Transcription factor-dependent nuclear localization of a transcriptional repressor in jasmonate hormone signaling

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The plant hormone jasmonate (JA) plays an important role in regulating growth, development and immunity. A key step in JA signaling is ligand-dependent assembly of a coreceptor complex consisting of the F-box protein COI1 and JAZ transcriptional repressors. Assembly of this receptor complex results in proteasome-mediated degradation of JAZ repressors, which at resting state bind to and repress the MYC transcription factors. Although the JA receptor complex is believed to function within the nucleus, how this receptor complex enters the nucleus and, more generally, the cell biology of jasmonate signaling are not well understood. In this study, we conducted mutational analysis of the C termini (containing the conserved Jas motif) of two JAZ repressors, JAZ1 and JAZ9. These analyses unexpectedly revealed different subcellular localization patterns of JAZ1ΔJas and JAZ9ΔJas, which were associated with differential interaction of JAZ1ΔJas and JAZ9ΔJas with MYC2 and differential repressor activity in vivo. Importantly, physical interaction with MYC2 appears to play an active role in the nuclear targeting of JAZ1 and JAZ9, and the nuclear localization of JAZ9 was compromised in *myc2* mutant plants. We identified a highly conserved arginine residue in the Jas motif that is critical for coupling MYC2 interaction with nuclear localization of JAZ9 and JAZ9 repressor function in vivo. Our results suggest a model for explaining why some JAZΔJas proteins, but not others, confer constitutive JA-insensitivity when overexpressed in plants. Results also provide evidence for a transcription factor-dependent mechanism for nuclear import of a cognate transcriptional repressor JAZ9 in plants.

biotic stress | E3 ubiquitin ligase | salicylic acid | plant immunity

Plants, being sessile organisms subjected to ever-changing environmental stresses, have evolved complex mechanisms to properly balance their growth and development with appropriate defense responses. One of the major plant hormones involved in regulating the balance between growth and defense belongs to a class of lipid-derived molecules, collectively termed jasmonates (JAs) (1). In healthy plants, JA signaling plays a role in reproductive development, photomorphogenesis, and other growth responses (2–6). Defense responses that are regulated by JA signaling are activated by environmental stress factors including tissue damage from herbivorous insects, pathogen attack, drought, and UV irradiation (7–12). Induction of the JA response pathway results in significant transcriptional reprogramming and typically shifts the balance from growth to defense-related cellular processes, through inhibition of gene expression involved in cell cycle progression and photosynthesis, and activation of defense-related genes (13–16).

In the past five years, exciting progress has been made in the understanding of JA signaling and how this stress hormone influences plant growth, development and defense. The bioactive JA ligand, (3R,7S)-jasmonoyl-L-isoleucine (JA-Ile), is perceived by a receptor complex consisting of CORONATINE-INSENSITIVE1 (COI1), the F-box subunit of the SCF^{COI1} ubiquitin ligase, and

members of the JASMONATE-ZIM DOMAIN (JAZ) family of transcriptional repressors (17–23). As an elegant example of host-pathogen coevolution, several strains of the plant pathogen *Pseudomonas syringae* have developed the ability to produce coronatine (COR), a structural mimic of JA-Ile that increases susceptibility of the host plant (24–27). *Arabidopsis* has 12 JAZ proteins, which share two conserved functional motifs: the ZIM motif in the central part of the protein and the Jas motif at the C terminus (28). The ZIM motif mediates homo- and heteromeric interactions among JAZ repressors and JAZ interactions with the adapters/corepressors NINJA and TPL proteins (29–31). The C-terminal Jas motif is necessary for interaction with the LRR domain of COI1 (17, 19, 20, 32, 33). A short peptide of 21 amino acids within the Jas motif defines the minimal "degron" that is sufficient for ligand-dependent formation of COI1-JAZ1 receptor complexes (19). In addition, the Jas motif mediates interaction between JAZ proteins and their target transcription factors (TFs) that control downstream responses (17). Among the most well characterized TFs targeted by JAZ are members of a basic-helix–loop–helix family, including MYC2, -3, and -4, that are key regulatory components of diverse aspects of JA-mediated physiological responses (17, 33–38).

The core JA signaling components, including a multimeric transcriptional corepression complex consisting of JAZ, NINJA, and TPL proteins bound to JA-responsive TFs and the COI1-JAZ receptor complex, are believed to function in the nucleus (31). However, direct evidence of nuclear localization (and underlying nuclear targeting mechanisms) for many of the key JA signaling components is lacking and, more generally, the cell biology of jasmonate signaling is poorly understood. Previous studies have shown that JAZ1 variants (JAZ1ΔJas) lacking the entire Jas motif or carrying alanine substitutions at specific arginine residues in the Jas motif are unable to interact with COI1 in a ligand-dependent manner and are therefore resistant to SCF^{COI1}-dependent degradation through the proteasome (20, 33). As a result, transgenic *Arabidopsis* plants that express these JAZ1 variants exhibit JA insensitivity (20, 32, 33). A similar JA-insensitive phenotype was observed for JAZ3 (JAZ3Δ-Jas) and JAZ10 (JAZ10ΔJas) (17, 23, 30, 39). However, it has also been shown that the Jas motif is required for JAZ3, but not JAZ1 or JAZ10, interaction with TFs such as MYC2, which should be a key step in TF-specific transcriptional repression (29, 30). Therefore, although transgenic overexpression of JAZΔJas proteins has been used as the primary means in the discovery of the repressor function of JAZ proteins, how JAZΔJas

