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A Critical Role for the TIFY Motif in Repression of Jasmonate Signaling by a Stabilized Splice Variant of the JASMONATE ZIM-Domain Protein JAZ10 in *Arabidopsis* [C][W]

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JASMONATE ZIM-domain (JAZ) proteins act as repressors of jasmonate (JA) signaling. Perception of bioactive JAs by the F-box protein CORONATINE INSENSITIVE1 (COI1) causes degradation of JAZs via the ubiquitin-proteasome pathway, which in turn activates the expression of genes involved in plant growth, development, and defense. JAZ proteins contain two highly conserved sequence regions: the Jas domain that interacts with COI1 to destabilize the repressor and the ZIM domain of unknown function. Here, we show that the conserved TIFY motif (TIFF/YXG) within the ZIM domain mediates homo- and heteromeric interactions between most *Arabidopsis thaliana* JAZs. We have also identified an alternatively spliced form (JAZ10.4) of JAZ10 that lacks the Jas domain and, as a consequence, is highly resistant to JA-induced degradation. Strong JA-insensitive phenotypes conferred by overexpression of JAZ10.4 were suppressed by mutations in the TIFY motif that block JAZ10.4–JAZ interactions. We conclude that JAZ10.4 functions to attenuate signal output in the presence of JA and further suggest that the dominant-negative action of this splice variant involves protein–protein interaction through the ZIM/TIFY domain. The ability of JAZ10.4 to interact with MYC2 is consistent with a model in which a JAZ10.4-containing protein complex directly represses the activity of transcription factors that promote expression of JA response genes.

INTRODUCTION

The lipid-derived hormone jasmonate (JA) regulates many physiological activities during the plant life cycle. JA is well known for its ability to promote plant defense responses against insect herbivores, necrotrophic pathogens, and various abiotic stresses as well (Pozo et al., 2004; Glazebrook, 2005; Browse and Howe, 2008; Howe and Jander, 2008). JA also plays an important role in normal plant growth and development, including cell division, cell fate determination, photomorphogenesis, and sexual reproduction (McConn and Browse, 1996; Li et al., 2004; Sineshchekov et al., 2004; Browse, 2005; Chen et al., 2007; Balbi and Devoto, 2008). An important attribute of JA is its ability to act both as a potent inhibitor of vegetative growth and as a positive regulator of reproductive and defensive processes (Yan et al., 2007; Howe and Jander, 2008; Mitra and Baldwin, 2008; Pauwels et al., 2008). These antagonistic activities of JA suggest a broader role for the hormone in dealing with the dilemma of plants to grow or defend (Herms and Mattson, 1992) in rapidly changing environments.

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JA exerts its many effects through large-scale changes in gene expression (Schenk et al., 2000; Sasaki et al., 2001; Goossens et al., 2003; Reymond et al., 2004; Devoto et al., 2005; Suzuki et al., 2005; Sasaki-Sekimoto et al., 2005; Uppalapati et al., 2005; Mandaokar et al., 2006; Schmidt and Baldwin, 2006). Activation of JA response genes is mediated in part by the basic helix-loop-helix transcription factor MYC2 (also known as JIN1) (Abe et al., 2003; Lorenzo et al., 2004; Laurie-Berry et al., 2006; Chini et al., 2007; Dombrecht et al., 2007; Takahashi et al., 2007). Current models of JA signaling indicate that, in the absence of JA, MYC2's function as a transcriptional activator is repressed by members of the JASMONATE ZIM-domain (JAZ) family of proteins that physically interact with MYC2 (Chini et al., 2007; Melotto et al., 2008). Elevated levels of JA trigger the release of MYC2 from this repression by increasing the rate of JAZ turnover. Bioactive JAs promote interaction of JAZ proteins with the CORONATINE INSENSITIVE1 (COI1) component of the ubiquitin E3 ligase SCF^{COI1} (Xie et al., 1998; Thines et al., 2007; Katsir et al., 2008b; Melotto et al., 2008). This interaction leads to ubiquitination and degradation of JAZs by the 26S proteasome. Ligand binding studies have shown that jasmonoyl-isoleucine (JA-Ile) and structurally related JA–amino acid conjugates stimulate COI1–JAZ interactions through the formation of a stable COI1/JA-Ile/JAZ complex (Katsir et al., 2008b). The bacterial toxin coronatine is a structural mimic of JA-Ile and a potent agonist of this intracellular receptor system (Katsir et al., 2008b; Melotto et al., 2008).

Several lines of evidence indicate that the C-terminal Jas domain plays a key role in destabilizing JAZ repressors in response to increased JA levels. JAZ–β-glucuronidase (GUS)

(Thines et al., 2007) and JAZ–green fluorescent protein (Chini et al., 2007) fusion proteins are degraded in JA-treated cells, whereas truncated JAZ proteins (referred to here as JAZΔJas) that lack the Jas domain are stable in the presence of the hormone. Ectopic expression of JAZΔJas proteins results in various JA-insensitive phenotypes, including male sterility, resistance to JA-mediated inhibition of root growth, reduced expression of JA response genes and secondary metabolites, susceptibility to insect feeding, and enhanced resistance to coronatine-producing strains of *Pseudomonas syringae* (Chini et al., 2007; Thines et al., 2007; Chung et al., 2008; Melotto et al., 2008; Shoji et al., 2008). Biochemical studies have shown that the Jas domain is sufficient for formation of COI1/JAZ complexes and associated ligand binding (Katsir et al., 2008b; Melotto et al., 2008). In addition, point mutations in the Jas domain that block hormone-induced COI1/JAZ interaction confer dominant JA insensitivity (Melotto et al., 2008).

A simple hypothesis to explain the dominant action of JAZΔ Jas proteins is that they interact with and repress the activity of MYC2. This idea can account for the JA-insensitive phenotype of plants that overexpress JAZ variants (e.g., JAZ1-A^{205}A^{206}) that fail to interact with COI1 but retain the ability to bind MYC2 (Melotto et al., 2008). However, this model does not explain the action of JAZΔJas proteins that do not interact with MYC2. Chini et al. (2007) reported that a truncated form of JAZ3 (also known as JAI3) that lacks the Jas domain interacts with COI1 in a JA-independent manner but fails to interact with MYC2. These workers proposed a poison complex model in which hormone-independent binding of JAZ3ΔJas (JAI3ΔC) to COI1 obstructs access of SCF^{COI1} to endogenous JAZs, thereby allowing endogenous JAZs to repress MYC2. Elucidation of how JAZΔJas proteins repress JA responses is critical for understanding the general mechanism of JA signaling.

JAZ proteins in *Arabidopsis thaliana* are encoded by 12 genes, designated *JAZ1* to *JAZ12*. The JAZ repressor model predicts that loss-of-function *jaz* mutations should result in constitutive or hypersensitive JA responses. Analysis of various *jaz* null mutants suggests functional redundancy between family members (Chini et al., 2007; Thines et al., 2007). Interestingly, however, transgenic plants silenced for the expression of *JAZ10* (also called *JAS1*) were more sensitive than the wild type to JA (Yan et al., 2007). These workers identified an alternatively spliced form of

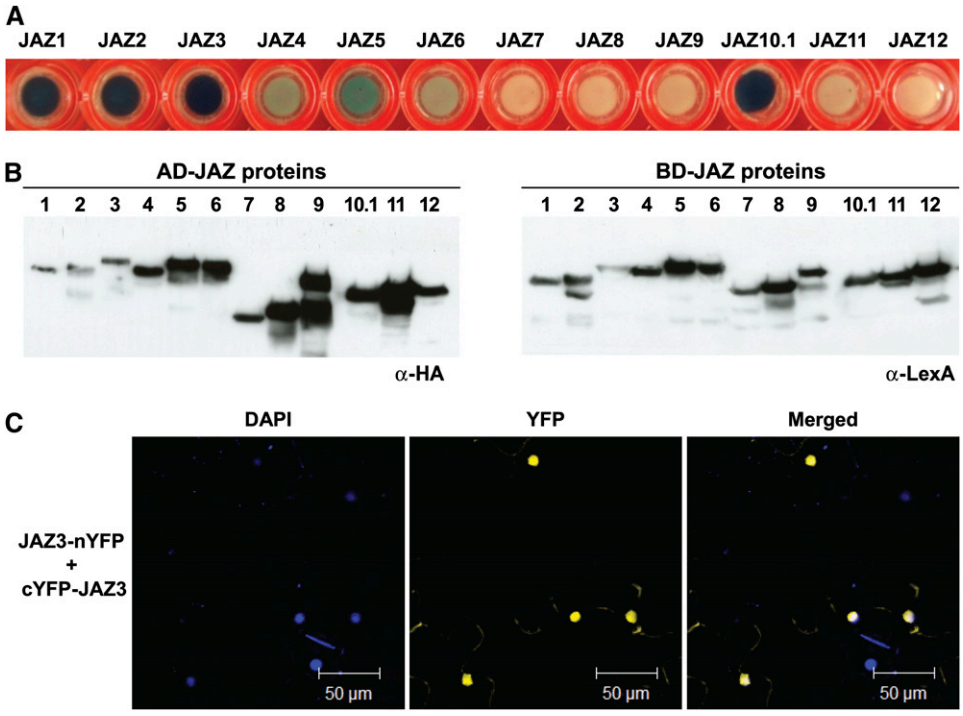

Figure 1. Homo- and Heteromeric Interaction of *Arabidopsis* JAZ Proteins.

(A) Y2H assay for homomeric JAZ interactions. Yeast strains coexpressing AD and BD fusions to each of the 12 full-length JAZ proteins were plated on media containing X-gal. Based on the intensity of LacZ-mediated blue-color formation, the strength of each interaction was rated as strong (e.g., JAZ1), medium (e.g., JAZ5), weak (e.g., JAZ6), or undetectable (e.g., JAZ8). See Table 1 for ratings on all interactions.

