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Plant J. Author manuscript; available in PMC 2011 August 1.

Published in final edited form as:
Plant J. 2010 August ; 63(4): 613–622. doi:10.1111/j.1365-313X.2010.04265.x.

Alternative splicing expands the repertoire of dominant JAZ repressors of jasmonate signaling

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SUMMARY

Jasmonates (JAs) are fatty acid-derived signaling compounds that control diverse aspects of plant growth, development, and immunity. The F-box protein COI1 functions both as a receptor for jasmonoyl-L-isoleucine (JA-Ile) and as the component of an E3-ubiquitin ligase complex (SCF^{COI1}) that targets JAZ transcriptional regulators for degradation. A key feature of JAZ proteins is the C-terminal Jas motif that mediates JA-Ile-dependent interaction with COI1. Here, we show that most *JAZ* genes from evolutionarily diverse plants contain a conserved intron that splits the Jas motif into 20 N-terminal and 7 C-terminal (X_5PY) amino acid submotifs. In most members of the Arabidopsis *JAZ* family, alternative splicing events involving retention of this intron generate proteins that are truncated before the X_5PY sequence. *In vitro* pull-down and yeast two-hybrid assays indicate that these splice variants have reduced capacity to form stable complexes with COI1 in the presence of the bioactive stereoisomer of the hormone, (3*R*,7*S*)-JA-Ile. cDNA overexpression studies showed that some, but not all, truncated splice variants are dominant repressors of JA signaling. We also show that strong constitutive expression of an intron-containing *JAZ10* genomic clone is sufficient to repress JA responses. These findings provide evidence for functional differences between JAZ isoforms, and establish a direct link between alternative splicing of *JAZ* pre-mRNA and dominant repression of JA signal output. We propose that production of dominant JAZ repressors by alternative splicing reduces the negative consequences associated with inappropriate or hyperactivation of the JA response pathway.

Keywords

Jasmonate; JAZ; alternative splicing; COI1; intron retention; coronatine

INTRODUCTION

Jasmonic acid (JA) and its derivatives (collectively referred to as JAs) play a central role in controlling growth, reproduction, and defense-related processes in higher plants (Browse, 2009; Devoto and Turner, 2003; Howe and Jander, 2008; Wasternack 2007). JAs exert their effects by activating large-scale changes in gene expression that depend in part on the transcription factor MYC2 (Boter et al., 2004; Dombrecht *et al*., 2007; Lorenzo *et al*., 2004).

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In resting cells containing low JA levels, JASMONATE ZIM-domain (JAZ) proteins negatively regulate gene expression by binding to MYC2 and other MYC2-related transcription factors (Chini *et al*., 2007; Melotto *et al*., 2008; Pauwels *et al*., 2010; Thines *et al*., 2007; Yan *et al*., 2007). JAZs belong to the TIFY family of proteins that is named for a highly conserved TIFYXG sequence motif located within the ZIM/TIFY domain (Vanholme *et al*., 2007). This domain functions to recruit transcriptional co-repressors (Pauwels *et al*., 2010), and also mediates homo- and heteromeric interactions between JAZ proteins (Chini *et al*., 2009; Chung and Howe, 2009). In response to cues that trigger JA synthesis, JAZ proteins are degraded by the ubiquitin-proteasome pathway, thereby releasing MYC2 from repression. The F-box protein CORONATINE INSENSITIVE 1 (COI1) functions both as a receptor for an amino acid-conjugated form of JA, jasmonoyl-L-isoleucine (JA-Ile), and as an adaptor protein that confers specificity of the SCF (SKP1-CUL1-F-box)-type ubiquitin ligase (SCF^{COI1}) for JAZ substrates (Katsir *et al*., 2008b; Xie *et al*., 1998; Yan *et al*., 2009). Genetic and biochemical evidence is consistent with a model in which COI1-mediated perception of JA-Ile leads to the formation of COI1/JAZ/JA-Ile complexes in which JAZs are ubiquitinated and subsequently degraded (Chini *et al*., 2007; Chico *et al*., 2008; Katsir *et al*., 2008a; Staswick, 2008; Thines *et al*., 2007; Saracco *et al*., 2009; Wasternack and Kombrink, 2010).

JAZ proteins contain a conserved ~27-amino-acid sequence, referred to as the Jas motif, that promotes JA-Ile-dependent interaction with COI1 (reviewed by Chung *et al*., 2009). Artificially truncated JAZs lacking this sequence are resistant to hormone-induced degradation and strongly repress JA responses in a dominant fashion (Chini *et al*., 2007; Chung *et al*., 2008; Shoji *et al*., 2008; Thines *et al*., 2007, Yan *et al*., 2007). The Jas motif also promotes JAZ interaction with MYC2 in a hormone-independent manner (Chini *et al*., 2007; Chini *et al*., 2009; Melotto *et al*., 2008), and has been implicated as a nuclear localization signal (NLS) as well (Grunewald *et al*., 2009). Basic amino acid residues near the N-terminal end of the Jas motif are required for JAZ interaction with COI1 but not MYC2 (Melotto *et al*., 2008), suggesting that sequence determinants for JAZ binding to these two proteins are not identical. Further elucidation of the various functions of the Jas motif is central to understanding the molecular mechanism of JA signaling.

The physiological importance of JA as both a growth inhibitor and a positive regulator of stress responses suggests a broader role for the hormone in controlling resource allocation between growth and defense-related processes (Baldwin, 1998; Yan *et al*., 2007; Zhang and Turner, 2008). Optimization of these antagonistic responses is thought to involve positive and negative feedback loops within the core JA signaling pathway. Rapid activation of *JAZ* gene expression in JA-stimulated cells (Chung *et al*., 2008; Koo *et al*., 2009; Thines *et al*., 2007) suggests that JA responses may be curtailed through *de novo* synthesis of JAZ repressors. Unlike most JAZs characterized to date, including JAZ1, JAZ3, and JAZ10, this type of negative feedback loop should, in theory, involve JAZ repressors that are relatively stable in the presence of JA-Ile. Recent studies indicate that alternative splice variants of JAZ10, which are more resistant to JA-induced degradation, may play a role in this form of negative feedback control (Chung and Howe, 2009). Ectopic expression of JAZ10 isoforms having a truncated Jas motif (JAZ10.3) or lacking the entire motif (JAZ10.4) confers dominant insensitivity to JA (Chung and Howe, 2009; Yan *et al*., 2007). A physiological role for truncated JAZ10 splice variants in attenuating hormone responses in wild-type plants is supported by the finding that plants silenced for *JAZ10* expression are hypersensitive to wounding and JA treatment (Yan *et al*., 2007). Whether this phenomenon is specific for JAZ10, or whether other JAZ splice variants constitute a general post-transcriptional mechanism for fine tuning the signaling pathway, is currently unknown.