proteins repress JA signaling has remained enigmatic (40). We report here that, in contrast to plants expressing JAZ1ΔJas, plants expressing JAZ9ΔJas do not exhibit obvious JA-insensitive phenotypes. This observation led us to conduct a series of experiments to understand the apparently different effects of the Jas motifs of JAZ1 and JAZ9 on JA signaling. Our results provide evidence for the existence of a TF-dependent mechanism for nuclear localization of cognate transcriptional repressor in plants, and show that nuclear localization of JAZ9 and interaction with MYC2 in the nucleus are coupled and are both required for repression of JA signaling.

Results

Constitutive Expression of Different JAZΔJas Proteins Results in Disparate JA-Insensitive Phenotypes. Several previous studies have reported the effects of ectopically expressing JAZΔJas proteins on JA signaling. Specifically, overexpression of JAZ1, JAZ3, and JAZ10 variants lacking the C-terminal Jas motif led to JA insensitivity, providing key evidence that JAZ proteins are transcriptional repressors (17, 20, 23, 30, 33). During investigation of the role of JAZ9 in JA signaling, we introduced an HA-tagged JAZ9 variant lacking the entire Jas motif (*35S:3×HA:JAZ9ΔJas*) into *Arabidopsis*, expecting a phenotypic effect on JA signaling similar to that in plants overexpressing JAZ1ΔJas (20, 33). Surprisingly, lines expressing this construct did not exhibit JA insensitivity as determined by root growth inhibition assays, in contrast to lines transformed with *35S:3×HA:JAZ1ΔJas* (*SI Appendix*, Fig. S1). One explanation for the lack of phenotype in *35S:3×HA:JAZ9ΔJas* lines is that JAZ9ΔJas maintains the ability to interact with COI1 in vivo, resulting in JAZ9ΔJas degradation in response to JA stimulus. However, in vivo degradation assays performed on 10-d-old transgenic seedlings revealed that both 3×HA:JAZ1ΔJas and 3×HA:JAZ9ΔJas were highly stable in comparison with the wild-type JAZ1 and JAZ9 fusion proteins (*SI Appendix*, Fig. S1). This finding is in agreement with previous yeast two-hybrid (Y2H) results indicating that JAZ1ΔJas and JAZ9ΔJas do not interact with COI1 in the presence of COR (33). Therefore, the observed phenotypic difference cannot be explained by differential stability of JAZ1ΔJas and JAZ9ΔJas.
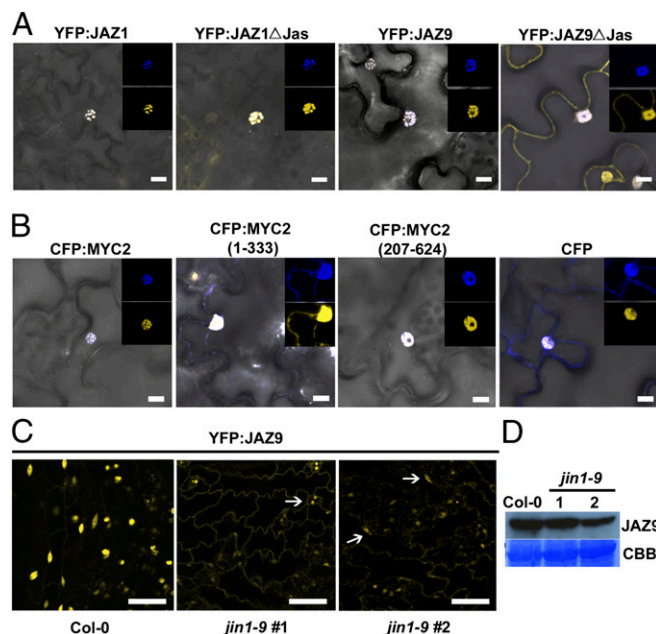
Role of MYC2 Interaction in Nuclear Localization of JAZ1 and JAZ9. We next compared the ability of JAZ1ΔJas and JAZ9ΔJas mutants to interact with MYC2 using Y2H assay. Here, we found a difference between JAZ1ΔJas and JAZ9ΔJas. Whereas JAZ1ΔJas retained the ability to interact with MYC2, albeit to a lesser extent than the wild-type JAZ1, JAZ9ΔJas did not interact with MYC2 (Fig. 1B and *SI Appendix*, Fig. S2). Next, we deleted 50 amino acids at a time from the C terminus of JAZ1ΔJas (Fig. 1A) and tested the ability of each to interact with MYC2 in Y2H. JAZ1ΔJas −50 and −100 retained interaction with MYC2 (Fig. 1B). In contrast, JAZ1ΔJas −150 lost the ability to interact with MYC2, indicating that a second MYC2 interacting motif is located in the N terminus before the conserved ZIM motif (Fig. 1B). In addition, we found a difference in the subcellular localization patterns of YFP:JAZ1ΔJas and YFP:JAZ9ΔJas in *Agrobacterium*-mediated transient expression experiments in *Nicotiana tabacum*. Both YFP:JAZ1 and YFP:JAZ9 were found to be localized to the nucleus, mostly in undefined subnuclear bodies, whereas the YFP alone was localized in both the nucleus and cytoplasm in a diffuse manner (*SI Appendix*, Fig. S3). YFP:JAZ1ΔJas could be readily detected both in the nucleus and in the cytoplasm, but lost the localization to subnuclear bodies and resembled the diffuse localization of the YFP control (*SI Appendix*, Fig. S3). YFP:JAZ9ΔJas was also detected in both the nucleus and the cytoplasm, but the signal in the nucleus was weaker compared with YFP:JAZ1ΔJas, even though YFP:JAZ1ΔJas and YFP:JAZ9ΔJas proteins were expressed to similar levels (*SI Appendix*, Figs. S3 and S4). These results provided an initial indication that there might be a correlation between the ability of JAZ proteins to interact with MYC2 and an enhanced localization to the nucleus. We also determined the subcellular localization of COI1 and NINJA and