(B) Immunoblot analysis of JAZ proteins in yeast strains used for Y2H assays shown in **(A)**. Each lane contains total protein extracted from cells expressing AD and BD fusions of the indicated JAZ protein (e.g., "1" corresponds to JAZ1). AD-JAZ and BD-JAZ fusion proteins were detected with anti-HA (left panel) and anti-LexA (right panel) antibodies, respectively.

(C) BiFC assay of JAZ3-JAZ3 homomeric interaction in planta. YFP fluorescence was detected in *N. tabacum* leaves coinfiltrated with *Agrobacterium* strains expressing JAZ3-nYFP and cYFP-JAZ3. 4',6-Diamidino-2-phenylindole (DAPI) staining shows the location of nuclei. The merged image shows colocalization of DAPI and YFP fluorescence.

JAZ10 (referred to here as JAZ10.3) that lacks seven amino acids from the C-terminal end of the Jas domain. Overexpression of this truncated protein, but not the Jas domain–containing full-length protein (JAZ10.1), conferred partial insensitivity to JA. These important findings strengthen the rationale for studies aimed at determining how JAZΔJas proteins regulate JA signal output.

JAZ proteins are classified as members of the tify family, also known as ZIM (Vanholme et al., 2007). The defining feature of all family members is the highly conserved TIFY motif (TIFF/YXG) located within a larger conserved region referred to as the ZIM (or TIFY) domain (Chini et al., 2007; Thines et al., 2007; Vanholme et al., 2007; Yan et al., 2007). The tify family in *Arabidopsis* includes the so-called ZIM and ZIM-like (ZML) transcription factors that are implicated in cell expansion (Shikata et al., 2004) as well as PEAPOD (PPD) proteins that regulate leaf size and shape (White, 2006). Unlike the ZIM and ZML proteins that possess a zinc-finger DNA binding domain, JAZs do not contain a known DNA binding domain. Rather, JAZs are proposed to exert their effects on gene expression through interaction with MYC2 (Chini et al., 2007) and perhaps other transcription factors. This idea is supported by the nuclear location of several JAZ family members (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007). The occurrence of the ZIM domain in proteins from higher and lower (i.e., moss) plants but not in green algae suggests an important role for JAZs in the evolution of land plants (Vanholme et al., 2007; Chico et al., 2008; Katsir et al., 2008a).

Here, we provide evidence that the ZIM domain mediates formation of homo- and heteromeric complexes involving most members of the *Arabidopsis* JAZ family. We identify a novel alternatively spliced form of JAZ10 (JAZ10.4) that, unlike the previously characterized JAZ10.3, lacks the entire Jas domain and confers severe JA insensitivity when overexpressed in *Arabidopsis*. We demonstrate that JAZ10.4 is stabilized against JA-induced degradation and, significantly, has the ability to interact with MYC2 and with other JAZ proteins. We also show that severe dominant JA-insensitive phenotypes resulting from JAZ10.4 overexpression are suppressed by mutations in the TIFY motif that block JAZ10.4–JAZ interactions. These results indicate that JAZ10.4 functions to dampen signal output in the presence of JA and that the repressive activity of this splice variant depends on the ZIM/TIFY domain. The data are consistent with a model in which a JAZ10.4-containing protein complex directly impairs the activity of transcription factors (e.g., MYC2) that control expression of JA response genes.

RESULTS

Homo- and Heteromeric Interaction of JAZ Proteins

We used the yeast two-hybrid (Y2H) system to determine the homomeric interaction potential of the 12 members of the *Arabidopsis* JAZ family. Each full-length JAZ was fused separately to the LexA DNA binding domain (BD) and the B42 activation domain (AD). Homodimeric interactions were assessed by coexpression of the BD and AD fusion proteins in yeast cells containing a *lacZ* reporter gene under the control of a LexA-dependent operator. As shown in Figure 1A, JAZ1, JAZ2, JAZ3, and JAZ10.1 strongly interacted as homodimers, as determined by strong LacZ reporter activity. Weaker homomeric interactions were observed for JAZ4, JAZ5, and JAZ6, whereas the remaining five family members (JAZ7, JAZ8, JAZ9, JAZ11, and JAZ12) failed to interact. Protein gel blot analysis showed that all JAZ fusion proteins were expressed in yeast cells (Figure 1B), indicating that the absence of interaction cannot be attributed to a lack of protein expression.

We next used a bimolecular fluorescence complementation (BiFC) assay to determine whether JAZ-JAZ homomeric complexes form in planta. JAZ3 was chosen for this analysis because it self-associates strongly in yeast (Figure 1A) and, when expressed as a fusion with green fluorescent protein, is localized to the nucleus (Chini et al., 2007). N- and C-terminal fragments of yellow fluorescent protein (YFP) (nYFP and cYFP, respectively)

Table 1. Summary of JAZ–JAZ Interactions in Yeast

	JAZ1	JAZ2	JAZ3	JAZ4	JAZ5	JAZ6	JAZ7	JAZ8	JAZ9	JAZ10.1	JAZ11	JAZ12
JAZ1	+++	**+++**	+++	+++	+	++	–	–	+	**+**	–	+++
JAZ2	**+++**	+++	+++	–	**+**	**+**	–	–	–	–	–	+
JAZ3	–	–	+++	**+**	–	–	–	–	–	–	–	–
JAZ4	–	–	**+++**	+	–	–	–	–	–	–	–	–
JAZ5	–	**++**	–	–	++	**+**	–	–	–	–	–	++
JAZ6	–	**+++**	–	–	**+**	+	–	–	–	**++**	–	**++**
JAZ7	–	–	–	–	–	–	–	–	–	–	–	–
JAZ8	+++	+++	+++	+++	+++	+++	–	–	+	+++	–	+++
JAZ9	–	+	+++	–	–	–	–	–	–	**+**	–	–
JAZ10.1	**+++**	+++	+++	+++	–	**++**	–	–	**+**	+++	–	+
JAZ11	+	++	+++	+	–	–	–	–	+	+ +	–	–
JAZ12	–	–	–	–	–	**+++**	–	–	–	–	–	–

Each of the 12 full-length *Arabidopsis* JAZs was tested in both AD (top row) and BD (left column) orientations against the remaining 11 family members (132 heteromeric combinations). A summary of results for JAZ homomeric interactions is also included. Based on the intensity of LacZ-mediated blue-color formation, the strength of each interaction was rated as strong (+++), medium (++), weak (+), or undetectable (–), as shown in Figure 1A. Heteromeric interactions observed in both AD and BD orientations are presented in bold.

were fused to either the N or C termini of JAZ3 to produce four constructs (JAZ3-nYFP, JAZ3-cYFP, nYFP-JAZ3, and cYFP-JAZ3). *Agrobacterium tumefaciens*–mediated transformation was used to coexpress each of the four possible combinations of nYFP and cYFP JAZ fusion constructs in epidermal cells of *Nicotiana tabacum*. Coexpression of JAZ3-nYFP and cYFP-JAZ3, but not any other combination tested, resulted in a YFP signal within the nucleus (Figure 1C). This finding confirms the Y2H results and further demonstrates that JAZ3 can self-associate in the plant nucleus.

We also employed Y2H assays to systematically test all possible JAZ-JAZ heteromeric interactions in both BD/AD orientations. Out of a total of 132 possible heteromeric combinations, we observed 47 interactions of varying strengths involving all family members except JAZ7 (Table 1). Nine of these interactions (JAZ1-JAZ2, JAZ1-JAZ10.1, JAZ2-JAZ5, JAZ2-JAZ6, JAZ3-JAZ4, JAZ5-JAZ6, JAZ6-JAZ10.1, JAZ6-JAZ12, and JAZ9-JAZ10.1) were detected in both BD/AD orientations. Several JAZ proteins that interacted strongly as homodimers tended to interact with a larger number of JAZs. For example, JAZ1 and JAZ3 interacted with nine and seven other family members, respectively, in one or both BD/AD orientations. We also found examples of proteins (e.g., JAZ8) that did not form homomeric complexes but did interact with several other family members. We conclude that most JAZ proteins in *Arabidopsis* form homodimers and heterodimers in yeast.

Involvement of the TIFY Motif in JAZ–JAZ Interactions

As an initial approach to identify sequence determinants that mediate JAZ-JAZ binding, we examined the ability of various deletion mutants of JAZ3 (Figure 2A) to interact with full-length JAZ3 and JAZ10.1. Deletion of 140 amino acids from the N terminus of JAZ3 (ΔN), including the weakly conserved N-terminal region observed by Thines et al. (2007), resulted in a slight reduction in the strength of homomeric and heteromeric

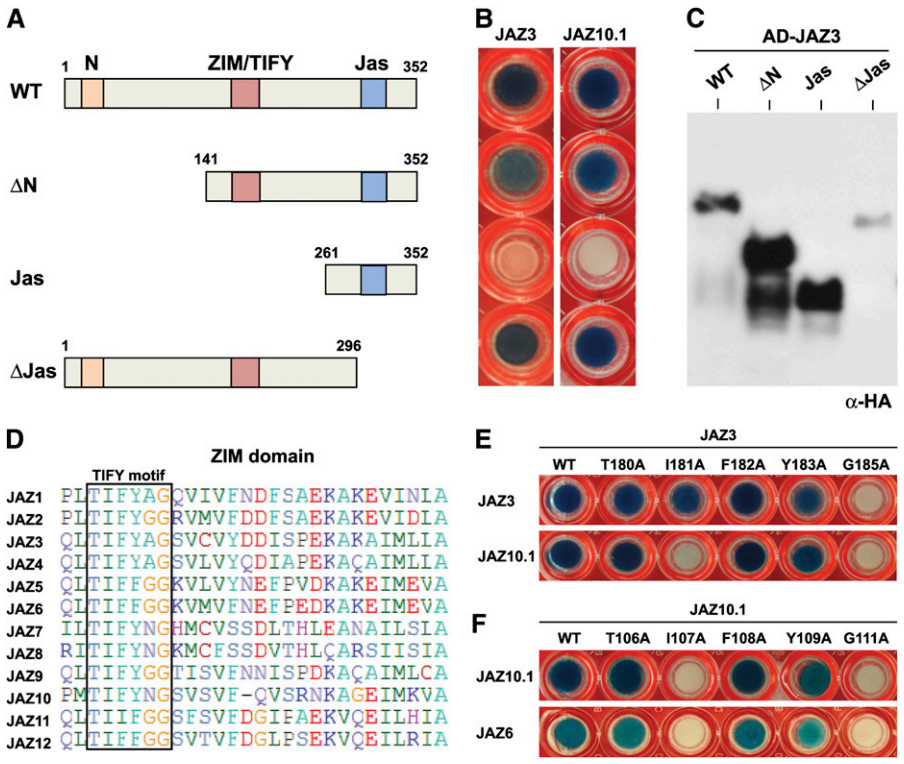

Figure 2. Requirement for the TIFY Motif in Homo- and Heteromeric Interaction of JAZ Proteins.

