Running title: Mechanism of repression by a JAZ10 splice variant

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Negative Feedback Control of Jasmonate Signaling by an Alternative Splice Variant of JAZ10[1]

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One-sentence summary: A transcriptional regulator generated by alternative splicing uses a cryptic binding site to negatively regulate bHLH-type transcription factors that promote jasmonate responses.

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Abstract

The plant hormone jasmonate (JA) activates gene expression by promoting ubiquitin-dependent degradation of JAZ transcriptional repressor proteins. A key feature of all JAZ proteins is the highly conserved Jas motif, which mediates both JAZ degradation and JAZ binding to the transcription factor MYC2. Rapid expression of *JAZ* genes in response to JA is thought to attenuate JA responses, but little is known about the mechanisms by which newly synthesized JAZ proteins exert repression in the presence of the hormone. Here, we show that desensitization to JA is mediated by an alternative splice variant (JAZ10.4) of JAZ10 that lacks the Jas motif. Unbiased protein-protein interaction screens identified three related bHLH transcription factors (MYC2, MYC3, and MYC4) and the co-repressor NINJA as JAZ10.4-binding partners. We show that the N-terminal region of JAZ10.4 contains a cryptic MYC2-binding site that resembles the Jas motif, and that the ZIM motif of JAZ10.4 functions as a transferable repressor domain whose activity is associated with recruitment of NINJA. Functional studies showed that expression of JAZ10.4 from the native *JAZ10* promoter complemented the JA-hypersensitive phenotype of a *jaz10* mutant. Moreover, treatment of these complemented lines with JA resulted in rapid accumulation of JAZ10.4 protein. Our results provide an explanation for how the unique domain architecture of JAZ10.4 links transcription factors to a co-repressor complex, and suggest how JA-induced transcription and alternative splicing of *JAZ10* pre-mRNA creates a regulatory circuit to attenuate JA responses.

INTRODUCTION

The small-molecule hormone jasmonate (JA) mediates plant responses to various environmental stresses and developmental cues. JA has been extensively characterized for its role in controlling reproductive development (McConn and Browse, 1996; Li et al., 2004; Nagpal et al., 2005; Browse, 2009) and defense responses to herbivores and pathogens (Howe et al., 1996; McConn et al., 1997; Kessler et al., 2004; Glazebrook, 2005; Howe and Jander, 2008). Increasing evidence indicates that this lipid-derived hormone also controls vegetative growth and cell differentiation responses (Staswick et al., 1992; Yan et al., 2007; Zhang and Turner, 2008; Yoshida et al., 2009; Chen et al., 2011; Yang et al., 2012). JA regulates gene expression by controlling the abundance of JAZ (Jasmonate ZIM domain) transcriptional repressor proteins (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007; Chung et al., 2009; Pauwels and Goossens, 2011). JAZs belong to the family of TIFY proteins that is defined by a highly conserved TIFYXG motif embedded within the ZIM domain (Vandholme et al., 2007). Low intracellular concentrations of the receptor-active form of JA, jasmonoyl-L-isoleucine (JA-Ile), permit JAZ proteins to accumulate in the nucleus, where they bind transcription factors (TFs) to actively repress JA-response genes. The best characterized targets of JAZ repressors are the basic helix-loop-helix TF MYC2 (also known as JASMONATE INSENSITIVE1, or JIN1) and its closely related paralogs MYC3 and MYC4 (Lorenzo et al., 2004; Chini et al., 2007; Melotto et al., 2008; Cheng et al., 2011; Fernández-Calvo et al., 2011; Niu et al., 2011; Song et al., 2011; Kazan and Manners, 2013). In response to tissue damage or other conditions that elevate JA-Ile levels, JAZ proteins are recruited to the F-box protein COI1 (CORONATINE INSENSITIVE1), which determines the specificity of the E3 ubiquitin ligase SCF^{COI1} for JAZ substrates (Thines et al., 2007; Katsir et al., 2008; Melotto et al., 2008; Fonseca et al., 2009; Koo and Howe, 2009; Yan et al., 2009; Sheard et al., 2010). Ubiquitin-dependent degradation of JAZ proteins relieves repression of TFs, thereby allowing expression of JA-response genes (Chini et al., 2007; Thines et al., 2007).

Recent studies have provided insight into the mechanisms by which TF-bound JAZ proteins repress transcription. NINJA (Novel Interactor of JAZ) was identified as an adaptor protein that physically links the ZIM domain of JAZ to the corepressor TOPLESS (TPL) and TPL-related (TPR) proteins. As is the case for other targets of TPL, NINJA contains an EAR (ERF-associated amphiphilic repression) motif that mediates interaction with TPL

(Szemenyei et al., 2008; Pauwels et al., 2010). Interestingly, some JAZs contain an EAR motif and thus may interact directly with TPL (Kagale et al., 2010; Consortium, 2011; Causier et al., 2012). It was recently demonstrated that the repressive function of JAZ8 depends on its TPL-interacting EAR motif (Shyu et al., 2012). Thus, whereas some JAZs repress transcription through NINJA (Pauwels et al., 2010), other JAZs such as JAZ8 may repress gene expression by a NINJA-independent mechanism in which corepressors are recruited directly to a TF-bound JAZ (Shyu et al., 2012).

Alternative splicing provides a mechanism to increase the repressive activity and functional diversity of JAZ proteins. The best example of this form of post-transcriptional regulation is splice variants of JAZ10 that are truncated in the C-terminal Jas motif, which mediates interaction with COI1 and MYC2 (Yan et al., 2007; Chung and Howe, 2009; Chung et al., 2010). Alternative splicing of *JAZ10* pre-mRNA generates three protein variants that differentially interact with COI1. The full-length JAZ10.1 isoform binds strongly to COI1 in the presence of JA-Ile, whereas C-terminally truncated splice variants interact weakly (JAZ10.3) or not at all (JAZ10.4) with COI1. JAZ10.3 and JAZ10.4 are more stable than JAZ10.1 in JA-stimulated cells and, as a consequence, exert dominant repression in transgenic overexpression assays (Yan et al., 2007; Chung and Howe, 2009; Chung et al., 2010). A role for JAZ10 splice variants in attenuating JA responses is supported by the fact that *jaz10* loss-of-function mutants (e.g., *jaz10-1*) are hypersensitive to JA, and that *JAZ10* expression is rapidly induced by JA (Yan et al., 2007; Chung et al., 2008; Demianski et al., 2012). *JAZ10* may also play a role in attenuating JA-mediated defense processes under environmental conditions that prioritize plant growth over defense (Moreno et al., 2009; Cerrudo et al., 2012).

Initial insight into the role of JAZs as transcriptional repressors came from experiments showing that expression of truncated JAZ proteins (referred to as JAZΔJas) lacking the Jas motif results in reduced sensitivity to JA (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007). This observation is explained by the fact that the Jas motif harbors an instability element (i.e., degron) that interacts with COI1 in a hormone-dependent manner. Thus, removal of this element creates a stable repressor (Katsir et al., 2008; Melotto et al., 2008; Sheard et al., 2010). Because the Jas motif also mediates TF binding, however, the mechanism by which JAZ10.4 and other JAZΔJas proteins interact with TFs to repress JA responses has

remained unclear (Chini et al., 2009; Wager and Browse, 2012). The strong JA-insensitive phenotype of JAZ10.4-overexpressing plants, together with the ability of JAZ10.4 to interact with MYC2 in yeast, suggest that sequence determinants outside the Jas motif promote binding to MYC2 (Chung and Howe, 2009). There is also evidence that JAZ1 can interact with MYC2 through sequences outside the Jas motif (Withers et al., 2012). Specific sequence determinants that mediate TF binding to JAZ10.4 and other JAZΔJas proteins have not been identified. In the present study, we discovered a cryptic MYC2-binding site at the N-terminus of JAZ10.4, providing a mechanism by which JAZ10.4 represses JA responses. We also describe a transgenic complementation assay to test the proposed function of JAZ10.4 in dampening JA responses. We use this system to show that the native *JAZ10* promoter is sufficient to drive JAZ10.4 protein expression and complement *jaz10* phenotypes in response to JA treatment. Our findings demonstrate how JA-induced transcription and alternative splicing of *JAZ10* establishes a negative feedback loop to attenuate JA responses.

RESULTS

JAZ10.4 Interacts with bHLH Transcription Factors and NINJA

As an unbiased approach to identify binding partners of JAZ10.4, we performed a high-throughput yeast two-hybrid (Y2H) screen for Arabidopsis proteins that interact with JAZ10.4. Sequence information obtained from 295 positive clones (among 3.2 x10^7 clones screened) produced a list of putative interacting proteins. Based on calculated confidence scores (Formstecher et al., 2005), five proteins were categorized as high-confidence interactors: MYC2, MYC3, MYC4, NINJA, and GENERAL REGULATING FACTOR 6 (GRF6, also known as 14-3-3λ/GF14λ) (Table I). GRF6 was not identified as a JAZ10.4-interacting protein *in planta* (see below) and thus was not characterized further. Sequencing of positive clones provided information on the minimal interaction domain of each prey protein (Formstecher et al., 2005). The results suggested that JAZ10.4 interacts with the N-terminal region of MYC2, MYC3 and MYC4, and C-terminal region of NINJA (Table I). These findings are in agreement with previous studies showing that most full-length JAZs (including JAZ10.1) interact with the N-terminus of MYC TFs (Cheng et al., 2011; Fernández-Calvo et al., 2011; Niu et al., 2011), and that JAZ1 interacts with the C-terminal region of NINJA (Pauwels et al., 2010).

As an alternative approach to identify JAZ10.4 interacting partners, we used mass spectrometry (MS) to identify proteins that co-purify with JAZ10.4 expressed in cultured Arabidopsis T87 cells. The utility of this system for studying JAZ proteins was assessed by analysis of cell lines expressing YFP fusions of each JAZ10 splice variant. Confocal microscopy and western blot analysis showed that JAZ10.3-YFP and JAZ10.4-YFP accumulate to higher levels than JAZ10.1-YFP in both control and JA-treated T87 cells (**Supplemental Figure 1**). This finding is consistent with previous studies showing that JAZ10.3 and JAZ10.4 are more stable than JAZ10.1 in JA-treated seedlings (Chung and Howe, 2009). MS-based analysis of coimmunoprecipitated JAZ10.4-YFP complexes (four independent replicates) identified NINJA as a major copurifying protein (Table II). MYC2, MYC3, and MYC4 were also identified as JAZ10.4-interacting partners in at least two of the four replicate experiments. We also identified JAZ12 and PEAPOD2 (PPD2) as components of the JAZ10.4 complex, which is in agreement with studies showing that JAZs form heteromeric complexes with other ZIM domain-containing proteins (Chini et al., 2009; Chung and Howe, 2009; Pauwels et al., 2010). These collective findings show that JAZ10.4 interacts *in vivo* with NINJA and the bHLH TFs MYC2, MYC3 and MYC4.

JAZ10.4 Contains a Cryptic MYC2-Interacting Domain that is Required for Repression of JA Responses

The ability of JAZ10.4 to interact with MYC2 was unexpected because previous studies have shown that the Jas motif, which is not present in JAZ10.4, is necessary and sufficient for interaction with MYC2 (Katsir et al., 2008; Melotto et al., 2008; Chini et al., 2009). We used Y2H assays to map the MYC2-interacting domain of JAZ10.4. The strength of JAZ10.4 interaction with MYC2 in this assay was comparable to that of the Jas motif-containing full-length splice variant, JAZ10.1 (Figure 1A). Deletion of the ZIM domain abolished JAZ10.4 dimerization but did not affect JAZ10.4 interaction with MYC2, as previously reported (Chung and Howe, 2009). However, constructs lacking the N-terminal 47 amino acids (JAZ10.4$^{\Delta 1\text{-}47}$) or lacking only amino acid residues 16-47 (JAZ10.4$^{\Delta 16\text{-}47}$) failed to bind MYC2. Systematic deletion analysis identified a 43-amino-acid fragment (JAZ10.4$^{16\text{-}58}$) that is sufficient for MYC2 interaction (Figure 1B). Interestingly, this cryptic MYC2-interacting domain (CMID) contains a nine-amino-acid sequence (FQKFLDRRR) that strongly

resembles the core of the Jas motif of JAZ10 and other JAZ proteins, including a tribasic cluster of residues (Figure 1C). In the Jas motif of JAZ9, the central R residue within the tribasic cluster (Figure 1C) is essential for interaction with MYC2 (Withers et al., 2012).

