Protoplasts were co-transformed with a GFP expression plasmid and a plasmid expressing the ORA59-GFP fusion and were treated for 4 hrs with JA or the solvent DMSO. Immunoblot analysis of total cellular protein with anti-GFP antibodies revealed that JA caused an increase in the amount of ORA59-GFP protein (Figure 9a). The amount of GFP, expressed from the same version of the *CaMV 35S* promoter, was not affected, demonstrating that the effect of JA on ORA59-GFP protein abundance did not occur at the transcriptional level.

To connect this observation in protoplasts to processes occurring in whole plants we monitored the levels of ORA59 protein in transgenic plants treated with JA for different periods

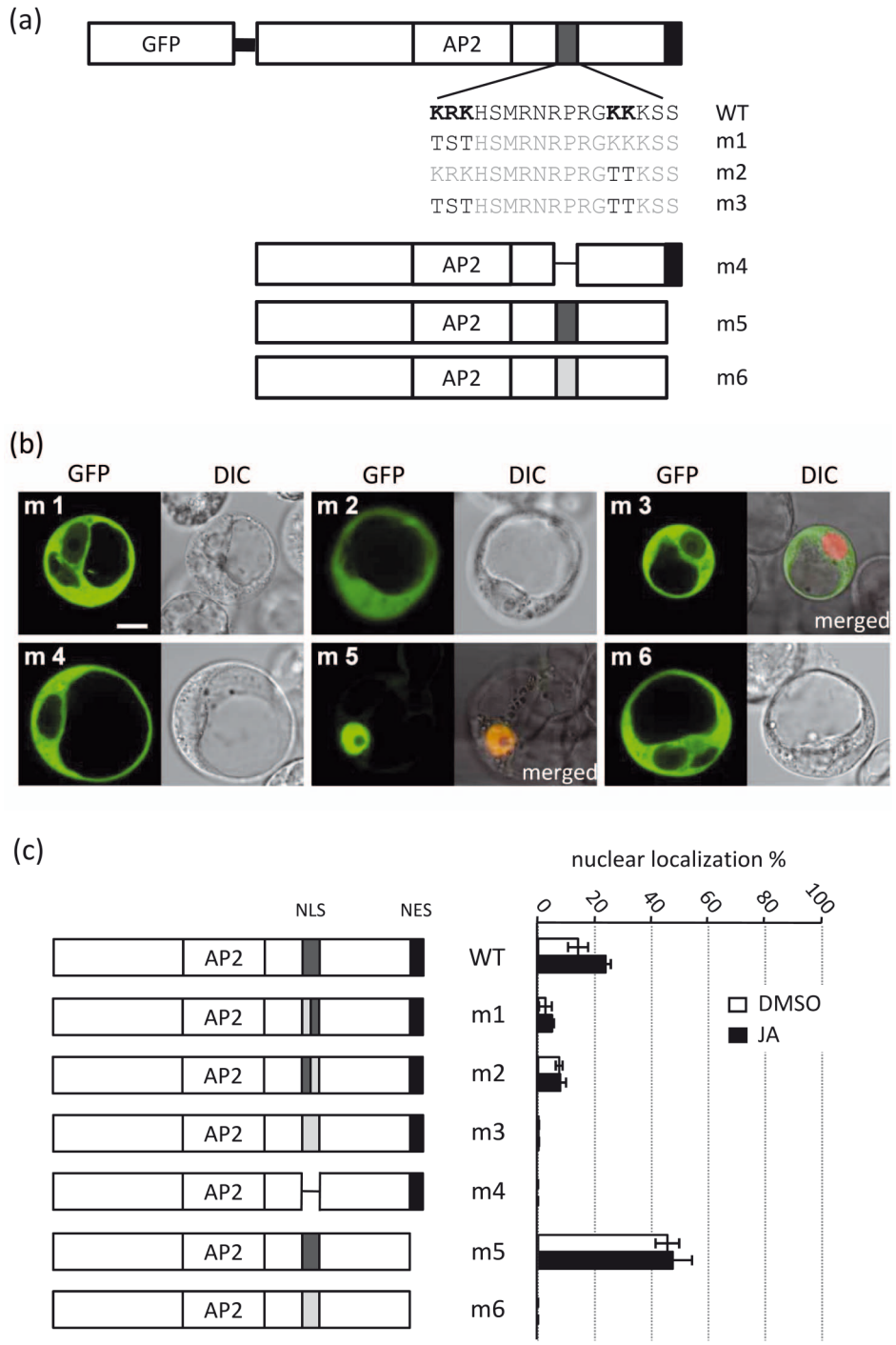


**Figure 7.** ORA59 localization is determined by an active NLS and NES. **(a)** Schematic overview of ORA59 derivatives with mutations in the putative bipartite nuclear localization

of time. Fourteen days-old T2 seedlings from two transgenic Arabidopsis lines expressing ORA59 tagged C-terminally with the influenza hemagglutinin (HA) epitope in an estradiol-inducible manner (XVE-*ORA59-HA*) were treated with estradiol for 4, 8 and 24 hrs in presence or absence of JA. Control samples were treated with the solvent DMSO. Treatment with estradiol alone strongly induced the expression of *PDF1.2* (Figure 9b), demonstrating that the ORA59-HA fusion protein was functional as a transcriptional activator. As shown in Figure 9c, the level of ORA59-HA markedly increased after 8 and 24 hrs of JA treatment. Although *PDF1.2* expression was induced in DMSO-treated plants as a result of the simultaneous estradiol treatment (data not shown), a significant increase in ORA59-HA protein was not detected (Figure 9b). This also suggests that in the absence of JA ORA59 was rapidly turned over.

**ORA59 is degraded by the 26S proteasome**

To test whether the low levels of ORA59 in DMSO treated protoplasts might be due to ORA59 degradation mediated by the 26S proteasome, we tested the effects of the proteasome inhibitor MG132 on ORA59 protein accumulation. Arabidopsis protoplasts transformed with *ORA59-GFP* expression plasmid were incubated in the dark for 16 hrs and then treated with MG132 or with the solvent DMSO for 2 hrs. Fluorescence microscopy showed that MG132, but not DMSO, increased nuclear and cytosolic abundance of ORA59-GFP in protoplasts (Figure 10a). Total protein was extracted from protoplasts co-transformed with *ORA59-GFP* and *GFP* expression plasmids and subjected to immunoblot analysis with anti-GFP antibodies. As shown in Figure 10c (left panel), MG132 treatment drastically increased GFP-ORA59 accumulation in protoplasts, indicating that GFP-ORA59 protein is subject to 26S proteasome-mediated degradation. Similar results were obtained with the stably transformed plant lines expressing ORA59-HA (data not shown).