(A) Schematic diagram of JAZ3 deletion constructs analyzed in **(B)** and **(C)**. The diagram shows the highly conserved Jas (blue) and ZIM (pink, with TIFY motif) domains as well as the weakly conserved sequence (orange, labeled "N") at the N terminus (Thines et al., 2007).
(B) Y2H assay to assess the interaction of each JAZ3 deletion construct (AD fusion) shown in **(A)** with full-length JAZ3 and JAZ10.1 (BD fusions).
(C) Immunoblot analysis of JAZ3 deletion proteins in yeast strains tested in **(B)**. AD-JAZ3 fusion proteins were detected with an anti-HA antibody.
(D) Amino acid sequence alignment of the ZIM domain in 12 *Arabidopsis* JAZs. The highly conserved TIFY motif (TIFF/YXG) is boxed. The in-color alignment depicts amino acids with similar hydrophobicity and was generated with the software BioEdit v 7.0.9.
(E) Ala-scanning mutagenesis of the JAZ3 TIFY motif. Wild-type and mutant forms (e.g., T180A) of JAZ3 (AD fusions) were tested in the Y2H system for interaction with wild-type JAZ3 and JAZ10.1 (BD fusions).
(F) Ala-scanning mutagenesis of the JAZ10.1 TIFY motif. Wild-type and mutant forms (e.g., T106A) of JAZ10.1 (AD fusions) were tested in the Y2H system for interaction with wild-type JAZ10.1 and JAZ6 (BD fusions).

interactions with JAZ3 and JAZ10.1, respectively (Figure 2B). A JAZ3 variant (Jas) that lacks both the N-terminal and ZIM domains was previously shown to interact with COI1 in a hormone-dependent manner (Melotto et al., 2008). This C-terminal fragment of JAZ3 failed to interact with either full-length JAZ3 or JAZ10.1 (Figure 2B). A third deletion construct (ΔJas) that lacks the Jas domain interacted strongly with full-length JAZ3 and JAZ10.1. Protein gel blot analysis confirmed that the JAZ3 deletion variants used in these experiments were expressed in yeast cells (Figure 2C). Taken together, these results indicate that the N-terminal region and the Jas domain are not required for JAZ3-JAZ interactions, thus implicating the ZIM domain as an important determinant for JAZ-JAZ binding.

To further investigate the role of the ZIM domain in JAZ–JAZ interaction, we performed Ala-scanning mutagenesis of the highly conserved TIFY motif found in the ZIM domain of JAZ3 and all other family members (Figure 2D). We assessed the interaction of the resulting mutant JAZ3 variants with both wild-type JAZ3 and JAZ10.1 in the Y2H system. Ala substitution of Gly-185 abolished the interaction with wild-type JAZ3, whereas replacement of Thr-180, Ile-181, Phe-182, and Tyr-183 did not affect this interaction (Figure 2E). Heteromeric interaction of JAZ3 with JAZ10.1 was also disrupted by the G185A mutation as well as by an I181A mutation in JAZ3 (Figure 2E). Site-directed mutagenesis of the TIFY motif in JAZ10.1 yielded similar results. In this case, Ala substitution of either Ile-107 or Gly-111 abrogated JAZ10.1 binding to itself and to JAZ6 (Figure 2F). Protein gel blot analysis showed that all Ala-substituted mutants of JAZ3 and JAZ10.1 were expressed in yeast (see Supplemental Figure 1 online). These findings establish an essential role for the TIFY motif in JAZ–JAZ interactions.

The ZIM domain was originally identified in ZIM, ZML1, and ZML2 members of the tify family (Shikata et al., 2004; Vanholme et al., 2007). All three of these proteins possess a GATA-type Zn-finger DNA binding motif and are thought to act as transcription factors. If the ZIM domain functions as a protein–protein interaction determinant, we reasoned that ZIM, ZML1, and ZML2 may interact with one another. Indeed, we found that each protein homo- and heterodimerized in yeast (see Supplemental Figure 2 online).

Characterization of a Novel JAZ10 Splice Variant That Lacks the Jas Domain

During efforts to isolate a full-length cDNA for *JAZ10*, we identified three distinct transcripts from this gene. These included the At5g13220.1 and At5g13220.3 transcripts that are predicted by The Arabidopsis Information Resource (TAIR) to encode the longest isoform (i.e., JAZ10.1) and the JAZ10.3/JAS1.3 isoform containing a partially truncated Jas domain (Yan et al., 2007). The third transcript we identified (designated At5g13220.4), which was not annotated in the TAIR database, is predicted to encode a 167–amino acid protein, designated JAZ10.4. The sequence of At5g13220.4 indicates that it is produced by the use of an

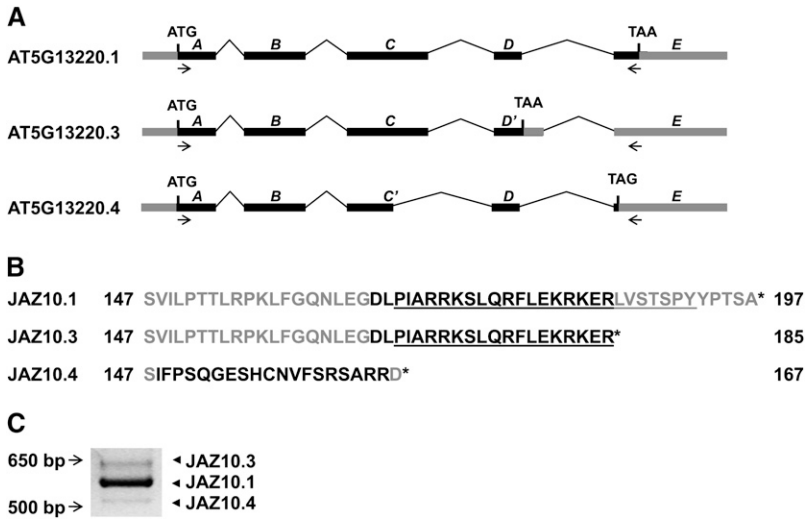

Figure 3. Identification of Three JAZ10 Variants Produced by Alternative Splicing.

(A) Schematic diagram of alternatively spliced transcripts At5g13220.1, At5g13220.3, and At5g13220.4 encoding JAZ10.1, JAZ10.3, and JAZ10.4, respectively. Thick lines (black, translated; gray, untranslated) represent exons (labeled *A* to *E*). Modified exons in At5g13220.3 and At5g13220.4 result from use of alternative splice sites marked D′ and C′, respectively.

(B) Alternative splicing differentially affects the sequence of the C-terminal Jas domain in the three JAZ10 splice variants. Amino acid sequences N-terminal to Ser-147 are identical in the three isoforms and are not shown. Amino acids comprising the Jas domain are underlined, whereas amino acids encoded by exon D are in bold. A frame shift in exon *D* of At5g13220.4 (resulting from alternative splicing of exon *C*) removes the Jas domain and adds 20 unrelated amino acid residues C-terminal to Ser-147. Asterisks indicate the stop codon.

(C) RT-PCR analysis of *JAZ10* transcripts in RNA isolated from wounded leaves. The location of primers used for RT-PCR is indicated by arrows in **(A)**. PCR products were separated by gel electrophoresis, and the resulting gel was stained with ethidium bromide. Arrows denote transcripts encoding the three JAZ10 splice variants.

alternative splice donor site in the third exon of the *JAZ10* gene (Figure 3A). This splicing event causes a frame-shift mutation that deletes the entire Jas domain and adds 20 amino acids C-terminal to Ser-147 (Figure 3B). RT-PCR was used to verify the occurrence of At5g13220.4 in wounded *Arabidopsis* leaves. As shown in Figure 3C, three RT-PCR products ranging in size between ~500 and 650 bp were distinguishable by agarose gel electrophoresis. Cloning and sequencing of these PCR products confirmed their identity as At5g13220.1, At5g13220.3, and At5g13220.4. RT-PCR experiments performed with transcript-specific primers also detected the At5g13220.3 and At5g13220.4 splice variants in wounded leaves (see Supplemental Figure 3 online).

