THE MOLECULAR MECHANISM OF JAZ PROTEINS IN REGULATING JASMONATE RESPONSES IN ARABIDOPSIS

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ABSTRACT

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Jasmonate (JA) is a lipid-derived phytohormone that regulates a broad range of physiological processes, including wound responses, defense against insect herbivores and pathogens, and plant growth and development. JASMONATE ZIM-DOMAIN (JAZ) proteins are negative regulators of transcription factors that repress JA-induced gene expression. The current JA signaling model indicates that JAZ proteins require the adaptor protein NOVEL INTERACTOR OF JAZ (NINJA) to recruit the co-repressor TOPLESS (TPL) and repress transcription of JA-response genes. Upon wounding or other biological cues that lead to accumulation of bioactive JA, JAZ proteins are targeted for ubiquitination by the F-box protein CORONATINE INSENSITIVE1 (COI1) and degraded by the 26S proteasome. Degradation of JAZ proteins results in induction of JA-responsive gene expression. There are 12 JAZ proteins in Arabidopsis, and it is unknown how they regulate such a wide range of biological processes. In this study, I first show that JAZ proteins exhibit differential stability upon JA treatment, with JAZ8 being a relatively stable repressor. The increased stability of JAZ8 is a consequence of a unique degron motif that differs from the canonical degron of most others JAZs. Secondly, I report that JAZ8 represses JA-induced gene expression through its N-terminal EAR (for EAR-associated amphiphilic repression) motif, and that the EAR motif is required for direct interaction of JAZ8 with TPL. This finding suggests that JAZ8 does not repress JA

responses through NINJA. Finally, I report the identification of a novel JAZ protein (called JAZ13) in Arabidopsis that is most closely related to JAZ7 and JAZ8. I provide genetic and biochemical evidence that JAZ13, like JAZ8, negatively regulates JA responses through a NINJA-independent pathway. Taken together, results from this study elucidate a novel mechanism to recruit TPL and repress JA responses, presumably for the purpose of fine-tuning myriad JA responses that balance plant defense and development.

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Thank you, who are reading this dissertation right now. It is extremely rewarding for a researcher to know that someone is interested in his or her work. I hope that research described in this dissertation contributes positively to the field, and that you may somehow benefit from it.

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CHAPTER ONE
LITERATURE REVIEW

1. THE BIOLOGY OF JASMONATES

Plants are faced with numerous environmental changes on a daily basis. The sessile nature of plants prevents them from escaping or hiding from environmental stresses such as insect herbivores or extreme weather conditions. Adaptive responses to environmental stimuli are thus important for plants to survive and successfully carry on their next generation. Plant hormones provide an important mechanism for plants to regulate growth and responses to stress. There are multiple plant hormones, each of which has distinct and overlapping roles in regulating plant growth, development and defense (reviewed in Santner et al., 2009). Jasmonates (JA) are a particularly interesting group of phytohormones because of their dual role in inhibiting growth and promoting defense and reproduction.

JA was first identified in the form of methyl-JA (MeJA) from jasmine essential oil extracts and later widely used in the perfume industry due to its pleasure scent (Demole and Lederer, 1962). Jasmonic acid was isolated from multiple plant species, and a biological role for JA as a growth inhibitor was described in 1981 (Yamane et al., 1981). Exogenous application of JA inhibits seedling growth, promotes senescence and represses photosynthesis (Ueda and Kato, 1980; Reinbothe et al., 1994;). JA also has roles in reproductive development in multiple species such as rice, maize, Arabidopsis and tomato. MeJA inhibits spikelet formation in rice(Kim et al., 2009). Mutations in a maize lipoxygenase *TASSELSEED1* (*TS1*) lead to disruption in stamen development that can be restored by applying JA (Acosta et al., 2009). Independent mutations in several steps of the JA biosynthetic pathway result in male sterility, in which Arabidopsis flowers of these mutants have shorter anther filaments and defects in pollen development (McConn and Browse, 1996; Sanders et al., 2000; Stintzi and Browse, 2000; Ishiguro et al., 2001). Exogenous JA restores fertility of these lines. On the other hand, mutations

in a key JA perception component *COI1* also leads to male sterility (Feys et al., 1994). Interestingly, mutations in the tomato ortholog of *COI1* result in female sterility and defects in male fertility as well (Li et al., 2001). Taken together, research on multiple plant species demonstrate a role for JA in inhibiting plant growth and promoting reproductive development.

The discovery of JA as a master regulator of plant defense was a key advance in understanding JA biology. Tomato protease inhibitors (PIs), which are involved in defense against insect herbivores, were shown to be highly expressed upon MeJA treatment (Farmer and Ryan, 1990). Indeed, mutations in genes regulating JA biosynthesis or signaling severely compromise defense against insect feeding (Howe et al., 1996; McConn and Browse, 1996). Many other roles for JA in plant defense have been characterized, including secondary metabolite production, trichome development, and defense against pathogens (Vijayan et al., 1998; Chen et al., 2006; Yoshida et al., 2009). All of these discoveries establish JA as a well known "wound hormone" (Koo and Howe, 2009). In addition to defense against biotic stresses, JA also has multiple roles in plant responses to abiotic stress. These roles include responses to UV light, ozone stress and other abiotic stresses (Conconi et al., 1996; Weiler et al., 1993; Dombrowski et al., 2003).

JA has dual roles in regulating growth and defense. It has been increasingly important to understand how multiple signaling pathways in a regulatory network crosstalk with each other to fine-tune signal outputs under different conditions. Understanding how JA regulates different responses to balance growth and defense is an important starting point toward this goal. Understanding of JA biology can be further applied to agricultural and industrial purposes, in which one can manipulate crops to become more resistant to insect herbivores and pathogens, which will increase crop yields. In addition to its role as an essential phytohormone, JA has been

shown to be an effective anti-cancer agent that inhibits tumor cell growth and promotes apoptosis (Xiao et al., 2011; Milrot et al., 2012). These results highlight the importance of JA research in both agricultural and medical research.

2. JA BIOSYNTHESIS AND METABOLISM

A detailed understanding of the biosynthesis of JA has been obtained mainly by research initiated from forward and reverse genetic screens in Arabidopsis and tomato. JA is synthesized via an octadecanoid pathway in two cellular components: the chloroplast and peroxisome (Figure 1.1). The first step of JA biosynthesis is in the chloroplast, which is composed of high levels of trienoic fatty acids. A role for chloroplast lipids in JA biosynthesis was demonstrated in experiments utilizing a triple mutant of fatty acid desaturases (*fad 3 fad7 fad8*). The *fad 3 fad7 fad8* mutant, which is defective in trienoic fatty acids, is male sterile, and exogenous application of JA restores fertility (McConn and Browse, 1996). In the second step of JA biosynthesis, α-linolenic acid (18:3) is released from membrane lipids by the lipase *DEFECTIVE IN ANTHER DEHISCENCE1* (*DAD1*). Similar to *fad 3 fad7 fad8*, *dad1* shows defects in male fertility that can be restored by exogenous JA (Ishiguro et al., 2001). α-Linolenic acid (18:3) is converted to 13-hydroxyperoxylinolenic acid by 13-lypoxygenases, such as *LOX2* (Bell et al., 1995). 13-Hydroxyperoxylinolenic acid is further metabolized to 12-oxo-phytodienoic acid (OPDA) by allene oxide synthase (AOS) and allene oxide cyclase (AOC) (Song and Brash, 1991; Ziegler et al., 2000). The production of OPDA completes the chloroplastic steps in JA biosynthesis.

Transport of OPDA from the chloroplast to peroxisome requires the ATP-binding cassette transporter protein COMATOSE (CTS) (Theodoulou et al., 2005). Upon transport to the

peroxisome, OPDA is further metabolized to 3-oxo-2-(cis-2'-pentenyl)-cyclopentane-1-octanoic acid (OPC 8:0) and 3-oxo-2-(cis-2'-pentenyl)-cyclopentane-1-octanoyl CoA (OPC 8:0-CoA) by peroxisomal OPDA reductases (OPR) and OPC-8:0 CoA ligases (OPCL) (Stintzi and Browse, 2000; Schneider et al., 2005). The final phase of JA biosynthesis involves three cycles of β-oxidation in the peroxisome by oxidases such as acyl CoA oxidases (ACX) and ABNORMAL INFLORESCENCE MERISTEM (AIM1) (Li et al., 2005; Delker et al., 2007; Schilmiller et al., 2007). Following export from the peroxisome, JA is further metabolized to various metabolites in the cytoplasm. Examples include methylation of JA by a methyl transferase to produce MeJA, conjugation of JA to isoleucine (Ile) to yield JA-Ile and hydroxylation of JA to produce 12-OH-JA (Seo et al., 2001; Staswick and Tiryaki, 2004; Koo et al., 2011).

Although many JA metabolites have been identified in plants, (3R, 7S)-JA-Ile is the only known receptor-active form of the hormone. JA-Ile levels are rapidly induced upon wounding, and JA-Ile is likely the most abundant conjugated form of JA in Arabidopsis (Koo et al., 2009). JA-Ile synthesis from JA and Ile is catalyzed by *JASMONATE RESISTANT 1* (*JAR1)* (Staswick and Tiryaki, 2004). Mutation of *JAR1* results in loss of JA-Ile accumulation and decreased sensitivity to JA in root growth inhibition assays. Application of JA-Ile, but not other JA metabolites, complements the *jar1* root growth phenotype (Staswick and Tiryaki, 2004). *jar1* plants also have defects in other JA responses such as insect feeding (Kang et al., 2006). These results collectively indicate that JA-Ile plays an essential role in JA signaling. JA-Ile can be further metabolized to a less active form, 12-OH-JA-Ile. This involves a subgroup of cytochrome P450 hydroxylases in the CYP94 family (Koo et al., 2011). Mutant lines of *CYP94B3* hyperaccumulate JA-Ile and are deficient in 12-OH-JA-Ile in response to wounding. *cyp94b3* lines exhibit increased sensitivity to JA-mediated root growth inhibition, and overexpression of

CYP94B3 results in decreased sensitivity to JA ((Kitaoka et al., 2011; Koo et al., 2011; Heitz et al., 2012). In vitro enzymatic assays show that CYP94B3 catalyzes oxidation steps or JA-Ile to produce 12OH-JA-Ile, which is less active than JA-Ile in promoting COI1 interaction with JAZ proteins (Koo et al., 2011; Heitz et al., 2012). These results firmly demonstrate that catabolism of JA-Ile is an essential pathway to deactivate JA-Ile.

Interestingly, several strains of the plant pathogen *Pseudomonas syringae* produce a phytotoxin called coronatine that is structurally similar to JA-Ile (Bender et al., 1999) (Figure 1.2). Indeed, coronatine and JA elicit many of the same responses, including inhibition of seedling growth (Feys et al., 1994). It is generally accepted that coronatine is a molecular mimic of JA-Ile and widely used in biochemical assays to approach questions related to JA signaling.

3. JASMONATE SIGNALING – BACKGROUND AND HISTORY

An important breakthrough in jasmonate research was the discovery of CORONATINE INSENSITIVE 1 (COI1). Application of coronatine to Arabidopsis growth medium results in inhibition of seedling growth, similar to effects of MeJA application. Seedlings treated with coronatine have shorter primary roots compared to those grown without coronatine. MeJA and coronatine also elicit chlorosis in vegetative tissues. Feys et al. (2004) reported an ethyl methane sulfonate (EMS) mutant screen to identify mutants that have reduced sensitivity to coronatine. The *coi1* mutant was identified as being insensitive to both coronatine and MeJA. In addition to seedling growth phenotypes, *coi1* plants also have shorter anther filaments and are male sterile. This is a phenotype commonly observed among mutants deficient in JA biosynthesis. Moreover, *coi1* is insensitive to the coronatine-producing bacterial pathogen *Pseudomonas syringae*. The

COI1 gene was identified by map-based cloning and shown to encode an F-box protein (Xie et al., 1998). Similar findings were reported in tomato; a screen for MeJA-insenstive tomato seedlings identified a mutant called *jasmonate insensitive 1* (*jai1*), that that lacks all known JA responses. Mapping of *jai1* revealed that it defines the tomato ortholog of Arabidopsis *COI1* (Li et al., 2004). F-box proteins are components of SCF (Skp/Cullin/F-box) E3-type ubiquitin ligases that provide targeting specificity for the SCF complex to recognize and target proteins for ubiquitination and degradation via the 26S proteasome pathway (Hershko and Ciechanover, 1998). Identification of COI1 as a F-box protein suggested that it may target repressors of jasmonate responses for degradation in response to a JA signal.

Transcription factors are also important components of the JA signaling pathway. One transcription factor that is involved in this process is *JASMONATE INSENSITIVE 1* (*JIN1*), also known as *MYC2*. Mutations in the MYC2 gene were identified in mutant screens for seedlings insensitive to jasmonate treatment (Lorenzo et al., 2004). *MYC2* belongs to a family of basic helix-loop-helix transcriptional regulators. Unlike *coi1* and mutants affected in JA biosynthesis, *jin1* mutants are fertile and do not exhibit obvious growth-related phenotypes. This indicates that other transcription factors are likely involved in other jasmonate responses. Knowledge of COI1 and MYC2 led to the following model: in the presence of JA, COI1 targets repressor proteins that work through an unknown mechanism to repress transcription factors such as MYC2.

4. JASMONATE ZIM-DOMAIN PROTEINS

4.1. Discovery of JAZ proteins

The missing link in the JA signaling pathway are the JASMONATE ZIM-DOMAIN (JAZ) proteins, which were identified nearly a decade after the discovery of COI1. Three independent groups identified JASMONATE ZIM-DOMAIN PROTEINS as JA-responsive transcriptional repressors (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007). Thines et al. (2007) identified *JAZ* genes in a microarray experiment comparing gene expression patterns in wild type (WT) and the JA-biosynthesis mutant *opr3*, treated with exogenous jasmonate. Seven genes of unknown function were identified in a list of rapidly induced genes in WT but not *opr3,* 0.5-hours after JA treatment. All seven genes contain a conserved ZIM domain. Homology search of the Arabidopsis genome identified five additional ZIM-domain-containing genes of unknown function. The 12 ZIM-domain containing proteins were named *JASMONATE ZIM DOMAIN* (*JAZ*) *1-12* (Figure 1.3). Loss-of-function and gain-of-function analyses of various JAZ family members did not reveal obvious JA-related phenotypes. However, overexpression of a form of JAZ1 lacking the C terminus (JAZ1Δ3A) resulted in several JA-insensitive phenotypes, including male sterility. This suggests that JAZ proteins are involved in JA signaling.

Chini et al. (2007) identified *jai3-1* (later renamed *jaz3*) in a mutant screen screening for seedlings with altered JA sensitivity. Map-based cloning revealed that *jai3-1* contains a G to A mutation in the *JAZ3* splicing-acceptor site of its fifth intron, resulting in deletion of the JAZ3 C-terminus. The *jai3-1* mutant is insensitive to JA-induced root growth inhibition, similar to *35S-JAZ1Δ3A* lines. Yan et al. (2007) identified *JASMONATE-ASSOCIATED1* (*JAS1*)/*JAZ10* using a transcript profiling approach similar to that described by Thines et al. (2007). Genes differentially expressed between WT and *aos* upon wounding were overexpressed in WT Arabidopsis plants to screen for JA-related phenotypes. Although overexpression of *JAZ10* did

not result in altered JA-responsiveness, overexpression of an alternative-spliced form of *JAZ10*, *JAZ10.3*, resulted in decreased sensitivity to JA.

JAZ proteins are hypothesized to be repressors that are targeted by COI1 for degradation in response to JA treatment (Chini et al., 2007; Thines et al., 2007). Indeed, JAZ1-GUS is degraded in a JA- and *COI1*-dependent manner. JAZ1-GUS degradation is also reduced when seedlings overexpressing JAZ1-GUS were treated with the protease inhibitor MG132. This suggests that JAZ1-GUS is degraded via the 26S proteasome pathway, consistent with known SCF ubiquitination-degradation pathways. Similarly, JAZ3-GFP is also rapidly degraded in a COI1-dependent manner upon JA treatment (Chini et al., 2007). Other JAZs, including JAZ6, JAZ9 and JAZ10 are also degraded in responses to JA treatment (Chini et al., 2007; Thines et al., 2007; Chung and Howe, 2009). Moreover, JAZ proteins physically interact with COI1 in the presence of JA-Ile (Thines et al., 2007). These results collectively demonstrate that JAZ proteins are direct targets of SCF^{COI1}.

Another important observation reported in Chini et al. (2007) is that JAZ3 interacts with the JA-responsive transcription factor MYC2 in yeast two-hybrid and in vitro pull-down assays. This discovery, along with other observations reported in these three pioneer papers (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007) leads to the following model: JAZ proteins are repressor proteins that bind to MYC2 in the absence of JA. Upon accumulation of bioactive JA, JAZ repressors are targeted for ubiquitination by COI1 and degraded via the 26S proteasome pathway to activate JA-induced gene expression (Figure 1.4).

4.2. Domain Features of JAZ Proteins

JAZ proteins share three conserved domains: an N-terminal Domain 1 (D1), a ZIM domain and a C-terminal Jas domain (Figure 1.5). The ZIM domain was first identified in the Zinc-finger protein expressed in inflorescence meristem (ZIM) (Nishii et al., 2000). A hallmark of ZIM domains is the TIFY sequence motif, hence the ZIM domain is sometimes referred as the TIFY domain (Vanholme et al., 2007). In silico approaches revealed the presence of TIFY family proteins in all species of land plants, but not the single-cellular green algae *Chlamydomonas rainhartii* or the multicellular green algae *Volvox carteri* (Bai et al., 2011). In the case of JAZ proteins, the ZIM domain has important roles in protein-protein interaction, specifically in mediating interaction with a NOVEL INTERACTOR OF JAZ (NINJA) corepressor adapter protein. The ZIM domain is also involved in interaction between JAZ proteins to form homo or heterodimers. Functional roles of JAZ-NINJA and JAZ-JAZ interactions mediated by the ZIM domain are discussed in later sections of this chapter.

The Jas domain is a 27-amino-acid motif located at the C-terminus of all 12 JAZ proteins. The Jas domain shares sequence similarity to the N-terminal region of CCT domains reported in CONSTANS (CO) (Strayer et al., 2000; Robson et al., 2001; Chung et al., 2009). Although the CCT domain in CO is reported to be involved in DNA binding, analyses of the Jas domain suggested different functions (Ben-Naim et al., 2006). A key feature of the Jas domain is the degron motif that facilitates JAZ-COI1 interaction (Melotto et al., 2008; Sheard et al., 2010). The Jas domain is also required for interaction with MYC2 and other transcription factors involved in JA signaling (Chini et al., 2007; Fernandez-Calvo et al., 2011; Qi et al., 2011; Song et al., 2011). Moreover, Grunewald et al (2009) suggested a role for the C-terminal region of Jas motif in nuclear localization of JAZ1 (Grunewald et al., 2009).

The N-terminal D1 is less conserved among JAZ proteins. It has been reported that JAZ1 interacts with DELLA proteins, which are important transcriptional repressors in regulating gibberellin responses (Hou et al., 2010; Yang et al., 2012). The N-terminal motif of JAZ1, which contained D1, is critical for JAZ1-DELLA interaction (Hou et al., 2010). The functional role of D1 in other JAZ proteins is not known.

4.3. JAZ-COI1 interaction – the JA-Ile receptor complex

One key feature of JAZ proteins is their role as targets of SCF^{COI1}. JAZ proteins interact with COI1 in a JA-Ile-dependent manner in yeast two-hybrid and in vitro pull-down assays (Thines et al., 2007; Katsir et al., 2008; Melotto et al., 2008). JAZ-COI1 interaction is also detected in the presence of coronatine, which is a molecular mimic of JA-Ile (Melotto et al., 2008). Coronatine binds specifically to COI1-JAZ complexes. The ability of JA-Ile to compete with COR shows that both are recognized by the same receptor (Katsir et al., 2008). These results suggest an important role for the COI1-JAZ complex in perceiving bioactive ligands.

JAZ3-COI1 interaction is dependent on the Jas domain. JAZ3 recombinant proteins lacking the Jas domain fail to interact with COI1 in in vitro pull down assays (Kastir et al., 2008). A JAZ3 C-terminal fragment containing the Jas domain is sufficient to interact with COI1 in a JA-Ile-dependent manner. Indeed, coronatine binding assays using purified COI1 and different fragments of the Jas domain of JAZ1 indicate that the N-terminal motif within the Jas domain is critical for coronatine binding (Sheard et al., 2010). A peptide containing 21 amino acids of the JAZ1 Jas motif, including an N-terminal ELPIAR motif, is sufficient to bind coronatine.

Mutation or deletion of the ELPIAR motif results in loss of coronatine binding. Sheard et al (2010) hence defined the first 21 amino acids within the Jas domain as the JAZ "degron" motif.

Structural analysis of COI1 revealed that the COI1-JAZ complex is the true receptor for JA-Ile (Sheard et al., 2010). COI1 was crystalized along with a JAZ1 degron peptide in the presence of the bioactive (3R, 7S) JA-Ile. The JA-Ile COI1-JAZ receptor complex is very similar to the auxin TIR1-Aux/IAA receptor complex, with the F-box protein COI1 forming a binding pocket for JA-Ile, and the JAZ degron forming a molecular clamp to secure ligand binding in the binding pocket. The LPIAR residues within the JAZ degron are responsible for direct contact with JA-Ile. This explains why the DLPIAR motif is required for COI1-JAZ1 to bind coronatine.

Interestingly, crystallization of the COI1-JAZ JA-Ile receptor complex revealed inositol pentakisphosphate (InsP5) as a cofactor in the receptor complex (Sheard et al., 2010). This is supported by the JA hypersensitive phenotype of the Arabidopsis *ipk1-1* mutant (Mosblech et al., 2011), which accumulates InsP4 and InsP5 due to a defect in the conversion of InsP5 to InsP6 by the *INOSITOL POLYPHOSPHATE KINASE1* (*IPK1*) (Stevenson-Paulik et al., 2005). It is interesting to speculate that biosynthesis and metabolism of InsP5 may provide a mechanism of regulating JA-Ile perception.

In summary, structural and biochemical analyses have demonstrated that the jasmonate receptor is composed of both COI1 and JAZ and a cofactor InsP5. The degron motif that mediates COI1-JAZ1-interaction has been defined. It is yet to be explored whether or not there is a role for the degron motif of JAZ proteins in planta.

4.4. Alternative Splice Variants of JAZ Proteins

Ectopic expression of artificially truncated JAZ proteins that lack the Jas motif results in decreased sensitivity to JA (Chini et al., 2007; Thines et al., 2007). Interestingly, many plants express natural JAZ splice variants (JAZΔJas) lacking the Jas motif (Chung et al., 2010). In most Arabidopsis genes, alternative splicing of a conserved intron within the Jas domain coding region prematurely truncates the highly conserved X_5PY submotif that defines the C-term end of the Jas domain. The resulting splice variants lacking the C-terminal X_5PY sequence are resistant to JA-Ile-promoted JAZ-COI1 interaction and, as a consequence, exert dominant repression on JA signaling (Chung and Howe, 2009; Chung et al., 2010). For example, overexpression of the JAZ10.3 splice variant results in decreased sensitivity to JA (Yan et al., 2007; Chung and Howe, 2009). Based on these findings, it was proposed that stable JAZ splice variants play a role in preventing plants from hyper-responding to JA. The biological role of stabilized JAZ splice variants and how they are removed from the cell are interesting future questions to investigate.

4.5. The JAZ-NINJA-TOPLESS Repression Complex

JAZ proteins repress JA-responsive gene expression by recruiting a Groucho/Tup1 corepressor TOPLESS (TPL) through an adapter protein NOVEL INTERACTOR OF JAZ (NINJA) (Pauwels et al., 2010). Tandem affinity purification (TAP) assays followed by mass spectrometry (MS) were used to identify JAZ1-interacting proteins in suspension-cultured Arabidopsis cells. Among the proteins identified with this approach were COI1, MYC2, JAZ12, and protein of unknown function, which was named NINJA. NINJA interacts with the ZIM domain of JAZ1. Overexpression of NINJA represses jasmonate-induced root growth inhibition, whereas knock-down of *NINJA* expression by RNAi leads to hypersensitivity to JA. These

results collectively indicate that NINJA is involved in repression of jasmonate responses, presumably through direct interaction with JAZ proteins.

Pauwels et al. (2010) searched for NINJA-interacting proteins using the same TAP-MS method, which resulted in identification of the corepressor TPL. This result is striking because TPL was previously shown to regulate auxin responses by interacting directly with Aux/IAA transcriptional repressors (Szemenyei et al., 2008). Indeed, *tpl-1*, which has strong temperature-sensitive auxin phenotypes, is hypersensitive to JA (Long et al., 2002; Pauwels et al., 2010). NINJA interacts with TPL through an ETHYLENE RESPONSIVE FACTOR-associated amphiphilic repressor (EAR) motif located near the N-terminal end of NINJA. Based on these findings, it was proposed that JAZ proteins repress JA responses by interacting simultaneously with MYC2 and, via the NINJA adaptor, TPL. The mechanism by which TPL represses expression of JA response genes is currently not known.

Interestingly, JAZ7 and JAZ8 do not interact with NINJA (Pauwels et al., 2010). Genome-wide analysis of EAR motif-containing proteins in Arabidopsis identified EAR motif sequences in JAZ5, JAZ6, JAZ7 and JAZ8 (Kagale et al., 2010). Whether or not JAZ proteins can repress through an alternative mechanism that directly recruits EAR-interacting corepressors, similar to the auxin repression pathway, remains an open question.

4.6. JAZ-JAZ Dimerization

JAZ proteins homo- and heterodimerize with other members in the JAZ family (Chini et al., 2009; Chung and Howe, 2009). JAZ-JAZ interaction is mediated through the ZIM domain. Ala-scanning of the TIFY motif of JAZ10 revealed that the I residue within the TIFY motif is

required for JAZ10 dimerization (Chung and Howe, 2009). Ectopic expression of a stabilized form of JAZ10, JAZ10.4, results in decreased sensitivity to JA. Overexpression of *JAZ10.4* $^{I \rightarrow A}$ compromises the JA-insensitive phenotype that was observed in *35S-JAZ10.4* lines. This result suggests that the TIFY motif is required for JAZ repression. Interestingly, the TIFY motif is also involved in JAZ-NINJA interaction (Pauwels et al., 2010). Whether the *JAZ10.4* $^{I \rightarrow A}$ overexpression phenotype is due to lack of JAZ-JAZ interaction, or to perturbation of the JAZ-NINJA interaction, remains to be elucidated. Detailed analyses of the ZIM domain structure is expected to provide important information about how JAZ proteins repress JA responses.

4.7. Involvement of Transcription Factors in Jasmonate Signaling

JAZ proteins regulate gene expression by physically interacting with JA-responsive transcription factors. Prior to JAZ identification, MYC2 was the only transcription factor known to have a direct role in promoting JA responses. Discovery of JAZ proteins led to rapid identification of novel transcription factors that regulate JA responses. MYC3 and MYC4 are two bHLH transcription factors closely related to MYC2 that interact with multiple JAZ proteins (Fernandez-Calvo et al., 2011). Domain mapping experiments indicate that MYC2/3/4 have an N-terminal JAZ-interacting domain (JID). Genetic analyses show that MYC3 and MYC4 play an important role in JA-mediated insect resistance, whereas MYC2 is mainly involved in regulating JA-mediated root growth inhibition.

Wounding or application of MeJA induces trichome formation (Yoshida et al., 2009). Trichome development is regulated by WD-repeat/bHLH/MYB complexes that involve a WD-repeat protein TRANSPARENT TESTA 1 (TTG1), three bHLH proteins (GLABRA3, GL3;

ENHANCER OF GLABRA3, EGL3; TRANSPARENT TESTA 8, TT8) and the MYB transcription factors MYB75 and GLABRA 1 (GL1). All three bHLH proteins contain a JID that interacts with at least eight distinct JAZ proteins (Qi et al., 2011). The two MYB proteins, MYB75 and GL1, interact with JAZ1, JAZ8 and JAZ11. GL1 also interacts with JAZ10. TTG1, on the other hand, does not reported interact with JAZ proteins. Interestingly, overexpression of JAZ1 abolishes formation of the WD-repeat/bHLH/MYB complex. Furthermore, *35S-JAZ1Δ3* transgenic lines show reduced JA-induced trichome formation and anthocyanin accumulation. These results collectively show that JAZ proteins interact with WD-repeat/bHLH/MYB complexes to disrupt the complex and repress trichome development.

In addition to bHLH transcription factors, JAZ proteins also interact with MYB transcription factors. MYB21 and MYB24 were identified as JAZ8-interactors via yeast two-hybrid screens (Song et al., 2011).Genetic analyses of MYB21 and MYB24 loss-of-function and gain-of-function lines revealed that MYB21 and MYB24 play a role in JA-regulated male fertility but not other JA responses such as insect resistance or anthocyanin accumulation.

To summarize, JAZ proteins interact with a wide array of transcription factors to regulate different JA signaling outputs. Understanding whether or not there are specificities for JAZ-TF interaction, and examining expression patterns of different JAZs and transcription factors will lead to greater understanding of how JAZ proteins regulate different JA responses.

4.8. Other JAZ-Interacting Proteins

JAZ proteins are important players in cross-regulation of JA and other signaling pathways. One example is JA and gibberellin (GA) cross-regulation through JAZ-DELLA

interaction. DELLA proteins function as transcriptional repressors that are rapidly degraded in the presence of GA (Dill et al., 2001). JAZ1 and JAZ9 interact with the DELLA protein REPRESSOR OF GAI-1 (RGA) (Hou et al., 2010; Yang et al., 2012). Interestingly, JA antagonizes the GA signaling pathway by delaying GA-induced DELLA degradation. Moreover, DELLAs compete with MYC2 for binding to JAZ1 to control the expression of MYC2-regulated target genes (Hou et al., 2010). *coi1* and JAZ9 overexpression mutant are hypersensitive to GA (Yang et al., 2012). These results collectively demonstrate that JA and GA crosstalk is regulated through JAZ-DELLA interaction, and that this may provide a way for plants to regulate the balance between growth and defense.

JAZ1 also interacts with ethylene-responsive transcription factors ETHYLENE INSENSITIVE 3 (EIN3) and its closest homolog EIN3-LIKE 1 (EIL1) (Zhu et al., 2011). EIN3 and EIL1 are transcription factors that are rapidly degraded in the absence of ethylene, and are stabilized in the presence of ethylene to regulate ethylene responses (An et al., 2010). JAZ proteins repress the function of EIN3 and EIL1 by recruiting a histone deacetylase HDA6 to repress EIN3/EIL1 activity (Zhu et al., 2011). These results suggest that EIN3/EIL1 responsive gene expression requires both ethylene and JA. This also indicates that JAZ proteins are important nodes to integrate JA and ethylene responses.

In summary, JAZ proteins interact with and modulate transcription factors in other hormone signaling pathways. JAZ binds to DELLA repressors to positively regulate GA responses, and interact with EIN3/EIL1 to repress ethylene responses. JAZ1 interaction with HDA6 also suggests an alternative mechanism for JAZ proteins to repress gene expression. It has become increasingly accepted that response to a particular signal is not the result of a single linear pathway, but integration of multiple signaling pathways. This phenomenon is referred as

"crosstalk" between signaling pathways (Vert and Chory, 2011). Crosstalk between different signaling pathways can be "direct" or "indirect", depending on whether or not there are shared components between different pathways that regulate a common signaling output (Vert and Chory, 2011). JAZ proteins have emerged as one of the best examples of direct crosstalk, in which JAZ proteins directly interact with signaling components in other pathways to regulate downstream responses.

4.9. Physiological Role of JAZ proteins

Although much has been revealed about the molecular mechanism of JAZ function, very little is known about the physiological role of individual JAZ proteins. Most *jaz* mutants reported to date have no obvious growth or development phenotypes, presumably due to functional redundancy of other JAZ protiens (Thines et al., 2007). Currently the only two JAZ loss-of-function lines that exhibit detectable phenotypes are described below. *jaz10-1* lines are hypersensitive to JA-induced root growth inhibition and *JAZ10* RNAi lines are also hypersensitive to JA-inhibited growth responses (Demianski et al., 2010; Yan et al., 2007). JAZ9, on the other hand, is involved in cross-regulating GA responses. *jaz9-1* and *jaz9-3* lines are late-flowering and have shorter petiole lengths, which are typical GA-insensitive phenotypes (Yang et al., 2012). Characterization of other *JAZ* loss-of-function mutants and higher-order mutants will provide more insight in how different JAZ proteins regulate diverse JA signaling outputs.

5. CURRENT MODEL FOR JA SIGNALING

The discovery and characterization of JAZ proteins has significantly sped up our understanding of the JA signaling pathway. Most importantly, JAZ proteins connected prior knowledge of the F-box protein COI1 to the transcription factor MYC2. The current JA signaling pathway is summarized in the following paragraph: (Figure 1.3)

In the absence of bioactive JA, JAZ proteins interact with multiple transcriptional regulators, including bHLH, MYB, and EIN3/EIL1, to repress JA-induced gene expression. JAZ proteins repress gene expression by recruiting the TOPLESS corepressor complex through the adapter protein NINJA. Upon wounding or other developmental cues that lead to accumulation of JA-Ile, JAZ proteins form receptor complexes with COI1 to perceive JA-Ile, and are further ubiquitinated and degraded through the 26S proteasome pathway. Degradation of JAZ proteins leads to activation of JA-responsive transcription factors, and induction of gene expression.

6. AIMS OF DISSERTATION

The main focus of this dissertation is to determine the molecular mechanism of JAZ function in response to JA. Firstly, I aim to determine whether or not all JAZ proteins interact with COI1 in a JA-Ile-dependent manner. I show that not all JAZ proteins interact with COI1 in the presence of JA-Ile, and JAZ8 is a weak COI1-interactor that is stable upon JA treatment. The results of this aim are described in Chapter 2 (Shyu et al., 2012). Another central aim is to understand the repression mechanism of JAZ8. I report that JAZ8 represses JA responses in a NINJA-independent pathway that is different from the NINJA-dependent pathway utilized by other JAZ proteins. Experimental results of this aim are illustrated in Chapter 2 (Shyu et al., 2012). Finally, I report identification and characterization of a novel JAZ protein, JAZ13, which

also represses JA responses via a NINJA-independent pathway. JAZ13 shares high sequence similarity to JAZ8, and groups with JAZ8 in the JAZ8 subclade of JAZ repressors. This research is described in Chapter 3. In summary, I show that the JAZ8 subclade of JAZ proteins consists of unique JAZ repressors that are stable in the presence of JA, and repress through NINJA-independent pathways to regulate JA responses.

FIGURES AND TABLES

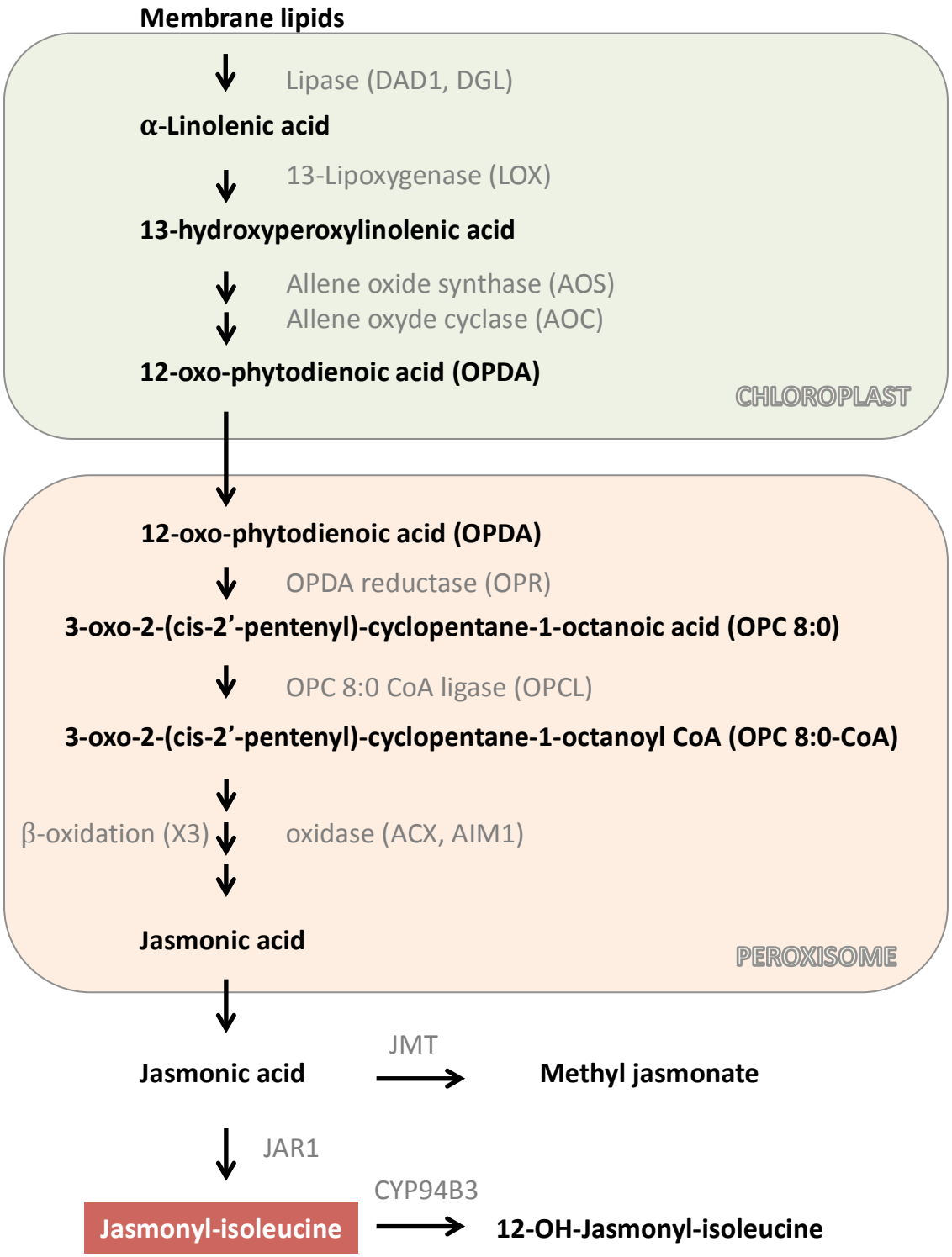

Figure 1.1. The Jasmonate Biosynthesis Pathway in Arabidopsis

Figure 1.1. The Jasmonate Biosynthesis Pathway in Arabidopsis – Continued.

For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

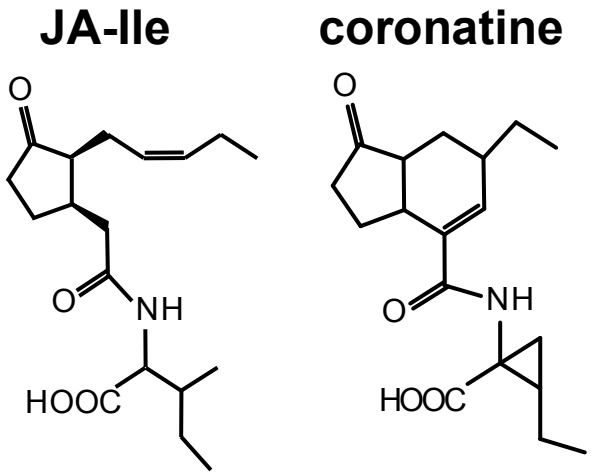

Figure 1.2. Chemical Structures of Jasmonyl-Isoleucine (JA-Ile) and Coronatine.

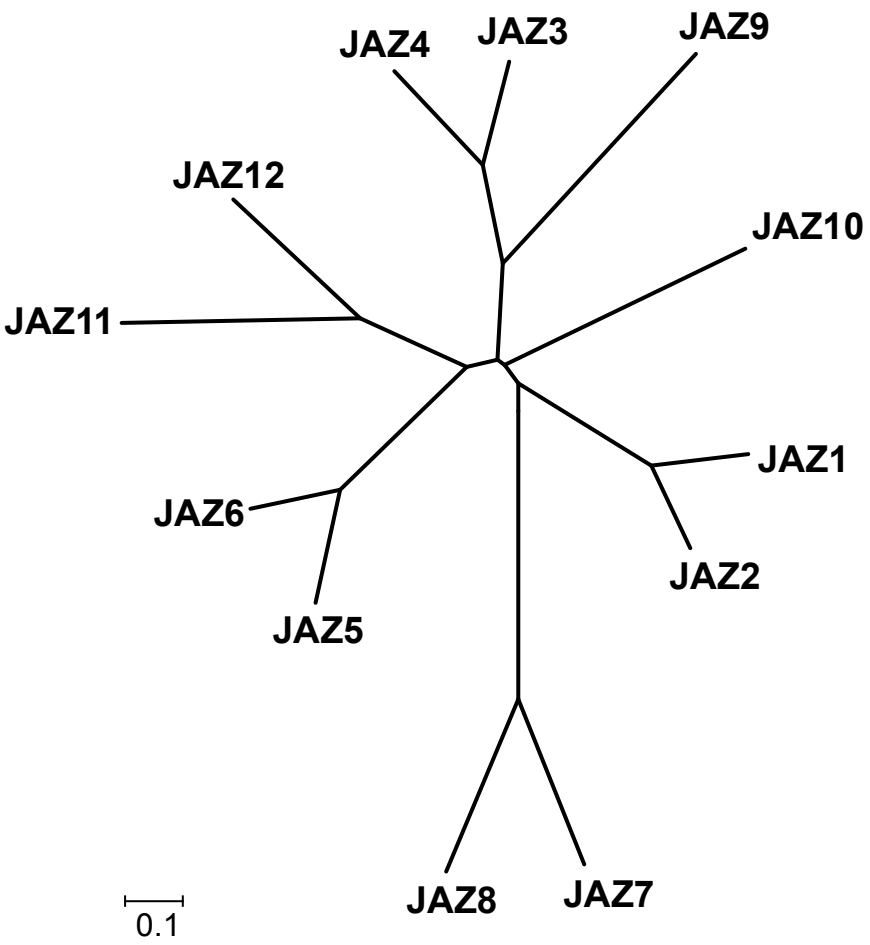

Figure 1.3. The JAZ Family of Proteins in Arabidopsis.