found that both YFP:COI1 and YFP:NINJA localize diffusely within the nucleus in our transient expression experiments (*SI Appendix*, Fig. S5). The nuclear localization of YFP:NINJA was in agreement with previously reported localization results (31).

The apparent correlation between the ability of JAZ proteins to interact with MYC2 and an enhanced localization to the nucleus prompted us to examine the possibility that MYC2 might play an active role in targeting JAZ repressors to the nucleus. MYC2 was transiently coexpressed together with either JAZ1 or JAZ9 in *N. tabacum* leaves. Both YFP:JAZ1 and YFP:JAZ9 colocalized with CFP:MYC2 in subnuclear bodies (Fig. 2A).

Fig. 1. Deletion of the Jas domain differentially affects interaction with MYC2. (A) A diagram of the coding sequences of JAZ1, JAZ1ΔJas, and the C-terminal truncations used to create yeast two-hybrid (Y2H) constructs. (B) MYC2 interaction with JAZ1, JAZ9, JAZ1ΔJas, JAZ9ΔJas, and C-terminal deletions of JAZ1ΔJas in Y2H assays. Yeast cultures cotransformed with BD:JAZ and AD:MYC2 (*Upper*) or GAL4AD (*Lower*) spotted on −LWH drop-out media. Protein interactions are indicated by colony growth.

Fig. 2. Nuclear localization of JAZ is influenced by interaction with MYC2. Overlay of bright-field and fluorescent images of YFP:JAZ and CFP:MYC2 (A), or YFP:JAZ9 and CFP:MYC2 or truncated derivatives of CFP:MYC2 (B), transiently coexpressed in *N. tabacum* epidermal cells. (*Insets*) Portions of images enlarged to show nuclei with CFP channel alone (*Upper*) and YFP channel alone (*Lower*). (Scale bar: 10 μm.) (C) Fluorescent images of leaf epidermal cells expressing YFP:JAZ9 in transgenic Col-0 or *jin1-9* (*myc2*) mutants. Arrows indicate nuclei. (Scale bar: 50 μm.) (D) Full-length YFP:JAZ9 protein was detected from each line by Western blot using a polyclonal antibody against GFP. CBB, Coomassie brilliant blue staining of PVDF membrane.

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Interestingly, coexpression of YFP:JAZ1ΔJas with CFP:MYC2 changed the localization of this truncated JAZ from both nuclear and cytoplasmic (*SI Appendix*, Fig. S3) to predominantly nuclear (Fig. 2*A*). In contrast, coexpression of YFP:JAZ9ΔJas with CFP: MYC2 did not have the same effect on the cytoplasmic location of YFP:JAZ9ΔJas (Fig. 2*A*). Thus, overexpression of CFP: MYC2 was sufficient to drive complete nuclear localization of YFP:JAZ1ΔJas (which retains interaction with MYC2), but not YFP:JAZ9ΔJas (which does not interact with MYC2).