We attempted to map the domain responsible for proteasome-mediated degradation (the “degron”) by analysing the stabilization of different deletion derivatives fused to GFP by treatment with MG132. In Figure 10b protein extracts from untreated protoplasts were run on the same gel allowing direct comparison of steady-state protein amounts. Full-length ORA59 was undetectable. All deletion derivatives were more stable than the full-length protein,

signal (NLS) and nuclear export signal position (NES). Bold letter case indicates substituted amino acids. The dark gray box represents the wild-type NLS, whereas the light gray box represents the double mutated NLS. **(b)** GFP-ORA59 mutant constructs (m1-m6) were transformed to Arabidopsis cell suspension protoplasts and examined by confocal laser scanning microscopy. Pictures of representative cells are shown. Merged pictures indicate nucleus of protoplasts co-transformed with NtKIS1a-DsRed. Scale bar = 10 µm. **(c)** Sixteen hrs after transformation Arabidopsis cell suspension protoplasts transformed with the indicated ORA59 mutant derivative fused to GFP were treated for 2 hrs with 0.1% DMSO or 50 µM JA. Values represent cells with nuclear localization as a percentage of the total number of GFP-expressing cells analysed by confocal laser scanning microscopy. For each data point at least 150 GFP-expressing protoplasts were analyzed. Bars represent the average from triplicate experiments ± SE.

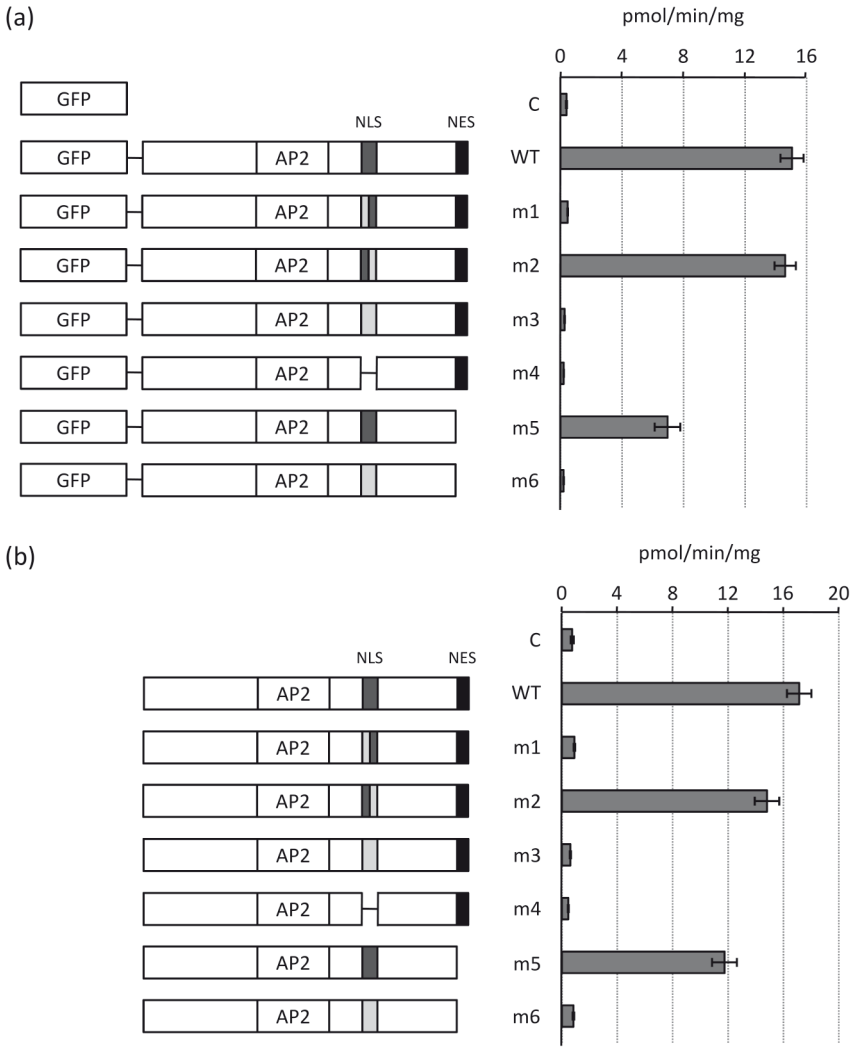


**Figure 8.** ORA59 activation potential is determined by nuclear localization signals. **(a)** Arabidopsis protoplasts were co-transformed with a *GUS* reporter gene driven by the 277 bp *PDF1.2* promoter and a *CaMV 35S* expression vector containing GFP fusions of wild-type (WT) or NLS/NES mutant versions of ORA59 or GFP alone, as indicated. **(b)** Same as (a) but with unfused proteins, as indicated. Empty vectors were used as control. Bars represent average GUS activity values from triplicate experiments corrected for protein concentrations ± SE.

indicating that there is not a single degron. Low amounts were detected of derivatives 1-180 and 81-244. Deletion derivatives 48-180, 48-139, 81-180 and 81-139 were almost as stable as GFP. This suggests that the Ser-rich domains are responsible for instability of ORA59. Next protein amounts were analyzed after treatment of transformed protoplasts with MG132 (Figure 10c). All

