

IDENTIFICATION OF A LINEAGE SPECIFIC *JAZ8*-LIKE GENE IN *ARABIDOPSIS THALIANA*

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Cell and Molecular Biology - Master of Science

2014

ABSTRACT

IDENTIFICATION OF A LINEAGE SPECIFIC JAZ8-LIKE GENE IN *ARABIDOPSIS THALIANA*

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Jasmonate (JA) is an oxylipin signaling molecule that plays a crucial role in inducing plant defense mechanisms in response to herbivore feeding. During herbivore feeding, JA accumulates and induces transcriptional changes through ubiquitin-dependent degradation of JAZMONATE ZIM DOMAIN (JAZ) transcriptional repressors. *A. thaliana* is known to have 12 members of the JAZ family. However, a BLAST search querying *JAZ8* revealed *At3G22275*, a gene of unknown function with sequence similarity to *JAZ8*. Striking domain conservation between *JAZ8* and *At3G22275* prompted an investigation to determine whether *At3G22275* functions as a transcriptional repressor of JA responses. *At3G22275* was found to interact with the JA-related transcription factor MYC2 and co-repressor TPL in a manner consistent with what has been previously reported for *JAZ8*. In addition, over-expression of *At3G22275* dampened JA-responsive gene expression and led to a JA-insensitive phenotype during root elongation and insect feeding. Complementary to the over-expression phenotype, a loss-of-function analysis revealed JA-hypersensitivity in *at3g22275/jaz10* double mutants. Interestingly, *At3G22275* homologs are only found within members of the Brassicaceae family while *JAZ8* homologs can be found among most land plants. Collectively, sequence similarities and experimental evidence supports the identification of *At3G22275* as “*JAZ13*,” a lineage-specific negative regulator of JA responses and a new member of the JAZ family of transcriptional repressors.

ACKNOWLEDGEMENTS

Work presented in this thesis is part of a manuscript in preparation and includes contributions from other authors. Dr. Christine Shyu provided invaluable foundation work for the project by performing the molecular cloning of JAZ13 constructs and generating transgenic *35S::JAZ13-HA Arabidopsis thaliana*. Dr. Shyu also performed initial Y2H screens reported here as experimental replicates. Dr. Yuki Yoshida performed genetic crosses to yield all combinations of *jaz7*, *jaz10* and *jaz13* and designed a strategy and began the introgression of the Vash-1 JAZ8 allele, *jaz8-V*, into Col-0 background.

Caitlin Thireault screened *35S::JAZ13-HA* lines for highly expressing homozygous lines and performed all subsequent experiments with this transgenic line; performed screening for the identification of all higher-order JAZ mutants reported here; assisted with the *jaz8-V* introgression by screening chromosomal markers to identify the best recombinants; performed genetic crosses and screening for all mutants containing *jaz8-V* allele; performed all experiments and generated all figures reported in this thesis. I would like to thank Dr. Sheng Yang He and Dr. Jian Yao who provided the *35S::JAZ8-HA* and *35S::JAZ9-HA* transgenic *Arabidopsis thaliana* which were used as controls in this manuscript.

This work would not be possible without the guidance and support provided by my mentor, Dr. Gregg Howe. I would like to thank him for the time and effort invested in my success. I would also like to thank past and present Howe lab members, who not only provided protocols and expertise, but also a welcoming and supportive research environment. I would like to specifically acknowledge Dr. Yuki Yoshida for orienting me in the lab and helping me

obtain the mutants described in this work; Dr. Christine Shyu for giving me a great head start on the project; Dr. Ian Major for being a statistical mastermind and for sharing his qRT-PCR expertise; Marcelo Campos (soon to be Dr. Campos) for always lending a helping hand and cheering me up when I was feeling down; as well as other lab members, Dr. Abe Koo, Dr. Javier Moreno, Dr. Koichi Sugimoto, and Qiang Guo, for being a source of intellectual growth and great discussion. I would also like to thank two undergraduate helpers; Eric Kastory and Kayla Moses, who saved me countless hours preparing media and cleaning up after me. Last but not least, I would like to thank my friends and family who were always a source of support and encouragement. I would like to especially thank my finance, Robert Loepp, who was always by my side and gave me strength when I needed it most.

TABLE OF CONTENTS

LIST OF TABLES.....	vii
LIST OF FIGURES.....	viii
KEY TO ABBREVIATIONS.....	ix
INTRODUCTION.....	1
Plant defense.....	1
The plant hormone jasmonate.....	2
Jasmonate biosynthesis.....	3
Elucidation of the jasmonate signaling pathway.....	5
Current signaling model.....	7
Signal attenuation.....	7
JAZ transcriptional repressors.....	8
JAZ stability.....	9
JAZ mediated repression.....	11
RESULTS.....	13
A new member of the JAZ family.....	13
At3G22275 expression is JA inducible.....	15
At3G22275 interacts with key JA-signaling components.....	15
Coronatine treatment induces degradation of JAZ13.....	16
Over expression of JAZ13 reduces JA sensitivity in Arabidopsis.....	17
Identification of JAZ7, JAZ8, JAZ10, and JAZ13 null mutants.....	18
jaz13/jaz10 double mutants display enhanced JA-sensitivity.....	20
DISCUSSION.....	21
At3G22275 is a member of the JAZ family of transcriptional repressors.....	21
JAZ13 is specific to the Brassicaceae family.....	24
JAZ natural variation.....	25
MATERIALS AND METHODS.....	27
Plant materials.....	27
Multiple sequence alignment and phylogenetic analysis.....	27
Molecular cloning.....	28
Yeast two-hybrid assay.....	28
Generation of transgenic lines.....	29
Plant treatments.....	29

Degradation assay.....	31
Generation of higher order JAZ mutants.....	31
Accession numbers.....	32
APPENDIX.....	33
REFERENCES.....	54

LIST OF TABLES

Table 1. PCR primers used in this study.....	52
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LIST OF FIGURES

Figure 1. Basic representation of JA metabolism and signaling pathway.....	34
Figure 2. At3G22275 shares sequence similarities with JAZ7 and JAZ8.....	35
Figure 3. Phylogenetic tree of all <i>Arabidopsis thaliana</i> TIFY family transcriptional regulators.....	36
Figure 4. At3G22275-like genes are only found in Brassicaceae.....	37
Figure 5. Sequence alignment of At3G22275-like predicted protein products.....	38
Figure 6. At3G22275 expression is induced by coronatine treatment.....	39
Figure 7. Y2H interaction with MYC2 and TPL.....	40
Figure 8. JAZ13 is degraded upon coronatine treatment.....	41
Figure 9. Over-expression of JAZ13 reduces MeJA sensitivity in the roots.....	42
Figure 10. JA-mediated defense responses are suppressed in <i>JAZ13-OE</i>	43
Figure 11. JA-responsive gene expression is dampened in <i>JAZ13-OE</i>	44
Figure 12. Higher order JAZ mutants display increased MeJA sensitivity in the roots.....	45
Figure 13. Gene structure and confirmation of mutants.....	46
Figure 14. Chromosomal locations of genes and markers included in this study.....	47
Figure 15. <i>jaz8-V</i> is not hypersensitive to MeJA induced root growth inhibition.....	48
Figure 16. Higher order JAZ mutants are phenotypically and developmentally similar to Col-0 in the absence of a stress.....	49
Figure 17. Addition of <i>jaz7-1</i> does not further enhance root phenotype of <i>jaz10</i> or <i>jaz10/13</i> mutants.....	50
Figure 18. Addition of <i>jaz8-V</i> does not further enhance root phenotype of <i>jaz10</i> or <i>jaz10/13</i> mutants.....	51

KEY TO ABBREVIATIONS

AOS	ALLENE OXIDE SYNTHASE
bHLH	Basic helix loop helix
COI1	CORONATINE INSENSITIVE 1
Col-0	<i>Arabidopsis thaliana</i> ecotype Columbia-0
COR	Coronatine
FPKM	fragment per kilobase mapped
JA	Jasmonic acid
JAR1	JASMONATE RESISTANT 1
JAZ	JASMONATE ZIM DOMAIN
JMT	JASMONIC ACID CARBOXYMETHYLTRANSFERASE
MeJA	Methyl-jasmonate
NINJA	NOVEL INTERACTOR OF JAZ
LOX3	LIPOXYGENASE 3
qPCR	Quantitative polymerase chain reaction
SA	Salicylic acid
SCF	Skip/CULLEN/F-BOX E3 ubiquitin ligase complex
SSLP	Simple sequence length polymorphism
TIR1	TRANSPORT INHIBITOR RESPONSE 1
TPL	TOPLESS
Vash-1	<i>Arabidopsis thaliana</i> ecotype Vashlovani-1

INTRODUCTION

Plant defense

The ability to perceive a threat and mount proper defenses is critical for plant survival, yet activation of defense responses is often resource intensive and can lead to suppression of other physiological processes. In the case of jasmonate (JA) signaling, activation of the JA pathway suppresses growth (Aldridge et al., 1971; Yamane et al., 1981; Yang et al., 2012; Attaran et al., 2014). Although constitutive JA activation could lead to a plant that is more resistant to insect feeding, prolonged growth suppression may hinder a plant's ability to compete for resources, such as sunlight and nutrients, and can limit energy available for reproduction resulting in an overall decrease in fitness. Therefore, in order to achieve maximum benefits from growth and defense, it is only beneficial activate defenses when appropriate and in the absence of stress allow resources to be devoted to growth and development. In addition, plant defense responses are regulated by several convening hormone signaling pathways that can act antagonistically to one another resulting in a complex network for the fine tuning of defense responses (Huot et al., 2014). A classic example of signal antagonism occurs between salicylic acid (SA) and jasmonate (JA). In general, SA is critical for defense against hemi-biotrophic and biotrophic pathogens while JA is critical for defense against necrotrophic pathogens and herbivores. Activation of one signaling pathway leads to a suppression of the other. As a result, favoring one defense pathway could dampen others

leading to a plant that is perhaps more resistant to herbivores but then more susceptible to attack by other types of pathogens or pests.

The plant hormone jasmonate

Jasmonate (JA) is an oxylipin plant hormone produced from membrane derived free fatty acids in the chloroplast. Most oxylipins are produced through serial modifications leading to a collection of structurally related molecules. Therefore, the term “jasmonate” does not refer to a specific molecule but rather a family of molecules; including the methyl ester and isoleucine conjugate of jasmonic acid, methyl jasmonate (MeJA) and JA-isoleucine (JA-Ile), respectively. MeJA was first identified as the compound responsible for the fragrance of jasmine flowers. Later, it was discovered that exogenous application of MeJA inhibited growth (Aldridge et al., 1971) leading to the classification of jasmonate as a plant hormone. JA-Ile is a particularly important jasmonate as it would eventually be identified as the predominantly bioactive JA (Fonseca et al., 2009; Staswick et al., 1992; Katsir et al., 2008).

In addition to the activation of defense in response to a variety of stimuli; including insect feeding, pathogen infection, UV damage, and various abiotic stresses (Vijayan et al., 1998; Weiler et al., 1993; Conconi et al., 1996; Dombrowski, 2003; Browse, 2009), JA has also been found to have multiple regulatory roles throughout growth and development; including root and shoot growth inhibition in seedlings (Staswick et al., 1992; Yamane et al., 1981), reproductive development (Feys et al., 1994; Li et al., 2004), and trichome development (Li et al., 2004; Yoshida et al., 2009). The multiple regulatory roles of JA, and specifically the role it

takes between growth suppression and defense activation, has led to the implication that JA, at least in part, regulates resource partitioning between growth and defense.

Jasmonate biosynthesis

Jasmonates accumulate within minutes in response to wounding or herbivore feeding (Chung et al., 2008; Glauser et al., 2008; Süza and Staswick, 2008; Koo and Howe, 2009; Koo et al., 2009) but the mechanism by which extracellular signals trigger JA biosynthesis remains unknown. Although JA biosynthetic genes are often induced by JA signaling, it has been observed that the enzymes required JA biosynthesis are present in unwounded tissue indicating that JA accumulation is likely regulated by substrate availability and that the induction of biosynthetic genes could be a positive feedback mechanism aimed to amplify the signal (Stenzel et al., 2003; Wasternack, 2007).

There is evidence that lipases, enzymes that catalyze the first step of JA biosynthesis by releasing linolenic acid from membrane galactolipids, could be regulated by environmental or developmental signals which could provide a mechanism for the induction of JA biosynthesis (Ito et al., 2007; Ishiguro et al., 2001). Interestingly, studies on Arabidopsis reproductive development has led to the identification of DAD1 (*DEFECTIVE IN ANther DEHISCENCE 1*), a developmentally responsive phospholipase A1 that influences JA production specifically in floral tissue (Ishiguro et al., 2001). *dad1* mutants display floral phenotypes indicative of JA-deficiency, including defects in anther elongation, pollen dehiscence, and floral opening. Interestingly, DAD1 is primarily expressed in the stamen over the course of floral development and therefore

JA-related phenotypes are restricted to the inflorescence, indicating that a separate lipase, or family of lipases, is responsible for the release of fatty acids in response to wounding (Ishiguro et al., 2001).

Downstream steps from linolenic acid have been well established over the years with elucidation aided by the identification of JA-deficient mutants (Creelman and Mullet, 2007; Mueller, 1997; Schaller, 2001). Within the plastid, a series of catalytic reactions facilitated by lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC) convert linolenic acid to the JA pre-cursor OPDA. OPDA is transported to the peroxisome where it undergoes several rounds of beta-oxidation to yield jasmonic acid. Jasmonic acid is released into the cytosol where a number of enzymes can catalyze various modifications to jasmonic acid, resulting in the production of various jasmonates (Wasternack et al., 2007; Koo et al., 2009; Koo et al., 2012). Importantly, JAR1 (JASMONATE INSENSITIVE 1) is a cytosolic enzyme that preferentially catalyzes the conjugation of jasmonic acid with isoleucine to generate JA-Ile (Staswick and Tiryaki, 2004; Suza and Staswick, 2008; Guranowski et al., 2007). Mutants of *JAR1* display a JA-insensitive phenotype and provided evidence for the important role of JA-Ile in JA signaling. Interestingly, despite reduced JA responsiveness, *jar1* plants are not completely JA insensitive and are still fertile indicating that other enzymes may be capable of compensating for the loss of JAR1 or that JAR1 is not required for all JA responses.