MYC2 has been shown to interact with JAZs via its N terminus (37). Protein sequence analysis using ProteinPredict software, which is based on the LOCtree and PredictNLS algorithms (41, 42), showed that the C-terminal half of MYC2 contains a monopartite nuclear localization signal (NLS), KRPKKRGRK[433-441]. We examined the localization of the N-terminal (1–333) and C-terminal (207–624) halves of MYC2 and the effects of the N-terminal and C-terminal halves of MYC2 on the localization of JAZ9. Consistent with the presence of a NLS in the C terminus, the C-terminal half of MYC2 was localized exclusively in the nucleus. In contrast, the N-terminal half was localized partially in the cytoplasm and partially in the nucleus (Fig. 2*B*). YFP-JAZ9 nuclear localization was not affected when coexpressed with the C terminus of MYC2, which does not interact with JAZ9 in Y2H (Fig. 2*B* and *SI Appendix*, Fig. S6). However, the cytoplasmically localized N terminus of MYC2, which interacts with JAZ9, trapped a portion of YFP:JAZ9 in the cytoplasm (Fig. 2*B* and *SI Appendix*, Fig. S6). This result further indicates that physical interaction with MYC2 affects JAZ9 localization in the cell.

We next asked the question about whether endogenous MYC2 is necessary for nuclear localization of full-length JAZ9. For this purpose, we transformed the *35S:YFP:JAZ9* construct into the *Arabidopsis jin1-9* (*myc2*) mutant (43). Confocal microscopy of the resulting lines revealed partial mis-localization of YFP:JAZ9 to the cytoplasm, in addition to the expected nuclear localization (Fig. 2*C*); this localization pattern contrasted with that of YFP: JAZ9 in wild-type Col-0 plants, where YFP:JAZ9 signal was located entirely in the nucleus. There are at least three MYC-family transcription factors (MYC2, MYC3, and MYC4) that have been shown to interact with JAZs and are involved in JA signaling (37, 44). Quantitative RT-PCR analysis showed that, under our growth conditions, *MYC2* and *MYC3* are highly expressed in leaves, whereas *MYC4* was expressed at a lower level (*SI Appendix*, Fig. S7). Taken together, the results provide genetic evidence that MYC2 is partially required for proper nuclear localization of JAZ9 and suggest that MYC3, and potentially MYC4, may also contribute to JAZ9 nuclear localization.

Arginine[Jas17] Is Critical for JAZ9 Nuclear Localization and Interaction with MYC2. Having established an active role for MYC2 in nuclear localization of JAZ9, we next sought to identify specific residues within the Jas motif that are required for this process. The minimal JAZ1 degron was previously identified in the crystal structure of the COI1-JAZ1 coreceptor complex (19). Because different JAZs are of different lengths, the specific amino acid positions within the conserved Jas motif vary greatly among different JAZs. We use a simplified nomenclature to describe amino acid positions within the Jas motif (Fig. 3*A*). The Jas motif contains clusters of basic amino acid residues that resemble the classic mono- and bipartite NLS (45–47). In addition, the highly conserved KRK(E/D)RX$_5$PY sequence in the C-terminal end of the Jas motif resembles a nonclassical NLS (46, 48), and has been implicated as a nuclear localization signal for JAZ1 (49). However, alternative splice variants of JAZ10 that lack the PY motif retain the ability to enter the nucleus (30, 39). To determine which, if any, of the motifs are involved in nuclear localization of JAZ9, we transiently expressed the wild-type JAZ9 and the RK[Jas6,7]AA or KRK[Jas16-18]AAA mutants as YFP fusions in *N. tabacum* leaf cells and analyzed their subcellular localization by confocal microscopy. YFP:JAZ9-RK[Jas6,7]AA retained the ability to accumulate in the nucleus and to form subnuclear bodies, whereas YFP:JAZ9-KRK[Jas16-18]AAA exhibited a cytoplasmic localization
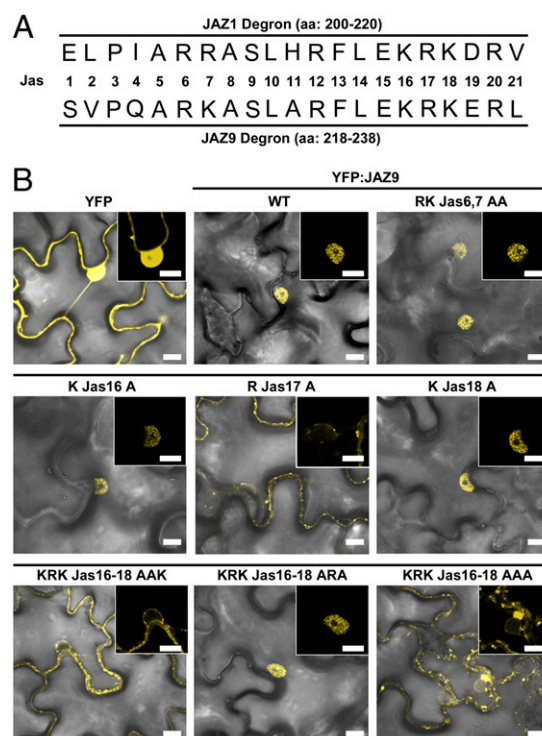

Fig. 3. Arginine[Jas17] in the Jas domain is critical for nuclear localization of JAZ9. (*A*) Amino acid sequences of the Jas degron from JAZ1 and JAZ9. Jas1 is the first amino acid in sequence. Subsequent amino acids are numbered sequentially. (*B*) Bright-field and fluorescent image overlay of YFP:JAZ9 variants transiently expressed in *N. tabacum* epidermal cells. Single, double, or triple alanine substitutions in the Jas motif of JAZ9 are indicated in each panel. (*Insets*) Portions of images enlarged to shown nuclei. (Scale bar: 10 μm.)