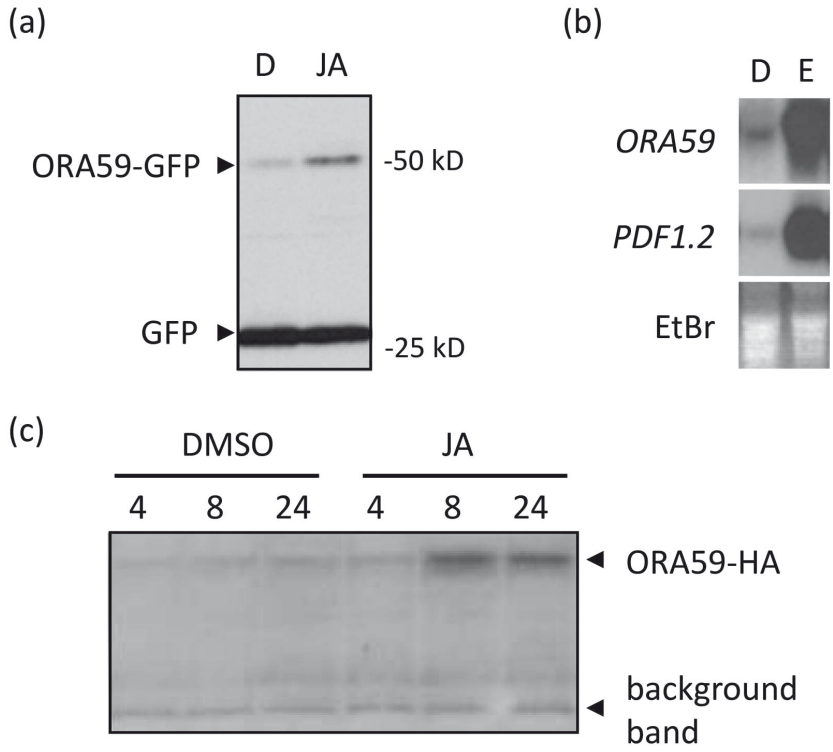


**Figure 9.** JA stabilizes the ORA59 protein. **(a)** Immunoblot analysis with anti-GFP antibodies of total protein extracts prepared from Arabidopsis cell suspension protoplasts co-expressing GFP and ORA59-GFP treated with 50 µM JA or 0.1% DMSO for 4 hrs. **(b)** Functional analysis of the XVE-ORA59-HA construct (line #18) treated with 2 µM estradiol (E) or 0.1% DMSO (D) for 16 hours. RNA blots were hybridized with the indicated probes. **(c)** Immunoblot analysis with anti-HA antibodies of total protein extracts from 14-days-old plants XVE-ORA59-HA (line #18) treated simultaneously with 2 µM estradiol and 50 µM JA or 0.1% DMSO. Intensities of the background band confirm equal protein loading.

deletion derivatives except GFP-81-139 were stabilized by MG132 to some degree. This shows that the AP2 domain does not harbour a degron function in contrast to all other regions present in the various derivatives. The other conclusion is that degradation did not occur uniquely in the nucleus since also deletion derivative GFP-48-139 that does not contain an NLS and did not accumulate in the nucleus (Figure 5b) was to some degree stabilized by MG132.

**Functional mapping of the activation domain in ORA59**

In many mammalian transcription factors the degron overlaps with the activation domain (Salghetti et al., 2000). In the “suicide” model degradation is proposed to be crucial for the

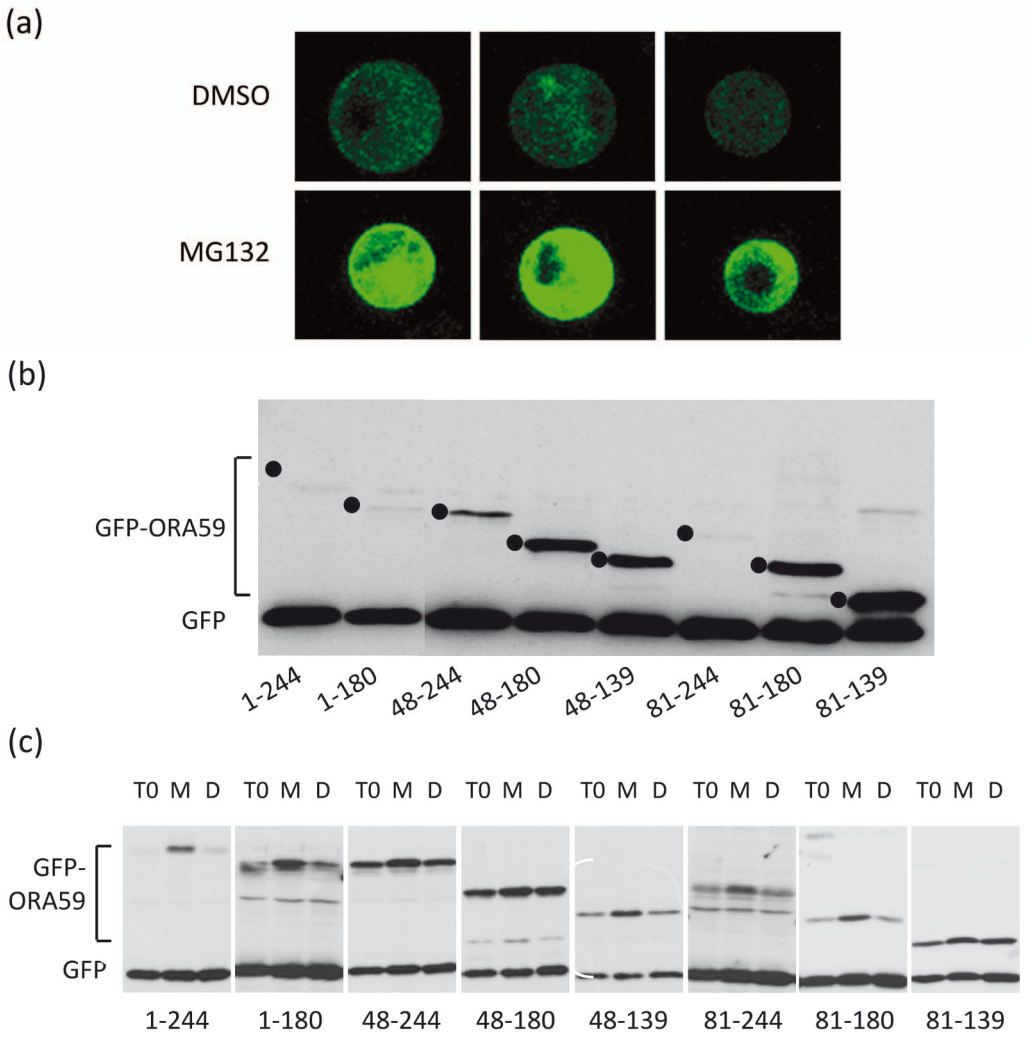


**Figure 10.** ORA59 is degraded by the 26S proteasome. **(a)** Confocal laser scanning microscopy images of Arabidopsis cell suspension protoplasts expressing ORA59-GFP. Sixteen hrs after transformation protoplasts were treated for 2 hrs with 50 µM MG132 or 0.1% DMSO. Pictures taken at identical confocal laser scanning microscope settings of three representative protoplasts are shown for each treatment. **(b)** Steady-state protein amounts of various ORA59 deletion derivatives in Arabidopsis cell suspension protoplasts 16 hrs after co-transformation with a GFP expression plasmid. Proteins were detected with anti-GFP antibodies. Positions of the GFP-ORA59 fusion proteins are indicated with dots. **(c)** Mapping of the degron in ORA59. Immunoblot analysis with anti-GFP antibodies of total protein extracts from Arabidopsis cell suspension protoplasts co-expressing GFP and deletion derivatives of ORA59 fused at their N-terminus to GFP. Arabidopsis protoplasts were harvested 16 hrs after transformation (T0) or treated for 4 hrs with the solvent DMSO at 0.1% (v/v) final concentration (D) or with 50 µM of the 26S proteasome inhibitor MG132 (M). The upper band is the full-length GFP-ORA59 fusion protein in those gels where multiple bands are visible in the region indicated with GFP-ORA59. Panels with different deletion derivatives were run on different gels and band intensities

transcription activating activity of the activation domain (Bach and Ostendorff, 2003). To determine whether a similar mechanism may apply for ORA59, we mapped the transcription activating domain in parallel with the mapping of the degron. As shown in Figure 11, deletion derivatives lacking the putative NLS were inactive in the trans-activation assay, reconfirming the importance of a functional NLS. Derivative 81-180 containing the AP2 domain and the NLS had a very low trans-activating activity, demonstrating that this part of ORA59 does not contain an activation domain. As with the degron we did not find a single domain responsible for transcription activation. Transcription activation functions were found to be present in the N-terminal region as well as in the C-terminal region. By comparing the activities of the GFP fusion derivatives 48-244 and 48-180 it appeared that the C-terminal Ser-rich region functions as an activation domain (Figure 11b). By comparing the activities of the GFP fusion derivatives 1-180 and 48-180 it appeared that the N-terminal Ser-rich region functions as an activation domain. The acidic region from positions 48-80 functioned only as an activation domain when deletion derivative 48-180 was not fused to GFP (compare to 81-180; Figure 11a).