Elucidation of the jasmonate signaling pathway

Identification of *coronatine insensitive 1 (coi1)* was critical for the elucidation of the JA signaling pathway. Unlike *jar1*, *coi1* was found to be completely insensitive to JA and male sterile (Feys et al., 1994; Xie et al., 1998). Exogenous application of JA was unable to rescue the phenotype, indicating that *coi1* was not a JA-biosynthetic mutant, but rather a JA-perception mutant. Interestingly, *coi1* was not originally identified during mutagenic screens for JA insensitivity, but rather during a screen for resistance to the bacterial produced phytotoxin coronatine (Feys et al., 1994). Coronatine is a potent JA-mimic secreted by the hemibiotrophic plant pathogen *Pseudomonas syringae* during infection to dampen SA mediated defenses by activating the JA pathway (Katsir et al., 2008; Geng et al., 2012) allowing for identification of JA signaling components.

Sequence analysis of *COI1* indicated that the gene encoded an F-box protein hypothesized to be part of an E3 ubiquitin ligase complex (Xie et al., 1998). At the time another plant hormone, auxin, was found to exert transcriptional changes through ubiquitin-dependent turnover of transcriptional repressors. During auxin signaling, the F-box protein TIR1 was found to be part of a complex with the Arabidopsis SKP-CULLEN-F-box (SCF) E3 ubiquitin ligase, referred to as SCF^{TIR1}. In this signaling pathway, bioactive auxin acts as a ligand promoting interaction between the TIR1 subunit of SCF^{TIR1} and AUX/IAA transcriptional repressors to promote ubiquitin dependent degradation of AUX/IAA repressors (Maraschin et al., 2009). Depletion of AUX/IAA transcriptional repressors allows activation and transcription of auxin response genes. Indeed as predicted, COI1 was found to be analogous to TIR1 and was part of

the E3 ubiquitin ligase complex, SCF^{COI1} (Xu et al., 2002). Despite identification of a potential receptor for JA signaling the targets of SCF^{COI1} remained elusive.

Identification of the SCF^{COI1} targets in Arabidopsis relied on the identification of JA-responsive genes in the stamen of a JA biosynthetic mutant, *opr3* (Thines et al., 2007). Eight genes of unknown function were found to be rapidly induced upon JA treatment. These unknown JA-responsive genes were found to contain several conserved domains which would later identified as the NT, ZIM, and Jas domains (Chini et al., 2007). In addition, they were also found to be negative regulators of JA activated gene expression and found to be degraded in a COI1 dependent manner upon activation of JA signaling by coronatine treatment (Thines et al., 2007; Chini et al. 2007; Yan et al. 2007). This family came to be known as the Jasmonate ZIM domain (JAZ) transcriptional repressors and provided another piece of the JA signaling pathway. A follow-up database search led to the identification of 4 additional genes with sequence similarity, leading to a total of 12 JAZs identified in Arabidopsis. A limitation during the original identification of the JAZ family was the Affymetrix ATH1 gene chip which does not contain all known Arabidopsis genes allowing for the possibility of missed JAZs.

Coronatine was found to stimulate JAZ-COI1 interaction to promote turn-over of the JAZs (Katsir et al., 2008). Based on the JA insensitivity seen in *jar1* mutants and structural similarities between coronatine and JA-Ile, it was proposed that JA-Ile might serve as the endogenous bioactive JA. Due to configurational stability, it was generally assumed that (-)-JA-L-isoleucine was the bioactive hormone. However, the bioactivity of (-)-JA-Ile was surprisingly low when compared to coronatine (Fonseca et al., 2009). Laboratory preparations of (-)-JA-Ile were found to contain trace amounts of the (+)-7-iso-JA-L-Ile epimer. Upon purification of the

two compounds, it was found that (-)-JA-Ile was unable to promote interaction between COI1 and JAZs and the limited activity observed was actually due to the (+)-7-iso-JA-L-Ile contamination (Fonseca et al., 2009). Although (+)-7-iso-JA-L-Ile has now been identified as the true endogenous ligand, JA-Ile used in laboratories tend to be racemic mixtures due to the cost and unstable nature of pure (+)-7-iso-JA-L-Ile.

Current signaling model

It has been long observed that the induction of jasmonate signaling leads to a signaling cascade spurred by transcriptional reprogramming. In the absence of JA, Jasmonate ZIM domain transcriptional repressors (JAZs) interact with transcription factors to repress JA-related gene expression. Upon wounding, JA-Ile accumulates and promotes JAZ interaction with the COI1 F-box subunit of the E3 ubiquitin ligase complex SCF^{COI1}. Interaction of JAZ-COI1 leads to ubiquitination and degradation of JAZ proteins via the 26S proteasome pathway (Katsir et al., 2008; Sheard et al., 2010). Turnover of JAZ repressors relieves repression on JA-related transcription factors allowing JA responsive gene expression to occur (Figure 1).

Signal attenuation

JA accumulation is known to be transient and correlates well with expression of primary JA response genes (Chung et al., 2008; Koo et al., 2009). The transient nature of JA is likely a mechanism to prevent over activation of JA responses. One mechanism that has been proposed

to help attenuate JA signaling is activation of competing metabolic pathways that convert the precursor molecule jasmonic acid into inactive jasmonates (Miersch et al., 2008). An example is the conversion of jasmonic acid to MeJA, a jasmonate that can induce JA responses when applied to plants, but does not itself stimulate JAZ-COI1 interaction (Thines et al., 2007). Studies involving ectopic expression of an *Arabidopsis* JA carboxyl methyltransferase (JMT) in *Nicotiana attenuata* demonstrated that an increased conversion of JA to MeJA reduces JA-Ile formation and, as might be predicted, influences JA-Ile-mediated physiological processes (Stitz et al., 2011). Another proposed mechanism for signal attenuation is catabolism of biologically active JA-Ile. A major route for inactivating JA-Ile is cytochrome P450-mediated ω -oxidation of JA-Ile to produce dicarboxy-JA-Ile (12-COOH-JA-Ile) (Heitz et al., 2012; Koo et al., 2012).

JAZ transcriptional repressors

Families of JAZ repressors tend to be large (>10) and are found ubiquitously among land plants. The *Arabidopsis thaliana* genome hosts a 12 member JAZ family, named sequentially JAZ1-JAZ12. The JAZs belong to the larger plant specific TIFY family of transcriptional regulators. TIFY proteins are named after their highly conserved TIFY (TIF[F/Y]XG) motif at the N terminus of a larger 36 amino acid TIFY domain, previously known as the ZIM domain (Vanholme et al., 2007; Bai et al., 2011). The ZIM/TIFY domain is important for JAZ mediated repression as well as protein-protein interaction (Chung and Howe, 2009; Pauwels et al., 2010). In addition to the ZIM/TIFY domain, JAZ proteins also share a conserved Jas domain at the C terminus (Thines et al., 2007). The Jas domain is a rather distinguishing feature for JAZs and

contains the degron, the amino acid sequence necessary for the formation of the JAZ-COI1 co-receptor 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, Fernández-Calvo et al., 2011), and the regulation of JAZ nuclear localization (Grunewald et al., 2009, Withers et al., 2012).

JAZ stability

Among the targets of JAZ repressors are the JAZ genes themselves, allowing JAZ gene expression to be rapidly induced upon JA-elicitation with transcript accumulation occurring in as little as 15 minutes (Thines et al., 2007; Chung et al., 2008). This is generally regarded as a mechanism for signal attenuation that allows the system to return to a state of repression upon activation (Chung et al., 2010; Shyu et al., 2012; Moreno et al., 2013). However, this mechanism alone does not fully explain how repression is restored post activation as newly synthesized JAZs would be rapidly turned over as long as bioactive hormone is present generating a futile cycle rather than restoring repression.

Interestingly, many JAZ genes produce splice variants that contain a truncation, deletion, or frame shift in the Jas domain allowing the production of JAZ repressors that do not interact with COI1 in the presence of the bioactive hormone allowing them to resist degradation (Chung et al., 2010). Furthermore, alternative splicing events that result in a modified Jas domain or degron sequence are conserved throughout the plant kingdom,

supporting that these isoforms are functional components of JA signaling (Chung et al., 2010). Upon JA-activation, alternative splice variants are induced along with their full length counterparts and have been suggested to offer a means for signal attenuation (Chung 2010; Moreno et al., 2013). Although unstable JAZs would be degraded in the presence of JA-Ile, stable JAZs may be allowed to accumulate over the course of JA-activation and help return the system to a state of repression. A well-studied example is JAZ10, which produces JAZ10.1, JAZ10.2/3, and JAZ10.4 isoforms. JAZ10.2/3 and JAZ10.4 are stable isoforms and confer JA-insensitivity when over-expressed in Arabidopsis, demonstrating an ability to act as stable repressors of JA responses (Chung et al., 2010). In addition, the *jaz10-1* mutant is one of the few JAZs that display a JA-hypersensitive phenotype. It has been suggested that *jaz10-1* is hypersensitive to JA treatment because the stable JAZ10 isoforms are absent allowing the plant to over react to JA stimulation (Chung et al., 2010; Moreno et al., 2013; Demianski et al., 2011).

While most JAZs share a well conserved degron sequence, JAZ7 and JAZ8 were found to contain non-canonical degron sequences suggesting that these two JAZs might display altered COI1 affinity. Indeed, JAZ8 has reduced affinity for COI1 in the presence of JA-Ile, but surprisingly retains interaction with COI1 in the presence of coronatine (Shyu et al., 2012). Reduced affinity with COI1 in the presence of JA-Ile suggests that JAZ8 could behave as a stable repressor of JA responses, similar to what has been observed with JAZ10.2/3 and JAZ10.4. Over expression of JAZ8 in Arabidopsis results in moderate insensitivity to MeJA treatment and increased sensitivity to insect feeding, supporting the conclusion that JAZ8 acts as a stable repressor of JA responses even in the presence of endogenous JAs. Interestingly, JAZ8-like genes with degenerate degrons can be found throughout the plant kingdom with homologs in

nearly all available eudicot species indicating that JAZ8 is a highly conserved component of the JA signaling pathway (Shyu et al., 2012). It could be hypothesized that JAZ8, similar to JAZ10.2/3 and JAZ10.4, functions as a stable repressor of JA responses to prevent over activation of JA signaling. In this case, interaction with COI1 in the presence of a phytotoxin may simply be a demonstration of the potency of coronatine. Alternatively, it is also possible that JAZ8 responds to a different endogenous ligand, one which is not produced in response to insect feeding, but currently there is little evidence in support of this.

JAZ mediated repression

Little is known on the mechanism by which the JAZs repress gene expression. Protein-protein interaction studies have provided some insight to JAZ mediated repression by identifying NOVEL INTERACTOR OF JAZ (NINJA) and Groucho/Tup1-type corepressor TOPLESS (TPL) as JAZ interactors (Pauwels et al., 2010). In the original model for JAZ mediated repression, it was suggested that NINJA was an adaptor protein facilitating interaction with TPL. In this model, the TIFY/ZIM domain of the JAZ allows interaction with NINJA while the presence of an EAR motif in NINJA allows recruitment of TPL (Pauwels et al., 2010). Recently, an alternative model for repression was formulated as four JAZs were found to contain endogenous EAR motifs. A closer look at JAZ8 co-repressor interactions revealed that, unlike the other JAZs, JAZ8 does not interact with NINJA and instead directly interacted with TPL through an N-terminal EAR motif. Furthermore, deletion of the EAR motif was found to abolish

the JA-insensitive phenotype found in transgenic plants overexpressing JAZ8 indicating that the EAR motif is required for JAZ8 mediated repression (Shyu et al., 2012).

Consistent with the idea that NINJA and TPL are important components for JAZ repression, Loss-of-function mutants of NINJA and TPL are hypersensitive to JA-induced root growth inhibition (Pauwels et al., 2010; Acosta et al., 2013). These results collectively suggest that JAZ proteins complex with co-repressors NINJA and/or TOPLESS in order to repress jasmonate responses (Shyu et al. 2012, Pauwels 2010).

RESULTS

A new member of the JAZ family

JAZ8 protein sequence was queried against NCBI's protein database using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHom). *At3G22275*, a gene of unknown function, was found within the top ten matches along with a number of known JAZs. A reciprocal blast search of *At3G22275* predicted protein product produced *JAZ8* as the top match following *JAZ7* and several other JAZ members. Although *At3G22275* overall does not share much sequence similarity to most of the JAZ family, A MUSCLE based amino acid sequence alignment between *At3G22275* and *JAZ7* and *JAZ8* revealed two conserved regions (Figure 2). *At3G22275* contained a discernable ZIM-like domain, a Jas-like domain, and interestingly, an EAR motif on the N-terminus similar to that found in *JAZ7* and *JAZ8*. Notably, unlike any other JAZ, *At3G22275* contains a "NAFY" sequence where the highly conserved "TIFY" motif typically resides. Perhaps the most unusual feature of *At3G22275* is the C-terminal tail which contains a serine rich region indicating a potential phosphorylation site. Phosphorylation data available from PhosPhAt4.0 (<http://phosphat.uni-hohenheim.de/>) was queried to determine whether there was any experimental evidence of phosphorylation of *At3G22275*. Indeed, 15 residues consisting mostly of serine were experimentally shown to be phosphorylated, but this data was neither confirmed or further investigated in this work (Heazlewood et al., 2008, Durek et al., 2010).

The similarities observed between At3G22275 and JAZ8 prompted a phylogenetic analysis of all the known TIFY proteins and At3G22275 to resolve the relationship between At3G22275 and the JAZs. The JAZs and other TIFY proteins clustered similar to what has been previously published (Browse, 2009; Cuéllar Pérez et al., 2014) (Figure 3). At3G22275 clustered reproducibly with the JAZ8 sub-family and did not cluster with the other TIFY proteins or with the out group.

Conservation of At3G22275 among plant species was investigated by searching all available plant genomes on Phytozome V9.1 (<http://www.phytozome.net/>). The predicted protein product of At3G22275 was used to query the 41 available Viridiplantae genomes. From the results, “At3G22275-like” genes were identified by the presence of an N terminal EAR motif, a “NAFY” sequence rather than “TIFY”, a non-canonical Jas domain, and a serine rich C-terminal region. Similarly, “JAZ7/8-like” genes were identified by querying the protein sequence of JAZ8 and identifying hits that contained an N terminal EAR motif, a ZIM domain, and degenerate degron similar to that of JAZ8. At3G22275-like genes were found among the available Brassicaceae genomes; *Arabidopsis thaliana*, *Arabidopsis lyrata*, *Thellungiella halophila*, *Brassica rapa*, *Brassica oleraceae*, *Capsella rubella*, *Capsella grandiflora*, and *Boechera stricta* but were not found in any other available species (Figure 4). In contrast, JAZ7/8-like genes were more widespread throughout the plant kingdom and could be found in almost all eudicots and several grasses (Figure 4). An alignment of “At3G22275-like” protein sequences revealed a high degree of conservation among the 8 species (Figure 5).