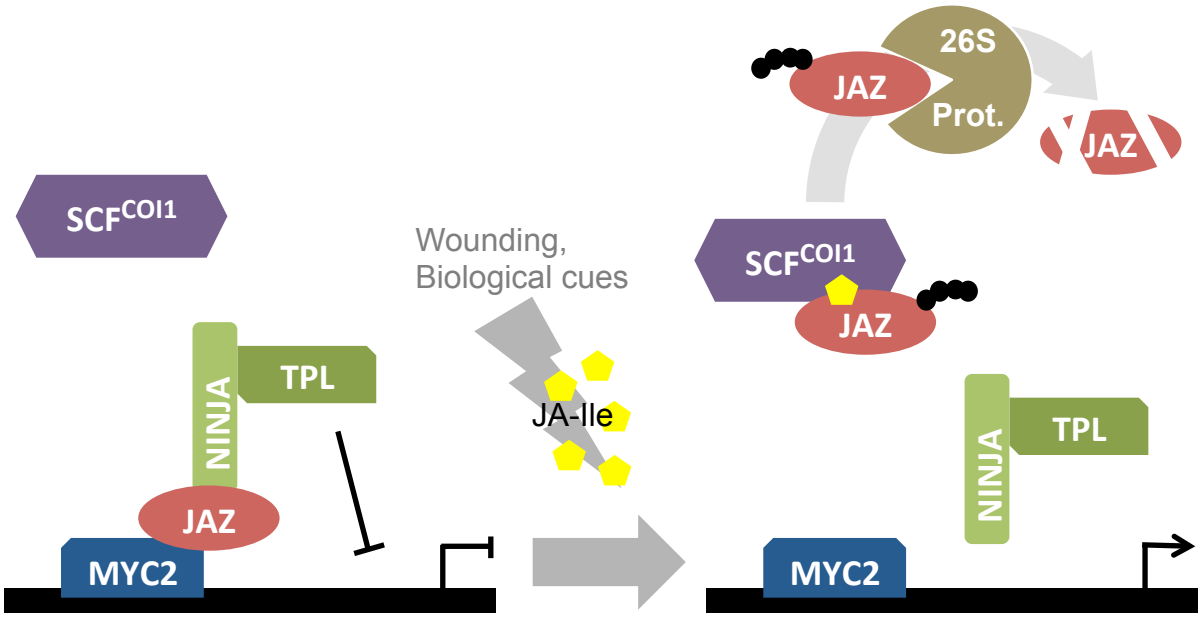

Figure 1.4. Current Jasmonate Signaling Model.

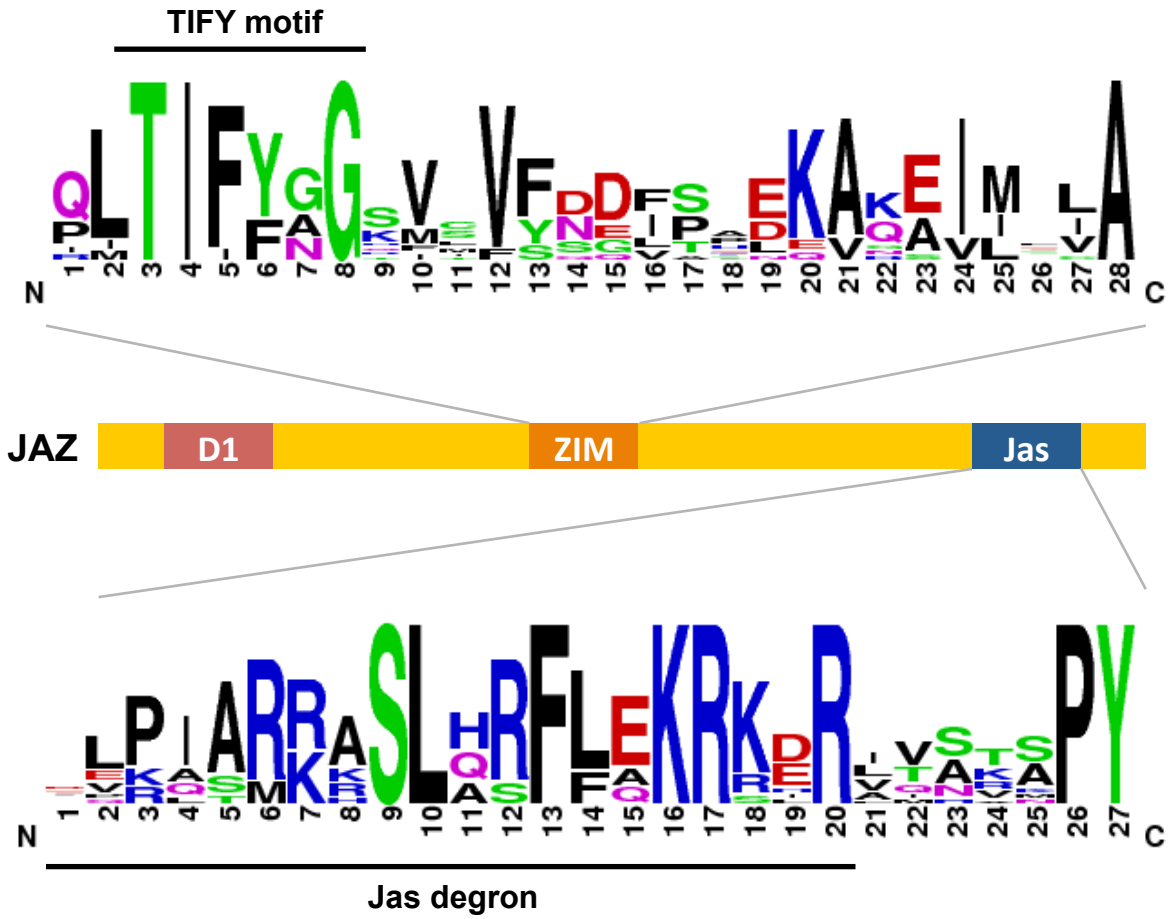

Figure 1.5. Domain Structure of JAZ Proteins.

Sequence logos of the conserved ZIM and Jas domains of 12 Arabidopsis JAZ proteins. Sequences used to generate the ZIM domain and Jas domain alignments were 28 and 27 amino acids, respectively, in length. The height of each stack of symbols indicates the sequence conservation at that position, whereas the height of symbols within the stack represents the relative frequency of the corresponding amino or nucleic acid at that position (Crooks et al., 2004).

REFERENCES

REFERENCES

Acosta IF, Laparra H, Romero SP, Schmelz E, Hamberg M, Mottinger JP, Moreno MA, Dellaporta SL (2009) tasselseed1 is a lipoxygenase affecting jasmonic acid signaling in sex determination of maize. Science **323**: 262–265

An F, Zhao Q, Ji Y, Li W, Jiang Z, Yu X, Zhang C, Han Y, He W, Liu Y, et al (2010) Ethylene-induced stabilization of ETHYLENE INSENSITIVE3 and EIN3-LIKE1 is mediated by proteasomal degradation of EIN3 binding F-box 1 and 2 that requires EIN2 in Arabidopsis. Plant Cell **22**: 2384–2401

Bai Y, Meng Y, Huang D, Qi Y (2011) Origin and evolutionary analysis of the plant-specific TIFY transcription factor family. Genomics **98**: 128–136

Bell E, Creelman RA, Mullet JE (1995) A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in Arabidopsis. Proc Natl Acad Sci U S A **92**: 8675–8679

Ben-Naim O, Eshed R, Parnis A, Teper-Bamnolker P, Shalit A, Coupland G, Samach A, Lifschitz E (2006) The CCAAT binding factor can mediate interactions between CONSTANS-like proteins and DNA. Plant J **46**: 462–476

Bender CL, Alarcón-Chaidez F, Gross DC (1999) Pseudomonas syringae phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. Microbiol Mol Biol Rev **63**: 266–292

Chen H, Jones AD, Howe GA (2006) Constitutive activation of the jasmonate signaling pathway enhances the production of secondary metabolites in tomato. FEBS Letters **580**: 2540–2546

Chini A, Fonseca S, Chico JM, Fernandez-Calvo P, Solano R (2009) The ZIM domain mediates homo- and heteromeric interactions between Arabidopsis JAZ proteins. The Plant Journal **59**: 77–87

Chini A, Fonseca S, Fernández G, Adie B, Chico JM, Lorenzo O, García-Casado G, López-Vidriero I, Lozano FM, Ponce MR, et al (2007) The JAZ family of repressors is the missing link in jasmonate signalling. Nature **448**: 666–671

Chung HS, Cooke TF, Depew CL, Patel LC, Ogawa N, Kobayashi Y, Howe GA (2010) Alternative splicing expands the repertoire of dominant JAZ repressors of jasmonate signaling. Plant J **63**: 613–622

Chung HS, Howe GA (2009) A Critical Role for the TIFY Motif in Repression of Jasmonate Signaling by a Stabilized Splice Variant of the JASMONATE ZIM-Domain Protein JAZ10 in Arabidopsis. Plant Cell **21**: 131–145

Chung HS, Niu Y, Browse J, Howe GA (2009) Top hits in contemporary JAZ: An update on

jasmonate signaling. Phytochemistry **70**: 1547–1559

Delker C, Zolman BK, Miersch O, Wasternack C (2007) Jasmonate biosynthesis in Arabidopsis thaliana requires peroxisomal β-oxidation enzymes – Additional proof by properties of pex6 and aim1. Phytochemistry **68**: 1642–1650

Demole E, Lederer E (1962) Isolement et détermination de la structure du jasmonate de méthyle, constituant odorant caractéristique de l'essence de jasmin - Demole - 2004 - Helvetica Chimica Acta - Wiley Online Library. Helvetica Chimica Acta

Dill A, Jung HS, Sun TP (2001) The DELLA motif is essential for gibberellin-induced degradation of RGA. Proc Natl Acad Sci U S A **98**: 14162–14167

Farmer EE, Ryan CA (1990) Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. Proc Natl Acad Sci U S A **87**: 7713–7716

Fernandez-Calvo P, Chini A, Fernandez-Barbero G, Chico J-M, Gimenez-Ibanez S, Geerinck J, Eeckhout D, Schweizer F, Godoy M, Franco-Zorrilla JM, et al (2011) The Arabidopsis bHLH Transcription Factors MYC3 and MYC4 Are Targets of JAZ Repressors and Act Additively with MYC2 in the Activation of Jasmonate Responses. Plant Cell **23**: 701–715

Feys B, Benedetti CE, Penfold CN, Turner JG (1994) Arabidopsis Mutants Selected for Resistance to the Phytotoxin Coronatine Are Male Sterile, Insensitive to Methyl Jasmonate, and Resistant to a Bacterial Pathogen. Plant Cell **6**: 751–759

Grunewald W, Vanholme B, Pauwels L, Plovie E, Inzé D, Gheysen G, Goossens A (2009) Expression of the Arabidopsis jasmonate signalling repressor JAZ1/TIFY10A is stimulated by auxin. EMBO Rep **10**: 923–928

Heitz T, Widemann E, Lugan R, Miesch L, Ullmann P, Désaubry L, Holder E, Grausem B, Kandel S, Miesch M, et al (2012) Cytochromes P450 CYP94C1 and CYP94B3 catalyze two successive oxidation steps of plant hormone Jasmonoyl-isoleucine for catabolic turnover. J Biol Chem **287**: 6296–6306

Hershko A, Ciechanover A (1998) The ubiquitin system. Annu Rev Biochem **67**: 425–479

Hou X, Lee LYC, Xia K, Yan Y, Yu H (2010) DELLAs Modulate Jasmonate Signaling via Competitive Binding to JAZs. CORD Conference Proceedings **19**: 884–894

Howe GA, Lightner J, Browse J, Ryan CA (1996) An octadecanoid pathway mutant (JL5) of tomato is compromised in signaling for defense against insect attack. Plant Cell **8**: 2067–2077

Ishiguro S, Kawai-Oda A, Ueda J, Nishida I, Okada K (2001) The DEFECTIVE IN ANTHER DEHISCIENCE gene encodes a novel phospholipase A1 catalyzing the initial step of jasmonic acid biosynthesis, which synchronizes pollen maturation, anther dehiscence, and flower opening in Arabidopsis. Plant Cell **13**: 2191–2209

Kagale S, Links MG, Rozwadowski K (2010) Genome-wide analysis of ethylene-responsive element binding factor-associated amphiphilic repression motif-containing transcriptional regulators in Arabidopsis. Plant Physiol **152**: 1109–1134

Kang J-HJ, Wang LL, Giri AA, Baldwin ITI (2006) Silencing threonine deaminase and JAR4 in Nicotiana attenuata impairs jasmonic acid-isoleucine-mediated defenses against Manduca sexta. Plant Cell **18**: 3303–3320

Katsir L, Schilmiller AL, Staswick PE, He SY, Howe GA (2008) COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. Proceedings of the National Academy of Sciences **105**: 7100–7105

Kim EH, Kim YS, Park S-H, Koo YJ, Choi YD, Chung Y-Y, Lee I-J, Kim J-K (2009) Methyl jasmonate reduces grain yield by mediating stress signals to alter spikelet development in rice. Plant Physiol **149**: 1751–1760

Kitaoka N, Matsubara T, Sato M, Takahashi K, Wakuta S, Kawaide H, Matsui H, Nabeta K, Matsuura H (2011) Arabidopsis CYP94B3 encodes jasmonyl-L-isoleucine 12-hydroxylase, a key enzyme in the oxidative catabolism of jasmonate. Plant Cell Physiol **52**: 1757–1765

Koo AJK, Cooke TF, Howe GA (2011) Cytochrome P450 CYP94B3 mediates catabolism and inactivation of the plant hormone jasmonoyl-L-isoleucine. Proceedings of the National Academy of Sciences **108**: 9298–9303

Koo AJK, Gao X, Jones AD, Howe GA (2009) A rapid wound signal activates the systemic synthesis of bioactive jasmonates in Arabidopsis. Plant J **59**: 974–986

Koo AJK, Howe GA (2009) The wound hormone jasmonate. Phytochemistry **70**: 1571–1580

Li C, Schilmiller AL, Liu G, Lee GI, Jayanty S, Sageman C, Vrebalov J, Giovannoni JJ, Yagi K, Kobayashi Y, et al (2005) Role of beta-oxidation in jasmonate biosynthesis and systemic wound signaling in tomato. Plant Cell **17**: 971–986

Li L, Li C, Howe GA (2001) Genetic analysis of wound signaling in tomato. Evidence for a dual role of jasmonic acid in defense and female fertility. Plant Physiol **127**: 1414–1417

Li L, Zhao Y, McCaig BC, Wingerd BA, Wang J, Whalon ME, Pichersky E, Howe GA (2004) The tomato homolog of CORONATINE-INSENSITIVE1 is required for the maternal control of seed maturation, jasmonate-signaled defense responses, and glandular trichome development. Plant Cell **16**: 126–143

Long JA, Woody S, Poethig S, Meyerowitz EM, Barton MK (2002) Transformation of shoots into roots in Arabidopsis embryos mutant at the TOPLESS locus. Development **129**: 2797–2806

Lorenzo OO, Chico JMJ, Sánchez-Serrano JJJ, Solano RR (2004) JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between

different jasmonate-regulated defense responses in Arabidopsis. Plant Cell **16**: 1938–1950

McConn M, Browse J (1996) The Critical Requirement for Linolenic Acid Is Pollen Development, Not Photosynthesis, in an Arabidopsis Mutant. Plant Cell **8**: 403–416

Melotto M, Mecey C, Niu Y, Chung HS, Katsir L, Yao J, Zeng W, Thines B, Staswick P, Browse J, et al (2008) A critical role of two positively charged amino acids in the Jas motif of Arabidopsis JAZ proteins in mediating coronatine- and jasmonoyl isoleucine-dependent interactions with the COI1 F-box protein. Plant J **55**: 979–988

Milrot E, Jackman A, Kniazhanski T, Gonen P, Flescher E, Sherman L (2012) Methyl jasmonate reduces the survival of cervical cancer cells and downregulates HPV E6 and E7, and survivin. Cancer Lett **319**: 31–38

Mosblech A, Thurow C, Gatz C, Feussner I, Heilmann I (2011) Jasmonic acid perception by COI1 involves inositol polyphosphates in Arabidopsis thaliana. Plant J **65**: 949–957

Nishii AA, Takemura MM, Fujita HH, Shikata MM, Yokota AA, Kohchi TT (2000) Characterization of a novel gene encoding a putative single zinc-finger protein, ZIM, expressed during the reproductive phase in Arabidopsis thaliana. Biosci Biotechnol Biochem **64**: 1402–1409

Pauwels L, Barbero GF, Geerinck J, Tilleman S, Grunewald W, Pérez AC, Chico J-M, Bossche RV, Sewell J, Gil E, et al (2010) NINJA connects the co-repressor TOPLESS to jasmonate signalling. Nature **464**: 788–791

Qi T, Song S, Ren Q, Wu D, Huang H, Chen Y, Fan M, Peng W, Ren C, Xie D (2011) The Jasmonate-ZIM-Domain Proteins Interact with the WD-Repeat/bHLH/MYB Complexes to Regulate Jasmonate-Mediated Anthocyanin Accumulation and Trichome Initiation in Arabidopsis thaliana. Plant Cell **23**: 1795–1814

Reinbothe S, Mollenhauer B, Reinbothe C (1994) JIPs and RIPs: the regulation of plant gene expression by jasmonates in response to environmental cues and pathogens. Plant Cell **6**: 1197–1209

Robson F, Costa MM, Hepworth SR, Vizir I, Piñeiro M, Reeves PH, Putterill J, Coupland G (2001) Functional importance of conserved domains in the flowering-time gene CONSTANS demonstrated by analysis of mutant alleles and transgenic plants. Plant J **28**: 619–631

Sanders PM, Lee PY, Biesgen C, Boone JD, Beals TP, Weiler EW, Goldberg RB (2000) The arabidopsis DELAYED DEHISCENCE1 gene encodes an enzyme in the jasmonic acid synthesis pathway. Plant Cell **12**: 1041–1061

Santner A, Calderon-Villalobos LIA, Estelle M (2009) Plant hormones are versatile chemical regulators of plant growth. Nature Chemical Biology **5**: 301–307

Schilmiller AL, Koo AJK, Howe GA (2007) Functional diversification of acyl-coenzyme A

oxidases in jasmonic acid biosynthesis and action. Plant Physiol **143**: 812–824

Schneider K, Kienow L, Schmelzer E, Colby T, Bartsch M, Miersch O, Wasternack C, Kombrink E, Stuible H-P (2005) A new type of peroxisomal acyl-coenzyme A synthetase from Arabidopsis thaliana has the catalytic capacity to activate biosynthetic precursors of jasmonic acid. J Biol Chem **280**: 13962–13972

Seo HS, Song JT, Cheong JJ, Lee YH, Lee YW, Hwang I, Lee JS, Choi YD (2001) Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses. Proc Natl Acad Sci U S A **98**: 4788–4793

Sheard LB, Tan X, Mao H, Withers J, Ben-Nissan G, Hinds TR, Kobayashi Y, Hsu F-F, Sharon M, Browse J, et al (2010) Jasmonate perception by inositol-phosphate-potentiated COI1-JAZ co-receptor. Nature **468**: 400–405

Song S, Qi T, Huang H, Ren Q, Wu D, Chang C, Peng W, Liu Y, Peng J, Xie D (2011) The Jasmonate-ZIM Domain Proteins Interact with the R2R3-MYB Transcription Factors MYB21 and MYB24 to Affect Jasmonate-Regulated Stamen Development in Arabidopsis. Plant Cell **23**: 1000–1013

Song WC, Brash AR (1991) Purification of an allene oxide synthase and identification of the enzyme as a cytochrome P-450. Science **253**: 781–784

Staswick PE, Tiryaki I (2004) The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in Arabidopsis. Plant Cell **16**: 2117–2127

Stevenson-Paulik JJ, Bastidas RJR, Chiou S-TS, Frye RAR, York JDJ (2005) Generation of phytate-free seeds in Arabidopsis through disruption of inositol polyphosphate kinases. Proc Natl Acad Sci U S A **102**: 12612–12617

Stintzi A, Browse J (2000) The Arabidopsis male-sterile mutant, opr3, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. Proc Natl Acad Sci U S A **97**: 10625–10630

Strayer C, Oyama T, Schultz TF, Raman R, Somers DE, Más P, Panda S, Kreps JA, Kay SA (2000) Cloning of the Arabidopsis clock gene TOC1, an autoregulatory response regulator homolog. Science **289**: 768–771

Szemenyei H, Hannon M, Long JA (2008) TOPLESS mediates auxin-dependent transcriptional repression during Arabidopsis embryogenesis. Science **319**: 1384–1386

Theodoulou FL, Job K, Slocombe SP, Footitt S, Holdsworth M, Baker A, Larson TR, Graham IA (2005) Jasmonic acid levels are reduced in COMATOSE ATP-binding cassette transporter mutants. Implications for transport of jasmonate precursors into peroxisomes. Plant Physiol **137**: 835–840

Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, Browse J (2007) JAZ repressor proteins are targets of the SCFCOI1 complex during

jasmonate signalling. Nature **448**: 661–665

Ueda JJ, Kato JJ (1980) Isolation and Identification of a Senescence-promoting Substance from Wormwood (Artemisia absinthium L.). Plant Physiol **66**: 246–249

Vanholme B, Grunewald W, Bateman A, Kohchi T, Gheysen G (2007) The tify family previously known as ZIM. Trends Plant Sci **12**: 239–244

Vert G, Chory J (2011) Crosstalk in cellular signaling: background noise or the real thing? Dev Cell **21**: 985–991

Vijayan P, Shockey J, Lévesque CA, Cook RJ, Browse J (1998) A role for jasmonate in pathogen defense of Arabidopsis. Proc Natl Acad Sci U S A **95**: 7209–7214

Xiao X-Y, Jiang G-S, Wang L, Lv L, Zeng F-Q (2011) Predominant enhancement of apoptosis induced by methyl jasmonate in bladder cancer cells: therapeutic effect of the Antp-conjugated Smac peptide. Anticancer Drugs **22**: 853–863

Xie DX, Feys BF, James S, Nieto-Rostro M, Turner JG (1998) COI1: an Arabidopsis gene required for jasmonate-regulated defense and fertility. Science **280**: 1091–1094

Yamane H, Takagi H, Abe H, Yokota T, Takahashi N (1981) Identification of Jasmonic Acid in Three Species of Higher Plants and Its Biological Activities. Plant Cell Physiol **22**: 689–697

Yan Y, Stolz S, Chételat A, Reymond P, Pagni M, Dubugnon L, Farmer EE (2007) A downstream mediator in the growth repression limb of the jasmonate pathway. Plant Cell **19**: 2470–2483

Yang D-L, Yao J, Mei C-S, Tong X-H, Zeng L-J, Li Q, Xiao L-T, Sun T-P, Li J, Deng X-W, et al (2012) Plant hormone jasmonate prioritizes defense over growth by interfering with gibberellin signaling cascade. Proc Natl Acad Sci U S A. doi: 10.1073/pnas.1201616109

Yoshida Y, Sano R, Wada T, Takabayashi J, Okada K (2009) Jasmonic acid control of GLABRA3 links inducible defense and trichome patterning in Arabidopsis. Development **136**: 1039–1048

Zhu Z, An F, Feng Y, Li P, Xue L, A M, Jiang Z, Kim J-M, To TK, Li W, et al (2011) Derepression of ethylene-stabilized transcription factors (EIN3/EIL1) mediates jasmonate and ethylene signaling synergy in Arabidopsis. Proc Natl Acad Sci U S A **108**: 12539–12544

Ziegler J, Stenzel I, Hause B, Maucher H, Hamberg M, Grimm R, Ganal M, Wasternack C (2000) Molecular cloning of allene oxide cyclase. The enzyme establishing the stereochemistry of octadecanoids and jasmonates. J Biol Chem **275**: 19132–19138

CHAPTER TWO
JAZ8 LACKS A CANONICAL DEGRON AND HAS AN EAR MOTIF THAT MEDIATES TRANSCRIPTIONAL REPRESSION OF JASMONATE RESPONSES IN ARABIDOPSIS

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C.S. and G.A.H. designed the project. P.F. designed and performed carrot protoplast assays. C.L.D., T.F.C., J.E.M., and L.K. performed pull down experiments. L.B.S. performed coronatine binding assays. C.S. performed all other experiments. All authors contributed to analysis and interpretation of results. C.S. and G.A.H. wrote the article with contributions and edits from P.F., L.B.S., N.Z., and J.B.

ABSTRACT

The lipid-derived hormone jasmonoyl-L-Ile (JA-Ile) initiates large-scale changes in gene expression by stabilizing the interaction of JASMONATE ZIM domain (JAZ) repressors with the F-box protein CORONATINE INSENSITIVE1 (COI1), which results in JAZ degradation by the ubiquitin-proteasome pathway. Recent structural studies show that the JAZ1 degradation signal (degron) includes a short conserved LPIAR motif that seals JA-Ile in its binding pocket at the COI1-JAZ interface. Here, we show that Arabidopsis thaliana JAZ8 lacks this motif and thus is unable to associate strongly with COI1 in the presence of JA-Ile. As a consequence, JAZ8 is stabilized against jasmonate (JA)-mediated degradation and, when ectopically expressed in Arabidopsis, represses JA-regulated growth and defense responses. These findings indicate that sequence variation in a hypervariable region of the degron affects JAZ stability and JA-regulated physiological responses. We also show that JAZ8-mediated repression depends on an LxLxL-type EAR (for ERF-associated amphiphilic repression) motif at the JAZ8 N terminus that binds the corepressor TOPLESS and represses transcriptional activation. JAZ8-mediated repression does not require the ZIM domain, which, in other JAZ proteins, recruits TOPLESS through the EAR motif– containing adaptor protein NINJA. These findings show that EAR repression domains in a subgroup of JAZ proteins repress gene expression through direct recruitment of corepressors to cognate transcription factors.

INTRODUCTION

The lipid-derived hormone jasmonoyl-L-isoleucine (JA-Ile) and structurally related jasmonates (collectively referred to as JAs) play an essential role in controlling plant growth, development, and responses to environmental stress. Among the major functions ascribed to JAs are activation of defense responses to insect attack and pathogen infection, reproductive development, and growth inhibition (Glazebrook, 2005; Wasternack, 2007; Howe and Jander, 2008; Browse, 2009). These general roles for the hormone suggest that JA signaling evolved as a mechanism to optimize plant fitness in rapidly changing terrestrial environments. This hypothesis is supported by emerging mechanistic evidence for extensive crosstalk between JA and other hormones that mediate developmental plasticity (Dombrecht et al., 2007; Moreno et al., 2009; Pieterse et al., 2009; Hou et al., 2010; Robson et al., 2010; Ballare, 2011; Kazan and Manners, 2011; Zhu et al., 2011). A greater understanding of the molecular mechanism of JA signaling is therefore expected to impact broad areas of plant biology (Howe, 2010; Kazan and Manners, 2012).

The discovery of JASMONATE ZIM-domain (JAZ) proteins spurred remarkable progress in understanding how JAs regulate large-scale changes in gene expression (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007; Pauwels and Goossens, 2011). JAZs belong to the plant-specific TIFY family of transcriptional regulators that are defined by the presence of a TIF[F/Y]XG motif within a larger (~28 amino acids) conserved region known as the ZIM (or TIFY) domain (Vanholme et al., 2007; Chung et al., 2009; Bai et al., 2011). JAZs are distinguished from other TIFY proteins by the presence of a multifunctional C-terminal region known as the Jas motif. Both the ZIM domain and Jas motif are required for JAZ-mediated repression of JA responses in cells containing low JA-Ile levels. The Jas motif interacts with

members of the bHLH (e.g., MYC2) and R2R3 MYB family of transcription factors that promote the expression of JA-response genes (Chini et al., 2007; Melotto et al., 2008; Cheng et al., 2011; Fernandez-Calvo et al., 2011; Niu et al., 2011; Qi et al., 2011; Song et al., 2011). The ZIM domain and its associated TIFY motif mediate JAZ interaction with an adaptor protein called NOVEL INTERACTOR OF JAZ (NINJA), which functions to recruit the transcription co-repessor TOPLESS (TPL) and TPL-related proteins (TPRs) (Pauwels et al., 2010; Pauwels and Goossens, 2011). As is the case for other proteins that repress transcription through TPL and associated chromatin remodeling enzymes (Ohta et al., 2001; Krogan and Long, 2009; Kagale and Rozwadowski), NINJA contains an EAR (ERF-associated amphiphilic repression) motif that is necessary and sufficient for TPL interaction (Pauwels et al., 2010). The ZIM domain also promotes homo- and heteromeric interactions between JAZ proteins, which in Arabidopsis are encoded by 12 genes (*JAZ1 – JAZ12*) (Chini et al., 2009; Chung and Howe, 2009; Chung et al., 2009). Alternative splicing of *JAZ* genes expands the repertoire and potential combinatorial diversity of JAZ-JAZ interactions (Yan et al., 2007; Chung and Howe, 2009; Chung et al., 2010). A current challenge is to determine the contribution of individual JAZ isoforms to the regulation of JA signaling.

JAZ-mediated repression is relieved in response to stimuli that activate the production of JA-Ile and subsequent degradation of JAZ proteins via the ubiquitin/26S proteasome pathway. JA-Ile initiates the signaling cascade by promoting interaction of JAZ with the F-box protein CORONATINE INSENSITIVE 1 (COI1). As the specificity determinant of the SCF (SKP1-CUL1-F-box)-type ubiquitin ligase SCF^{COI1}, COI1 binding to many JAZ proteins is stimulated directly by JA-Ile (Xie et al., 1998; Thines et al., 2007; Katsir et al., 2008b; Melotto et al., 2008; Fonseca et al., 2009; Yan et al., 2009). Recent structural studies revealed that the N-terminal 20

amino acids of the Jas motif adopt a bipartite structure consisting of a loop region that interacts with both COI1 and JA-Ile, and an α-helix that contacts the surface of COI1 adjacent to the ligand binding pocket (Sheard et al., 2010). Point mutations affecting conserved basic amino acids in the loop region of JAZ1 and JAZ9 abrogate COI1 binding, and also enhance the repression activity of ectopically expressed JAZ1 (Melotto et al., 2008). High-affinity ligand binding to the COI1-JAZ1 coreceptor complex also requires an inositol pentakisphosphate (IP5) cofactor that interacts with COI1 and JAZ near the hormone-binding pocket (Sheard et al., 2010).

Initial insight into the identity of JAZ proteins as repressors came from the observation that truncated JAZs lacking the Jas motif are resistant to JA-mediated degradation and, when expressed in Arabidopsis, confer dominant insensitivity to exogenous JA (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007). A role for JAZ10 as a negative regulator is supported by the phenotype of *jaz10* loss-of-function mutants, which exhibit increased sensitivity to JA (Yan et al., 2007; Demianski et al., 2011). The repressive function of *JAZ10* appears to result from the action of alternative splice variants that lack either a portion of the Jas motif (JAZ10.3) or the entire Jas motif (JAZ10.4) (Yan et al., 2007; Chung and Howe, 2009; Chung et al., 2010). These naturally occurring truncated JAZs have reduced capacity to interact with COI1 and thus are stabilized against hormone-dependent degradation (Chung and Howe, 2009). Ectopic expression of JAZ10.3 and JAZ10.4, which retain the ability to interact with MYC2, results in reduced sensitivity to JA. Transgenic lines overexpressing a *JAZ10* genomic clone also show reduced sensitivity to JA, presumably as a consequence of overproduction of JAZ10.3 and JAZ10.4 (Chung et al., 2010).

Although it is established that dominant repression by JAZ10 splice variants and artificially truncated JAZs results from decreased association with COI1, a role for full-length (i.e., Jas motif containing) JAZs as transcriptional repressors remains to be shown. For example, JA-insensitive phenotypes have not been observed in transgenic lines of Arabidopsis that overexpress full-length JAZ1, JAZ2, JAZ3, or JAZ10 (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007; Chung and Howe, 2009; Chung et al., 2010). Other than *jaz10*, the only *jaz* mutant reported to exhibit enhanced sensitivity to JA is an RNA interference (RNAi) line of *JAZ1* (Grunewald et al., 2009). This phenotype, however, was not observed in a T-DNA insertion mutant (*jaz1-1*) that was characterized as a likely null mutant (Demianski et al., 2011). The lack of obvious JA-hypersensitive phenotypes among most *jaz* mutants reported to date may result from functional redundancy between JAZ proteins. Determining the role of specific JAZ isoforms in transcriptional repression is needed to further understand how JAs control myriad responses during the plant life cycle.

Here, we establish a role for full-length JAZ8 as a transcriptional repressor, and describe two novel features of the protein that are critical for repression activity. First, we show that JAZ8 lacks the canonical degron that promotes JA-Ile-dependent interaction of other JAZ proteins with COI1. Second, we demonstrate that an LxLxL-type EAR motif near the N-terminus of JAZ8 mediates transcriptional repression. Significantly, JAZ8-mediated repression does not require the ZIM domain, which in other JAZs recruits TPL through the EAR motif of NINJA. Based on these findings, we propose that JAZ8 and other EAR motif-containing JAZ proteins directly recruit corepressors to transcription factors as a mechanism for repressing JA responses.

RESULTS

JAZ8 Weakly Associates with COI1 in the Presence of Known Receptor Ligands

We used in vitro pull-down assays to investigate the relationship between sequence diversity in the Jas motif and hormone-dependent binding of full-length JAZ proteins to COI1. A phylogenetic tree constructed from the 27-amino acid Jas motif of all 12 Arabidopsis JAZs divided the family into six groups: JAZ1/2; JAZ3/4/9; JAZ5/6; JAZ7/8; JAZ10.1; JAZ11/12 (Supplemental Figure 2.1). Representative full-length proteins from each group were expressed in *E. coli* as MBP-JAZ-6xHis fusions, and tested for their ability to interact with COI1 in the presence or absence of the active form of the hormone, (3*R*,7*S*)-JA-Ile. At a concentration of 1 μM, JA-Ile stimulated binding of COI1 to JAZ2.1, JAZ3.1, JAZ6, JAZ10.1, and JAZ12, but not JAZ8 (Figure 2.1A). Higher concentrations of JA-Ile increased the amount of COI1 binding to JAZ10.1, but failed to promote interaction with JAZ8 (Figure 2.1B). The phytotoxin coronatine, which is a potent agonist of the COI1-JAZ receptor system (Katsir et al., 2008b; Melotto et al., 2008; Yan et al., 2009; Sheard et al., 2010), stimulated low-level, dose-dependent binding of COI1 to JAZ8 (Figure 2.1C). Saturation binding assays confirmed that ^{3}H-coronatine binds specifically to COI1-JAZ8 receptor complexes, albeit with much lower affinity (91.4 nM) than that observed for COI1-JAZ10.1 complexes (7.0 nM) (Figure 2.1D).

JAZ8 Attenuates JA Responses and is Resistant to JA-mediated Degradation in Vivo

Truncated JAZ proteins that fail to interact with COI1 in the presence of JA-Ile can repress JA signaling when overexpressed in Arabidopsis (Thines et al., 2007; Melotto et al., 2008; Chung et al., 2010). To determine whether full-length JAZ8 exerts dominant repression in vivo, we

constructed transgenic lines of Arabidopsis that express the *JAZ8* cDNA under the control of the *Cauliflower Mosaic Virus* 35S promoter. T1 plants (24 independent lines) selected for the presence of the *35S*:*JAZ8* transgene did not exhibit obvious defects in flower development or fertility. Subsequently, homozygous lines (T3 generation) that were selected for high expression of the transgene (Materials and Methods) were tested in root growth inhibition assays. All *35S*:*JAZ8* lines (n = 5) selected in this manner were insensitive to JA-mediated root growth inhibition, and were significantly more insensitive to JA than the *myc2* mutant, *jin1-7* (Supplemental Figure 2) (Lorenzo et al., 2004). Dose-response experiments performed with a representative *35S*:*JAZ8* homozygous line (#24) showed that JAZ8 overexpression confers insensitivity to a broad range of concentrations of exogenous MeJA, but does not affect root growth in seedlings grown on medium lacking the compound (Figure 2.2A). Consistent with this finding, RNA blot analysis demonstrated that *35S*:*JAZ8* seedlings are impaired in the expression of the JA-responsive genes *VSP2* and *JAZ7* (Figure 2.2B). To test the effect of JAZ8 overexpression on JA-mediated defense responses, we compared the weight gain of *Spodoptera exigua* larvae grown on adult WT and *35S*:*JAZ8* plants. Insects reared on *35S*:*JAZ8* lines for either 9 or 14 d were significantly heavier (P<0.0001) than insects grown on the WT (Figure 2.2C and D). The increased mass of caterpillars grown on *35S*:*JAZ8* plants was associated with increased consumption of leaf tissue (Supplemental Figure 2.3). In summary, these results show that overexpression of full-length JAZ8 impairs JA-mediated root growth inhibition and defense responses, as well as JA-dependent gene expression.

To determine whether the JA-insensitive phenotype of *35S*:*JAZ8* plants is associated with increased JAZ8 stability in vivo, we used confocal laser-scanning microscopy to compare JA-mediated changes in fluorescence of JAZ8-YFP and JAZ10.1-YFP fusion proteins that were

stably expressed in Arabidopsis. Root growth inhibition assays showed that *35S*:*JAZ8-YFP* seedlings, similar to *35S*:*JAZ8* lines, exhibit reduced sensitivity to exogenous MeJA (Supplemental Figure 4A). In the absence of JA treatment, a nuclear-localized YFP signal was observed in both *35S*:*JAZ10.1-YFP* and *35S*:*JAZ8-YFP* seedlings (Figure 3A). Within 10 min of treatment with 50 μM MeJA, this signal was largely eliminated in *35S*:*JAZ10.1-YFP* seedlings, as previously reported (Chung and Howe, 2009). The nuclear-localized YFP signal in roots of *35S*:*JAZ8-YFP* seedlings persisted up to 30 min after MeJA treatment. Consistent with the ability of coronatine to associate with COI1-JAZ8 complexes in vitro (Figure 2.1), exogenous coronatine stimulated JAZ8-YFP turnover in vivo (Figure 2.3B). This effect was partially inhibited by pre-treatment of seedlings with the 26S proteasome inhibitor MG132. These findings show that JAZ8 is more resistant than JAZ10.1 to JA-mediated degradation in vivo, but can be degraded in response to ligands (i.e., coronatine) that promote COI1-JAZ8 association.

JAZ8 Lacks a Canonical JA-Ile Degron

We performed domain swap experiments to test whether the observed functional differences between JAZ8 and JAZ10.1 can be attributed to sequence variation in the Jas motif (Figure 2.4A). For this purpose, we generated constructs encoding chimeric proteins in which the Jas motif and remaining C-terminal amino acids (two and five amino acids for JAZ8 and JAZ10.1, respectively) of JAZ8 and JAZ10.1 were reciprocally exchanged as shown schematically in Figure 4B. In vitro pull-down assays performed with the resulting fusion proteins showed that the Jas motif of JAZ10.1 confers on JAZ8 the ability to interact with COI1 in the presence of 2.5 μM JA-Ile (Figure 2.4C). Moreover, binding of COI1 to the JAZ10-Jas8 chimera, in which the C-terminal region of JAZ10.1 is replaced by the Jas motif from JAZ8, was not stimulated by JA-

Ile. To determine whether these changes in hormone-dependent COI1 interaction impact JA-regulated physiological responses, we constitutively expressed the chimeric proteins in Arabidopsis. Seedlings that express the JAZ8-Jas10 chimera were fully sensitive to JA. Conversely, lines expressing JAZ10-Jas8 exhibited a JA-insensitive root growth phenotype that was indistinguishable from that of *35S*:*JAZ8* plants (Figure 2.4D). We conclude that the Jas motif of JAZ8 lacks sequence determinants that are required for JA-Ile-dependent binding to COI1.

The COI1-interacting degron of JAZ1 includes a six-amino acid (LPIARR) loop region that encloses JA-Ile in its binding pocket (Sheard et al., 2010). This sequence is largely conserved in other JAZs (e.g., JAZ10.1) that strongly interact with COI1 in the presence of JA-Ile (Figure 4A). The sequence (PKASMK) of the corresponding region in JAZ8 differs markedly from the canonical LPIARR motif. To determine whether this variation is responsible for the weak COI1-JAZ8 association observed in vitro (Figure 2.1), we constructed a modified version ($JAZ8^{LPIAR}$) of JAZ8 in which five amino acids (PKASM) in the loop region were replaced with the canonical LPIAR. In vitro pull-down assays showed that this substitution is sufficient to confer JA-Ile-dependent interaction of JAZ8 with CO11 (Figure 2.5A). Consistent with this finding, the PKASM→LPIAR substitution completely suppressed the JA-insensitive root growth phenotype of *35S*:*JAZ8* seedlings (Figure 2.5B). The ability of JAZ8 to interact with MYC2 (Chini et al., 2009) raised the possibility that the PKASM→LPIAR substitution impairs this interaction, which could account for the JA-sensitive phenotype of *35S*:$JAZ8^{LPIAR}$ seedlings. Yeast two-hybrid assays showed that $JAZ8^{LPIAR}$, like JAZ8, interacts with MYC2, thus excluding this possibility (Supplemental Figure 2.5). Fluorescence microscopy showed that a $JAZ8^{LPIAR}$-YFP fusion protein accumulates in the nucleus (Figure 2.5C), further indicating that

the substitution does not impair expression or localization of the protein. Imaging of *35S*:*JAZ8-YFP* and *35S*:*JAZ8*^{LPIAR}*-YFP* seedlings treated with MeJA (for 15 min) demonstrated that JAZ8^{LPIAR}-YFP is more sensitive than JAZ8-YFP to JA-mediated degradation. The ability of MG132 to block JA-mediated turnover of JAZ8^{LPIAR}-YFP showed that degradation of JAZ8^{LPIAR} is dependent on the 26S proteasome. We conclude that the non-canonical degron sequence (PKASM) in JAZ8 does not effectively interact with COI1 in a JA-Ile-dependent manner, and that this sequence is important for JAZ8's function as a dominant repressor of JA signaling.

These results led us to investigate whether JAZ proteins in other plant species have a JAZ8-like Jas motif. Phylogenetic analyses indicate that angiosperm JAZs cluster into five groups, including those represented by JAZ7/8 (group IV) and JAZ10 (group III) (Bai et al., 2011). Alignment of the predicted Jas motif in group IV proteins from diverse plant species showed that Ser and Met residues within the PKASM motif are highly conserved in other members of this group, whereas the PKA sequence is more variable (Figure 2.6A). Comparison of this motif with a consensus Jas motif constructed from JAZ10 orthologs showed that these two groups of JAZs differ markedly in the N-terminal degron sequence, but not in other regions of the Jas motif (Figure 2.6A).

An EAR Motif in JAZ8 Mediates Transcriptional Repression

Previous genome-wide sequence searches identified an LxLxL-type EAR motif in Arabidopsis JAZ8 and its close relative, JAZ7 (Kagale et al., 2010). This sequence (LELRL) is located close to the N-terminus of JAZ8 and is highly conserved among group IV JAZs from diverse plant species (Figure 6B) (Bai et al., 2011). To investigate the functional relevance of the motif in JAZ8-mediated repression of JA responses, we generated transgenic lines that overexpress a

mutant version (JAZ8ΔEAR) of JAZ8 in which the LELRL sequence was deleted. Root growth assays performed with multiple homozygous *35S:JAZ8ΔEAR* lines showed that deletion of the EAR motif suppresses the JA-insensitive phenotype conferred by JAZ8 overexpression (Figure 2.6C). Similar results were obtained with lines expressing YFP-tagged JAZ8 proteins in which the LELRL motif was deleted (JAZ8ΔEAR-YFP) or substituted with Ala residues (JAZ8$^{LELRL\rightarrow 5xA}$-YFP) (Supplemental Figure 2.4B). Fluorescence microscopy showed that these JAZ8-YFP derivatives are expressed in these lines and, like JAZ8-YFP, accumulate in the nucleus (Supplemental Figure 2.6). These results support the hypothesis that the conserved LELRL motif performs a critical role in JAZ8-mediated inhibition of JA responses.