In general activities of GFP fusion proteins were similar to those of the unfused deletion derivatives, with the differences that derivative 48-244 was relatively active as a GFP fusion, and that the activity of 48-180 was considerably lower as a GFP fusion. Interestingly the activation strength of ORA59 deletion derivatives was not related to their abundance in protoplasts. Full-length ORA59 fused to GFP for example was a strong activator (Figure 11b), whereas the protein was undetectable by Western blotting (Figure 10b). Deletion derivative 48-180 on the other hand was almost inactive as a transcription activator when fused to GFP (Figure 11b), whereas the protein was as abundant as the co-expressed GFP (Figure 10b).

**ORA59 is nuclear and cytosolic in transgenic plants**

In order to assess ORA59 localization in Arabidopsis plants, transgenic lines overexpressing ORA59 fused C-terminally to GFP were generated. Independent lines with high overexpression of functional GFP-ORA59 were selected by measuring the expression levels of *ORA59* and its target gene *PDF1.2* (Figure 12a). Selected lines were also evaluated for levels of GFP-ORA59 protein accumulation by immunoblotting with anti-GFP antibodies, but it turned out to be undetectable. As shown in Figure 6, MG132 stabilized ORA59. Therefore seedlings were treated with MG132 or the solvent DMSO for 16 h prior to protein extraction. Among the lines tested, a low level of GFP-ORA59 protein could be detected only in line # 21 after MG132 treatment (Figure 12b). Lines # 3, 5 and 21 were further analyzed by confocal laser scanning microscopy, but GFP signal was difficult to detect. JA and MG132 improved the signal strength, but it remained difficult to detect GFP fluorescence. Remarkably, incubation of 4-7 days old seedlings in water for 24 h after

cannot be directly compared. Use Figure 9B for direct comparison of steady-state protein levels without treatment or use GFP band intensities to estimate relative amounts of the fusion proteins.

germination in solid medium slightly improved the GFP signal. Under these conditions, ORA59-GFP was shown to be localized both in the nucleus and in the cytoplasm of root and leaf cells of line # 21 (Figure 12c).

**Discussion**

The goal of the studies described here was to get insight in JA signal transduction steps affecting ORA59 activity at the protein level. As a first step we determined whether the ORA59 target gene *PDF1.2* is an immediate-early JA-responsive gene. If so, it would indicate that pre-existing ORA59 protein is activated in response to JA. However it turned out that JA-responsive *PDF1.2* expression depended on *de novo* protein synthesis. It is possible that the missing protein which needs to be synthesized is ORA59 itself. If so, JA could regulate *PDF1.2* expression by simply increasing ORA59 abundance at the transcriptional level. In such a scenario ORA59 is formally not a component of JA signal transduction (Pauw and Memelink, 2005). However, our data show that JA directly affected ORA59 protein activity by inducing stabilization and nuclear localization, and this establishes ORA59 as a component of JA signal transduction. Our results show that JA controls ORA59 at multiple levels, i.e. via transcriptional induction and by acting on de novo synthesized protein.

Domain mapping of ORA59 showed that stabilization and nuclear localization were conferred by more than one single domain. The conclusions from the different domain mapping experiments are summarized in Table 1. Transcriptional activation of the *PDF1.2* promoter required either the N-terminal or the C-terminal Ser-rich region. Comparison of *PDF1.2* trans-activation levels by derivatives 1-180, 48-244, 81-244 and NES deletion m5 indicate that the acidic domain is the N-terminal region partially important for activation, while the NES is responsible for the activation of the C-terminal region. Similarly, in untreated protoplasts either Ser-rich domain caused instability of the ORA59 deletion derivative resulting in low steady state protein levels. The full-length ORA59 protein containing both Ser-rich domains was by far the most unstable protein among all ORA59 derivatives analyzed.

For the interpretation of the nuclear localization studies, comparison of ORA59 and ERF1 might be instructional. Both proteins act as transcriptional activators of the *PDF1.2* promoter (Pré et al., 2008). ERF1 was also stabilized by MG132 (data not shown), but was constitutively nuclear localized. Comparison of the protein sequences shows that ORA59 and ERF1 share relatively high amino acid identity throughout their protein sequences except for the C-terminal Ser-rich domain, which is totally absent in ERF1. However in the nuclear localization studies the C-terminal Ser-rich domain did not emerge as a unique functional domain. Removal of either the N-terminal or the C-terminal region caused the constitutive nuclear accumulation of the deletion derivative in a significant fraction of the cells, while the presence of both determined a stronger cytoplasmic retention.

ORA59 contains active NLS and NES sequences, resulting in protein shuttling. Removal of