At3G22275 expression is JA inducible

Transcriptional activation in response to wounding is a well-known characteristic of JA-signaling components (Reymond et al., 2000; Koo et al., 2009; Herde et al., 2013). As a first step to determine whether *At3G22275* could be involved with JA-signaling, gene expression in response to JA treatment was investigated. A recently published RNA-seq data set following transcriptional response of seedlings treated with the powerful JA agonist coronatine (COR) over time (Attaran et al., 2014) allowed for an in depth look at *At3G22275* expression and allowed comparison with the close relatives *JAZ7* and *JAZ8*. *At3G22275* displays strong gene induction in response to COR treatment supporting that transcription is JA inducible (Figure 6A). *At3G22275* expression seems to mimic most closely that of *JAZ7* which appear to be experience a spike in activation followed by strong repression (Figure 6A,C). In contrast, *JAZ8* appears to be rapidly induced, but not as rapidly repressed as *JAZ7* and *At3G22275* (Figure 6B). Collectively, the expression of *At3G22275* is JA inducible with an expression pattern similar to that of other JAZ repressors.

At3G22275 interacts with key JA-signaling components

To further investigate the possibility of *At3G22275* functioning as a repressor of JA responses, we investigated possible domain dependent interactions with MYC2, TPL, and NINJA. Previous yeast two hybrid screens have shown that *JAZ8* interacts with MYC2 in a Jas domain dependent manner and interacts with TPL in an EAR dependent manner (Shyu et al.,

2012). Full length At3G22275 as well as two derivatives, one with a deleted Jas domain (At3G22275 Δ Jas) and one with a deleted EAR motif (At3G22275 Δ EAR), were tested for interaction with MYC2, TPL, and NINJA. In addition, JAZ8, JAZ8 Δ Jas, JAZ8 Δ EAR, and JAZ10 were included for comparison purposes. As previously reported, JAZ8 interacts with MYC2 in a Jas dependent manner and TPL in an EAR dependent manner (Shyu et al., 2012). Similar to JAZ8, At3G22275 was also found to interact with MYC2 in a Jas dependent manner and TPL in an EAR dependent manner (Figure 7). In contrast to JAZ10, neither JAZ8 nor At3G22275 interacts with NINJA (Figure 7). Collectively, these results demonstrate that the region identified as the Jas-like domain of At3G22275 is likely a true Jas domain and, similar to JAZ8, At3G22275-mediated repression is likely NINJA-independent due to direct TPL interaction.

Taken together, the presence of distinct conserved domains, JA inducible gene transcription, and protein interactions with MYC2 and TPL thus far support the identification of At3G22275 as a previously undescribed JAZ repressor. Throughout the remainder of this work At3G22275 will be referred to as “JAZ13”.

Coronatine treatment induces degradation of JAZ13

The degron sequence within the Jas domain is a critical site for COI1 interaction. JAZ8 contains a degenerate degron which reduces COI1 interaction in the presence of JA-Ile, allowing JAZ8 to act as a stable repressor during MeJA treatment or wounding. Although JAZ8 resists degradation in the presence of endogenous JAs, the powerful JA agonist coronatine is capable of inducing interaction between JAZ8-COI1 to induce JAZ8 degradation. JAZ13 contains a

degron sequence similar to that of JAZ8 which may indicate similar interactions with COI1. Therefore, coronatine treatment was used to investigate ligand dependent degradation of JAZ13 and protein stability. Transgenic plants over-expressing HA-epitope tagged JAZ13 and JAZ8 were treated with coronatine and protein degradation was monitored by western blot. Similar to JAZ8, the signal for JAZ13 was found to be diminished after one hour of coronatine treatment (Figure 8) indicating the protein is destabilized in the presence of coronatine. Although this does not provide direct evidence of COI1 interaction, the current understanding of JA signaling and ligand dependent turn-over of JAZ repressors would support JAZ13 degradation to be mediated by COI1.

Over expression of JAZ13 reduces JA sensitivity in Arabidopsis

To assess JAZ13's ability to act as a stable repressor of JA responses, *35S::JAZ13-HA* was overexpressed in *Arabidopsis thaliana*. 10 independent *35S::JAZ13-HA* (*JAZ13-OE*) transgenic Arabidopsis lines were isolated and surveyed for JA-sensitivity. No obvious growth or developmental phenotypes were observed in the absence of a stress, but when the plants were grown on MeJA containing media they were found to have reduced JA-responsiveness in the roots (Fig. 9A-B). Line #26-3 was selected for further analysis because it displayed the most striking phenotype and was found to have the highest accumulation of JAZ13-HA as determined by western blot (Figure 9C). To determine whether JA-insensitivity seen in the roots could also be observed in the rosette tissue, an insect feeding assay was performed. *Spodoptera exigua* larvae were allowed to feed on 9 week old plants for 8 days. Larvae harvested from *JAZ13-OE*

plants were found to be nearly double in size as compared to those reared on WT Col-0 (fig 10A,C). In addition anthocyanin accumulation, a compound which can be seen as a purple pigmentation on the petioles, was greatly suppressed on *JAZ13-OE* as compared to the wild type control (fig 10B,D-E). Collectively, these results indicate a suppression of JA-mediated defense responses in plants over-expressing *JAZ13*. To further investigate the potential role of *JAZ13* as a transcriptional repressor, quantitative-PCR (qPCR) was used to investigate JA-responsive gene activation after wounding of mature rosette leaves. Transcript abundance for three primary response genes, *MYC2*, *LOX3*, and *AOS*, were monitored relative to housekeeping gene *PP2A* (Fig 11C-E). While WT Col-0 plants exhibit a typical response to wounding, *JAZ13-OE* displays markedly reduced expression of these three primary response genes, indicating that the JA insensitivity observed in the *JAZ13-OE* is likely a result of dampened JA induced gene expression.

Identification of JAZ7, JAZ8, JAZ10, and JAZ13 null mutants

To further investigate the function of *JAZ13*, a T-DNA insertion null mutant *jaz13* (*jaz13-GK*, *GK_193G07*) was identified and assessed for JA-sensitivity using a root growth assay. *jaz13* did not appear to display MeJA hypersensitivity in the roots as compared to WT Col-0. This result was unsurprising as most JAZs do not display a phenotype when knocked-out, presumably due to functional redundancy or compensation in expression by remaining JAZs. In an attempt to overcome redundancy, we sought to identify null mutants within the subfamily of *JAZ13*. In addition, *jaz10* (*jaz10-1*), was also included in our genetic analysis to determine

whether any of the JAZ8 subfamily members could enhance the MeJA hypersensitivity previously reported for *jaz10* (Moreno et al., 2013). *JAZ7*, *JAZ8*, *JAZ10*, and *JAZ13* reside on separate chromosomes which would allow for a classic genetic approach for the generation of higher order mutants (figure 14A). *jaz10-1* and *jaz7-1*, referred to here as *jaz10* and *jaz7*, respectively, were previously identified and characterized (Sehr et al. 2010; Demainski et al., 2010; Moreno et al., 2013). Although *jaz10* was reported to display JA hypersensitivity, *jaz7* displayed similar JA responsiveness as WT.

While attempting to identify a suitable T-DNA insertion mutant for JAZ8, a mutant with an insertion within the gene body was not found. Instead, we directed our focus to natural variation and used genomic data made available by the 1001 Arabidopsis genomes project (<http://signal.salk.edu/atg1001/>) to attempt to identify a natural variant null allele. To our surprise, a JAZ8 allele containing a non-sense mutation immediately following the TIFY motif was identified in the Vash-1 ecotype of Arabidopsis. Previous work has demonstrated that JAZ8's Jas domain is required for MYC2 interaction and that deletion of the Jas domain eliminates JAZ8's ability to act as a stable repressor of JA responses (Shyu et al., 2012). The truncated protein product of JAZ8 from the Vash-1 ecotype would lack a Jas domain, indicating that the protein product is likely unable to act as a repressor.

The JAZ8 Vash-1 allele, referred to here as *jaz8-V* (*jaz8-Vashlovani*), was confirmed through Sanger sequencing of the gene and introduced into Col-0 background through a process of marker assisted introgression. JA sensitivity of introgressed *jaz8-V* was investigated by MeJA induced root growth inhibition and, similar to *jaz7* and *jaz13*, was found to have similar JA sensitivity as WT (figure 15).

jaz13/jaz10 double mutants display enhanced JA-sensitivity

The *jaz10* mutant has previously been reported to display enhanced root sensitivity to MeJA treatment. Although the *jaz13* single mutant did not display MeJA hypersensitivity, when combined with *jaz10* in the *jaz10/13* double mutant root sensitivity to MeJA was enhanced as compared to the *jaz10* single mutant (Figure 12). Interestingly, neither the addition of *jaz7* or *jaz8* further enhanced the root phenotype seen in *jaz10*, or *jaz10/13*. Furthermore, the *jaz7/8/10/13* quadruple mutant was also not found to enhance the root phenotype seen in the *jaz10/13* mutant, indicating that JAZ10 and JAZ13 may play a more significant role in JA-mediated root growth inhibition as compared to JAZ7 and JAZ8.

DISCUSSION

At3G22275 is a member of the JAZ family of transcriptional repressors

Initial identification of JAZ repressors in Arabidopsis relied on profiling of JA-responsive genes in the stamen of JA-treated *opr3* mutants (Thines et al., 2007). Eight rapidly induced genes of unknown function encoded proteins that contained several conserved domains identified as the NT, ZIM, and Jas. A follow-up database search led to the identification of 4 additional family members, leading to a total of 12 JAZs in Arabidopsis. During the original identification of the JAZ family the Affymetrix ATH1 gene chip for microarray based studies did not contain *At3G22275*, thus impeding the identification of the gene as a JAZ family member. Similar to the original identification of the JAZs, a sequence based search uncovered *JAZ13* (*At3G22275*), a protein of unknown function with sequence similarity to *JAZ8*. The expression of this unknown gene was found to be strongly induced upon coronatine treatment indicating that gene expression is JA responsive, a common characteristic of JA metabolic and signaling genes. Furthermore, a recent study probing the Arabidopsis genome for JA inducible genes targeted by JAM1 and MYC2, negative and positive transcriptional regulators of JA-signaling, respectively, identified *At3G22275* (*JAZ13*) to be highly responsive to JA treatment and directly targeted by both MYC2 and JAM1 along with many other JAZs and known JA responsive genes (Nakata et al., 2013). This finding not only supports JA responsive transcriptional activation, but also demonstrates direct targeting of *JAZ13* by JA-signaling components.

The expression profile of *JAZ13* seems to mimic most closely that of *JAZ7* which exhibits a spike in activation followed by a reduction in transcript accumulation. The sharp peak in expression of *JAZ13* and *JAZ7* could represent a rapid mechanism of repression following activation. It may also be an indication of differences in transcript stability of JA responsive genes. Since the discovery of mRNA, the importance of mRNA stability has been implicated as a possible mechanism to regulate gene expression (Jacob and Monod, 1961). In plants, transcript degradation is an important regulatory mechanism for certain physiological processes (Lidder et al., 2005) and there is evidence that cold stress can influence the stability and half-life of mRNAs (Chiba et al., 2013). Although the impact of transcript stability during herbivore stress or JA signaling has yet to be investigated, in a system that relies on transcriptional changes to induce specific responses, transcript stability could offer an additional level of regulation.

In addition to sequence and expression based evidence, protein interactions of *JAZ13* also support the identification as a JAZ family member. *JAZ13* interacts with key JA signaling component such as MYC2 and TPL in a domain dependent manner similarly to what is observed with *JAZ8*. Notably, the EAR motif of *JAZ13* was found to facilitate direct interaction with TPL, indicating a NINJA-independent mechanism of repression.

Phenotypically, over-expression of *JAZ13* supports involvement in the JA signaling pathway. *JAZ13-OE* transgenic *Arabidopsis* plant exhibit decreased JA-sensitivity during insect feeding as well as exogenous MeJA treatment. Interestingly, while the observed root phenotype was modest, the biological phenotype from insect feeding was rather striking. *JAZ13-OE* plants were not only more susceptible to *S. exigua* feeding, but appeared to suppress anthocyanin accumulation in the petiole as well. The JAZs have been implicated as important

regulatory components for secondary metabolites such as glucosinolates and anthocyanin. Interestingly, several dominant alleles of bHLH transcription factors have been described to hyper accumulate glucosinolates or anthocyanins (Frerigmann and Gigolashvili, 2014; Pattanaik et al., 2008). The causative mutation appears to abolish JAZ interaction (Frerigmann and Gigolashvili, 2014) allowing the transcription factors to act independently of JA. While independence from JAZ repression leads to an increase in secondary metabolite production, an over-expressed JAZ repressor might be expected to suppress the production of secondary metabolites, as is seen here with JAZ13.

To further probe the involvement of JAZ13 in the JA signaling pathway, induction of primary JA-response genes was monitored in *JAZ13-OE* post wounding to determine whether the observed JA-insensitivity could be explained by a dampening of JA-induced transcriptional responses. The JA-responsive genes *MYC2*, *LOX3*, and *AOS* all showed markedly reduced expression post wounding in *JAZ13-OE* as compared to Col-0. This result suggests that the JA insensitivity observed in *JAZ13-OE* is likely the result of dampened transcriptional activation and is consistent with a role for JAZ13 as a transcriptional repressor of JA responses.

Although most JAZs do not display a phenotype when overexpressed, over-expression of JAZ8 was reported to confer JA-insensitivity during root growth inhibition assays as well as insect feeding, a phenotype attributed to JAZ8's decreased affinity for COI1. Given the over-expression phenotype of *JAZ13* and sequence similarity between the degron of *JAZ13* and *JAZ8*, it is likely that JAZ13 also has reduced affinity for COI1 in the presence of bioactive JA and is able to act as a stable repressor of JA-responses when over-expressed. It is worth noting that *JAZ13* expression is relatively low when compared to *JAZ8* and *JAZ9* over-expression lines

suggesting that even moderate over-expression of *JAZ13* is capable of producing a JA-insensitive phenotype.