To assess whether the tribasic cluster (RRR^{32-34}) in the JAZ10.4 CMID plays a role in MYC2 binding, we mutated each Arg residue, individually and in combination, to an Ala and tested the resulting constructs for interaction with MYC2. Single and double R→A substitutions had no effect on MYC2 binding in yeast. However, substitution of all three residues (RRR→AAA) abolished MYC2 binding, but did not affect JAZ10.4 dimerization (Figure 2A). We also expressed wild type and mutant ($JAZ10.4^{RRR\rightarrow AAA}$) versions of JAZ10.4 in *Escherichia coli* as maltose-binding protein (MBP)-JAZ-6xHis fusions, and tested these proteins in pull-down assays for their ability to bind a cMyc-tagged derivative of MYC2 expressed in Arabidopsis. The results verified that the RRR^{32-34} motif of JAZ10.4 is required for MYC2 binding (Figure 2B). To determine whether the CMID of JAZ10.4 is sufficient for MYC2 binding, we fused the N-terminal 78 amino acids of JAZ10.4 ($JAZ10^{1-78}$) to the C-terminus of a JAZ3 derivative (JAZ3ΔJas) that lacks the entire Jas motif and thus does not interact with MYC2 (Chini et al., 2009). Pull-down assays showed that fusion of $JAZ10^{1-78}$ to the C-terminus of JAZ3ΔJas partially restores interaction with MYC2 (Figure 2C). Introduction of the RRR^{32-34}→AAA mutation into the full-length JAZ10.1 isoform had no effect on MYC2 binding, presumably because $JAZ10.1^{RRR\rightarrow AAA}$ contains an intact Jas motif (Supplemental Figure 2).

To determine whether the CMID is required for JAZ10.4 function *in vivo*, we compared JA responses in transgenic lines (*35S:JAZ10.4-YFP* and *35S:JAZ10.4*$^{RRR\rightarrow AAA}$*-YFP*) that overexpress YFP fusions of JAZ10.4 and $JAZ10.4^{RRR\rightarrow AAA}$. In agreement with previous studies (Chung and Howe, 2009), 24% of all *35S:JAZ10.4-YFP* plants tested (n=73 independent T1 lines) exhibited hallmark characteristics of JA-associated male sterility, including short anther filaments and reduced anther dehiscence (Figure 3A). Root growth inhibition assays also showed that *35S:JAZ10.4-YFP* seedlings were strongly insensitive to JA (Figure 3B). All *35S:JAZ10.4*$^{RRR\rightarrow AAA}$*-YFP* lines (n=50 independent T1 lines) were fully fertile and showed no signs of JA-associated male sterility. Experiments performed with T3 seedlings that are homozygous for the transgene further showed that the sensitivity of *35S:JAZ10.4*$^{RRR\rightarrow AAA}$*-YFP* seedlings to JA is comparable to that of WT seedlings (Figure 3B

and 3C). Confocal microscopy analysis showed that both JAZ10.4-YFP and JAZ10.4$^{RRR\rightarrow AAA}$-YFP localize to the nucleus of trichome cells (Supplemental Figure 3). JAZ10.4$^{RRR\rightarrow AAA}$-YFP also showed nuclear localization in roots, although the signal was more diffuse (presumably cytosolic) than that of JAZ10.4-YFP. This suggests that the inability of JAZ10.4$^{RRR\rightarrow AAA}$-YFP to repress JA responses does not result from mislocalization of the JAZ fusion protein. Taken together, the results indicate that the N-terminal region of JAZ10.4 contains a cryptic MYC2-binding site that is required for JAZ10.4-mediated repression of JA responses.

The ZIM Domain of JAZ10.4 Interacts with NINJA to Repress JA Responses

Identification of NINJA as a JAZ10.4-interacting protein (Tables I and II) suggests that repression of JA responses by JAZ10.4 involves recruitment of the NINJA-TPL co-repressor complex to the ZIM domain of JAZ10.4 (Pauwels et al., 2010). To address this question, we tested whether the ZIM domain (residues 104-130) of JAZ10 is capable of conferring repressive activity on a JAZ that binds MYC2 but fails to associate with the NINJA-TPL co-repressor complex. We took advantage of the fact that repression of JA responses by JAZ8 does not depend on its ZIM domain, but rather relies on an EAR motif at the N-terminus of JAZ8 that directly binds TPL. A mutant (JAZ8ΔEAR) of JAZ8 lacking the EAR motif does not interact with TPL and is unable to repress JA responses (Figure 4A) (Shyu et al., 2012). A chimeric protein (JAZ8ΔEAR-ZIM10) in which the ZIM domain of JAZ8ΔEAR was replaced with the ZIM domain of JAZ10 exhibited strong interaction with NINJA in yeast (Figure 4A). In root growth assays, transgenic lines that express JAZ8ΔEAR-ZIM10 from the *35S* promoter exhibited a JA-insensitive phenotype identical to that of JAZ8-overexpressing lines (Figure 4B). Thus, the ZIM domain of JAZ10 restores repression activity to a JAZ variant (JAZ8ΔEAR) that binds MYC2 but is unable to recruit the co-repressor complex.

The highly conserved Ile residue (I107 in the TIFY motif) of the ZIM domain is required both for JAZ10.4 dimerization and repression of JA responses by JAZ10.4 (Chung and Howe, 2009). Given that NINJA represses JA responses through its interaction with the ZIM domain of JAZ (Pauwels et al., 2010), we tested whether I107 of JAZ10.4 may also be important for interaction with NINJA. Y2H assays showed that whereas substitution of I107 to Ala eliminates JAZ10.4 dimerization, this mutation reduced but did not abolish JAZ10.4

interaction with NINJA (Figure 5A). A T106A mutation had no effect on JAZ10.4 binding to either JAZ10.4 or NINJA, suggesting that the effect is specific for I107. *In vitro* pull-down assays confirmed that I107A eliminates JAZ10.4 dimerization and reduces the extent to which JAZ10.4 interacts with NINJA (Figure 5B and C). MS analysis of protein complexes immunopurified from T87 cells provided additional evidence that I107A disrupts the ability of NINJA to copurify with JAZ10.4 (Table II). Based on the number of assigned mass spectra corresponding to NINJA and JAZ10.4, we estimated that the amount of NINJA copurifying with JAZ10.4^{I107A} was less than 20% of that copurifying with WT JAZ10.4 (Table II). These findings show that I107 is not only critical for JAZ10.4 dimerization, but also suggest a key role for this residue in stabilizing the JAZ10.4–NINJA interaction.

JA-induced Expression of JAZ10.4 Attenuates JA Responses

To further test the hypothesis that JAZ10.4 participates in a negative feedback loop to restrain JA responses, we tested whether expression of this splice variant from the native *JAZ10* promoter is sufficient to attenuate JA responses. For these experiments, we used a 2.0 kb *JAZ10* promoter fragment that confers JA-inducible expression of a *GUS* reporter gene (Sehr et al., 2010). A hemagglutinin (HA) epitope-tag was added to the N-terminus of JAZ10.4 to facilitate protein detection in plant tissues. The resulting *pJAZ10:HA-JAZ10.4* transgene was introduced into the *jaz10-1* mutant that is hypersensitive to JA-induced root growth inhibition (Demianski et al., 2012). Of eight lines that were confirmed to contain a single segregating copy of the transgene, all were significantly less sensitive to exogenous MeJA (20 μM) than *jaz10-1* seedlings. Further analysis of five representative *pJAZ10:HA-JAZ10.4* lines showed that all but one line (4.1) were also less sensitive than *jaz10-1* to 10 μM MeJA (Figure 6A). On medium containing 20 μM MeJA, root growth of some lines (e.g., 14.4) was significantly less sensitive than that of WT seedlings (Figure 6A). This finding suggests that differences in the expression level of JAZ10.4 between transgenic lines may modulate sensitivity to JA.

We used immunoblot analysis to test whether the level of JA-induced accumulation of JAZ10.4 in specific transgenic lines correlates with observed differences in root growth sensitivity to JA. Lines that exhibited either WT-like (line 4.1) or reduced (line 14.4) sensitivity to the hormone were grown in liquid MS medium and treated with 50 μM MeJA. At various times after treatment, seedlings were harvested for western blot analysis with an

anti-HA antibody (Figure 6B). We detected accumulation of JAZ10.4 protein in both lines within 1 hr of treatment. In the case of line 4.1, JAZ10.4 protein levels steadily increased during the 8 h time course. In the moderately JA-insensitive 14.4 line, however, JA treatment resulted in much stronger accumulation of JAZ10.4, with protein levels peaking at 2 h and then declining slowly at later time points. These results show that JAZ10.4, when expressed from the native *JAZ10* promoter, rapidly accumulates in response to JA treatment, and that the strength of JAZ10.4 expression inversely correlates with the level of sensitivity to JA.

DISCUSSION

Domain Architecture of JAZ10.4 Repressor

In our initial studies of *JAZ10* alternative splicing, the strong repressive activity of JAZ10.4 was enigmatic because this isoform lacks the MYC2-interacting Jas motif (Chung and Howe, 2009). In the present study, we show that repression by JAZ10.4 depends on a cryptic MYC2-interacting domain (CMID) located near the N-terminus of JAZ10.4. The ability of JAZ10.4 to interact with MYC3 and MYC4 in yeast and *in planta* indicates that this JAZ splice variant likely targets the core MYC2/3/4 triad of TFs that control primary JA responses in leaves and roots (Cheng et al., 2011; Fernández-Calvo et al., 2011; Niu et al., 2011). The minimal CMID defined by deletion analysis contains a sequence (FQKFLDRRR) that has striking similarity to the central region of the conserved Jas motif (Figure 1C). We show that a tribasic amino acid cluster (RRR) within this sequence is required both for MYC2 binding and dominant repressor function *in planta*. The RRR motif may therefore be functionally equivalent to the highly conserved KRK sequence of the Jas motif, which was recently shown to be critical for binding of JAZ9 to MYC2 and nuclear localization of JAZ9 (Withers et al., 2012). This conclusion is supported by the finding that the CMID restores, at least in part, the ability of JAZ3ΔJas to interact with MYC2. The apparent increase in partitioning of JAZ10.4$^{RRR\rightarrow AAA}$-YFP to the cytosol is consistent with the recent finding that MYC2 interaction correlates with the efficiency of nuclear localization of JAZ1 and JAZ9 (Withers et al., 2012). Our results indicate, however, that accumulation of JAZ10.4$^{RRR\rightarrow AAA}$-YFP in the nucleus is not strictly dependent on MYC2 interaction with the CMID. The fact that MYC2 interacts with the RRR-mutated form of JAZ10.1 (JAZ10.1$^{RRR\rightarrow AAA}$) suggests that two MYC binding sites (Jas and CMID) on JAZ10.1 may act redundantly in recruitment of TFs. It is also

possible that these sites engage multiple TFs simultaneously to modulate the repressive activity of JAZ10.1 and JAZ10.3, which contain full-length and truncated Jas motifs, respectively. It is noteworthy that the CMID encompasses a sequence region (referred to as domain 1) that is weakly conserved between various members of the Arabidopsis JAZ family (Thines et al., 2007). Inspection of the domain 1 sequence indicates that the Jas motif-like sequence within the CMID is not well conserved in other Arabidopsis JAZ proteins. The presence of JAZ10 orthologs in other plant species, however, suggests that production of JA-resistant truncated JAZs via alternative splicing may be a general mechanism to dampen JA responses.