(Fig. 3*B*). These nuclear and cytoplasmic localization patterns were further confirmed in transgenic *Arabidopsis* expressing the same YFP:JAZ9 fusions (*SI Appendix*, Fig. S8), thus implicating the KRK motif as a critical element for nuclear localization of JAZ9. We noticed that the mis-localization of YFP:JAZ9-KRK[Jas16-18]AAA to the cytoplasm was more complete than YFP:JAZ9ΔJas (*SI Appendix*, Fig. S3), indicating that the residual nuclear localization of YFP:JAZ9ΔJas may be caused by some nonspecific diffusion because YFP:JAZ9ΔJas is smaller than full-length YFP:JAZ9-KRK[Jas16-18]AAA.

To further delineate the KRK[Jas16-18] motif in nuclear localization of JAZ9, we next analyzed single, double and triple alanine mutations of KRK[Jas16-18]. Single alanine point mutations of K[Jas16] or K[Jas18] had no effect on nuclear localization, as evidenced by the formation of subnuclear bodies (Fig. 3*B*). However, R[Jas17]A completely excluded the fusion protein from the nucleus; all double and triple amino acid substitutions that included the R[Jas17]A mutation had the same effect (Fig. 3*B*). Moreover, with only R[Jas17] present in the KRK motif (i.e., KRK[Jas16-18]ARA), the fusion protein became properly localized to subnuclear bodies (Fig. 3*B*). The results from these experiments revealed a key role of R[Jas17] in the correct nuclear localization of YFP:JAZ9.

The identification of R[Jas17] as a critical determinant of JAZ9 nuclear localization provided us with another opportunity to test the hypothesis that JAZ9 nuclear localization is coupled to the physical interaction with MYC2. Results from Y2H assays revealed that KRK[Jas16-18] triple alanine mutations disrupted interaction with MYC2. However, JAZ9-KRK[Jas16-18]ARA had normal interaction with MYC2. Therefore, within the KRK sequence, R[Jas17] was necessary and sufficient for mediating interaction with MYC2 (Fig. 4*A*).

Next, we examined the ability of various mutants to colocalize with CFP:MYC2 upon transient expression in *N. tabacum*.
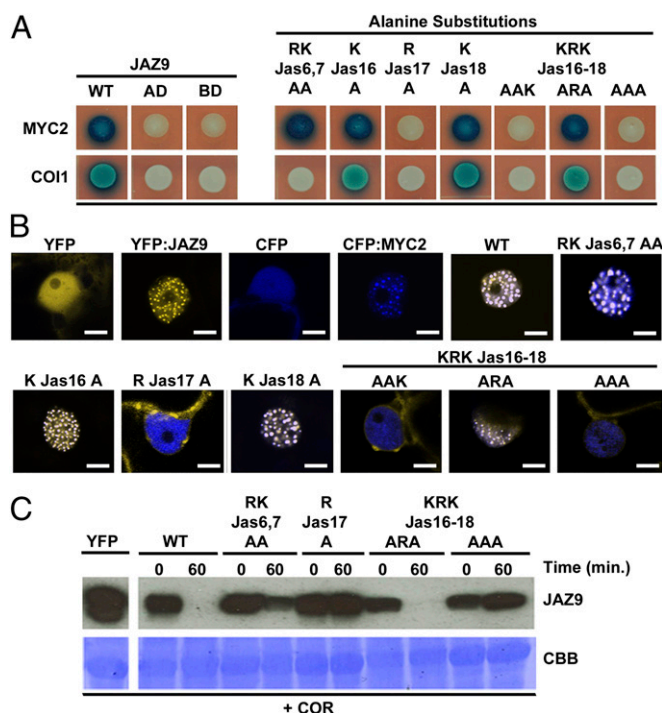
Fig. 4. Arginine[Jas17] is critical for JAZ9 interaction, stability, and colocalization with MYC2. (*A*) Yeast two-hybrid assay. Yeast cultures cotransformed with AD:MYC2 and BD:JAZ9 variants (*Upper*), or BD:COI1 and AD:JAZ9 variants (*Lower*), were spotted on −UWH drop-out media supplemented with X-Gal (and 10μM COR for COI1 interaction). Blue color indicates protein interaction. (*B*) Colocalization of YFP:JAZ9 variants and CFP:MYC2 transiently expressed in *N. tabacum* epidermal cells. Fluorescent images of YFP:JAZ9, CFP:MYC2, and vector controls, and dual channel overlay of YFP:JAZ9 carrying the indicated alanine substitutions coexpressed with CFP:MYC2. (Scale bar: 5 μm.) (*C*) In vivo JAZ protein degradation assay. Stability of YFP:JAZ9 variants expressed in *Arabidopsis* was determined by Western blot 1 h after 1 μM COR treatment. CBB, Coomassie brilliant blue staining of PVDF membrane.