The lack of a phenotype in single *JAZ* mutants has hindered the identification of specific roles for the JAZs, leading to the assumption that they are functionally redundant. Our higher order genetic analysis was aimed at overcoming functional redundancy by generating higher order *JAZ* mutants with *JAZ13* and its two close paralogs *JAZ7* and *JAZ8*. Interestingly, our analysis reveals that *Arabidopsis thaliana* appears to tolerate the loss of multiple *JAZ* repressors with little consequence. Certain *JAZ* null mutants display JA hypersensitivity, such as *jaz10*, and here we report the *jaz10/jaz13* double mutant is further enhanced in JA sensitivity, but remarkably JA sensitivity is not further increased in triple or quadruple mutants containing *jaz7* and/or *jaz8*. Functional loss of *jaz7* or *jaz8* could be compensation by remaining JAZs, or it may indicate that certain *JAZ* members are more important to specific JA phenotypes than others. These results warrant an investigation of other JA regulated responses such as insect feeding, pathogen infection, or drought tolerance with higher order mutants to resolve the individual contribution of JAZs to a variety of stress responses to provide a better understanding of how response specificity is achieved.

JAZ13 is specific to the Brassicaceae family

The unique features of *JAZ13*, such as the NAFY motif and serine rich region, led to questions of potential biological function and conservation throughout the plant kingdom. A search for *JAZ13*-like genes was carried out by querying the predicted *A. thaliana* *JAZ13* product

in all available genomes on Phytozome v9.1 (<http://www.phytozome.net/>). In striking contrast with *JAZ8*, which has been previously reported to be found in most land plants (Shyu et al., 2012), *JAZ13*-like genes were only found in Brassicaceae, a family that includes a number of cultivated vegetable crops as well as *Arabidopsis thaliana*.

Brassicaceae members almost exclusively rely on the production of resource intensive chemical defense compounds known as glucosinolates for defenses against herbivores (Fahey et al., 2001; Züst et al., 2012). Glucosinolates are energetically costly and have been predicted to increase photosynthetic demands by at least 15% (Bekaert et al., 2012). A potential benefit of acquiring an extra JAZ repressor could its origin in the growth versus defense dilemma of resource partitioning. An additional stable JAZ could help exert tighter repression on JA responses to prevent defense activation unless absolutely necessary. Interestingly, the accumulation of chemical defense compounds, including glucosinolates, has been correlated with a reduction in reproductive fitness (Berenbaum et al., 1986; Mitchell-Olds et al. 1996; Stowe 1998; Strauss et al., 1999). Although the ecological importance of this correlation has yet to be determined, it is a strong indication that overly defended plants have reduced fitness.

JAZ natural variation

Plant species have been found to display genetic variation across geographic locations (Linhart and Grant, 1996). Reciprocal transplant experiments have shown that local adaptation often allows plants that contain native genetic variation to outperform those containing foreign genetic variation (Fournier-level et al., 2011; Leimu et al., 2008). Most work investigating

geographic genetic variation has focused on abiotic factors, such as climate and soil (Hancock et al., 2011), but genes involved with defense have been observed to contain remarkably high levels of polymorphisms (Bergelson et al., 2003; Clark et al., 2007) and it has been shown that herbivore feeding is also capable of driving geographic genetic patterns (Züst et al., 2012). Defense variation often highlights the growth versus defense dilemma in plants. As might be predicted, in the absence of herbivores growth provides a strong competitive edge and genetic variation favoring low defense and fast growth will outcompete heavily defended, slow growing plants. However, in the presence of herbivores, the slow growing yet more defended plants have the competitive edge and may then outcompete faster growing plants due to increased resistance to insect feeding (Züst et al., 2012).

The discovery of the *JAZ8* allele in the Vash-1 ecotype suggests that *JAZ8* is dispensable, or it may be a case of local adaptation. The Vash-1 ecotype was collected on a shrubby river bank in a semi-arid region of Vashlovani Nature Reserve in Kakheti, Georgia. Although we report that *JAZ8* Vash-1 allele (*jaz8-V*) does not appear to have any developmental phenotypes or enhanced JA-sensitivity when introgressed into Col-0 background, it is possible that *jaz8-V* has a phenotype under conditions we have not yet investigated. In this case, the geographic location could favor genetic variation that results in a more highly defended plant against an unknown stress. Indeed, jasmonate responses implicated in adaptation to semi-arid environments such as UV radiation and drought stress (Conconi et al., 1996, Seo et al., 2011). Future investigation of abiotic stress may be an interesting area of study with this allele.

MATERIALS AND METHODS

Plant materials

Arabidopsis thaliana ecotype Colombia-0 (Col-0) was used as wild type for all experiments. Long day growth conditions consisted of 16h day 8h night cycle at 20°C while short day growth conditions consisted of 8h day 16h night cycle at 20°C. Experiments with constant light conditions were maintained at 19°C. All mutant and transgenic lines reported are in Col-0 background, or in the case of *jaz8-V* introgressed into Col-0 background. T-DNA insertion GK_193G07 (*jaz13-GK*) and Vash-1 seed was obtained from the Arabidopsis Biological Resource Center (ABRC) at the Ohio State University. *jaz7-1* and *jaz10-1* were also obtained from the ABRC and previously confirmed (Moreno et al., 2013; Sehr et al., 2010). 35S::JAZ9-HA and 35S::JAZ8-HA were obtained from Dr. Sheng Yang He at Michigan State University (Withers et al., 2012).

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 et al., 2004) in software MEGA5. Evolutionary history was inferred using the Maximum likelihood method (MLM) based on the JTT matrix-based model using MEGA5 software (Saitou and Nei, 1987).

Molecular cloning

JAZ13 was cloned from a PCR reaction with JAZ13 sequence-specific primers (JAZ13_pENTR_FP and JAZ13_XhoI_RP) using cDNA synthesized from wild type wounded mature leaf RNA as template. PCR reactions were performed using Pfu Turbo DNA polymerase (Stratagene). JAZ13 was cloned and sequenced in pENTR-TOPO vector (Invitrogen) and further cloned into pGILDA and pB42AD vectors for yeast two-hybrid assays using the Gateway system (Invitrogen). JAZ7 and JAZ8 were similarly cloned into pGEMT-Easy (Promega) for sequencing and subcloned into pGILDA and pB42AD as previously described (Shyu et al., 2012). A complete list of primers used in this study is provided in Table 1.

Yeast two-hybrid assay

Yeast two-hybrid assays were performed with the Matchmaker LexA system (Clontech). Bait and prey vectors were co-transformed 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 determining the presence of blue pigmentation (β -galactosidase) on SD-galactose plates with proper amino acid supplements. After 48 hours of incubation at 30°C cultures were transferred to 4°C. Photographic images were taken after 24 hours at 4°C.

Generation of transgenic lines

Transgenic *JAZ13* lines were made in the pEARLYGATE-HA vector. 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 μ M/mL). T2 seed was sown on MeJA containing media, the 11 most resistant plants were transplanted. 8 progeny from each independent line were transplanted and propagated for identification of homozygous T3 lines and further MeJA root growth inhibition analysis. Of these 10 independent lines, *JAZ13* expression was confirmed by α -HA western blot in the four independent lines most resistant to MeJA treatment (root growth data on these four lines shown). See table 1 for primer details.

Plant treatments

Seedlings were plated on ½ MS agar medium containing sucrose (0.8%) with or without 25 μ M MeJA for 8 days under continuous light, unless otherwise indicated. Primary root lengths were measured using Image J software (<http://rsbweb.nih.gov/ij/index.html>). Each genotype was tested at a minimum of three times with similar results.

Insect feeding assay was performed with 9 week-old Col-0 wild-type and *35S:JAZ13-HA* transgenic plants. *Spodoptera exigua* eggs were obtained from Benzon Research and hatched at 30°C. Four newly hatched larvae were transferred to fully expanded rosette leaves on each of 15 plants per genotype. Insect challenged and unchallenged control plants were maintained

under short day growth conditions. Three independent experiments were performed and produced similar results.

Wounding experiments were performed with 6 week old plants grown under short day growth conditions. Fully expanded mature rosette leaves were wounded twice across the midvein with a hemostat. Tissue from wounded and unwounded plants was collected 1 hour after wounding. One biological replicate consisted of 6 leaves pooled from 3 different plants. RNA was extracted using an RNeasy Kit (Qiagen) as per manufacturer's instruction. cDNA was reverse transcribed from 100 ng of total RNA with random primers using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Resulting cDNA was diluted to 0.5 ng/ μ L with RNase-free water. qPCR was performed on an ABI 7500 Fast qPCR instrument (Applied Biosystems) on Fast Optical 96-well plates (Applied Biosystems) using Power SYBR Green (Applied Biosystems). Standard reactions were run with the following conditions: 50°C for 2 min, 95°C for 10 min, then 40 cycles of 15 s at 95°C for denaturation and 60 s at 60°C for annealing and polymerization. C^T values of the three technical replicates were averaged to produce a value for each biological replicate. *Protein Phosphatase 2A (PP2A)* previously reported to have stable expression in Arabidopsis (Vandesompele et al., 2002) was used as a reference gene. Data was normalized to *PP2A* expression by $\log_2$ -transformation of the difference in expression (C^T) of the gene of interest and *PP2A*. Graphs display the average of 3 biological replicates. Student's T-test was used to determine statistical differences between Col-0 and *JAZ13-OE*. This experiment was independently repeated twice with similar results.

Degradation assay

35S::JAZ8-HA, 35S::JAZ9-HA (Withers et al., 2012), and 35S::JAZ13-HA were liquid grown in ½ MS media under long day conditions for 12 days. Plants were treated with 25 µM MeJA, 10 µM coronatine, or an 80% MeOH mock treatment. Tissue was harvested 1 hour post treatment and immediately frozen in liquid N₂. Tissue was ground with liquid N₂ and a plant protein extraction buffer (Chung et al., 2010). Lysate was cleared by centrifugation at 14,000 RPM in 4°C. Total protein was quantified using Bradfords reagent (BioRad). 15µg total protein was loaded for JAZ8-HA and JAZ9-HA samples while 50-150µg total protein was loaded for JAZ13-HA samples. Western blot was performed using a monoclonal mouse α-HA (Cell Signaling) 1:1,000 and secondary goat α-MOUSE (Cell Signaling) 1:13,000.

Generation of higher order JAZ mutants

jaz8-Vash was first introgressed into Col-0 background before generating higher order mutants. The C to A mutation in *jaz8-V* generates a CAPS (cleaved amplified polymorphic sequence) marker allowing for the differentiation of *jaz8-V* from WT through AflIII digest of full length *JAZ8* PCR product. After each backcross to Col-0, approximately 100 offspring were screened for *jaz8-V* and several SSLP (simple sequence length polymorphism) markers differentiating Col-0 and Vash-1 regions through-out the genome (figure 14B) in order to determine recombinants retaining *jaz8-V* with the highest Col-0 background. After 4 backcrosses, all SSLP markers screened as Col-0. A total of 5 backcrosses were performed.

After Col-0 introgression, *jaz8* was confirmed to contain the nonsense mutation by PCR of cDNA from wounded tissue followed by AflIII digestion (figure 13). Higher order mutants were

generated through classic genetic crosses between *jaz7*, *jaz10*, *jaz13*, and *jaz8* and mutants were identified through allele specific PCR screening. Higher order mutants were generating using classic genetic crosses beginning with *jaz7-1*, *jaz10-1*, *jaz8-V*, and *jaz13-GK*. Genotypes were confirmed by PCR with gene and T-DNA specific primers, or by CAPS marker for *jaz8-V*. cDNA from wounded *jaz8/10/13* mutants was extracted using RNeasy kit (Qiagen) and cDNA from synthesized and used as a template for allele specific PCR to confirm the presence or absence of full length transcripts for *jaz8-V* and *jaz13-1*. See table 1 for primer details.

Accession numbers

Arabidopsis Genome Initiative numbers described in this work: 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), TPL (AT1G15750), PP2A (AT1G69960), LOX3 (AT1G17420), MYC2 (AT1G17420), AOS (AT5G42650), ZIM (AT5G42650), ZML1 (AT3G21175), ZML2 (AT1G51600), PPD1 (AT4G14713), PPD2 (AT4G14720), and TIFY8 (AT4G32570)

APPENDIX

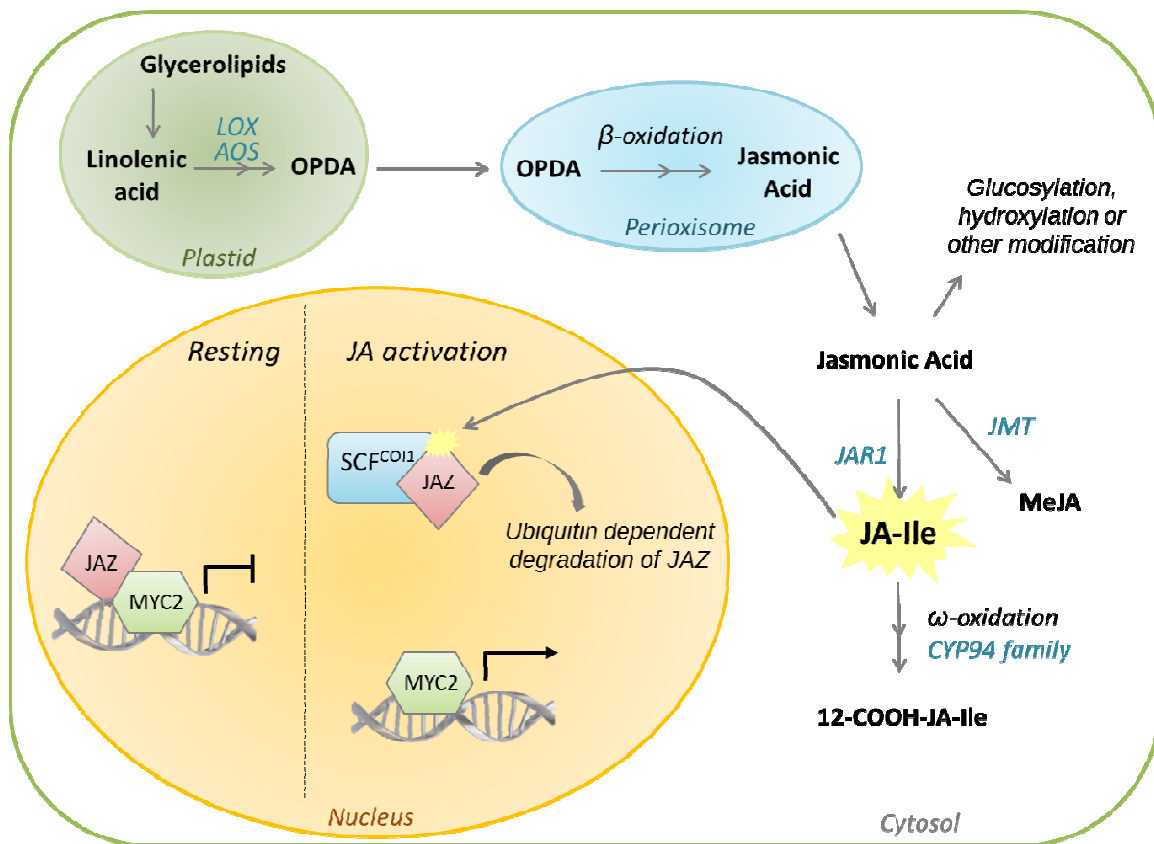


Figure 1. Basic representation of JA metabolism and signaling pathway. Biosynthesis begins in the plastid (often the chloroplast) where glycerolipids are cleaved to release linolenic acid. Linolenic acid is converted to OPDA through a series of enzymatic reactions. OPDA is then transported to the peroxisome where it undergoes several rounds of β -oxidation, producing jasmonic acid. Many modifications may occur to jasmonic acid in the cytosol to produce a variety of jasmonates. Importantly, conjugation with isoleucine produces the predominantly bioactive jasmonate, JA-Ile. Jasmonate metabolism is induced upon wounding. As JA-Ile accumulates, it can induce transcriptional reprogramming by promoting JAZ-SCF^{COI1} interaction in the nucleus leading to ubiquitin dependent degradation of JAZ repressors through the 26S proteasome pathway. Degradation of JAZs allows transcription of JA response genes by removing repression of JA related transcription factors, such as MYC2. Enzymes mentioned in this thesis are in blue italic font.