We next used a heterologous carrot protoplast transfection system (Tiwari et al., 2004) to test directly whether JAZ8 functions as a transcriptional repressor. This assay relied on the use of a *GUS* (ß-glucuronidase) reporter gene [*35S(-46)LexA(2x)-Gal4(2x):GUS*] that is driven by a 35S minimal promoter harboring LexA and Gal4 DNA-binding sites. The reporter was cotransfected with two effector constructs: one encoding a fusion protein (LD-VP16) consisting of the LexA DNA binding domain (LD) fused to the VP16 activation domain, and a second effector encoding the Gal4 DNA binding domain (GD) fused to either full-length JAZ8 (GD-JAZ8) or the N-terminal region of JAZ8 (GD-JAZ8-N). Cotransfection of the *GUS* reporter with LD-VP16 and a construct encoding only GD resulted in strong activation (~13-fold) of the reporter in comparison to the GD construct alone (Figure 2.7A). The EAR motif repression domain of IAA17 was used as a positive control for transcriptional repression; cotransfection of this effector (GD-IAA17[I]) resulted in strong reduction in reporter gene expression, as previously reported (Tiwari et al., 2004). GD-JAZ8 repressed LD-VP16-mediated transcription of the reporter to a level similar to that obtained with GD-IAA17[I]. Cotransfection with GD-

JAZ-N, which encodes the EAR motif-containing N-terminal 44 amino acids of JAZ8, also resulted in strong repression (Figure 2.7A). These findings show that JAZ8 directly represses a transcriptional activator, and that the repression activity resides within the N-terminal 44 amino acids of JAZ8.

Ala-scanning mutagenesis was used to determine whether the LELRL motif of JAZ8 is required for transcriptional repression. Individual Ala substitutions within the LELRL motif resulted in partial or complete loss of repression by GD-JAZ-N in the protoplast transfection assay (Figure 2.7B). The effect of mutating any one of the three Leu residues was stronger than that of mutating the intervening hydrophilic residues. The Leu→Ala substitution completely impaired GD-JAZ8-N repression activity, suggesting that this residue is important for recruitment of co-repressors. Similar findings were reported for the characterization of EAR motifs in SUPERMAN and IAA17 repressors (Hiratsu et al., 2003; Tiwari et al., 2004).

Mechanism of Repression by JAZ8

We used the yeast two-hybrid (Y2H) system to investigate how interaction of JAZ8 with other components of the JA signaling module may contribute to the mechanism of JAZ8-mediated repression. We first investigated the interaction of JAZ8 with the bHLH transcription factor MYC2, which plays a major role in controlling JA-dependent root growth inhibition (Lorenzo et al., 2004; Fernandez-Calvo et al., 2011). Full-length JAZ8 interacted with MYC2 (Figure 2.8A), as previously reported (Chini et al., 2009). A JAZ8ΔJas construct lacking the Jas motif did not bind MYC2 but maintained the ability to heterodimerize with JAZs. Moreover, a 36-amino acid C-terminal fragment containing the Jas motif was sufficient for binding of MYC2.

Consistent with previous Y2H studies (Pauwels et al., 2010; Arabidopsis Interactome Mapping Consortium, 2011), full-length JAZ8 interacted with TPL but not with NINJA (Figure 8B). Deletion of the ZIM domain abolished JAZ8 heterodimerization with JAZ1 and all other JAZs tested (Supplemental Figure 2.7), but did not affect the interaction with MYC2 or TPL. Removal of the LELRL motif (JAZ8ΔEAR construct), or substitution of this sequence with five Ala residues (JAZ8$^{LELRL\rightarrow 5xA}$), eliminated the interaction with TPL but did not affect binding to JAZ1 or MYC2 (Figure 2.8B). A construct (JAZ8ΔEZ) that lacks both the EAR and ZIM domains interacted with MYC2 but not with TPL or JAZ1. These collective results show that the EAR, ZIM, and Jas motifs mediate JAZ8 interaction with TPL, other JAZ proteins, and MYC2, respectively.

Pauwels et al (2010) showed that although JAZ8 does not interact with NINJA in the Y2H system, the two proteins do interact in pull-down assays performed with GFP-tagged NINJA expressed in planta. We used a yeast-expressed, HA-tagged NINJA derivative to re-examine this question. Whereas JAZ8-His and JAZ10.1-His recovered comparable amounts of HA-JAZ1, the amount of HA-NINJA recovered by JAZ8-His was much less than that associated with JAZ10.1-His (Figure 2.8C). The amount of HA-NINJA bound by JAZ8-His, however, was consistently greater than that recovered by MBP-His alone. This finding, together with the results of Pauwels et al (2010), raised the possibility that JAZ8-mediated repression may depend in part on recruitment of TPL via JAZ8's ZIM domain. To test this hypothesis, we constructed transgenic lines (*35S:JAZ8ΔZIM*) that overexpress JAZ8ΔZIM. Multiple independent T1 lines showing high transgene expression were selected for use in root growth inhibition assays. The mean root length ratio (0.72 ± 0.01; n=127) exhibited by seedlings from eight independent homozygous *35S:JAZ8ΔZIM* lines was not significantly different from that of *35S:JAZ8*

seedlings (0.70 ± 0.04; *n*=23) (Figure 2.9A). As an additional control, we constructed transgenic lines that express the JAZ8ΔEZ construct (Figure 2.8B) in which both the EAR and ZIM motifs of JAZ8 are deleted. Eleven independent lines that express the *35S:JAZ8ΔEZ* transgene to high levels were selected for root growth inhibition assays. The mean root length ratio of seedlings from all lines was not different (P-value = 0.62, Student's t-test) from that of WT seedlings (Figure 2.9B), indicating that overexpression of JAZ8ΔEZ does not significantly repress JA signaling. These results show that the ZIM domain is not required for repression of JA-mediated root growth inhibition by ectopically expressed JAZ8.

DISCUSSION

Sequence Diversity in the JAZ Degron Modulates JA Responsiveness

Our results provide new insight into sequence determinants within the Jas motif that promote hormone-dependent interaction of JAZ proteins with COI1. Recent structural studies showed that the JAZ1 degron is located in the N-terminal region of the Jas motif, and includes a hexapeptide motif (LPIARR) that seals JA-Ile at the COI1-JAZ1 interface (Sheard et al., 2010). The C-terminal end (ARR) of the motif makes direct contact with JA-Ile, whereas N-terminal residues (LPI) clamp down on the COI1 surface to trap the hormone in place. These structural studies are supported by genetic evidence showing that mutations in the C-terminal basic residues (RR) result in dominant repression of JA responses by JAZ1 (Melotto et al., 2008). Here, we show that the LPIARR sequence is largely conserved in JAZ proteins (including JAZ2, JAZ3, JAZ9, JAZ10.1, and JAZ12) that strongly interact with COI1 in the presence of JA-Ile. This finding is

consistent with the rapid JA-mediated turnover of this group of JAZs (Chini et al., 2007; Thines et al., 2007; Chung and Howe, 2009; Grunewald et al., 2009; Pauwels et al., 2010).

The importance of the LPIARR motif as a JAZ degradation signal is highlighted by the finding that JAZ8, which lacks this motif, does not readily associate with COI1 in the presence of JA-Ile. The weak COI1-JAZ8 interaction was associated with increased stability of JAZ8 in JA-treated cells and the ability of JAZ8 to repress JA responses in *35S:JAZ8* plants. Domain swap and site-directed mutagenesis experiments demonstrated that these unique features of JAZ8 are attributed to sequence variation in the degron region; replacement of PKASM in the Jas motif of JAZ8 with LPIAR conferred JA-Ile-dependent interaction with COI1, and also decreased the stability of JAZ8. The PKASM→LPIAR substitution also suppressed the JA-insensitive phenotype of *35S:JAZ8* plants, thus establishing a direct link between sequence variation in the ligand-contacting region of the degron and JA signal output. These findings show that the LPIARR motif functions in vivo as a critical part of the JAZ degron, thus confirming and extending our previous structural studies (Sheard et al., 2010).

The central ($SLX_2FX_2KRX_2R$) and C-terminal (X_5PY) regions of the Jas motif are highly conserved among all JAZs, whereas the N-terminal amino acids corresponding to the degron loop are much more variable (Figure 2.6A) (Chung et al., 2009; Sheard et al, 2010). Our work on JAZ8 indicates that sequence variation in the N-terminus reflects the existence of functionally distinct JAZ subgroups that differ in their stability. These subgroups include the canonical LPIAR(R/K)-containing JAZs (e.g., JAZ1) that interact robustly with COI1 in the presence of JA-Ile, and X_3SMK-containing JAZs such as JAZ8 that do not strongly associate with COI1 under these conditions. The degron loop region of JAZ5 and JAZ6 (Figure 2.4A) is unusual in that it contains the canonical C-terminal residues (IARR) that contact JA-Ile (Sheard et al.,

2010), but lacks the N-terminal residues (LP) that clamp the hormone in the binding pocket. Interestingly, this sequence variation correlates with the reduced capacity of JAZ6 to recover COI1 in pull-down assays (Figure 2.1A). Differential association of JAZ isoforms with COI1, mediated by sequence variation in the degron, may enable plants to perceive and respond appropriately to a wide dynamic range of JA-Ile. Our results support a scenario in which the intracellular level of JA-Ile dictates which JAZs in a given cell associate with COI1. It is likely, for example, that some JAZs are recruited to COI1 in response to low JA-Ile concentrations, whereas other JAZ associate with COI1 only in the presence of high JA-Ile levels. Sequence variation in the auxin degron has also been shown to contribute to the diversification of Aux/IAA proteins (Dreher et al., 2006; Sato and Yamamoto, 2008), further highlighting the conserved nature of the COI1-JAZ and TIR1-Aux/IAA receptor systems (Katsir et al., 2008a).

The mechanisms by which JAZ8 and other stable JAZs are removed from cells remain unknown. One hypothesis is that stable JAZs are eliminated via COI1-independent proteolytic pathways, perhaps involving other F-box proteins. However, the ability of coronatine to destabilize JAZ8 in vivo, together with the inhibitory effect of MG132 on this process, suggests that JAZ8 can be degraded by a pathway involving COI1 and the 26S proteasome. Given that coronatine functions as a potent agonist of the JA-Ile receptor, it is possible that JAZ8 is slowly degraded by a COI1-dependent route in the presence of very high intracellular levels of JA-Ile. Our results also raise the possibility that recruitment of JAZ8 to COI1 is mediated by a small molecule other than (3*R*,7*S*)-JA-Ile. The ability of JAZ8 to associate with COI1 in the presence of coronatine demonstrates that JAZ8 is capable of interacting with COI1 in a ligand-dependent manner. Although we cannot exclude the possibility that this interaction is mediated by a cryptic COI1-binding site located outside the Jas motif, the most straightforward interpretation is that the

non-canonical loop region of JAZ8 is a weak substrate for coronatine-triggered COI1 association. The putative degron loop of JAZ8 contains a KK motif (Figure 4A) that may partially fulfill the function of the critical dibasic motif (R205R206) in JAZ1, which interacts directly with the carboxyl group of the ligand at the bottom of the binding pocket (Sheard et al., 2010). Among the small molecules that could potentially promote COI1-JAZ8 association are metabolic precursors or derivatives of JA-Ile. Several studies have provided evidence that the JA-Ile precursors 12-oxo-phytodienoic acid and jasmonic acid elicit JA-related responses without their prior conversion to JA-Ile (Hopke et al., 1994; Blechert et al., 1999; Miersch et al., 1999; Stintzi et al., 2001; Ribot et al., 2008; Wang et al., 2008). To date, however, the only naturally occurring JA derivatives known to promote COI1-JAZ binding are JA-Ile, structurally related JA-amino acid conjugates (e.g., JA-Leu), and 12-hydroxy-JA-Ile (Thines et al., 2007; Katsir et al., 2008b; Melotto et al., 2008; Chini et al., 2009; Koo et al., 2011).

The expression pattern of *JAZ8* in WT plants provides insight into the physiological role of JAZ8 as a repressor of JA responses. The most striking aspect of *JAZ8* expression is its rapid and strong induction in response to exogenous JA and other stress-related cues that activate the pathway (Figure 2B; Thines et al., 2007; Chini et al., 2007; Chung et al., 2008). In mature Arabidopsis leaves, for example, *JAZ8* and its paralog, *JAZ7*, are highly expressed in response to mechanical wounding and infection with coronatine-producing strains of *P. syringae* (Chung et al., 2008; Koo et al., 2009; Demianski et al., 2011). The enhanced expression of *JAZ8* in wounded leaves of a *cyp94b3* mutant that hyperaccumulates JA-Ile further suggests that expression of JAZ8 is promoted by high endogenous levels of JA-Ile (Koo et al., 2011). Given that the repressive activity of JAZ8 is dominant and persists in the presence of JA-Ile, stress-induced expression of JAZ8 is expected to result in attenuation of JA responses, consistent with

the JA-insensitive phenotype exhibited by *35S:JAZ8* plants. We therefore propose that JAZ8, like the alternative splice variants of JAZ10 (Yan et al., 2007; Chung and Howe, 2009; Chung et al., 2010), is a component of a JA-triggered negative feedback loop to prevent runaway activation of JA responses that could lead to inhibition of plant growth and reduced fitness (Zhang and Turner, 2008).

Repression of JA Responses by EAR Motif-Containing JAZ Proteins

The recent identification of NINJA provided an important advance in understanding how JAZ proteins repress JA-dependent transcriptional responses (Pauwels et al., 2010). NINJA negatively regulates the expression of JA response genes by bridging the ZIM domain of target JAZs to members of the TPL family of corepressors. As is the case for other TPL-interacting proteins (Szemenyei et al., 2008; Krogan and Long, 2009), NINJA contains an EAR motif that functions to recruit TPL. Our results indicate that JAZ8 employs a distinct and more direct mechanism to repress JA-dependent transcription; through its N-terminal EAR and C-terminal Jas motifs, we propose that JAZ8 provides a direct link between TPL and cognate transcription factors. Three independent lines of evidence demonstrate a key role for the EAR motif in JAZ8-mediated repression. First, we show that JAZ8 interacts with TPL in yeast and that a canonical LxLxL-type EAR motif at the N-terminus of JAZ8 is required for this interaction. These findings are in agreement with a recent large-scale Y2H study in which TPL was shown to interact with JAZ8 (Arabidopsis Interactome Mapping Consortium, 2011). Second, we demonstrate that the EAR motif is required for JAZ8-mediated repression of JA-dependent root growth inhibition in *35S:JAZ8* plants. Finally, we show that JAZ8 represses VP16-mediated transcriptional activation in a protoplast transfection assay and, moreover, that the EAR motif is important for the

mechanism of repression. To our knowledge, this is the first direct demonstration that a full-length JAZ protein functions as a transcriptional repressor.

A key role for the EAR motif in JAZ8-mediated repression is supported by our finding that *35S:JAZ8* and *35S:JAZ8ΔZIM* lines exhibit a similar level of insensitivity to JA. This finding shows that the ZIM domain is not strictly required for repression by ectopically expressed JAZ8, thus suggesting that JAZ8 action under these conditions depends neither on NINJA nor on interaction with other JAZs. Such a ZIM/TIFY-independent mechanism of repression differs from that of JAZ10.4, whose function as a dominant repressor is blocked by point mutations within the ZIM domain (i.e., TIFY motif) that disrupt JAZ10.4 homo- and heterodimerization (Chung and Howe, 2009). In this context, JAZ8ΔZIM appears to define the minimal domain architecture of a JAZ repressor, which in this case consists only of an EAR repression domain and a transcription factor-binding Jas motif. Given the ability of JAZ8 to interact with NINJA in pull-down assays (Pauwels et al., 2010; this study), we cannot exclude the hypothesis that JAZ8 is capable of repressing gene expression through both a NINJA-independent pathway involving direct binding of TPL/TPR to JAZ8's EAR motif, as well as a NINJA-dependent pathway in which the ZIM domain of JAZ8 recruits TPL/TPR via NINJA. Our collective results, however, favor the hypothesis that JAZ8 represses gene expression mainly via a direct, NINJA-independent mechanism.

The bHLH transcription factor MYC2 plays a major role in JA-mediated root growth inhibition (Fernandez-Calvo et al., 2011). We found that the JA-insensitive root growth phenotype of JAZ8-overexpressing plants is stronger than that of a *myc2* mutant (*jin1-7*). This finding, together with the fact that JAZ8 physically associates with MYC2 (Cheng et al., 2011; Fernandez-Calvo et al., 2011) (this study), suggests that JAZ8 targets MYC2 for repression.

JAZ8 also interacts with the MYC2-related proteins MYC3 and MYC4, which serve a minor but significant role in activating JA responses in the root (Cheng et al., 2011; Fernandez-Calvo et al., 2011; Niu et al., 2011), as well as MYB-type transcription factors (MYB21 and MYB24) implicated in JA-regulated male fertility (Song et al., 2011). In contrast to male sterile phenotype of transgenic lines that overexpress various truncated JAZ proteins (Thines et al., 2007; Melotto et al., 2008; Chung and Howe, 2009), we did not observe reproductive defects associated with overexpression of JAZ8. Identification of transcription factors and physiological processes that are regulated by endogenous JAZ8 is an important question for future studies.

Genome-wide sequence surveys showed that JAZ5, JAZ6, and JAZ7 also contain predicted EAR motifs (Kagale et al., 2010). Moreover, proteome-wide mapping of the Arabidopsis interactome identified JAZ5 and JAZ6 as TPL-binding proteins in yeast (Arabidopsis Interactome Mapping Consortium, 2011; Causier et al., 2012). It thus seems likely that repression of JA-responsive genes through direct JAZ-TPL coupling is not unique to JAZ8. Several reported features of JAZ7 are reminiscent of JAZ8, including the N-terminal location of the EAR motif and the ability to interact with MYC-related transcription factors but not NINJA (Pauwels et al., 2010; Cheng et al., 2011; Fernandez-Calvo et al., 2011). JAZ5 and JAZ6 both interact with NINJA but are distinct in that they each contain two predicted EAR motifs in the central and C-terminal regions of the protein (Kagale et al., 2010; Pauwels et al., 2010; Fernandez-Calvo et al., 2011). These observations support our general conclusion that different subgroups of JAZ employ distinct domain architectures to recruit corepressors to cognate transcription factors. Future work aimed at delineating the mechanisms and evolutionary origins of NINJA-dependent and NINJA-independent JAZ repression pathways should help to unravel the complexities of JA biology.

MATERIAL AND METHODS

Plant Material & Growth Conditions

Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used as the wild-type (WT) parent for all experiments. Plants were grown in soil under controlled conditions and were transformed with *Agrobacterium tumefaciens* as previously described (Chung et al., 2008; Chung and Howe, 2009). *35S:JAZ10.1* and *35S:JAZ10.1-YFP* transgenic lines were described previously (Chung and Howe, 2009). The *jin1-7* mutant (SALK_040500) was previously described (Lorenzo et al., 2004). Lines that express transgenes to high levels were identified as follows. Seedlings (T_1 generation) of transformed lines were screened on MS agar medium containing sucrose (0.8%), kanamycin (50 μg/mL), and vancomycin (100 μg/mL). For each construct, 32 independent T_1 plants were transferred to soil for evaluation of transgene expression level by Northern blot analysis. RNA isolated from untreated leaf tissue was hybridized with a *JAZ8* cDNA as a probe as previously described (Chung et al., 2008). Selected high-expressing lines (typically five per construct) were further propagated for identification of homozygous T_3 lines. Lines expressing JAZ8-YFP fusion proteins were screened at the seedling stage by fluorescence microscopy (Axio Scope, Carl Zeiss).

Plant Treatments

The effect of exogenous JA on root growth inhibition was determined as described previously (Chung and Howe, 2009), with minor modifications. Surface-sterilized and stratified seeds were sown on square petri plates containing MS agar medium (0.8% agar, Caisson Labs) supplemented with 0.8% sucrose (w/v) and the indicated concentration of MeJA (Sigma-Aldrich). WT and transgenic lines were grown on the same plate to control for plate-to-plate

variation. Petri plates were incubated vertically in a growth chamber maintained at 21°C and continuous light, unless otherwise indicated. Following eight d of growth, the length of primary roots was measured using Image J software (http://rsbweb.nih.gov/ij/index.html). Root length ratios (% inhibition by MeJA) were calculated by dividing the average the root length of seedlings grown on MeJA-containing medium by the average root length of seedlings of the same genotype grown in the absence of MeJA.

Spodoptera exigua eggs were obtained from Benzon Research and hatched at 30°C. Newly hatched larvae were transferred to fully expanded rosette leaves of 5-week-old WT and *35S:JAZ8* transgenic (line 24) plants. Eight larvae were reared on each of 12 plants per genotype. Insect-challenged and unchallenged control plants were maintained under standard growth conditions (Chung et al., 2008). Larval weights were measured at various times after challenge, and weighed larvae were returned to their plant of origin. The experiment was independently repeated four times with similar results. Northern blot analysis of RNA isolated from liquid-grown seedlings was performed as previously described (Chung et al., 2008).

Transgene Constructs and Site-Directed Mutagenesis

A complete list of constructs and primers used to generate transgenic lines is provided in Supplemental Table 1 online. Three PCR reactions were used to generate constructs encoding the JAZ8-JAZ10 chimeric proteins. In the case of the JAZ8-Jas10 chimera, one reaction used the *JAZ8* cDNA as a template with primer set JAZ8-XbaI-FP and JAZ8-Jas10-RP to amplify a portion (amino acids 1-100) of the JAZ8 coding sequence that lacks the Jas motif. A second PCR reaction used the *JAZ10* cDNA as a template with a primer set (JAZ8-Jas10-FP and JAZ10-KpnI-RP) to amplify the Jas motif-containing region of JAZ10 (amino acids 166-205). The PCR

products from these two reactions contain complementary sequences (built into the primers) that allow the products to anneal as template in a third PCR reaction with primer set JAZ8-XbaI-FP and JAZ10-KpnI-RP. The resulting chimeric product was cloned into pGEM-T Easy (Promega) for sequencing and subcloning into the final destination vector. A similar approach was used to construct the JAZ10-Jas8 chimera, using the primer sets listed in Supplemental Table 1.

The JAZ8ΔZIM construct was generated using three PCR reactions. The first PCR reaction used JAZ8 cDNA as a template with a primer set (JAZ8-NcoI-FP and JAZ8-deltaZIM-RP) to amplify the N-terminal region (amino acids 1-44) of JAZ8. A second PCR was used to amplify the C-terminal region of JAZ8 (amino acids 72-131) with primers JAZ8-deltaZIM-FP and JAZ8-XhoI-RP. The two PCR products contain complementary sequences built into the primers (JAZ8-deltaZIM-FP and JAZ8-deltaZIM-RP). In a third PCR reaction (primers JAZ8-NcoI-FP and JAZ8-XhoI-RP), the two PCR products were annealed as a template to generate JAZ8ΔZIM (i.e., JAZ8 lacking amino acids 45-71). The resulting product was cloned into pGEM-T Easy for sequencing and subcloning into Y2H and overexpression vectors. The JAZ8ΔEAR construct was generated in two PCR reactions. The first reaction used the *JAZ8* cDNA as a template with a primer set (JAZ8-deltaEAR-FP and JAZ8-pENTR-RP) that amplifies a portion of the JAZ8 coding region lacking the EAR motif. This PCR product was then used as template in the second PCR (primers JAZ8-pENTR-FP and JAZ8-pENTR-RP) to generate the *JAZ8ΔEAR* product, which was subsequently cloned into pENTR/D-TOPO (Invitrogen) and pGEM-T Easy for sequencing and subcloning.

Site-directed mutagenesis of the Jas and EAR motif of JAZ8 was performed with Pfu Turbo DNA Polymerase (Stratagene) as previously described (Chung and Howe, 2009). PCR reactions were performed with *JAZ8* cDNA in the vector pGEM-T Easy. PCR-amplified

products were treated with restriction enzyme *Dpn*I to remove the parental plasmid. The presence of the desired mutation was verified by DNA sequencing.

In Vivo Degradation of JAZ-YFP Fusion Proteins

Transgenic seedlings expressing JAZ-YFP fusion proteins were grown on MS plates (containing kanamycin) and then transferred to water (0.6 ml) 48-well microtiter plates. Seedlings were incubated in the presence or absence of MeJA or coronatine at room temperature on an orbital shaker (70 rpm). YFP fluorescence was analyzed on an Olympus Fluoview confocal microscope, with imaging software provided by the manufacturer. In some experiments, seedlings were pretreated with water or 100 μM MG132 (Sigma-Aldrich) for ~75 min prior to the addition of MeJA. Images shown in a single panel were taken at the same exposure time and microscope parameters, unless otherwise indicated in the figure legend.

Protein-Protein Interaction Assays

JAZ cDNAs were cloned into pRMG-nMAL to produce plasmids that encode maltose binding protein (MBP) and hexa-histidine (His_6)-tagged fusion proteins, referred to as JAZ-His (Thines et al., 2007). Primers used for amplifying *JAZ* cDNAs are listed in Supplemental Table 1. JAZ-His fusion proteins were expressed in *Escherichia coli* and purified by Ni-affinity chromatography as previously described (Katsir et al., 2008b; Chung et al., 2010). Leaf extracts prepared from a transgenic line of Arabidopsis that expresses Myc-tagged AtCOI1 were used as the source of COI1 (Melotto et al., 2008; Chung et al., 2010). In vitro COI1-JAZ pull-down assays were performed with chemically synthesized (3*R*,7*S*)-JA-Ile or coronatine as previously described (Chung et al., 2010). Purified JAZ-COI1 complexes were separated by SDS-PAGE on

a 10% acrylamide gel, transferred to polyvinylidene fluoride (PVDF) membrane, and probed with an anti-c-Myc antibody (Roche). In vitro JAZ8-NINJA and JAZ8-JAZ1 interaction assays were performed with purified JAZ-His protein (25 μg) and crude extract (600 μg) from yeast cells expressing N-terminal HA-tagged derivatives of NINJA or JAZ1, which were cloned into the yeast expression vector pB42AD (Clontech) using primers listed in Supplemental Table 1. JAZ-His complexes were recovered by Ni-affinity chromatography, protein-protein interactions were detected with an anti-HA antibody (Covance), as described above.

Yeast two-hybrid (Y2H) assays were performed with the Matchmaker LexA system (Clontech) as previously described (Melotto et al., 2008; Chung and Howe, 2009). JAZ8 and JAZ8-deletion constructs were subcloned into the pGILDA "bait" vector to generate translational fusions with the LexA DNA-binding domain. Full-length cDNAs encoding MYC2, other JAZs, NINJA, and TPL were subcloned into the pB42AD "prey" vector to generate fusions with B42 activation domain (AD). Bait and prey vectors were co-transformed into yeast (*Saccharomyces cerevisae*) strain EGY48 using the frozen-EZ yeast transformation II kit (Zymo Research). Transformants were selected for colorimetric detection (β-galactosidase) of protein-protein interaction as described by Chung and Howe (2009). Photographic images of Y2H plates were taken after 48 h of incubation at 30°C.

Coronatine Binding Assays

Full-length Arabidopsis COI1 and ASK1 were co-expressed as a glutathione S-transferase (GST)-fusion protein and an untagged protein, respectively, in Hi5 suspension insect cells. The COI1-ASK1 complex was isolated from the soluble cell lysate by glutathione affinity chromatography. Full-length JAZ substrate proteins were expressed as 6xHis-fusion proteins in

Escherichia coli and purified on Ni-NTA resin with subsequent dialysis into 20 mM Tris-HCl, pH 8.0, 200 mM NaCl, and 10% glycerol. Radioligand binding was assayed with purified COI1-ASK1 complex (2 mg) and JAZ proteins at a 1:3 molar ratio. Reactions were prepared in 100 mL final volume and in a binding buffer containing 20 mM Tris-HCl, 200 mM NaCl, and 10% glycerol. Saturation binding experiments were conducted with serial dilutions of ^{3}H-coronatine in binding buffer. Non-specific binding was determined in the presence of 300 mM coronatine (Sigma-Aldrich). Specific binding was calculated by subtracting non-specific binding from total binding. Following incubation with mixing at 4°C, all samples were collected with a cell harvester (Brandel, Gaithersburg, MD) on polyethyleneimine (Sigma)-treated filters. Samples were incubated in liquid scintillation fluid for >1 h before counting with a Packard Tri-Carb 2200 CA liquid scintillation analyzer (Packard Instrument Co. Inc., Rockville, MD). Saturation binding experiments were analyzed by non-linear regression using GraphPad Prism version 5.00 for MacOSX. ^{3}H-coronatine was synthesized commercially as previously described (Katsir et al., 2008b).

Carrot Protoplasts Transactivation Assay

The effector constructs *35S*:*GD* (GD: Gal4 DNA binding domain), *35S*:*GD-IAA17[I]* (IAA17/AXR3 domain I, amino acids 1 to 29), and *35S*:*LD-VP16* (LD: LexA DNA binding domain; VP16: *Herpes simplex virus* VP16 activation domain) were all driven by the CaMV 35S promoter and have been described previously (Tiwari et al., 2004). The *35S*:*GD* vector was converted into a Gateway destination version (pGD-RfA) by inserting the reading frame A cassette (RfA, Invitrogen) downstream of the sequence encoding the Gal4 DNA binding domain. *JAZ8* (amino acids 1 to 131) and *JAZ8-N* (N-terminus; amino acids 1 to 44) coding regions were

amplified by RT-PCR using RNA from Arabidopsis seedlings as template and the primer pairs listed in Supplementary Table S1. cDNAs were cloned into pDONR/Zeo via Gateway BP cloning reactions (Invitrogen). *GD-JAZ8* and *GD-JAZ8-N* effector constructs were generated by recombination between pDNOR clones described above and pGD-RfA via Gateway LR cloning reactions. The reporter constructs were *35S*:*LexA(2x)-Gal4(2x):GUS,* which is driven by CaMV 35S -46 minimal promoter containing LexA and Gal4 DNA binding sites, and *35S*:*LUC* have been described previously (Tiwari et al., 2004). To generate *JAZ8* effector constructs encoding mutated proteins (L9A, E10A, L11A, R12A, L13A and F14A), the *JAZ8-N* coding region was mutagenized with a Quickchange II site-directed mutagenesis kit (Stratagene) and a complementary pair of primers (Supplementary Table S1) containing the relevant mutation. All mutations were confirmed by DNA sequencing. Reporter and effector plasmids were isolated from the *E. coli* strain ER2925 (New England Biolabs) using Wizard midiprep DNA purification kit (Promega).

Isolation of protoplasts from carrot (*Daucus carota*) suspension culture cells, transfections, luciferase (LUC), and GUS assays have been described (Niu et al., 2011). GUS activities for each measurement were normalized by cotransfection with a *35S*:*LUC* reporter gene (Liu et al., 1994). GUS activity measurements were performed with 4-methylumbelliferyl-β-glucuronide (Sigma-Aldrich) as a substrate, and excitation and emission wavelengths of 365 and 455 nm, respectively. LUC activity was determined with a Luciferase Assay System (Promega), using an emission wavelength of 550 nm and a photomultiplier gain of 775 V. Fluorescence and luminescence were measured with a 96-well luminescence spectrometer LS-50B (Perkin Elmer). Three transfections were performed for each construct and the measurements were averaged. All transfections were repeated two or three times using

independent protoplast preparations. Results from representative experiments are reported. Significant differences were determined by Student´s t-test using Graphpad Prism version 4.00 for Windows software (GraphPad Software). Differences between mean values are reported as significant at $P<0.01$.

Multiple Sequence Alignments

JAZ8-related sequences were identified using Basic Local Alignment Search Tools (BLASTP and TBLASTN) and the Phytozome database (http://www.phytozome.net/), with JAZ8 as the query sequence. Of the resulting 43 sequences identified from 18 monocot and dicot species, 35 proteins contained a complete ZIM domain and Jas motif and were used to generate the Weblogo (Crooks et al., 2004) shown in Figure 6. Manual annotation identified 30 JAZ8-like proteins that contain a canonical an EAR motif at the N-terminus, and these sequences were used to generate the Weblogo shown in Figure 6. A similar approach was used to identify 97 JAZ10-like proteins for construction of the Weblogo.

Accession Numbers

Arabidopsis Genome Initiative numbers described in this article are listed below: ACT8 (At1g49240), COI1 (At2g39940), IAA17 (At1g04250), JAZ1 (At1g19180), JAZ2 (At1g74950), JAZ3 (At3g17880), JAZ4 (At1g48500), JAZ5 (At1g17380), JAZ6 (At1g72450), JAZ7 (At2g34600), JAZ8 (At1g30135), JAZ9 (At1g70700), JAZ10.1 (At5g13220.1), JAZ11 (At3g43440), JAZ12 (At5g20900), MYC2 (At1g32640), NINJA (At4g28910), TPL (At1g15750), and VSP2 (At5g24770).

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FIGURE AND TABLES

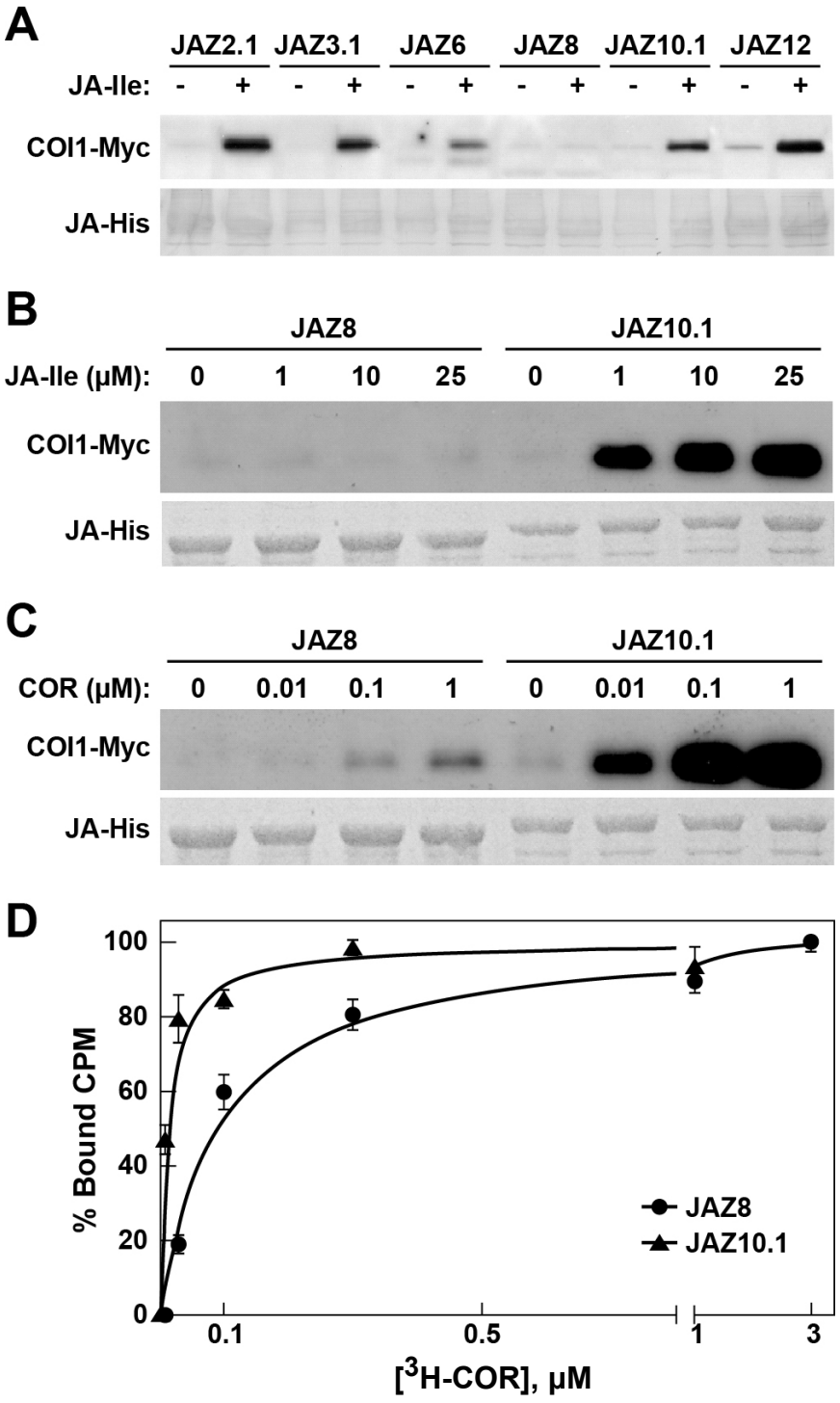


Figure 2.1. JAZ Proteins Differentially Interact with COI1 in the Presence of JA-Ile.

Figure 2.1. JAZ Proteins Differentially Interact with COI1 in the Presence of JA-Ile. – Continued.

(A) JA-Ile differentially promotes COI1 interaction with various JAZ proteins. Pull-down assays were performed using crude leaf extracts from *35S:COI1-Myc* transgenic plants and purified recombinant JAZ-His proteins. Reactions were supplemented (+) with 1 μM (3*R*,7*S*)-JA-Ile or an equivalent volume of assay buffer (-). Protein bound to JAZ-His was separated by SDS-PAGE and analyzed by immunoblotting with anti-Myc antibody for the presence of COI1-Myc. The blotted membrane was stained with Coomassie blue to visualize the amount of JAZ-His loaded.
(B) JAZ8 does not associate with COI1 in the presence of JA-Ile. Pull-down assays with purified JAZ8-His and JAZ10.1-His were performed as described in (A). Reaction mixtures were supplemented with the indicated concentration of (3*R*,7*S*)-JA-Ile.
(C) JAZ8 weakly associates with COI1 in the presence of coronatine. Pull-down assays were performed as described in (A). Reactions were supplemented with the indicated concentration of coronatine (COR).
(D) Saturation binding of ^{3}H-labeled coronatine to COI1-JAZ8 (circle) and COI1-JAZ10.1 (triangle) complexes. Data show the mean ± SE of 2 replicates performed in duplicate.

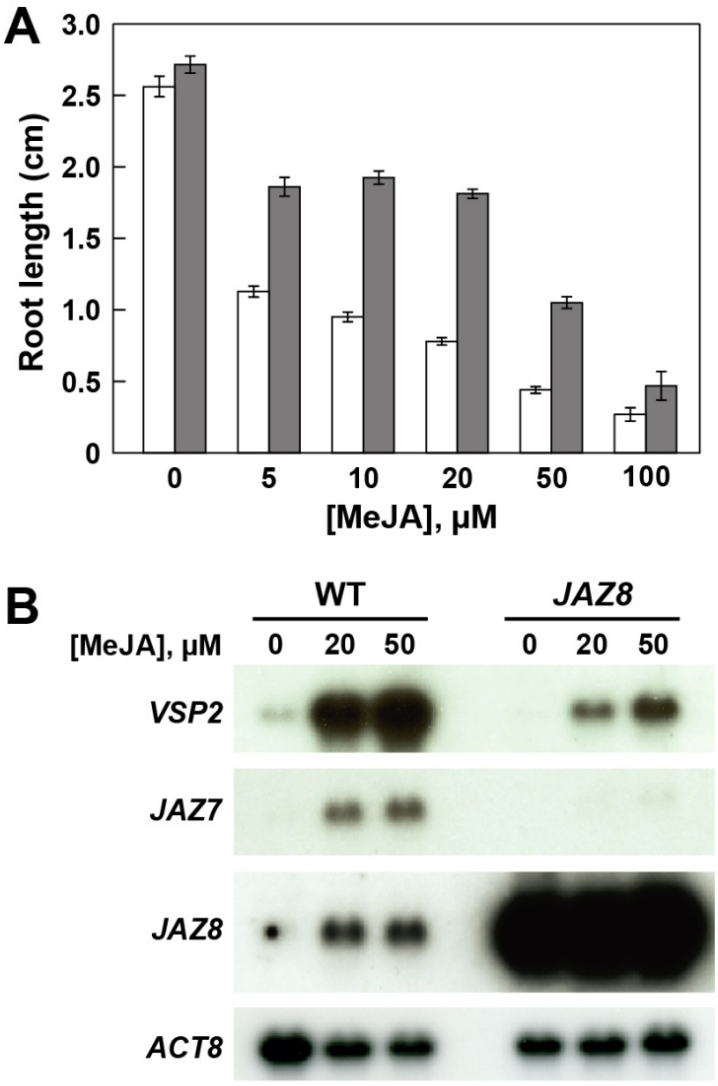


Figure 2.2. Overexpression of JAZ8 Results in Decreased Sensitivity to JA.

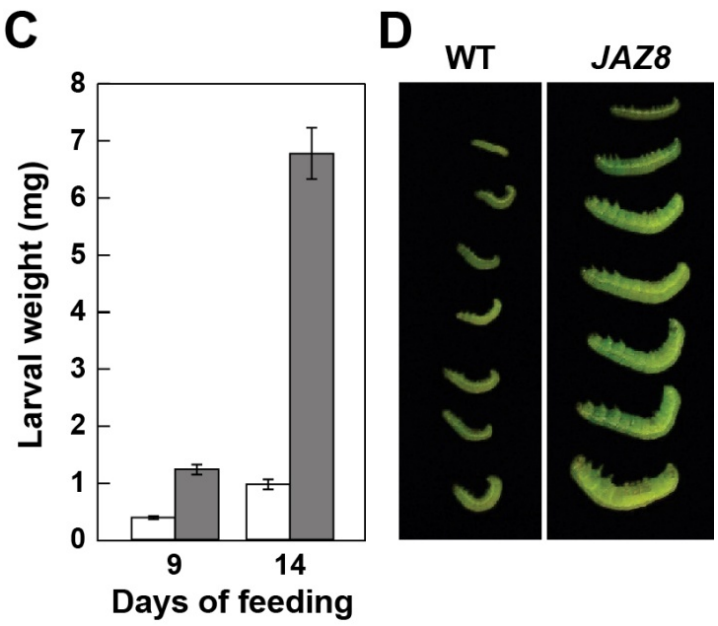


Figure 2.2. Overexpression of JAZ8 Results in Decreased Sensitivity to JA. – Continued.

(A) Root growth inhibition assay of wild-type (WT; open bars) and *35S:JAZ8* (filled bars) seedlings grown on MS media containing the indicated concentration of MeJA. Root length measurements were made 6 d after seed germination. Data show the mean ± SE ($n > 20$ seedlings per genotype for each concentration of MeJA except 100 μM, in which $n = 6$). Asterisks denote statistically significant differences ($P<0.05$, Student's t-test) between the two plant genotypes at the indicated concentration of MeJA.
(B) JA-responsive gene expression in wild-type (WT) and *35S:JAZ8* (*JAZ8*) seedlings. Seedlings were grown in liquid MS medium for nine d and then treated for two hr with the indicated concentration of MeJA or a mock control ("0"). Total RNA isolated from the treated seedlings was subjected to Northern blot analysis with the indicated probes. A *JAZ8* probe was used to verify overexpression of *JAZ8* in the transgenic line, and an *ACTIN8* (*ACT8*) probe was used as a loading control.
(C) Weight of *S. exigua* larvae reared on WT (open bars) or *35S:JAZ8* (filled bars) plants for 9 or 14 d. Data show the mean ± SE (n = 56 to 65 larvae per plant genotype). The experiment was repeated four times with similar results. Data from a representative experiment are shown.
(D) Photograph of representative *S. exigua* larvae recovered from WT and *35S:JAZ8* (*JAZ8*) plants after 14 d of feeding.