The role of the Jas domain as a JAZ destabilization element (Katsir et al., 2008b; Melotto et al., 2008) led us to test the hypothesis that JAZ10.1, JAZ10.3, and JAZ10.4 differentially interact with COI1. We assessed the three isoforms for their ability to interact with COI1 in a ligand-dependent manner in the Y2H system. Coronatine, a potent signal for COI1–JAZ interactions (Katsir et al., 2008b; Melotto et al., 2008), stimulated JAZ10.1 binding to COI1 in a dose-dependent manner (Figure 4A). JAZ10.3 interacted with COI1 in the presence of relatively high concentrations of coronatine (e.g., 200 μM), whereas JAZ10.4 did not interact with COI1 at any concentration of coronatine tested. Quantification of β-galactosidase activity confirmed that JAZ10.3 weakly interacts with COI1 in comparison to JAZ10.1 and that JAZ10.4 fails to bind COI1 in the presence of 200 μM coronatine (Figure 4B). Protein gel blot analysis showed that all three JAZ10 proteins were produced in yeast cells (Figure 4C). We also performed Y2H assays to determine whether the JAZ10 splice variants interact with the basic helix-loop-helix transcription factor, MYC2. As shown in Figure 4D, all three JAZ10 isoforms interacted with MYC2 in the absence of coronatine. These findings indicate that the Jas domain is critical for interaction of JAZ10 splice variants (e.g., JAZ10.1) with COI1 but is not required for binding of JAZ10 isoforms to MYC2.

To further investigate potential functional differences between the JAZ10 isoforms, we tested whether JAZ10.3 and JAZ10.4, like JAZ10.1 (Figure 1, Table 1), form homo- and heterodimers in yeast. As shown in Figure 4E, the JAZ interaction pattern of JAZ10.3 was identical to that of JAZ10.1, with both variants partnering with eight of the 14 JAZ proteins tested. In this BD/AD orientation, JAZ10.4 interacted with the same set of eight JAZs and also formed heterodimers with JAZ2 and JAZ3 (Figure 4E). Protein gel blot analysis of protein extracts from yeast strains

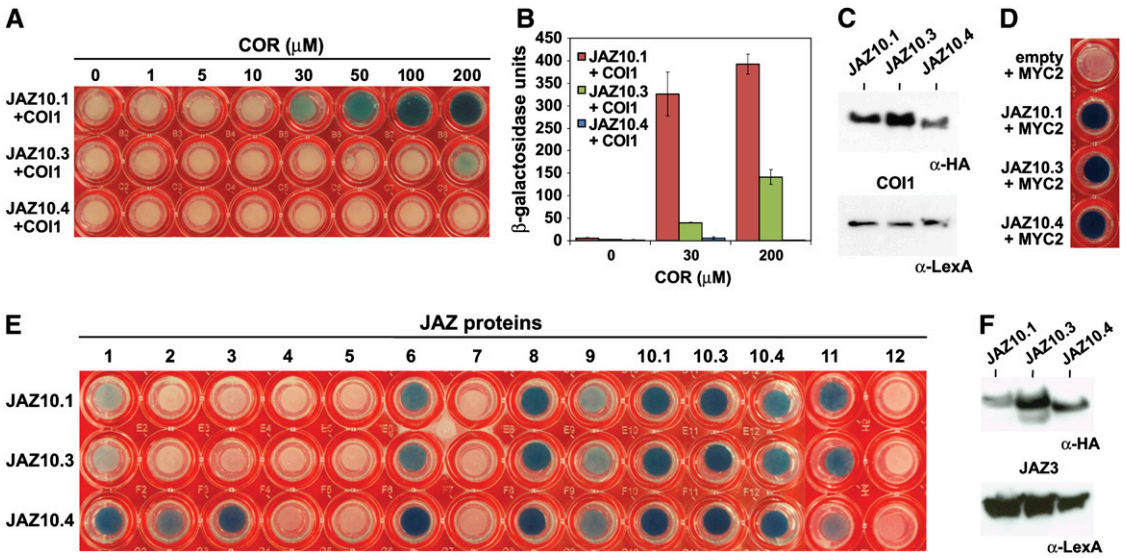

Figure 4. Protein–Protein Interaction Characteristics of JAZ10 Isoforms.

(A) Coronatine-dependent interaction of three JAZ10 splice variants with COI1 in the Y2H system. Yeast strains cotransformed with AD-JAZ10 isoforms (JAZ10.1, JAZ10.3, or JAZ10.4) and BD-COI1 were plated on media containing the indicated concentration of coronatine (COR).

(B) Quantification of β-galactosidase activity in yeast strains shown in **(A)**. Each strain was grown in the absence of coronatine ("0") or in the presence of 30 or 200 μM coronatine (COR). Data show the mean ± SD of triplicate technical replicates.

(C) Immunoblot analysis of JAZ10 and COI1 proteins in yeast cells tested in **(A)**. JAZ10 splice variants and COI1 were detected with anti-HA and anti-LexA antibodies, respectively.

(D) Interaction of three JAZ10 splice variants with MYC2 in the Y2H system. Yeast strains cotransformed with AD-MYC2 and BD-JAZ10 variants (JAZ10.1, JAZ10.3, and JAZ10.4) were plated on media containing X-gal. A yeast strain cotransformed with AD-MYC2 and an empty BD vector (pGILDA) is shown as a control (empty + MYC2).

(E) Homo- and heteromeric interaction of JAZ10 splice variants. Yeast strains cotransformed with one of the three JAZ10 isoforms (as an AD fusion) and other members (as a BD fusion) of the JAZ family were plated on media containing X-gal.

(F) Protein gel blot analysis of JAZ10 isoforms and JAZ3 in yeast strains used for the Y2H experiment shown in **(E)**. JAZ10 splice variants and JAZ3 were detected with anti-HA and anti-LexA antibodies, respectively.

cotransformed with JAZ3 and JAZ10 isoforms showed that each JAZ protein was expressed (Figure 4F). We conclude that the three JAZ10 splice variants interact with a similar set of JAZs in the Y2H system.

Previous studies have shown that several JAZ family members, including JAZ10.1 and JAZ10.3, are located in the nucleus (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007). To determine whether the modified C terminus of JAZ10.4 affects the targeting of the protein to the nucleus, we used confocal laser microscopy to examine the subcellular location of a JAZ10.4-YFP fusion protein transiently expressed in tobacco epidermal cells. Control experiments showed that whereas YFP alone partitions to both the cytosol and the nucleus, JAZ10.1-YFP and JAZ10.3-YFP are located predominantly in the nucleus, as previously reported (Yan et al., 2007) (Figure 5). JAZ10.4-YFP exhibited a nuclear localization pattern that was similar to that of the other two isoforms. We conclude that the COI1-noninteracting splice variant JAZ10.4 localizes to the nucleus.

Overexpression of JAZ10 Splice Variants Differentially Affects JA Signal Output

The absence of a Jas domain in JAZ10.4 indicated to us that this splice variant might exert dominant-negative effects on JA signaling, as previously observed for JAZ10.3 (Yan et al., 2007) and other JAZs in which the Jas domain was removed by targeted deletion or mutagenesis (Chini et al., 2007; Thines et al., 2007; Melotto et al., 2008; Shoji et al., 2008). To investigate this possibility, we assessed JA-related phenotypes in transgenic plants overexpressing JAZ10.1, JAZ10.3, or JAZ10.4. As previously reported by Yan et al. (2007), overexpression of JAZ10.1 and JAZ10.3 did not have overt effects on plant fertility. By contrast, we observed that ~30% (49 of 150 plants examined) of T1 plants expressing a *35S-JAZ10.4* transgene were male sterile as a result of defects in filament elongation and anther dehiscence (Figures 6A to 6D). These flower phenotypes, which are hallmarks of strong JA synthesis and perception mutants (Feys et al., 1994; McConn and Browse, 1996; Sanders et al., 2000; Stintzi and Browse, 2000; Ishiguro et al., 2001; Park et al., 2002), were not rescued by exogenous methyl-JA (MeJA) (data not shown). Fertilization of sterile *35S-JAZ10.4* flowers with wild-type pollen yielded F1 progeny in which the sterile phenotype strictly cosegregated with the *35S-JAZ10.4* transgene. To determine whether overexpression of JAZ10.4 affects other JA responses, we compared JA-mediated root growth inhibition in *35S-JAZ10.4* seedlings to that of wild-type, *coi1-1*, *35S-JAZ10.1*, and *35S-JAZ10.3* seedlings (Figures 6E and 6F). As reported by Yan et al. (2007), root growth of *35S-JAZ10.3* seedlings was moderately resistant to JA, whereas *35S-JAZ10.1* roots were as sensitive as wild-type seedlings to the hormone. *35S-JAZ10.4* seedlings exhibited a strong JA-insensitive root growth phenotype, which was comparable to that of *coi1-1* seedlings.

Differential Stability of JAZ10 Splice Variants in Vivo

The strong JA-insensitive phenotypes conferred by JAZ10.4 overexpression, together with the inability of this isoform to

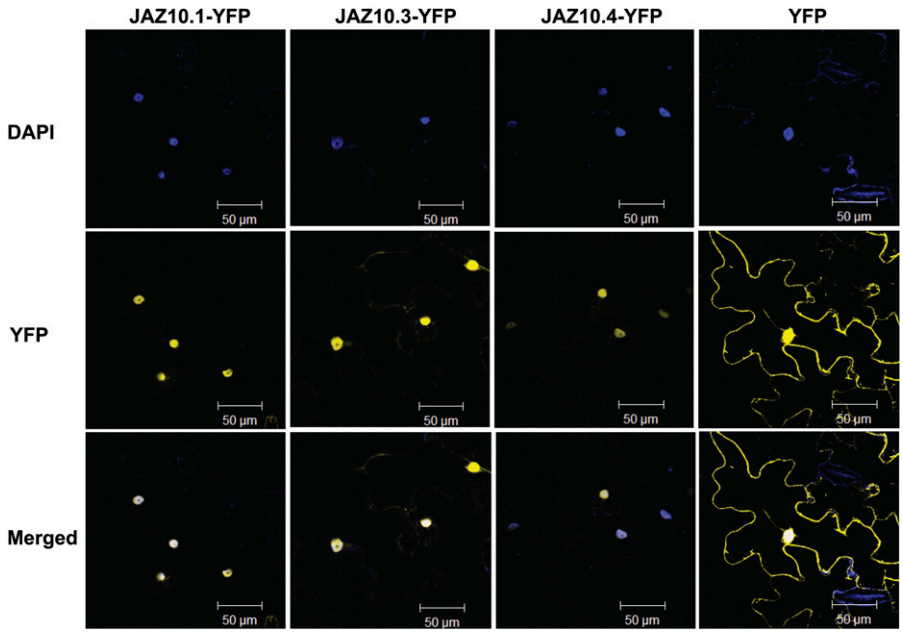

Figure 5. Subcellular Localization of JAZ10 Splice Variants.