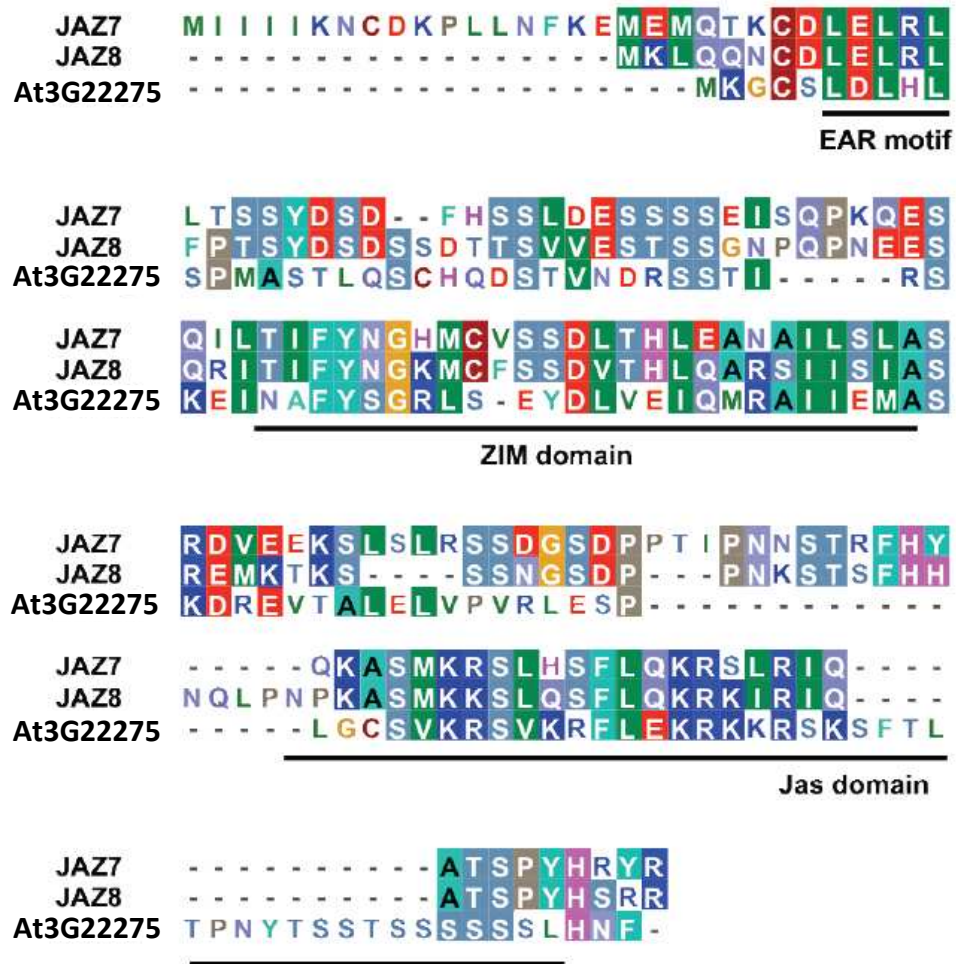


Figure 2. At3G22275 shares sequence similarities with JAZ7 and JAZ8. Amino acid based sequence alignment of JAZ7, JAZ8, and At3G22275 using default parameters with MUSCLE sequence alignment tool in MEGA5 software (<http://www.megasoftware.net/>).

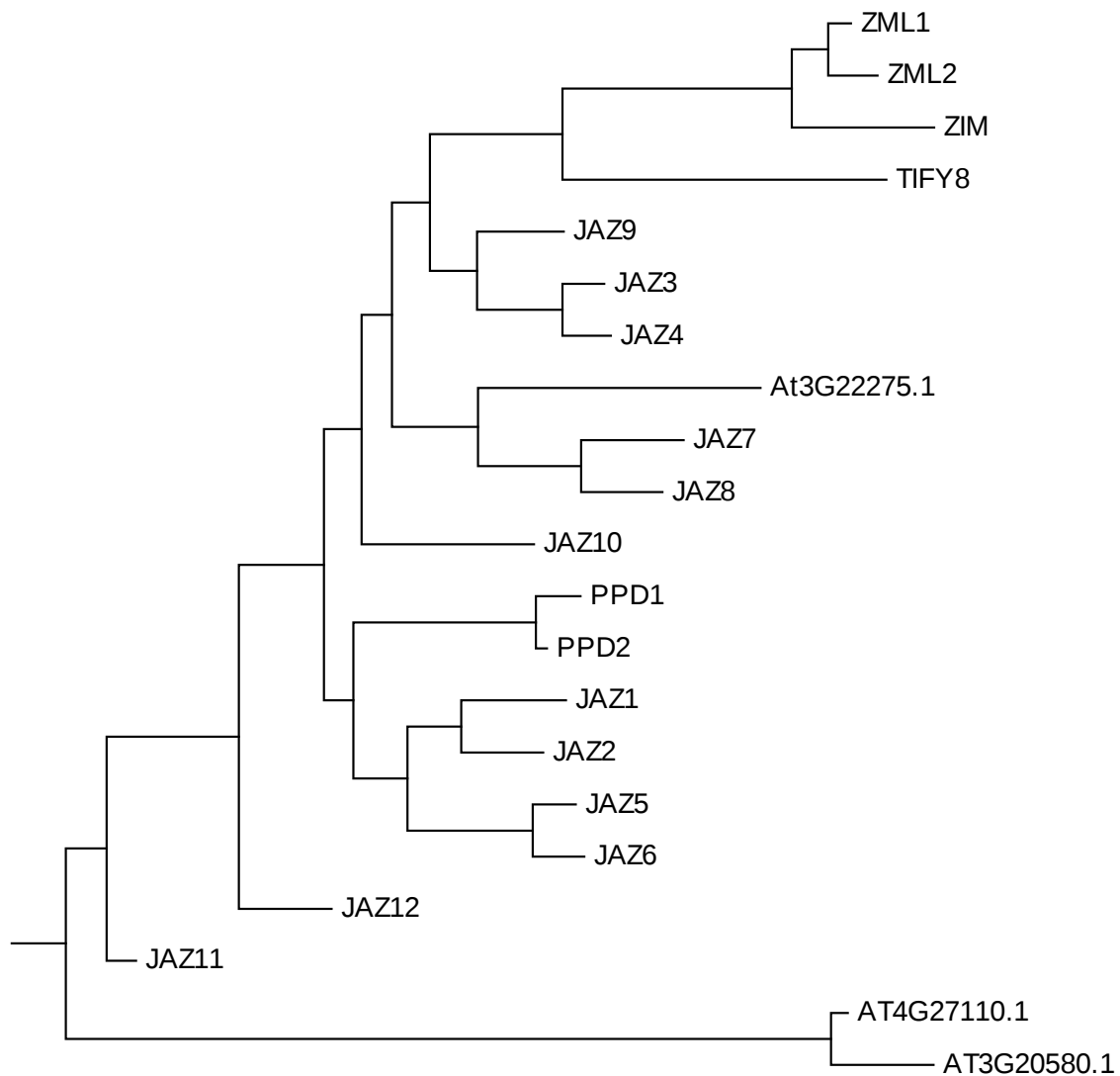


Figure 3. Phylogenetic tree of all *Arabidopsis thaliana* TIFY family transcriptional regulators. Evolutionary history was inferred using the Maximum likelihood method (MLM) based on the JTT matrix-based model using MEGA5 software (<http://www.megasoftware.net/>). At4G27110 and At3G20580 contain a “TIFY” sequence but are not considered a part of the TIFY family and have been included as an out-group.



Figure 4. At3G22275-like genes are only found in Brassicaceae. Tree view of all available genomes on Phytozome V9.1 (<http://www.phytozome.net/>). Species that contain (A) JAZ7/8-like or (B) At3G22275-like genes are highlighted in green.

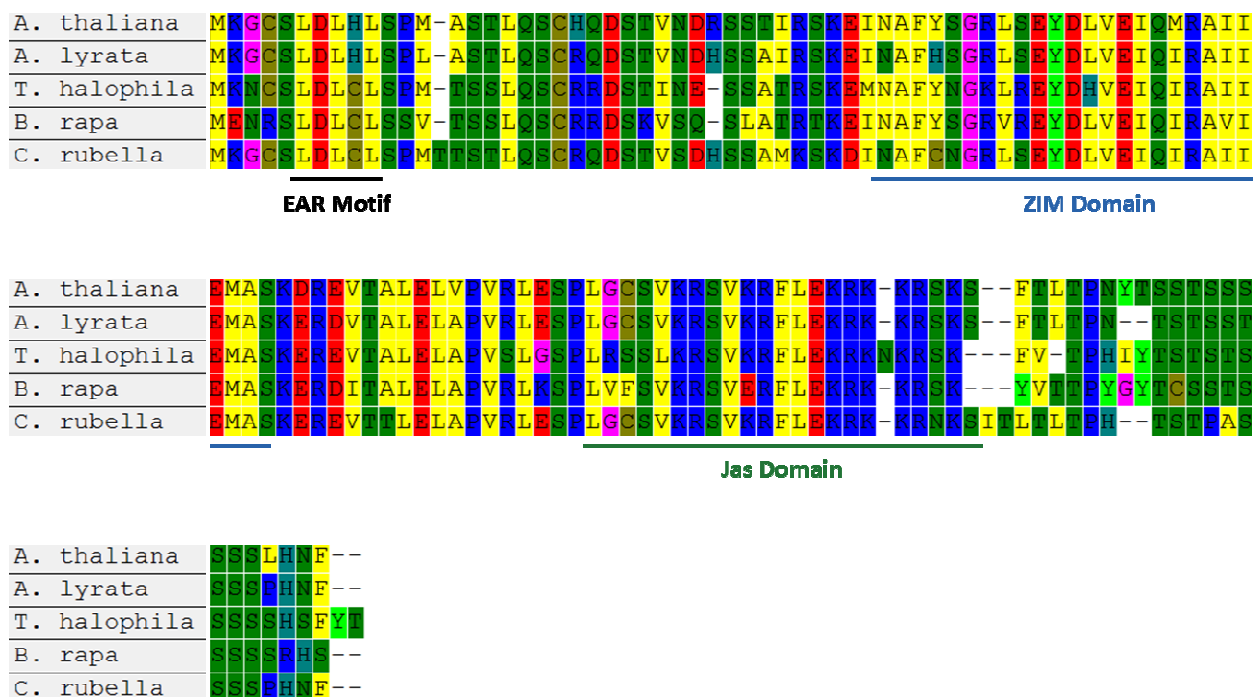


Figure 5. Sequence alignment of At3G22275-like predicted protein products. Amino acid based sequence alignment was performed using default parameters with the MUSCLE sequence alignment tool in MEGA5 software (<http://www.megasoftware.net/>)

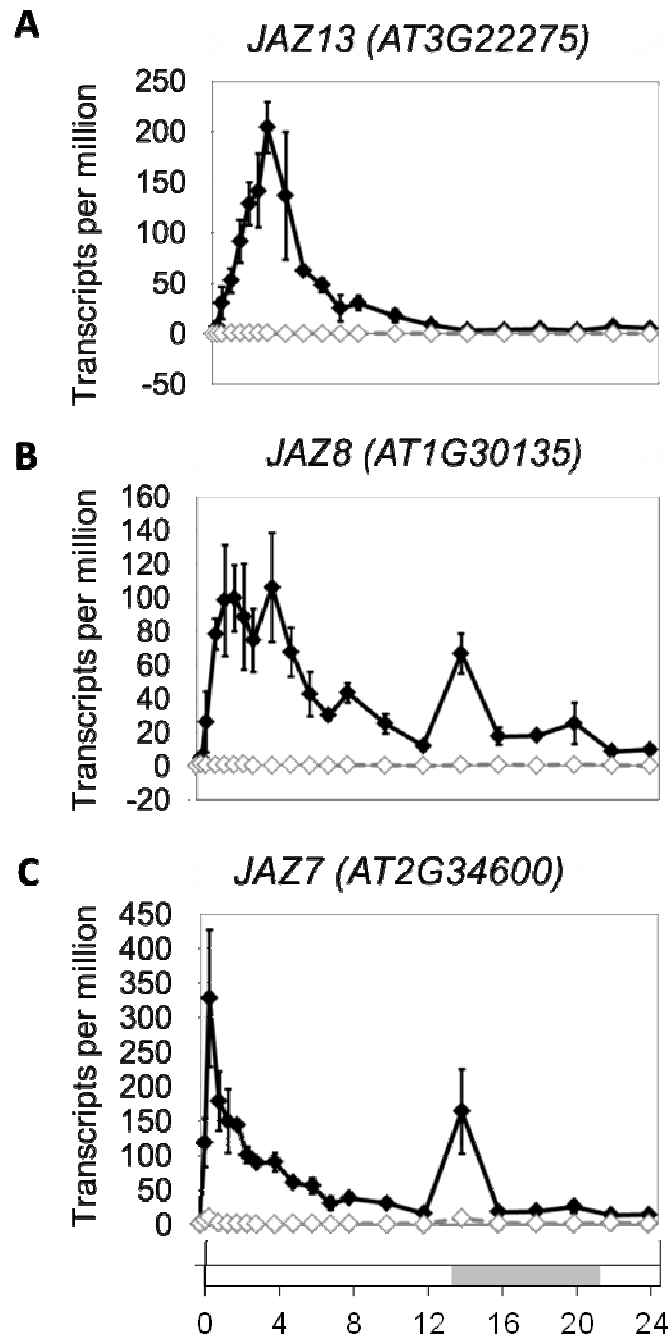


Figure 6. *At3G22275* expression is induced by coronatine treatment. Transcript accumulation over time in coronatine treated seedlings for *At3G22275* (A) *JAZ8* (B) and *JAZ7* (C). Coronatine and mock treatments are shown in black and grey, respectively.