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Here, we report that the Jas motif coding region in most *JAZ* genes from diverse plant species is highly conserved, and includes a homologous intron that divides the motif into segments of ~20 N-terminal and 7 C-terminal (X_5PY) amino acids. We show: 1) that most *JAZ* genes in Arabidopsis are subject to alternative splicing events in which retention of this intron generates truncated proteins lacking the X_5PY and other C-terminal amino acids; 2) that these truncated JAZs retain the ability to interact with MYC2 but have reduced capacity to form complexes with COI1 in comparison to their respective full-length isoforms; 3) that overexpression of cDNAs encoding some but not all truncated splice variants results in dominant repression of JA responses; and 4) that high-level constitutive expression of the intron-containing *JAZ10* gene is sufficient to generate alternatively spliced *JAZ10* transcripts and strong phenotypic repression of JA responses. These findings suggest that alternative splicing of *JAZ* genes provides a general mechanism to reduce the fitness costs associated with over-stimulation of the signaling pathway.

RESULTS

Alternative splicing of a conserved intron generates transcripts encoding truncated JAZ proteins

We observed that the intron/exon organization of the Jas motif-coding region is conserved in most Arabidopsis *JAZ* genes (Figure 1a). In 9 of the 12 family members, the 27-amino-acid motif is split into 20 N-terminal and 7 C-terminal (X_5PY) amino acid segments by an intron (the Jas intron) located in phase 2 of the codon specifying the Arg residue (R_{20}) at position 20 (Figure 1b). The 3'-most exon in all nine of these genes encodes the X_5PY sequence and additional C-terminal residues of the protein. Interestingly, in seven of the Jas intron-containing genes, the entire coding capacity of the upstream exon (the Jas exon) specifies ~20 amino acids that comprise the N-terminal portion of the motif (Figure 1a and b).

Retention of the 5' splice site of the Jas intron during pre-mRNA processing of *JAZ2*, *JAZ3*, *JAZ4*, *JAZ6*, *JAZ10*, and *JAZ11* is predicted to generate a premature stop codon (PTC) immediately or shortly (within six codons) after R_{20} (Figure 1c). The Arabidopsis Information Resource (TAIR) database contains EST-supported gene models for *JAZ4* (At1g48500.2) and *JAZ10* (At5g13220.2 and At5g13220.3) transcripts that are spliced in this manner. Reverse transcriptase-mediated PCR (RT-PCR) performed with RNA from seedlings that had been treated with methyl-JA (MeJA) to induce *JAZ* expression identified fully spliced (i.e., lacking the Jas intron) mRNAs for all nine genes that contain the Jas intron (Figure 2). RT-PCR experiments performed with the same RNA and gene-specific primers that anneal within the Jas intron amplified alternatively spliced forms of *JAZ2*, *JAZ3*, *JAZ4*, *JAZ6*, *JAZ9*, *JAZ10*, and *JAZ12* mRNA (Figure 2). Sequencing of these PCR products showed that the transcripts contain the 5' end of the Jas intron but not other intron sequences, thereby excluding the possibility of genomic DNA contamination in the PCR reactions. Thus, most *JAZ* genes (except *JAZ1*, *JAZ7*, and *JAZ8*) expressed in JA-treated Arabidopsis seedlings produce alternatively spliced mRNAs predicted to encode truncated proteins that lack the X_5PY sequence and additional C-terminal amino acids. We henceforth refer to these splice variants as ΔPY JAZs.

To gain additional insight into the functional significance of the Jas intron, we sought to determine whether *JAZ* genes from phylogenetically diverse plant species contain this sequence. Analysis of select genome sequence databases (see Experimental Procedures) showed that most (~76%) *JAZ*-related genes in rice (*Oryza sativa*), grapevine (*Vitis vinifera*), poplar (*Populus trichocarpa*), *Brachypodium distachyon*, moss (*Physcomitrella patens*), and the lycophyte *Selaginella moellendorffii* contain a homologous intron at this position (i.e., phase 2 of the R_{20} codon) within the gene (Table 1 and Table S1). Three EST-supported gene models (Bradi3g10820.2, Bradi4g31240.2, and Bradi5g24410.4; Table S1)

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for transcripts encoding ΔPY JAZs in *B. distachyon* provide evidence that alternative splicing events involving the Jas intron are conserved between monocots and dicots. As is the case for Arabidopsis *JAZ2*, *JAZ6 JAZ10*, and *JAZ11* (Figure 1c), hypothetical retention of the 5' donor splice site of the Jas intron in 59% of the non-Arabidopsis *JAZ*-related genes generates a PTC immediately after the R_{20} codon in which the intron resides (Table 1 and Table S1). The high proportion of predicted transcripts with a PTC at this position appears to be a consequence of the compositional bias of 5' splice sites, which in plants have a consensus sequence AG/GTAA (stop codon underlined) (Reddy, 2007).

ΔPY JAZ proteins differentially associate with the COI1

We used *in vitro* pull-down (PD) assays to study the hormone-dependent interaction of Arabidopsis ΔPY isoforms with COI1. This assay was first tested with splice variants of JAZ10, which previously were shown to differentially associate with COI1 in yeast cells grown in the presence of coronatine, a potent agonist of the JA-Ile receptor (Chung and Howe, 2009; Katsir *et al*., 2008b; Yan *et al*., 2009). A biologically active form of JA-Ile, (3*R*,7*S*)-JA-Ile (Fonseca *et al*., 2009), strongly stimulated the ability of recombinant JAZ10.1-His to recover epitope-tagged COI1 (AtCOI1-Myc) from crude leaf extracts (Figure 3a). In comparison to the full-length JAZ10.1 isoform, the ΔPY splice variant JAZ10.3 recovered only minor amounts of COI1-Myc in the presence of the highest (500 nM) concentration of JA-Ile tested. (3*R*,7*S*)-JA-Ile did not stimulate recovery of COI1 by JAZ10.4, which lacks the entire Jas motif as a consequence of an alternative splicing event within the third exon (Chung and Howe, 2009).

Genes belonging to three (A, B, and C) of the four JAZ phylogenetic clades in Arabidopsis contain the Jas intron (Figure 1a). To determine whether the weak COI1 interaction observed with JAZ10.3 (clade C) is a general property of ΔPY splice variants, we performed PD assays with representative members of clades A (JAZ3) and B (JAZ2). In accordance with established gene models in TAIR, we refer to proteins produced from transcripts encoding full-length JAZ2 and JAZ3 as JAZ2.1 and JAZ3.1, respectively, and the corresponding ΔPY isoforms as JAZ2.2 and JAZ3.4 (Figure 3b). The capacity of JAZ2.1-His and JAZ2.2-His to recover COI1 from leaf extracts in the presence of (3*R*,7*S*)-JA-Ile was very similar to that of JAZ10.1 and JAZ10.3, respectively. For example, the amount of COI1 bound by JAZ2.1-His in the presence of 10 nM JA-Ile was comparable to that bound by JAZ2.2 in the presence of 500 nM JA-Ile (Figure 3c). (3*R*,7*S*)-JA-Ile stimulated strong interaction of JAZ3.1-His with COI1, as previously reported (Fonseca *et al*., 2009; Melotto *et al*., 2008), and also acted in a dose-dependent manner to promote COI1 binding to JAZ3.4-His (Figure 3d). The amount of COI1-Myc bound to JAZ3.4 at a given hormone concentration, however, was reproducibly less than that recovered by JAZ3.1-His.