JAZ10-YFP fusion proteins were transiently expressed by *Agrobacterium*-mediated transformation of tobacco epidermal cells. YFP fluorescence was detected with a confocal laser scanning microscope. DAPI fluorescence was used as a marker for the nucleus. YFP alone (not fused to JAZ10) is located in both the nucleus and the cytosol.

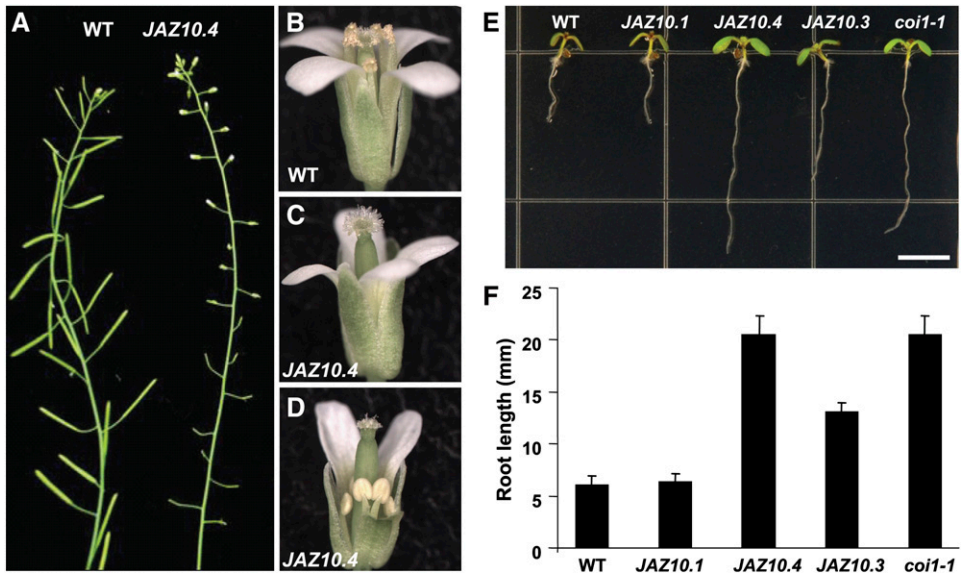

Figure 6. Overexpression of JAZ10 Splice Variants Differentially Affects JA Responses.

(A) to **(D)** JAZ10.4-overexpressing plants are male sterile.
(A) Wild-type (left) and *35S-JAZ10.4* (right) inflorescence.
(B) to **(D)** Close-up view of a wild-type flower **(B)** and JAZ10.4 flowers (**[C]** and **[D]**) that have short filaments and nondehisced anthers.
(E) Differential effect of JAZ10 isoforms on JA-mediated inhibition of root growth. Germinated seedlings of the indicated genotype were grown for 7 d on MS medium containing 50 μM MeJA. Bar = 5 mm.
(F) Quantification of JA-induced root growth inhibition of seedlings shown in **(E)**. Data show the mean ± SD (*n* = 15 seedlings per genotype).
[See online article for color version of this figure.]

interact with COI1, suggested that JAZ10.4 is resistant to JA-induced protein degradation. To test this idea, we generated stable transgenic lines expressing JAZ10.1-YFP, JAZ10.3-YFP, and JAZ10.4-YFP fusion proteins under control of the 35S promoter. Root growth inhibition assays showed that the JA responsiveness of each line was essentially identical to that of JAZ10.1-, JAZ10.3-, and JAZ10.4-overexpresing plants; the root length of wild-type, *35S-JAZ10.1-YFP*, *35S-JAZ10.3-YFP*, and *35S-JAZ10.4-YFP* seedlings grown on Murashige and Skoog (MS) plates containing 50 μM MeJA was 5.6 ± 1.0 mm, 5.4 ± 0.8 mm, 11.5 ± 0.5 mm, and 16.9 ± 1.3 mm, respectively. To investigate the hormone-dependent stability of each JAZ10 isoform in vivo, we monitored YFP fluorescence in roots of 7-d-old transgenic seedlings that were either treated (for 2 h) or not treated with MeJA. In the absence of MeJA, a nuclear-localized YFP signal was observed in all three genotypes (Figure 7A). Analysis of several independent lines (at least five lines per construct) showed that the strength of the YFP signal in *35S-JAZ10.1-YFP* plants was consistently less than that in *35S-JAZ10.3-YFP* and *35S-JAZ10.4-YFP* seedlings (Figure 7A, Mock). Treatment with 1 μM MeJA eliminated nuclear YFP fluorescence in *35S-JAZ10.1-YFP* roots but did not obviously affect the signal in *35S-JAZ10.3-YFP* and *35S-JAZ10.4-YFP* seedlings. Treatment of seedlings with 100 μM MeJA resulted in loss of the JAZ10.3-YFP nuclear signal, although a diffuse (presumably cytosolic) fluorescence pattern in roots was still detected. The fluorescence pattern in *35S-JAZ10.4-YFP* seedlings was unaffected by 100 μM MeJA. We conclude that the relative stability of the three JAZ10 splice variants in hormone-stimulated plants is JAZ10.4 > JAZ10.3 > JAZ10.1 and that JAZ10.4 is highly resistant to JA-induced degradation. Pretreatment of seedlings with MG132, a specific inhibitor of the 26S proteasome, impaired JA-induced turnover of JAZ10.1-YFP (Figure 7B). This finding indicates that JA-induced degradation of this Jas domain–containing isoform is mediated by the 26S-proteasome pathway.

The Dominant-Negative Effect of JAZ10.4 Requires a Functional TIFY Motif

We next investigated whether the strong repression of JA responses by JAZ10.4 is dependent on a functional TIFY motif. To address this question, we generated transgenic lines that overproduce JAZ10.4$^{I->A}$ and JAZ10.4$^{G->A}$ proteins harboring I107A or G111A point mutations, respectively, in the TIFY motif of JAZ10.4. Although these mutations abrogate JAZ10.1 interaction with other JAZs in the Y2H system (Figure 2F), it was necessary to determine the effect of these mutations on JAZ10.4–JAZ interactions. As shown in Figure 8A, JAZ10.4$^{I->A}$ lost the ability to interact with JAZ6, JAZ10.1, JAZ10.3, and JAZ10.4. JAZ10.4$^{G->A}$ also failed to bind JAZ10.4 but did retain ability to interact with JAZ6, JAZ10.1, and JAZ10.3. Protein gel blot analysis of protein extracts from yeast strains cotransformed with JAZ10.1 and mutant forms of JAZ10.4 showed that the JAZ fusion proteins were expressed (Figure 8B). These findings, together with results of experiments to assess JAZ10.4$^{G->A}$ and

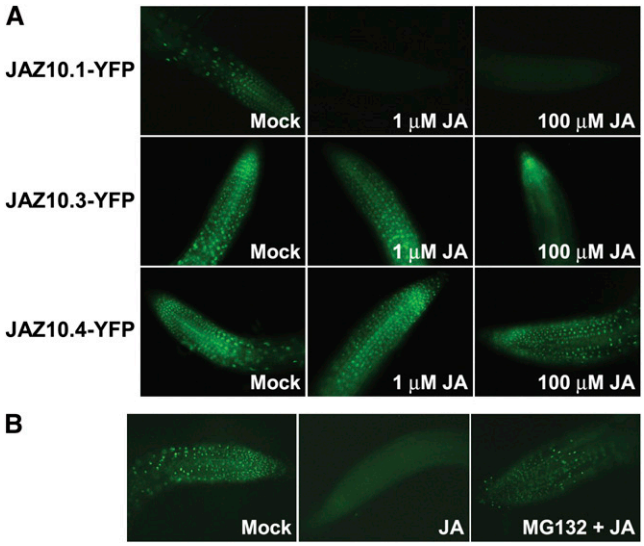

Figure 7. Stability of JAZ10 Splice Variants in Vivo.

(A) Differential stability of three JAZ10 splice variants in response to JA. Transgenic seedlings expressing JAZ10.1-YFP, JAZ10.3-YFP, or JAZ10.4-YFP fusion proteins were treated either with water (Mock) or with the indicated concentration of MeJA (JA). Two hours after treatment, YFP signal in root tissue was visualized by fluorescence microscopy. The exposure times for each image in **(A)** and **(B)** were identical.

(B) Proteasome-dependent degradation of JAZ10.1-YFP. Seedlings expressing the *35S-JAZ10.1-YFP* transgene were pretreated with water or the 26S proteasome–specific inhibitor MG132 (50 μM) for 2 h, at which time seedlings were treated with either MeJA (JA; 10 μM) or water (Mock) for 2 h. YFP signal in root tissue was visualized by fluorescence microscopy.

JAZ10.4$^{I->A}$ interaction with other members of the JAZ family (see Supplemental Figure 4 online), indicate that I107A is more effective than G111A at disrupting JAZ10.4 heterodimerization with other JAZs.