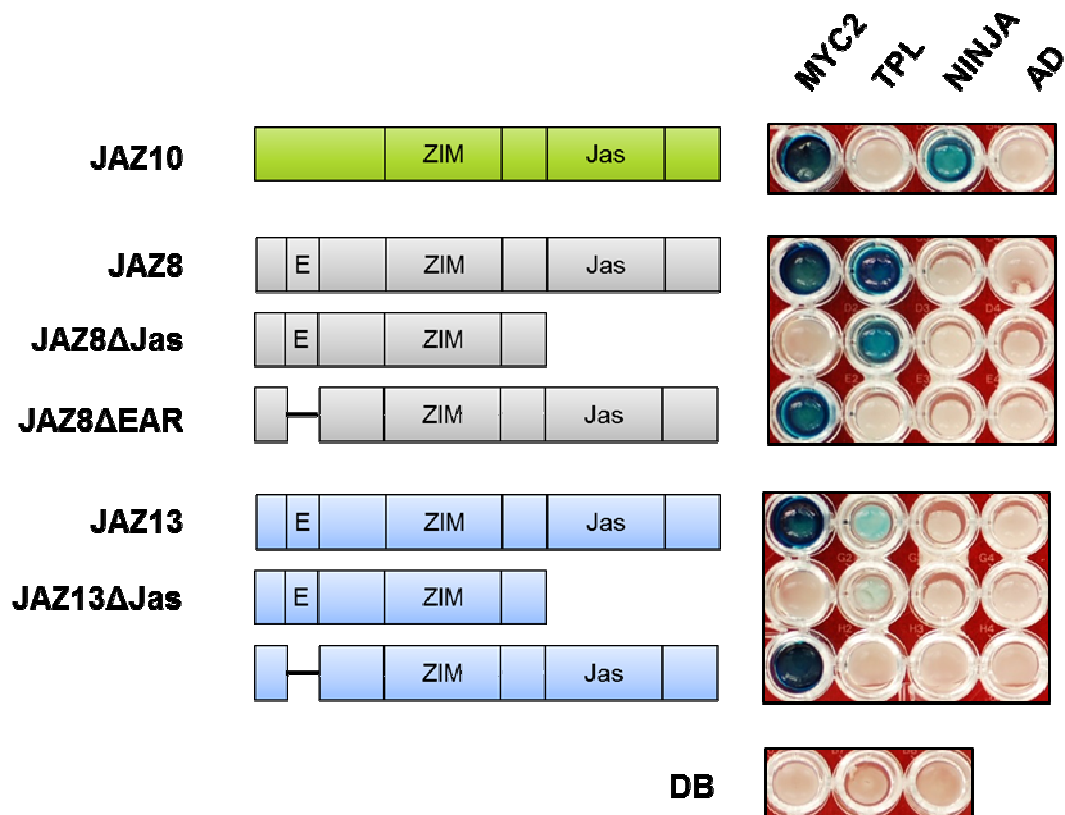


Figure 7. Y2H interaction with MYC2 and TPL. Yeast-two hybrid screen with full length and truncated JAZs (bait, indicated left) co-transformed with full length *MYC2*, *TOPLESS*, *NINJA*, or empty vector (AD) (prey). Unlike JAZ10.1, neither JAZ8 nor At3G22275 interacts with NINJA and instead directly interacts with TPL. Similarly to JAZ8, deletion of the Jas domain or EAR motif of At3G22275 abolishes interaction with MYC2 and TPL, respectively.

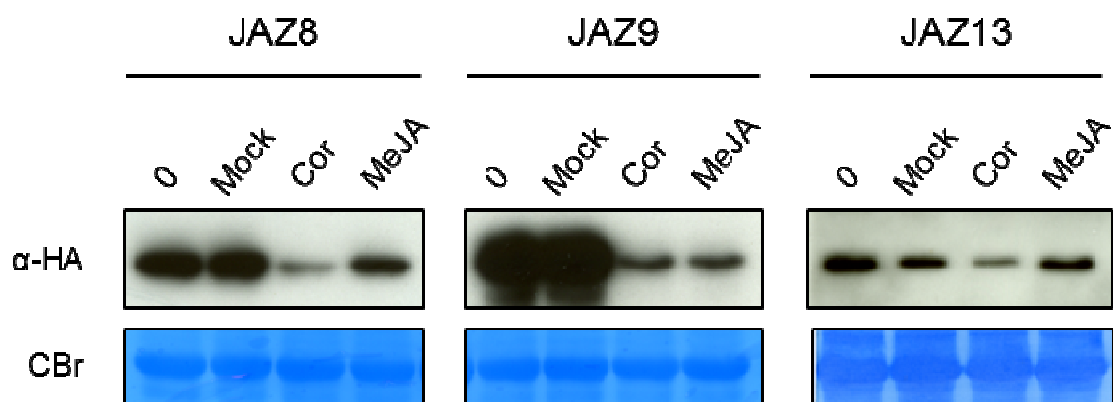


Figure 8. JAZ13 is degraded upon coronatine treatment. Western blot detecting degradation of JAZ8, JAZ9, and JAZ13 in liquid grown *35S::JAZ8-HA*, *35S::JAZ9-HA* and *35S::JAZ13-HA* arabidopsis seedlings, respectively. “0” sample is an untreated control. All other samples were treated for one hour with either a mock solution (Mock), 10 μ M coronatine (Cor), or 25 μ M methyljasmonate (MeJA). Total protein was extracted from whole seedlings and quantified. Total protein (100 μ g) was loaded for *35S::JAZ13-HA* whereas 10 μ g total protein was loaded for *35S::JAZ8-HA* and *35S::JAZ9-HA* samples. JAZ8 degradation was induced by Cor treatment, but was resistant to MeJA treatment. In contrast, JAZ9 degradation was readily induced by both MeJA and Cor treatments. JAZ13 followed a similar pattern to JAZ8 in that Cor treatment induced degradation, but it was resistant to MeJA treatment. CBr- Coomassie Brilliant Blue stained membrane to show equal loading.

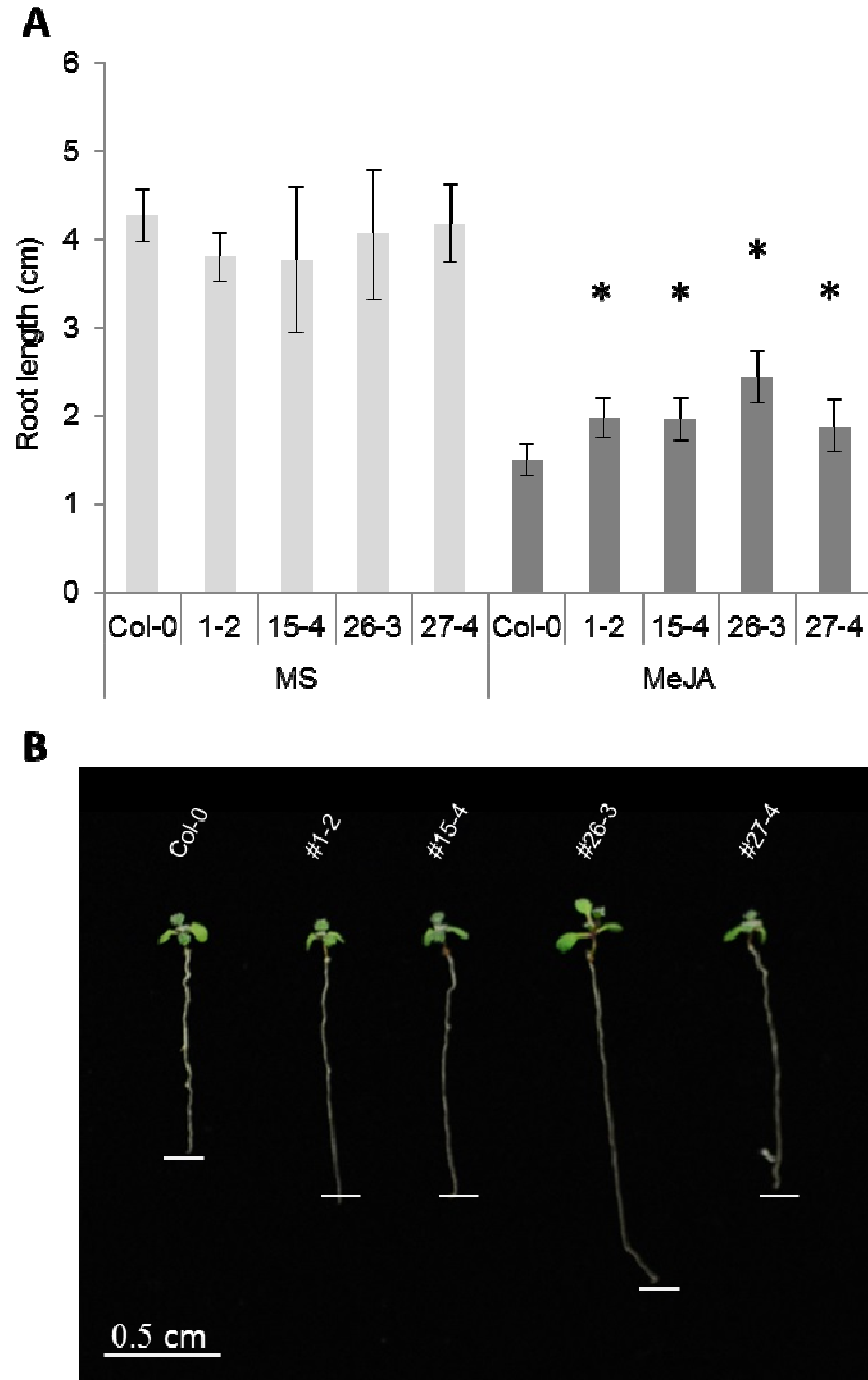


Figure 9. Over-expression of JAZ13 reduces MeJA sensitivity in the roots. (A) Root growth assay comparing WT Col-0 with four independent homozygous T3 35S::JAZ13-HA (*JAZ13-OE*) transgenic *Arabidopsis* lines grown in the absence (MS) or presence of 25μM MeJA (MeJA) for 10 days. (B) Representative 10d *JAZ13-OE* seedlings grown on media containing 25μM MeJA. ANOVA-Tukey HSD statistical analysis was used to determine significance with a cutoff of $P < 0.01$.

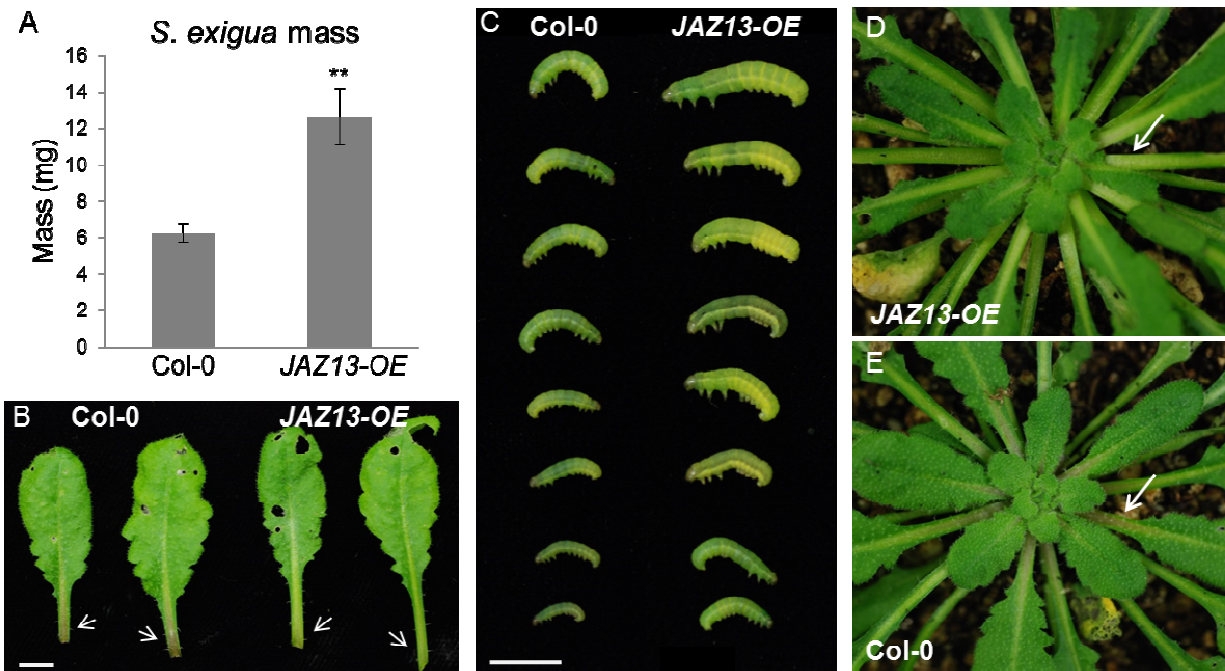


Figure 10. JA-mediated defense responses are suppressed in *JAZ13-OE*. (A) Graph displaying *S. exigua* larval mass in mg after 8 days of feeding on *JAZ13-OE* or WT Col-0. N= average of 3-4 insects over 12 plants per genotype. P-value determined from Students T-Test < 0.01. Error bars represent standard error. (C) Picture of representative *S. exigua* after 8 days of feeding on either Col-0 or *JAZ13-OE*. Anthocyanin accumulation (white arrows) is suppressed in (B) Leaves and (D-E) rosettes after 8 days of *S. exigua* feeding in *JAZ13-OE*. Scale bars represent 0.5cm.