Transcriptional repression by JAZ proteins is thought to involve recruitment of TPL and TPR co-repressors to JAZ-TF complexes bound to promoter regions of JA-response genes (Pauwels and Goossens, 2011). Genetic and biochemical evidence supports the existence of two mechanisms to explain how JAZs are linked to TPL/TPRs. One mechanism invokes interaction of the ZIM domain with NINJA, which contains an EAR motif that interacts with TPL/TRRs (Pauwels et al., 2010). Alternatively, and as exemplified by JAZ8, a subset of JAZs contain an EAR motif that allows direct binding to TPL independently of the ZIM domain and NINJA (Shyu et al., 2012). A third group of JAZ proteins (JAZ5 and JAZ6) have the potential to interact with co-repressors via both NINJA-dependent and -independent mechanisms, but this remains to be confirmed (Kagale et al., 2010; Pauwels and Goossens, 2011). Our results indicate that JAZ10.4 represses JA responses through the former NINJA/ZIM-dependent pathway. In support of this conclusion, mutation of I107 within the conserved TIFY motif abolished the ability of ectopically expressed JAZ10.4 to confer JA insensitivity (Chung and Howe, 2009; this study). Various protein-protein interaction assays, including MS analysis of JAZ10.4 protein complexes purified from Arabidopsis cells, showed that I107A strongly diminishes the ability of JAZ10.4 to interact with NINJA. An essential role for the ZIM domain in JAZ10.4 repression is also supported by domain swap experiments showing that the ZIM domain of JAZ10 confers repressive activity on a modified JAZ (JAZ8ΔEAR) that binds MYC2 but fails to interact with NINJA. Overexpression of JAZ8ΔEAR-ZIM10 and JAZ8 has similar quantitative effects on JA-mediated root growth inhibition, suggesting that our engineered NINJA-dependent JAZ8 repressor is functionally equivalent to the wild-type JAZ8 repressor (Figure 4). In contrast to JAZ10.4, however,

overexpression of JAZ8ΔEAR-ZIM10 or JAZ8 does not result in male sterility. This finding suggests that JAZ10.4 may be more stable than JAZ8 or, alternatively, that JAZ10.4 and JAZ8 target different sets of TFs in the respective overexpressing lines. These findings highlight the modular nature of JAZ functional domains and provide proof-of-concept for the idea of using synthetic JAZ repressors to control specific JA responses.

An important feature of the ZIM domain is its ability to mediate JAZ interaction with both NINJA and other TIFY proteins (Vanholme et al., 2007; Chini et al., 2009; Chung and Howe, 2009). Using cell extracts from Arabidopsis T87 cells, we found that JAZ10.4 co-purifies not only with MYC TFs and NINJA, but also with JAZ12 and PPD2. This finding is in agreement with tandem affinity purification (TAP) tagging screens that identified NINJA, MYC TFs, and various TIFY proteins as components of JAZ-containing multiprotein complexes (Pauwels et al., 2010; Fernández-Calvo et al., 2011). Our *in vivo* and *in vitro* studies of the JAZ10.4^{I107A} mutant further showed that the TIFY motif is required for JAZ10.4 interaction with both NINJA and other JAZ proteins (Chung and Howe, 2009). Although the MS data indicates that JAZ10.4^{I107A} retains the ability to interact with MYC TFs, we cannot exclude the possibility that this mutation alters the stability of JAZ10.4-TF complexes as a consequence of changes in the overall structure of JAZ10.4. Nevertheless, our findings suggest that JAZ-JAZ and JAZ-NINJA interactions involve common sequence determinants, and raise the question of how JAZ homo- and heteromeric interactions affect JAZ repressor activity (Howe, 2010). JAZ8 is unique in this context because, despite the presence of a conserved TIFY motif, it interacts very weakly with NINJA but yet retains the ability to heterodimerize with other JAZs (Pauwels et al., 2010; Shyu et al., 2012; this study). The differential interaction of JAZ8 and JAZ10 with NINJA indicates that unidentified sequence determinants within the ZIM domain play an important role in JAZ-NINJA coupling.

Negative Feedback Regulation by JAZ10.4

In comparison to most other JAZ proteins in Arabidopsis, a distinguishing feature of JAZ10.4 is its enhanced stability in cells containing high levels of JA. The increased stability of JAZ10.4 is a direct consequence of an alternative splicing event that results in loss of the COI1-interacting degron and, as a consequence, resistance to JA-induced degradation (Chung and Howe, 2009). In contrast to labile JAZs, the strength of repression

by JAZ10.4 is expected to increase as the expression of *JAZ10* increases. Consistent with this idea, overexpression of either the *JAZ10.4* cDNA or a *JAZ10* genomic clone from the constitutive CaMV 35S promoter results in strong JA-insensitive root phenotypes (Chung and Howe, 2009; Chung et al., 2010). Given that *JAZ10* expression is tightly controlled by the JA pathway (Yan et al., 2007; Chung et al., 2008; Fernández-Calvo et al., 2011; Demianski et al., 2012), an unresolved question has been whether JAZ10.4 accumulates in JA-stimulated wild-type plants and, if so, whether its induced expression is sufficient to attenuate JA responses. Here, we demonstrate that expression of JAZ10.4 from the native *JAZ10* promoter does in fact complement the JA-hypersensitive phenotype of *jaz10-1* seedlings. We also show that the reduced sensitivity of *pJAZ10:HA-JAZ10.4* lines to exogenous JA correlates with the level of JAZ10.4 protein in JA-stimulated seedlings. These collective findings strongly support a role for JAZ10.4 as an endogenous negative regulator of JA signaling.

Because the expression of *JAZ10.4* in *pJAZ10:HA-JAZ10.4* lines does not depend on alternative splicing of *JAZ10* pre-mRNA, the level of JAZ10.4 in these lines may be greater than that produced in wild-type plants. We attempted to account for this possibility by characterizing multiple independent transgenic lines that exhibit a range of sensitivity to JA. When grown on relatively low levels of exogenous JA (10 μM), all lines tested were less sensitive to JA than the JA-hypersensitive *jaz10-1* mutant, and for the most part resembled wild-type seedlings. At higher concentrations of JA, some lines were significantly less sensitive to JA than the wild type. Based on the level of JAZ10.4 accumulation in representative low expressing (i.e., 4.1) and high expressing (e.g., 14.4) lines, we attribute variation in hormone sensitivity to differences in the strength of JAZ10.4 expression. Regardless of this variation, the fact that all *pJAZ10:HA-JAZ10.4* lines were less sensitive to JA than *jaz10-1* seedlings suggests that the hypersensitive phenotype of *jaz10-1* results in part from loss of production of JAZ10.4.

Our results support a model of negative feedback inhibition by JAZ10.4. At low JA levels, one or more JAZ repressors interact with MYC TFs to inhibit the expression of *JAZ10* and shut off production of JAZ10 splice variants. This is supported by the fact that *JAZ10* expression is nearly abolished in a *myc2/3/4* triple mutant (Fernández-Calvo et al., 2011), and the existence of putative MYC2/3/4-binding G-box elements in the promoter of

JAZ10 (Chini et al., 2007). Additionally, cycloheximide induction experiments showed that *JAZ10* is a primary rather than secondary JA-response gene (Chung et al., 2008). In response to environmental or developmental cues that result in increased JA-Ile levels, we propose that JAZ repressors bound to the promoter of *JAZ10* are degraded, thus allowing transcriptional activation of *JAZ10* by MYC2/3/4. The identity of specific JAZ proteins that silence the expression of *JAZ10* and other primary response genes under low JA conditions remains to be determined. Following JA-induced transcription of *JAZ10*, *JAZ10* pre-mRNA is subject to alternative splicing events that give rise to JAZ10.1, JAZ10.3 and JAZ10.4. The relative abundance of each JAZ10 isoform will depend not only on the efficiency of the respective alternative splicing events but also the differential stability of the proteins in JA-stimulated cells (Chung and Howe, 2009). Indeed, we found that JAZ10.4 rapidly accumulates in response to JA treatment and that accumulation of the protein correlates with reduced sensitivity of seedlings to JA. Based on the ability of JAZ10.4 to repress JA responses and to bind MYC2/3/4, JAZ10.4 likely restrains the expression of genes controlled by these transcription factors. Additional experiments are needed to test this hypothesis. Given that JA-induced expression of *JAZ10* is dependent on MYC2/3/4 (Fernández-Calvo et al., 2011), it is possible that JAZ10.4 negatively regulates its own production by binding to MYC2/3/4 and inhibiting *JAZ10* transcription. This function of JAZ10 in feedback control of JA signaling supports the emerging view that alternative splicing plays a fundamental role in plant adaptation to environmental stress (Reddy, 2007; Barbazuk et al., 2008).

It is well established that plants, like animals, have evolved various mechanisms to desensitize cells to the presence of a hormone, thereby restraining the duration and amplitude of hormone-induced responses. Among the proposed mechanisms involved in attenuation of JA responses are JA-induced removal of JA-Ile (Kitaoka et al., 2011; Koo et al., 2011; Heitz et al., 2012; Koo and Howe, 2012; Woldemariam et al., 2012), JA-induced expression of MYC2-related TFs that negatively regulate JA responses (Nakata et al., 2013), and, as described here, JA-induced synthesis of stable JAZ repressors (Chung and Howe, 2009; Chung et al., 2010; Shyu et al., 2012). Negative regulatory feedback by JAZ10.4 and other stable JAZs may provide stability to the JA network by limiting the range over which the concentrations of signaling components, including MYC-related TFs, JAZs, and JA-Ile,

fluctuate during the JA response. These JAZs may be important for curtailing JA-related defense responses that are energetically demanding or toxic to the cell, or for maintaining appropriate growth rates in fluctuating environmental conditions (Moreno et al., 2009; Cerrudo et al., 2012; Yang et al., 2012). The ability of JAZ10 to physically interact with Arabidopsis DELLA proteins (Yang et al., 2012), which mediate growth repression, raises the interesting possibility that stable JAZ10 splice variants may act synergistically to promote growth in the presence of high JA levels. Tissue- or cell-specific accumulation of JA-resistant JAZs may provide a mechanism to generate spatial heterogeneity in growth and defense responses (Kessler and Baldwin, 2002; Melotto et al., 2006). A better understanding of JAZ protein expression patterns promises to provide insight into how these key regulators enhance plant fitness in challenging and continuously changing environments.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana plants were grown at 21°C under long-day conditions as described by Chung and Howe (2009). Columbia (Col-0) ecotype was used as the wild-type genetic background for all experiments. The *jaz10-1* mutant (SAIL_92_D08) was obtained from the Arabidopsis Biological Resource Center (ABRC). The *35S:JAZ8 ΔEAR* line was previously described by Shyu et al. (2012). JA-mediated root growth inhibition assays were performed as described previously (Chung and Howe, 2009; Shyu et al., 2012).

Yeast Two-Hybrid Analysis

JAZ10.4 was used as the bait in a yeast two-hybrid (Y2H) screen performed by Hybrigenics (Paris, France). A cDNA encoding JAZ10.4 was cloned into pB27 as a C-terminal fusion to LexA (N-LexA-JAZ10.4-C) and subsequently used to screen a random-primed cDNA library prepared from one-week-old Arabidopsis seedlings. Thirty two million clones (3.2-fold coverage of the library) were screened using a mating approach with yeast strains HGX13 and L40ΔGal4 as previously described (Fromont-Racine et al., 1997). His+ colonies (295 total) were selected on a medium lacking Trp, Leu, and His, and supplemented with 0.5 mM 3-aminotriazole to reduce autoactivation by the "bait" protein. cDNA fragments

corresponding to positive "prey" clones were amplified by PCR and sequenced at their 5' and 3' junctions. The resulting sequences were searched against GenBank and assigned a quality score indicative of the confidence of interaction (Formstecher et al., 2005). Only those interactions with the highest confidence values are reported in this paper.

All other Y2H assays were performed with the Matchmaker LexA system (Clontech) as previously described (Melotto et al., 2008; Chung and Howe, 2009). JAZ10.4 constructs were subcloned into the pGILDA vector to generate translational fusions with the LexA DNA-binding domain (BD). Full-length cDNAs encoding MYC2 and NINJA were subcloned into the pB42AD vector to generate fusions with B42 activation domain (AD). Various bait-prey pairs were co-transformed into yeast (*Saccharomyces cerevisae*) strain EGY48 using the frozen-EZ yeast transformation II kit (Zymo Research). Photographic images of Y2H plates were taken after one or two day of incubation at 30°C (Chung and Howe, 2009). Y2H assays were replicated at least three times. A complete list of primers and constructs used for cloning into pGILDA and pB42AD are described in Supplemental Table I.