We used the yeast two-hybrid (Y2H) system as a second approach to assess the relative ability of ΔPY splice variants to interact with COI1. Dose-dependent COI1-JAZ2.1 and COI1-JAZ3.1 interactions were observed in yeast cells grown in the presence of increasing concentrations of (3*R*,7*S*)-JA-Ile (Figure 3e). These interactions were not observed in yeast cells grown in the presence of 100 μM of the *trans* stereoisomer (3*R*,7*R*)-JA-Ile (Figure S1), which is a largely inactive form of the hormone (Fonseca *et al*., 2009). Remarkably, bioactive (3*R*,7*S*)-JA-Ile failed to promote detectable interaction of COI1 with JAZ2.2 and JAZ3.4 in yeast (Figure 3e). Coronatine strongly stimulated COI1 binding to JAZ2.1 and JAZ3.1 in yeast, and was significantly more active in these assays than our synthetic preparation of (3*R*,7*S*)-JA-Ile (Figure 3f). COI1 interacted weakly with JAZ2.2 in the presence of high concentrations (100 μM) of coronatine. COI1 interacted more strongly with JAZ3.1 than with JAZ3.4 in the presence of low concentrations (e.g., 5–10 μM) of coronatine, whereas discrimination between these two splice variants by COI1 was not observed at higher concentrations (e.g., 100 μM) of coronatine (Figure 3f). Western blot

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analysis showed that all JAZ proteins were expressed in yeast (Figure S2). These findings show that ΔPY splice variants of JAZ2 and JAZ3, like JAZ10, have reduced capacity to form complexes with COI1 in the presence of the receptor-active form JA-Ile and the potent agonist coronatine.

We hypothesized that the reduced ability of ΔPY JAZs to associate with COI1 may reflect a role for the highly conserved PY sequence in promoting COI1-JAZ interactions. To test this idea, we substituted the PY sequence in JAZ2.1 and JAZ10.1 with Ala residues, and tested the resulting proteins in PD assays for their ability to bind COI1. The results showed that the PY→AA mutation did not diminish the ability of JAZ2.1-His and JAZ10.1-His to recover COI1-Myc from crude leaf of extracts in the presence of JA-Ile or coronatine (Figure 4), indicating that the PY motif is not required for these COI1-JAZ interactions.

Attenuation of JA signaling by JAZ splice variants

The reduced capacity of JA-Ile to stimulate COI1 interaction with JAZ2.2 and JAZ3.4 led us to hypothesize that these splice variants may act to repress JA responses *in vivo*. To address this question, we overexpressed cDNAs encoding full-length and ΔPY variants of JAZ2 and JAZ3 in Arabidopsis, and tested the resulting transgenic lines for altered responsiveness to exogenous methyl-JA (MeJA) using a root growth-inhibition assay. Overexpression of cDNAs encoding JAZ2.1, JAZ3.1, and JAZ3.4 did not obviously affect MeJA-induced inhibition of root growth in progeny of 10 independent lines tested for each construct (data not shown). In the case of JAZ2.2, however, 7 of 15 independent T1 lines expressing the *35S–JAZ2.2* transgene produced progeny that were significantly less sensitive than wild-type seedlings to MeJA (Figure 5). Root-length assays showed that the sensitivity of *35S–JAZ2.2* homozygous seedlings to MeJA is similar to or slightly less than that of *35S–JAZ10.3* seedlings, which overexpress the ΔPY variant of JAZ10 (Figure S3). *35S–JAZ2.2* homozygous lines did not display obvious defects in fertility (data not shown). Y2H assays showed that JAZ2.2, like the full-length JAZ2.1 protein (Chini *et al*., 2009), retains the ability to interact with MYC2 (Figure 5c). These results suggest that JAZ2.2 may exert its dominant effect through inhibition of MYC2 or related transcription factors.

Previous functional analysis of JAZ splice variants relied on overexpression of individual cDNAs encoding truncated JAZ isoforms (Chung and Howe, 2009; Yan *et al*., 2007; Figure 5). To further address the functional significance of *JAZ* alternative splicing in JA signaling, we transformed Arabidopsis with a full-length genomic *JAZ10* sequence under the control of the CaMV 35S promoter. Fifteen of 20 independent T1 lines expressing the *35S–JAZ10G* transgene produced progeny that were strongly insensitive to root growth inhibition by MeJA (Figure 6a). Root length measurements performed with 9-day-old seedlings showed that the strength of the JA-insensitive phenotype in three representative T2 lines was greater than that of *35S–JAZ10*.3 seedlings, and comparable to *35S–JAZ10.4* seedlings that express the COI1-noninteracting JAZ10.4 isoform (Figure 6b). In contrast to the male-sterile phenotype of *35S–JAZ10.4* plants (Chung and Howe, 2009), reproductive defects were not observed in any of the 50 independent *35S–JAZ10G* lines tested, including homozygous lines (data not shown). RT-PCR experiments confirmed that *JAZ10.1*, *JAZ10.3*, and *JAZ10.4* transcripts derived from the transgene are expressed in *35S–JAZ10G* seedlings (Figure 6c). These findings establish a causal link between strong expression of *JAZ10*, alternative splicing of *JAZ10* pre-mRNA, and functional repression of JA responses.

DISCUSSION

A large proportion of intron-containing plant genes are subject to alternative splicing (Barbazuk *et al*., 2008; Campbell *et al*. 2006; Filichkin *et al*., 2010; Reddy, 2007; Wang and Brendel, 2006). Examples of evolutionarily conserved splicing events that have functional

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significance in plant biology, however, are scarce (Filichkin *et al*., 2010; Ner-Gaon *et al*., 2007). Here, we analyzed splicing events affecting the JAZ family of proteins that, together with COI1 and MYC2, comprise the core JA signaling pathway in vegetative tissues. Focus was placed on the analysis of splicing events that alter the protein's C-terminal Jas motif, which mediates interaction with COI1, MYC2, and possibly JA-Ile as well. We show that the intron/exon organization of the Jas motif-coding region in most *JAZ* genes consists of two exons separated by an intron (the Jas intron) that is found in most *JAZ*-related genes from diverse plant species. The presence of the Jas intron in *JAZ* orthologs from *P. patens* indicates that the sequence arose early in the evolution of the *JAZ* gene family, and has been retained during the time since *P. patens* diverged from higher plants approximately 400 million years ago (Rensing *et al*., 2008). Evolutionary conservation of this gene architecture implies an important biological function for the Jas intron; our results provide evidence that alternative splicing events affecting this intron expand the functional repertoire of JAZ proteins to modulate JA signaling. These findings provide a counter example to the view that alternative splicing has only a limited role in expanding the diversity of plant proteomes (Severing *et al*., 2009).