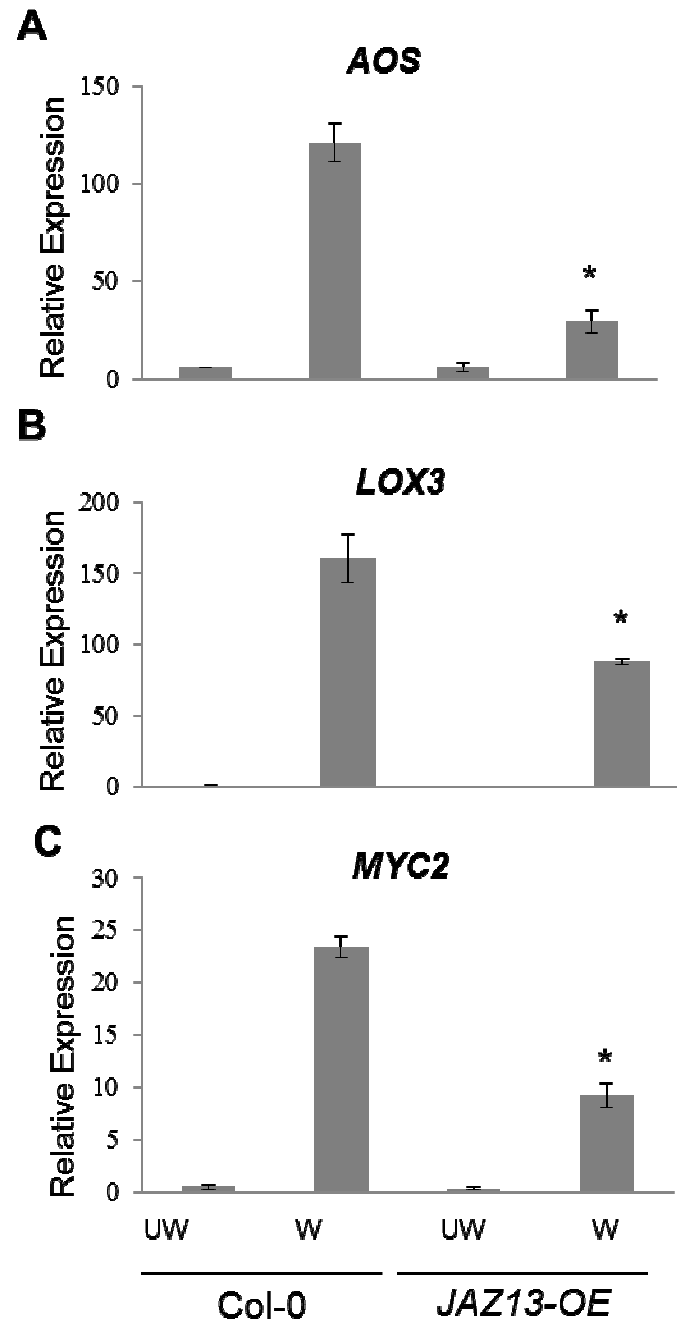


Figure 11. JA-responsive gene expression is dampened in *JAZ13-OE*. qPCR determined gene expression of primary JA-response genes 1 hour post wounding of mature rosette leaf tissue (A-C). Data shown is relative gene expression to *PP2A* internal control. Statistical significance was determined by student's T-test comparison between WT and *JAZ13-OE*. Asterisks indicate a P-value < 0.01.

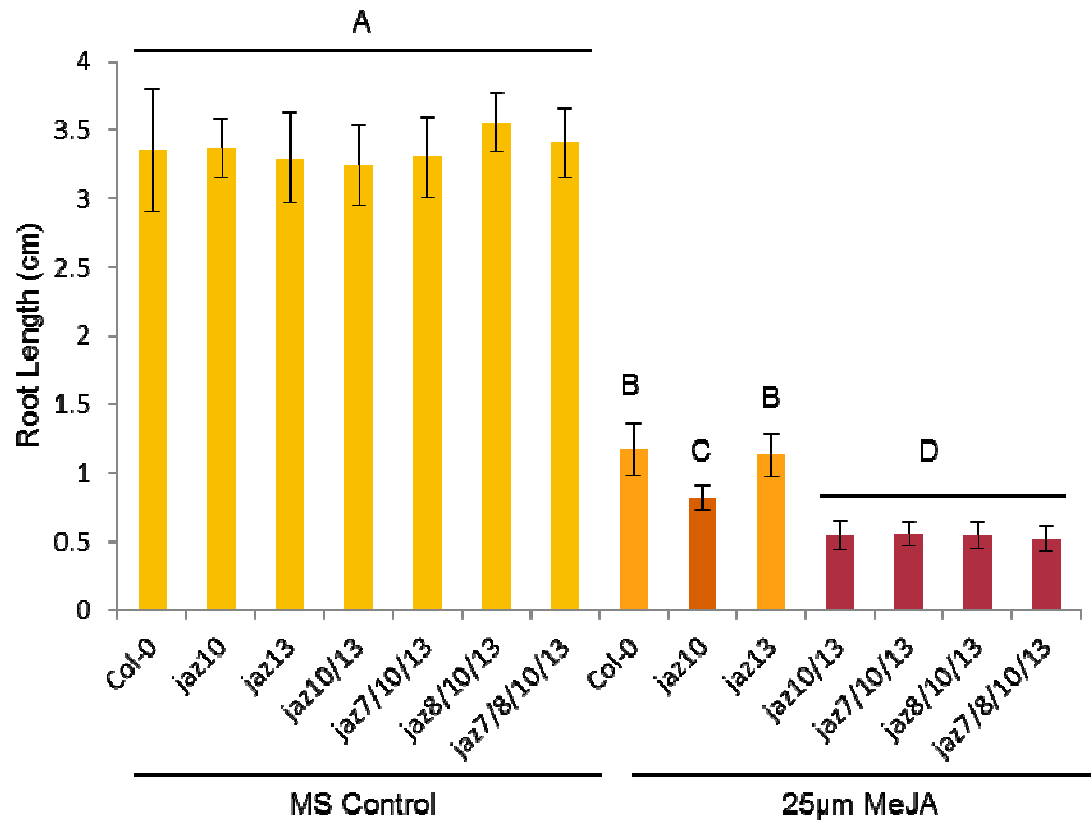


Figure 12. Higher order JAZ mutants display increased MeJA sensitivity in the roots. Seedlings were grown upright in 24hr light in the absence (MS) or presence of 25µm MeJA (MeJA). Root length was measured using imageJ 8 days post sowing. N=16-20 seedlings. ANOVA-TUKEY HSD statistical analysis was used to determine significance with a cutoff of $P < 0.001$.

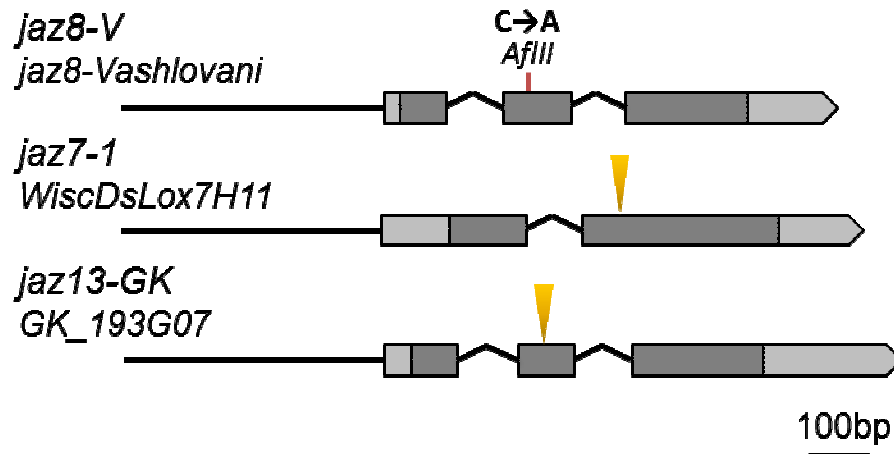
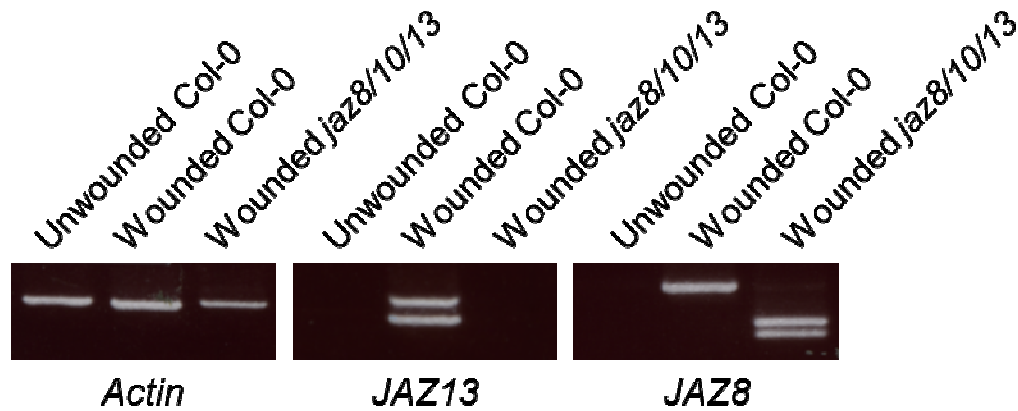
A**B**

Figure 13. Gene structure and confirmation of mutants. (A) Basic gene structure of mutants used in the study. *jaz7-1* (*WiscDsLox7H11*), *jaz13-GK* (*GK_193G07*), and *Vashlovani-1* (*Vash-1*) seed were ordered from The Arabidopsis Biological Resource Center (ABRC) at The Ohio State University. *jaz8-V* was generated through the introgression of the *JAZ8* *Vash-1* allele in a WT Col-0 background. (B) Transcripts produced by the *jaz13-GK* and *jaz8-V* alleles. cDNA was synthesized from RNA harvested from unwounded Col-0, and one hour post wounding Col-0 (wounded Col-0), and *jaz8/10/13* (wounded *jaz8/10/13*) leaf tissue. Transcripts for *JAZ13* and *Actin* were amplified by gene specific PCR. Transcripts for *JAZ8* were amplified by gene specific PCR and then digested with *AflIII* to distinguish the WT allele and *jaz8-V* allele. *Actin* is constitutively expressed and is detected in all three samples (*Actin*). *JAZ* transcripts are wound inducible and are not detected in the unwounded Col-0 (unwounded Col-0). Upon wounding, full length *JAZ13* and WT *jaz8-V* transcripts are detected in Col-0 (wounded Col-0). As expected, *JAZ13* was not detected in the wounded *jaz8/10/13* mutant indicating that the T-DNA insertion prevents the production of full length transcripts. Full length *JAZ8* transcripts accumulate in the *jaz8/10/13* mutant, however *AflIII* digestion results in two smaller bands confirming the presence of the *jaz8-V* nonsense mutation.

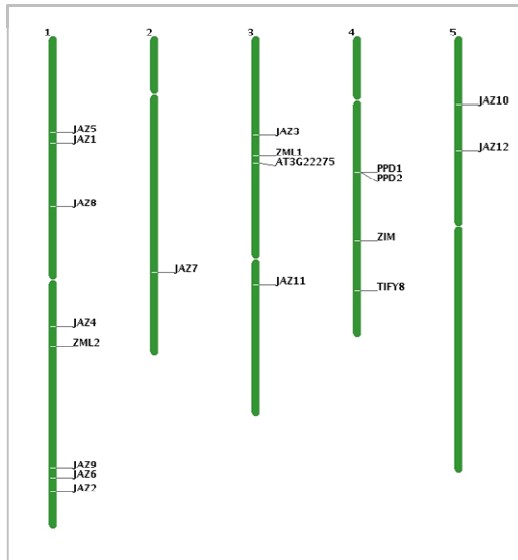
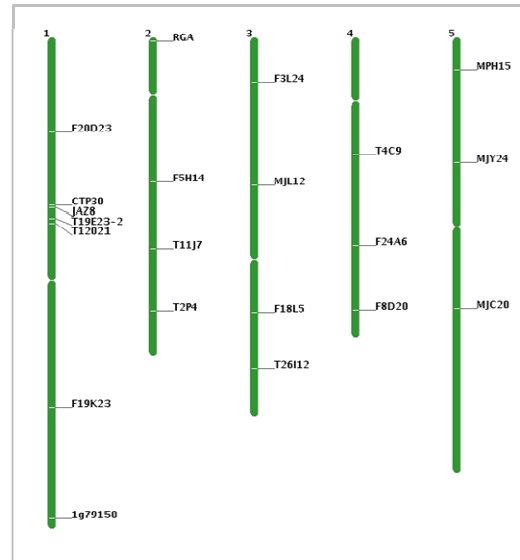
A**B**

Figure 14. Chromosomal locations of genes and markers included in this study. (A) Chromosomal location of TIFY family proteins in *A. thaliana*. **(B)** Chromosomal location of SSLP markers between Col-0 and Vash-1 ecotype screened during introgression.

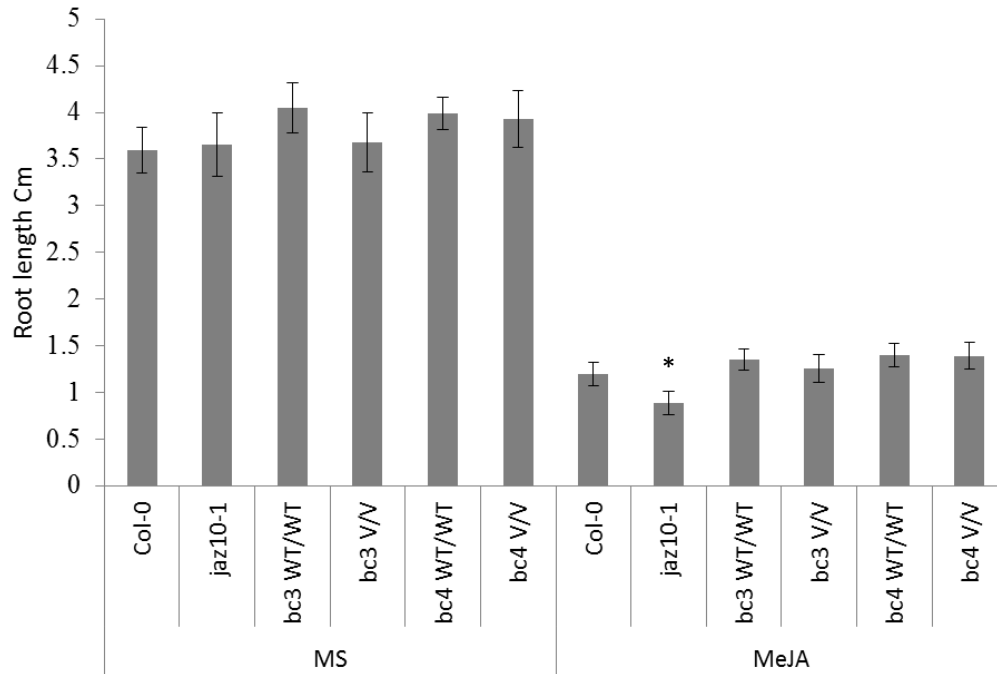


Figure 15. *jaz8-V* is not hypersensitive to MeJA induced root growth inhibition. Segregating seed for backcross 3 and backcross 4 *jaz8-V* generations were sown and genotyped after 8 days of growth upright in 24hr light in the absence (MS) or presence of 25 μ m MeJA (MeJA) alongside Col-0 and *jaz10-1*. Homozygous *jaz8-V* (V/V) and homozygous wild-type JAZ8 (WT/WT) were included in the analysis for both BC3 and BC4 lines. Root length was measured using imageJ. Asterisk denotes a significant difference from WT Col-0. T-test statistical analysis (P-value<0.01) was used to pairwise compare Col-0 with each genotype.