Transgene Constructs

All PCR reactions were performed with KAPA HIFI Polymerase (Kapa Biosystems) following manufacturer instructions. All primer sets used for PCR are listed in Supplemental Table I. For cloning with Gateway vectors (Invitrogen), PCR amplicons were purified and cloned into pEntr-D-Topo using pENTR™ Directional TOPO® Cloning Kit (Invitrogen). Sequence-verified clones were transferred to the corresponding destination vector (Supplementary Table I) using the Gateway LR Clonase II Enzyme Mix (Invitrogen). JAZ10.4 and JAZ10.4$^{RRR\rightarrow AAA}$ constructs were cloned by Gateway reaction into the pEarley101 destination vector to generate a C-terminal fusion protein with YFP (Earley et al., 2006). We followed a two-step selection process to obtain lines that express these fusion proteins at relatively high levels. First, transgenic seedlings were screened on MS agar plates for resistance to kanamycin (50 μg/mL). Second, a Leica M165FC Fluorescence Stereomicroscope was used to screen the resulting kan-resistant lines for seedlings that accumulate the JAZ-YFP fusion protein in the nucleus.

Expression of the N-terminal hemagglutinin (HA)-tagged JAZ10.4 protein from the native *JAZ10* promoter was achieved as follows. A 2.0 kb *JAZ10* promoter sequence, similar to that described in Sehr et al (2010), was amplified by PCR using *Xho*I pre-digested T31B5

bacterial artificial chromosome DNA as a template. The *JAZ10* promoter was cloned directionally into pEntr-D-Topo (to generate pEntr-JAZ10promoter) using forward and reverse primers carrying *Not*I restriction sites (Supplemental Table I). *JAZ10.4* cDNA was amplified with a primer set designed to add an HA-epitope tag to the N-terminus of the protein and the resulting amplicon was cloned into pEntr-D-Topo (to give pEntr-HA-JAZ10.4). The *JAZ10* promoter was released from pEntr-JAZ10promoter using *Not*I restriction enzyme and ligated to the *Not*I-linearized pEntr-HA-JAZ10.4 using Rapid Ligation Kit (Roche). An LR Clonase (Invitrogen) reaction was used to transfer this construct into the final destination vector pGWB401 (Nakagawa et al., 2007). The final construct was confirmed by sequencing and transformed into *Agrobacterium tumefaciens* strain C58C1 for generation of transgenic Arabidopsis plants (Chung and Howe, 2009). Kanamycin-resistant T1 seedlings were selected in agar plates containing 50 μg/mL of the antibiotic. T2 seedlings were used to select lines containing a single T-DNA insertion based on a 3:1 segregation ratio of the antibiotic resistance marker. T3 seedlings were used to screen for lines that were homozygous for the transgene.

The *MYC2* cDNA was PCR-amplified using cDNA synthesized from total RNA isolated from rosette leaves of five-week-old Arabidopsis Col-0 plants. Oligonucleotide primer sequences are described in Supplemental Table I. The resulting *MYC2* amplicon was cloned into pENTR/D-TOPO (Invitrogen) and transferred into the pJYP006 binary vector to create a vector (*35S:Myc-MYC2*) in which the N-terminus of MYC2 is fused to nine repeats of the c-Myc epitope. This vector was introduced into *Agrobacterium tumefaciens* strain GV3100 for transformation of the *jin1-9* mutant. T1 plants were grown on soil for 7 d and sprayed with a solution containing 0.3 μM glufosinate (Finale, AgrEvo Environmental Health, NJ, USA). T2 plants containing a single T-DNA insertion (as determined by 3:1 segregation of glufosinate resistance) were used for Western blot analysis to confirm the expression of Myc-MYC2. A homozygous T3 line was used as a source of leaf extracts for *in vitro* pull-down assays.

We used four PCR reactions to generate the JAZ8ΔEAR-ZIM10 construct. In one reaction, *JAZ8ΔEAR* cDNA was used as a template with primer set JAZ8-pENTR-FP and JAZ8-ZIM10-N-RP to amplify cDNA encoding the first 44 amino acids of JAZ8ΔEAR. Another reaction also used *JAZ8ΔEAR* as a template with primer set JAZ8-ZIM10-C-FP and

JAZ8-pENTR-RP to amplify cDNA encoding the last 59 amino acids of JAZ8ΔEAR (amino acids 73-131). In the third PCR reaction, the amplicon from the first PCR reaction (JAZ8ΔEAR N-terminal 44 amino acids) and *JAZ10.1* cDNA were both used as templates with primer set JAZ8-pENTR-FP and JAZ10-KpnI-RP to generate a JAZ8ΔEAR-JAZ10 chimera. Finally, the *JAZ8ΔEAR-JAZ10* chimera and cDNA encoding the last 59 amino acids of JAZ8ΔEAR (amplicon from the second PCR reaction) were used as templates with primer set JAZ8-pENTR-FP and JAZ8-pENTR-RP to generate the final *JAZ8ΔEAR-ZIM10* construct. *JAZ8ΔEAR-ZIM10* was cloned into vector pENTR-TOPO for sequencing and subcloning. Following Agrobacterium-mediated transformation, 32 independent T1 plants were transferred to soil and RNA was extracted from leaf tissue. *JAZ8ΔEAR-ZIM10* gene-specific primers (JAZ8-pENTR-FP and JAZ8-pENTR-RP) were used in RT-PCR to confirm expression of the transgene. Ten lines were selected and further propagated for identification of homozygous T3 lines.

Site-Directed Mutagenesis

Single and multiple amino acid residues within JAZ proteins were substituted to Ala with the Quick-Change II site-directed mutagenesis kit (Stratagene), as previously described (Chung and Howe, 2009; Shyu et al., 2012). PCR reactions were performed with Pfu Turbo DNA Poylmerase to generate constructs listed in Supplemental Table I. pGEM-T vector harboring the wild-type *JAZ* cDNA was used as template for mutagenesis. All constructs were sequenced to confirm the corresponding mutation.

***In vitro* Pull-Down Assays**

Cloning, expression and purification of recombinant JAZ proteins as maltose-binding protein (MBP)- and hexa-histidine (His)-tagged fusions (referred as JAZ-His) was done as previously described (Thines et al., 2007; Chung et al., 2010). Primers used for cloning are described in Supplemental Table I. JAZ-MYC2 interactions were analyzed in pull-down assays employing purified JAZ-His proteins and an epitope-tagged (9X c-Myc) derivative of MYC2, which was expressed under the control of CaMV 35S promoter in the *jin1-9* mutant background of Arabidopsis as described above. Leaf tissue from this transgenic line (*35S:Myc-MYC2*) was ground to a fine powder in liquid nitrogen and extracted in 2 mL of homogenization buffer (Chung et al., 2010) per g ground tissue with the addition of Complete Mini protease inhibitor

tablet-EDTA free (Roche) and 50μM MG132 (Sigma-Aldrich). The extract was centrifuged at 20,200 x *g* for 15 min at 4°C, and the resulting supernatant was subjected to a second centrifugation round under same conditions. *In vitro* pull-down assays were done as previously described (Chung et al., 2010), using 25 μg recombinant JAZ-His and 0.5 mg protein of *35S:Myc-MYC2* leaf extract. MYC2 binding was assessed by western blot analysis using an anti-cMyc antibody (Covance). All *in vitro* pull-down assays were repeated at least three times.

Analysis of JAZ10 Protein Complexes Isolated from Arabidopsis T87 Cells

Arabidopsis T87 cells (Axelos et al., 1992) were obtained from RIKEN BioResource Center. Cells were transformed with *Agrobacterium tumefaciens* strain EHA105 as described by Held et al. (2012). All transgenic Arabidopsis cell lines were generated by transformation with vector pVKgw-N-YFP, which fuses YFP to the N-terminus of JAZ10 isoforms (Held et al., 2012). Expression of YFP-JAZ10 proteins in T87 cells was assessed by western blot analysis using an anti-GFP antibody (Molecular Probes). Selected T87 cell lines were inoculated into 50 mL of fresh medium and grown for 4 days to exponential phase (Held et al., 2012). For experiments involving JA treatment, a solution of MeJA (Sigma-Aldrich) was added to a final concentration of 50 μM to exponentially growing cells. Cells were harvested 2 hr later on Whatman filter paper using a vacuum trap connected to a ceramic funnel. The fresh cell mass was immediately estimated and frozen in liquid nitrogen. A small aliquot of cells in the original flask was set aside for imaging by confocal microscopy (Olympus FluoView 1000 Laser Scanning Confocal Microscope). Frozen cells were ground with a mortar and pestle to a fine powder in liquid nitrogen. Protein was extracted by addition of 2 mL lysis buffer (25 mM Tris-HCl, pH 7.5, 15 mM MgCl, 150 mM NaCl, 0.01% Tween 20, 50 μM MG132, 1 mM PMSF, 14.6 μM β-Mercaptoethanol, 1 tablet of MiniProtean cocktail (Roche) per 10 mL lysis buffer) per g liquid N_2-ground cell powder. The resulting mixture was thawed with gentle rocking at 4°C for 30 min. and then centrifuged at 26,890 x *g* at 4°C for 30 min. The supernatant was decanted to a pre-chilled tube and GFP-Trap_A resin (ChromoTek GmbH, Martinsried, Germany) was added (10 μL pre-washed resin per 10 g of cell powder). Prior to this step, GFP-Trap resin was washed three times with 500 μL dilution buffer (identical to lysis buffer but lacking Tween 20). The crude cell extract/GFP-Trap resin mixture was

incubated for 2 hr at 4°C with gentle rocking and then centrifuged at 900 x *g* for 5 min in a swinging bucket centrifuge (GS-6R, Beckman) at 4°C. The resulting supernatant was transferred to a pre-chilled tube for a second round of affinity purification using the conditions described above. Protein-bound resin from both rounds of purification was pooled and washed three times with 500 µL ice-cold dilution buffer.

Antibody-bound proteins were digested on-bead by incubation for 6 hr at 37°C in 10 µL of a solution containing trypsin (5 ng/ µL) and 50 mM ammonium biocarbonate. The solution was acidified by the addition of 5% formic acid and then centrifuged at 14,000 x *g*. Peptide-containing supernatant was removed and concentrated by solid-phase extraction with OMIX tips (www.varian.com). Purified peptides were then re-suspended in 20 µL of a solution containing 2% acetonitrile and 0.1% trifluoroacetic acid. An aliquot (10 µL) of this fraction was injected by a Waters nanoAcquity Sample Manager (www.waters.com) and loaded for 5 min onto a Waters Symmetry C18 peptide trap (5 µm, 180 µm x 20 mm) at 4 µL/ min in 5% acetonitrile/ 0.1% formic acid. The bound peptides were eluted onto a Waters BEH C18 nanoAcquity column (1.7 µm, 100 µm x 100 mm) over 35 min with a gradient of 5% B to 30% B in 21 min, ramped up to 90% B at 23 min and held for 1 min, then dropped back to 5% B at 24.1 min using a Waters nanoAcquity UPLC (Buffer A = 99.9% water/ 0.1% formic acid, Buffer B = 99.9% acetonitrile/ 0.1% formic acid) with an initial flow rate of 0.8 µL/ min. Eluted peptides were sprayed into a ThermoFisher LTQ Linear Ion trap mass spectrometer outfitted with a MICHROM Bioresources ADVANCE nano-spray source. The top five ions in each survey scan were then subjected to data-dependant zoom scans followed by low energy collision induced dissociation (CID) and the resulting MS/MS spectra were converted to peptide lists with BioWorks Browser v 3.3.1 (ThermoFisher) using the default LTQ instrument parameters to filter out non-peptide signals recorded during data acquisition. Peptide lists were searched against a custom protein sequence database consisting of the *Arabidopsis thaliana* (TAIR10, www.arabidopsis.org) combined with common laboratory contaminants (downloaded from www.ncbi.nlm.nih.gov) using the Mascot searching algorithm, v2.4 (www.matrixscience.com). The Mascot output was then analyzed with Scaffold, v3.6.0 (www.proteomesoftware.com) to probabilistically validate protein identifications using the ProteinProphet computer algorithm (Nesvizhskii et al., 2003). Only those peptides satisfying the Scaffold 95% confidence filter were reported.