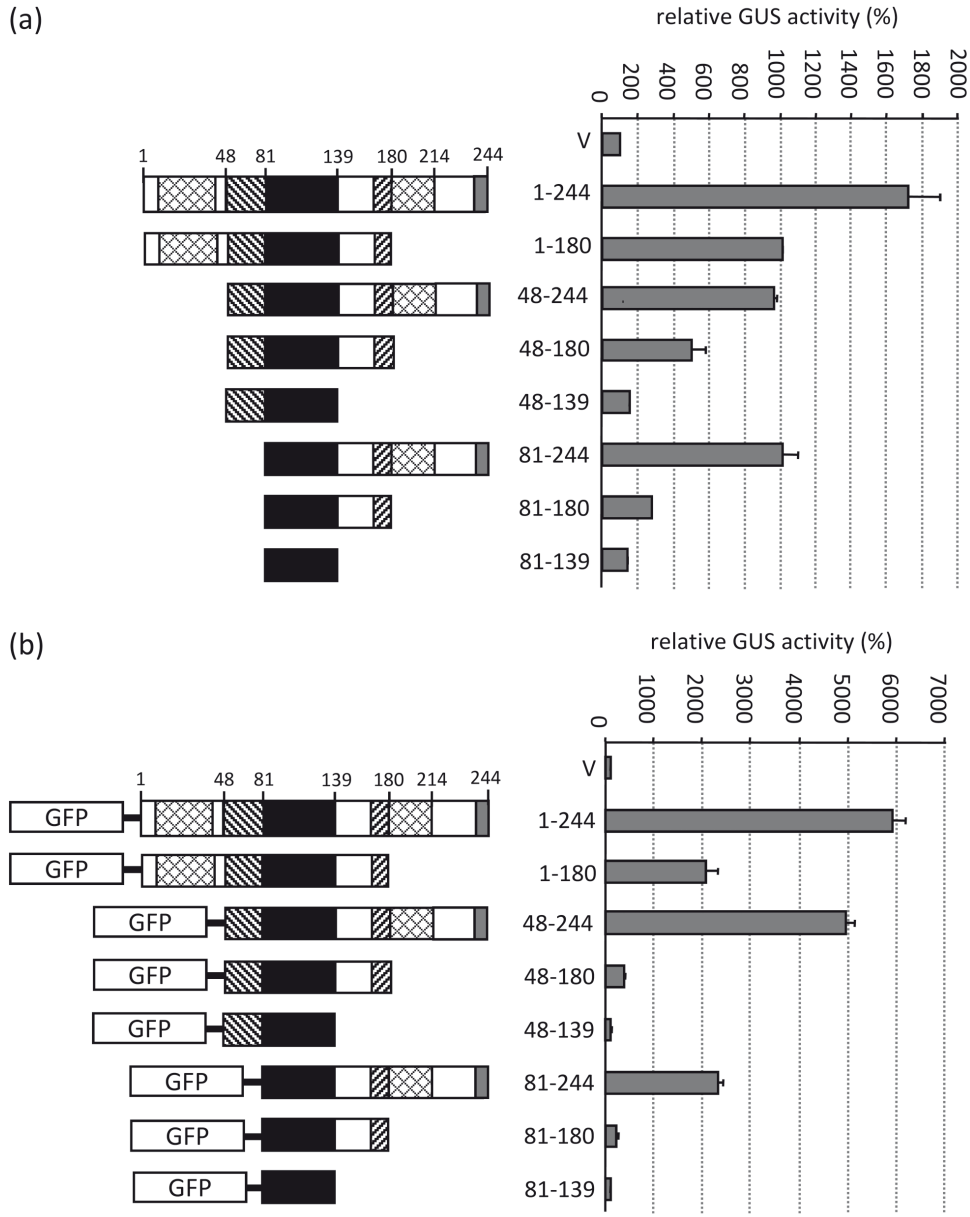


**Figure 11.** Functional mapping of the activation domain in ORA59. **(a)** Arabidopsis protoplasts were co-transformed with a *GUS* reporter gene driven by the 277 bp *PDF1.2* promoter and a *CaMV 35S* expression vector containing full-length (FL) ORA59 or one of the deletion derivatives as indicated. A reference plasmid carrying the *Renilla LUC* gene fused to the *CaMV 35S* promoter was co-transformed in all experiments to correct for differences in transformation and protein extraction efficiencies. Bars represent average GUS/LUC ratios from triplicate experiments ± SE expressed relative to the vector control set at 100%. **(b)** Same as in (a) but with fusions between ORA59 derivatives and GFP.

the NES resulted in nuclear localization in about 50% of the cells, whereas derivative 81-180, lacking in addition to the NES several other regions, showed nearly 100% nuclear localization. This indicates that one or more of these other regions are involved in cytoplasmic retention. Surprisingly removal of the NES, although resulting in a 2-fold increase in nuclear localization did not result in a concomitant increase in trans-activation of the *PDF1.2* promoter, but instead caused a 2-fold decrease. This suggests that active shuttling contributes to ORA59 activity, but how this comes about at the molecular level is unclear.

A highly surprising finding was that nuclear localization of ORA59 in response to JA did not require a functional COI1 protein. The COI1 protein is required for all known JA responses including induction of *ORA59* and *PDF1.2* gene expression (Lorenzo et al., 2003; Atallah, 2005; Pré et al., 2008). COI1 is the jasmonate receptor which directly interacts with an Ile-conjugated form of jasmonic acid (Chung et al., 2009). Upon binding of JA-Ile COI1 interacts with the JAZ repressors and related JAZ proteins leading to their degradation (Chini et al, 2007; Thines et al., 2007) in a scenario which shows strong similarity with auxin-responsive degradation of AUX/IAA repressor proteins via the auxin receptor/F-box protein TIR1 (Guilfoyle, 2007). The fact that other biologically active jasmonates besides JA-Ile exist and do not bind COI1 and that ORA59 relocalization is COI1-independent indicates that at least one other JA receptor exists. The way in which this novel JA receptor functions could be similar to COI1 action on the JAZ family members. This receptor could direct the degradation of a repressor protein which retains ORA59 in the cytoplasm by masking the NLS. Alternatively this putative receptor could activate a protein that masks the NES and due to disruption of the nuclear-cytoplasmic shuttling cycle ORA59 would accumulate in the nucleus.

The nucleocytoplasmic shuttling of ORA59 and its (in)stability shows striking similarities with the well studied regulation of the tumor supressor p53 in mammals. In normal cells under non-stressed conditions, p53 is a short-lived transcription factor which shuttles between the nucleus and the cytoplasm (Woods and Vousden, 2001) and is maintained in a latent form. Nuclear p53 levels are tightly regulated by the critical interacting protein Mdm2 or Hdm2 in human cells, an E3 ubiquitin ligase that promotes p53 nuclear export and cytoplasmic turnover. Studies suggest the presence of a cytoplasmic sequestration domain (CSD) in the C-terminus of p53 that inhibits association of importin with the C-terminal NLS1 (Liang et al., 1998; Liang et al., 1999), thereby preventing p53 nuclear import. In response to cellular stresses, p53 becomes transiently stabilized and translocates to the nucleus, where it forms a tetrameric complex which masks the C-terminal NES, thus trapping p53 in the nucleus (Stommel et al., 1999). An additional regulation is mediated by phosphorylation of the N-terminal NES in p53, which blocks p53 nuclear export (Zhang and Xiong, 2001).