Again, we observed a strong correlation between nuclear localization of YFP:JAZ9 and the ability of these fusion proteins to interact with MYC2 and to colocalize with CFP:MYC2. Specifically, YFP:JAZ9-RK[Jas6,7]AA, which interacted with MYC2 in Y2H assays, was colocalized with CFP:MYC2 in subnuclear bodies, similar to YFP:JAZ9 and CFP:MYC2 (Fig. 4*B*). Single lysine mutations in the KRK[Jas16-18] motif had no effect on nuclear import or colocalization with CFP:MYC2. However, any variation of YFP:JAZ9 lacking the critical amino acid R[Jas17] was excluded from the nucleus, whereas the CFP:MYC2 signal remained nuclear. Furthermore, within the KRK sequence R[Jas17] was sufficient for JAZ9 colocalization with CFP:MYC2 to subnuclear bodies (Fig. 4*B*). Consistent with findings reported in a previous study (33), we found that RK[Jas6,7] are not required for JAZ9 interaction with MYC2 (Fig. 4*A*). This result can now be explained by the fact that, of the basic amino acids in the Jas motif, only R[Jas17] is critical for JAZ9 interaction with MYC2.

We also determined the ability of JAZ9 mutants to interact with COI1 in Y2H experiments. Consistent with previous results (33), JAZ9-RK[Jas6,7]AA did not interact with COI1, and again, we found that the R[Jas17]A mutation disrupted interaction (Fig. 4*A*). These results indicated that alanine substitutions of both JAZ9-RK[Jas6,7] and -R[Jas17] would result in increased stability. As expected, in vivo protein degradation experiments in transgenic *Arabidopsis* seedlings showed that whereas YFP:JAZ9 and YFP:JAZ9-KRK[Jas16-18]ARA are completely degraded within 1 h after treatment with coronatine, YFP:JAZ9-RK[Jas6,7]AA and YFP:JAZ9-R[Jas17]A were resistant to coronatine-mediated degrada-

tion (Fig. 4*C*). This finding is consistent with the ability of JAZ9 and JAZ9-KRK[Jas16-18]ARA, but not JAZ9-RK[Jas6,7]AA or JAZ9-R[Jas17]A, to interact with COI1 (Fig. 4*C*) (33).

R[Jas17] Is Required for JAZ9 to Repress JA Responses *in Planta*. Our results suggest a model in which the repressor function of JAZ9 protein *in planta* requires two inseparable R[Jas17]-mediated processes: (*i*) MYC2-assisted nuclear localization of JAZ9 protein and (*ii*) physical interaction with MYC2. If this model is correct, we expect that (*i*) directing JAZ9ΔJas (SCF[COI1] degradation-resistant, but not MYC2-interacting) to the nucleus may not be sufficient to confer JA insensitivity, but (*ii*) JAZ9-RK[Jas6,7]AA (SCF[COI1] degradation-resistant, MYC2-interacting, and nuclear-localized) should confer JA insensitivity when transgenically expressed in wild-type plants. To test these predictions, we fused JAZ9ΔJas to mCherry (mCH) and VirD2NLS, a well characterized nuclear localization signal from the *Agrobacterium tumefaciens* VirD2 protein (50, 51), and expressed these constructs in Col-0 plants. As expected, mCH:JAZ9ΔJas:NLS was localized in the nucleus (*SI Appendix*, Fig. S9). However, Col-0 plants expressing mCH:JAZ9ΔJas:NLS remained sensitive to JA, as indicated by inhibition of root elongation in seedlings grown on MS agar supplemented with 10 μM methyl-JA (MeJA; *SI Appendix*, Fig. S9). This result demonstrates that redirecting JAZ9ΔJas to the nucleus with a classical NLS is not sufficient to repress JA signaling.

In contrast, transgenic Col-0 seedlings expressing YFP:JAZ9-RK[Jas6,7]AA were partially insensitive (26% root growth inhibition, compared with 62–65% root growth inhibition for Col-0), whereas transgenic expression of YFP:JAZ9-KRK[Jas16-18]AAA did not affect root or rosette growth sensitivity to JA (Fig. 5*A* and *SI Appendix*, Fig. S10). We also performed disease assays to evaluate whether these plants are altered in response to the coronatine-producing strain *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000. We found typical symptom development on wild-type Col-0 and transgenic plants expressing YFP, YFP:JAZ9, or YFP:JAZ9-KRK[Jas16-18]AAA (*SI Appendix*, Fig. S10). In contrast, plants overexpressing YFP:JAZ9-RK[Jas6,7]AA showed significant resistance to *Pst* DC3000, comparable to that seen in the *coi1-30* mutant (Fig. 5*B* and *SI Appendix*, Fig. S10). Bacterial counts were as much as 100 times lower than that in Col-0, and leaves retained a disease-free appearance (Fig. 5*B* and *SI Appendix*, Fig. S10). These findings indicate that the ability of JAZ9 to strongly repress JA responses in planta requires nuclear localization, MYC2 interaction, and resistance to SCF[COI1]-dependent degradation.