Supplemental Material

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

Supplemental Figure 1. Expression of JAZ10 splice variants in Arabidopsis T87 cell cultures.

Supplemental Figure 2. The RRR motif is required for interaction of MYC2 with JAZ10.4 but not JAZ10.1.

Supplemental Figure 3. Subcellular localization of JAZ10.4 and JAZ10.4$^{RRR\rightarrow AAA}$-YFP.

Supplemental Table I. Description of oligonucleotide primers used in this study.

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FIGURE LEGENDS

Figure 1. The N-terminal region of JAZ10.4 has a cryptic MYC2-interacting domain. A, Y2H assays of JAZ10.4 deletion constructs (DNA binding-domain "bait" fusions) with MYC2 and JAZ10.4 (activation domain "prey" fusions). Yeast strains co-expressing the indicated bait and prey proteins were plated on media containing X-Gal. Blue-color formation in streaked yeast cells is indicative of protein-protein interaction. Photographic images were taken after 30 h of incubation at 30°C. JAZ10.1 is included as a positive control. The ZIM (Z) and Jas domains are indicated. The solid black line denotes the C-terminal region that is specific for the JAZ10.4 isoform. B, C-terminal deletion constructs of JAZ10.4 were tested for interaction with MYC2 as described in A. C, Sequence similarity between the conserved Jas motif of JAZ10 and JAZ9 and the cryptic MYC2-interacting domain (CMID) near the N-terminus of JAZ10. The underlined sequence shows the region of conservation, which includes a tribasic motif (bolded).

Figure 2. A RRR motif in the N-terminal region of JAZ10.4 is required for interaction with MYC2. A, Site-directed mutagenesis of the RRR motif within the cryptic MYC2-interacting domain (CMID) of JAZ10.4. The indicated R→A substitution mutants of JAZ10.4 were tested for interaction with MYC2 or JAZ10.4 as a positive control. Y2H assays were performed as described in Figure 1. B, *In vitro* pull-down assay of JAZ10.4-MYC2 interaction. Assays were performed using the indicated JAZ10.4-His recombinant proteins and crude extracts from leaves of wild-type (WT) or *35S:cMyc-MYC2* transgenic (T) plants. Protein bound to JAZ-His was separated by SDS-PAGE and analyzed by immunoblotting (anti-cMyc antibody) for the presence of cMyc-MYC2. The Coomassie-blue stained gel shows total input protein as a loading control. C, The cryptic MYC2-interacting domain (CMID) of JAZ10.4 is sufficient for MYC2 binding. *In vitro* pull-down assays were performed as described in B using JAZ3, JAZ3ΔJas, or a chimeric JAZ (JAZ3ΔJas-J10$^{1-78}$) in which the CMID of JAZ10.4 was fused to the C-terminus of JAZ3ΔJas.

Figure 3. The RRR motif is required for JAZ10.4-mediated repression of JA responses. A, The photograph shows silique development in wild-type (WT) and transgenic lines that overexpress JAZ10.4 or JAZ10.4$^{RRR\rightarrow AAA}$. B, The photograph shows seedlings of the indicated genotype grown for 10 d on MS medium containing 20 μM MeJA. C, Quantification of JA-induced root growth inhibition in WT, *35S:JAZ10.4* (*10.4*), and eight independent *JAZ10.4*$^{RRR\rightarrow AAA}$ lines. Seedlings were grown for 10 d on MS agar plates containing or not containing 20 μM MeJA. The root length ratio was calculated by dividing the average root length of MeJA-treated seedlings by the average root length of seedlings of the same genotype grown in the absence of MeJA. Data show the mean ± SE (n=12 seedlings/genotype). The asterisk denotes a significant difference ($P < 0.05$, Student's test) in comparison to WT.

Figure 4. The ZIM domain of JAZ10 interacts with NINJA and is required for repression of JA responses. A, Y2H assays depicting interaction of JAZ10-JAZ8 chimeric proteins (DNA binding-domain fusions, DB) with MYC2, JAZ1, NINJA (NJA), and TPL (activation domain fusions, AD). Empty vectors were included as negative controls. Sequence regions derived from JAZ10 and JAZ8 are shown in white and gray, respectively, together with various protein domains. E, EAR motif. Y2H assays were performed as described in Figure 1, except that the photographic image was taken after 48 h of incubation of yeast cells at 30°C. B, Overexpression of a JAZ8ΔEAR-ZIM10 chimeric protein confers insensitivity to JA in root growth assays. Seedlings were grown for 8 d on MS agar plates containing or not containing 20 μM MeJA, and root length ratios were calculated as described in Figure 3C. Data show the mean ± SE of at least 11 seedlings per genotype. The asterisk denotes a significant difference ($P < 0.05$, Student's test) in comparison to WT.

Figure 5. Isoleucine^{107} in the TIFY motif is involved in JAZ10.4 interaction with both JAZ10 and NINJA. A, Y2H assay depicting the role of Thr106 and Ile107 (within the TIFY motif) in JAZ10.4 dimerization and JAZ10.4 interaction with NINJA. Y2H assays were performed as described in Figure 1, except that the photographic image was taken after 24 h of incubation of yeast cells at 30°C. B, *In vitro* pull-down assay of JAZ10.4-JAZ10.4 interaction. Assays were performed using the indicated wild-type (JAZ10.4) and mutant (JAZ10.4^{I107A}) recombinant proteins and crude extract from a yeast strain expressing a JAZ10.4-HA fusion

protein. Protein bound to JAZ-His was separated by SDS-PAGE and analyzed by immunoblotting with an anti-HA antibody. A Coomassie-blue stained gel is shown as a loading control. C, *In vitro* pull-down assay of JAZ10.4-NINJA interaction. Assays were performed as described in panel B except for the use of a crude extract from a yeast strain expressing a NINJA-HA fusion protein.

Figure 6. JA-induced expression of JAZ10.4 complements the JA-hypersensitive phenotype of *jaz10-1*. A, Root growth inhibition assay of wild-type (WT), *jaz10-1* (*jaz10*), and five independent lines in which the *pJAZ10:HA-JAZ10.4* transgene was introduced into the *jaz10-1* mutant background. Root length was measured in 8-d-old seedlings grown on MS medium containing 0, 10, or 20 μM MeJA. The root length ratio was calculated by dividing the average root length of seedlings grown in the presence of either 10 μM (gray bar) or 20 μM (black bar) MeJA by the average root length of seedlings of the same genotype grown in the absence of MeJA. Data show the mean ± SE of 12-14 seedlings/genotype. Asterisks indicate significant differences in root length ($P<0.05$, Student's test) in comparisons between WT and *jaz10* seedlings grown at the same concentration of MeJA, or between WT and *pJAZ10:HA-JAZ10.4* seedlings grown at the same concentration of MeJA. B, Accumulation of HA-JAZ10.4 protein in response to JA treatment. Ten-d-old liquid-grown seedlings of the indicated *pJAZ10:HA-JAZ10.4* line were treated with 50 μM MeJA and harvested at various times (h) thereafter. As controls, seedlings were harvested immediately prior to treatment ("0") and 8 h after mock treatment ("C"). Total protein was subject to immunoblot analysis with an anti-HA antibody to detect HA-JAZ10.4. A Coomassie-blue stained gel of protein extracts was used as a loading control (CB).

Table I. List of high confidence JAZ10.4-interacting proteins identified in a Y2H screen.

[1]Number of positive clones for which sequence information was obtained.

[2]The Selected Interaction Domain (SID) defines the minimum interaction domain of the prey identified as the amino acid sequence shared by all prey fragments matching with the same reference protein.

Protein	**AGI code**	**Length**	**No. clones[1]**	**SID[2]**
MYC2	At1g32640	623	11	51-272
MYC3	At5g46760	592	34	40-279
MYC4	At4g17880	589	100	46-246
NINJA	At4g28910	425	22	229-425
GRF6	At5g10450	273	15	37-248

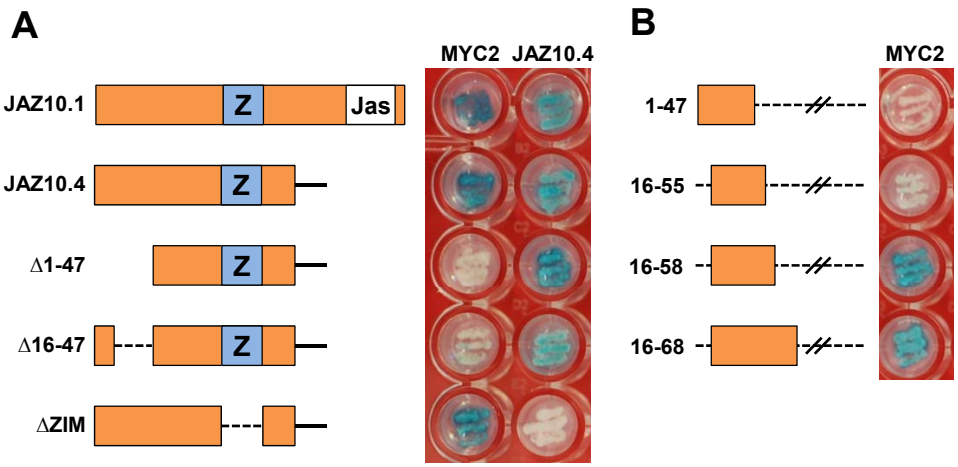
Table II. List of JAZ10.4-interacting proteins identified by immunoaffinity purification of JAZ10.4-YFP followed by mass spectrometry.

[1]JAZ10.4-containing complexes were purified from T87 Arabidopsis cell cultures expressing either JAZ10.4-YFP (JAZ10.4) or JAZ10.4^{I107A}-YFP (JAZ10.4 ^{I107A}) as "bait". Data are shown for four and two independent experiments (rep) with JAZ10.4 and JAZ10.4 ^{I107A}, respectively. Data entries denote the number of assigned spectra / number unique peptides for each of the listed copurifying proteins. Only peptides with ≥ 95% confidence are shown. None of the listed proteins were identified in control cultures expressing YFP.

[2] Indicates identification of the bait protein (JAZ10.4 or JAZ10.4 ^{I107A}) by MS/MS.

[3] nd, not detected.

Prey protein	**AGI code**	**Bait protein[1]**					
		JAZ10.4				**JAZ10.4^{I107A}**	
		rep 1	**rep 2**	**rep 3**	**rep 4**	**rep 1**	**rep 2**
JAZ10[2]	At5g13220	25/17	42/10	34/12	56/9	28/7	47/6
NINJA	At4g28910	21/10	22/10	39/11	34/11	nd[3]	6/5
MYC2	At1g32640	nd	nd	3/2	16/7	nd	nd
MYC3	At5g46760	5/3	nd	17/6	18/8	nd	5/3
MYC4	At4g17880	nd	nd	3/1	4/4	nd	6/4
PPD2	At4g14720	4/4	8/5	2/2	8/5	nd	nd
JAZ12	At5g20900	nd	4/2	6/3	3/1	nd	3/2



C **JAZ10 CMID** ^{22}PKPKFQKFLD**RRR**SFRDI^{39}

JAZ10 Jas motif ^{166}DLPIARRKSLQRFLE**KRK**ERLVSTSPY^{192}

JAZ9 Jas motif ^{218}SVPQARKASLARFLE**KRK**ERLMSAMPY^{244}

Figure 1. The N-terminal region of JAZ10.4 has a cryptic MYC2-interacting domain.

A, Y2H assays of JAZ10.4 deletion constructs (DNA binding-domain "bait" fusions) with MYC2 and JAZ10.4 (activation domain "prey" fusions). Yeast strains co-expressing the indicated bait and prey proteins were plated on media containing X-Gal. Blue-color formation in streaked yeast cells is indicative of protein-protein interaction. Photographic images were taken after 30 h of incubation at 30°C. JAZ10.1 is included as a positive control. The ZIM (Z) and Jas domains are indicated. The solid black line denotes the C-terminal region that is specific for the JAZ10.4 isoform.