The ~20 N-terminal amino acids of the Jas motif in many JAZ proteins are encoded by a dedicated exon, which we refer to as the Jas exon. The modular and conserved nature of this coding sequence implies an important role in JAZ function. It is intriguing that the amino acid sequence specified by the Jas exon resembles the N-terminal portion of the CCT (CO, CO-like, TOC1) motif found in proteins that control plant circadian rhythm and other responses to environmental cues (Chung *et al*., 2009). This sequence similarity accounts for the annotation of the Jas motif as a CCT-like domain in Pfam (http://pfam.sanger.ac.uk/) and InterPro (http://www.ebi.ac.uk/Databases/) databases. An evolutionary link between CO/COL/TOC1 and JAZ proteins is supported by the fact that ZIM and ZIM-like members of the TIFY family contain a CCT domain.

Alternative splicing events involving retention of the 5' splice site of the Jas intron generate transcripts in which a PTC effectively removes the X_5PY sequence and other amino acids encoded by the 3' exon. PTC-containing (PTC$^+$) transcripts may be targeted for destruction by the nonsense-mediated mRNA decay (NMD) pathway (Reddy, 2007). Among the features that trigger transcripts for NMD are PTCs located >50–55 nucleotides upstream of an exon-exon junction and abnormally long 3' untranslated regions (Chang *et al*., 2007; Hori and Watanabe, 2007). Although additional work is needed to determine the role of the NMD pathway in regulating *JAZ* mRNA accumulation, RT-PCR experiments showed that several PTC$^+$ *JAZ* transcripts are expressed in Arabidopsis. At least some these transcripts produce JAZ isoforms that, when ectopically expressed, repress JA responses (Chung and Howe, 2009; Yan *et al*., 2007; this study). Given the widespread occurrence of the Jas intron in the plant kingdom and the high frequency of intron retention in plants (Filichkin *et al*., 2010; Ner-Gaon *et al*., 2004; Wang and Brendel, 2006), it seems likely that *JAZ* genes in other plants are spliced in a similar manner. Evidence for cross-species conservation ΔPY splice variants comes from three sequence-supported *JAZ* gene models in *B. distachyon* (Table S1).

The JA-resistant phenotype of *35S–JAZ10G* plants establishes a direct link between alternative splicing of *JAZ* pre-mRNA and dominant attenuation of JA signal output. High-level constitutive expression of *JAZ10* from the 35S promoter appears to be sufficient to generate a pool of pre-mRNA that is subject to alternative splicing and production of the dominant JAZ10.3 and JAZ10.4 isoforms. In wild-type plants, transcription of *JAZ* genes is rapidly and strongly induced in response to cues that trigger the synthesis of bioactive JAs (Chung *et al*., 2008; Koo *et al*., 2009; Thines *et al*., 2007; Yan *et al*., 2007). Alternative splicing of the resulting *JAZ* pre-mRNA, together with the enhanced stability of truncated

splice variants, could provide a mechanism to accumulate dominant JAZ repressors during the early phase of JA-mediated stress responses. This model is consistent with studies showing coordinate accumulation of *JAZ10.1* and *JAZ10.3* transcripts in wounded Arabidopsis leaves (Yan *et al*., 2007). It is also possible that alternative splicing of *JAZ* pre-mRNA is controlled in a cell- or tissue-type specific manner by *trans*-acting factors of the spliceosome machinery. The fact that roots of *35S–JAZ10.4* and *35S–JAZ10G* plants are both strongly insensitive to JA-induced growth inhibition, whereas only *35S–JAZ10.4* plants are male sterile, raises the possibility that JAZ10.4 is not normally produced in male reproductive tissues. It is also possible that *JAZ10.4* transcript levels are higher in *35S–JAZ10.4* plants than in *35S–JAZ10G* plants.

Our results indicate that repression of JA responses by ΔPY JAZs results in part from decreased interaction with COI1 and, as a consequence, increased JAZ stability in the presence of JA-Ile. This finding implies a role for the X_5PY sequence or additional amino acids at the protein C-terminus in promoting COI1-JAZ complex formation. Site-directed mutagenesis experiments excluded the possibility that the highly conserved PY is required for this aspect of JAZ2 and JAZ10 function. We also found that coronatine promotes robust binding of COI1 to JAZ3.4, which lacks the PY sequence, in PD and Y2H assays. Ectopic expression of ΔPY isoforms of JAZ10 (JAZ10.3) and JAZ2 (JAZ2.2) conferred an obvious JA-insensitive phenotype, whereas overexpression of JAZ3.4 did not, indicating that some but not all ΔPY isoforms exert strong dominant effects. However, because the ligand-dependent interaction of COI1 with JAZ3.1 was stronger than that with JAZ3.4, it is possible that JAZ3.4 affects JA responses in a manner that was not detected in our analysis.

In considering potential functional differences between the dominant JAZ2.2/JAZ10.3 proteins and JAZ3.4, it is worth noting that the latter isoform contains five amino acids C-terminal to the KRKD/ER sequence, whereas JAZ2.2/JAZ10.3 and most other predicted ΔPY splice variants are truncated immediately after KRKD/ER (Table 1). We speculate that the C-terminal extension of JAZ3.4 may contribute indirectly to the formation of COI1-JAZ complexes by stabilizing the structure of the KRKD/ER region, which is predicted to have α-helical character (Chung *et al*., 2009). The most straightforward interpretation of our results, together with other structure-function studies (Melotto *et al*., 2008), is that the primary sequence determinant for COI1 binding resides in the N-terminal region of the Jas motif (i.e., residues encoded by the Jas exon), and that the C-terminal end of the Jas motif (i.e., X_5PY) contributes indirectly to ligand binding by stabilizing the COI1-ligand-JAZ ternary complex.

The highly conserved nature of the X_5PY sequence suggests that it may contribute to other aspects of JAZ function as well. Recent studies with JAZ1 indicate that the X_5PY motif may function as part of a NLS for targeting to or within the nucleus (Grunewald *et al*., 2009). A similar sequence motif ($RX_{2–5}$PY) is part of an NLS in yeast and human proteins (Lee *et al*., 2006). It should be noted, however, that JAZ proteins lacking either the entire Jas motif or the X_5PY submotif retain the ability to modulate JA signaling, presumably by interacting with signaling components in the nucleus (Chini *et al*., 2007; Chung and Howe, 2009; Thines *et al*., 2007; Yan *et al*., 2007; this study).