Discussion

JA is an important hormone that regulates diverse physiological processes in plants, ranging from growth and development to immunity against biotic and abiotic stresses. Recent studies from many laboratories have contributed to several significant advances in the dissection of the COI1-JAZ-MYC core signaling module and molecular connections to different downstream cellular pathways (7, 52–58). Although the COI1-JAZ-MYC signaling module is thought to perceive ligands and function inside the nucleus, direct evidence supporting this model and the underlying mechanisms for the nuclear entry of various signaling components has remained largely enigmatic. In this study, we addressed this important issue and show that COI1, JAZ1, JAZ9, MYC2, and NINJA are constitutively localized in the nucleus. In particular, our results revealed a TF-dependent mechanism for the nuclear localization of JAZ9 and suggest a model for explaining why some JAZΔJas proteins, but not others, confer constitutive JA-insensitivity when overexpressed in plants, a puzzle that has remained unresolved since the discovery of JAZ repressors.

All JAZ proteins studied to date have been shown to be localized in the nucleus and contain a highly conserved Jas motif at the C terminus (20, 23, 30, 49, 59). How the Jas motif mediates nuclear import, however, is not understood. The Jas motif contains clusters of basic amino acids conserved among the JAZ proteins (RK[Jas6,7] and KRKER[Jas16-20], separated by eight amino
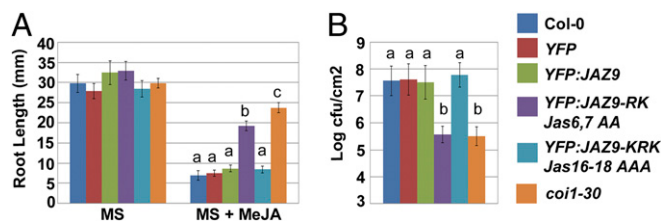
Fig. 5. Trangenic expression of JAZ9-RK^{Jas6,7}AA confers JA-insensitive phenotypes. (*A*) Quantification of root growth. Seedlings were grown in the absence or presence of 10 μM MeJA for 10 d, and root length was quantified using ImageJ software. Data are the means from 15 plants; error bars represent SD. Letters on columns indicate statistically significant differences (*P* < 0.01, Tukey's HSD test). (*B*) Five-week-old plants were syringe-infiltrated with *Pst* DC3000 at a concentration of 10^6 cfu/mL. Bacterial enumeration was conducted 3 d postinoculation. Data are the mean cfus counted from four individual leaves per genotype; error bars represent SD. Letters on columns indicate statistically significant differences (*P* < 0.01, Tukey's HSD test).

acids in JAZ9), which superficially resembles a putative bipartite NLS. Importantly, this stretch of basic amino acids, plus five additional nonbasic amino acids in the Jas motif of JAZ1, was capable of driving GFP into the nucleus (60). Unexpectedly, our results show that none of the basic amino acids, except for R^{Jas17}, in the Jas motif of JAZ9, is required for nuclear localization (Fig. 3*B*). Because clustered, basic amino acids are essential for the function of an NLS (45, 61), our results cast doubt on the idea that the Jas motif functions as an NLS per se. Instead, we found that the requirement of R^{Jas17} for JAZ9 nuclear entry is correlated with its requirement for JAZ9 interaction with MYC2. First, overexpression of nuclear-localized CFP:MYC2 in *N. tabacum* cells is sufficient to drive complete nuclear localization of YFP:JAZ1ΔJas (which retains interaction with MYC2), but not YFP:JAZ9ΔJas (which does not interact with MYC2) (Fig. 2*A*). Second, CFP:MYC2 lacking its C-terminal NLS (KRPKKRGRK$^{433-441}$) not only was partially localized in the cytoplasm, but also trapped a substantial portion of YFP:JAZ9 in the cytoplasm (Fig. 2*B* and *SI Appendix*, Fig. S6). Third, the nuclear localization of YFP:JAZ9 is compromised in the *myc2* mutant plants (Fig. 2*C*). Altogether, these results suggest that MYC2, but not JAZ9, plays an active role in determining the nuclear localization of both MYC2 and JAZ9 in the cell. We therefore hypothesize that the Jas motif (in particular, R^{Jas17}) is necessary for the nuclear localization of JAZ9 because it is essential for physical interaction with MYC2. This hypothesis is further supported by protein sequence analysis using ProteinPredict, which shows a monopartite NLS, KRPKKRGRK$^{433-441}$, in the C-terminal half of MYC2, but failed to identify a predictable NLS in JAZ9.

It remains to be determined whether the TF-dependent mechanism for nuclear localization would be generally applicable to other JAZ repressors, although interaction with MYC2 and related TFs is a common property of all characterized JAZ repressors. Furthermore, although our results indicate the physical interaction with MYC2 (and presumably other TFs) being a major factor in directing the nuclear localization of JAZ9, it is possible that other MYC2-dependent processes also influence this process. For example, MYC2 may positively regulate the expression of the components of the nuclear import machinery as an additional mechanism of assisting the nuclear localization of JAZs. However, examination of publicly available *Arabidopsis* gene expression databases (36, 62) did not reveal obvious MYC2-dependent regulation of the expression of such genes.