B, C-terminal deletion constructs of JAZ10.4 were tested for interaction with MYC2 as described in A.

C, Sequence similarity between the conserved Jas motif of JAZ10 and JAZ9 and the cryptic MYC2-interacting domain (CMID) near the N-terminus of JAZ10. The underlined sequence shows the region of conservation, which includes a tribasic motif (bolded).

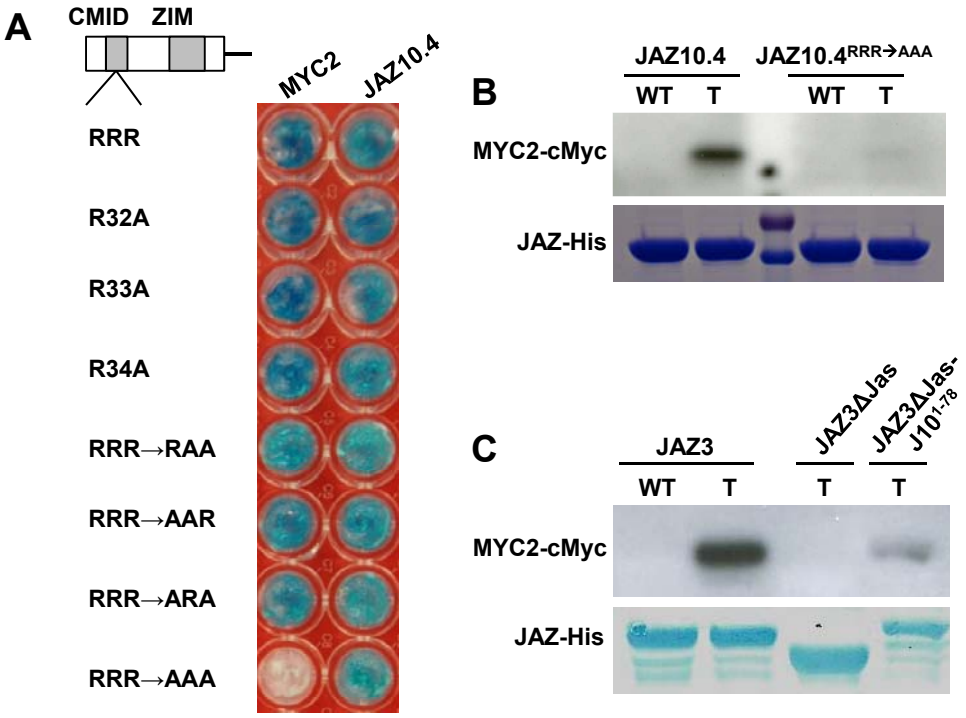


Figure 2. A RRR motif in the N-terminal region of JAZ10.4 is required for interaction with MYC2.

A, Site-directed mutagenesis of the RRR motif within the cryptic MYC2-interaction domain (CMID) of JAZ10.4. The indicated R→A substitution mutants of JAZ10.4 were tested for interaction with MYC2 or JAZ10.4 as a positive control. Y2H assays were performed as described in Figure 1.

B, *In vitro* pull-down assay of JAZ10.4-MYC2 interaction. Assays were performed using the indicated wild-type and mutant JAZ10.4-His recombinant proteins and crude extracts from leaves of wild-type (WT) or *35S-cMyc-MYC2* transgenic (T) plants. Protein bound to JAZ-His was separated by SDS-PAGE and analyzed by immunoblotting (anti-cMyc antibody) for the presence of cMyc-MYC2. The Coomassie-blue stained gel shows total input protein as a loading control.

C, The cryptic MYC2-binding domain (CMID) of JAZ10.4 is sufficient for MYC2 binding. *In vitro* pull-down assays were performed as described in B using JAZ3, JAZ3ΔJas, or a chimeric JAZ (JAZ3ΔJas-JAZ10$^{1-78}$) in which the CMID of JAZ10.4 was fused to the C-terminus of JAZ3ΔJas.

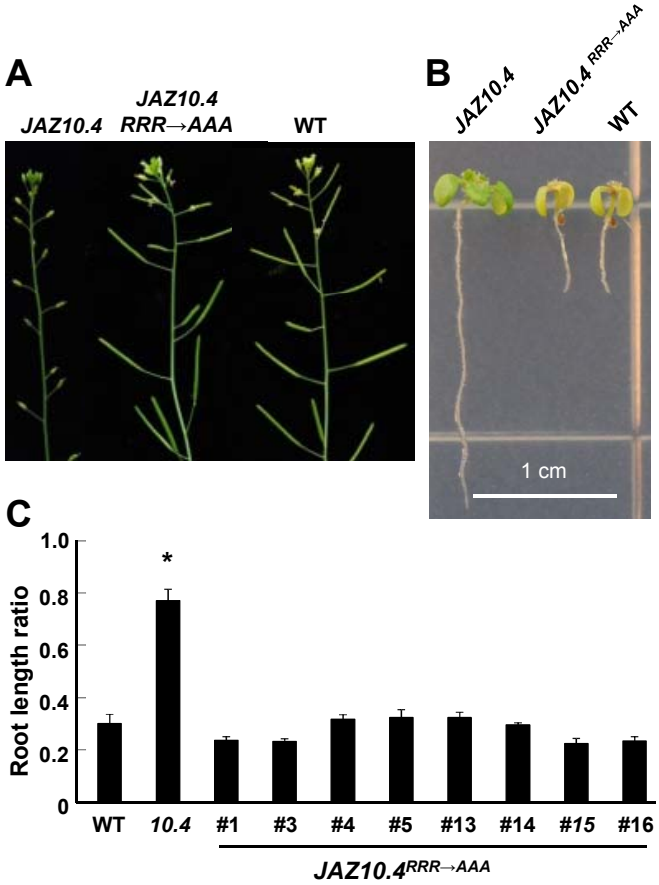


Figure 3. The RRR motif is required for JAZ10.4-mediated repression of JA responses.

A, The photograph shows silique development in wild-type (WT) and transgenic lines that overexpress JAZ10.4 or JAZ10.4$^{RRR→AAA}$.

B, The photograph shows seedlings of the indicated genotype grown for 10 d on MS medium containing 20 µM MeJA.

C, Quantification of JA-induced root growth inhibition in WT, *35S-JAZ10.4* (*10.4*), and eight independent *JAZ10.4*$^{RRR→AAA}$ lines. Seedlings were grown for 10 d on MS agar plates containing or not containing 20 µM MeJA. The root length ratio was calculated by dividing the average root length of MeJA-treated seedlings by the average root length of seedlings of the same genotype grown in the absence of MeJA. Data show the mean ± SE (n=12 seedlings/genotype). The asterisk denotes a significant difference ($P < 0.05$, Student's test) in comparison to WT.

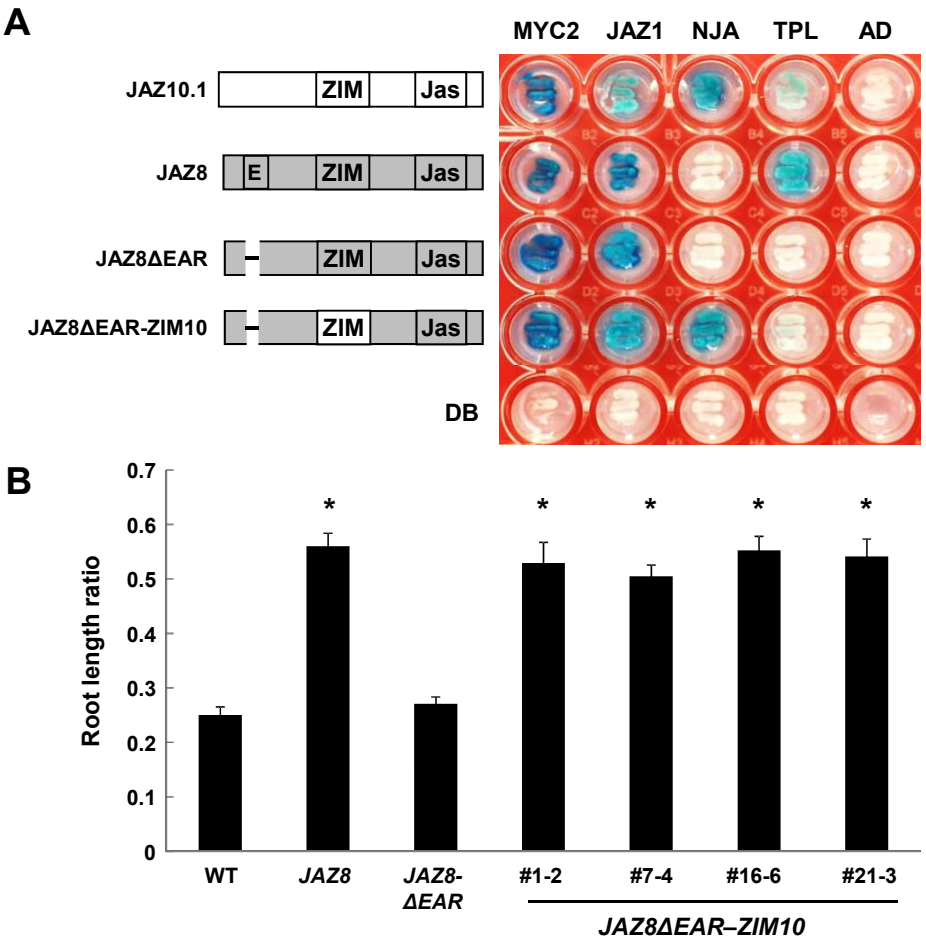


Figure 4. The ZIM domain of JAZ10 interacts with NINJA and is required for repression of JA responses.

A, Y2H assays depicting interaction of JAZ10-JAZ8 chimeric proteins (DNA binding-domain fusions, DB) with MYC2, JAZ1, NINJA (NJA), and TPL (activation domain fusions, AD). Empty vectors were included as negative controls. Sequence regions derived from JAZ10 and JAZ8 are shown in white and gray, respectively, together with various protein domains. E, EAR motif. Y2H assays were performed as described in Figure 1, except that the photographic image was taken after 48 h of incubation of yeast cells at 30°C.

B, Overexpression of a JAZ8ΔEAR-ZIM10 chimeric protein confers insensitivity to JA in root growth assays. Seedlings were grown for 8 d on MS agar plates containing or not containing 20 μM MeJA, and root length ratios were calculated as described in Figure 3C. Data show the mean ± SE of at least 11 seedlings per genotype. The asterisk denotes a significant difference ($P < 0.05$, Student's test) in comparison to WT.

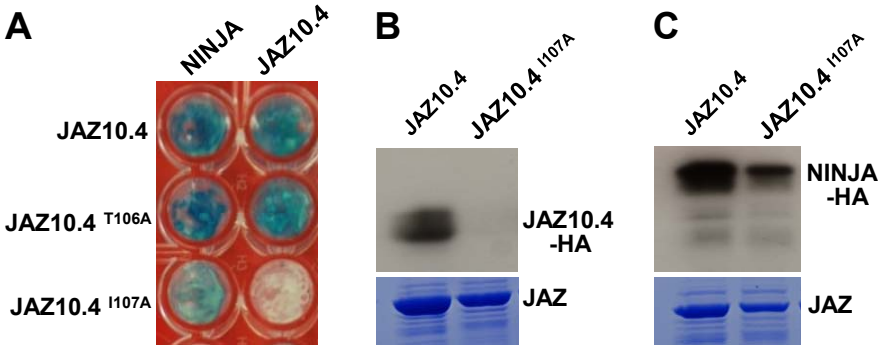


Figure 5. Isoleucine^{107} in the TIFY motif is involved in JAZ10.4 interaction with both JAZ and NINJA.

A, Y2H assay depicting the role of Thr106 and Ile107 (within the TIFY motif) in JAZ10.4 dimerization and JAZ10.4 interaction with NINJA. Y2H assays were performed as described in Figure 1, except that the photographic image was taken after 24 h of incubation of yeast cells at 30°C.