Our results are consistent with the view that alternative splicing plays a role in optimizing plant adaptation to stress (Barbazuk *et al*., 2008; Filichkin *et al*., 2010; Reddy, 2007). In contrast to our understanding of how stress-regulated genes are activated in plants, relatively little is known about how these responses are restrained once they are initiated (Kazan, 2006). In wounded Arabidopsis leaves, down-regulation of response genes during a time period when JA-Ile levels remain high suggests that stressed tissues become desensitized to the hormone (Koo *et al*., 2009; Koo and Howe, 2009). Epimerization of receptor-active (3*R*,

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7*S*)-JA-Ile to the inactive (3*R*,7*R*)-JA-Ile isomer was proposed as a mechanism to inactivate signaling (Fonseca *et al*., 2009). Measurement of JA-Ile stereoisomers in wounded tomato leaves, however, indicates that endogenous pools of (3*R*,7*S*)-JA-Ile are not readily epimerized *in planta* (Suza *et al*., 2010).

Our data support an alternative hypothesis in which stable ΔPY JAZ isoforms act to restrain the transcription of JA-response genes in the presence of (3*R*,7*S*)-JA-Ile. Dominant isoforms such as JAZ2.2 and JAZ10.3 retain the ability to interact with MYC2, presumably through the truncated Jas motif, whereas JAZ10.4 appears to bind MYC2 through a region outside the Jas motif (Chung and Howe, 2010). These endogenous repressors contain an intact ZIM/TIFY domain and thus may actively repress gene expression through the NINJA/TOPLESS transcriptional co-repressor complex (Pauwels *et al*., 2010). Fitness costs resulting from inappropriate or hyperactivation of JA responses (Baldwin, 1998; Moreno *et al*., 2009; Staswick *et al*., 1992; Yan *et al*., 2007; Zhang and Turner, 2008) may have provided selective pressure to evolve dominant JAZ isoforms through alternative splicing events within the Jas intron. Other endogenous JAZ repressors (e.g. JAZ10.4) are generated by alternative splicing events outside the Jas intron (Chung and Howe, 2009). Thus, multiple mechanisms may contribute to expanding the functional repertoire of JAZ proteins to fine tune JA responses.

EXPERIMENTAL PROCEDURES

Biological materials

Growth conditions for *Arabidopsis thaliana* (ecotype Col-0), *Agrobacterium tumefaciens*-mediated transformation, and root growth inhibition assays were conducted as previously described (Chung and Howe, 2009).

Identification of *JAZ* gene sequences

Rice *JAZ* genes were previously described (Ye *et al*., 2009). Genome sequences for poplar (Tuskan *et al*., 2006), *Brachypodium* (International Brachypodium Initiative, 2010), *Physcomitrella* (Rensing *et al*., 2008), *Selaginella* (http://genome.jgi-psf.org/Selmo1/Selmo1.home.html), and grapevine (Jaillon *et al*., 2007), were searched with BLASTP and TBLASTN for matches to Arabidopsis JAZ1. Retrieved sequences were used to re-query the database for that species. Following removal of redundant sequences, protein sequences were manually annotated for the presence of the ZIM/TIFY and Jas motifs. No attempt was made to discriminate between genes encoding JAZ and PEAPOD (PPD) proteins, both of which contain ZIM/TIFY and Jas-like motifs. The intron/exon structure of all identified genes was assessed with the genome browser in the respective database, as well as ClustalW-based alignment of the genomic DNA sequence with the predicted coding sequence of the gene. The conserved intron/exon organization of the Jas motif coding region was verified with software that predicts common introns within orthologous genes (http://ciwog.gdcb.iastate.edu/) (Wilkerson *et al*., 2009) and with comparative genome analysis tools available at http://www.phytozome.net/. The consensus sequence for the Jas motif in Arabidopsis JAZs was determined with the web-based application WebLogo (Crooks *et al*., 2004).

Molecular biology procedures

RT-PCR was used to identify Arabidopsis *JAZ* transcripts that either contain or do not contain the Jas intron. RT-PCR reactions were performed with RNA prepared from 12-d-old wild-type seedlings treated with 100 μM MeJA for 2 h (Chung *et al*., 2008), Taq DNA polymerase (Invitrogen), and the transcript-specific primer sets listed in Table S2. PCR products were cloned into pGEM-T Easy (Promega) for sequencing with T7 and SP6

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primers. A PCR product containing the full-length *JAZ10* genomic sequence was amplified from Arabidopsis genomic DNA using Pfu Turbo DNA polymerase (Stratagene) and the primers listed in Table S2. The resulting ~1.9-kb product was subcloned into pGEM-T Easy (Promega), followed by recloning into the *Bam*HI site of a modified pBI121 binary vector (Schilmiller *et al*., 2007). *35S–JAZ10G* transgenic plants harboring this construct were generated as described above. PCR-based site-directed mutagenesis of the PY submotif in JAZ2.1 and JAZ10.1 was performed as previously described (Chung and Howe, 2009). Primers for these reactions are listed in Table S2.

COI1-JAZ interaction assays

Appropriate restriction sites were added to *JAZ* cDNAs (Chung and Howe, 2009) by PCR amplification with the primer sets listed in Table S2. PCR products were cloned into the corresponding site of pRMG-nMAL to produce plasmids encoding MBP-JAZ-His_6 fusion proteins (referred to as JAZ-His). JAZ fusion proteins were expressed in *E. coli* and purified by Ni-affinity chromatography as described previously (Katsir *et al*., 2008b; Thines *et al*., 2007), with the following minor modifications. The lysis buffer consisted of 50 mM Na-phosphate, pH 7.8, 500 mM NaCl, 0.1% Tween-20, 0.1 mM phenylmethyl sulfonyl fluoride, and 15 mM imidazole. The wash buffer was identical to the lysis buffer but contained 25 mM imidazole. Leaf extract from a transgenic Arabidopsis line expressing AtCOI1–9xMyc (Melotto 2008) was used as the source of COI1. Soluble protein was extracted from rosette leaves (ground in liquid nitrogen) in binding buffer (50 mM Na-phosphate, pH 7.7, 100 mM NaCl, 10% glycerol, 0.1% Tween-20, 25 mM imidazole, 20 mM β-mercaptoethanol, and Roche Complete Mini protease inhibitor tablet-EDTA free) and clarified by centrifugation at 15,000 × g at 4°C for 10 min. Each PD assay contained 1 mg leaf protein and 25 μg recombinant JAZ–His in a total volume of 200 μL. Reactions were incubated for 30 min at 4°C in the absence or presence of JA-Ile or coronatine. Following the addition of 60 μL Ni-NTA Resin (Qiagen), the reaction was incubated for an additional 30 min at 4°C. Ni-NTA resin was recovered by centrifugation on a spin column (Biorad) and washed three times with 250 μL binding buffer. The affinity resin was eluted with 30 μL of a solution containing 350 mM imidazole. The eluted protein was separated by SDS-PAGE on a 10% gel, transferred to PVDF membrane, and probed with an anti-c-Myc antibody (Roche). For Y2H analysis, *JAZ* cDNAs were amplified using the primer sets listed in Table S2, and subsequently cloned into pB42AD. Y2H assays were performed as described previously (Chung and Howe, 2009). Stereoisomers of JA-Ile were chemically synthesized as described by Ogawa and Kobayashi (Ogawa and Kobayashi, 2008). Coronatine was purchased from Sigma-Aldrich.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Sheng Yang He (Michigan State University) for providing the Arabidopsis line expressing AtCOI1-9xMyc and Todd Mockler (Oregon State University) for sharing unpublished data on alternatively spliced *JAZ* transcripts in Arabidopsis and *Brachypodium*. We thank Abe Koo for mass spectrometry analysis of JA-Ile isomers, and Laura Sheard and Ning Zheng (University of Washington) for helpful discussions during the course of the research. This work was supported by the National Institutes of Health (grant R01GM57795) and the Chemical Sciences, Geosciences and Biosciences Division, Office of Basic Energy Sciences, Office of Science, U.S. Department of Energy (grant DE–FG02–91ER20021).