Having established the effect of I107A and G111A on JAZ10.4–JAZ interactions in yeast, we assessed JA-mediated fertility phenotypes in transgenic plants that overexpress JAZ10.4$^{I->A}$ and JAZ10.4$^{G->A}$. Of 150 independent *35S-JAZ10.4$^{I->A}$* plants (T1 generation) examined, none showed defects in filament elongation, anther dehiscence, silique development, or viable seed production (Figures 8C and 8D), indicating that I107A completely suppresses JAZ10.4-mediated male sterility. In a population of 40 independent T1 *35S-JAZ10.4$^{G->A}$* plants, 12 individuals exhibited reduced fertility; these plants produced only a few mature siliques during the latter stage of reproductive development (Figure 8C). Some flowers produced by this group of semifertile *35S-JAZ10.4$^{G->A}$* plants were indistinguishable from wild-type flowers, whereas other *35S-JAZ10.4$^{G->A}$* flowers had anther filaments that failed to fully elongate (Figures 8E and 8F).

We also studied the effect of TIFY point mutations (I107A and G111A) on the JA-resistant root growth phenotype that results from JAZ10.4 overexpression. In agreement with the ability of I107A to strongly suppress JAZ10.4-mediated sterility, *35S-JAZ10.4$^{I->A}$* roots were as sensitive to JA-induced growth arrest as wild-type roots (Figures 8G and 8H). *35S-JAZ10.4$^{G->A}$* roots, by contrast, were less sensitive to JA than wild-type roots but significantly more sensitive ($P < 0.0001$; Student's *t* test) than *35S-JAZ10.4* roots. These results show that the ability of JAZ10.4 to repress JA signaling in various tissues is strongly suppressed by I107A and moderately suppressed by G111A.

To test the possibility that normal JA responsiveness of *35S-JAZ10.4$^{I->A}$* plants results from low expression of the JAZ10.4$^{I->A}$ mutant protein in planta, we analyzed the level of GUS activity in stable transgenic lines that constitutively express either JAZ10.4-GUS or JAZ10.4$^{I->A}$-GUS fusion proteins. In several independent transgenic lines tested (at least five lines per construct), seedlings expressing the *35S-JAZ10.4$^{I->A}$-GUS* transgene exhibited a pattern of strong blue staining (in roots, cotyledons, and leaves) that was similar to that of *35S-JAZ10.4-GUS* seedlings (Figure 9A), indicating that the JAZ10.4$^{I->A}$-GUS fusion protein is expressed. *35S-JAZ10.4-GUS* plants were male sterile and highly insensitive to JA-induced root growth arrest (Figures 9B and 9C). This finding shows that JAZ10.4-GUS, like JAZ10.4, strongly represses JA signal output. Plants overexpressing JAZ10.4$^{I->A}$-GUS, however, were fully fertile and exhibited normal sensitivity to exogenous JA (Figures 9B and 9C). We also examined the subcellular localization of JAZ10.4-YFP and JAZ10.4$^{I->A}$-YFP fusion proteins to determine whether I107A affects subcellular protein distribution. Following transient *Agrobacterium*-mediated expression of these proteins in tobacco leaf epidermal cells, laser scanning confocal microscopy revealed that both JAZ10.4-YFP and JAZ10.4$^{I->A}$-YFP are targeted to the nucleus (Figure 9D). These results indicate that the I107A mutant form of JAZ10.4 is expressed and targeted to the nucleus.

DISCUSSION

Functional Diversification of JAZ Proteins by Alternative Splicing

Alternative splicing plays an important role in increasing protein diversity and, ultimately, biological complexity. Our characterization of functionally distinct JAZ10 splice variants adds to a growing body of literature indicating that alternative splicing of pre-mRNAs provides a general mechanism to alter the proteome in ways that optimize plant adaptation to stress (Reddy, 2007; Barbazuk et al., 2008). In addition to the JAZ10.1 and JAZ10.3 isoforms described by Yan et al. (2007), we identified a third JAZ10 splice variant (JAZ10.4) that lacks the entire Jas domain. Several observations indicate that differential association of JAZ10 variants with COI1 affects the stability and action of each isoform in different ways. We showed that JAZ10.1 interacts with COI1 in a ligand-dependent manner, whereas JAZ10.4 fails to bind COI1. JAZ10.3, which contains a partially truncated Jas domain, interacted weakly with COI1. Analysis of JAZ10-YFP reporter lines showed that JAZ10.1 is readily degraded via the 26S proteasome pathway in response to JA treatment, whereas JAZ10.4 is highly resistant to hormone-induced destruction (Figure 7). Consistent with COI1 interaction studies performed

Figure 8. The TIFY Motif Is Required for Repression of JA Responses by JAZ10.4.

in yeast, high concentrations of JA were required to induce turnover of JAZ10.3-YFP in planta. The differential stability of JAZ10 splice variants can explain the JA-related phenotypes resulting from ectopic expression of each isoform. Overexpression of JAZ10.3 and JAZ10.4 conferred weak and strong JA-insensitive phenotypes, respectively, whereas *35S-JAZ10.1* plants that produce the full-length protein did not exhibit altered sensitivity to JA. We conclude that functional differences between JAZ10 splice variants can be attributed to sequence alterations in the Jas domain that affect COI1 binding and, as a consequence, the stability of each isoform. Gene prediction programs (www.softberry.com) suggest that *JAZ10* orthologs in rice (*Oryza sativa*) (gi:115458122) and poplar (Populus trichocarpa) (Joint Genome Institute gene model 548076) are subject to alternative splicing events that alter the Jas domain. Thus, diversification of JAZ function through alternative splicing may be widespread in the plant kingdom.

Several Jas domain–containing JAZ proteins have been shown to be degraded in a JA-dependent manner (Chini et al., 2007; Thines et al., 2007; this study). Presumably, these proteins act to repress JA responses in unstimulated cells that contain low levels of the hormone and are degraded in the presence of high JA levels. By contrast, our results indicate that JAZ10.4 is an endogenous repressor of JA signal output in JA-stimulated cells. This hypothesis is consistent with the observation that *JAZ10* RNA interference–silenced plants are hypersensitive to JA (Yan et al., 2007), whereas null mutations in several other *JAZ* genes apparently do not have this effect (Chini et al., 2007; Thines et al., 2007). It seems likely that the increased sensitivity of *JAZ10*-silenced lines to JA results from a deficiency in stabilized JAZs (e.g., JAZ10.4) rather than from reduced expression of the more labile JAZ10.1. Sequence variation in the Jas domain could potentially give rise to JAZ family members having a wide range of stabilities, which could provide a mechanism to modulate the amplitude and duration of JA responses. The ability of JAZ10.4 to

(A) Y2H analysis of the effect of I107A and G111A TIFY mutations on the interaction of JAZ10.4 with JAZ6, JAZ10.1, JAZ10.3, and JAZ10.4. Yeast strains cotransformed with AD fusions of JAZ10.4 (or JAZ10.4$^{I->A}$ and JAZ10.4$^{G->A}$ mutants) and BD-JAZ fusions (JAZ6, JAZ10.1, JAZ10.3, and JAZ10.4) were plated on X-gal–containing medium.

(B) Immunoblot analysis of JAZ proteins in yeast cells cotransformed with AD fusions of JAZ10.4 (or JAZ10.4$^{I->A}$ and JAZ10.4$^{G->A}$ mutants) and BD-JAZ10.1, which were tested in **(A)**. AD- and BD-JAZ fusions were detected with anti-HA and anti-LexA antibodies, respectively.

(C) to **(F)** Mutations (I107A and G111A) in the TIFY motif differentially lack the male-sterile phenotype conferred by overexpression of wild-type JAZ10.4. Complete lack of sterility is shown by the inflorescence (**[C]**, left) and floral **(D)** phenotype of *35S-JAZ10.4*$^{I->A}$ plants. Partial lack of JAZ10.4-mediated sterility is indicated by development of a few mature siliques on the inflorescence of *35S-JAZ10.4*$^{G->A}$ plants (**[C]** and **[D]**, right). *35S-JAZ10.4*$^{G->A}$ plants produced a mixture of wild-type-like flowers **(E)** and flowers with shortened anther filaments **(F)**.

(G) Differential effect of I107A and G111A mutations on JAZ10.4-mediated changes in root growth in the presence of JA. Seedlings of the indicated genotype were grown for 6 d on MS medium containing 50 μM MeJA. Bar = 5 mm.

(H) Measurement of primary root length of seedlings shown in **(G)**. Data are the mean ± SD (n = 19 seedlings per genotype).

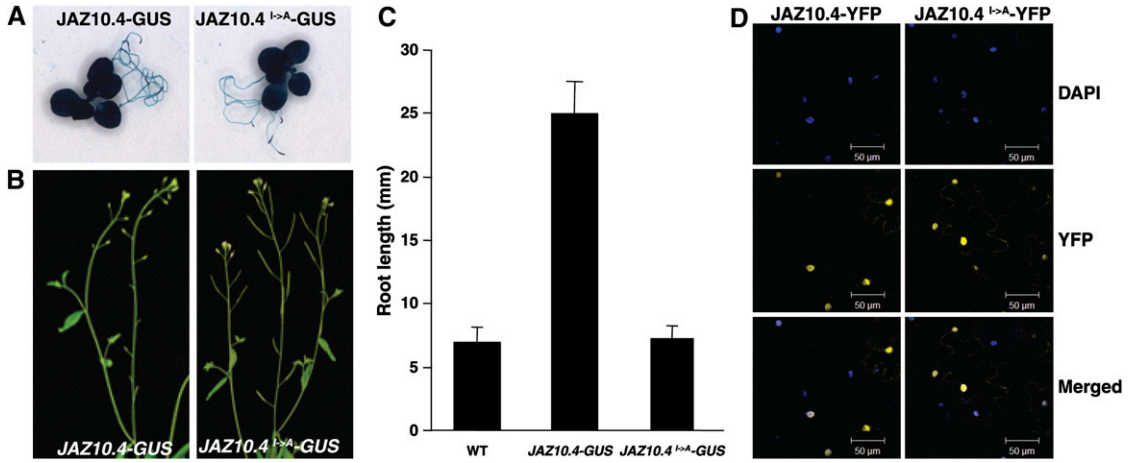

Figure 9. The I107A Mutant Form of JAZ10.4 Is Expressed and Targeted to the Nucleus.