B, *In vitro* pull-down assay of JAZ10.4-JAZ10.4 interaction. Assays were performed using the indicated wild-type (JAZ10.4) and mutant (JAZ10.4^{I107A}) recombinant proteins and crude extract from a yeast strain expressing a JAZ10.4-HA fusion protein. Protein bound to JAZ-His was separated by SDS-PAGE and analyzed by immunoblotting with an anti-HA antibody. A Coomassie-blue stained gel is shown as a loading control.

C, *In vitro* pull-down assay of JAZ10.4-NINJA interaction. Assays were performed as described in Figure 5B except for the use of a crude extract from yeast strain expressing a NINJA-HA fusion protein.

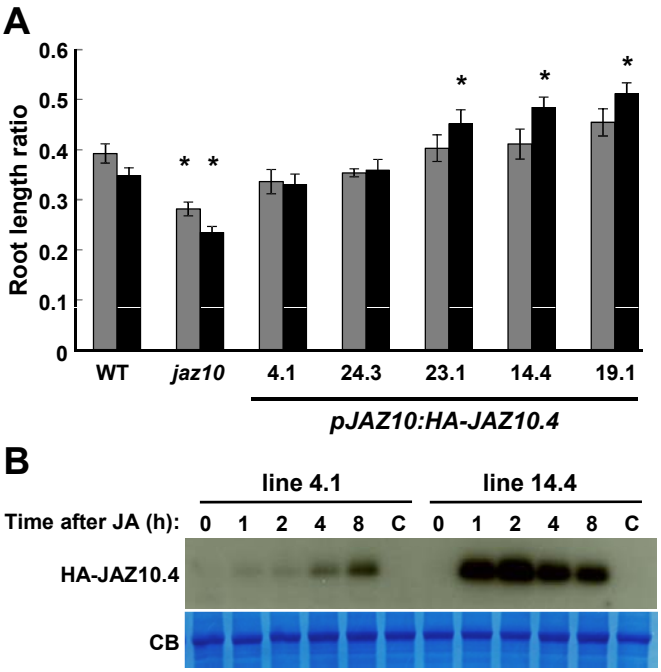
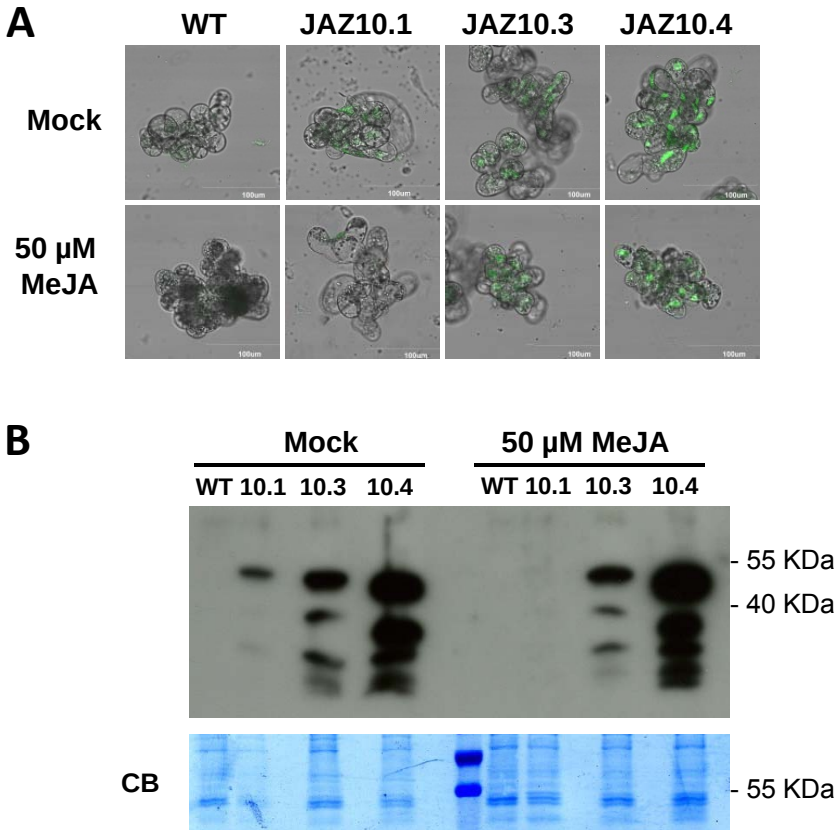


Figure 6. JA-induced expression of JAZ10.4 complements the JA-hypersensitive phenotype of *jaz10-1*.

A, Root growth inhibition assay of wild-type (WT), *jaz10-1* (*jaz10*), and five independent lines in which the *pJAZ10:HA-JAZ10.4* transgene was introduced into the *jaz10-1* mutant background. Root length was measured in 8-d-old seedlings grown on MS medium containing 0, 10, or 20 μM MeJA. The root length ratio was calculated by dividing the average root length of seedlings grown in the presence of either 10 μM (gray bar) or 20 μM (black bar) MeJA by the average root length of seedlings of the same genotype grown in the absence of MeJA. Data show the mean ± SE of 12-14 seedlings/genotype. Asterisks indicate significant differences in root length ($P<0.05$, Student's test) in comparisons between WT and *jaz10* seedlings grown at the same concentration of MeJA, or between WT and *pJAZ10:HA-JAZ10.4* seedlings grown at the same concentration of MeJA.

B, Accumulation of HA-JAZ10.4 protein in response to JA treatment. Ten-d-old liquid-grown seedlings of the indicated *pJAZ10:HA-JAZ10.4* line were treated with 50 μM MeJA and harvested at various times (h) thereafter. As controls, seedlings were harvested immediately prior to treatment ("0") and 8 h after mock treatment ("C"). Total protein was subject to immunoblot analysis with an anti-HA antibody to detect HA-JAZ10.4. A Coomassie-blue stained gel of protein extracts was used as a loading control (CB).

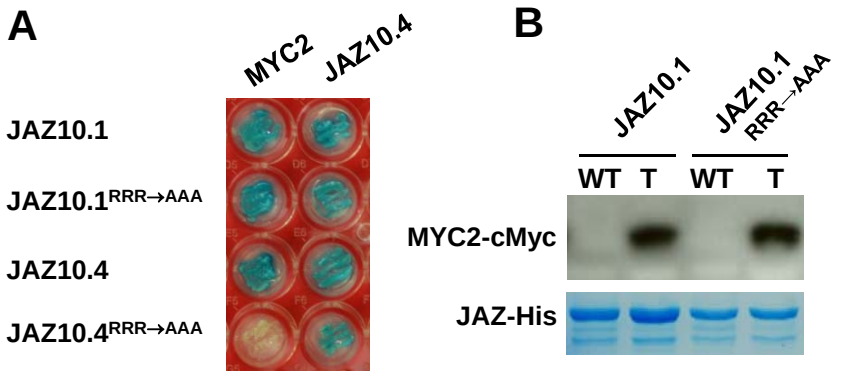
Supplemental data for Moreno et al.



Supplemental Figure 1. Expression of JAZ10 splice variants in Arabidopsis T87 cell cultures.

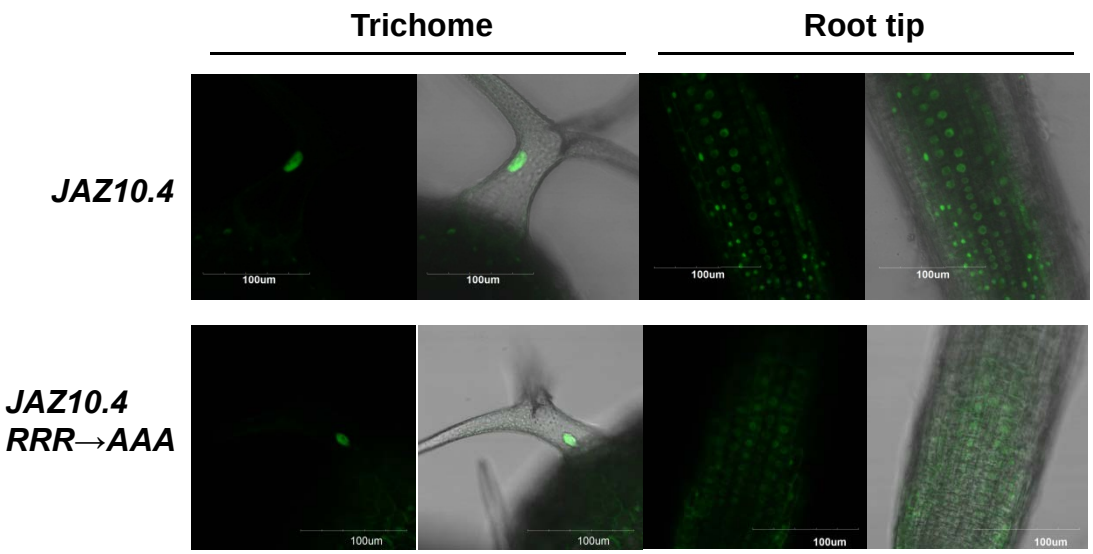
A, JAZ10 splice isoforms were stably expressed as YFP fusion proteins in suspension cultured Arabidopsis T87 cells. Cells were treated with 50 µM MeJA or a mock control for 2 h, and then imaged by confocal fluorescence microscopy. Untransformed T87 cells (WT) were used as a control. Scale bar = 100 µm.

B, JAZ10.3 and JAZ10.4 isoforms are resistant to JA-induced degradation in Arabidopsis T87 cells. The indicated JAZ10 splice isoforms were stably expressed as YFP fusion proteins in suspension Arabidopsis T87 cells (YFP-JAZ10.1, 10.1; YFP-JAZ10.3, 10.3; YFP-JAZ10.4, 10.4). Cells were treated as described in panel A, and crude cell extracts were prepared. As a control, extract was prepared from a parallel culture of untransformed (WT) T87 cells. YFP-JAZ10 fusion proteins in cell lysates were affinity purified on GFP-Trap resin. Resin-bound protein was eluted and analyzed by Western blot using an anti-GFP antibody. Lower molecular weight bands in the YFP-JAZ10.3 and YFP-JAZ10.4 samples represent proteolysis products of the fusion protein. The Coomassie-blue stained PVDF membrane is shown as a loading control.



Supplemental Figure 2. The RRR motif is required for interaction of MYC2 with JAZ10.4 but not JAZ10.1.
A, Y2H assay of wild-type and mutant (RRR→AAA) forms of JAZ10.1 and JAZ10.4 (DNA binding-domain "bait" fusions) with MYC2 and JAZ10.4 (activation domain "prey" fusions). Conditions of the assay were as described in Figure 1A.
B, *In vitro* pull-down assay to test interaction of JAZ10.1 with MYC2. Assays were performed using the indicated wild-type and mutant forms JAZ10.1-His recombinant proteins and crude extracts from leaves of wild-type (WT) or *35S:cMyc-MYC2* transgenic (T) plants. Protein bound to JAZ10.1-His was separated by SDS-PAGE and analyzed by immunoblotting (anti-cMyc antibody) for the presence of cMyc-MYC2. The Coomassie-blue stained gel shows total input protein as a loading control.

Supplemental data for Moreno et al.



Supplemental Figure 3. Subcellular localization of JAZ10.4 and JAZ10.4$^{RRR→AAA}$-YFP.

Confocal fluorescence microscopy was used to determine the subcellular localization of wild-type (JAZ10.4-YFP) and mutant (JAZ10.4$^{RRR→AAA}$-YFP) forms of JAZ10.4 in trichome (left two panels) or root (right two panels) cells of 10-d-old transgenic seedlings. Left photograph is the confocal image for detection of YFP fusion protein. The photograph on the right is the corresponding merge of confocal and transmitted light images. Scale bar = 100 μm.

Supplemental Table I. List of oligonucleotide primers used in this study.