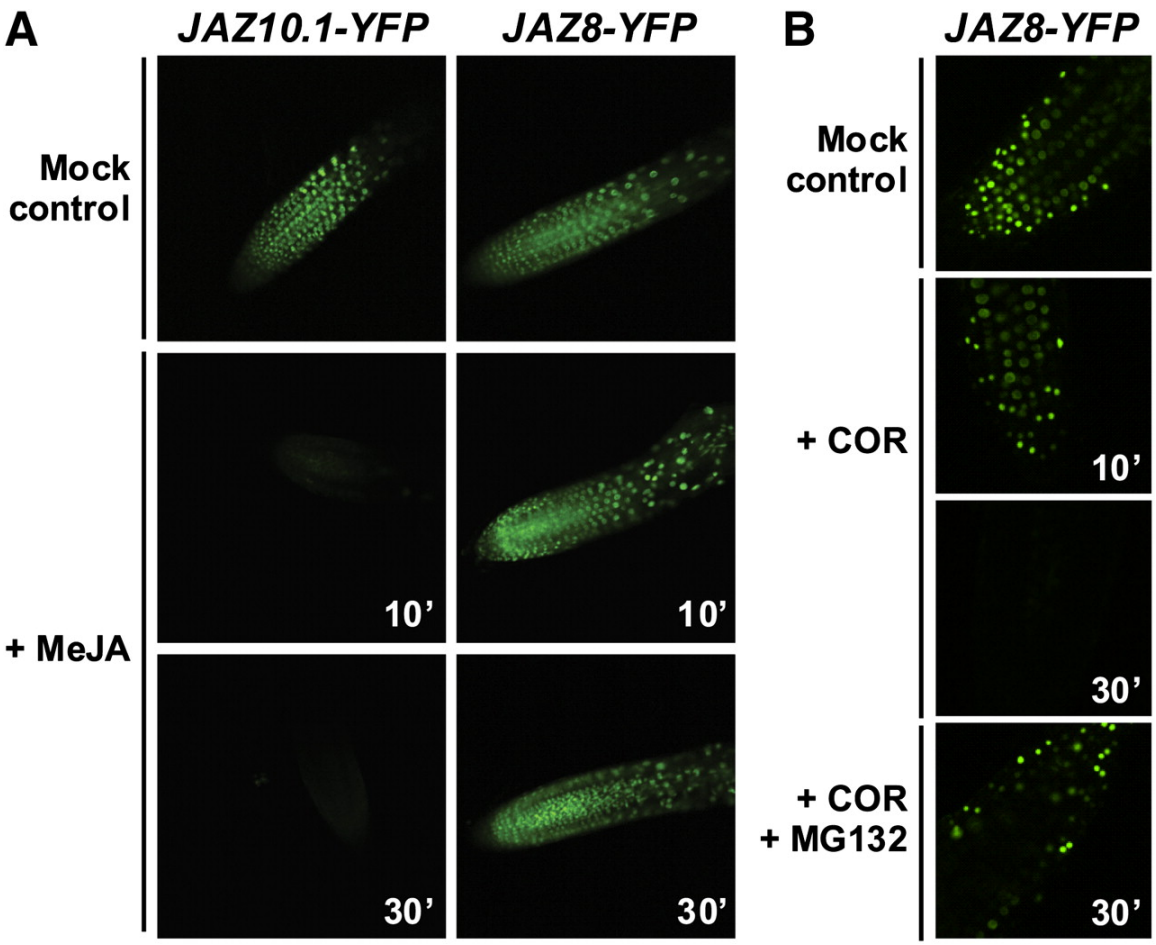


Figure 2.3. JAZ8 is Resistant to JA-Mediated Degradation in Vivo.

(A) Differential stability of JAZ8 and JAZ10.1 in the presence of JA. Transgenic seedlings (seven-d-old) expressing JAZ8-YFP or JAZ10.1-YFP fusion proteins were treated either with water (Mock) or with 50 μM MeJA for the indicated amount of time prior to imaging of roots by confocal fluorescence microscopy. Image settings used for seedlings of the same genotype at different time points were identical.
(B) Coronatine stimulates JAZ8 turnover in a 26S proteasome-dependent manner. Transgenic seedlings (four-d-old) expressing *JAZ8-YFP* were pretreated with water or the 26S proteasome inhibitor MG132 (100 μM) for 80 min, at which time seedlings were treated with coronatine (1 μM) for an additional 10 or 30 min. Seedlings treated with both coronatine and MG132 were imaged at the 30 min time point. YFP signal in root tissue was visualized by confocal microscopy. Microscope settings were identical for all images.

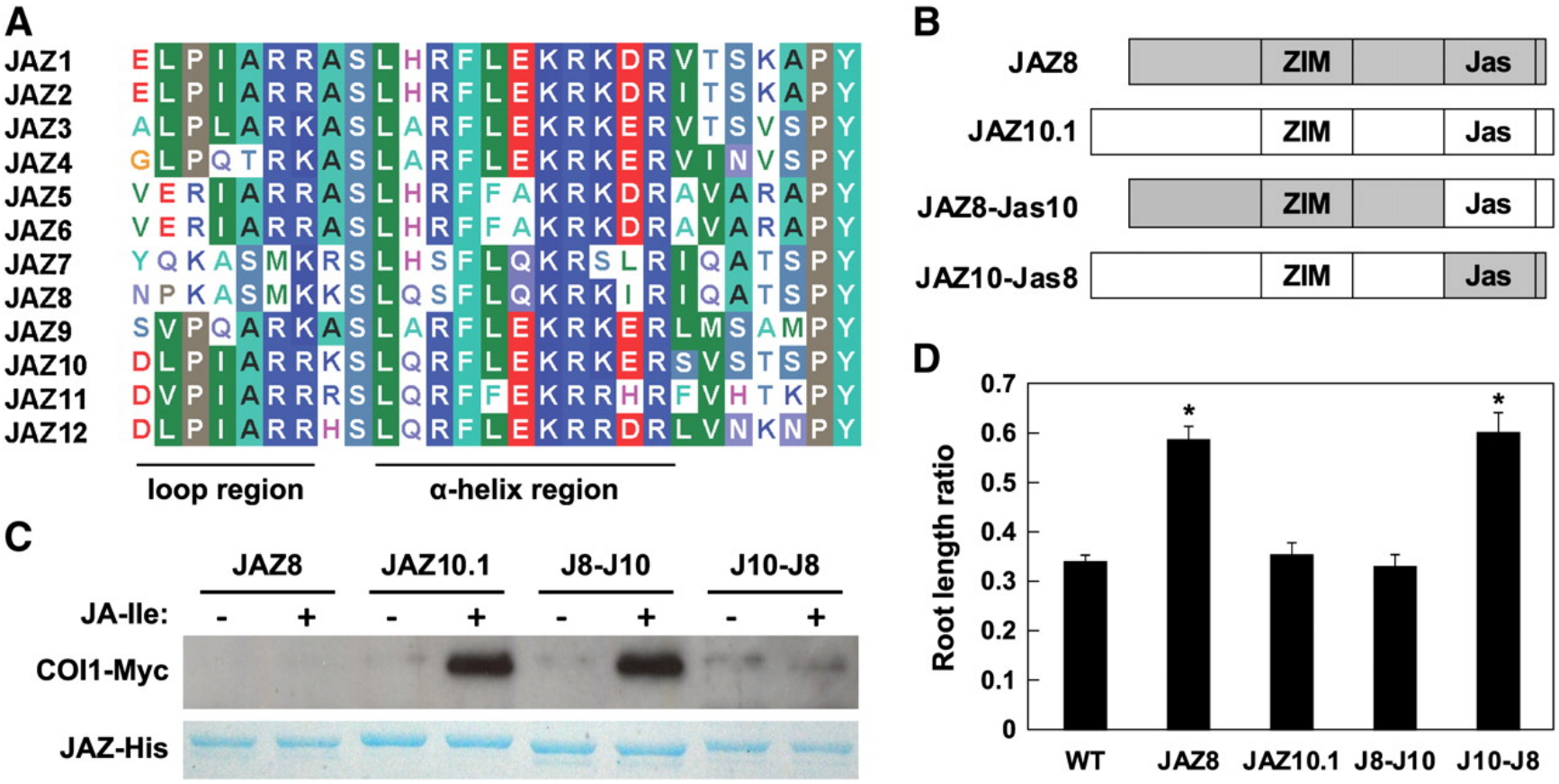


Figure 2.4. A Jas Domain Swap Converts JAZ8 to a COI1-Interacting Protein That Does Not Exert Dominant Repression of JA Responses.

Figure 2.4. A Jas Domain Swap Converts JAZ8 to a COI1-Interacting Protein That Does Not Exert Dominant Repression of JA Responses. – Continued.

(A) Amino acid alignment of the Jas motif in 12 Arabidopsis full-length JAZ proteins. Structural features within the Jas motif that physically associate with COI1 and JA-Ile (Sheard et al., 2010) are indicated.
(B) Schematic diagram of chimeric proteins constructed by swapping of the Jas motif. Sequence regions derived from JAZ8 and JAZ10.1 are shown in grey and white, respectively.
(C) Pull-down assays performed with parental (JAZ8 and JAZ10.1) and chimeric (JAZ10-Jas8, J10-J8; JAZ8-Jas10, J8-J10) proteins in the presence (+) or absence (-) of 2.5 μM JA-Ile.
(D) JA-mediated root growth inhibition in WT and transgenic seedlings (*35S:JAZ8*, JAZ8; *35S:JAZ10.1*, JAZ10.1; *35S:JAZ8-Jas10*, J8-J10; and *35S:JAZ10-Jas8*, J10-J8). Seedlings were grown for 6 d on MS medium supplemented or not supplemented with 20 μM MeJA. The root length ratio was calculated by dividing the average the root length of seedlings grown on MeJA-containing medium by the average root length of seedlings of the same genotype grown in the absence of MeJA. Data points show the mean ± SE (n = 18 to 24 seedlings per data point). Asterisks denote significant differences ($P<0.05$, Student's t-test) in comparisons between the indicated transgenic line and WT.

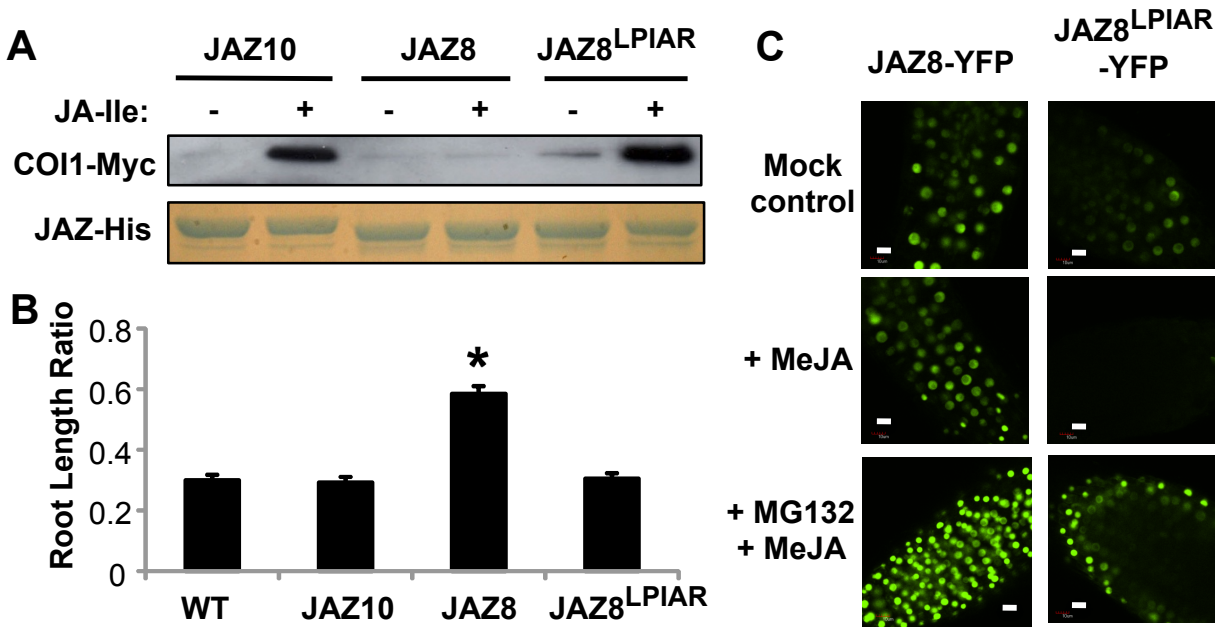


Figure 2.5. The Canonical LPIAR Degron Promotes JAZ8 Binding to COI1, Destabilizes JAZ8, and Restores JA Responsiveness.

(A) JAZ8^{LPIAR} interacts with COI1 in a JA-Ile-dependent manner. Pull-down reactions were performed in the presence (+) or absence (-) of 2.5 µM JA-Ile.

(B) Transgenic lines overexpressing JAZ8^{LPIAR} are sensitive to JA. Root growth assays were performed with WT, *35S:JAZ10* (*JAZ10.1*), *35S:JAZ8* (*JAZ8*), and *35S:JAZ8^{LPIAR}* (*JAZ8^{LPIAR}*) seedlings. Data show the mean ± SE for each genotype (n = 20). Asterisk denotes significant differences ($P<0.05$, Student's t-test) in comparisons between the indicated transgenic line and WT.

(C) JAZ8^{LPIAR}-YFP is degraded in vivo in response to JA treatment. Six-d-old transgenic seedlings expressing *JAZ8-YFP* (left) or *JAZ8^{LPIAR}-YFP* (right) were pretreated with water or the 26S proteasome inhibitor MG132 (100 µM) for 75 min, at which time seedlings were treated with either MeJA ("+MeJA", 50 µM) or water (Mock control) for an additional 15 min. YFP signal in root tissue was visualized by fluorescence microscopy. Microscope settings were identical for all images. Scale bars = 50 µm.

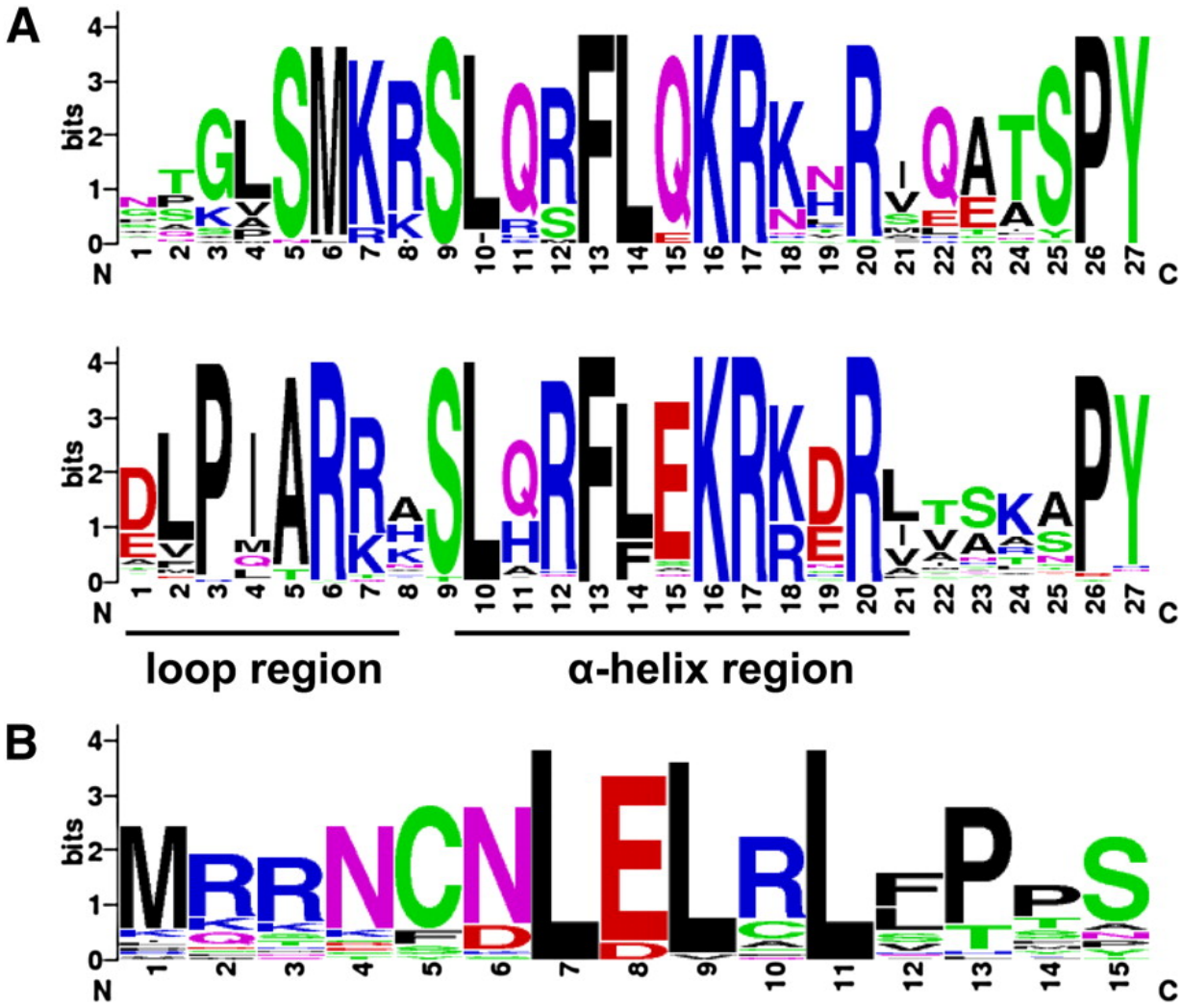


Figure 2.6. The EAR Motif of JAZ8 is Required for Repression of JA Responses.

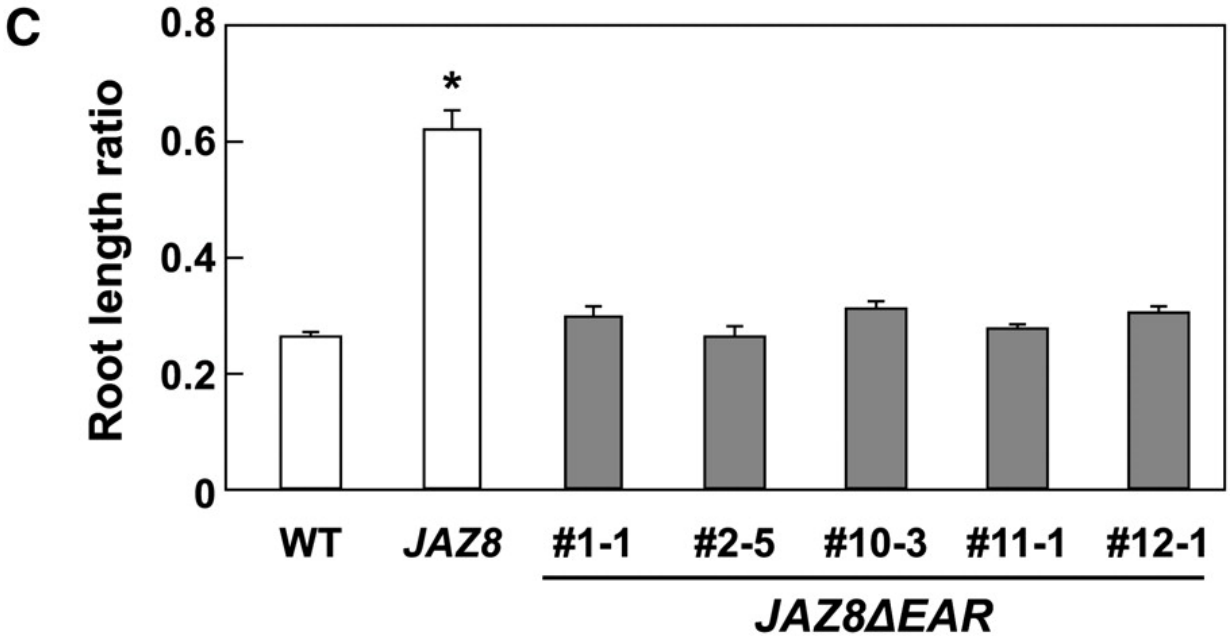


Figure 2.6. The EAR Motif of JAZ8 is Required for Repression of JA Responses. – Continued.

(A) Consensus sequence of the Jas motif in JAZ8 (upper) and JAZ10 (lower) orthologs from diverse plant species. The height of each stack of symbols indicates the sequence conservation at that position, whereas the height of symbols within the stack represents the relative frequency of the corresponding amino or nucleic acid at that position (Crooks et al., 2004).
(B) Consensus sequence of the N-terminal 15-amino acids from JAZ8-like proteins from diverse plant species.
(C) Comparison of JA-mediated root growth inhibition in *35S:JAZ8* and *35S:JAZ8ΔEAR* seedlings. Wild-type (WT), *35S:JAZ8* (line #24), and *35S:JAZ8ΔEAR* (five independent homozygous lines) were grown for 8 d on medium supplemented or not supplemented with 20 μM MeJA. Root length ratios were calculated as described in the legend of Figure 4D. Data show the mean ± SE for each genotype (n >14 seedlings per genotype). Asterisks denote a significant difference (P<0.05, Student's t-test) in comparisons between transgenic and WT seedlings. The mean root length ratio calculated for seedlings (n = 92) from all five independent *35S:JAZ8ΔEAR* lines was not significantly different (P-value = 0.068, Student's t-test) from that of WT.

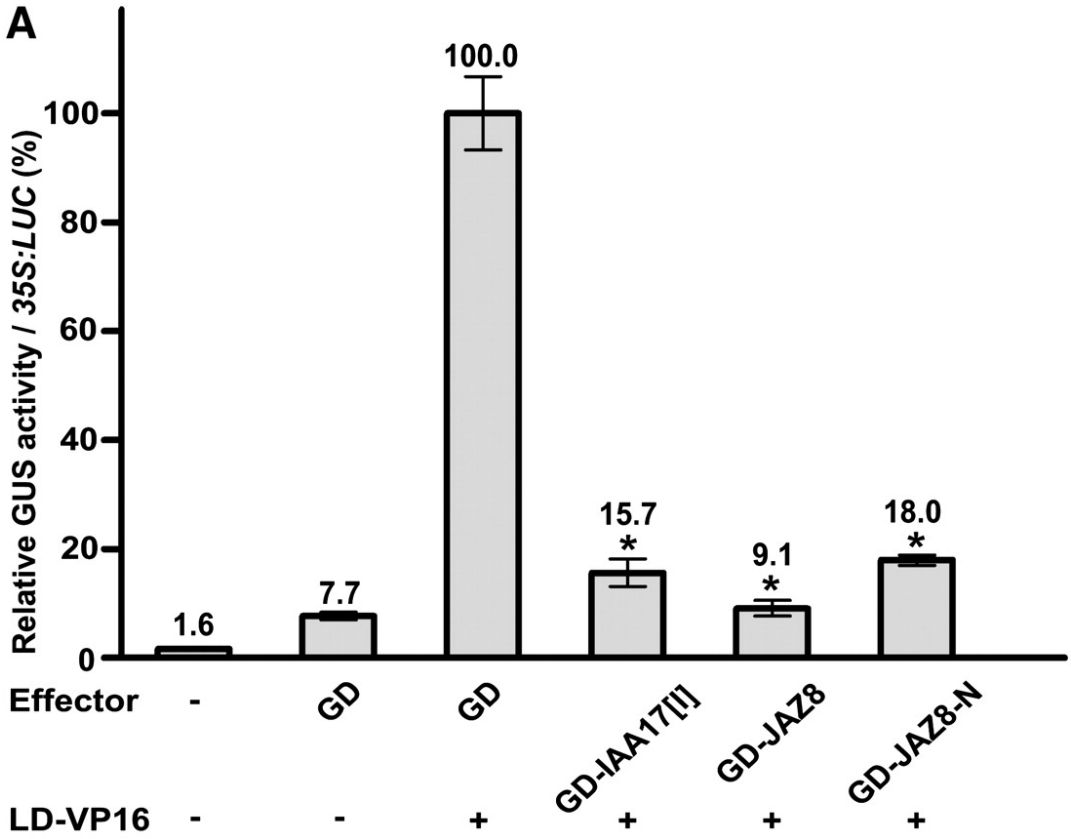


Figure 2.7. The EAR Motif is Required for Transcriptional Repression by JAZ8.

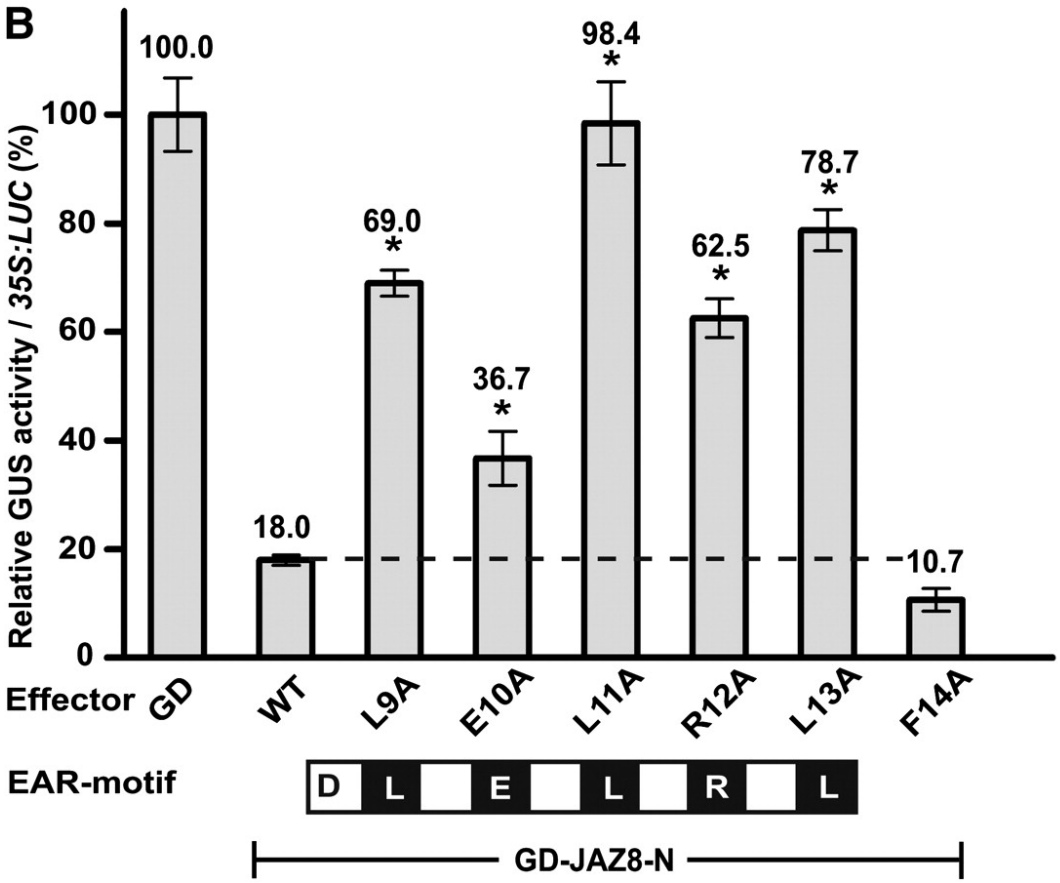


Figure 2.7. The EAR Motif is Required for Transcriptional Repression by JAZ8. – Continued.

(A) Carrot protoplasts were transfected with the *GUS* reporter [*35S(-46)LexA(2x)-Gal4(2x):GUS*] alone or in combination with the indicated effector and LD-VP16 constructs as described in the text. GUS activities are expressed relative to the sample in which LD-VP16 was cotransfected with the GD effector (Gal4 DNA binding domain alone; set to 100%). GUS activities were normalized by cotransfection with a *35S:LUC* construct. Asterisks denote statistically significant differences ($P<0.01$, Student's t-test) in comparisons to the LD-VP16 + GD activation control. Data show the mean ± SD of three replicate assays.
(B) Carrot protoplasts were cotransfected with the *GUS* reporter gene described in (A), *LD-VP16*, and the indicated effector construct. L9A, E10A, L11A, R12A and L13A correspond to single Ala-substitution mutations in the EAR motif of JAZ8-N (black boxes). The F14A mutation is located C-terminal to the EAR motif and serves as a control. GUS activities are expressed as described in (A). Asterisks denote statistically significant differences ($P<0.01$, Student's t-test) in comparisons to the unmodified GD-JAZ8-N construct. Data points show the mean ± SD of three replicate assays.

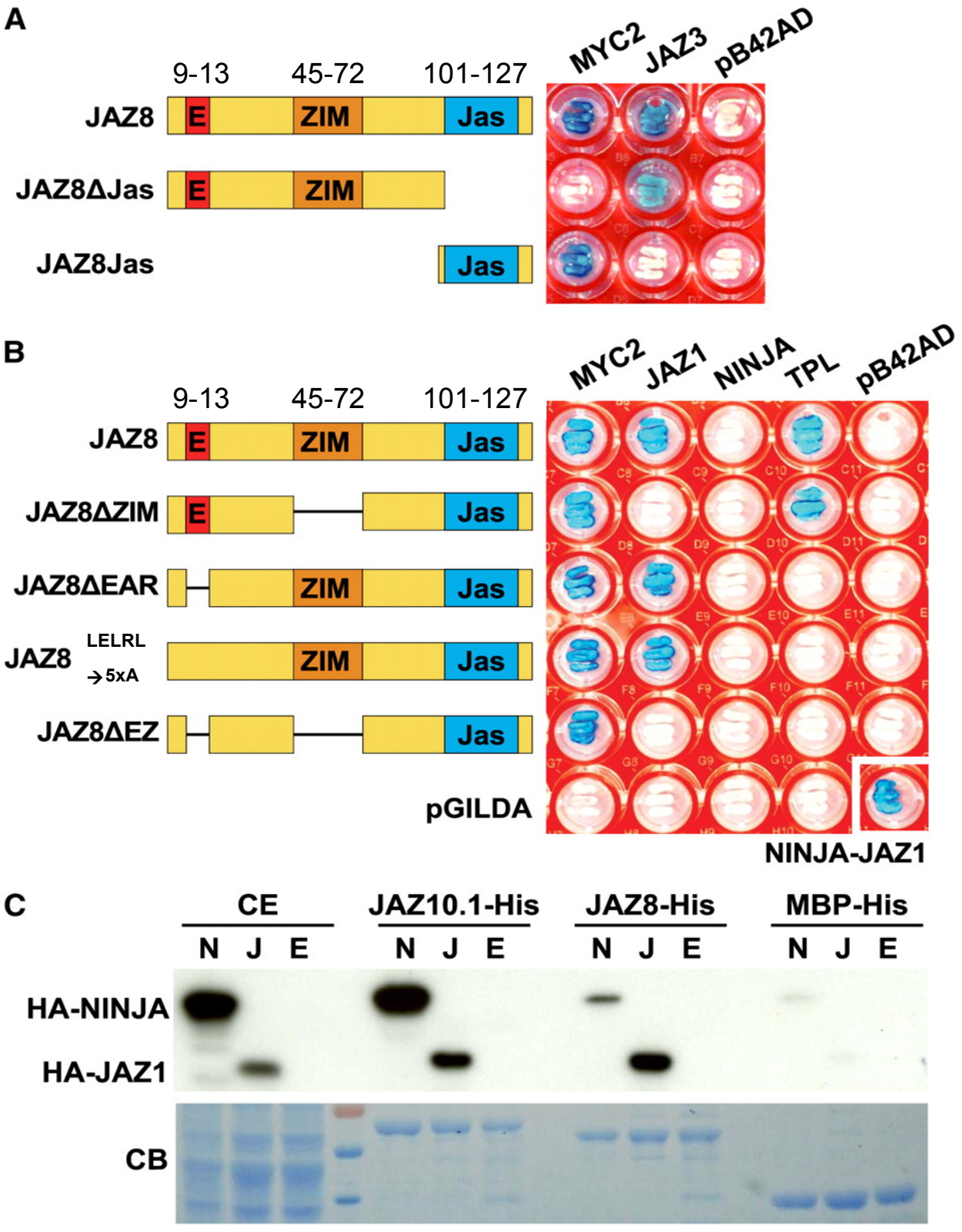


Figure 2.8. Protein-Protein Interaction Domains in JAZ8.

Figure 2.8. Protein-Protein Interaction Domains in JAZ8. – Continued.

(A) Yeast two-hybrid (Y2H) assay of JAZ8 and JAZ8 deletion derivatives with MYC2 and JAZ3. Yeast strains expressing both the bait (JAZ8) and prey (MYC2 or JAZ3) proteins were plated on media containing X-gal. LacZ-mediated blue-color formation is indicative of protein-protein interaction. JAZ8 proteins were co-expressed with an empty prey vector (pB42AD) as a negative control. Photographic images of yeast cells were taken after 48 h of incubation at 30°C.
(B) Y2H analysis of JAZ8 deletion proteins with MYC2, JAZ1, NINJA and TPL. Yeast strains expressing both the bait (JAZ8 or JAZ8 deletion) and prey (MYC2, JAZ1, NINJA, or TPL) proteins were tested as described in (A). Empty bait (pGILDA) and prey (pB42AD) vectors were used as negative controls. As a positive control for NINJA interaction, yeast cells were cotransformed with pB42AD-NINJA and pGILDA-JAZ1 (lower right).
(C) JAZ8 interacts weakly with NINJA in vitro. Purified JAZ-His proteins (fused to MBP) were incubated with crude extract from yeast cells expressing HA-tagged derivatives of NINJA (N), JAZ1 (J), or an empty vector control (E). MBP-His was used as a control for specificity. Purified protein complexes were separated by SDS-PAGE and probed with an anti-HA antibody for the presence of HA-tagged NINJA (HA-NINJA) or JAZ1 (HA-JAZ1). Lower panel shows a Coomassie blue (CB)-stained gel to visualize the amount of protein loaded. CE, crude yeast extracts which were used as an input control. Protein molecular weight markers were run in the fourth lane from the left.

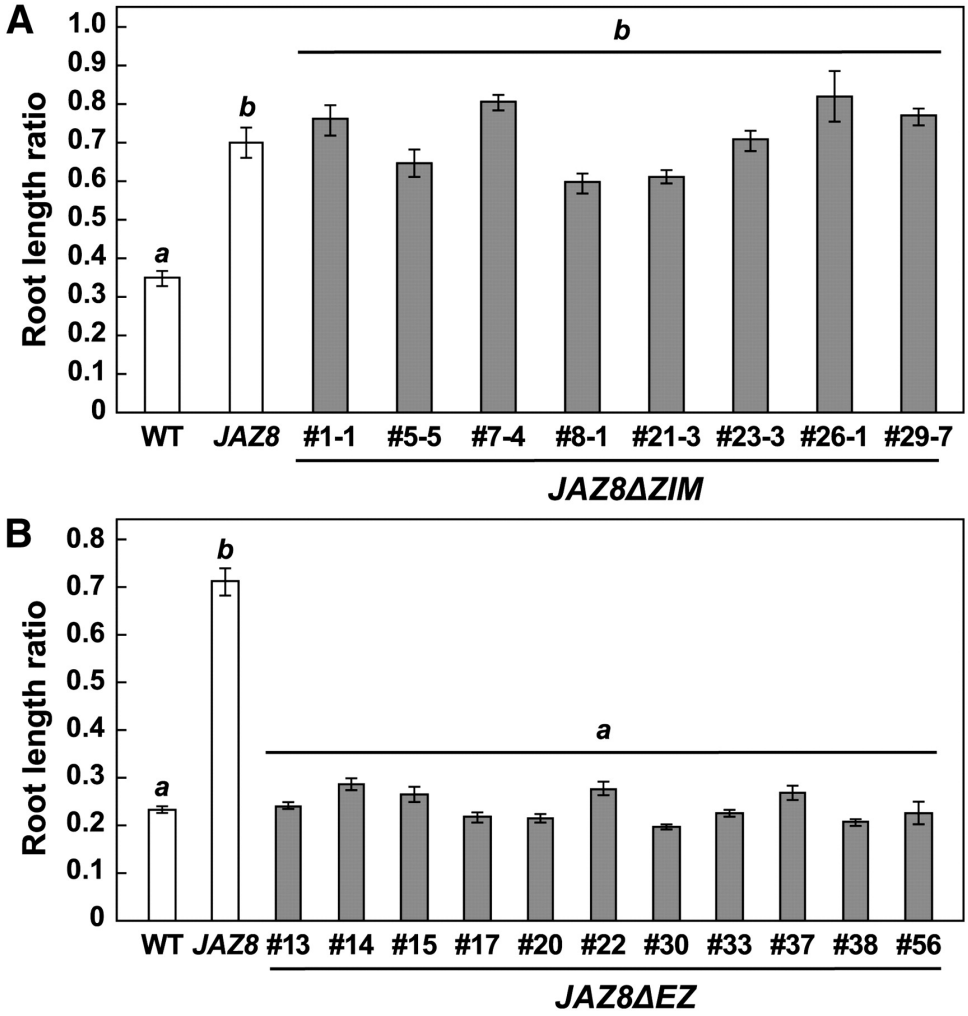
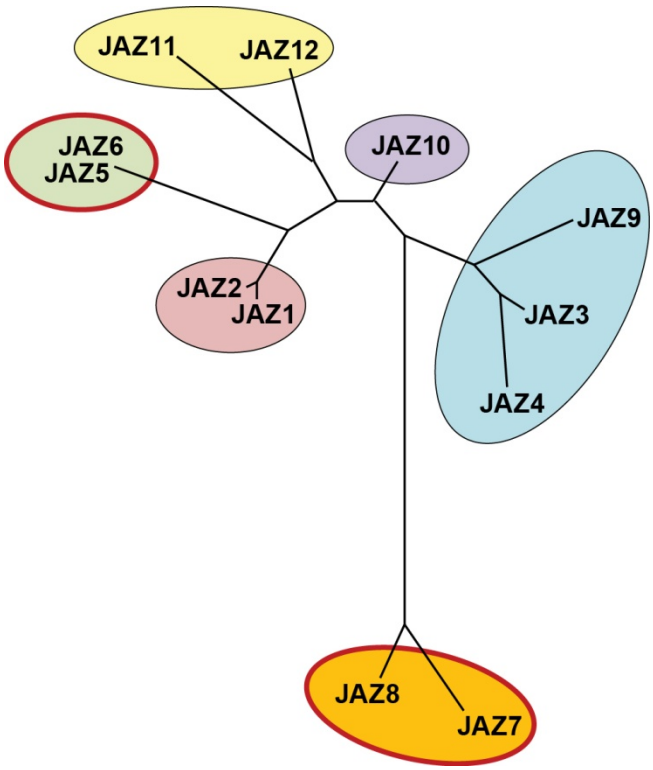


Figure 2.9. The ZIM Domain is not Required for JAZ8-mediated Repression of JA-induced Root Growth Inhibition.

(A) *35S:JAZ8ΔZIM* plants are insensitive to JA-mediated root growth inhibition. Assays were performed with WT, *35S:JAZ8* (*JAZ8*; line #24), and eight independent *35S:JAZ8ΔZIM* (*JAZ8ΔZIM*; gray bars) lines that are homozygous for the transgene. Root length ratios were calculated as described in the legend of Figure 4D. Data show the mean ± SE for each genotype (n = 12 to 24 seedlings per genotype). The mean root length ratio of seedlings (n = 127) from all eight *35S:JAZ8ΔZIM* lines was not significantly different than that of *35S:JAZ8* seedlings (P-value = 0.69, Student's t-test). Means with different italicized letters are significantly different at P<0.001.

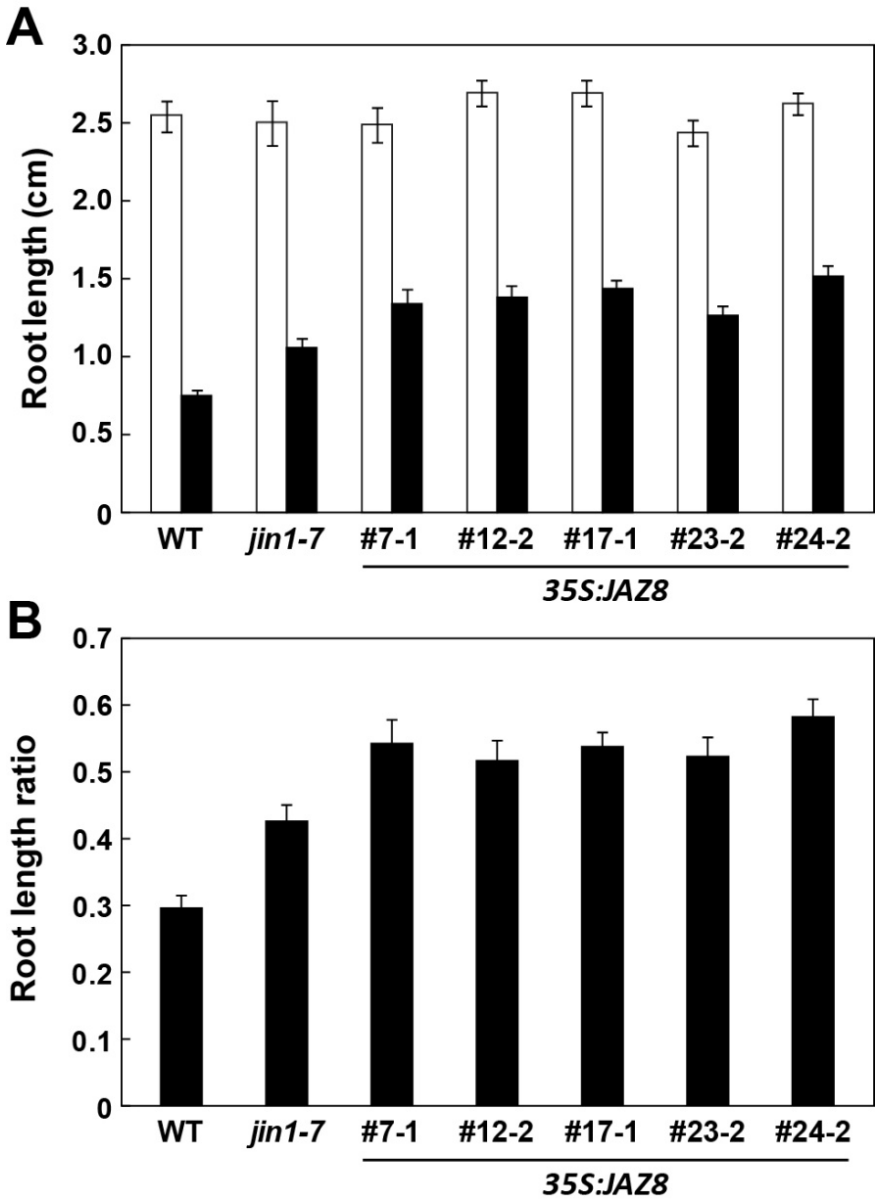
(B) JA-mediated root growth phenotype of *35S:JAZ8ΔEZ* lines expressing a JAZ8 derivative lacking both the EAR and ZIM motifs. Assays were performed as described in panel (A), and included eleven independent *35S:JAZ8ΔEZ* T2 lines (*JAZ8ΔEZ*; gray bars). Data show the mean ± SE for each genotype (n = 13 to 22 seedlings per genotype). The mean root length ratio of seedlings (n = 212) from all eleven *35S:JAZ8ΔEZ* lines was not significantly different than that of WT seedlings (P-value = 0.62, Student's t-test). Means with different italicized letters are significantly different at P<0.001.