Our favourite hypothesis is as follows. In the absence of JA signaling, cellular ORA59 protein levels are kept low by recognition by a specific F-box protein and proteasome-mediated degradation. The low residual level of protein is retained in the cytoplasm by interaction with a repressor protein. Perception of a biologically active jasmonate by a receptor distinct from COI1 leads to disruption of the interactions between ORA59 and the repressor protein. JA perception

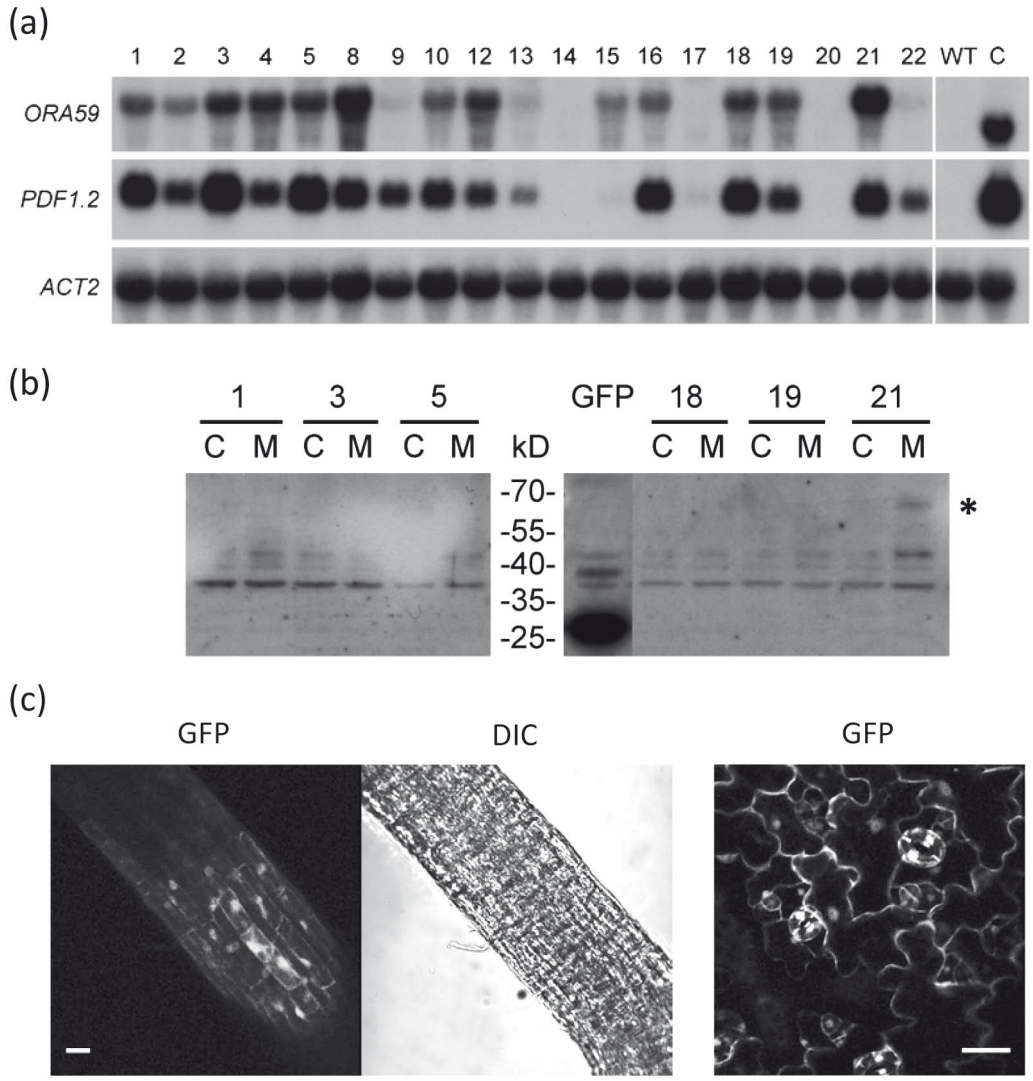


**Figure 12.** ORA59 is nuclear and cytosolic in transgenic plants. **(a)** Northern blot analysis. Fourteen-days-old seedlings from several *35S:ORA59-GFP* transgenic lines were screened by RNA blot hybridization with the indicated probes. A transgenic line (#2) expressing unfused ORA59 from the *35S* promoter was used as control. The ACTIN2 probe was used to verify equal loading. **(b)** Western blot analysis. Fourteen-days old seedlings were treated with 50 µM MG132 (M) or 0.1% DSMO (C) for 16 hours. Protein samples were separated by SDS-PAGE followed by Western blotting and immuno-probing with anti-GFP antibody. The asterisk indicates the position of the ORA59-GFP fusion protein. Positions of protein size markers are indicated in k Dalton. **(c)** Confocal laser scanning microscopy images of five-days-old *35S:ORA59-GFP* Arabidopsis seedlings of line #21, after 24 h incubation in water. Representative pictures of root and leaf tissues are shown. Scale bar = 25 µm.

by COI1 or the unknown receptor also disrupts the interaction with the F-box protein. Both events lead to increased amounts of ORA59 protein in the nucleus. One way to confirm this hypothesis is by identifying the F-box protein and the repressor protein, which might be feasible by yeast two-hybrid screening or TAP-tag purification of ORA59 complexes from plants.

**Table 1.** Summary of ORA59 domain mapping in different assays.

| | act. PDF1.2 unfused[a] | act. PDF1.2 nGFP[a] | stability[b] | stabilized by MG132[c] | % nuclear DMSO | % nuclear JA |
|---|---|---|---|---|---|---|
| 1-244 | + | + | - | + | 19 | 46 |
| 1-180 | + | + | - | ± | 38 | 57 |
| 48-244 | + | + | ± | ± | 24 | 30 |
| 48-180 | ± | - | + | ± | 84 | 84 |
| 48-139 | - | - | + | ± | 2 | 1 |
| 81-244 | + | + | - | ± | 50 | 56 |
| 81-180 | - | - | + | ± | 97 | 97 |
| 81-139 | - | - | + | - | 20 | 16 |

[a] Scores of transcriptional activating activity on the *PDF1.2* promoter as unfused proteins or as fusions with GFP at the N-terminal end.
[b] Relative stability compared to co-expressed GFP in untreated protoplasts.
[c] Scores whether MG132 caused an increase in protein amount compared to DMSO treatment.