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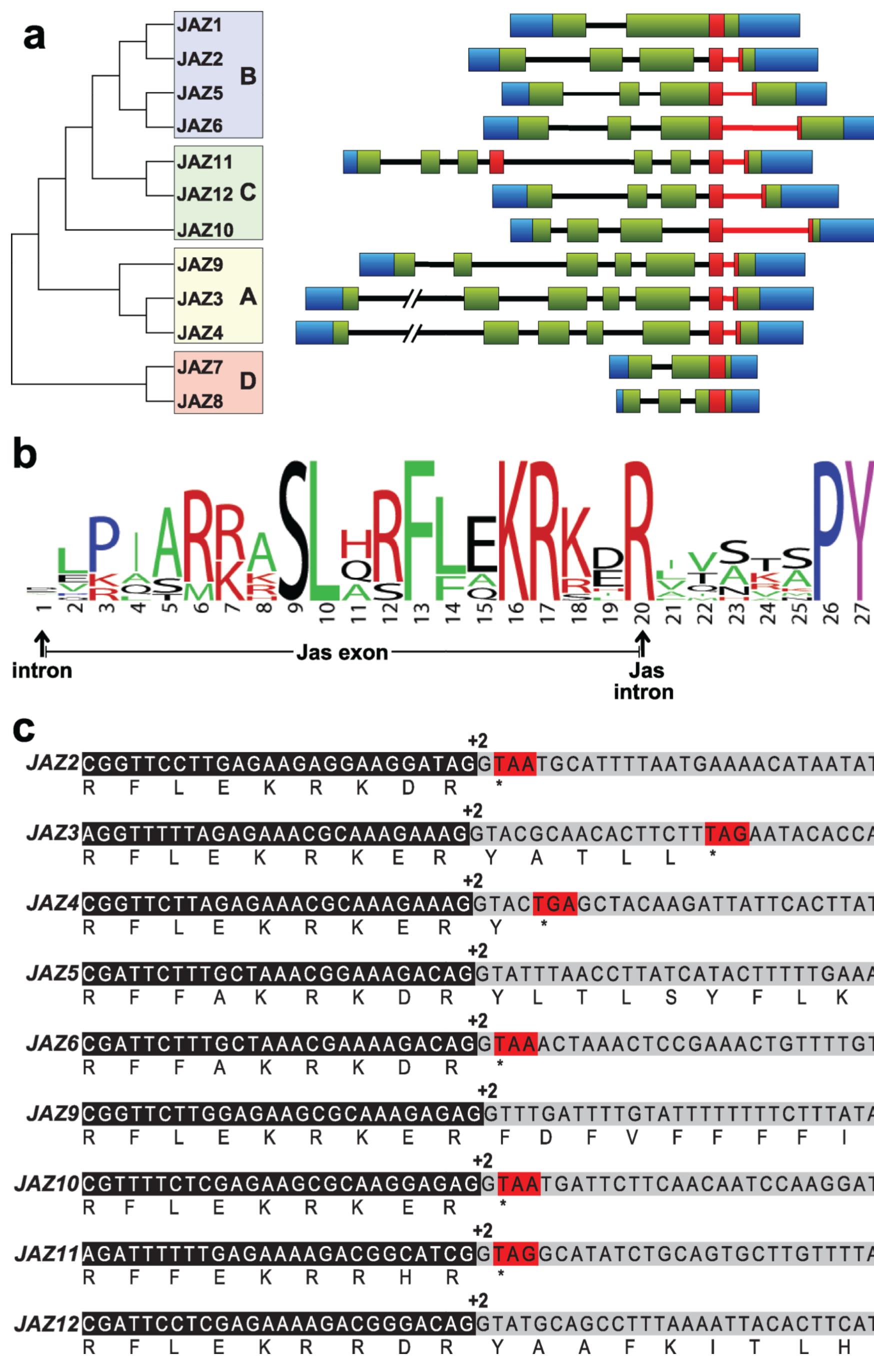

Figure 1.

Bipartite organization of the Jas domain-coding region in Arabidopsis *JAZ* genes.

(a) Left, Phylogenetic tree constructed from the amino acid sequence of 12 full-length JAZs, showing four (A–D) subclades of proteins. Right, intron/exon organization of the corresponding genes. Thick green and blue bars indicate coding regions and non-coding untranslated regions in exons, respectively. The Jas motif coding-region is depicted in red. Introns are depicted by a thin black horizontal line or, in the case of the Jas intron, a red line.

(b) Consensus sequence of the Jas motif in 12 Arabidopsis JAZ. The arrow indicates the location of homologous introns. The Jas intron is positioned invariably in phase 2 of the codon specifying Arg at position 20 of the motif.

(c) Sequence of the Jas exon/intron junction in nine Arabidopsis *JAZ* genes that contain the intron. Exon and intron sequences are highlighted in black and gray, respectively, together with the predicted amino acid sequence. In the case of *JAZ2 JAZ3 JAZ4 JAZ6 JAZ10*, and *JAZ11*, an in-frame stop codon (red) is encountered close to the 5' end of the Jas intron, if the intron is retained.

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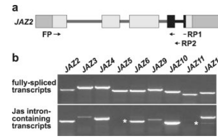

Figure 2.

RT-PCR-based detection of Jas intron retention in various *JAZ* transcripts.

(a) Primer design strategy showing *JAZ2* as an example. Structural elements of the gene include the Jas motif coding-region (black box) and Jas intron (black line). Light grey and dark grey bars indicate coding regions and non-coding untranslated regions in exons, respectively. Arrows indicate forward (FP) and reverse (RP) primers used for RT-PCR assays designed to amplify transcripts in which the Jas intron is spliced out or retained. The RP1 primer spans the exon/exon junction and thus hybridizes to transcripts in which the Jas intron is removed. The RP2 primer hybridizes to a gene-specific sequence near the 5' end of the Jas intron, thereby amplifying transcripts in which the 5' splice site of the intron is retained. Primer sequences are listed in Table S2.

(b) RNA extracted from MeJA-treated Arabidopsis seedlings was used as a template for RT-PCR (see Experimental Procedures). Gene-specific FP-RP1 primer sets amplified fully-spliced transcripts (upper panel), whereas FP-RP2 primer sets amplified transcripts in which the 5' end of the Jas intron was retained (lower panel). PCR-amplified products were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide. For those RT-PCR assays yielding more than one main product, asterisks denote the transcript containing the Jas intron but not other introns, as determined by DNA sequencing of cloned products.