Supplemental Figure 2.1. Phylogenetic Tree Constructed from the Jas Motif of Arabidopsis JAZ Proteins.

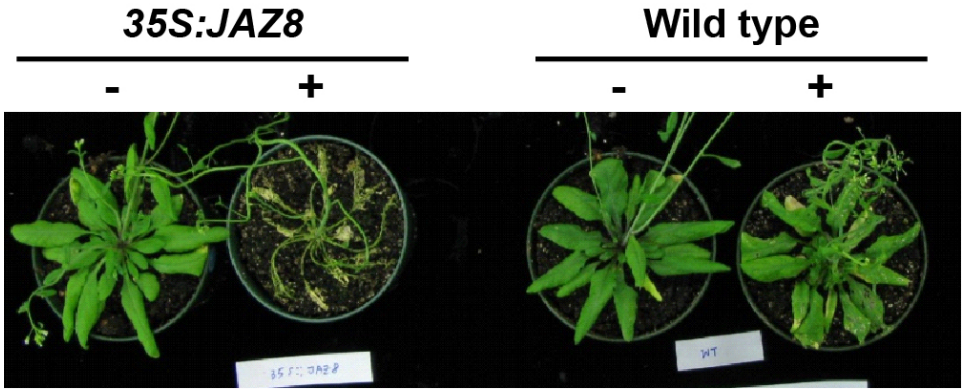
The 27-amino-acid Jas motif in all 12 Arabidopsis JAZ proteins was aligned with MUSCLE (Edgar, 2004). The phylogenetic tree was generated with the Neighbor-joining method using MEGA5 software (http://www.megasoftware.net). Red circles indicate JAZ proteins that have a predicted EAR motif.

Reference **Edgar, R.C.** (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. **32:**1792-1797.



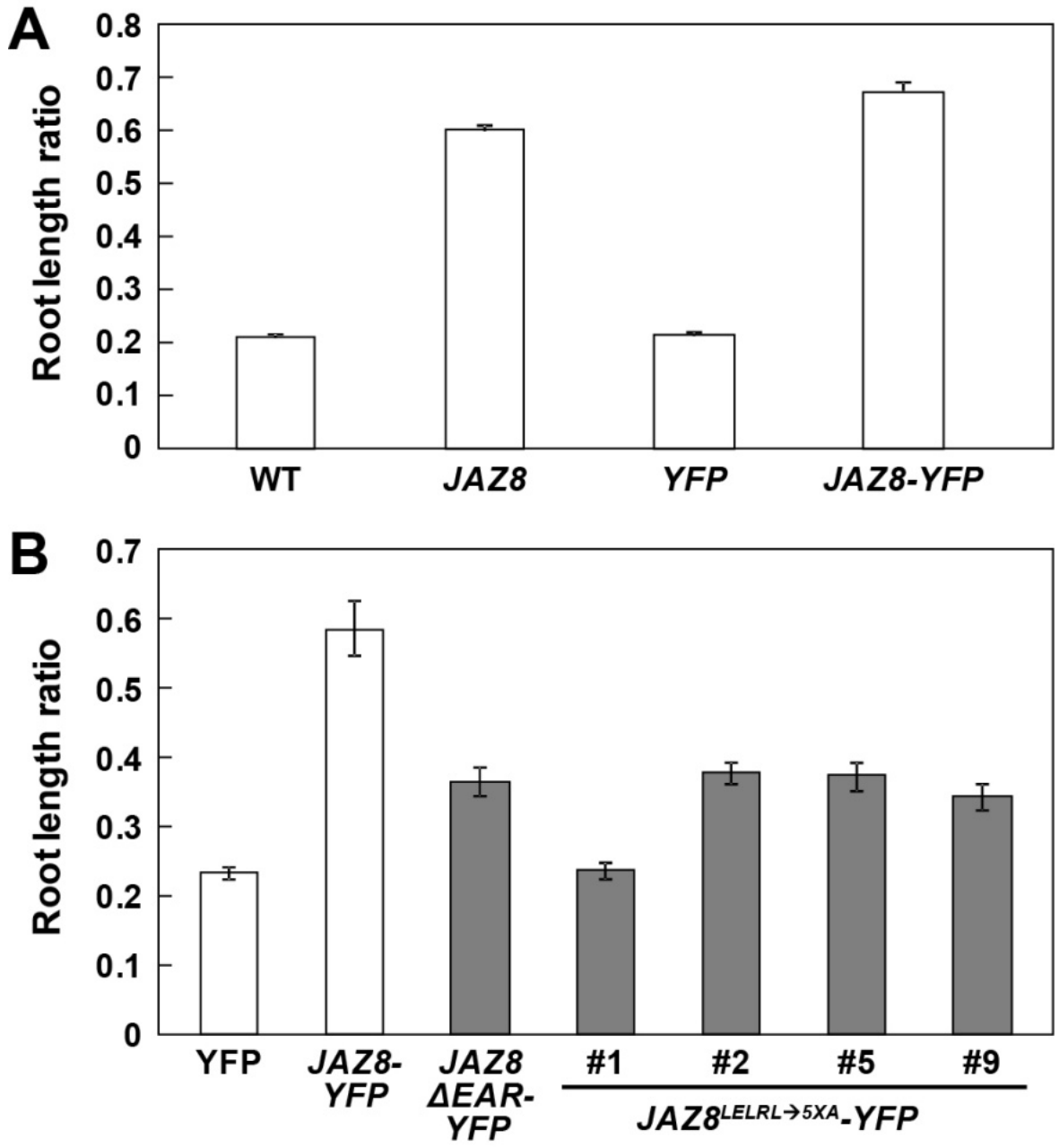
Supplemental Figure 2.2. JA-Mediated Root Growth Inhibition in Independent *35S:JAZ8* Lines.

(A) Five independent homozygous *35S:JAZ8* lines, which were selected for high expression of the transgene, were grown together with WT and *jin1-7* seedlings on MS medium supplemented (filled bars) or not supplemented (open bars) with 20 μM MeJA. Plants were grown for 8 d on a 16-h light and 8-h dark cycle. Data show the mean ± SE for each genotype (n >18 seedlings per genotype). The mean root length of each *35S:JAZ8* line was significantly longer ($P<0.05$, Student's t-test) than that of the *jin1-7* mutant in the presence of MeJA.
(B) Data shown in (A) were plotted as root length ratio, which was calculated by dividing the average the root length of seedlings grown on MeJA-containing medium by the mean root length of seedlings of the same genotype grown in the absence of MeJA. Means with different italicized letters are significantly different at $P<0.05$.



Supplemental Figure 2.3. *35S:JAZ8* Plants are Compromised in Resistance to Herbivory by *S. exigua* Larvae.

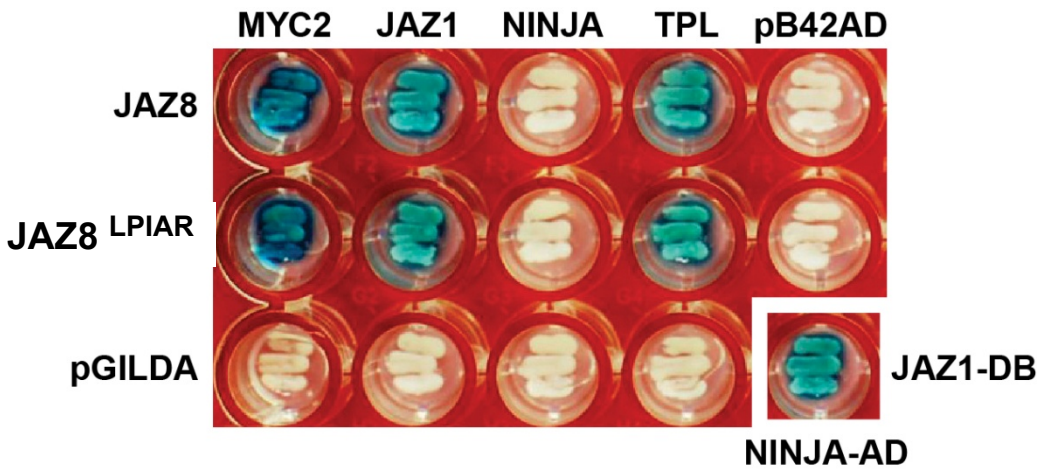
Six-week-old *35S:JAZ8* (line #24) and wild-type (WT) plants were challenged (+) for 14 d with *Spodoptera exigua* larva. A separate set of control plants grown side-by-side with the challenged plants were not infested (-) with larvae. The photograph was taken at the end of the feeding trial.



Supplemental Figure 2.4. JA-Mediated Root Growth Inhibition in *35S:JAZ8-YFP* Lines.

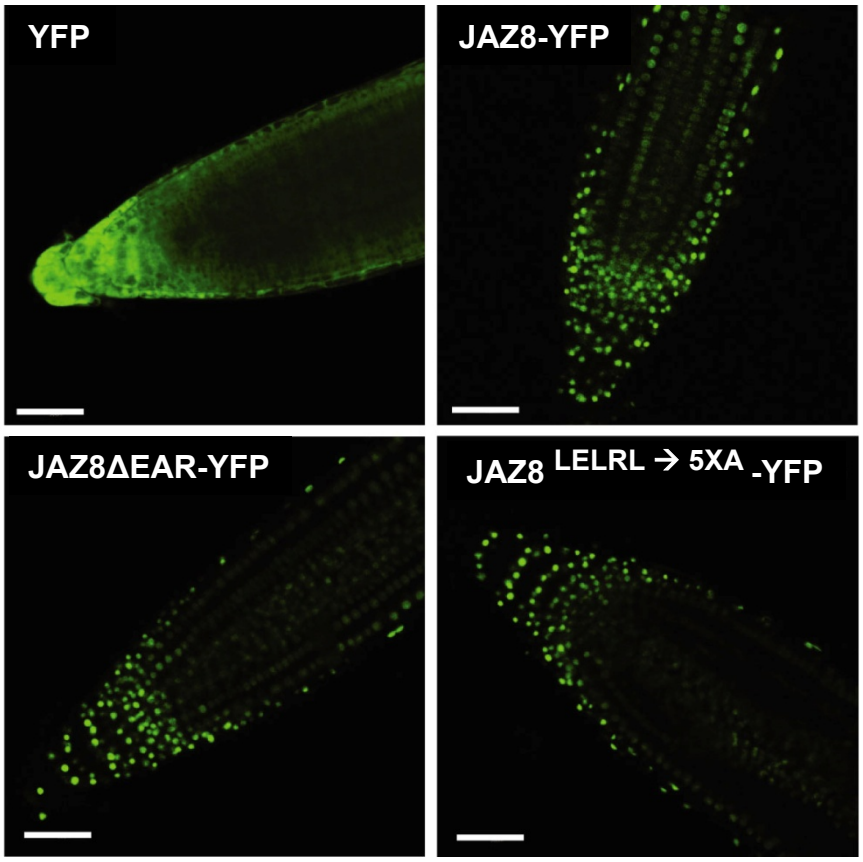
Supplemental Figure 2.4. JA-Mediated Root Growth Inhibition in *35S:JAZ8-YFP* Lines. – Continued.

(A) *35S:JAZ8-YFP* plants are insensitive to MeJA. Wild-type (WT), *35S:JAZ8* (line #24; *JAZ8*), *35S:YFP* (*YFP*), and *35S:JAZ8-YFP* (*JAZ8-YFP*) seedlings were grown on MS medium supplemented with or without 25 μM MeJA for 8 d under continuous light. Root length ratios were calculated as described in the legend of Supplemental Figure 2B. Data show the mean ± SE for each genotype (n > 20 seedlings per genotype). Asterisks indicate significant differences (P<0.05, Student's t-test) in comparisons between the indicated transgenic line and WT.
(B) Mutation of the EAR motif suppresses the JA-insensitive root growth phenotype of *35S:JAZ-YFP* seedlings. Root growth inhibition assays were performed with *35S:YFP* (*YFP*), *35S:JAZ8-YFP* (*JAZ8-YFP*), *35S:JAZ8ΔEAR-YFP* (*JAZ8ΔEAR-YFP*; gray bars), and four independent *35S:JAZ8*$^{LELRL\rightarrow 5xA}$*-YFP* (*JAZ8*$^{LELRL\rightarrow 5xA}$*-YFP*; gray bars) homozygous lines (T3 generation). Seedlings were grown for 8 d on MS medium containing or lacking 20 μM MeJA. Data were plotted as root length ratio as described in panel (A). Data show the mean ± SE for each genotype (n > 23 seedlings per genotype). Means with different italicized letters are significantly different at P<0.05. The root length ratio of *35S:JAZ8*$^{LELRL\rightarrow 5xA}$*-YFP* seedlings was calculated as the mean of all four independent *35S:JAZ8*$^{LELRL\rightarrow 5xA}$*-YFP* lines.



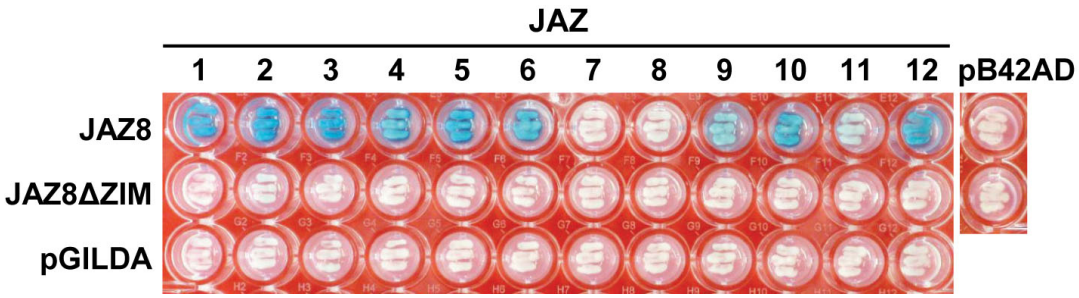
Supplemental Figure 2.5. Substitution of PKASM to LPIAR does not Affect the Ability of JAZ8 to Interact with MYC2, JAZ1, or TPL.

Yeast strains expressing both the bait (JAZ8 or JAZ8^{LPIAR}) and prey (MYC2, JAZ1, NINJA or TPL) were plated on SD-Gal medium containing X-gal. Blue color indicates protein-protein interaction. Photographic image was taken 48 h after incubation of yeast cells at 30°C. As a positive control for NINJA interaction, yeast cells were cotransformed with pB42AD-NINJA and pGILDA-JAZ1 (lower right).



Supplemental Figure 2.6. Nuclear Localization of JAZ8-YFP Fusion Proteins.

Root tips of 9-day-old seedlings expressing the indicated JAZ-YFP reporter were imaged with an Olympus Fluoview confocal microscope. Transgenic lines were *35S:YFP* (YFP), *35S:JAZ8-YFP* (JAZ8-YFP), *35S:JAZ8ΔEAR-YFP* #1-1 (JAZ8ΔEAR-YFP), and *35S:JAZ8*$_{LELRL→5xA}$*-YFP* #5-1 (JAZ8$_{LELRL→5xA}$-YFP). Scale bar = 50 μm.



Supplemental Figure 2.7. Yeast Two-hybrid Analysis of JAZ8 and JAZ8ΔZIM Interactions with other Arabidopsis JAZ proteins.

Yeast strains expressing both the bait (JAZ8 or JAZ8ΔZIM) and prey (JAZ1 – JAZ12) were plated on plates containing X-gal. Blue colors indicate protein-protein interaction. Photographic images were taken 48 h after incubation of yeast cells at 30°C.

Supplemental Table 2.1 List of oligonucleotide primers used in this study.

Primers in this section used for Y2H and overexpression constructs

INSERT	VECTOR	PRIMER NAME	SEQUENCE
JAZ8	pGILDA & pB42AD	JAZ8_NcoI_FP	5'-CCATGGATGAAGCTACAGCAAAATTGTG-3'
		JAZ8_XhoI_RP	5'-CTCGAGTTATCGTCGTGAATGGTACG-3'
	pBI-TONY	JAZ8_XbaI_FP	5'-ATATTCTAGAATGAAGCTACAGCAAAATTGTG-3'
		JAZ8_KpnI_RP	5'-ATATGGTACCTTATCGTCGTGAATGGTACG-3'
	pBI-EYFP	JAZ8_XbaI_FP	5'-ATATTCTAGAATGAAGCTACAGCAAAATTGTG-3'
		JAZ8ns_XhoI_RP	5'-CTCGAGTCGTCGTGAATGGTACGGTG-3'
	pENTR-TOPO	JAZ8_pENTR_FP	5'-CACCATGAAGCTACAGCAAAATTGTG-3'
		JAZ8_pENTR_RP	5'-TTATCGTCGTGAATGGTACGGTG-3'
JAZ8-Jas10	pGILDA & pB42AD	JAZ8_XbaI_FP	5'-ATATTCTAGAATGAAGCTACAGCAAAATTGTG-3'
		JAZ10_NcoI_RP	5'-CCATGGTTAGGCCGATGTCGGATAGTAAG-3'
	pBITONY	JAZ8_XbaI_FP	5'-ATATTCTAGAATGAAGCTACAGCAAAATTGTG-3'
		JAZ10_KpnI_RP	5'-GGTACCTTAGGCCGATGTCGGATAGTAAG-3'
	pBI-EYFP	JAZ8_XbaI_FP	5'-ATATTCTAGAATGAAGCTACAGCAAAATTGTG-3'
		JAZ10ns_KpnI_RP	5'-GGTACCGGCCGATGTCGGATAGTAAGGAG-3'
JAZ10-Jas8	pGILDA & pB42AD	JAZ10_NcoI_FP	5'-CCATGGATGTCGAAAGCTACCATAGAAC-3'
		JAZ8_XhoI_RP	5'-CTCGAGTTATCGTCGTGAATGGTACG-3'
	pBITONY	JAZ10_BamHI_FP	5'-GGATCCATGTCGAAAGCTACCATAGAAC-3'
		JAZ8_XhoI_RP	5'-CTCGAGTTATCGTCGTGAATGGTACG-3'
	pBI-EYFP	JAZ10_BamHI_FP	5'-GGATCCATGTCGAAAGCTACCATAGAAC-3'
		JAZ8ns_XhoI_RP	5'-CTCGAGTCGTCGTGAATGGTACGGTG-3'
JAZ8ΔZIM	pGILDA & pB42AD	JAZ8_NcoI_FP	5'-CCATGGATGAAGCTACAGCAAAATTGTG-3'
		JAZ8_XhoI_RP	5'-CTCGAGTTATCGTCGTGAATGGTACG-3'
	pBI-TONY	JAZ8_XbaI_FP	5'-ATATTCTAGAATGAAGCTACAGCAAAATTGTG-3'

Supplemental Table 2.1 - Continued.

		JAZ8_KpnI_RP	5'-ATATGGTACCTTATCGTCGTGAATGGTACG-3'
	pBI-EYFP	JAZ8_XbaI_FP	5'-ATATTCTAGAATGAAGCTACAGCAAAATTGTG-3'
		JAZ8ns_XhoI_RP	5'-CTCGAGTCGTCGTGAATGGTACGGTG-3'
JAZΔEAR	pBI-TONY	JAZ8_XbaI_FP	5'-ATATTCTAGAATGAAGCTACAGCAAAATTGTG-3'
		JAZ8_XhoI_RP	5'-CTCGAGTTATCGTCGTGAATGGTACG-3'
	pBI-EYFP	JAZ8_XbaI_FP	5'-ATATTCTAGAATGAAGCTACAGCAAAATTGTG-3'
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JAZ8ΔEZ	pBI-TONY	JAZ8_XbaI_FP	5'-ATATTCTAGAATGAAGCTACAGCAAAATTGTG-3'
		JAZ8_XhoI_RP	5'-CTCGAGTTATCGTCGTGAATGGTACG-3'
	pBI-EYFP	JAZ8_XbaI_FP	5'-ATATTCTAGAATGAAGCTACAGCAAAATTGTG-3'
		JAZ8ns_XhoI_RP	5'-CTCGAGTCGTCGTGAATGGTACGGTG-3'

Primers in this section used for MBP-JAZ-His constructs

INSERT	VECTOR	PRIMER NAME	SEQUENCE
JAZ6	pRMG-nMAL	AtJAZ6-5'NotI	5'-GCGCGGCCGCCATGTCAACGGGACAAGCGC-3'
		AtJAZ6-3'XhoI	5'-CGGCTCGAGAAGCTTGAGTTCAAGGTTTTTGG-3'
JAZ7	pRMG-nMAL	NotI-C-JAZ7-FP	5'-GCGCGGCCGCCATCATCATCAAAAACTGCGACAAGCC-3'
		JAZ7_NotI_FP	5'-GCGGCCGCATGATCATCATC-3'
		JAZ7_XhoI_RP	5'-CTCGAGCTATCGGTAACGGTGGTAAG-3'
JAZ8	pRMG-nMAL	JAZ8_NotI_FP	5'-GCGGCCGCATGAAGCTACAGC-3'
		JAZ8 XhoI RP	5'-CTCGAGTTATCGTCGTGAATGGTACG
JAZ12	pRMG-nMAL	AtJAZ12-NotI F	5'-GCGCGGCCGCCACTAAGGTGAAAGATGAG-3'
		AtJAZ12-SalI R	5'-TCGGTCGACAGCAGTTGGAAATTCCTCC-3'
JAZ8-Jas10	pRMG-nMAL	JAZ8_NotI_FP	5'-GCGGCCGCATGAAGCTACAGC-3'

Supplemental Table 2.1 - Continued.

		AtJAZ10-SalI	5'-TCGGTCGACGGCCGATGTCGGATAG-3'
JAZ10-Jas8	pRMG-nMAL	AtJAZ10-NotI JAZ8 XhoI RP	5'-GCGCGGCCGCCTCGAAAGCTACCATAGAACTCG-3' 5'-CTCGAGTTATCGTCGTGAATGGTACG
JAZ8^{LPIAR}	pRMG-nMAL	JAZ8_NotI_FP JAZ8 XhoI RP	5'-GCGGCCGCATGAAGCTACAGC-3' 5'-CTCGAGTTATCGTCGTGAATGGTACG

Primers in this section used for chimeric proteins and deletion constructs

CONSTRUCT	PRIMER NAME	SEQUENCE
JAZ8-Jas10	JAZ8_XbaI_FP JAZ8_Jas10_RP	5'-ATATTCTAGAATGAAGCTACAGCAAAATTGTG-3' 5'-GCGATGGGAAGATCTGGAAGCTGATTATGATGAAATG-3'
	JAZ8_Jas10_FP JAZ10_KpnI_RP	5'-CATAATCAGCTTCCAGATCTTCCCATCGCAAGGAG-3' 5'-GGTACCTTAGGCCGATGTCGGATAGTAAG-3'
	JAZ8_XbaI_FP JAZ10_KpnI_RP	5'-ATATTCTAGAATGAAGCTACAGCAAAATTGTG-3' 5'-GGTACCTTAGGCCGATGTCGGATAGTAAG-3'
JAZ10-Jas8	JAZ10_BamHI_FP JAZ10_Jas8_RP	5'-GGATCCATGTCGAAAGCTACCATAGAAC-3' 5'-GATGCTTTTGGATTTCCTTCTAGATTCTGGCCAAAG-3'
	JAZ10_Jas8_FP JAZ8_XhoI_RP	5'-GAATCTAGAAGGAAATCCAAAAGCATCAATGAAAAAATC-3' 5'-CTCGAGTTATCGTCGTGAATGGTACG-3'
	JAZ10_BamHI_FP JAZ8_XhoI_RP	5'-GGATCCATGTCGAAAGCTACCATAGAAC-3' 5'-CTCGAGTTATCGTCGTGAATGGTACG-3'
JAZ8ΔZIM	JAZ8_NcoI_FP JAZ8_deltaZIM_RP	5'-CCATGGATGAAGCTACAGCAAAATTGTG-3' 5'-GGTTTTCATTTCTCTGCTTTGAGATTCTTCATTTGG-3'
	JAZ8_deltaZIM_FP JAZ8_XhoI_RP	5'-CCAAATGAAGAATCTCAAAGCAGAGAAATGAAAACC-3' 5'-CTCGAGTTATCGTCGTGAATGGTACG-3'

Supplemental Table 2.1 - Continued.

	JAZ8_NcoI_FP	5'-CCATGGATGAAGCTACAGCAAAATTGTG-3'
	JAZ8_XhoI_RP	5'-CTCGAGTTATCGTCGTGAATGGTACG-3'
JAZ8ΔEAR	JAZ8_deltaEAR_FP	5'-GCTACAGCAAAATTGTGAC TTTCCCACTTCTTATGATTC-3'
	JAZ8_pENTR_RP	5'-TTATCGTCGTGAATGGTACGGTG-3'
	JAZ8_pENTR_FP	5'-CACCATGAAGCTACAGCAAAATTGTG-3'
	JAZ8_pENTR_RP	5'-TTATCGTCGTGAATGGTACGGTG-3'
JAZ8ΔEZ	JAZ8_deltaEAR_FP	5'-GCTACAGCAAAATTGTGAC TTTCCCACTTCTTATGATTC-3'
	JAZ8_pENTR_RP	5'-TTATCGTCGTGAATGGTACGGTG-3'
	JAZ8_pENTR_FP	5'-CACCATGAAGCTACAGCAAAATTGTG-3'
	JAZ8_pENTR_RP	5'-TTATCGTCGTGAATGGTACGGTG-3'
JAZ8ΔJas	JAZ8_NcoI_FP	5'-CCATGGATGAAGCTACAGCAAAATTGTG-3'
	JAZ8_XhoI_306R	5'-CTCGAGTTATGGATTTGGAAGCTGATTATG-3'
JAZ8-Jas	JAZ8_ATG133_NcoI_FP	5'-CCATGGATGCATAATCAGCTTCCAAATCC-3'
	JAZ8_XhoI_RP	5'-CTCGAGTTATCGTCGTGAATGGTACG-3'

Primers in this section used for site-directed mutagenesis

CONSTRUCT	PRIMER NAME	SEQUENCE
JAZ8$^{LPIAR→AAAAA}$	JAZ8_AR_FP	5'-CCAAATCCAAAAGCAGCAAGGAAAAAATCTCTCC-3'
	JAZ8_AR_RP	5'-GGAGAGATTTTTTCCTTGCTGCTTTTGGATTTGG-3'
	JAZ8_IAR_FP	5'-CCAAATCCAAATCCAAAAATCGCAAGGAAAAAATCTCTC-3'
	JAZ8_IAR_RP	5'-GAGAGATTTTTTCCTTGCGATTTTTGGATTTGGATTTGG-3'
	JAZ8_PIAR_FP	5'-CTTCCAAATCCAAATCCACCCATCGCAAGGAAAAAATC-3'
	JAZ8_PIAR_RP	5'-GATTTTTTCCTTGCGATGGGTGGATTTGGATTTGGAAG-3'

Supplemental Table 2.1 - Continued.

	JAZ8_LPIAR_FP	5'-CATAATCAGCTTCCA AATCTTCCCATCGCAAGGAAAAAATC-3'
	JAZ8_LPIAR_RP	5'-GATTTTTTCCTTGCGATGGGAAGATTTGGAAGCTGATTATG-3'
JAZ8$^{LELRL\rightarrow AAAAA}$	JAZ8_LEtoAA_FP	5'-GCTACAGCAAAATTGTGACGCGGCACTTCGTCTTTTTCCCAC-3'
	JAZ8_LEtoAA_FP	5'-GTGGGAAAAAGACGAAGTGCCGCGTCACAATTTTGCTGTAGC-3'
	JAZ8_LELRLtoAAAAA_FP	5'-GCAAAATTGTGACGCGGCAGCTGCTGCTTTTCCCACTTCTTATGATTC-3'
	JAZ8_LELRLtoAAAAA_RP	5'-GAATCATAAGAAGTGGGAAAAGCAGCAGCTGCCGCGTCACAATTTTGC-3'

Primers in this section used in carrot protoplast assay

CONSTRUCT	PRIMER NAME	SEQUENCE
35S::GD-JAZ8	JAZ8-F	5´-GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGAAGCTACAGCAAAATTGTGACTTGG-3´
		5´-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCATCGTCGTGAATGGTACGGTGAAGTAG-3´

Supplemental Table 2.1 - Continued.

35S::GD-JAZ8ΔJas	JAZ8-F	5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGAAGCTACAGCAAAATTGTGACTTGG-3′
	JAZ8-Jas-R	5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAATTTGGAAGCTGATTATGATGAAATGAGG-3′
35S::GD-JAZ8-N	JAZ8-F	5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGAAGCTACAGCAAAATTGTGACTTGG-3′
	JAZ8[I]-R	5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTATTGAGATTCTTCATTTGGTTGTGGATTT-3
JAZ8-L9A	JAZ8-L9A-F	5′-AAGCTACAGCAAAATTGTGACGCGGAACTTCGTCTTTTTCCCAC-3′
	JAZ8-L9A-R	5′-GTGGGAAAAAGACGAAGTTCCGCGTCACAATTTTGCTGTAGCTT-3′
JAZ8-L11A	JAZ8-L11A-F	5′-GCTACAGCAAAATTGTGACTTGGAAGCTCGTCTTTTTCCCACT-3′
	JAZ8-L11A-R	5′-AGTGGGAAAAAGACGAGCTTCCAAGTCACAATTTTGCTGTAGC-3′
JAZ8-L13A	JAZ8-L13A-F	5′-CAAAATTGTGACTTGGAACTTCGTGCTTTTCCCACTTCTTATGATTCTGA-3′
	JAZ8-L13A-R	5′-TCAGAATCATAAGAAGTGGGAAAAGCACGAAGTTCCAAGTCACAATTTTG-3′
JAZ8-F14A	JAZ8-F14A-F	5′-ACAGCAAAATTGTGACTTGGAACTTCGTCTTGCTCCCACTTCTTATGATT-3′
	JAZ8-F14A-R	5′-AATCATAAGAAGTGGGAGCAAGACGAAGTTCCAAGTCACAATTTTGCTGT-3′
JAZ8-AAA	JAZ8-AAA-F	5′-CGCATATGAAGCTACAGCAAAATTGTGACGCGGCCGCTCGTCTTTTTCCCACTTCTTATGATTCTGATTCTT-3′
	JAZ8-AAA-R	5′-AAGAATCAGAATCATAAGAAGTGGGAAAAAGACGAGCGGCCGCGTCACAATTTTGCTGTAGCTTCATATGCG-3′

Supplemental Table 2.2. JAZ genes used for construction of consensus motifs shown in Figure 2.6.

JAZ8-like sequences used in Figure 2.6A

SPECIES	GENE ID	JAS MOTIF
Arabidopsis lyrata	881314	NPKASMKKSLQSFLQKRKSRIQATSPY
Arabidopsis lyrata	333262	NQKVSMKRSLRSFLQKRNVRIQATSPY
Arabidopsis lyrata	482423	NQKVSMKRSLRSFLQKRNVRIQATSPY
Glycine max	Glyma05g27280.1	STGLSMKRSLQRFLQKRKNRVQETSPY
Glycine max	Glyma08g27280.1	GTGLSMKRSLQRFLQKRKNRVQETSPY
Glycine max	Glyma13g29070.1	GTGLSMRKSLQRFLQKRKNRVQEASPY
Glycine max	Glyma15g09980.1	GTGPSMRKSLQRFLQKRRNRVQEASPY
Carica papaya	supercontig_17.12	ANGLSMKRSLQRFLQKRKHSIRATSPY
Ricinus communis	29693.m001988	STGLSMKRSIQRFLQKRKHRAQATSPY
Ricinus communis	29693.m001989	NNGLSMKRSLQRFLQKRNHRIQATCPY
Cucumis sativus	Cucsa.047800.1	TPGLSMKKSLQRFLQKRKHRVQATSPY
Cucumis sativus	Cucsa.054580.1	EASPSMRRSLQRFLQKRKLRIQTLSPY
Cucumis sativus	Cucsa.054630.1	GSGLSMKRSLQRFLQKRKNRIQSASPY
Cucumis sativus	Cucsa.179960.1	AGFSIKKSLQKFLQRRKMRRIRTMSPY
Medicago truncatula	Medtr2g024430.1	GPGLSMKRSLQRFLQKRKNRVQEASPY
Medicago truncatula	Medtr4g154880.1	QSGLSMKRSLQRFLQKRKNRVQEASPY
Prunus persica	ppa013410m	VAGMSMKRSLQRFLQKRKHRVQATSPY
Prunus persica	ppa018461m	PPGVSMKRSLQSFLQKRKKRSQEASPY
Mimulus guttatas	mgv1a016361m	TSGLSMKRSLQRFLEKRKTRLQCTSPY
Mimulus guttatas	mgv1a016202m	SSVVSMKRSLKMFLQKRKTRSQASSPY
Vitus Vinifera	GSVIVT01021514001	ATGLSMKRSLQRFLQKRKNRMEATSPY
Vitus Vinifera	GSVIVT01021516001	PTGLSMKRSLQRFLQKRKNRMEATSPY
Vitus Vinifera	GSVIVT01021519001	PTGLSMKRSLRSFLQKRKNRIEATSPY
Manihot esculenta	cassava4.1_026855m	VYSPNMKISLQRFLEKRNHRIQTTYPY
Manihot esculenta	cassava4.1_019045m	AAGLSMKRSIQRFLQKRKHRVQENSPY
Manihot esculenta	cassava4.1_018315m	NTALSMKRSLQRFLQKRNNRMQATYPY
Citrus sinensis	orange1.1g046141m	NSGLSMKRSLQRFLQKRKHRSLATSPY
Poplus trichocarpa	POPTR_0006s02410.1	PPGLSLKRSLQRFLQKRKKRAEAISPY
Eucalyptus grandis	Egrandis_v1_0.031045m	STGLSMKRSLQRFLQKRQHRSQATSPY
Eucalyptus grandis	Egrandis_v1_0.049073m	GTGLSMKRSLQRFLQKRKLRFHATSPY
Citrus clementina	clementine0.9_025662m	NSGLSMKRSLQRFLQKRKHRSLATSPY
Arabidopsis thaliana	AT2G34600.1	YQKASMKRSLHSFLQKRSLRIQATSPY
Arabidopsis thaliana	AT1G30135.1	NPKASMKKSLQSFLQKRKIRIQATSPY

Supplemental Table 2.2. Continued.

JAZ10-like sequences used for Figure 2.6A

SPECIES	GENE ID	JAS MOTIF
Sorghum bicolor	Sb01g045180	DMPLARKVSLKRFLEKRKNRLTAADPY
Sorghum bicolor	Sb02g025720	DLPIARRNSLHRFLEKRKDRITAKAPY
Sorghum bicolor	Sb06g031060	DLPIARRHSLQRFLEKRRDRIVSKAPY
Selaginella moellendorffii	439249	DLPQARKASLHRFLEKRKDRLFAKSDK
Arabidopsis lyrata	488996	DLPIARRHSLQRFLEKRRDRLVNKNPY
Arabidopsis lyrata	936941	DVPIARRRSLQRFLEKRRDRFVHTNPY
Arabidopsis lyrata	476379	VERIARRASLHRFFAKRKDRAVARAPY
Arabidopsis lyrata	472142	ELPIARRASLHRFLEKRKDRVTSKAPY
Arabidopsis lyrata	476633	ELPIARRASLHRFLEKRKDRITSKAPY
Arabidopsis lyrata	488138	DLPIARRKSLQRFLEKRKERLVSTSPY
Glycine max	Glyma09g09100.1	EFPIARRHSLQRFLEKRRDRLGSKAPY
Glycine max	Glyma09g09100.2	EFPIARRHSLQRFLEKRRDRLGSKAPY
Glycine max	Glyma13g17180.1	DLPIARKASLHRFLSKRKDRIAAKAPY
Glycine max	Glyma13g17640.2	EFPLARRQSLQRFLEKRRNRLANKSPH
Glycine max	Glyma15g19840.4	HLPIARKASLHRFLEKRKDRIASKAPY
Glycine max	Glyma15g20670.1	EFPIARRHSLQRFLEKRRDRLGSKTPY
Glycine max	Glyma17g05540.1	ELPIARKVSLHRFLSKRKDRIASKAPY
Carica papaya	supercontig_113.51	NLPIARKISLQHFIEKRKSRVMSQSPY
Brachypodium distachyon	Bradi1g72600.1	EMPMARKASLQRFLEKRKSRLAAADPY
Brachypodium distachyon	Bradi3g10820.1	DLPIARKASLHRFLEKRKDRLHAKAPY
Brachypodium distachyon	Bradi4g31240.1	DLPIARRNSLHRFLEKRKGRVIAKAPY
Brachypodium distachyon	Bradi5g08650.1	DIPLARTKSLQQFLVKRKERLTHLGPY
Brachypodium distachyon	Bradi5g24410.2	DLPIARRHSLQRFLEKRRDRIVSKAPY
Ricinus communis	29727.m000494	DLPIARRASLHRFFEKRKDRAAAKAPY
Ricinus communis	29765.m000754	DLPIARKLSLQHFLEKRRRRRTGKSPY
Ricinus communis	30128.m009047	DLPIARRKSLQRFLEKRKERLTSASPY
Oryza sativa	Os03g08320	DMPIARKVSLQRFLEKRKNRIVVAEPL
Oryza sativa	Os04g32480	KEPLTRTKSLQRFLSKRKERLTSLGPY
Oryza sativa	Os09g26780	DLPIARRNSLHRFLEKRKGRMNANAPY
Cucumis sativus	Cucsa.141940	ALPMARKASIQRFLEKRKDRLTPRTPY
Cucumis sativus	Cucsa.149450	DLPIARRASLHRFFEKRKDRVAARGPY
Cucumis sativus	Cucsa.349280	DLPIARKKSLQRFLEKRKERLTTASPY
Medicago truncatula	Medtr5g013690	DLPMTRKASLHRFLEKRKDRIAAKAPY
Prunus persica	ppa011370m	DLPIARRASLHKFLAKRKERVAAIAPY
Prunus persica	ppa011303m	EFPIARRHSLQRFLEKRRDRLVSKNPY
Prunus persica	ppa011173m	DLPIARRKSLQRFLEKRKERLNSVSPF
Mimulus guttatas	mgv1a012664m	YLPIARKKSLARFLEKRKDRITANAPY

Supplemental Table 2.2. Continued.

JAZ10-like sequences used for Figure 2.6A – CONTINUED

SPECIES	GENE ID	JAS MOTIF
Mimulus guttatas	mgv1a015572m	DLPITRRISLQRFLEKRRERLIMLSPY
Mimulus guttatas	mgv1a015559m	DLPITRRISLQRFLEKRRERLIMLSPY
Vitus Vinifera	GSVIVT01000967001	DLPLARRKSLHRFLEKRKERLTSVYPY
Vitus Vinifera	GSVIVT01015042001	ELPIARKASLHRFLEKRKDRITARAPY
Vitus Vinifera	GSVIVT01023256001	DFPIARKSSLQRFLEKRRDRITSRSPY
Manihot esculenta	cassava4.1_013723m	DLPIARRASLHRFLEKRKDRITARAPY
Manihot esculenta	cassava4.1_031135m	GLPIVTRVSLHRFFEKRKERVASKAPY
Manihot esculenta	cassava4.1_016821m	DLPIARRKSLQRFLEKRKERLTSLSPY
Manihot esculenta	cassava4.1_016877m	DLPIARRKSLQRFLEKRKERLTSLSPY
Manihot esculenta	cassava4.1_015933m	ELPIARRHSLQRFFEKRRDRLYSKSPY
Manihot esculenta	cassava4.1_014096m	DLPIARRASLHRFLEKRKDRVASKAPY
Manihot esculenta	cassava4.1_017020m	DLPIARRKSLQRFLEKRKERLTLAFPH
Citrus sinensis	orange1.1g027340m	ELPMARRHSLQRFFEKRRDRLVSKNPY
Citrus sinensis	orange1.1g027356m	ELPMARRHSLQRFFEKRRDRLVSKNPY
Citrus sinensis	orange1.1g028845m	ELPMARRHSLQRFFEKRRDRLVSKNPY
Citrus sinensis	orange1.1g030011m	ELPMARRHSLQRFFEKRRDRLVSKNPY
Citrus sinensis	orange1.1g028982m	TVPIARRASLHRFFEKRKDRAIARAPY
Aquilegia coerulea	AcoGoldSmith_v1.011636m	DLPFARKKSLQRFLEKRKERLISVTPY
Poplus trichocarpa	POPTR_0006s23390.1	ELPIARRHSLQRFFEKRRDRLVSKSPY
Poplus trichocarpa	POPTR_0018s08300.1	ELPIARRQSLQRFFKKRRDRLVSKSPY
Poplus trichocarpa	POPTR_0003s16350.1	DLPIARRKSLQRFLEKRKERLTSATPY
Poplus trichocarpa	POPTR_0003s06670.1	DVPHARRASLHRFFSKRKDRVTARAPY
Poplus trichocarpa	POPTR_0012s04220.1	AVPQARKASLARFLEKRKERVTQTSPY
Poplus trichocarpa	POPTR_0012s04220.2	AVPQARKASLARFLEKRKERVTQTSPY
Poplus trichocarpa	POPTR_0012s04220.3	AVPQARKASLARFLEKRKERVTQTSPY
Poplus trichocarpa	POPTR_0001s13240.1	DLPIARRKSLQRFLEKRKGRLTSVSPY
Eucalyptus grandis	Eucgr.F02865.1	TLPQARRATLVRFLEKRKDRLSSDIYN
Eucalyptus grandis	Eucgr.F02865.2	TLPQARRATLVRFLEKRKDRLSSDIYN
Eucalyptus grandis	Eucgr.G01954.1	DLPLTRRKSLERFFEKRKERLTFASPY
Eucalyptus grandis	Eucgr.B03545.1	YLPIARRKSLQRFLEKRKERLTSASPY
Eucalyptus grandis	Eucgr.C00753.1	DLPIARRHSLQRFFEKRRDRLVNKAPY
Eucalyptus grandis	Eucgr.C00753.2	DLPIARRHSLQRFFEKRRDRLVNKAPY
Eucalyptus grandis	Eucgr.H00537.1	ALPQARQASLARFLEKRKERAMTTSPY
Eucalyptus grandis	Eucgr.H00537.2	ALPQARQASLARFLEKRKERAMTTSPY
Citrus clementina	clementine0.9_021045m	ELPMARRHSLQRFFEKRRDRLVSKNPY
Citrus clementina	clementine0.9_021054m	ELPMARRHSLQRFFEKRRDRLVSKNPY
Citrus clementina	clementine0.9_022268m	TVPIARRASLHRFFEKRKDRAIARAPY
Citrus clementina	clementine0.9_023056m	DLPIARRKSLQRFLEKRKERNDMESDI

Supplemental Table 2.2. Continued.