(A) Histochemical GUS staining of *35S-JAZ10.4-GUS* and *35S-JAZ10.4*$^{I->A}$*-GUS* transgenic seedlings. Seedlings were grown on MS medium containing kanamycin (50 μg/mL) for 14 d prior to transferring to a 48-well microtiter plate for GUS staining.

(B) The male-sterile phenotype conferred by overexpression of JAZ10.4-GUS (left) is not shown by plants overexpressing the I107A mutant form of JAZ10.4-GUS (right).

(C) The JA-insensitive root growth phenotype conferred by overexpression of JAZ10.4-GUS is not shown by plants overexpressing the I107A mutant form of JAZ10.4-GUS.

(D) Subcellular localization of JAZ10.4-YFP and JAZ10.4$^{I->A}$-YFP fusion proteins that were transiently expressed by *Agrobacterium*-mediated transformation of tobacco epidermal cells. YFP and DAPI fluorescence was detected by confocal laser scanning microscopy.

strongly attenuate signal output, for example, may be important for reigning in JA responses that are energetically demanding and potentially damaging to the cell. It is also possible that JAZ10.4 or other stabilized JAZs attenuate JA-mediated growth inhibition under environmental conditions where rapid growth is required to compete for limited resources (Izaguirre et al., 2006; Yan et al., 2007, Zhang and Turner, 2008).

An important question that remains to be addressed is how the production of JAZ10.4 is regulated in wild-type plants. Our results support a scenario in which negative feedback control of signal output by JAZ10.4 (and perhaps JAZ10.3) is initiated upon transcriptional activation of the *JAZ10* gene in response to wounding or other inductive cues that trigger JA synthesis (Yan et al., 2007; Chung et al., 2008). It is possible that wounding regulates alternative splicing of *JAZ10* pre-mRNA in a manner that favors the accumulation of a specific transcript (Bove et al., 2008). However, our data and those of Yan et al. (2007) show that all three transcripts accumulate in wounded leaves. Assuming that all three JAZ10 isoforms are synthesized at the same rate in the same JA-stimulated cell, the relative abundance of each isoform would be determined largely by the rate of JA-induced turnover. Based on the results of JAZ10-YFP degradation assays (Figure 7), we predict that JAZ10.4 (and perhaps JAZ10.3) should accumulate to higher levels than JAZ10.1 and other labile JAZs in JA-stimulated cells.

The ZIM Domain Mediates Protein–Protein Interaction

The ZIM domain is the defining feature of the tify family that contains JAZ, PPD, and ZIM/ZML proteins (Vanholme et al., 2007). Here, we present several lines of evidence to indicate that the ZIM domain mediates homo- and heteromeric protein–protein interactions among JAZ proteins. We have identified nine *Arabidopsis* JAZs (including the three JAZ10 splice variants) that self-associate in yeast. BiFC experiments showed that JAZ3 homomeric complexes accumulate in the plant nucleus. Our Y2H results also revealed an extensive network of JAZ heterodimers; in an experiment to test all 66 possible heterodimeric combinations between 12 full-length *Arabidopsis* JAZs (Table 1), we identified 38 heterodimeric interactions involving most but not all JAZs. Given that protein dimerization is a common regulatory mechanism in signal transduction (Klemm et al., 1998), it is conceivable that combinatorial interactions among various JAZs play a role in generating diverse JA signal outputs. Additional work is needed to determine the physiological relevance of these interactions and to assess whether differences in the interaction potential of various JAZs have functional significance.

Site-directed mutagenesis studies showed that the TIFY motif is a key determinant of JAZ homo- and heteromerization. Because the ZIM domain contains several highly conserved residues in addition to the TIFY motif (Figure 2D), it is likely that other regions of the domain are also important for protein–protein interaction. Y2H assays with JAZ3 deletion constructs and JAZ10.4 showed that JAZ-JAZ binding does not require the N-terminal region or the C-terminal Jas domain. Although this finding supports the notion that the ZIM domain is the primary determinant for intermolecular contact between JAZ proteins, it remains to be determined whether the ZIM domain is a self-stabilizing structure that is sufficient for protein–protein interaction. The ability of ZIM and ZML proteins to homo- and

heterodimize in yeast (see Supplemental Figure 2 online) shows that protein–protein interaction extends to other members of the tify family. This finding raises the possibility that JAZs functionally interact with PPD and ZIM/ZML proteins.

Our results extend the functional analogy between JAZ proteins and auxin/indole-3-acetic acid (Aux/IAA) proteins that are substrates for the SCF^{TIR1} E3 ligase component of the auxin response pathway. JAZ proteins appear to share several key features with Aux/IAA repressor proteins, including hormone-induced rapid degradation by the SCF/ubiquitin/26S proteasome pathway, interaction with transcription factors in the nucleus, and rapid induction of their corresponding genes in response to increased hormone levels (Chini et al., 2007; Thines et al., 2007; Chico et al., 2008; Chung et al., 2008; Katsir et al., 2008b). A role for the ZIM domain in JAZ–JAZ interactions is reminiscent of the ability of Aux/IAA proteins to homo- and heterodimerize (Kim et al., 1997). Sequence determinants (domains III and IV) that mediate these interactions are conserved in AUXIN RESPONSE FACTOR transcription factors that control expression of auxin response genes, which enables Aux/IAAs to bind to and repress AUXIN RESPONSE FACTOR activity (Kim et al., 1997; Ulmasov et al., 1999). Available evidence indicates that the ZIM/TIFY domain is not conserved in MYC2 and is not required for MYC2 binding to JAZ proteins (Chini et al., 2007). Nevertheless, the possibility that the ZIM domain mediates JAZ interaction with proteins outside the tify family cannot be excluded.

A Role for the ZIM Domain in Regulating JAZ Function

We took advantage of the strong JA-insensitive phenotype of *35S-JAZ10.4* plants to study the functional significance of the ZIM domain in JA signaling. The main conclusion of these experiments is that mutations (e.g., I107A) in the TIFY motif that disrupt JAZ10.4 interaction with other JAZs also abrogate the dominant-negative effects of JAZ10.4 on JA signal output. Importantly, functional analysis of JAZ10.4 TIFY mutants ($JAZ10.4^{I->A}$ and $JAZ10.4^{G->A}$) showed that the capacity of JAZ10.4 to interact with other JAZs in yeast is tightly correlated with the strength of the JAZ10.4-mediated dominant-negative effect. Because mutation of the TIFY motif did not appear to affect the accumulation or nuclear localization of JAZ10.4, the most straightforward interpretation of these results is that the repressive activity of JAZ10.4 depends on the ability of its ZIM domain to interact with another protein. Our Y2H analyses support the hypothesis that JAZ10.4 interacts in planta either with itself or with another tify protein to repress JA signaling. Interestingly, Ouellet and colleagues (2001) showed that a mutation (in domain III) that impairs the ability of an Aux/IAA protein to homo- and heterodimerize suppresses auxin-related phenotypes conferred by an intragenic mutation that stabilizes the Aux/IAA against auxin-induced degradation. Thus, the repressive activity of stabilized JAZ and Aux/IAA variants depends on their ability to interact with other family members or with downstream transcription factors.

Our results raise the possibility that a network of JAZ-JAZ complexes differentially interact with transcription factors to control the specificity and strength of JA signal output. Among the important factors that would determine the composition and stiochiometry of JAZ-JAZ dimers (or higher order oligomers) in any given cell type are (1) the rate of JAZ synthesis, (2) the rate of JAZ degradation, and (3) the interaction capacity of each JAZ expressed in that cell type. Inductive cues that increase the synthesis of bioactive JAs are predicted to remodel JAZ-JAZ partnering by increasing the rate of de novo JAZ synthesis and the selective degradation of unstable JAZs. We suggest that complexes containing stabilized JAZs (e.g., JAZ10.4) will accumulate in JA-stimulated cells and, eventually, attenuate expression of JA response genes.

Ectopic expression of JAZ10.4 likely perturbs the normal balance of JAZ proteins by creating a situation in which JAZ10.4 predominates over more labile JAZ isoforms. A simple model to explain the requirement for the TIFY motif in JAZ10.4-mediated repression of signal output is that JAZ10.4 homo- or heteromeric complexes are the functional unit for direct repression of MYC2. The ability of JAZ10.4 to interact with MYC2 (Figure 4D) in the Y2H system is consistent with this model, as is a site-directed mutagenesis study indicating that the COI1 and MYC2 interaction surfaces of JAZ9 are likely not identical (Melotto et al., 2008). This model may also explain the dominant-negative action of JAZ1ΔJas (Thines et al., 2007) and other JAZΔJas proteins that have the capacity to form homo- and heterodimers (Table 1). This scenario, however, would not appear to explain the repressive action of JAZ3ΔJas, which was shown to interact with COI1 but not with MYC2 (Chini et al., 2007). These workers proposed a model in which binding of JAZ3ΔJas to SCF^{COI1} impairs the turnover of endogenous JAZs that would accumulate and repress MYC2 activity. The necessary biochemical and genetic tools are now available to rigorously test these models of JAZΔJas action. Further study of naturally occurring JAZ isoforms that evade degradation by the JA/COI1 pathway promises to provide new insight into the molecular mechanisms underlying the control of JA responses.