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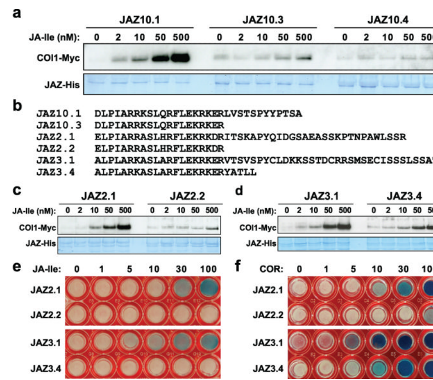

Figure 3.

ΔPY JAZ isoforms differentially associate with COI1 in the presence of receptor ligands.

(a) Hormone-dependent interaction of JAZ10 splice variants with COI1. Pull-down assays were performed using recombinant JAZ10 splice variants and crude leaf extracts from *35S-COI1-Myc* leaves as a source of COI1. JAZ10.4 lacks the entire Jas motif. Reaction mixtures were supplemented with the indicated concentration of (3*R*,7*S*)-JA-Ile, or an equivalent volume of assay buffer ("0"). Protein bound to JAZ10-His was separated by SDS-PAGE and analyzed by immunoblotting with anti-Myc antibody for the presence of COI1-Myc. As a loading control, the immunoblotted membrane was stained with Coomassie blue to detect JAZ-His.

(b) Effect of alternative splicing of the Jas intron on the sequence of the Jas motif in full-length (JAZ10.1, JAZ2.1, and JAZ3.1) and ΔPY (JAZ10.3, JAZ2.2, JAZ3.4) isoforms of JAZ10, JAZ2, and JAZ3.

(c, d) Hormone-dependent interaction of full-length (JAZ2.1 and JAZ3.1) and ΔPY (JAZ2.2 and JAZ3.4) splice variants with COI1. Experiments were performed as described in panel a.

(e) JA-Ile-dependent interaction of JAZ2 (upper) and JAZ3 (lower) splice variants with COI1 in the Y2H system. Yeast strains expressing both COI1 (as a DNA binding domain fusion) and the indicated JAZ splice variant (as an activation domain fusion) were plated on media containing X-gal and the indicated concentration (μM) of (3*R*,7*S*)-JA-Ile. LacZ-mediated blue-color formation is indicative of the strength of the COI1-JAZ interaction.

(f) Coronatine-dependent interaction of JAZ2 (upper) and JAZ3 (lower) splice variants with COI1 in the Y2H system. Experiments were performed as described in panel *e*, except that coronatine (COR) was included at the indicated concentration (μM) in the yeast medium.

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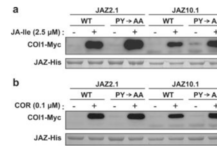

Figure 4.

The PY submotif of JAZ2.1 and JAZ10.1 is not required for COI1 interaction.

(a, b) Site-directed mutagenesis was used to change the PY diamino acid sequence in the Jas motif of JAZ2.1 (position 227–228) and JAZ10.1 (position 191–192) to AA. The resulting wild-type (WT) and mutant (PY→AA) proteins were expressed in *E. coli* as MBP-His fusions. Pull-down reactions containing purified JAZs and protein extract from leaves of the *35S-AtCOI1-Myc* transgenic line were supplemented (lanes labeled “+”) with 2.5 μM (3*R*, 7*S*)-JA-Ile (panel a) or 0.1 μM coronatine (panel b). Control reactions contained an equivalent amount of binding buffer (lanes labeled “−”). Reactions were incubated and processed as described in the legend for Figure 3a. The Coomassie blue-stained gel shows the input of MBP-JAZ-His fusion protein.

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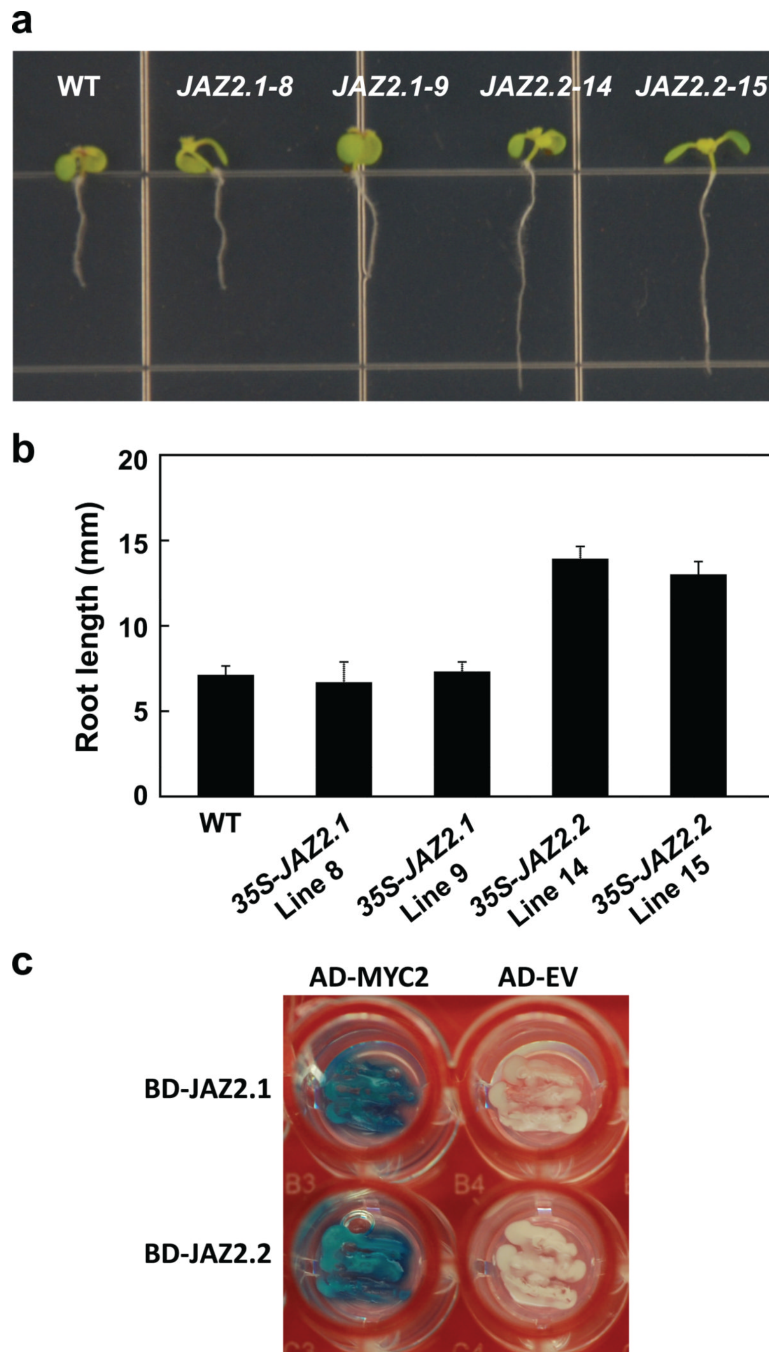

Figure 5.