Transgenic overexpression of JAZΔJas proteins has been used as the primary means in the discovery of the repressor function of JAZ proteins because these derivatives are resistant to SCF^{COI1}-dependent degradation and confer JA-insensitive phenotypes (17, 30, 33, 39). However, the mechanism by which JAZΔJas confers the dominant-negative repressor function is

still unresolved (17, 20, 29, 40). Our results now provide an explanation for why some, but not other, JAZΔJas proteins confer constitutive JA-insensitivity when overexpressed in plants, and suggest a model in which the ability of a JAZΔJas to confer JA insensitivity is linked to three features: TF-interacting, nuclear-localized, and resistant to SCF^{COI1}-dependent degradation. First, we found that JAZ9ΔJas failed to repress JA signaling (*SI Appendix*, Figs. S1 and S9). This result was in contrast to strong JA insensitivity caused by overexpression of JAZ1ΔJas (20, 33). By investigating the capability of JAZ1ΔJas and JAZ9ΔJas to interact with MYC2, we have determined that interaction with MYC2 is a key feature that is associated with eliciting JA-insensitivity, as JAZ1ΔJas, but not JAZ9ΔJas, is still able to interact with MYC2 (Fig. 1*B* and *SI Appendix*, Fig. S2). Second, confocal microscopic examination revealed that YFP:JAZ9 is colocalized with CFP:MYC2 in the nucleus, whereas JAZ9ΔJas and those Jas motif mutants that do not confer JA insensitivity were not found to be colocalized with CFP:MYC2, but instead localized mostly in the cytosol, despite being resistant to degradation in response to coronatine (Figs. 3 and 4; and *SI Appendix*, Fig. S8). Third, we were able to identify two COI1-interacting residues, RK^{Jas6,7}, that when mutated to alanine, created a JAZ9 derivative that is nuclear localized, resistant to degradation, still interacts with MYC2, and is colocalized with MYC2 in the nucleus (Figs. 3 and 4; and *SI Appendix*, Fig. S8). This JAZ9 derivative now exerts JA insensitive phenotypes when transgenically overexpressed (Fig. 5 and *SI Appendix*, Fig. S10). On the other hand, we found that simply directing JAZ9ΔJas (resistant to SCF^{COI1}-dependent degradation, but not interacting with MYC2) into the nucleus by fusion to a NLS was not sufficient to create jasmonate insensitive phenotypes (*SI Appendix*, Fig. S9). The TF interaction-based model of repression not only could explain the lack of dominant-negative effect of JAZ9ΔJas, but also the ability of JAZ1ΔJas and JAZ10ΔJas to confer JA-insensitivity because both of them still interact with MYC2, indicating more than one MYC2-interacting region in these particular JAZs (30). However, this model could not yet explain the ability of JAZ3ΔJas to confer JA-insensitive phenotypes because the Jas motif is required for JAZ3 interaction with MYC2, but JAZ3ΔJas confers JA insensitivity (17, 29). It is possible that TFs other than MYC2 are biologically relevant targets of JAZ3, or that JAZ3ΔJas confers JA-insensitivity through heterodimerization with other nuclear-localized and MYC2-interacting JAZ repressors, as proposed (29).

To our knowledge, TF-dependent nuclear localization of cognate transcriptional repressors has not been reported in plants or other eukaryotic systems. However, Pfeiffer et al. (63) recently reported that phytochrome-interacting factor (PIF) TFs facilitate the nuclear import of a light receptor protein, phytochrome B, as a critical step in regulating light signaling. Thus, at least two major signaling systems in plants, light signaling and JA signaling, use the TF-dependent nuclear localization mechanism. It is possible that plants have evolved this mechanism to enable immediate targeting of TFs for repression by cognate repressors, possibly right after they are synthesized in the cytoplasm. This mechanism may be important if the TPL transcriptional repression complex is rate-limiting in the nucleus because of its involvement in many signaling processes (31). TF-dependent import of JAZs could efficiently target the NINJA/TPL transcriptional repression complex to only TF-bound JAZs, but not "nonproductive" TF-free JAZs, which could occur if JAZs were imported independently. Regardless, the TF-dependent nuclear import mechanism may be needed to ensure maximal and immediate repression of JA signaling to minimize unnecessary growth inhibition and other undesirable side effects that are known to accompany the activation of JA signaling (1).

Materials and Methods

All experiments reported in this work were performed three or more times with similar results. Confocal microscopy was conducted on leaf tissues from *N. tabacum* (transient expression) and *Arabidopsis thaliana* (stable expression). Dual-channel sequential imaging was used to capture images during

colocalization experiments. Detailed experimental protocol and information about creating gene constructs and transgenic *Arabidopsis* can be found in *SI Appendix*.

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