Construction of Y2H vectors

INSERT	VECTOR	PRIMER NAME	SEQUENCE 5'-3'	NOTE
JAZ10.4	pGilda	JAZ10.4_EcoRI_FP	GAATTCATGTCGAAAGCTACCATAGAACTC	
		JAZ10.4_NcoI_RP	CCATGGCTAATCTCTCCTTGCGCTTCTCGAGAAAACG	
Δ1-47	pGilda	JAZ10.4 48-167_EcoRI_FP	GAATTCATGCCGGAGATTATCAAATCGCTGTTAGC	
		JAZ10.4_NcoI_RP	CCATGGCTAATCTCTCCTTGCGCTTCTCGAGAAAACG	
Δ16-47	pGilda	JAZ10.4 without D1 N-term_RP	AACAGCGATTTGATAATCTCCGGCTTCTCAAGTCCGAGGAAATC	
		JAZ10.4 without D1 C-term_FP	GATTTCCTCGGACTTGAGAAGCCGGAGATTATCAAATCGCTGTT	
ΔZIM	pGilda	JAZ10.4 without ZIM C-term_FP	CCGAACTCGTTTCGGGAACTGTTAATGAAGCAGCATCTAAG	
		JAZ10.4 without ZIM N-term_RP	CTTAGATGCTGCTTCATTAACAGTTCCCGAAACGAGTTCGG	
1-47	pGilda	JAZ10.4 16_EcoRI_FP	GAATTCATGAAACAAACCAACAACGCTCCTAAGCC	
		JAZ10.4 1-47 _NcoI_RP	CCATGGCTAATCGATTTTCGAAATCGCACC	
16-55	pGilda	JAZ10.4 16_EcoRI_FP	GAATTCATGAAACAAACCAACAACGCTCCTAAGCC	
		JAZ10.4 1-55_NcoI_RP	CCATGGCTAAACAGCGATTTGATAATCTCCGGATC	
16-58	pGilda	JAZ10.4 16_EcoRI_FP	GAATTCATGAAACAAACCAACAACGCTCCTAAGCC	
		JAZ10.4 1-58_NcoI_RP	ATCATACCATGGCTATCCAGTGGAAGCTAAC	
16-68	pGilda	JAZ10.4 16_EcoRI_FP	GAATTCATGAAACAAACCAACAACGCTCCTAAGCC	
		JAZ10.4 1-68_NcoI_RP	CCATGGCTAAGATTTAGCCGATGAATCGGAATTG	
NINJA	pB42AD	NINJA_pEntr_EcoRI_FP	CACCGAATTCATGGACGATGATAATGGGCTC	
		NINJA_BglII_pB42AD_RP	AGATCTTCAGGTGTGAGCTGACGCTGC	
NINJA	pGilda	NINJA_pEntr_EcoRI_FP	CACCGAATTCATGGACGATGATAATGGGCTC	
		NINJA_NotI_pGilda_RP	TTTTTGCGGCCGCTCAGGTGTGAGCTGACGCTGC	
JAZ10.4_R32A	pGilda	JAZ10.4_R32toA_FP	GAAATTTCTCGATGCCCGTCGTAGTTTCCGAGATA	
		JAZ10.4_R32toA_RP	TATCTCGGAAACTACGACGGGCATCGAGAAATTTC	
JAZ10.4_R33A	pGilda	JAZ10.4_R33toA_FP	TTTCTCGATCGCGCTCGTAGTTTCCGAGAT	
		JAZ10.4_R33toA_RP	ATCTCGGAAACTACGAGCGCGATCGAGAAA	
JAZ10.4_R34A	pGilda	JAZ10.4_R34toA_FP	CTCGATCGCCGTGCTAGTTTCCGAGATA	
		JAZ10.4_R34toA_RP	TATCTCGGAAACTAGCACGGCGATCGAG	
JAZ10.4_RRR→AAR	pGilda	JAZ10.4_RRRtoAAR_FP	CAGAAATTTCTCGATGCCGCTCGTAGTTTCCGAGAT	
		JAZ10.4_RRRtoAAR_RP	ATCTCGGAAACTACGAGCGGCATCGAGAAATTTCTG	
JAZ10.4_RRR→ARA	pGilda	JAZ10.4_RRRtoARA_FP	CAGAAATTTCTCGATGCCCGTCGTAGTTTCCGAGATATT	
		JAZ10.4_RRRtoARA_RP	AATATCTCGGAAACTAGCACGGGCATCGAGAAATTTCTG	
JAZ10.4_RRR→RAA	pGilda	JAZ10.4_RRRtoRAA_FP	AAATTTCTCGATCGCGCTGCTAGTTTCCGAGATAT	
		JAZ10.4_RRRtoRAA_RP	ATATCTCGGAAACTAGCAGCGCGATCGAGAAATTT	
JAZ10.4_RRR→AAA	pGilda	JAZ10.4_RRRtoAAA_FP	CAGAAATTTCTCGATGCCGCTGCTAGTTTCCGAGATATTC	
		JAZ10.4_RRRtoAAA_RP	GAATATCTCGGAAACTAGCAGCGGCATCGAGAAATTTCTG	
JAZ10.4_T106A	pGilda	AD-JAZ10.1 T106A _FP	GGAACTGTTCCTATGGCGATTTTCTACAATG	Described in Chung & Howe (2009)
		AD-JAZ10.1 T106A _RP	CATTGTAGAAAATCGCCATAGGAACAGTTC	
JAZ10.4_I107A	pGilda	AD-JAZ10.1 I107A _FP	ACTGTTCCTATGACGGCTTTCTACAATGGAAG	Described in Chung & Howe (2009)
		AD-JAZ10.1 I107A _RP	CTTCCATTGTAGAAAGCCGTCATAGGAACAGT	
JAZ8ΔEAR-ZIM10	JAZ8ΔEAR	JAZ8-pENTR-FP JAZ8-ZIM10-N-RP	CACCATGAAGCTACAGCAAAATTGTG GTAGAAAATCGTCATAGGTTGAGATTCTTCATTTGG	1st PCR reaction to generate chimera
	JAZ8ΔEAR	JAZ8-ZIM10-C-FP JAZ8-pENTR-RP	GGTGAAATTATGAAGGTCGCTAGCAGAGAAATGAAAACCAAG TTATCGTCGTGAATGGTACGGTG	2nd PCR reaction to generate chimera
	JAZ8ΔEAR (1-44 aa) JAZ10.1	JAZ8-pENTR-FP JAZ10-KpnI-RP	CACCATGAAGCTACAGCAAAATTGTG GGTACCTTAGGCCGATGTCGGATAGTAAG	3rd PCR reaction to generate chimera. PCR product named JAZ8ΔEAR-JAZ10.
	JAZ8ΔEAR-JAZ10 JAZ8ΔEAR (73-121 aa)	JAZ8-pENTR-FP JAZ8-pENTR-RP	CACCATGAAGCTACAGCAAAATTGTG TTATCGTCGTGAATGGTACGGTG	Final PCR reaction to generate chimera
JAZ10.1	pGilda			Described in Chung & Howe (2009)
MYC2	pB42AD			Described in Chung & Howe (2009)
TOP-LESS	pB42AD			Described in Shyu et al. (2012)

Expression MBP-JAZ-HIS recombinant proteins

INSERT	VECTOR	PRIMER NAME	SEQUENCE 5'-3'	NOTE
JAZ10.1	pRMG-nMAL	JAZ10.1_NotI_FP	GCGCGGCCGCCTCGAAAGCTACCATAGAACTCG	Described in Chung et al. (2010)
		JAZ10.1_XhoI_RP	TCGGTCGACGGCCGATGTCGGATAG	
JAZ10.4	pRMG-nMAL	JAZ10.4_NotI_FP	GCGCGGCCGCCTCGAAAGCTACCATAGAAC	Described in Chung et al. (2010)
		JAZ10.4_XhoI_RP	TCGCTCGAGATCTCTCCTTGCGCTTCTTG	
JAZ10.4_I107A	pRMG-nMAL	JAZ10.4_NotI_FP	GCGCGGCCGCCTCGAAAGCTACCATAGAAC	
		JAZ10.4_XhoI_RP	TCGCTCGAGATCTCTCCTTGCGCTTCTTG	
JAZ10.1_RRR→AAA	pRMG-nMAL	JAZ10.1_NotI_FP	GCGCGGCCGCCTCGAAAGCTACCATAGAACTCG	
		JAZ10.1_XhoI_RP	TCGGTCGACGGCCGATGTCGGATAG	
JAZ10.4_RRR→AAA	pRMG-nMAL	JAZ10.4_NotI_FP	GCGCGGCCGCCTCGAAAGCTACCATAGAAC	
		JAZ10.4_XhoI_RP	TCGCTCGAGATCTCTCCTTGCGCTTCTTG	
JAZ3-ΔJas		JAZ3-ΔJas_NotI_FP	GCGGCCGCGATGGAGAGAGATTTTCTCGGGTTGGGTT	
		JAZ3-deltaJas_XhoI_RP	CTCGAGCACTGTTGGAGCCATTACATTGGTAG	
1-78 JAZ10		JAZ10.4_D1_XhoI_FP_J3deltaJas	CTCGAGATGTCGAAAGCTACCATAGAACTC	
		JAZ10.4_D1_XhoI_RP_J3deltaJas	CTCGAGATCTTCCCTCGGAGTAGACG	
JAZ3-ΔJas+ 1-78 JAZ10	pRMG-nMAL	JAZ3-ΔJas_NotI_FP	GCGGCCGCGATGGAGAGAGATTTTCTCGGGTTGGGTT	
		JAZ10.4_D1_XhoI_RP_J3deltaJas	CTCGAGATCTTCCCTCGGAGTAGACG	

Expression of YFP-JAZ10 variants in Arabidopsis T87 cells

INSERT	VECTOR	PRIMER NAME	SEQUENCE 5'-3'	NOTE
JAZ10.1	pVKgw-N-YFP	JAZ10G_For_pEntr	CACCATGTCGAAAGCTACCATAGAACTCGATTTCCTCGG	
		JAZ10.1_Rev_pEntr	TTAGGCCGATGTCGGATAGTAAGGAGATGTTG	
JAZ10.3	pVKgw-N-YFP	JAZ10G_For_pEntr	CACCATGTCGAAAGCTACCATAGAACTCGATTTCCTCGG	
		JAZ10.3_Rev_pEntr	TTACCTCTCCTTGCGCTTCTCGAGAAAACGTTGC	
JAZ10.4	pVKgw-N-YFP	JAZ10G_For_pEntr	CACCATGTCGAAAGCTACCATAGAACTCGATTTCCTCGG	
		JAZ10.4_Rev_pEntr	CTAATCTCTCCTTGCGCTTCTCGAGAAAACGTTG	

Other transgenic Arabidopsis lines

INSERT	VECTOR	PRIMER NAME	SEQUENCE 5'-3'	NOTE
JAZ10.4-YFP	pEntr D-TOPO	JAZ10G_For_pEntr	CACCATGTCGAAAGCTACCATAGAACTCGATTTCCTCGG	pEarley-101 was the final destination vector
		cJAZ10.4_pEntr_RP	ATCTCTCCTTGCGCTTCTCGAGAAAACGTTG	
JAZ10.4 RRR→AAA -YFP	pEntr D-TOPO	JAZ10G_For_pEntr	CACCATGTCGAAAGCTACCATAGAACTCGATTTCCTCGG	pEarley-101 was the final destination vector
		cJAZ10.4_pEntr_RP	ATCTCTCCTTGCGCTTCTCGAGAAAACGTTG	
JAZ10 promoter	pEntr D-TOPO	JAZ10pro NotI GW FP	CACCGCGGCCGCGACTTTGGCGAGCAAACC	
		JAZ10pro NotI GW RP	GCGGCCGCCTTCTTTGATCTTATTAGAAAGTG	
HA-JAZ10.4	pEntr D-TOPO	JAZ10CDS HA NotI GW FP	CACCATGTACCCTTATGATGTGCCAGATTATGCCTCTTCGAAAGCTAC	pGW401 was the final destination vector
		JAZ10.4 RP	CTAATCTCTCCTTGCGCTTCTCGAGAAAACG	
MYC2	pEntr D-TOPO	AtMYC2-F	CACCCATATGACTGATTACCGGCTACA	pJYP006 was the final destination vector
		AtMYC2-R	CAATTGTTAACCGATTTTTGAAATCAAAC	
JAZ8ΔEAR-ZIM10	pBI-Tony	JAZ8_XbaI_FP	ATATTCTAGAATGAAGCTACAGCAAAATTGTG	Described in Shyu et al. (2012)
		JAZ8_KpnI_RP	ATATGGTACCTTATCGTCGTGAATGGTACG	