JAZ10-like sequences used for Figure 2.6A – CONTINUED

SPECIES	GENE ID	JAS MOTIF
Zea mays	GRMZM2G116614_T01	DLPIARRNSLHRFLEKRKDRITAKAPY
Zea mays	GRMZM2G145407_T01	DMPLTRTKSLQQFLQKRKERLSGPGPY
Zea mays	GRMZM2G086920_T02	DLPIARRHSLQRFLEKRRDRVVSKAPY
Zea mays	GRMZM2G005954_T01	DLPIARRNSLHRFLEKRKDRITAKAPY
Zea mays	GRMZM2G005954_T02	DLPIARRNSLHRFLEKRKDRITAKAPY
Zea mays	GRMZM2G024680_T01	MPPIARKLTLQNFLRKRKNRIAGTDDA
Zea mays	GRMZM2G143402_T03	DLPIARRHSLQRFLEKRRDRVVSKAPY
Zea mays	GRMZM2G143402_T01	DLPIARRHSLQRFLEKRRDRVVSKAPY
Zea mays	GRMZM2G143402_T02	DLPIARRHSLQRFLEKRRDRVVSKAPY
Arabidopsis thaliana	AT1G72450.1	VERIARRASLHRFFAKRKDRAVARAPY
Arabidopsis thaliana	AT1G70700.2	SVPQARKASLARFLEKRKERLMSAMPY
Arabidopsis thaliana	AT1G74950.1	ELPIARRASLHRFLEKRKDRITSKAPY
Arabidopsis thaliana	AT1G19180.1	ELPIARRASLHRFLEKRKDRVTSKAPY
Arabidopsis thaliana	AT1G19180.2	ELPIARRASLHRFLEKRKDRVTSKAPY
Arabidopsis thaliana	AT3G43440.1	DVPIARRRSLQRFFEKRRHRFVHTKPY
Arabidopsis thaliana	AT3G43440.2	DVPIARRRSLQRFFEKRRHRFVHTKPY
Arabidopsis thaliana	AT5G20900.1	DLPIARRHSLQRFLEKRRDRLVNKNPY
Setaria italica	Si037628m	DLPIARKASLQRFLQKRKHRINAAEPY
Setaria italica	Si023275m	DLPIARRHSLQRFLEKRRDRIVNKAPY

Supplemental Table 2.2. Continued.

JAZ8-like sequences used in Figure 2.6B

SPECIES	GENE ID	EAR MOTIF
Arabidopsis lyrata	881314	LELRL
Arabidopsis lyrata	333262	LELRL
Glycine max	Glyma05g27280.1	LELRL
Glycine max	Glyma08g27280.1	LELRL
Glycine max	Glyma13g29070.1	LELAL
Glycine max	Glyma15g09980.1	LELAL
Carica papaya	evm.model.supercontig_17.12	LELRL
Ricinus communis	29693.m001989	LELRL
Cucumis sativus	Cucsa.047800.1	LELRL
Cucumis sativus	Cucsa.054580.1	LELCL
Cucumis sativus	Cucsa.054630.1	LELRL
Cucumis sativus	Cucsa.179960.1	LELGL
Medicago truncatula	Medtr2g024430.1	LELCL
Medicago truncatula	Medtr4g154880.1	LELCL
Prunus persica	ppa013410m	LELQL
Mimulus guttatas	mgv1a016361m	LELPL
Mimulus guttatas	mgv1a016202m	LDLRL
Vitus Vinifera	GSVIVT01021514001	LELRL
Vitus Vinifera	GSVIVT01021516001	LEVRL
Manihot esculenta	cassava4.1_026855m	LELRL
Manihot esculenta	cassava4.1_019045m	LELRL
Manihot esculenta	cassava4.1_018315m	LELRL
Citrus sinensis	orange1.1g046141m	LELRL
Aquilegia coerulea	AcoGoldSmith_v1.025874m	LDLCL
Poplus trichocarpa	POPTR_0006s02410.1	LDLCL
Poplus trichocarpa	POPTR_0011s02260.1	LELRL
Eucalyptus grandis	Egrandis_v1_0.031045m	LELRL
Citrus clementina	clementine0.9_025662m	LELRL
Arabidopsis thaliana	AT2G34600.1	LELRL
Arabidopsis thaliana	AT1G30135.1	LELRL

REFERENCES

REFERENCES

Bai, Y., Meng, Y., Huang, D., Qi, Y., and Chen, M. (2011). Origin and evolutionary analysis of the plant-specific TIFY transcription factor family. Genomics **98:** 128-136.

Ballare, C.L. (2011). Jasmonate-induced defenses: a tale of intelligence, collaborators and rascals. Trends Plant Sci. **16:** 249-257.

Blechert, S., Bockelmann, C., Fusslein, M., Von Schrader, T., Stelmach, B., Niesel, U., and Weiler, E.W. (1999). Structure-activity analyses reveal the existence of two separate groups of active octadecanoids in elicitation of the tendril-coiling response of Bryonia dioica Jacq. Planta **207:** 470-479.

Browse, J. (2009). Jasmonate passes muster: a receptor and targets for the defense hormone. Annu. Rev. Plant Biol. **60:** 183-205.

Causier B, Ashworth M, Guo W, Davies B. (2012). The TOPLESS interactome: A framework for gene repression in Arabidopsis. Plant Physiol. doi:10.1104/pp.111.186999.

Cheng, Z., Sun, L., Qi, T., Zhang, B., Peng, W., Liu, Y., and Xie, D. (2011). The bHLH transcription factor MYC3 interacts with the Jasmonate ZIM-domain proteins to mediate jasmonate response in Arabidopsis. Mol. Plant **4:** 279-288.

Chini, A., Fonseca, S., Chico, J.M., Fernandez-Calvo, P., and Solano, R. (2009). The ZIM domain mediates homo- and heteromeric interactions between Arabidopsis JAZ proteins. Plant J. **59:** 77-87.

Chini, A., Fonseca, S., Fernandez, G., Adie, B., Chico, J.M., Lorenzo, O., Garcia-Casado, G., Lopez-Vidriero, I., Lozano, F.M., Ponce, M.R., Micol, J.L., and Solano, R. (2007). The JAZ family of repressors is the missing link in jasmonate signalling. Nature **448:** 666-671.

Chung, H.S., and Howe, G.A. (2009). A critical role for the TIFY motif in repression of jasmonate signaling by a stabilized splice variant of the JASMONATE ZIM-domain protein JAZ10 in Arabidopsis. Plant Cell **21:** 131-145.

Chung, H.S., Niu, Y., Browse, J., and Howe, G.A. (2009). Top hits in contemporary JAZ: an update on jasmonate signaling. Phytochemistry **70:** 1547-1559.

Chung, H.S., Koo, A.J.K., Gao, X., Jayanty, S., Thines, B., Jones, A.D., and Howe, G.A. (2008). Regulation and function of Arabidopsis *JASMONATE ZIM*-domain genes in response to wounding and herbivory Plant Physiol. **146:** 952-964.

Chung, H.S., Cooke, T.F., Depew, C.L., Patel, L.C., Ogawa, N., Kobayashi, Y., and Howe, G.A. (2010). Alternative splicing expands the repertoire of dominant JAZ repressors of jasmonate signaling. Plant J. **63:** 613-622.

Arabidopsis Interactome Mapping Consortium. (2011). Evidence for network evolution in an Arabidopsis interactome map. Science **333:** 601-607.

Crooks, G.E., Hon, G., Chandonia, J.M., and Brenner, S.E. (2004). WebLogo: A sequence logo generator. Genome Research **14:** 1188-1190.

Demianski, A.J., Chung, K.M., and Kunkel, B.N. (2012). Analysis of Arabidopsis JAZ gene expression during Pseudomonas syringae pathogenesis. Mol. Plant Pathol. **13:** 46-57.

Dombrecht, B., Xue, G.P., Sprague, S.J., Kirkegaard, J.A., Ross, J.J., Reid, J.B., Fitt, G.P., Sewelam, N., Schenk, P.M., Manners, J.M., and Kazan, K. (2007). MYC2 differentially modulates diverse jasmonate-dependent functions in Arabidopsis. Plant Cell **19:** 2225-2245.

Dreher, K.A., Brown, J., Saw, R.E., and Callis, J. (2006). The Arabidopsis Aux/IAA protein family has diversified in degradation and auxin responsiveness. Plant Cell **18:** 699-714.

Fernández-Calvo, P., Chini, A., Fernández-Barbero, G., Chico, J.M., Gimenez-Ibanez, S., Geerinck, J., Eeckhout, D., Schweizer, F., Godoy, M., Franco-Zorrilla, J.M., Pauwels, L., Witters, E., Puga, M.I., Paz-Ares, J., Goossens, A., Reymond, P., De Jaeger, G., and Solano, R. (2011). The Arabidopsis bHLH transcription factors MYC3 and MYC4 are targets of JAZ repressors and act additively with MYC2 in the activation of jasmonate responses. Plant Cell **23:** 701-715.

Fonseca, S., Chini, A., Hamberg, M., Adie, B., Porzel, A., Kramell, R., Miersch, O., Wasternack, C., and Solano, R. (2009). (+)-7-iso-Jasmonoyl-L-isoleucine is the endogenous bioactive jasmonate. Nat. Chem. Biol. **5:** 344-350.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu. Rev. Phytopathol. **43:** 205-227.

Grunewald, W., Vanholme, B., Pauwels, L., Plovie, E., Inze, D., Gheysen, G., and Goossens, A. (2009). Expression of the Arabidopsis jasmonate signalling repressor JAZ1/TIFY10A is stimulated by auxin. EMBO Rep. **10:** 923-928.

Hiratsu, K., Matsui, K., Koyama, T., and Ohme-Takagi, M. (2003). Dominant repression of target genes by chimeric repressors that include the EAR motif, a repression domain, in Arabidopsis. Plant J. **34:** 733-739.

Hopke, J., Donath, J., Blechert, S., and Boland, W. (1994). Herbivore-induced volatiles: the emission of acyclic homoterpenes from leaves of *Phaseolus lunatus* and *Zea mays* can be triggered by a β-glucosidase and jasmonic acid. FEBS Lett. **352:** 146-150.

Hou, X., Lee, L.Y., Xia, K., Yan, Y., and Yu, H. (2010). DELLAs modulate jasmonate signaling via competitive binding to JAZs. Dev. Cell **19:** 884-894.

Howe G.A. (2010).Ubiquitin ligase-coupled receptors extend their reach to jasmonate. Plant Physiol. **154:** 471-474.

Howe, G.A., and Jander, G. (2008). Plant immunity to insect herbivores. Annu. Rev. Plant Biol. **59:** 41-66.

Kagale, S., and Rozwadowski, K. (2011). EAR motif-mediated transcriptional repression in plants: an underlying mechanism for epigenetic regulation of gene expression. Epigenetics **6:** 141-146.

Kagale, S., Links, M.G., and Rozwadowski, K. (2010). Genome-wide analysis of ethylene-responsive element binding factor-associated amphiphilic repression motif-containing transcriptional regulators in Arabidopsis. Plant Physiol. **152:** 1109-1134.

Katsir, L., Chung, H.S., Koo, A.J., and Howe, G.A. (2008a). Jasmonate signaling: a conserved mechanism of hormone sensing. Curr. Opin. Plant Biol. **11:** 428-435.

Katsir, L., Schilmiller, A.L., Staswick, P.E., He, S.Y., and Howe, G.A. (2008b). COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. Proc. Natl. Acad. Sci. USA **105:** 7100-7105.

Kazan, K., and Manners, J.M. (2011). The interplay between light and jasmonate signalling during defence and development. J. Exp. Bot. **62:** 4087-4100.

Kazan, K., and Manners, J.M. (2012). JAZ repressors and the orchestration of phytohormone crosstalk. Trends Plant Sci. doi:10.1016/j.tplants.2011.10.006.

Koo, A.J., Cooke, T.F., and Howe, G.A. (2011). Cytochrome P450 CYP94B3 mediates catabolism and inactivation of the plant hormone jasmonoyl-L-isoleucine. Proc. Natl. Acad. Sci. USA **108:** 9298-9303.

Koo, A.J.K., Gao, X.L., Jones, A.D., and Howe, G.A. (2009). A rapid wound signal activates the systemic synthesis of bioactive jasmonates in Arabidopsis. Plant J. **59:** 974-986.

Krogan, N.T., and Long, J.A. (2009). Why so repressed? Turning off transcription during plant growth and development. Curr. Opin. Plant Biol. **12:** 628-636.

Liu, Z.B., Ulmasov, T., Shi, X., Hagen, G., and Guilfoyle, T.J. (1994). Soybean GH3 promoter contains multiple auxin-inducible elements. Plant Cell **6:** 645-657.

Lorenzo, O., Chico, J.M., Sanchez-Serrano, J.J., and Solano, R. (2004). JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. Plant Cell **16:** 1938-1950.

Melotto, M., Mecey, C., Niu, Y., Chung, H.S., Katsir, L., Yao, J., Zeng, W., Thines, B., Staswick, P., Browse, J., Howe, G.A., and He, S.Y. (2008). A critical role of two positively charged amino acids in the Jas motif of Arabidopsis JAZ proteins in mediating coronatine- and jasmonoyl isoleucine-dependent interactions with the COI1 F-box protein. Plant J. **55:** 979-988.

Miersch, O., Kramell, R., Parthier, B., and Wasternack, C. (1999). Structure-activity relations of substituted, deleted or stereospecifically altered jasmonic acid in gene expression of barley leaves. Phytochemistry **50:** 353-361.

Moreno, J.E., Tao, Y., Chory, J., and Ballare, C.L. (2009). Ecological modulation of plant defense via phytochrome control of jasmonate sensitivity. Proc. Natl. Acad. Sci. USA **106:** 4935-4940.

Niu, Y.J., Figueroa, P., and Browse, J. (2011). Characterization of JAZ-interacting bHLH transcription factors that regulate jasmonate responses in Arabidopsis. J. Exp. Bot. **62:** 2143-2154.

Ohta, M., Matsui, K., Hiratsu, K., Shinshi, H., and Ohme-Takagi, M. (2001). Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. Plant Cell **13:** 1959-1968.

Pauwels, L., Barbero, G.F., Geerinck, J., Tilleman, S., Grunewald, W., Perez, A.C., Chico, J.M., Bossche, R.V., Sewell, J., Gil, E., Garcia-Casado, G., Witters, E., Inze, D., Long, J.A., De Jaeger, G., Solano, R., and Goossens, A. (2010). NINJA connects the co-repressor TOPLESS to jasmonate signalling. Nature **464:** 788-791.

Pauwels, L., and Goossens, A. (2011). The JAZ proteins: A crucial interface in the jasmonate signaling cascade. Plant Cell **23:** 3089-3100.

Pieterse, C.M.J., Leon-Reyes, A., Van der Ent, S., and Van Wees, S.C.M. (2009). Networking by small-molecule hormones in plant immunity. Nat. Chem. Biol. **5:** 308-316.

Qi, T., Song, S., Ren, Q., Wu, D., Huang, H., Chen, Y., Fan, M., Peng, W., Ren, C., and Xie, D. (2011). The jasmonate-ZIM-domain proteins interact with the WD-repeat/bHLH/MYB complexes to regulate jasmonate-mediated anthocyanin accumulation and trichome initiation in *Arabidopsis thaliana*. Plant Cell **23:** 1795-1814.

Ribot, C., Zimmerli, C., Farmer, E.E., Reymond, P., and Poirier, Y. (2008). Induction of the Arabidopsis PHO1;H10 gene by 12-oxo-phytodienoic acid but not jasmonic acid via a CORONATINE INSENSITIVE1-dependent pathway. Plant Physiol. **147:** 696-706.

Robson, F., Okamoto, H., Patrick, E., Harris, S.R., Wasternack, C., Brearley, C., and Turner, J.G. (2010). Jasmonate and phytochrome A signaling in Arabidopsis wound and shade responses are integrated through JAZ1 stability. Plant Cell **22:** 1143-1160.

Sato, A., and Yamamoto, K.T. (2008). Overexpression of the non-canonical Aux/IAA genes causes auxin-related aberrant phenotypes in Arabidopsis. Physiol. Plant **133:** 397-405.

Sheard, L.B., Tan, X., Mao, H.B., Withers, J., Ben-Nissan, G., Hinds, T.R., Kobayashi, Y., Hsu, F.F., Sharon, M., Browse, J., He, S.Y., Rizo, J., Howe, G.A., and Zheng, N. (2010). Jasmonate perception by inositol-phosphate-potentiated COI1-JAZ co-receptor. Nature **468:** 400-405.

Song, S., Qi, T., Huang, H., Ren, Q., Wu, D., Chang, C., Peng, W., Liu, Y., Peng, J., and Xie, D. (2011). The Jasmonate-ZIM domain proteins interact with the R2R3-MYB transcription factors MYB21 and MYB24 to affect jasmonate-regulated stamen development in Arabidopsis. Plant Cell **23:** 1000-1013.

Stintzi, A., Weber, H., Reymond, P., Browse, J., and Farmer, E.E. (2001). Plant defense in the absence of jasmonic acid: the role of cyclopentenones. Proc. Natl. Acad. Sci. USA **98:** 12837-12842.

Szemenyei, H., Hannon, M., and Long, J.A. (2008). TOPLESS mediates auxin-dependent transcriptional repression during Arabidopsis embryogenesis. Science **319:** 1384-1386.

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., Nomura, K., He, S.Y., Howe, G.A., and Browse, J. (2007). JAZ repressor proteins are targets of the SCF^{COI1} complex during jasmonate signalling. Nature **448:** 661-665.

Tiwari, S.B., Hagen, G., and Guilfoyle, T.J. (2004). Aux/IAA proteins contain a potent transcriptional repression domain. Plant Cell **16:** 533-543.

Vanholme, B., Grunewald, W., Bateman, A., Kohchi, T., and Gheysen, G. (2007). The tify family previously known as ZIM. Trends Plant Sci. **12:** 239-244.

Wang, L., Allmann, S., Wu, J., and Baldwin, I.T. (2008). Comparisons of LOX3- and JAR4/6-silenced plants reveal that JA and JA-AA conjugates play different roles in herbivore resistance of *Nicotiana attenuata* Plant Physiol. **146:** 904-915.

Wasternack, C. (2007). Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. Ann. Bot. (Lond) **100:** 681-697.

Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M., and Turner, J.G. (1998). COI1: an Arabidopsis gene required for jasmonate-regulated defense and fertility. Science **280:** 1091-1094.

Yan, J.B., Zhang, C., Gu, M., Bai, Z.Y., Zhang, W.G., Qi, T.C., Cheng, Z.W., Peng, W., Luo, H.B., Nan, F.J., Wang, Z., and Xie, D.X. (2009). The Arabidopsis CORONATINE INSENSITIVE1 protein is a jasmonate receptor. Plant Cell **21:** 2220-2236.

Yan, Y., Stolz, S., Chetelat, A., Reymond, P., Pagni, M., Dubugnon, L., and Farmer, E.E. (2007). A downstream mediator in the growth repression limb of the jasmonate pathway. Plant Cell **19:** 2470-2483.

Zhang, Y., and Turner, J.G. (2008). Wound-induced endogenous jasmonates stunt plant growth by inhibiting mitosis. PLoS One **3:** e3699.

Zhu, Z., An, F., Feng, Y., Li, P., Xue, L., A, M., Jiang, Z., Kim, J.M., To, T.K., Li, W., Zhang, X., Yu, Q., Dong, Z., Chen, W.Q., Seki, M., Zhou, J.M., and Guo, H. (2011). Derepression of ethylene-stabilized transcription factors (EIN3/EIL1) mediates jasmonate and ethylene signaling synergy in Arabidopsis. Proc. Natl. Acad. Sci. USA. **108:** 12539-12544.

CHAPTER THREE
THE JAZ8 SUBCLADE OF JAZ PROTEINS REPRESS JA SIGNALING THOUGH A NINJA-INDEPENDENT PATHWAY

ABSTRACT

Jasmonoyl-L-Ile (JA-Ile) is a lipid-derived hormone that regulates a broad range of gene expression in response to wounding, insect herbivores and developmental cues. The JASMONATE ZIM-DOMAIN (JAZ) proteins are repressors of JA-responsive gene expression. Twelve JAZ proteins were identified in Arabidopsis. The current JA signaling model suggests that JAZ proteins interact with an adapter protein NOVEL INTERACTOR OF JAZ (NINJA) through the JAZ ZIM domain to recruit the corepressor TOPLESS and repress JA responses. In contrast to the current JAZ repression model, JAZ8 represses JA responses through an N-terminal EAR motif, in a NINJA-independent manner. JAZ8 does not interact with NINJA in yeast, but directly interacts with TOPLESS in the absence of NINJA. Here, we report the identification and characterization of a thirteenth member (JAZ13) in the Arabidopsis JAZ family of proteins. JAZ13 shares high sequence similarity with JAZ8. JAZ13 contains a conserved EAR motif, as well as non-canonical ZIM and Jas motifs that are present in all JAZ members. JAZ13 negatively regulates jasmonate responses. Overexpression of *JAZ13* results in decreased sensitivity to JA. JAZ13 does not interact with NINJA but does interact with TOPLESS through the EAR motif, suggesting that JAZ13 also represses JA responses through a NINJA-independent mechanism. The JAZ8 subclade of JAZ proteins, which includes JAZ7, JAZ8 and JAZ13, all interact with MYC2 strongly in yeast. We show that this interaction is through the Jas motif and we identify a critical Arg residue in the Jas motif of JAZ8 that is required for JAZ8-MYC2 interaction and the repressive activity of JAZ8. Collectively, these results suggest that the JAZ8 subfamily of JAZ proteins work through a common NINJA-independent pathway to repress the JA-mediated transcriptional activity of MYC2. Our results

strengthen the importance of NINJA-independent repression mechanisms in regulating defense responses in Arabidopsis.

INTRODUCTION

Jasmonates, collectively known as JAs, are lipid-derived phytohormones involved in multiple aspects of plant growth, as well as responses to the environment. JAs were first identified as growth inhibitors (Aldridge et al., 1971), and reported to have multiple roles in regulating growth and development, including growth inhibition of seedlings (Yamane et al., 1981), male fertility in Arabidopsis, female fertility in tomato, and trichome development (Feys et al., 1994; Li et al., 2004; Yoshida et al., 2009). JAs also have important roles in regulating plant defense (Farmer et al., 1992). JAs are important activators of defense against insects, pathogen infection, UV light and other abiotic stresses (Weiler et al., 1993; Conconi et al., 1996; Dombrowski, 2003; Browse, 2009). These reports collectively suggest that JAs have a role in regulating a broad range of responses balancing plant growth and defense (Kazan and Manners, 2012; Ballaré, 2011). Understanding the molecular mechanism of this process is expected to expand our current knowledge in JA biology.

JASMONATE ZIM-DOMAIN (JAZ) proteins are key players in the JA signaling pathway that repress JA responses. In the absence of JA-Ile, JAZs interact with transcription factors to repress JA-induced gene expression. Upon accumulation of JA-Ile, JAZ proteins interact with the F-box protein CORONATINE INSENSITIVE 1 (COI1) to form a JA-Ile receptor complex (Katsir et al., 2008; Sheard et al., 2010). Interaction of JAZ-COI1 leads to ubiquitination and degradation of JAZ proteins via the 26S proteasome pathway. Turnover of

JAZ repressors result in activation of JAZ-interacting transcriptional activators to induce JA-responsive gene expression

In Arabidopsis, the 12-member JAZ family of proteins belongs to the larger plant specific TIFY family of transcriptional regulators. TIFY proteins are named for their conserved TIF[F/Y]XG motif at the N terminus of a larger conserved motif known as the ZIM domain (Vanholme et al., 2007; Bai et al., 2011). The ZIM domain is important for JAZ repression and protein-protein interaction (Chung and Howe, 2009; Pauwels et al., 2010). In addition to the ZIM domain, JAZ proteins also share a conserved Jas motif at the C terminus, and a less conserved Domain 1 at the N terminus (Thines et al., 2007). The Jas motif contains the degron sequence that promotes JAZ-COI1 interaction and instability of JAZ substrates of SCF^{COI1} complex (Sheard et al., 2010; Shyu et al., 2012). The Jas motif is also required for interaction with basic helix-loop-helix (bHLH) and R2R3 MYB transcription factors such as MYC2 (Chini et al., 2007; Chini et al., 2009; Qi et al., 2011; Song et al., 2011; Shyu et al., 2012), and regulation of JAZ nuclear localization (Grunewald et al., 2009). The N-terminal Domain 1, on the other hand, is involved in JAZ1-DELLA interactions that connect jasmonate signaling with the gibberellin signaling pathway (Hou et al., 2010).

The current JAZ repression model suggests that JAZ proteins interact with NOVEL INTERACTOR OF JAZ (NINJA) to recruit a Groucho/Tup1-type corepressor TOPLESS (TPL) to represses gene expression (Pauwels et al., 2010). NINJA was identified as a JAZ-interacting protein in a tandem-affinity purification assay followed by mass spectrometry with JAZ1 as bait. The TIFY motif of JAZ1 is important for interaction with NINJA. TPL was then identified using the same assay with NINJA as bait. The EAR motif of NINJA is required for NINJA-TPL

interaction. Loss-of-function mutants of NINJA and TPL are hypersensitive to JA-induced root growth inhibition. These results collectively suggest that JAZ proteins repress jasmonate responses through a NINJA-TOPLESS corepressor complex.

Recent studies suggest an alternative model for JAZ repression independent of NINJA (Shyu et al., 2012). The non-NINJA-interacting protein JAZ8 contains an N-terminal EAR motif that is required for repression of JA responses in transgenic plants that overexpress JAZ8 (Shyu et al., 2012). JAZ8 interacts with TPL in in the absence of NINJA. This interaction requires the EAR motif of JAZ8. Furthermore, although the ZIM domain was shown to be important for NINJA interaction with JAZ1, deletion of the ZIM domain of JAZ8 had no effect in JAZ8-mediated repression of jasmonate responses (Shyu et al., 2012). These results lead to an alternative repression model in which JAZ8 directly recruits corepressors through its EAR motif to repress expression of JA-response genes.

JAZ repression of JA responses also depends on direct interaction with transcription factors. JAZ proteins interact with various transcription factors in the MYB R2R3 and basic helix-loop-helix (bHLH) family to regulate different JA responses (Chini et al., 2007; Fernandez-Calvo et al., 2011; Niu et al., 2011; Qi et al., 2011; Song et al., 2011; Shyu et al., 2012). JAZ8 interacts with many of these transcription factors, suggesting an important role for JAZ8 in regulating diverse JA responses. Most JAZ proteins, including JAZ8, interact with transcription factors through the C-terminal Jas motif (Chini et al., 2009; Qi et al., 2011; Song et al., 2011). In addition, the Jas motif is also involved in JAZ-COI1 interaction (Sheard et al., 2010; Shyu et al., 2012) and localization of JAZ proteins (Grunewald et al., 2009). The N-terminal motif of Jas domains is critical for JAZ-COI1 interaction, and the C-terminal region of Jas domains is

important for JAZ1 localization (Sheard et al., 2010; Shyu et al., 2012). Sequence determinants within the Jas motif that mediate JAZ-MYC2 interaction remains to be determined.

Here, we report identification of a novel JAZ protein, JAZ13, which shares high sequence similarity with JAZ8. JAZ13 interacts with MYC2 and TPL, but not NINJA. We also show that members in the NINJA-independent JAZ8 subfamily require their Jas motif to interact with MYC2. We identify critical amino acids responsible for JAZ8-MYC2 interaction and show that this interaction is required for JAZ repressive activity. These results suggest that the JAZ8 subclade of JAZ repressors use a common NINJA-independent pathway to repress JA responses. Based on these findings, we propose that NINJA-independent repression mechanisms are important for JAZ proteins to regulate defense responses in Arabidopsis.

RESULTS

Identification of a novel JAZ protein, JAZ13

We asked the question whether or not there are other genes in the Arabidopsis genome that share similar functions with NINJA-independent JAZ repressors. A BLAST search performed with JAZ8 as a query sequence identified a previously uncharacterized protein (At3g22275) of unknown function that shared 21% and 23% amino acid identity with JAZ7 and JAZ8, respectively. A multiple sequence alignment of JAZ7, JAZ8 and At3g22275 revealed that At3g22275 contains: (i) an LxLxL-type EAR motif at the N-terminus; (ii) a central ZIM-like domain having a non-canonical TIFYXG motif (NAFYXG), and (iii) a Jas-like motif at the C-terminus (Figure 3.1). There are two conserved submotifs within the Jas motif of JAZ proteins: the Jas degron and a C-terminal PY motif. At3g22275 shares a similar Jas degron submotif as

JAZ7 and JAZ8, but does not have a conserved PY submotif at the C-terminus. Phylogenetic analysis showed that At3g22275, which we tentatively designated as JAZ13, groups closely with JAZ7 and JAZ8 as a distinct subclade (Figure 3.2).

JAZ13 Expression is Induced by JA

Genes involved in jasmonate responses are typically induced by wounding or other stress-related cues that activate JA synthesis (Chung et al., 2008). To understand whether or not *JAZ13* expression is regulated by wounding and JA treatment, we measured gene expression patterns of *JAZ7*, *JAZ8* and *JAZ13* in wounded wild-type plants. Consistent with previous results (Chung et al., 2008), *JAZ7* and *JAZ8* expression was not detectable in unwound plants and rapidly induced 30 minutes after mechanical wounding. *JAZ7* and *JAZ8* expression peaked at 1 hour after wounding and then decreased at time points thereafter (Figure 3.3A). Similar to *JAZ7* and *JAZ8*, *JAZ13* transcript levels were very low in unwound plants but rapidly induced 1 hour after wounding. *JAZ13* expression was also strongly induced in seedlings treated with 20 μM MeJA or 0.1 μM coronatine, resembling the expression pattern as *JAZ7* and *JAZ8* (Figure 3.3B). These results show that *JAZ13* is highly responsive to activation of the JA signaling pathway and is tightly co-expressed with *JAZ7* and *JAZ8*.

JAZ13 functions to repress jasmonate responses

To determine whether JAZ13 has a functional role in regulating JA responses, we generated stable transgenic lines expressing a full-length *JAZ13* cDNA driven by the cauliflower

mosaic virus (CaMV) 35S promoter. We selected 32 independent kanamycin-resistant T1 lines and transferred them to soil. We used RNA gel-blot analysis to select 10 lines showing high levels of transgene expression (Supplemental Figure 3.1). None of these lines exhibited obvious defects in flower morphology or fertility. However, all ten lines (T2 generation) showed decreased sensitivity to MeJA-induced root growth inhibition in comparison with wild type (WT) (Figure 3.4). This finding demonstrates that ectopic expression of JAZ13 negatively regulates the JA response pathway.

JAZ8 is known to repress jasmonate responses through its N-terminal EAR motif, presumably through interaction with TPL (Shyu et al., 2012). We therefore tested whether the N-terminal EAR motif of JAZ13 interacts with TPL. In yeast two-hybrid assays, JAZ13 interacted with TPL but not NINJA (Figure 3.5). We generated JAZ13 constructs lacking the entire EAR motif, or substituted the conserved Leucine (L) residues in the EAR motif with Alanine (A), to test the dependence of the TPL interaction on the EAR motif. The constructs were named JAZ13ΔEAR and JAZ13$^{\text{LDLHL}\rightarrow\text{ADAHA}}$, respectively. The results showed that neither JAZ13ΔEAR nor JAZ13$^{\text{LDLHL}\rightarrow\text{ADAHA}}$ interact with TPL (Figure 3.5), indicating that the EAR motif is required for JAZ13-TPL interaction.

The Jas motif is critical for JAZ7/8/13 interaction with MYC2 and this interaction is required for JAZ8 to repress JA responses

JAZ proteins interact with JA-inducible transcription factors, such as MYC2, to regulate JA responses. Yeast two-hybrid analysis of JAZ13 shows that JAZ13 strongly interacts with

MYC2 (Figure 3.5). This strengthens the hypothesis of JAZ13 being involved in regulation of JA signaling. JAZ8 interacts through its Jas motif with MYC2 and other bHLH and MYB family transcription factors (Qi et al., 2011; Song et al., 2011; Shyu et al., 2012). To determine whether or not the Jas motif is required for other members in the JAZ8 subgroup to interact with transcription factors, we generated deletion constructs of JAZ7 and JAZ13 lacking the Jas motif, and tested their interaction with MYC2 in yeast. Indeed, JAZ7ΔJas and JAZ13ΔJas failed to interact with MYC2 in this assay (Figure 3.6). To further define the MYC2-interacting region of the JAZ8 subclade of proteins, we generated nested deletions at the C-terminal end of the JAZ8 Jas motif and tested these mutants for interaction with MYC2. Yeast two-hybrid assays showed that removal of 9, 11, or 13 amino acids from the JAZ8 C-terminus (constructs JAZ8ΔC122, JAZ8ΔC120 and JAZ8ΔC118, respectively) do not significantly affect interaction with MYC2 (Figure 3.7A). Strikingly, however, further deletion of three amino acids (KRK; JAZ8ΔC115) abolished the JAZ8-MYC2 interaction. This finding, together with the highly conserved nature of the KRK motif in all JAZ proteins, suggested that these residues perform a critical role of TF binding. Indeed, site-directed mutagenesis showed that Ala substitution of the central R residue (mutant JAZ8^{R117A}) is sufficient to abrogate JAZ8 binding to MYC2 (Figure 3.7A).

We next tested whether JAZ8^{R117A} retains the ability to repress JA responses in vivo. Among 32 independent *35S-* *JAZ8*^{R117A} transgenic lines identified, RNA-blot analysis showed that seven lines the transgene to high levels (Supplemental Figure 3.2). Root growth assays showed that all seven lines (T2 generation) were as sensitive as WT seedlings to MeJA-induced root growth inhibition (Figure 3.7B). Similar results were obtained with transgenic lines overexpressing JAZ8^{R117A}-YFP (Supplemental Figure 3.3). Interestingly, confocal microscopy

showed that although JAZ8-YFP is exclusively localized in the nucleus, JAZ8^{R117A}-YFP is localized in both the nucleus and cytoplasm, similar to the YFP only control (Supplemental Figure 3.4). We conclude that the R117A mutation affects both interaction with MYC2 and localization of the protein, which correlates with in the inability of JAZ8 ^{R117A} to repress JA.

T-DNA Knockout lines of *JAZ7*, *JAZ8* and *JAZ13* are sensitive to JA

To begin to investigate the physiological role of the JAZ7/8/13 clade, we obtained T-DNA insertion lines that are defective in each of the corresponding genes (Figure 3.8A). *jaz7-1* (WiscDsLox7H11) contains a T-DNA insertion in the second exon of *JAZ7* and has been previously reported to be a null mutant (Thines et al., 2007). The WiscDsLox255G12 line, which we designated *jaz8-1*, harbors the T-DNA insertion 134 bp upstream of the translational start site. The SALK_149961 line (designated *jaz13-1*) carries a T-DNA insertion in the promoter region of *JAZ13*, 203 bp upstream of the translational start site (Figure 3.8A). We used RNA blot analysis to assess the effect of each T-DNA insertion on the wound-induced expression of the corresponding transcript. Consistent with previous reports (Thines et al., 2007), *JAZ7* transcripts were undetectable in wounded leaves of *jaz7-1*. Likewise, *JAZ8* and *JAZ13* mRNAs also failed to accumulate in wounded leaves of the *jaz8-1* and *jaz13-1* mutants, respectively (Figure 3.8B). These results show that *jaz7-1*, *jaz8-1* and *jaz13-1* are null or severe loss of function alleles for the respective genes, and thus are suitable for use in physiological assays.

Obvious morphological phenotypes related to growth and development were not observed in either of the three mutant lines. We also tested the effect of *jaz7-1*, *jaz8-1* and *jaz13-*

1 on JA-induced root growth inhibition in MS medium containing 20 uM MeJA. The sensitivity of all three mutants was not significantly different from that of wild-type seedlings (Figure 3.9). Interestingly, *JAZ7* expression levels were higher in *jaz8-1* and *jaz13-1* (Figure 3.8B). *JAZ8* expression is also slightly higher in *jaz7-1* and *jaz13-1*. This supports the hypothesis of negative feedback of *JAZ7/8* expression by JAZ7/8/13 proteins (Chung et al., 2008).

DISCUSSION

JAZ proteins are key components in the JA signaling pathway regulating multiple biological processes balancing growth and defense. There are 12 JAZ members in Arabidopsis, and the current model suggests two independent models for JAZ proteins to repress JA-induced gene expression: a NINJA-dependent pathway in which JAZ proteins recruit corepressor TPL through an adaptor protein NINJA; and a NINJA-independent pathway in which JAZ proteins directly repress transcriptional responses through a TPL-interacting EAR motif, in the absence of NINJA (Pauwels et al., 2010; Shyu et al., 2012). Here, we report the identification of a thirteenth member in the JAZ family, and show that it represses JA responses and recruits TPL through a NINJA-independent pathway.

JAZ13, A New Member in the Arabidopsis JAZ Family

Key features of JAZ proteins include their function to repress JA-induced gene expression, their conserved ZIM domain and Jas motif, and their ability to interact with transcription factors (TFs) involved in JA signaling. JAZ13 shares all of these features (further

discussed below), thus we designate it as a novel JAZ family member. Another important feature is that JAZ proteins are targeted for ubiquitination by COI1, and degraded through the 26S proteasome pathway. This was shown not to be the case in some JAZ proteins, in which JAZ8 and some JAZ alternative splice variants lack the canonical degron motif that mediates JAZ-COI1 interaction. These JAZ variants are more stable upon JA treatment, and overexpression of these noncanonical JAZ proteins lead to decreased sensitivity to JA (Yan et al., 2007; Chung and Howe, 2009; Chung et al., 2010; Shyu et al., 2012). Similar observations were made in *35S-JAZ13* lines, in which *35S-JAZ13* seedlings were less sensitive to JA-induced root growth inhibition, compared to WT seedlings. These results demonstrate a role for JAZ13 as a negative regulator in JA signaling, that is presumably stable upon JA treatment.

JAZ13 shares high sequence similarity with JAZ7 and JAZ8. JAZ13 contains an LxLxL EAR motif at the N-terminus, which represents a key feature of NINJA-independent JAZ repressors (Shyu et al., 2012). JAZ13 interacts with TPL but not NINJA, and JAZ13 interaction with TPL is dependent on its EAR motif. These results demonstrate that domain functions are similar between JAZ13 and JAZ8. Overexpression of JAZ13 leads to repression of JA responses, presumably due to direct recruitment of TPL. Whether or not JAZ13 repression requires its EAR motif remains to be examined.

The ZIM domain and Jas domain sequences of JAZ13 are less conserved among other JAZ family members. This presumably explains why JAZ13 was not identified previously. JAZ proteins share a very conserved TIFY motif at the N-terminus of ZIM domains, which is also a key feature in the TIFY family of proteins (Vanholme et al., 2007). The ZIM domain, specifically the TIFY motif within the ZIM domain, is important for JAZ homo- and hetero-dimerization (Chini et al., 2009; Chung and Howe, 2009). Site-directed mutagenesis of the I

residue within the TIFY motif of JAZ10 resulted in abolishment of JAZ10 homo- and heterodimerization with other JAZ proteins (Chung and Howe, 2009). JAZ13 contains a NAFY motif instead of a TIFY motif (Figure 3.1). Blast search of JAZ13 ZIM-like sequences reveals that this NAFY-containing ZIM domain is not found in other proteins in Arabidopsis. Yeast two-hybrid analysis shows that JAZ13 does not interact with other JAZ proteins, presumably due to the diversified NAFY motif. In addition to its role in JAZ dimerization, the ZIM domain in most JAZ proteins plays an important role to mediate JAZ-NINJA interaction (Pauwels et al., 2010). The only two JAZ proteins that do not interact with NINJA are JAZ7 and JAZ8 (Pauwels et al., 2010). JAZ13 also does not interact with NINJA, supporting the hypothesis of JAZ13 repressing through a NINJA-independent pathway.

The Jas motif of canonical JAZ proteins is composed of three conserved sub-regions: a N-terminal LPIAR(R/K) motif, a central ($SLX_2FX_2KRX_2R$) and a C-terminal (X_5PY) region. The first two sub-regions compose the Jas degron, which mediates JAZ-COI1 interaction (Sheard et al., 2010). Interestingly, the LPIAR(R/K) is highly diversified among JAZ proteins, in which stable JAZs such as JAZ8 contain a $X_3S(M/V)K$ motif instead (Shyu et al., 2012). JAZ13 shares a similar degron motif as JAZ8, with $X_3S(M/V)K$ and $SLX_2FX_2KRX_2R$ regions. This suggests that JAZ13 may be a weak COI1-interactor in the presence of JA-Ile, thus stable upon JA treatment. Indeed, overexpression of JAZ13 leads to decreased sensitivity, similar to what has been observed for overexpression of stable JAZ variants such as JAZ8 and JAZ splice variants (Chung and Howe, 2009; Chung et al., 2010; Shyu et al., 2012). The C-terminal (X_5PY) region is the least conserved domain in JAZ13, when comparing with other JAZ proteins. Instead of the highly conserved X_5PY motif, JAZ13 contains a long stretch of Serine (S) and Threonine (T)

residues at the C-terminus of its Jas motif. Phosphorylation of serine and threonine residues is an important posttranslational modification pathway that regulates many aspects in plant signaling (Nakagami et al., 2012). Whether phosphorylation plays a role in regulation of JAZ13 function is an exciting area to explore.

The NINJA-Independent JAZ8 Subclade

Identification of JAZ13 expands the repertoire of JAZ proteins and strengthens the importance of NINJA-independent JAZ repressors. NINJA-independent JAZ repressors have important features that are distinct from other JAZ proteins: an N-terminus EAR motif that is required for TPL-interaction and JAZ repression, and a diversified ZIM domain that does not mediate JAZ-NINJA interaction. They also contain a diversified degron motif that causes low affinity of JAZ-COI1 interaction in the presence of JA-Ile (Shyu et al., 2012). Currently three JAZ members belong to the NINJA-independent subclade in Arabidopsis: JAZ7, JAZ8 and the newly identified JAZ13. Blast search of JAZ8-like sequences reveals that NINJA-independent JAZ proteins are also present in other species (Shyu et al., 2012). All of these JAZ8-like sequences contain an EAR motif at the N-terminus and a diversified degron motif with conserved X_3S(M/V)K sequences. Although the EAR motif of NINJA-independent JAZ repressors was shown to be required for both TPL-interaction and JAZ repression, we would like to point out that our results do not exclude the possibility of NINJA-independent JAZ proteins repressing though other EAR motif-interacting corepressors, such as mSin3-associated polypeptide 18 (SAP18) (Song and Galbraith, 2006; Kagale and Rozwadowski, 2011). Tandem affinity purification (TAP) assays followed by mass spectrometry (MS) would be helpful to

determine JAZ8- or JAZ13-interacting proteins in vivo to determine the composition of NINJA-independent repression complexes.