Ectopic expression of JAZ2.2 attenuates jasmonate responses.

(a) Photograph of wild-type (WT), *35S-JAZ2.1*, and *35S-JAZ2.2* seedlings grown for 9 d on MS medium containing 50 μM MeJA. Seedlings from two independent lines for each construct are shown.

(b) Quantification of MeJA-induced root growth inhibition of seedlings shown in (a). Data show the mean ± SD (n = 15 seedlings per genotype).

(c) JAZ2.1 and JAZ2.2 interact with MYC2 in yeast. Yeast strains co-transformed with plasmids encoding MYC2 (as an activation domain fusion; AD) and the indicated JAZ2 splice variant (as a DNA binding domain fusion; BD) were plated on media containing X-

gal. LacZ-mediated blue-color formation is indicative of JAZ-MYC2 interaction. Yeast strains expressing BD-JAZ and an empty AD vector (AD-EV) control did not exhibit visible blue-color formation.

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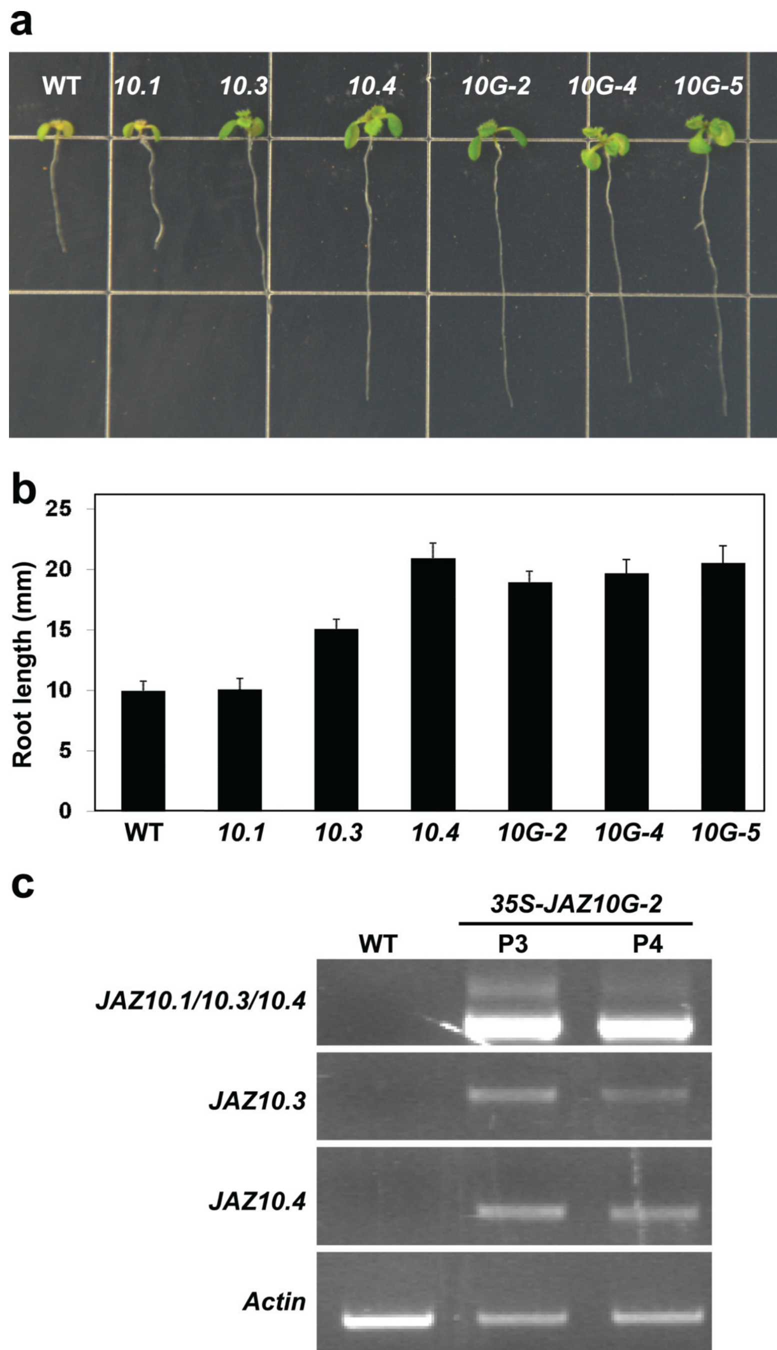

Figure 6.

Overexpression of a *JAZ10* genomic clone attenuates jasmonate signaling.

(a) Photograph of wild-type (WT) and transgenic seedlings of the indicated genotype grown for 9 d on MS medium containing 50 μM MeJA. *10.1 10.3*, and *10.4* refer to transgenic lines overexpressing cDNAs for JAZ10.1, JAZ10.3, and JAZ10.4, respectively (Chung and Howe, 2009), which were grown together with three independent *35S-JAZ10G* lines (*10G-2, 10G-4*, and *10G-5*).

(b) Quantification of MeJA-induced root growth inhibition of seedlings shown in (*a*). Data show the mean ± SD of at least 11 seedlings per genotype.

(c) Semi-quantitative RT-PCR analysis of *JAZ10* transcripts in 2-week-old *35S-JAZ10G* seedlings (line 2) grown on MS medium. Expression of transcripts derived from the *35S-JAZ10G* transgene was assessed using primer sets (Table S2) that amplify all three *JAZ10* transcripts (top panel), *JAZ10.3* only (second panel), or *JAZ10.4* only (third panel). PCR reactions contained a forward primer that hybridizes specifically to the *35S-JAZ10G* transgene, and were limited to 22 cycles. The experiment was repeated with RNA isolated from two independent sets of *35S-JAZ10G-2* plants (P3 and P4). A primer set that amplifies an *actin* transcript (lower panel) was used as a control.

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Table 1

The Jas intron is highly conserved in *JAZ*-related genes from diverse plant species

Species	No. *JAZ* genes[1]	No. *JAZ* genes with the Jas intron	Arg_{20}-PTC[2]
Physcomitrella patens	9	7	3
Selaginella moellendorffii	9	9	7
Arabidopsis thaliana	12	9	4
Brachypodium distachyon	11	7	4
Oryza sativa	15	9	5
Populus trichocarpa	14	12	6
Vitis vinifera	9	7	5
Total	79	60	34

[1]Indicates the number of *JAZ* genes in Arabidopsis and rice (*O. sativa*) or, for all other species, the number of group II *TIFY* genes (which includes *JAZ* and *PPD* genes) identified in genome databases.

[2]Indicates the number of genes in which a premature stop codon (PTC) occurs immediately after the codon in which the Jas intron resides. This codon typically (but not invariably) codes for Arg (Table S1).