Figure 16. Higher order JAZ mutants are phenotypically and developmentally similar to Col-0 in the absence of a stress. Images show 4 week old representative plants grown in short day conditions. Genotype indicated below image.

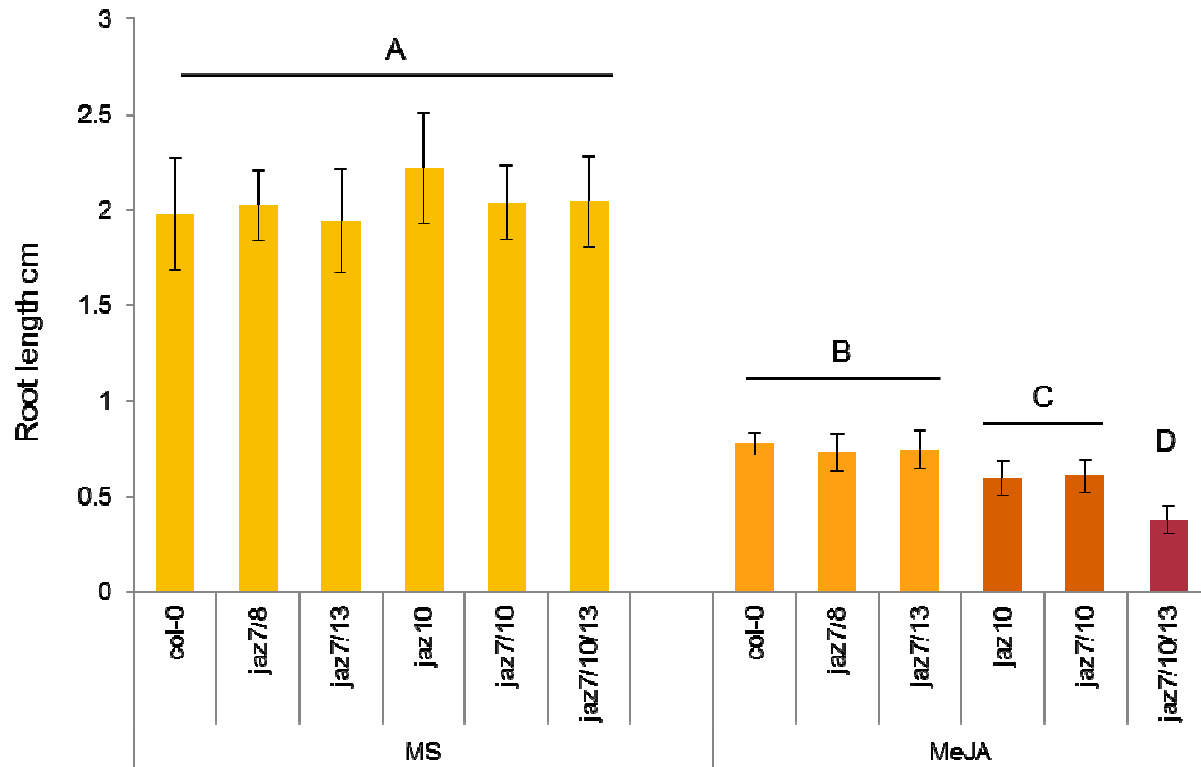


Figure 17. Addition of *jaz7-1* does not further enhance root phenotype of *jaz10* or *jaz10/13* mutants. Seedlings were grown upright in the absence (MS) or presence of 25 μM MeJA (MeJA). Root length was measured using imageJ after 7 days post sowing in 24 hour light. ANOVA-TUKEY HSD statistical analysis was used to determine significance with a cutoff of $P < 0.001$.

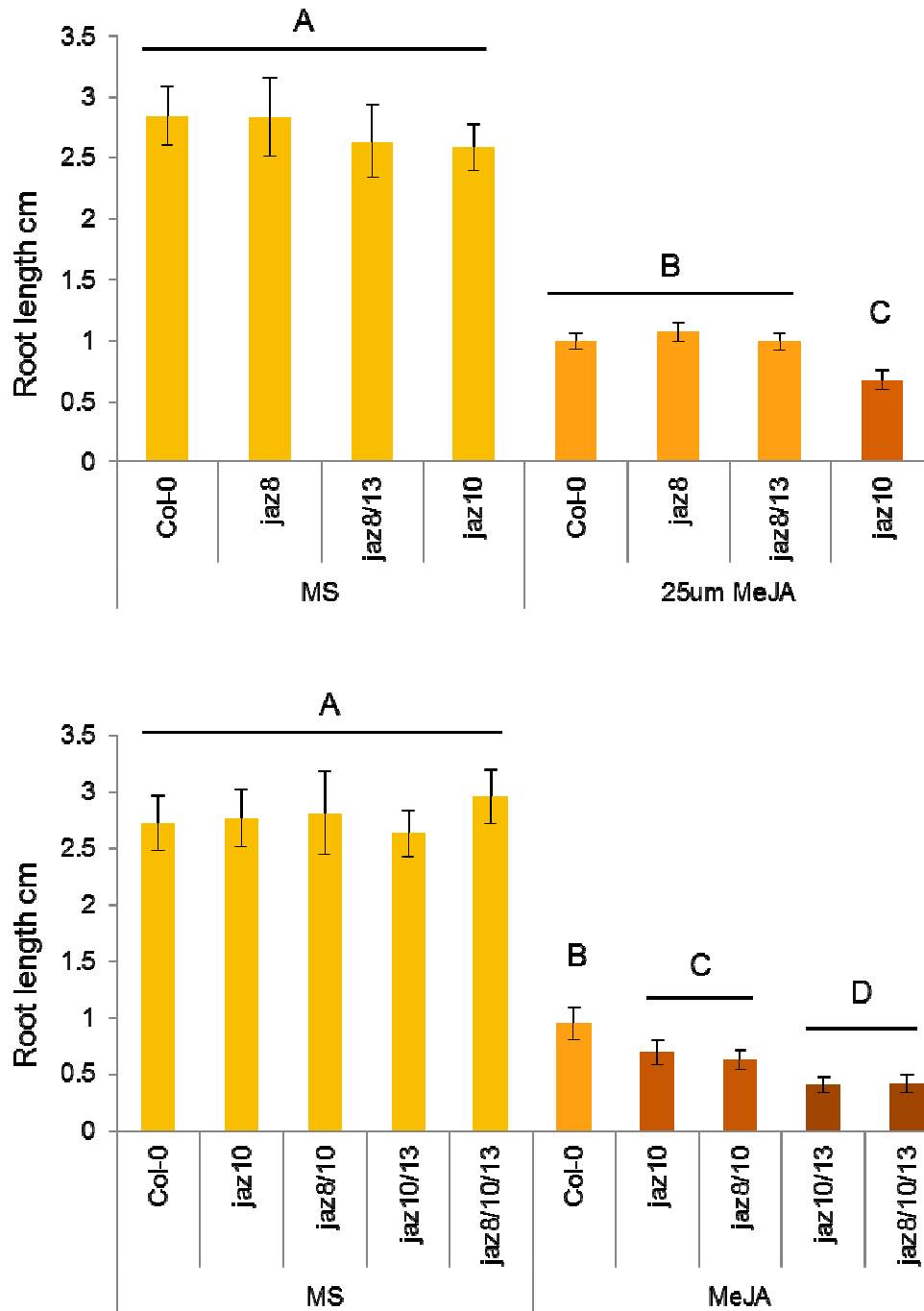


Figure 18. Addition of *jaz8-V* does not further enhance root phenotype of *jaz10* or *jaz10/13* mutants. Seedlings were grown upright in the absence (MS) or presence of 25µm MeJA (MeJA). Root length was measured using imageJ after 8 days post sowing in 24 hour light. ANOVA-TUKEY HSD statistical analysis was used to determine significance with a cutoff of $P < 0.001$.

Table 1. PCR Primers used in this study**Molecular cloning primers**

Primer ID	Sequence (5'-3')
JAZ13 JAZ13_pENTR_FP	CACCATGAAGGGTTGCAGCTTAG
JAZ13_KpnI_RP	GGTACCTTAGAAATTATGAAGAGAGGAGG
JAZ13_XhoI_FP	CTCGAGATGAAGGGTTGCAGCTTAG
JAZ13_KpnI_RP	GGTACCTTAGAAATTATGAAGAGAGGAGG
JAZ13ΔEAR	CACCATGAAGGGTTGCAGCTCTCCAATGGCCTCTACG
JAZ13dEAR_pENTR_FP	GGTACCTTAGAAATTATGAAGAGAGGAGG
JAZ13_KpnI_RP	GGTACCTTAGAAATTATGAAGAGAGGAGG
JAZ13ΔJas JAZ13_pENTR_FP	CACCATGAAGGGTTGCAGCTTAG
JAZ13ns_XhoI_249R_stop	CTCGAGTTATAACGGTGATTCCAGTCTCACCG

qPCR primers

Primer ID	Sequence (5'-3')
AOS F	GGAGAACTCACGATGGGAGCGATT
AOS R	GCGTCGTGGCTTTTCGATAACCAGA
PP2A F	AAGCAGCGTAATCGGTAGG
PP2A R	GCACAGCAATCGGGTATAAAG
MYC2 F	AGAAACTCCAAATCAAGAACCAGCTC
MYC2 R	CCGGTTTAATCGAAGAACACGAAGAC
LOX3 F	CCTAGACCGGATTAATGCGCTAGAC
LOX3 R	GACCGATGTTTTGGACCATGGGG

Genotype primers

Primer ID	Target	Sequence (5'-3')
JAZ14-f4	jaz13-GK (GK_193G07)	GCACGTGACCAAATTTGCAGA
JAZ14-r4		TGAAGAGAGGAGGATGATGAGGA
35S-fseq1		AAACCTCCTCGGATTCCATTGC
JAZ8-f3	jaz8-Vashlovani-1*	TGTCCTAAGAGTCCGCCGTTGT
JAZ8-r3		TTTGGAGGATCCGACCCGTTTG
JAZ7-f1		ATATCTCGAGATGATCATCATCAAAAAC
JAZ7-r1	jaz7-1 (WiscDsLox7H11)	CTCGAGCTATCGGTAACGGTGGTAAG
pWiscDsLox-p745_alt		GTCCGCAATGTGTTATTAAGTTGTC
JAZ10-f1		ATTTCTCGATCGCCGTCGTAGT
JAZ10-r1	jaz10-1 (SAIL_92_D08)	GCCAAAGAGCTTTGGTCTTAGAGTG
pCSA110-LB4		GTCTAAGCGTCAATTTGTTTACACC

* Digest PCR product with AflIII

Table 1 (cont'd)
Primers for SSLP ecotype markers

Primer ID	Target	Sequence (5'-3')
F20D23-f2 F20D23-r1	chr1	GAGTTCGATAGTTTTTCAGAGAGATGC ACTCTTCCTCTTCTTTATCGTCACG
T17H3-f1 T17H3-r1	chr1	GGCCCAATATACGACGTCCATCA TGGGTAGGCGACAAGAAGGAAG
T12O21-f1 T12O21-r1	chr1	TTTTTTCGGCGAAGTGGATG GGTTTATTTATCGACCGCGATAG
F19K23-f1 F19K23-r1	chr1	GACAACAAAACCCTGTTGTTTCTGAGC TCCTCTAGTCATTTCTCATAAGATCCAC
At1g79150-f1 At1g79150-r1	chr1	TCATGACTTTGAAGGCGACAAG CGAAAGATGCAGACAGAGACAC
10444578F 10445831R	chr1	GGACAACATTCAAAGCAATATCCGC GTCAATTAATTACAAATGTTATGGAG
RGA-f1 RGA-r1	chr2	AGACGGTGGAGGTAACATGGA AGTCAACCAGGATAACAGAACGAG
F5H14-f1 F5H14-r1	chr2	AATTCGAAGCGTGAAGCAAAAGAATTCG ACCTTGGAACCCAACATCAACAGG
T11J7-f1 T11J7-r1	chr2	GTCCAGTCTTTTTTGACAAGTAGTCTTTGC ACAATACACATGACATTAATTGCATCGTCG
T2P4-f1 T2P4-r1	chr2	CAAACTAAACTAGTCCCACTGTCTG GCCTAAACGTGATCAACTAAACAGC
F3L24-f2 F3L24-r2	chr3	TACCGGTTTGGCTTTCCCTTTG TCCGTCAAAGCTGTCTGGTATC
MJL12-f1 MJL12-r1	chr3	ATGAGTTTAGATCATCAAGATCGGAGG CCAACCGAATGAACCAAAAACCATCG
T26I12-f1 T26I12-r1	chr3	ACCGGTGTATTCCAGTTTCAGATTACC GGTCAAGGTAATAAACCAAAAACCCATTTGC
F4C21-f1 F4C21-r1	chr4	GCGGCTGTGTTAGTGTACTC GAAAAATGTGTGCCACACACTC
T4C9-f1 T4C9-r1	chr4	GACCAAGCTTCGTTATCGAAGATAACC AAAGAGAACTCACCGGCATACC
F24A6-f1 F24A6-r1	chr4	CGGATGCATGCAGACAAGTGAG CTGCTCCTGCACCATTTAGACC
F8D20-f1 F8D20-r1	chr4	TAATTTGTCTCCCTGTGTAACTTGC GTAGGTAACGAGATCCAGATCTTCC
MPH15-f1 MPH15-r1	chr5	CCCTTGATGAGGAAGTAATGGGA GATTGGAAGGAACACTGGAAGT
MYJ24-f1 MYJ24-r1	chr5	GTTTTATTGGTGGTGTGAGAGGAATGG TATCTCTGTGCGTTGTGAGTGTGTTGG
MJC20-f1 MJC20-r1	chr5	TTTGGTGGACCATAGAGATTGATTGG CTTTGTACTTTTACTCGGTTGATGACG

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