Although much has been reported on the molecular mechanism of JAZ action, little is known about the physiological role of JAZ proteins. Currently knockout or knockdown lines of *JAZ1* and *JAZ10* have been reported to be hypersensitive to JA treatment in growth inhibition assays (Yan et al., 2007; Grunewald et al., 2009; Demianski et al., 2012). *jaz10-1*, a null allele for *JAZ10*, has also been reported to have enhanced susceptibility to *Pseudomonas syringae* DC3000 (Demianski et al., 2012). All of the knockout or knockdown lines characterized so far are all JAZ proteins that undergo NINJA-dependent pathways to repress jasmonate responses. It would be interesting to determine whether NINJA-dependent and NINJA-independent repression mechanisms result in different signaling outputs. Here we report identification of knockout lines of all members in the NINJA-independent clade of JAZ proteins. Root growth inhibition assays do not indicate any difference in wild type and *jaz7-1*, *jaz8-1* and *jaz13-1* lines possibly due to redundancy. Other jasmonate-related bioassays would help gain more insight in unveiling the physiological role of JAZ7, JAZ8 and JAZ13. Furthermore, generation of high-order mutants within subclades of JAZ family members will strongly benefit understanding of physiological roles of different JAZ proteins.

Role of JAZ-TF Interaction in Regulating JA Responses

JAZ proteins interact with many transcription factors that are involved in regulating JA responses, but sequence determinants that mediate JAZ-TF interaction were not known. Furthermore, whether or not JAZ-TF interaction is required for JAZ repression has not been

reported previously. Our results demonstrate that the R residue within the $SLX_2FX_2KRX_2R$ region of the Jas motif is critical for JAZ8-MYC2 interaction, and also JAZ8 repression. Although the interaction pattern is different, all JAZ proteins interact with JA-related transcription factors, such as MYC2 (reviewed in Pauwels and Goossens, 2011). It has been shown in the case of multiple JAZ proteins (JAZ3, JAZ8 and JAZ11) that JAZ-TF interaction is mediated through the Jas domain (Chini et al., 2009; Qi et al., 2011; Song et al., 2011; Shyu et al., 2012). Furthermore, the $SLX_2FX_2KRX_2R$ region, especially the KRK submotif, is extremely conserved among the JAZ family members. We suggest that the importance of the R residue within the KRK submotif to mediate JAZ-MYC2 interaction can be widely applied to other JAZ members. Interestingly, mutation of the R117 residue in JAZ8 not only led to abolishment of JAZ8-MYC2 interaction, but also mislocalization of JAZ8. Although most of $JAZ8^{R117A}$-YFP was detected in the nucleus, $JAZ8^{R117A}$-YFP was also detected in the cytosol. Overexpression of $JAZ8^{R117A}$ results in restoration of JA sensitivity. Whether this is due to loss-of-interaction with MYC2, or mislocalization of JAZ8 remains to be examined.

MATERIALS AND METHODS

Plant Materials

Arabidopsis thaliana ecotype Colombia-0 (Col-0) was used as wild type for all experiments. All mutants and transgenic lines reported in this article are in the Col-0 background. T-DNA

insertion lines WiscDsLox255G12 (*jaz8-1*) and SALK_149961 (*jaz13-1*) were obtained from the Arabidopsis Biological Resource Center (ABRC) (Alonso et al., 2003). *jaz7-1* (WiscDsLox7H11) was previously described (Thines et al., 2007).

Molecular Cloning and Generation of Constructs

JAZ13 was cloned from a PCR reaction with *JAZ13* sequence-specific primers (JAZ13_pENTR_FP and JAZ13_XhoI_RP) using wild type wounded mature leaf RNA as template. PCR reactions were performed using Pfu Turbo DNA polymerase (Stratagene). *JAZ13* was cloned and sequenced in the pENTR-TOPO vector (Invitrogen) and further cloned into pGILDA and pB42AD vectors for further yeast two-hybrid assays using the Gateway system (Invitrogen). JAZ8-related constructs were cloned into pGEMT-Easy (Promega) for sequencing and subcloning into pGILDA and pB42AD. A complete list of constructs and primers used in this article is provided in Supplemental Table 1.

Site-Directed Mutagenesis

All site-directed mutagenesis assays were performed using Pfu Turbo DNA polymerase (Stratagene) as previously described (Chung and Howe, 2009). PCR reactions were performed using templates in pGILDA vectors. PCR-amplified products were treated with the restriction enzyme DpnI to remove the parental plasmid, and transformed into *E. coli* strain DH5α for further amplification. Presence of the desired mutation was verified by DNA sequencing.

Protein-Protein Interaction assays

Yeast two-hybrid assays were performed with the Matchmaker LexA system (Clontech). Full length cDNA of *JAZ10* and *JAZ8*-related sequences were cloned into the pGILDA bait vector to generate fusion proteins with the LexA DNA binding domain. *MYC2*, other *JAZ*s, *NINJA* and *TPL* full length cDNA were cloned into the pB42AD prey vector to generate fusion proteins with the B42 activation domain (AD). Bait and prey vectors were cotransformed into yeast strain EGY48 using the Frozen-EZ yeast Transformation II Kit (Zymo Research). Transformants were selected on SD-glucose plates containing amino acid supplements lacking uracil (U), tryptophan (T) and histidine (H) after 48 hours of incubation at 30°C. Protein-protein interaction was detected by adding X-gal and selection for blue colors (β-galactosidase) on SD-galactose plates with proper amino acid supplements. Photographic images were taken after 48 hours of incubation at 30°C.

Generation of Transgenic Lines

All constructs for generating transgenic lines were made in the pBI-AS vector. The pBI-AS vector is a modified pBI121 vector, as described previously (Schilmiller et al., 2007). Sequenced overexpression constructs were transformed into *Agrobacterium tumefaciens* strain C58C1, and further transformed into wild type plants using the floral dip method (Clough and Bent, 1998). T1 seedlings were screened on Murashige and Skoog (MS) agar medium containing sucrose (0.8%) and kanamycin (50 μM/mL) and vancomycin (100 μM/mL). For each construct, 32 kanamycin-resistant lines were selected for RNA gel-blot analysis to screen for lines expressing high levels of the transgene. RNA extracted from untreated leaf tissue was hybridized with the

transgene cDNA in RNA gel-blot assays as previously described (Shyu et al., 2012). High-expression lines were further used for root growth inhibition assays and propagated for identification of homozygous T3 lines. YFP-tagged lines were identified by fluorescence microscopy at the T1 seedling stage (Axio Scope; Carl Zeiss).

Root Growth Inhibition Assays

Seedlings were plated on MS agar medium containing sucrose (0.8%) with or without 20 μM MeJA for 8 days under continuous light unless otherwise noted. Primary root lengths were measured using Image J (http://rsbweb.nih.gov/ij/indext.html). Root growth ratios indicate primary root lengths of seedlings grown on MeJA-containing plates, divided by the average of primary root lengths of seedlings of the same genotype grown on MS only plates.

RNA Gel-Blot Analysis

Primers used to amplify cDNA probes are described in Supplemental Table 1. PCR reactions were performed using Pfu Turbo DNA polymerase (Stratagene) and amplified products were cloned and sequenced in pGEMT-Easy (Promega). RNA extraction was performed using RNeast Plant Mini Kit (Qiagen) or the phenol/chloroform extraction method as described in Li et al., 2002. Gel-blot analyses were performed as described previously (Li et al., 2002).

Multiple Sequence Alignment and Phylogenetic Analysis

All sequences were obtained from The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org). Multiple sequence alignments were performed using the MUSCLE method (Edgar, 2004) in software BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Phylogenetic trees were generated with the Neighbor-joining Method (Saitou and Nei, 1987) (Tamura et al., 2011).

Accession Numbers

Arabidopsis Genome Initiative numbers described in this article are listed below: ACT8 (At1g49240), COI1 (At2g39940), JAZ1 (At1g19180), JAZ2 (At1g74950), JAZ3 (At3g17880), JAZ4 (At1g48500), JAZ5 (At1g17380), JAZ6 (At1g72450), JAZ7 (At2g34600), JAZ8 (At1g30135), JAZ9 (At1g70700), JAZ10 (At5g13220.1), JAZ11 (At3g43440), JAZ12 (At5g20900), JAZ13 (At3g22275), MYC2 (At1g32640), NINJA (At4g28910) and TPL (At1g15750).

ACKNOWLEDGEMENTS

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FIGURES AND TABLES

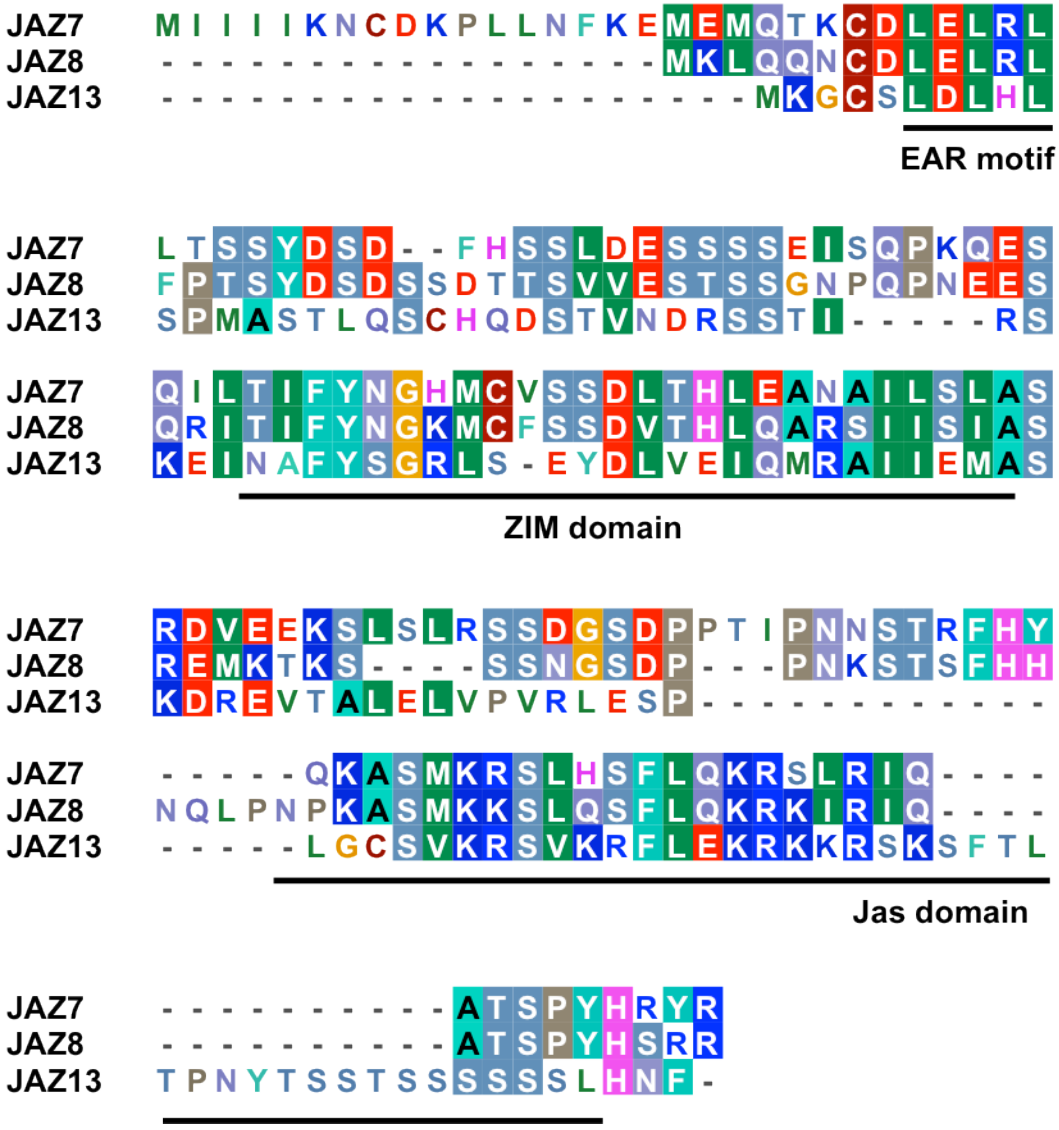


Figure 3.1. Amino acid sequence alignment of full length JAZ7, JAZ8 and JAZ13.

Full length amino acid sequences of JAZ7, JAZ8 and JAZ13 were aligned by MUSCLE using the BioEdit software. Conserved EAR, ZIM and Jas motifs are underlined.

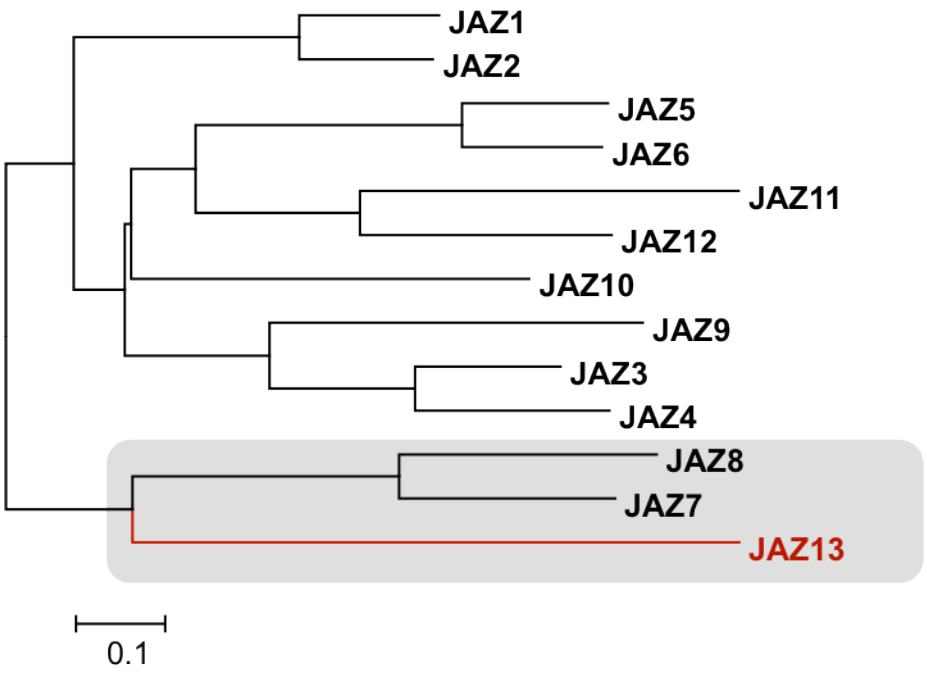


Figure 3.2. Phylogenetic tree of the JAZ family of proteins in Arabidopsis.

Full-length amino acid sequences were used to generate phylogenetic tree by the Neighbor-Joining method. Evolutionary analyses were conducted in MEGA5.

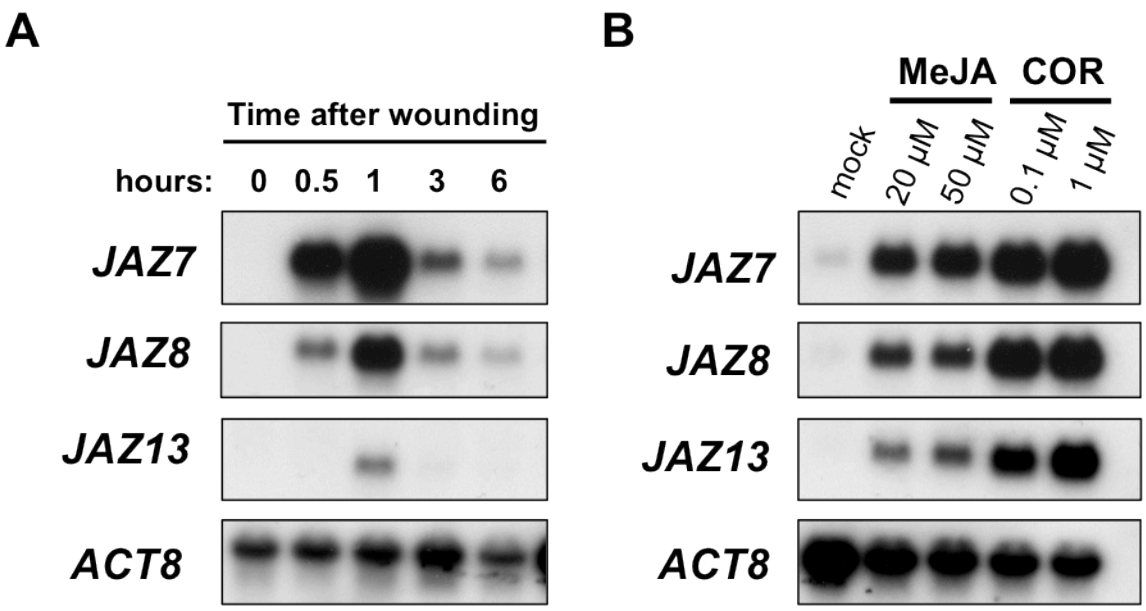


Figure 3.3. Induction of *JAZ7*, *JAZ8* and *JAZ13* in response to wounding, MeJA and coronatine treatment.

(A) RNA gel blot analysis of RNA extracted from 4-week-old mature leaf tissue of WT plants untreated or treated with mechanical wounding at the indicated time after wounding (hours). (B) RNA gel-blot analysis of 9-day-old WT seedling RNA grown in liquid MS medium and treated with the indicated concentration of MeJA, coronatine or a mock control. An *ACTIN8* (*ACT8*) probe was used as a control.

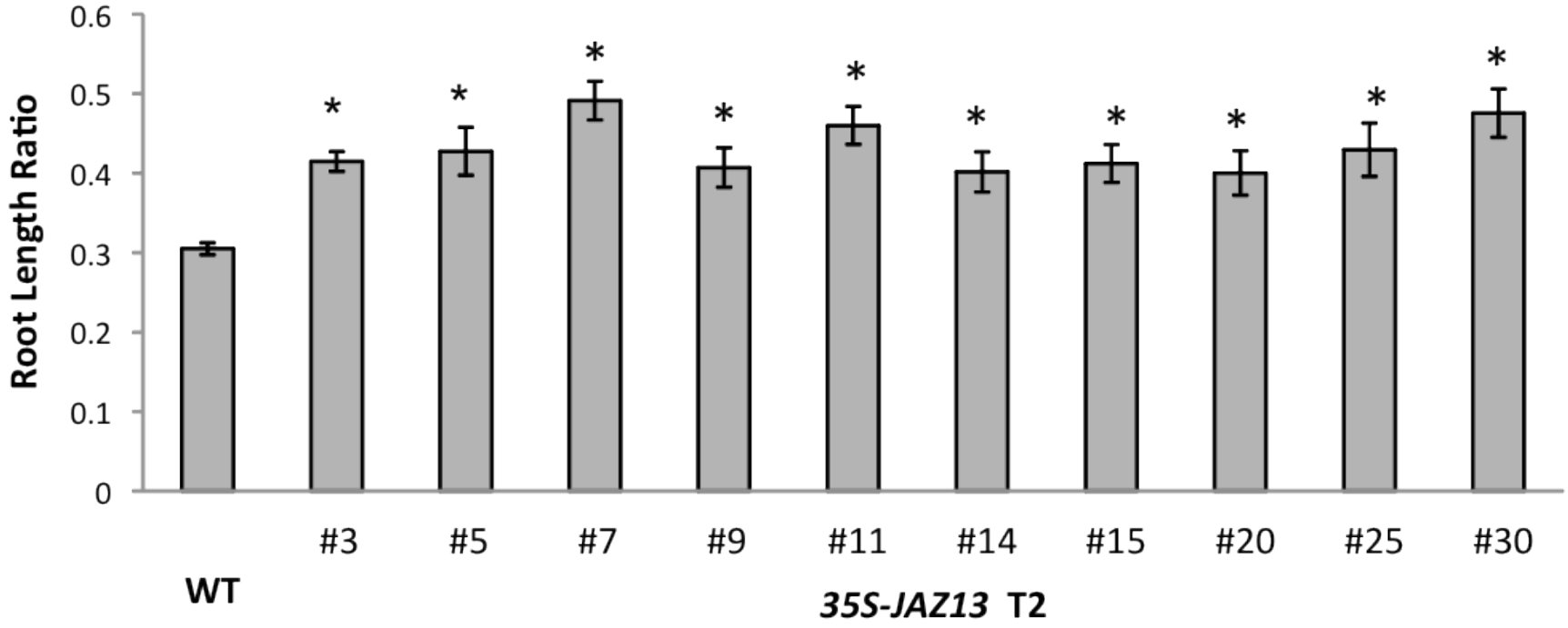


Figure 3.4. Overexpression of *JAZ13* leads to decreased sensitivity to JA-induced root growth inhibition.

Figure 3.4. Overexpression of *JAZ13* leads to decreased sensitivity to JA-induced root growth inhibition. –Continued.

T2 seedlings were grown under continuous light on MS plates containing ± 20 μM MeJA for 8 days. The root length ratio was calculated by dividing the average root length of seedlings grown on MeJA-containing medium by the average root length of seedlings of the same genotype grown in the absence of MeJA. Data points show the mean ± SE (n = 17-22 per data point). Asterisks indicate significant differences (p value < 0.05, Student's t-test) in comparisons between the indicated transgenic line and WT.

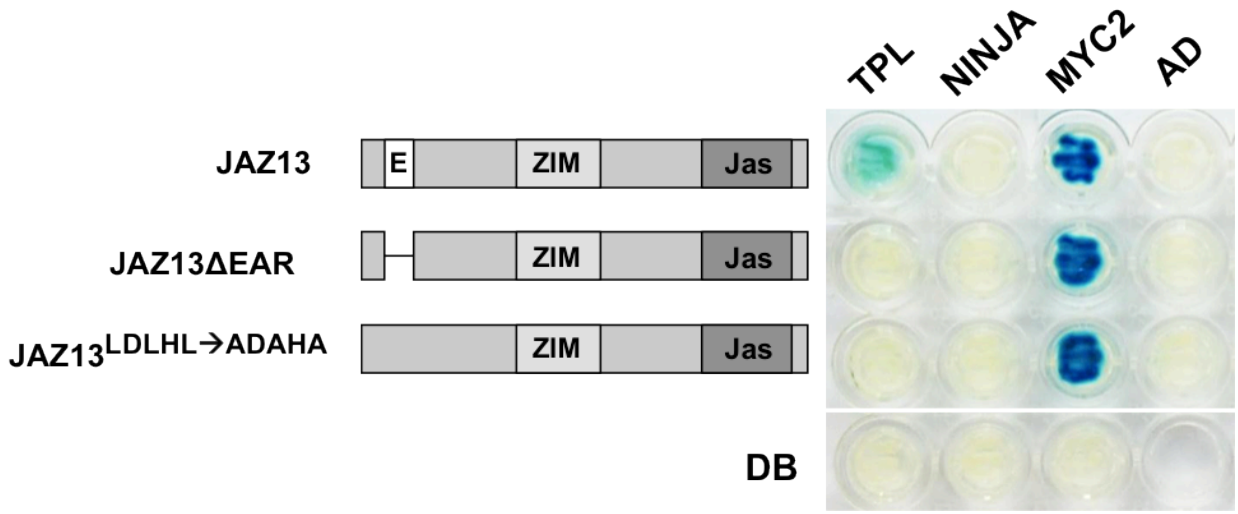


Figure 3.5. The EAR motif of JAZ13 is required for interaction with TPL.

Yeast two-hybrid analysis of JAZ13 deletion proteins with TPL, NINJA and MYC2. Yeast strains expressing both the bait (JAZ13 or JAZ13 deletions) and prey (TPL, NINJA or MYC2) were tested on media containing X-Gal. LacZ-mediated blue-color formation is indicative of protein-protein interaction. Images were taken 48 hours after incubation at 30 °C. DB indicates empty bait vector. AD indicates empty prey vector.

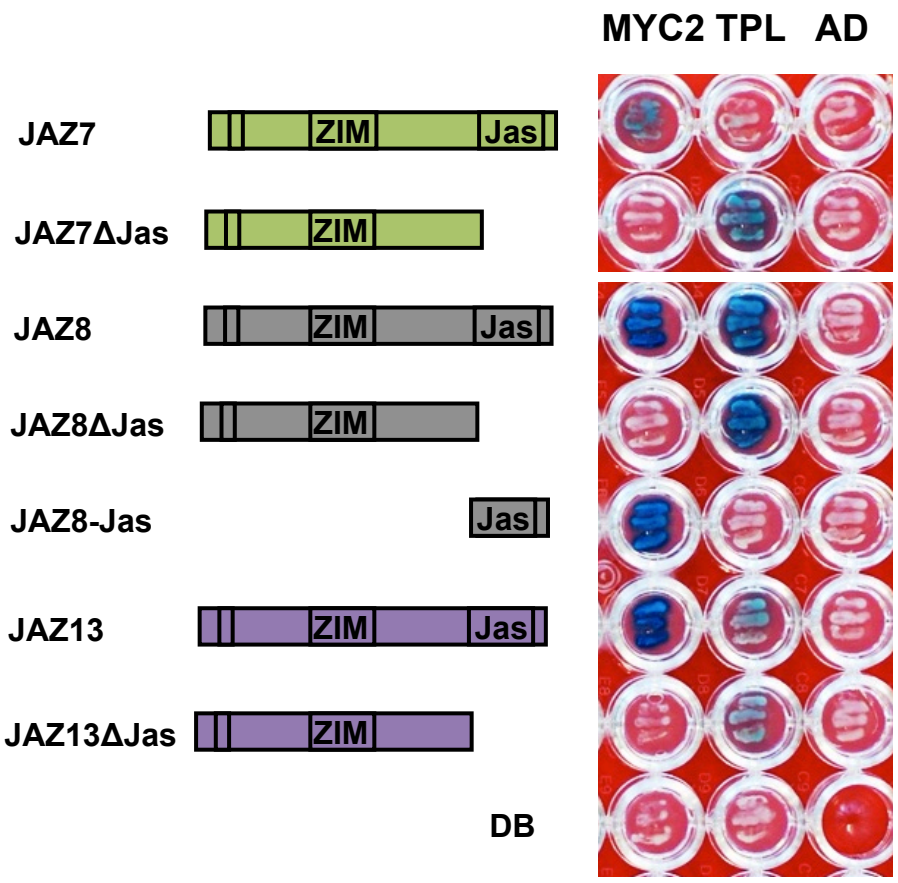


Figure 3.6. The Jas motif is required for interaction of JAZ7, JAZ8 and JAZ13 with MYC2.

Yeast two-hybrid analysis of yeast strains coexpressed with JAZ related sequences fused with DB and MYC2 or TPL fused with AD. Experiments were performed as described in Material & Methods and Figure 3.5.

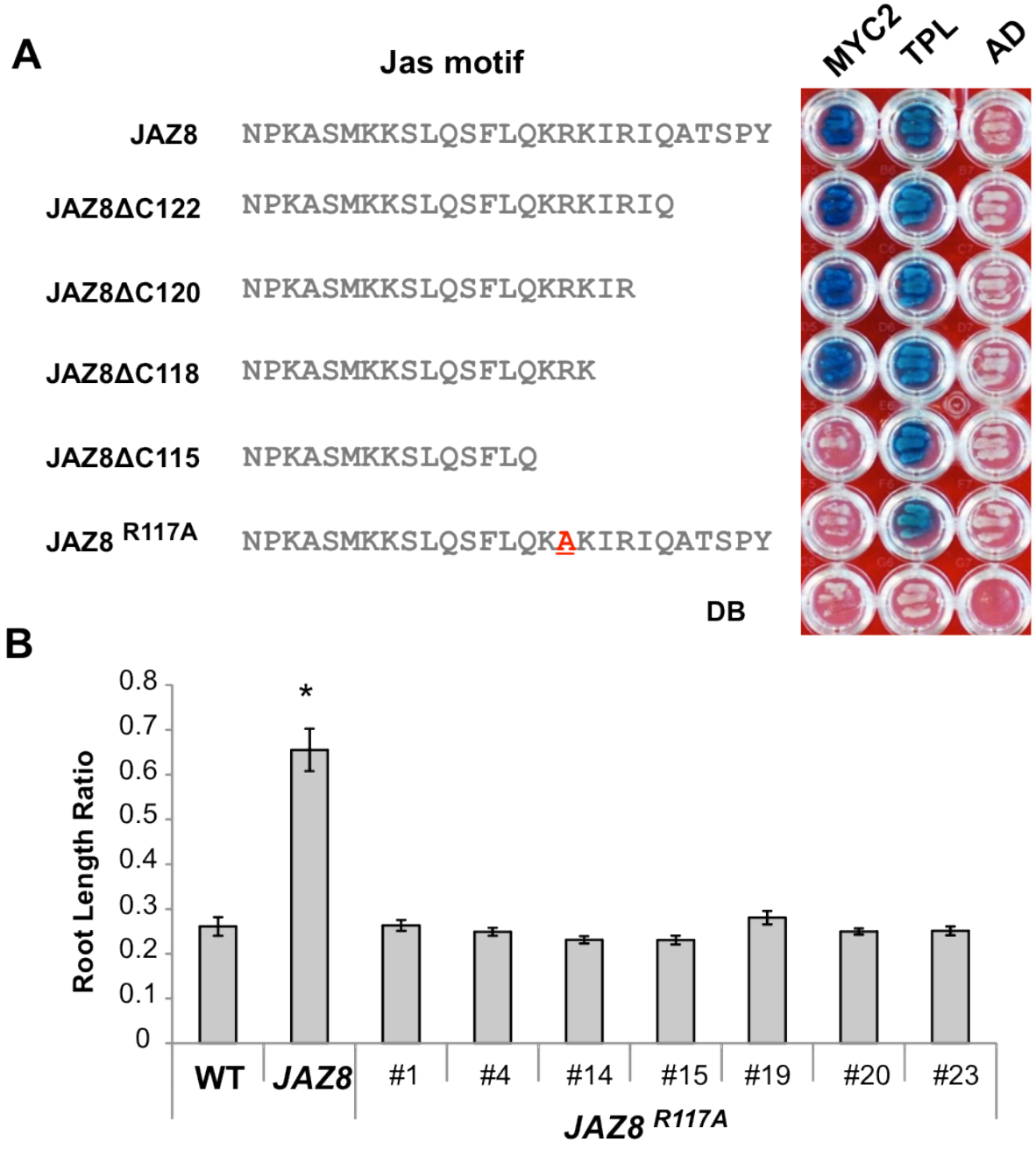
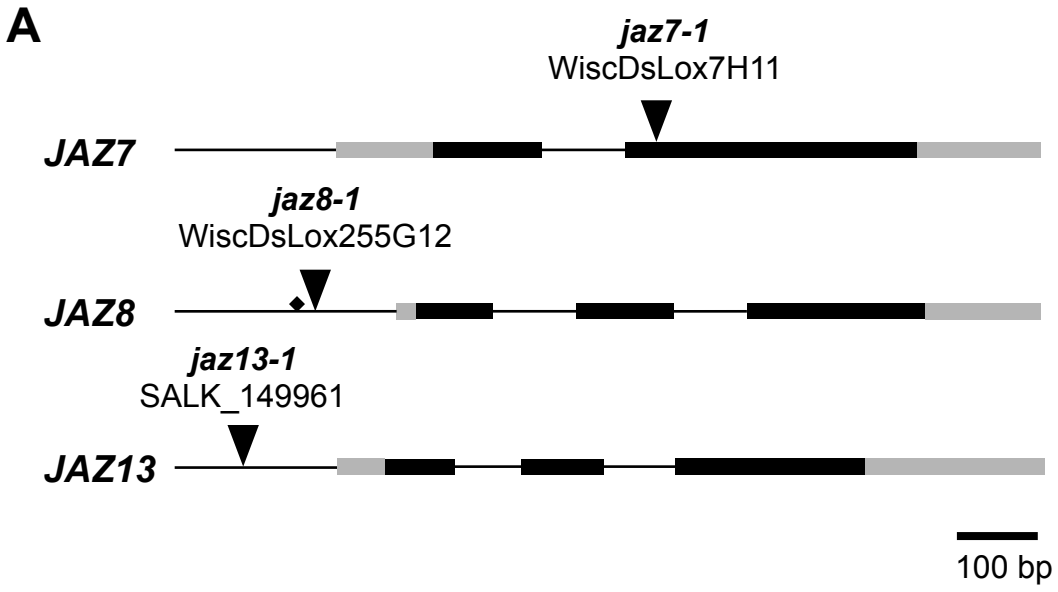


Figure 3.7. The Jas domain is required for JAZ-MYC2 interaction and JAZ repression of JA responses.

Figure 3.7. The Jas domain is required for JAZ-MYC2 interaction and JAZ repression of JA responses. –Continued.

(A) The R117 residue is critical for JAZ8-MYC2 interaction. (B) Transgenic lines overexpressing *JAZ8*^{R117A} are sensitive to JA-induced root growth inhibition. Seedlings were grown on ± 20 μM MeJA containing MS plates for 9 days under continuous light. The root length ratio was calculated by dividing the average root length of seedlings grown on MeJA-containing medium by the average root length of seedlings of the same genotype grown in the absence of MeJA. Data points show the mean ± SE (n = 7-20 per data point). Asterisks indicate significant differences (p value < 0.05, Student's t-test) in comparisons between the indicated transgenic line and WT.



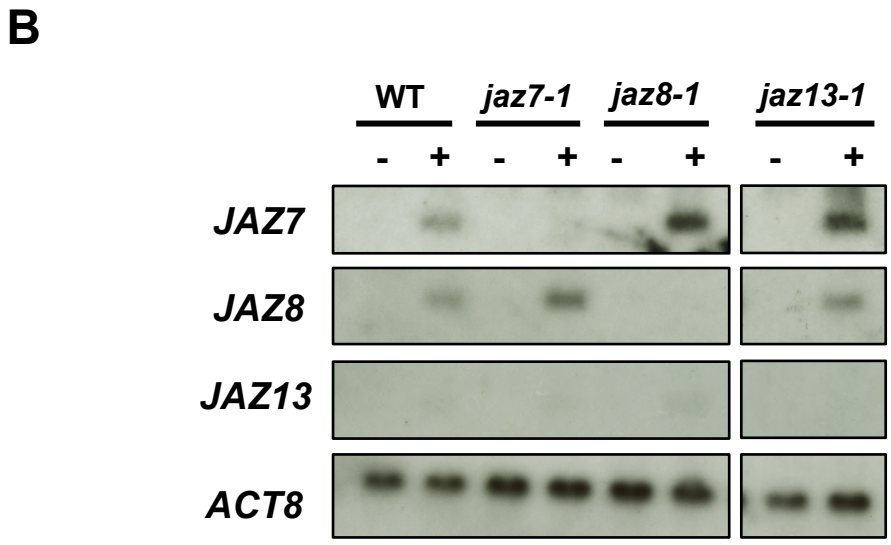


Figure 3.8. Characterization of knockout lines of *JAZ7*, *JAZ8* and *JAZ13*. (A) T-DNA insertion lines of the JAZ8 subclade of JAZ proteins.

Black boxes indicate exons, gray boxes indicate 5' or 3' UTR. Black lines indicate introns or untranslated genomic sequences. Black diamond indicates a G-box cis-regulatory element. Scale bar = 100 bp. (B) RNA gel blot analysis of *jaz7-1*, *jaz8-1* and *JAZ13-1* upon wounding. Fully expanded leaves of 4-week-old mature plants were used for RNA extraction and RNA gel blot analysis. Samples were collected prior to wounding (-) or 1 hour after wounding (+). Probes containing full-length cDNA of *JAZ7*, *JAZ8* and *JAZ13* were used to detect target gene expression. *ACTIN8* (*ACT8*) was used as a loading control.

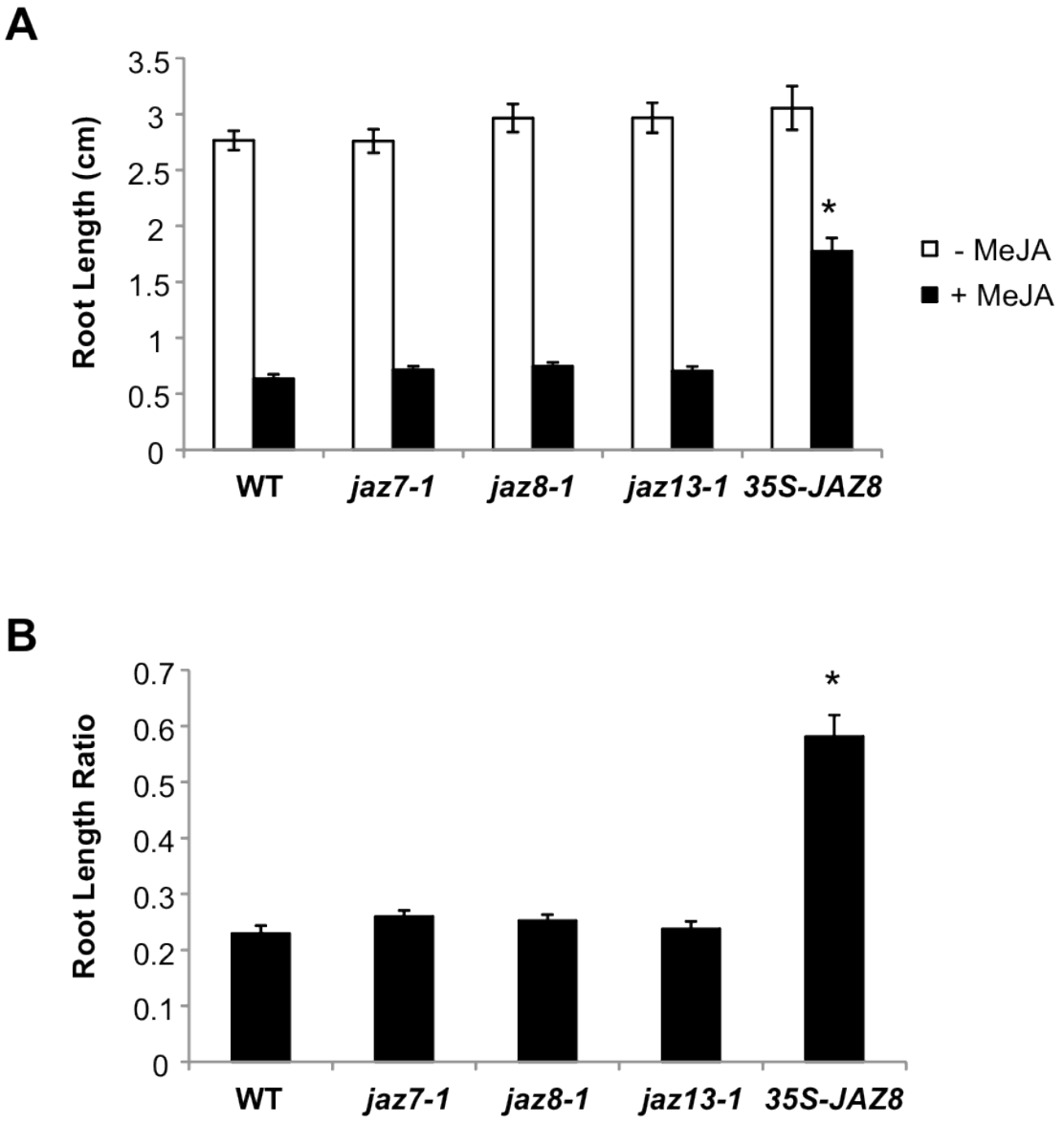
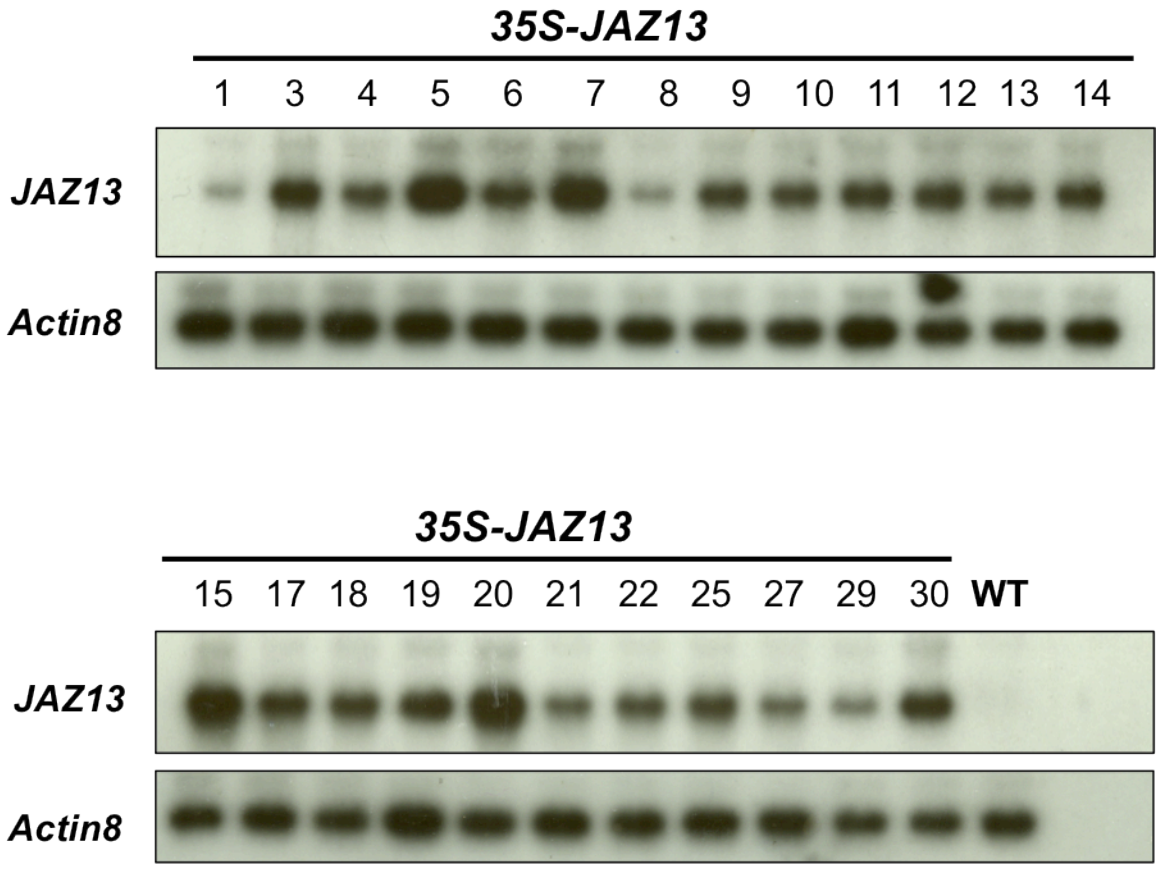


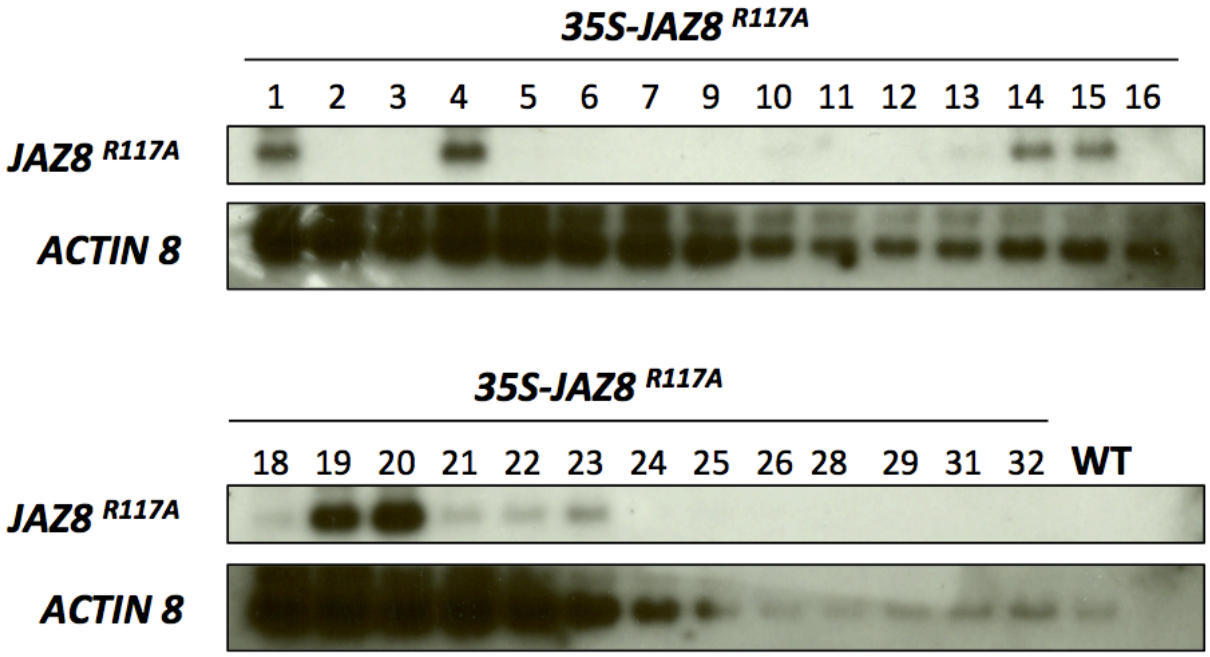
Figure 3.9. Root growth inhibition assay of *jaz7-1*, *jaz8-1* and *jaz13-1* lines.