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild type for all experiments. Soil-grown plants were maintained in a growth chamber at 21°C under 16 h light (100 $\mu E\ m^{-2}\ s^{-1}$) and 8 h dark. *Arabidopsis* was transformed with *Agrobacterium tumefaciens* (strain C58C1) using the floral dip method (Clough and Bent, 1998). A list of transgenic plants used in this study is provided in Supplemental Table 1 online. Transformed lines (T1 generation) were selected on MS plates containing kanamycin (50 μg/mL) and vancomycin (100 μg/mL). At least 40 independent T1 plants per genotype were transferred to soil for subsequent phenotypic analysis. We identified homozygous lines by testing T3 progeny for resistance to kanamycin. We propagated male-sterile *35S-JAZ10.4* lines by outcrossing to wild-type pollen. F1 progeny containing the transgene were selected on MS medium plates containing kanamycin (50 μg/mL). *Nicotiana tabacum* (cv Petit Havana) was grown in a growth chamber maintained under 17 h of light (200 $\mu E/m^{-2}/s$) at 28°C and 7 h of dark at 18°C.

Y2H Assays

Y2H assays were performed with the Matchmaker LexA system (Clontech). Full-length *JAZ* cDNAs were isolated as previously described

(Chung et al., 2008). cDNAs encoding JAZ10 splice variants were generated from RNA prepared from rosette leaves collected 1.5 h after mechanical wounding. RT-PCR reactions were performed with Pfu Turbo DNA polymerase (Stratagene) and the primer sets listed in Supplemental Table 1 online. *JAZ* cDNAs were subcloned into the Matchmaker pGILDA vector to generate fusions of the bait protein with the LexA DNA BD. These cDNAs were also subcloned into the pB42AD vector to generate fusions of the prey protein with the B42 AD. Yeast transformation and selection of transformants were conducted as described by Melotto et al. (2008). JAZ–JAZ interaction assays were performed as follows. A 1-mL culture of yeast transformants in SD-glucose medium (BD Biosciences) supplemented with -Ura/-His/-Trp dropout solution was grown to an OD_{600} of 1.0 to 1.2. Cells were recovered by centrifugation and resuspended in 0.4 mL of distilled water. Four microliters of the resulting cell suspension was placed on SD-galactose/raffinose (-Ura/-His/-Trp)-inducing media (in 96-well plates) containing 80 μg/mL of X-gal. To test the interaction of JAZ10 variants with COI1, coronatine (Sigma-Aldrich) was added to the medium before the yeast cells were plated. All photographic images of Y2H plates were taken after 48 h of incubation of the plate at 30°C. Expression of the AD and BD fusion proteins was detected by protein gel blot analysis using anti-hemagglutinin (HA; Covance) and anti-LexA (Upstate) antibodies, respectively. β-Galactosidase activity in yeast cells was measured with liquid cultures as described by the manufacturer (Clontech) using ortho-nitrophenyl-β-D-galactopyranoside as a substrate.

Site-Directed Mutagenesis

Ala-scanning mutagenesis of the TIFY motif was performed with Pfu Turbo DNA Polymerase according to the instructions provided with the QuikChange XL site-directed mutagenesis kit (Stratagene). PCR reactions were performed with *JAZ3*, *JAZ10.1*, and *JAZ10.4* cDNAs in pB42AD or pGEM-T Easy vectors, and the mutagenic primers are listed in Supplemental Table 1 online. The presence of the desired mutation was confirmed by DNA sequencing.

Subcellular Protein Localization

The pBI-eYFP binary vector was prepared by subcloning the eYFP coding sequence into the *Kpn*I and *Sac*I restriction sites of the pBI-TS binary vector (Koo et al., 2006; Schilmiller et al., 2007), which is a modified version of pBI121. The PCR-amplified open reading frames (without a stop codon) of *JAZ10.1*, *JAZ10.3*, *JAZ10.4*, and *JAZ10.4*$^{I->A}$ were subcloned into pGEM-T Easy (Promega), followed by recloning into the *Bam*HI site of pBI-eYFP. The resulting constructs encode fusion proteins in which the JAZ C terminus is fused in frame to the N terminus of eYFP. Oligonucleotide primers used to generate these constructs are listed in Supplemental Table 1 online. *Agrobacterium* (strain C58C1) harboring binary vector constructs were grown overnight at 28°C in YEP medium containing 50 μg/mL kanamycin and 50 μg/mL rifampicin. Bacterial cells were resuspended in induction medium (10 mM MES, pH 5.6, 10 mM $MgCl_2$, and 150 μM acetosyringone) and incubated for 1.5 h at room temperature prior to infiltration. Leaves of 5-week-old *Nicotiana tabacum* plants were syringe-infiltrated with the bacterial suspension (OD_{600} = 0.2), and plants were maintained in a growth chamber for 2 d. Confocal microscopy was performed with a Zeiss LSM 510 META microscope (Carl Zeiss) and imaging software provided by the manufacturer. To visualize nuclei, tobacco leaves that were previously infected with *Agrobacterium* were syringe-infiltrated 2 h prior to imaging with a solution containing 10 μg/mL DAPI (Sigma-Aldrich). YFP and DAPI fluorescence were monitored simultaneously by excitation at 514 nm (argon laser) and 405 nm (diode laser), respectively. YFP and DAPI fluorescence was detected after passage through band-pass 530- to 600-nm and 474- to 525-nm emission filters, respectively.

BiFC Assays

The full-length JAZ3 coding sequence was cloned into the pSY728, pSY738, pSY735, and pSY736 vectors (Bracha-Drori et al., 2004) to generate JAZ3-nYFP, JAZ3-cYFP, nYFP-JAZ3, and cYFP-JAZ3, respectively. See Supplemental Table 1 online for primer information. These constructs were subcloned into the *Xho*I and *Sal*I restriction sites of pBI-TS and transformed into *Agrobacterium* strain C58C1. Mixtures of two *Agrobacterium* strains were coinfiltrated into tobacco leaves, such that each of the four possible combinations of nYFP and cYFP JAZ3-fusion constructs was coexpressed. Imaging of protein–protein interaction was performed by confocal laser microscopy as described above.

In Vivo Protein Degradation Assay

Seedlings of transgenic plants expressing JAZ10.1-YFP, JAZ10.3-YFP, and JAZ10.4-YFP fusion proteins grown for 7 d on MS plates were transferred to a 48-well microtiter plate for JA treatment. Seedlings were incubated with JA (0, 1, and 100 μM MeJA) for 2 h at room temperature on an orbital shaker with low speed (70 rpm). To test the effect of proteasome inhibition on protein stability, seedlings expressing JAZ10.1-YFP were pretreated with water or 50 μM MG132 (Sigma-Aldrich) for 2 h prior to 10 μM MeJA treatment. Fluorescence of JAZ10-YFP fusion proteins was analyzed with a Zeiss Axio Scope fluorescence microscope (Carl Zeiss). Images were taken using the software AxioVision 4.7 provided by the manufacturer. All images shown in a single panel in Figure 7 were taken with the same exposure time and software settings.

Root Growth Inhibition Assay

Seeds were surface-sterilized with 30% (v/v) commercial bleach for 15 min and washed 10 times with sterile distilled water. Seeds of each genotype were placed on square Petri plates (Fisher) containing MS medium that was supplemented with 50 μM MeJA. Plates were incubated at 4°C for 4 d in darkness and then incubated under normal growth conditions for the remainder of the experiment. Plates were oriented in a vertical position 6 to 7 d prior to measurement of primary root length. Wild-type (Col-0) and *coi1-1* seeds (or other appropriate lines) were included as controls for JA-sensitive and JA-resistant phenotypes, respectively.

Histochemical Staining

Seedlings grown on MS medium as described in the legend to Figure 9 were transferred to a 48-well microtiter plate for GUS staining. Seedlings were vacuum-infiltrated with GUS staining buffer (50 mM KPO_4, pH 7.2, 2 mM potassium ferricyanide, 2 mM potassium ferrocyanide, 0.1% [v/v] Triton X-100, and 2 mM X-Gluc) for 15 min and then incubated for 12 h at 37°C. A graded series of ethanol washes (up to 70% ethanol) was used to remove chlorophyll, as described previously (Schilmiller et al., 2007). Photographic images were taken with a Leica MZ16 dissecting microscope. Strong GUS staining was also observed in several (at least five per construct) independent *35S-JAZ10.4-GUS* and *35S-JAZ10.4*$^{I->A}$*-GUS* lines within 2 to 3 h of incubation with the X-Gluc substrate.

Accession Numbers

The nucleotide sequence of At5g13220.4 encoding JAZ10.4 has been deposited in GenBank under accession number FJ417331. Arabidopsis Genome Initiative numbers for genes described in this article are as follows: COI1 (At2g39940), JAZ1 (At1g19180), JAZ2 (At1g74950), JAZ3 (At3g17860), JAZ4 (At1g48500), JAZ5 (At1g17380), JAZ6 (At1g72450), JAZ7 (At2g34600), JAZ8 (At1g30135), JAZ9 (At1g70700), JAZ10 (At5g13220), JAZ11 (At3g43440), JAZ12 (At5g20900), MYC2

(At1g32640), ZIM (At4g24470), ZML1 (At3g21175), and ZML2 (At1g51600).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Protein Gel Blot Analysis of JAZ3 and JAZ10.1 Proteins with Point Mutations in the TIFY Motif.

Supplemental Figure 2. Y2H Analysis of Homo- and Heteromeric Interactions between *Arabidopsis* ZIM, ZIM-Like1 (ZML1), and ZML2 Proteins.

Supplemental Figure 3. Detection of Transcripts Encoding JAZ10.3 and JAZ10.4 Using Transcript-Specific Primers.

Supplemental Figure 4. Yeast Two-Hybrid Analysis of the Effect of I107A and G111A TIFY Mutations on the Interaction of JAZ10.4 with Other Members of the JAZ Family.

Supplemental Table 1. List of Plasmid Constructs and Primers Used in This Study.

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