## Materials and Methods

### Constructs and transient expression assays

For the construction of GFP-ORA37 and GFP-ORA59, the *ORA37* and *ORA59* open reading frames (ORF) were excised from pIC20H-ORA37 and pBluescript SK+-ORA59 with *Bam*HI and *Eco*RI/*Spe*I respectively and cloned into pTH2$^{BN}$ digested with *Bam*HI/*Bgl*II and *Eco*RI/*Spe*I respectively. pTH2$^{BN}$ is a derivative of pTH2 (Niwa et al., 1999; Chiu et al., 1996) lacking the stop codon of GFP (Kuijt et al., 2004). For the construction of GFP-ERF1 the *ERF1* ORF was excised from pIC20H-ERF1 with *Bgl*II and cloned into pBluescript SK+ digested with *Bam*HI, then *ERF1* was excised from pBluescript SK+-ERF1 with *Eco*RI/*Spe*I and cloned into pTH2$^{BN}$ digested with *Eco*RI/*Spe*I to generate GFP-ERF1. ORA59 deletion derivatives were amplified with pIC20H-ORA59 as template and primer sets 5'-CGG AAT TCA AAA TGG AAT ATC AAA CTA ACT TC-3' and 5'-CGG GAT CCT TAT TTC TTC TTT CCT CTA GGA CG-3' for 1-180, 5'-CGG AAT TCA AAA TGC CTA CTG ATA ACT ACT G-3' and 5'-CGG GAT CCT CAA GAA CAT GAT CTC ATA AG-3' for 48-244, 5'-CGG AAT TCA AAA TGC CTA CTG ATA ACT ACT G-3' and 5'-CGG GAT CCT TAT TTC TTC TTT CCT CTA GGA CG-3' for 48-180, 5'-CGG AAT TCA AAA TGC CTA CTG ATA ACT ACT G-3' and 5'-CGG GAT CCT TAG GGG AAA TTG AGT ACT GCG AGG-3' for 48-139, 5'-CGG AAT TCA AAA TGT CAT ACA GAG GAG TGA GG-3' and 5'-CGG

GAT CCT CAA GAA CAT GAT CTC ATA AG-3' for 81-244, 5'-CGG AAT TCA AAA TGT CAT ACA GAG GAG TGA GG-3' and 5'-CGG GAT CCT TAT TTC TTC TTT CCT CTA GGA CG-3' for 81-180, 5'-CGG AAT TCA AAA TGT CAT ACA GAG GAG TGA GG-3' and 5'-CGG GAT CCT TAG GGG AAA TTG AGT ACT GCG AGG-3' for 81-139. All ORA59 deletion derivatives were cloned in pGEM-T Easy (Promega, Madison, WI) and then inserts were excised with *Bam*HI and *Eco*RI and cloned in pRT101 (Töpfer et al., 1987). For N-terminal GFP fusions ORA59 deletion derivatives were excised from pGEM-T Easy with *Eco*RI and *Spe*I and cloned in pTH2$^{BN}$. The ORA59-GFP fusion was created by removal of the stop codon and in frame fusion with GFP. *ORA59* was amplified by PCR with the primer set 5'-ACG CGT CGA CAA AAT GGA ATA TCA AAC TAA CTT C- 3' and 5'- CCG CTC GAG CCT TGA GAA CAT GAT CTC ATA AG-3'and cloned in pGEM-T Easy. The *ORA59-ΔSTOP* insert was excised with *Sal*I/*Xho*I and cloned into pBluescript SK+ digested with *Sal*I. Then *ORA59-ΔSTOP* was excised from pBluescript SK+ with *Sal*I/*Eco*RI and cloned into pTH2$^{SN}$ (another derivative of pTH2; Kuijt et al., 2004). *ORA59-ΔSTOP* was excised from pTH2$^{SN}$ with *Sal*I/*Nco*I and cloned into pTH2. Details about the cloning of *ORA59* and *ERF1* in pRT101 and about the fusion between the *PDF1.2* promoter derivative SF and the *GUS* reporter gene are described in Chapter 2. Mutations of ORA59-NLS were generated according to the QuickChange Site-Directed Mutagenesis protocol (Stratagene) using pBluescript SK+-ORA59 as template and primers 5'-GTG ATA GCC TTG ACG TCG ACA CAC TCC ATG AGA-3' and 5'-TCT CAT GGA GTG TGT CGA CGT CAA GGC TAT CAC-3' for m1; 5'-GAA ACC GTC CTA GAG GAA CGA CGA CCT CGA GTT CTT CTT CGA CGT TG-3' and 5'- CAA CGT CGA AGA AGA ACT CGA GGT CGT CGT TCC TCT AGG ACG GTT TC-3' for m2 and m3 (using m1 in pGEM-T Easy as template); Deletions m1 and m2 were PCR amplified with 5'- CGG AAT TCA AAA TGG AAT ATC AAA CTA ACT TC-3' and 5'-CG GGA TCC TCA AGA ACA TGA TCT CAT AAG-3' and ligated in pGEM-T-Easy. The NLS was deleted by excision of an *Nde*I/*Nco*I m3 fragment from pGEM-T-Easy, subsequently digested with *Sal*I and *Xho*I. Digested fragment was ligated in pGEM-T-Easy digested with *Nde*I and *Nco*I. Deletion of the NES was generated with primer set 5'- CGG AAT TCA AAA TGG AAT ATC AAA CTA ACT TC-3' and 5'- GAC TAC TAG TTC AAA GAA CCA CAA GTG TTG TAT TAC-3' for m5 and m6 (using m3 as template). Deletions m1, m2 m3 and m4 were excised with *Eco*RI and *Bam*HI from pGEM-T Easy and cloned in pRT101 digested with *Eco*RI and *Bam*HI. Deletions m5 and m6 were digested with *Eco*RI from pGEM-T Easy and cloned in pRT101 digested with *Eco*RI, respectively. Deletions m1, m5 and m6 were excised with *Eco*RI and *Spe*I from pRT101 and cloned in pTH2$^{BN}$ digested with *Eco*RI and *Spe*I. Deletion m2 was excised with *Bgl*II and m3 and m4 with *Eco*RI from pRT101 and cloned in pTH2$^{BN}$ digested with *Bam*HI and *Eco*RI, respectively.

Protoplasts prepared from *Arabidopsis thaliana* cell suspension culture ecotype Col-0 (Axelos et al., 1992) were co-transformed with a reporter plasmid carrying *PDF1.2*-promoter-*GUS*, effector plasmids carrying *ERF1, GFP-ERF1*, *ORA59*, *GFP-ORA59* or *ORA59-GFP* genes fused to the *CaMV 35S* promoter and the p2rL7 plasmid (De Sutter et al., 2005) carrying the *Renilla reniformis luciferase* (*LUC*) gene under the control of the *CaMV 35S* promoter. As controls, co-transformations of *PDF1.2*-promoter-*GUS* with the empty pRT101 or pTH2 vectors and the p2rL7 plasmid were carried out. Protoplasts were transformed using polyethylene glycol

as described previously (Schirawski et al., 2000) with the three constructs in a ratio of 2:2:6 (µg *GUS*:*LUC*:effector plasmid). Arabidopsis leaf protoplasts were prepared and transformed as described (Sheen, 2002). Protoplasts were harvested 18 hours after transformation and frozen in liquid nitrogen. GUS and LUC activity assays were performed as described by van der Fits and Memelink (1997) and Dyer et al. (2000) with minor modifications. GUS activities from triplicate transformations were related to LUC activities in the same samples or to total protein content, determined by Bradford, to correct for differences in transformation and protein extraction efficiencies.