(A) Primary root length of *jaz7-1*, *jaz8-1* and *jaz13-1* lines grown on ± 20 μM MeJA containing MS plates. WT and *35S-JAZ8* were used as controls. Seedlings were grown on ± 20 μM MeJA containing MS plates for 8 days under continuous light. Data points show the mean ± SE (n = 17-20 per data point). (B) Root length ratio of the identical experiment described in (A). The root length ratio was calculated by dividing the average root length of seedlings grown on MeJA-containing medium by the average root length of seedlings of the same genotype grown in the absence of MeJA. Asterisks indicate significant differences (p value < 0.05, Student's t-test) in comparisons between the indicated transgenic line and WT.



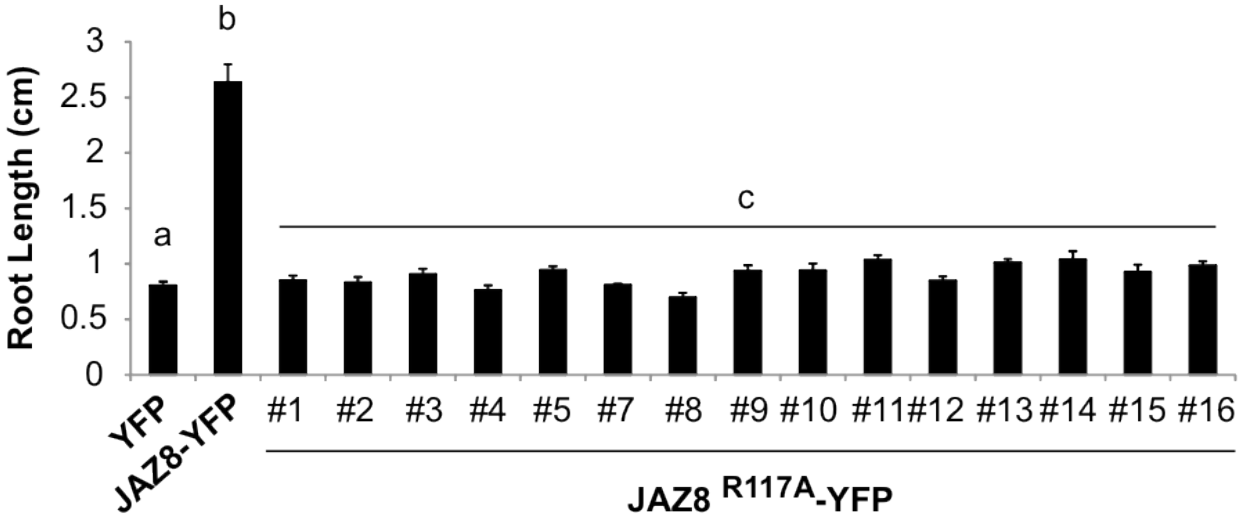
Supplemental Figure 3.1. Identification of *35S-JAZ13* transgenic lines overexpressing *JAZ13*.

RNA gel blot analysis of RNA extracted from T1 *35S-JAZ13* mature leaf tissues. Each number represents independent lines resistant to kanamycin. WT plants grown side by side to the transgenic lines were used as a control. No wound or JA-related treatment was done before harvesting samples.



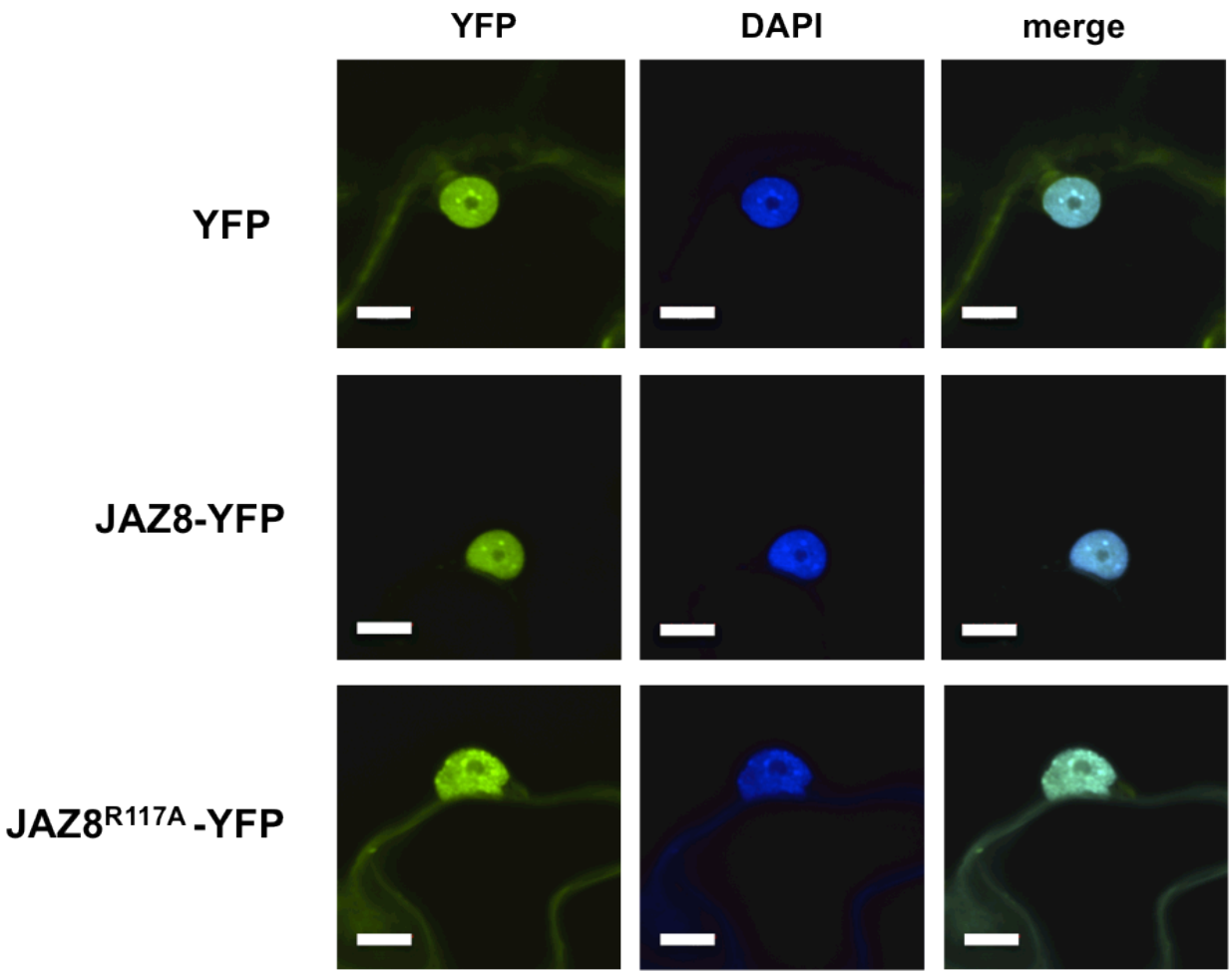
Supplemental Figure 3.2. Identification of *35S-JAZ8*^{R117A} transgenic lines overexpressing *JAZ8*^{R117A}.

RNA gel blot analysis of RNA extracted from T2 *35S-JAZ8* ^{R117A} 2-week-old seedlings. Each number represents independent lines resistant to kanamycin. WT plants grown side by side to the transgenic lines were used as a control. No wound or JA-related treatment was done before harvesting samples. Lines #1, #4, #14, #15, #19, #20 and #23 were selected for further analyses.



Supplemental Figure 3.3. Characterization of *35S-JAZ8 ^{R117A} –YFP* transgenic lines.

Transgenic lines overexpressing JAZ8^{R117A} – YFP are sensitive to JA-induced root growth inhibition. Primary root length of *35S-JAZ8^{R117A}-YFP* lines grown on 20 µM MeJA containing MS plates. *35S-YFP* and *35S-JAZ8-YFP* were used as controls. Seedlings were grown on plates for 10 days under continuous light. N = 15-28. b was significantly different than a (p value < 0.0001). c was significantly different than b (p = 0.02).



Supplemental Figure 3.4. JAZ8 ^{R117A} –YFP localization in *N. tabacum*.

JAZ8 ^{R117A} –YFP transiently expressed in *N. tabacum* leaves. YFP and JAZ8-YFP was used as controls. Confocal images were taken 48 hours after infiltration. DAPI (4',6-diamidino-2-phenylindole) was applied 2 hours before imaging to locate the nucleus.

Supplemental Table 3.1. List of oligonucleotide primers used in this study.

INSERT	PRIMER NAME	PRIMER SEQUENCE
JAZ14	JAZ14_pENTR_FP JAZ14_KpnI_RP	5'-CACCATGAAGGGTTGCAGCTTAG-3' 5'-GGTACCTTAGAAATTATGAAGAGAGGAGG-3'
JAZ14	JAZ14_XhoI_FP JAZ14_KpnI_RP	5'-CTCGAGATGAAGGGTTGCAGCTTAG-3' 5'-GGTACCTTAGAAATTATGAAGAGAGGAGG-3'
JAZ14ΔEAR	JAZ14dEAR_pENTR_FP JAZ14_KpnI_RP	5'-CACCATGAAGGGTTGCAGCTCTCCAATGGCCTCTACG-3' 5'-GGTACCTTAGAAATTATGAAGAGAGGAGG-3'
JAZ14 ADAHA	JAZ14_ADAHA_FP JAZ14_ADAHA_RP	5'- CACCATGAAGGGTTGCAGCGCAGATGCTCACGCATCTCCAATGGCCTCT ACGC-3' 5'- GCGTAGAGGCCATTGGAGATGCGTGAGCATCTGCGCTGCAACCCTTCAT GGTG-3'
JAZ7ΔJas	JAZ7_NcoI_FP JAZ7dJas_XhoI_RP	5'-CCATGGATGATCATCATCATCAAAAAC-3' 5'-CTCGAGCTATTGATAATGAAATCGAGTCG-3'
JAZ7-Jas	JAZ7Jas_NcoI_FP JAZ7_XhoI_RP	5'-CCATGGATGTCGACTCGATTTCATTATC-3' 5'-CGAGCTATCGGTAACGGTGGTAAG-3'
JAZ14ΔJas	JAZ14_pENTR_FP JAZ14ns_XhoI_249R_stop	5'-CACCATGAAGGGTTGCAGCTTAG-3' 5'-CTCGAGTTATAACGGTGATTCCAGTCTCACCG-3'
JAZ8ΔC122	JAZ8_NcoI_FP JAZ8dC122_s_XhoI_RP	5'-CCATGGATGAAGCTACAGCAAAATTGTG-3' 5'-CTCGAGTTAAATTCGAATTTTCCGTTTCTG-3'
JAZ8ΔC120	JAZ8_NcoI_FP JAZ8dC120_s_XhoI_RP	5'-CCATGGATGAAGCTACAGCAAAATTGTG-3' 5'-CTCGAGTTAAATTTTCCGTTTCTCAAG-3'
JAZ8ΔC118	JAZ8_NcoI_FP JAZ8dC118_s_XhoI_RP	5'-CCATGGATGAAGCTACAGCAAAATTGTG-3' 5'-CTCGAGTTACCGTTTCTGAAGAAAAC-3'
JAZ8ΔC115	JAZ8_NcoI_FP	5'-CCATGGATGAAGCTACAGCAAAATTGTG-3'

Supplemental Table 3.1. – Continued.

	JAZ8dC115_s_XhoI_RP	5'-CTCGAGTTAAAGAAAACTTTGGAG-3'
JAZ8 R117A	JAZ8_R117A_FP	5'-CCAAAGTTTTCTTCAGAAAGCGAAAATTCGAATTCAAGC-3'
	JAZ8_R117A_RP	5'-GCTTGAATTCGAATTTTCGCTTTCTGAAGAAAACTTTGG-3'
JAZ8 R117A	JAZ8_XhoI_FP	5'-CTCGAGATGAAGCTACAGCAAAATTGTG-3'
	JAZ8_KpnI_RP	5'-ATATGGTACCTTATCGTCGTGAATGGTACG-3'
JAZ8 R117A	JAZ8_XhoI_FP	5'-CTCGAGATGAAGCTACAGCAAAATTGTG-3'
	JAZ8ns_KpnI_RP	5'-ATATGGTACCTCGTCGTGAATGGTACG-3'

REFERENCES

REFERENCES

Aldridge DC, Galt S, Giles D, Turner WB (1971) Metabolites of Lasiodiplodia theobromae. J Chem Soc, C 1623–1627

Bai Y, Meng Y, Huang D, Qi Y (2011) Origin and evolutionary analysis of the plant-specific TIFY transcription factor family. Genomics **98**: 128–136

Ballaré CL (2011) Jasmonate-induced defenses: a tale of intelligence, collaborators and rascals. Trends Plant Sci **16**: 249–257

Browse J (2009) Jasmonate passes muster: a receptor and targets for the defense hormone. Annu Rev Plant Biol **60**: 183–205

Chini A, Fonseca S, Chico JM, Fernandez-Calvo P, Solano R (2009) The ZIM domain mediates homo- and heteromeric interactions between Arabidopsis JAZ proteins. The Plant Journal **59**: 77–87

Chini A, Fonseca S, Fernández G, Adie B, Chico JM, Lorenzo O, García-Casado G, López-Vidriero I, Lozano FM, Ponce MR, et al (2007) The JAZ family of repressors is the missing link in jasmonate signalling. Nature **448**: 666–671

Chung HS, Cooke TF, Depew CL, Patel LC, Ogawa N, Kobayashi Y, Howe GA (2010) Alternative splicing expands the repertoire of dominant JAZ repressors of jasmonate signaling. Plant J **63**: 613–622

Chung HS, Howe GA (2009) A Critical Role for the TIFY Motif in Repression of Jasmonate Signaling by a Stabilized Splice Variant of the JASMONATE ZIM-Domain Protein JAZ10 in Arabidopsis. Plant Cell **21**: 131–145

Chung HS, Koo AJK, Gao X, Jayanty S, Thines B, Jones AD, Howe GA (2008) Regulation and Function of Arabidopsis JASMONATE ZIM-Domain Genes in Response to Wounding and Herbivory. Plant Physiol **146**: 952–964

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J **16**: 735–743

Conconi A, Smerdon MJ, Howe GA, Ryan CA (1996) The octadecanoid signalling pathway in plants mediates a response to ultraviolet radiation. Nature **383**: 826–829

Demianski AJ, Chung KM, Kunkel BN (2012) Analysis of Arabidopsis JAZ gene expression during Pseudomonas syringae pathogenesis. Mol Plant Pathol **13**: 46–57

Dombrowski JE (2003) Salt stress activation of wound-related genes in tomato plants. Plant Physiol **132**: 2098–2107

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high

throughput. Nucleic Acids Res **32**: 1792–1797

Farmer EE, Johnson RR, Ryan CA (1992) Regulation of expression of proteinase inhibitor genes by methyl jasmonate and jasmonic Acid. Plant Physiol **98**: 995–1002

Fernandez-Calvo P, Chini A, Fernandez-Barbero G, Chico J-M, Gimenez-Ibanez S, Geerinck J, Eeckhout D, Schweizer F, Godoy M, Franco-Zorrilla JM, et al (2011) The Arabidopsis bHLH Transcription Factors MYC3 and MYC4 Are Targets of JAZ Repressors and Act Additively with MYC2 in the Activation of Jasmonate Responses. Plant Cell **23**: 701–715

Feys B, Benedetti CE, Penfold CN, Turner JG (1994) Arabidopsis Mutants Selected for Resistance to the Phytotoxin Coronatine Are Male Sterile, Insensitive to Methyl Jasmonate, and Resistant to a Bacterial Pathogen. Plant Cell **6**: 751–759

Grunewald W, Vanholme B, Pauwels L, Plovie E, Inzé D, Gheysen G, Goossens A (2009) Expression of the Arabidopsis jasmonate signalling repressor JAZ1/TIFY10A is stimulated by auxin. EMBO Rep **10**: 923–928

Hou X, Lee LYC, Xia K, Yan Y, Yu H (2010) DELLAs Modulate Jasmonate Signaling via Competitive Binding to JAZs. CORD Conference Proceedings **19**: 884–894

Kagale S, Rozwadowski K (2011) EAR motif-mediated transcriptional repression in plants: an underlying mechanism for epigenetic regulation of gene expression. Epigenetics **6**: 141–146

Katsir L, Schilmiller AL, Staswick PE, He SY, Howe GA (2008) COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. Proceedings of the National Academy of Sciences **105**: 7100–7105

Kazan K, Manners JM (2012) JAZ repressors and the orchestration of phytohormone crosstalk. Trends Plant Sci **17**: 22–31

Li C, Williams MM, Loh Y-T, Lee GI, Howe GA (2002) Resistance of cultivated tomato to cell content-feeding herbivores is regulated by the octadecanoid-signaling pathway. Plant Physiol **130**: 494–503

Li L, Zhao Y, McCaig BC, Wingerd BA, Wang J, Whalon ME, Pichersky E, Howe GA (2004) The tomato homolog of CORONATINE-INSENSITIVE1 is required for the maternal control of seed maturation, jasmonate-signaled defense responses, and glandular trichome development. Plant Cell **16**: 126–143

Nakagami H, Sugiyama N, Ishihama Y, Shirasu K (2012) Shotguns in the front line: phosphoproteomics in plants. Plant Cell Physiol **53**: 118–124

Niu YY, Figueroa PP, Browse JJ (2011) Characterization of JAZ-interacting bHLH transcription factors that regulate jasmonate responses in Arabidopsis. CORD Conference Proceedings **62**: 2143–2154

Pauwels L, Barbero GF, Geerinck J, Tilleman S, Grunewald W, Pérez AC, Chico J-M, Bossche RV, Sewell J, Gil E, et al (2010) NINJA connects the co-repressor TOPLESS to jasmonate signalling. Nature **464**: 788–791

Pauwels L, Goossens A (2011) The JAZ Proteins: A Crucial Interface in the Jasmonate Signaling Cascade. Plant Cell **23**: 3089–3100

Qi T, Song S, Ren Q, Wu D, Huang H, Chen Y, Fan M, Peng W, Ren C, Xie D (2011) The Jasmonate-ZIM-Domain Proteins Interact with the WD-Repeat/bHLH/MYB Complexes to Regulate Jasmonate-Mediated Anthocyanin Accumulation and Trichome Initiation in Arabidopsis thaliana. Plant Cell **23**: 1795–1814

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol **4**: 406–425

Schilmiller AL, Koo AJK, Howe GA (2007) Functional diversification of acyl-coenzyme A oxidases in jasmonic acid biosynthesis and action. Plant Physiol **143**: 812–824

Sheard LB, Tan X, Mao H, Withers J, Ben-Nissan G, Hinds TR, Kobayashi Y, Hsu F-F, Sharon M, Browse J, et al (2010) Jasmonate perception by inositol-phosphate-potentiated COI1-JAZ co-receptor. Nature **468**: 400–405

Shyu C, Figueroa P, Depew CL, Cooke TF, Sheard LB, Moreno JE, Katsir L, Zheng N, Browse J, Howe GA (2012) JAZ8 lacks a canonical degron and has an EAR motif that mediates transcriptional repression of jasmonate responses in Arabidopsis. Plant Cell **24**: 536–550

Song C-P, Galbraith DW (2006) AtSAP18, an orthologue of human SAP18, is involved in the regulation of salt stress and mediates transcriptional repression in Arabidopsis. Plant Mol Biol **60**: 241–257

Song S, Qi T, Huang H, Ren Q, Wu D, Chang C, Peng W, Liu Y, Peng J, Xie D (2011) The Jasmonate-ZIM Domain Proteins Interact with the R2R3-MYB Transcription Factors MYB21 and MYB24 to Affect Jasmonate-Regulated Stamen Development in Arabidopsis. Plant Cell **23**: 1000–1013

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol **28**: 2731–2739

Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, Browse J (2007) JAZ repressor proteins are targets of the SCFCOI1 complex during jasmonate signalling. Nature **448**: 661–665

Vanholme B, Grunewald W, Bateman A, Kohchi T, Gheysen G (2007) The tify family previously known as ZIM. Trends Plant Sci **12**: 239–244

Weiler E, Albrecht T, Groth B, Xia Z, Luxem M, Liss H, Andert L, Spengler P (1993)

Evidence for the Involvement of Jasmonates and Their Octadecanoid Precursors in the Tendril Coiling Response of Bryonia-Dioica. Phytochemistry **32**: 591–600

Yan Y, Stolz S, Chételat A, Reymond P, Pagni M, Dubugnon L, Farmer EE (2007) A downstream mediator in the growth repression limb of the jasmonate pathway. Plant Cell **19**: 2470–2483

Yoshida Y, Sano R, Wada T, Takabayashi J, Okada K (2009) Jasmonic acid control of GLABRA3 links inducible defense and trichome patterning in Arabidopsis. Development **136**: 1039–1048

CHAPTER FOUR
SUMMARY AND FUTURE PERSPECTIVES

SUMMARY OF DISSERTATION

Identification of the JASMONATE ZIM DOMAIN (JAZ) family of proteins has spurred remarkable progress towards understanding of jasmonate (JA) signaling. At the time this dissertation research was initiated, little was known about the molecular mechanism of JAZ function. CORONATINE INSENSITIVE 1 (COI1)-JAZ interaction was shown to require JA-Ile as a molecular ligand, but sequence determinants of COI1-JAZ interaction were not identified. Moreover, 12 JAZ proteins were hypothesized to have similar mechanisms in repressing JA responses. In this dissertation research, I characterize JAZ8 as a weak interactor of COI1, and I determine the sequence motifs that are responsible for COI1-JAZ interaction and JAZ stability in vivo (Shyu et al., 2012). I report that JAZ8 represses JA responses through a NOVEL INTERACTOR OF JAZ (NINJA)-independent pathway, which is unique from the known NINJA-dependent repression mechanism (Shyu et al., 2012). I also report the identification of a previously unidentified JAZ protein, JAZ13. JAZ13 shares high sequence similarity with JAZ8, and represses JA responses through the NINJA-independent repression pathway. My findings suggest that JAZ proteins regulate JA-induced gene expression through diverse mechanisms to generate different signaling outputs.

I demonstrate that JAZ8 is a stable JAZ repressor in Chapter Two of this dissertation (Shyu et al., 2012). In vitro pull down assays show that different JAZ proteins have different interaction affinities to COI1 in the presence of JA-Ile. JAZ8 does not interact with COI1 in the presence of JA-Ile. JAZ8-YFP was stable upon MeJA treatment compared to JAZ10.1-YFP. Moreover, overexpression of JAZ8 leads to decreased sensitivity to JA, indicating that JAZ8 is a stable repressor of JA responses. I further show that JAZ8 stability is due to it's diversified

degron motif. Domain swap of the N-terminal Jas degron of JAZ10.1 into JAZ8 results in promotion of JAZ8^{LPIAR}-COI1 interaction and rapid degradation of JAZ8^{LPIAR}-YFP in the presence of MeJA. These results collectively demonstrate that JAZ proteins have different stabilities in response to JA, due to their diversified degron.

I report that JAZ8 represses JA-induced gene expression through a previous uncharacterized NINJA-independent pathway. Experiments demonstrating this finding are recorded in Chapter Two. JAZ8 contains a EAR motif at the N-terminus, in which the EAR motif is required for JAZ8's repressive activity (Kagale et al., 2010; Shyu et al., 2012). JAZ8 interacts with corepressor TOPLESS in the absence of NINJA (Arabidopsis Interactome Mapping Consortium, 2011; Shyu et al., 2012). Furthermore, the ZIM domain, which is important for JAZ-NINJA interaction in other JAZ members, is not required for JAZ8 to repress JA responses. These findings illustrate that JAZ8 recruits TOPLESS and represses JA responses through a NINJA-independent pathway, different from the previous demonstrated NINJA-dependent repression mechanism. This is significant because it indicates that JAZ proteins repress JA-induced gene expression through alternative mechanisms.

Finally, I identify and characterize a previously undiscovered JAZ member, JAZ13. Research related to JAZ13 is recorded in Chapter Three of this dissertation. JAZ13 was identified from a BLAST search using JAZ8 amino acid sequences as query. JAZ13 has high sequence similarities with JAZ8, and shares a conserved EAR motif at the N-terminus. JAZ13 also has sequence motifs similar to the ZIM domain and Jas motif of JAZ8. Overexpression of JAZ13 leads to decreased sensitivity to JA, indicating JAZ13 has a negative role in regulating JA responses. Interestingly, JAZ13 interacts with TOPLESS in a NINJA-independent manner, suggesting that JAZ13 represses JA responses through a NINJA-independent pathway. I also

report that the JAZ8 subclade of JAZ repressors requires interaction with MYC2 to repress JA responses. I identified sequence determinants for JAZ8-MYC2 interaction, and show that mutations in the JAZ8-MYC2 interaction motif abolish JAZ8 repression. Findings in this chapter strengthen the importance of NINJA-independent repression mechanisms, and expand the repertoire of JAZ proteins.

To summarize, research in this dissertation demonstrates an alternative mechanism for JAZ repression. I show that JAZ proteins differ in stability and repression mechanisms to regulate JA responses. JAZ8 is a stabilized JAZ protein, and JAZ8 represses JA responses through a NINJA-independent pathway. Identification and characterization of JAZ13 reveals that the JAZ8 subclade of JAZ proteins represses JA responses in a NINJA-independent manner. Taken together, I report the identification of a novel repression mechanism, presumably for the purpose of fine-tuning myriad JA responses to balance growth and defense.

FUTURE PERSPECTIVES

In the past five years, many exciting discoveries have drastically advanced our understanding towards JA biology. JAZ proteins were identified as substrates of COI1 (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007). (3R, 7S) JA-Ile was identified as the bioactive form of JA (Fonseca et al., 2009). Crystal structure of the COI1-JAZ JA-Ile receptor complex was resolved (Sheard et al., 2010). Domain analyses of JAZs were done to understand the molecular mechanism of JAZ function, and NINJA and TOPLESS were identified as important components in the JAZ repression complex (Melotto et al., 2008; Chini et al., 2009; Chung and

Howe, 2009; Pauwels et al., 2010; Shyu et al., 2012). Furthermore, many transcription factors were identified as JAZ-interactors, connecting JA perception with different transcriptional signaling outputs (Hou et al., 2010; Cheng et al., 2011; Fernandez-Calvo et al., 2011; Qi et al., 2011; Song et al., 2011; Zhu et al., 2011; Yang et al., 2012). These findings collectively place JAZ proteins as core internodes that link JA-Ile perception to diverse JA signaling outputs. However, as all exciting discoveries do, advancement in JAZ research raises many questions that must now be answered.

It is not known how stable JAZ repressors are turned over. In Chapter Two of the dissertation I demonstrated that not all JAZ proteins interact with COI1 in a JA-Ile-dependent manner, and JAZ8 is a weak COI1-interactor (Shyu et al., 2012). JAZ8-YFP is stable upon MeJA treatment. In addition to JAZ8, alternative splice variants of JAZ10 (JAZ10.3 and JAZ10.4) are stable upon MeJA treatment compared to JAZ10.1 (Chung and Howe, 2009). Other JAZ proteins such as JAZ2 and JAZ6 also produce alternative splice variants that interact weakly with COI1 (Chung et al., 2010). Although JA-Ile is characterized as the bioactive ligand that can promote JAZ-COI1 interaction, it is interesting to point out that JA-amino acid conjugates that are structurally similar to JA-Ile also promote JAZ-COI1 interaction in in vitro pull down experiments (Katsir et al., 2008). A variety of JA derivatives are present endogenously, and many of which are wound-inducible (Koo et al., 2009). It would be interesting to examine whether or not other JA derivatives can promote JAZ-COI1 interaction and JAZ degradation. JAZ8 has a diversified degron motif that prevents JAZ8-COI1 interaction in the presence of JA-Ile (Shyu et al., 2012). Therefore it is reasonable to propose that JAZ8 may form a binding pocket that does not recognize JA-Ile, but interacts with other ligands. Indeed, JAZ8 interacts with COI1 in the presence of high concentrations of coronatine (Shyu et al., 2012). Whether or

not endogenous ligands other than JA-Ile can promote JAZ8 degradation is not known. Some alternative splice variants, on the other hand, lack the entire Jas degron. How these stable splice variants are turned over still remains to be elucidated. In the case of Aux/IAA repressors in auxin signaling, it is known that different Aux/IAA proteins have different half-lives (Dreher et al., 2006). Examining the half-life of JAZ proteins may also provide alternative insights to JAZ stability. Taken together, whether or not other endogenous ligands are able to promote JAZ degradation, and examining the half-life of JAZ proteins are expected to advance our knowledge in JAZ signaling.

Composition of the JAZ repression complex still remains to be elucidated. The current JA signaling model suggests that the JAZ repression complex involves MYC2, NINJA and TPL (Pauwels et al., 2010). However, this model is mainly based on protein-protein interaction assays that restricts to examining one protein-protein pair at a time. Very little is known about the endogenous JAZ repression complex. Currently two repression models are proposed for JAZ proteins: a NINJA-dependent pathway that JAZ proteins recruit TPL via NINJA, and a NINJA-independent pathway in which JAZ repressors directly interact with TPL through a EAR motif (Pauwels et al., 2010; Shyu et al., 2012). Interestingly, JAZ5 and JAZ6 interact with NINJA, but also contain TPL-interacting EAR motifs (Kagale et al., 2010; Pauwels et al., 2010). Whether or not JAZ5/6 repression requires NINJA or TPL is not known. Understanding the composition of JAZ repression complexes involving JAZ5 and JAZ6 will advance understanding towards the JAZ repression machinery. On the other hand, JAZ proteins form homo- and heterodimers (Chini et al., 2009; Chung and Howe, 2009). Transcription factors and TPL also homodimerize (Szemenyei et al., 2008; Fernandez-Calvo et al., 2011). The role of dimerization and how dimerization contributes to JAZ repression is not known. Geerinck et al. (2010) isolated the

JAZ1 complex by tandem affinity purification (TAP) of JAZ1-TAP overexpressing Arabidopsis cell lines and showed that the JAZ1-TAP complex assembles into protein complexes that are up to 1 MDa (Geerinck et al., 2010). Mass spectrometry analyses of these protein complexes are expected to provide more insight to composition of the JAZ repression complex.

How JAZ proteins control the specificity of JA signaling outputs is largely unknown. Although overexpression of stable JAZ proteins result in decreased sensitivity to JA, *35S-JAZ10.4* lines are male sterile, whereas *35S-JAZ8* are fertile (Chung and Howe, 2009; Shyu et al., 2012). This suggests that different JAZ proteins may repress gene expression of different target genes. One hypothesis is that JAZ repressors are expressed in various tissues at different developmental stages to regulate a myriad of responses. Little is known about expression patterns of Arabidopsis JAZ repressors. Most *JAZ*s in Arabidopsis have a very low basal expression level, and are rapidly induced in mature leaf tissues or whole seedlings upon wounding or JA treatment (Chung et al., 2008). Microarray analyses of *JAZ* genes in *Nicotiana attenuata* showed that different *JAZ* genes have different expression patterns (Oh et al., 2012). Interestingly, *NaJAZg*, which is an ortholog of Arabidopsis *JAZ3* and *JAZ4*, is highly expressed in root tissues, but not leaf tissues, whereas other *NaJAZ* genes are present in leaf tissues. These findings support the hypothesis in which regulation of JA signaling outputs is due to the presence of different JAZ proteins at different tissues and/or developmental stages. Another hypothesis is that JAZ proteins interact with different transcription factors (TFs) to regulate different signaling outputs. Multiple transcription factors interact with JAZ proteins. Interestingly not all JAZ proteins have similar interaction patterns with these transcription factors (Fernandez-Calvo et al., 2011; Qi et al., 2011; Song et al., 2011). Sequence determinants of specificity for JAZ-TF interaction are largely unknown. Examination of JAZ and TF expression patterns and chromatin

immunoprecipitation (ChIP) analyses of JAZ-associated DNA motifs will greatly accelerate understanding towards JAZ target specificity. Moreover, characterization of *jaz* mutants will also advance understanding towards the biological role of different JAZ proteins.

Multiple stable JAZ isomers are characterized, but little is known about the physiological relevance of stable JAZ proteins. One proposed hypothesis is that stable JAZ proteins function to attenuate JA responses to prevent plants from over-responding to JA-Ile. Upon wounding or other biological cues that cause high levels of JA-Ile to accumulate, liable JAZ proteins are rapidly degraded, resulting in accumulation of stable JAZ proteins such as JAZ8. Stable JAZ proteins then interact with transcription factors to turn of JA responses. Another hypothesis is that stable JAZ proteins are only expressed in certain tissues to provide specificity in signaling outputs. Results supporting this hypothesis are that *NaJAZ* genes have differential expression patterns upon wounding (Oh et al., 2012). It is also possible that a combination of the two models exist simultaneously, to provide a complex regulation of JA signaling outputs. Live cell imaging of native promoter driven JAZ-reporter lines in combination with characterization of JAZ targets (as mentioned in the previous chapter) are expected to provide more understanding to the physiological relevance of stable JAZ proteins.

Taken together, characterization of JAZ proteins has introduced us into a new era of understanding the molecular mechanism of JA signaling. Large-scale gene expression analysis and protein-protein interaction techniques played a significant role in identification of new members in the JA signaling pathway and revealing the molecular mechanism of JAZ function. Continued work that expands our knowledge in JA perception, composition of the JAZ repressor complex, and target specificity of different JAZ proteins, is required to answer the big question

of how plants utilize JA to balance growth and defense in response to myriad environmental conditions.

REFERENCES

REFERENCES

Arabidopsis Interactome Mapping Consortium (2011) Evidence for network evolution in an Arabidopsis interactome map. Science **333**: 601–607

Cheng Z, Sun L, Qi T, Zhang B, Peng W, Liu Y, Xie D (2011) The bHLH Transcription Factor MYC3 Interacts with the Jasmonate ZIM-Domain Proteins to Mediate Jasmonate Response in Arabidopsis. Molecular Plant **4**: 279–288

Chini A, Fonseca S, Chico JM, Fernandez-Calvo P, Solano R (2009) The ZIM domain mediates homo- and heteromeric interactions between Arabidopsis JAZ proteins. The Plant Journal **59**: 77–87

Chini A, Fonseca S, Fernández G, Adie B, Chico JM, Lorenzo O, García-Casado G, López-Vidriero I, Lozano FM, Ponce MR, et al (2007) The JAZ family of repressors is the missing link in jasmonate signalling. Nature **448**: 666–671

Chung HS, Cooke TF, Depew CL, Patel LC, Ogawa N, Kobayashi Y, Howe GA (2010) Alternative splicing expands the repertoire of dominant JAZ repressors of jasmonate signaling. Plant J **63**: 613–622

Chung HS, Howe GA (2009) A Critical Role for the TIFY Motif in Repression of Jasmonate Signaling by a Stabilized Splice Variant of the JASMONATE ZIM-Domain Protein JAZ10 in Arabidopsis. Plant Cell **21**: 131–145

Chung HS, Koo AJK, Gao X, Jayanty S, Thines B, Jones AD, Howe GA (2008) Regulation and Function of Arabidopsis JASMONATE ZIM-Domain Genes in Response to Wounding and Herbivory. Plant Physiol **146**: 952–964

Dreher KA, Brown J, Saw RE, Callis J (2006) The Arabidopsis Aux/IAA protein family has diversified in degradation and auxin responsiveness. Plant Cell **18**: 699–714

Fernandez-Calvo P, Chini A, Fernandez-Barbero G, Chico J-M, Gimenez-Ibanez S, Geerinck J, Eeckhout D, Schweizer F, Godoy M, Franco-Zorrilla JM, et al (2011) The Arabidopsis bHLH Transcription Factors MYC3 and MYC4 Are Targets of JAZ Repressors and Act Additively with MYC2 in the Activation of Jasmonate Responses. Plant Cell **23**: 701–715

Fonseca S, Chini A, Hamberg M, Adie B, Porzel A, Kramell R, Miersch O, Wasternack C, Solano R (2009) (+)-7-iso-Jasmonoyl-L-isoleucine is the endogenous bioactive jasmonate. Nature Chemical Biology **5**: 344–350

Geerinck J, Pauwels L, De Jaeger G, Goossens A (2010) Dissection of the one-MegaDalton JAZ1 protein complex. Plant Signal Behav **5**: 1039–1041

Hou X, Lee LYC, Xia K, Yan Y, Yu H (2010) DELLAs Modulate Jasmonate Signaling via Competitive Binding to JAZs. CORD Conference Proceedings **19**: 884–894

Kagale S, Links MG, Rozwadowski K (2010) Genome-wide analysis of ethylene-responsive element binding factor-associated amphiphilic repression motif-containing transcriptional regulators in Arabidopsis. Plant Physiol **152**: 1109–1134

Katsir L, Schilmiller AL, Staswick PE, He SY, Howe GA (2008) COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. Proceedings of the National Academy of Sciences **105**: 7100–7105

Koo AJK, Gao X, Jones AD, Howe GA (2009) A rapid wound signal activates the systemic synthesis of bioactive jasmonates in Arabidopsis. Plant J **59**: 974–986

Melotto M, Mecey C, Niu Y, Chung HS, Katsir L, Yao J, Zeng W, Thines B, Staswick P, Browse J, et al (2008) A critical role of two positively charged amino acids in the Jas motif of Arabidopsis JAZ proteins in mediating coronatine- and jasmonoyl isoleucine-dependent interactions with the COI1 F-box protein. Plant J **55**: 979–988

Oh Y, Baldwin IT, Galis I (2012) NaJAZh regulates a subset of defense responses against herbivores and spontaneous leaf necrosis in Nicotiana attenuata plants. Plant Physiol –

Pauwels L, Barbero GF, Geerinck J, Tilleman S, Grunewald W, Pérez AC, Chico J-M, Bossche RV, Sewell J, Gil E, et al (2010) NINJA connects the co-repressor TOPLESS to jasmonate signalling. Nature **464**: 788–791

Qi T, Song S, Ren Q, Wu D, Huang H, Chen Y, Fan M, Peng W, Ren C, Xie D (2011) The Jasmonate-ZIM-Domain Proteins Interact with the WD-Repeat/bHLH/MYB Complexes to Regulate Jasmonate-Mediated Anthocyanin Accumulation and Trichome Initiation in Arabidopsis thaliana. Plant Cell **23**: 1795–1814

Sheard LB, Tan X, Mao H, Withers J, Ben-Nissan G, Hinds TR, Kobayashi Y, Hsu F-F, Sharon M, Browse J, et al (2010) Jasmonate perception by inositol-phosphate-potentiated COI1-JAZ co-receptor. Nature **468**: 400–405

Shyu C, Figueroa P, Depew CL, Cooke TF, Sheard LB, Moreno JE, Katsir L, Zheng N, Browse J, Howe GA (2012) JAZ8 lacks a canonical degron and has an EAR motif that mediates transcriptional repression of jasmonate responses in Arabidopsis. Plant Cell **24**: 536–550

Song S, Qi T, Huang H, Ren Q, Wu D, Chang C, Peng W, Liu Y, Peng J, Xie D (2011) The Jasmonate-ZIM Domain Proteins Interact with the R2R3-MYB Transcription Factors MYB21 and MYB24 to Affect Jasmonate-Regulated Stamen Development in Arabidopsis. Plant Cell **23**: 1000–1013

Szemenyei H, Hannon M, Long JA (2008) TOPLESS mediates auxin-dependent transcriptional repression during Arabidopsis embryogenesis. Science **319**: 1384–1386

Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, Browse J (2007) JAZ repressor proteins are targets of the SCFCOI1 complex during jasmonate signalling. Nature **448**: 661–665

Yan Y, Stolz S, Chételat A, Reymond P, Pagni M, Dubugnon L, Farmer EE (2007) A downstream mediator in the growth repression limb of the jasmonate pathway. Plant Cell **19**: 2470–2483

Yang D-L, Yao J, Mei C-S, Tong X-H, Zeng L-J, Li Q, Xiao L-T, Sun T-P, Li J, Deng X-W, et al (2012) Plant hormone jasmonate prioritizes defense over growth by interfering with gibberellin signaling cascade. Proc Natl Acad Sci U S A. doi: 10.1073/pnas.1201616109

Zhu Z, An F, Feng Y, Li P, Xue L, A M, Jiang Z, Kim J-M, To TK, Li W, et al (2011) Derepression of ethylene-stabilized transcription factors (EIN3/EIL1) mediates jasmonate and ethylene signaling synergy in Arabidopsis. Proc Natl Acad Sci U S A **108**: 12539–12544