**Microscopy**

Arabidopsis cell suspension protoplasts were transformed with 10 µg of GFP-fusion plasmid DNA, after which the protoplasts were incubated for at least 16 hours in the dark. *Catharanthus roseus* cell suspensions were grown and transformed by particle bombardment as described (van der Fits and Memelink, 1997). Confocal laser scanning microscopy was performed by placing the cells on slides in a drop of water and examination of GFP fluorescence using an Axioplan upright microscope (Zeiss, Germany) equipped with a BIORAD MRC1024ES scanhead with a krypton/argon laser. ORA59-GFP seedlings were germinated in solid MA medium for 4 to 7 days-old and incubated 24h in water prior to microscopy analysis.

For visualization of GFP the excitation wavelength was 488 nm while the emitted fluorescence was collected after passage through a broad band pass filter (500-550 nm). The resulting signal was amplified, digitalized and the consistent picture reconstituted by Leica software.

**Protein extraction**

Protoplasts were ground in 50 µl of cold protein extraction buffer (50 mM HEPES-KOH pH 7.2, 100 mM NaCl, 5 mM EDTA, 5 mM EGTA, 50 mM β-glycerophosphate, 50 mM NaF, 1% Triton X-100, 1 mM $Na_3VO_4$, 5 µg/ml leupeptin, 5 µg/ml antipain, 5 mM DTT and 1 mM phenylmethylsulfonyl-fluoride (PMSF)). Fourteen-days old seedlings were frozen in liquid nitrogen and then ground in 100 µl of TAP protein extraction buffer (20 mM Tris pH 8.0, 150 mM NaCl, 2.5 mM EDTA pH 8.0, 0.1% Igepal, 10 mM 2-mercaptoethanol, 2 mM benzamidine, 1 mM PMSF, 20 mM NaF, protease inhibitor cocktail Complete Mini (Roche)). After centrifugation at 12000 rpm for 15 min at 4°C, supernatants were transferred into clean tubes, frozen in liquid nitrogen, and stored at -80°C. Protein concentrations were determined using Bio-Rad protein assay reagent with bovine serum albumin as the standard.

**Immunoblot analysis**

Protein extracts were separated on 10% (w/v) SDS-PAA gels and transferred to Protran nitrocellulose (Schleicher&Schuell) by semidry blotting. After blocking 1 hr in Tris-buffered saline-Tween (TBST; 20 mM Tris-HCl pH 7.6, 140 mM NaCl and 0.05% Tween 20) with 5 % non-fat dry milk at room temperature, the Western blots were incubated overnight with anti-HA peroxidase antibodies (1:2000, Roche) or with anti-GFP antibodies (1:5000) in TBST with 3 % bovine serum

albumin. After 1 hr incubation at room temperature the blots were washed 4 x with TBST. After incubation with anti-GFP antibodies, blots were incubated for 1 hr with anti-rabbit IgG antibodies linked to peroxidase (1:10000) in TBST and 5% non-fat dry milk, followed by 4 washings. Finally, the blots were incubated 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 (Fuji, Tokyo, Japan).

**Biological materials, growth conditions and treatments**

*Arabidopsis thaliana* wild-type plants, *coi1-1* mutant plants, and all transgenic plants are in the genetic background of ecotype Col-0. Seeds were surface-sterilized by incubation for 1 minute in 70 % ethanol, 15 minutes in 50% bleach, and five rinses with sterile water. Alternatively, seeds were surface-sterilized in a closed container with chlorine gas for three hours (http://plantpath.wisc.edu/~afb/vapster.html).

Surface-sterilized seeds were transferred to 250 ml Erlenmeyer flasks containing 50 ml MA medium (Masson and Paszkowski, 1992) or grown on plates containing MA medium supplemented with 0.6% w/v agar. Transgenic plants from T2 or T3 generations were selected on solid MA medium containing 100 mg/L timentin and 20 mg/L hygromycin for ORA59-overexpressing plants. Following stratification for 3 days at 4°C, seeds were incubated at 21°C in a growth chamber (16 h light/8 h dark, 2500 lux) for 10 days or otherwise as indicated. Seeds in liquid medium were placed on a shaker at 120 rpm. Transgenic plants carrying an XVE expression module containing the *ORA59-HA* gene were treated with 2 µM estradiol (Sigma) dissolved in DMSO (0.1% final concentration). Seedlings were treated for different time periods with 50 µM JA (Sigma-Aldrich) or 50 µM MG132 dissolved in DMSO at a final concentration of 0.1%. Control seedlings were treated with 0.2% DMSO. Methyl-jasmonate (Bedoukian Research Inc.), JA (Sigma-Aldrich, St. Louis, MO), MG132 (Sigma-Aldrich) were diluted in dimethylsulfoxide (DMSO). Leptomycin B (Biomol) was diluted in ethanol.

**Binary constructs and plant transformation**

For the construction of the XVE-*ORA59-HA* lines, the *ORA59-HA* cassette was created by removing the stop codon and in frame fusion with a double HA tag. *ORA59-HA* was amplified by PCR with the primer set 5'-GGG GTA CCA AAA TGG AAT ATC AAA CTA ACT TC-3' and 5'-CGG GAT CCT TAA GCG TAA TCT GGA ACA TCG TAT GGG TAA CCA GCG TAA TCT GGA ACA TCG TAT GGG TAG AGC TCT TGA GAA CAT GAT CTC ATA AG-3', and was first cloned as a *Kpn*I/*Bam*HI fragment into pRT101. The expression cassette was transferred as a *Xho*I/*Xba*I fragment to pER8 (Zuo et al., 2000) digested with *Xho*I/*Spe*I. For the generation of *35S:ORA59-GFP* plants the expression cassette was transferred from $pTH2^{BN}$ as a *Hind*III/*Eco*RI fragment to pCAMBIA1300 (acc No. AF234296) digested with *Xho*I/*Spe*I. Binary vectors were introduced into *A. 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.

**RNA extraction and Northern blot analyses**

For each treatment, 15 to 20 10-days-old seedlings were transferred from plates with solidified MA medium 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. Seedlings were first treated for 10 min with 100 µM cycloheximide (CHX) dissolved in DMSO (0.1% final concentration) and then JA (Sigma-Aldrich) dissolved in DMSO at a final concentration of 50 µM or JA combined with 1 mM of the ethylene releaser ethephon (Sigma-Aldrich) dissolved in 50 mM sodium phosphate pH 7 (0.5 mM final concentration) were added for times as indicated. Total RNA was extracted from frozen tissues by hot phenol/chloroform extraction followed by overnight precipitation with 2 M lithium chloride and two washes with 70% ethanol, and resuspended in water. As described by Memelink et al. (1994), 10 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). 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 to Fuji X-ray films.

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