The Role of *JAZ10* in the Regulation of Jasmonate Signaling

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ABSTRACT

Jasmonic acid and its precursors and derivatives (collectively referred as jasmonates) are plant hormones involved in the regulation of a multitude of developmental processes ranging from seed germination, growth, flower development and senescence to defense against several biotic and abiotic stresses. Even though these molecules are essential for plant development, the knowledge about the jasmonate-signaling pathway was superficial until recently. A major breakthrough in the study of jasmonates was the discovery of the JAZ proteins, the missing repressors of the jasmonate-signaling pathway that links the hormone-receptor complex to the jasmonate-associated transcription factors. Although numerous studies have uncovered molecular partners and the mechanism of repression for some *JAZ* genes, the individual functions of all twelve *JAZ* family members remain to be elucidated. It is also unclear if different JAZ proteins are involved in specific jasmonate responses and if they repress different subsets of genes. The present proposal focuses on *JAZ10,* suggesting it as an ideal model to further understand the jasmonate-signaling pathway. The *JAZ10* gene produces three unique protein isoforms that show, among other differences, variable levels of stability in the presence of the bioactive hormone. Based on this evidence I hypothesize that each isoform works as a different repressive unit possibly associated with different sets of jasmonate responses, further performing different roles in hormone signaling. One possibility is that the *JAZ10* splice isoforms can be employed to regulate different sets of jasmonate-responsive genes. Furthermore, *JAZ10* is also the only member of the *JAZ* gene family in which a knockout mutant shows a clear jasmonate-hypersensitive phenotype. This mutant can be used as a platform not only to uncover the functions of *JAZ10*, but also to create high-order mutants with other JAZ knockouts and evaluate functional redundancy. Understanding the molecular mechanisms behind the synthesis and signaling of jasmonates is a key step for understanding how plants adjust their development and respond to surrounding signals. For the large scope of action of jasmonates as regulators of plant development, results obtained with the present proposal will not only help uncover many steps involved in the jasmonate signaling but likely impact and benefit broad areas of plant biology.

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Common abbreviations

amiRNAs – artificial microRNAs
ChIP – Chromatin Immunoprecipitation
COI1 – CORONATINE INSENSITIVE1
D1 – Domain1
EAR – ETHYLENE RESPONSIVE FACTOR-associated amphiphilic repressor
JA – Jasmonic acid
JA-Ile – (+)-7-*iso*-Jasmonoyl-L-isoleucine
JAZ – Jasmonate ZIM-domain
MeJA – Methyl-jasmonate
NINJA – NOVEL INTERACTOR OF JAZ
SCF – Skp/Cullin/F-box
TF – Transcription factor
TPL – TOPLESS
WT – Wild Type
ZIM - Zinc-finger Inflorescence Meristem

I. HYPOTHESES AND SPECIFIC AIMS.

The range of action of jasmonates in plant's life cycle is vast as many processes, from growth to stress responses, are controlled by the dynamics of these molecules. Therefore, elucidation of the signaling mechanism of jasmonates is essential to our understanding of how plants alter their development to respond to a myriad of external signals. To further characterize the function of a specific JAZ protein is a key step to comprehend how a hormone with so many different functions can trigger such specific responses throughout the plant. To this end, the present proposal seeks to analyze the role of *JAZ10* in the regulation of jasmonate signaling. Three protein isoforms, with different C-terminals ends, are generated from alternative splicing of the *JAZ10* gene and each exhibits distinct patterns of jasmonate-dependent degradation. Recent findings from the Howe lab showed that different phenotypes are obtained when overexpressing each of these splice variants *in planta*. **I hypothesize that (I) the JAZ10 splice isoforms work as different repressive elements with different functions in the jasmonate-signaling pathway and (II) that *JAZ10* shares functions with other members of the JAZ family.** To evaluate the roles of each splice isoforms I will use two different approaches: First I will analyze the functions of each splice variant throughout plant development and defense (Aim 1). Then, I will check if the different isoforms can repress different sets of JA-responsive genes (Aim 2). Moreover, using the obvious JA-hypersensitive phenotype of the *JAZ10* knockout mutant, *jaz10-1*, I will ascertain if *JAZ10* shares biological functions with other JAZ proteins (Aim 3). These roles will only be observable following the combined removal of *JAZ10* and other *JAZ* genes from the system. The three aims of the present proposal are summarized below:

Aim 1. Examine the specific roles of *JAZ10* splice isoforms in the jasmonate signaling.
To determine if each splice isoforms of *JAZ10* have a different in jasmonate signaling, two approaches will be used: 1) Complementation of the *jaz10-1* mutant with each splice variant of *JAZ10* and; 2) Phenotypic characterization of plants silenced for specific splice isoforms.

Aim 2. Evaluate if different *JAZ10* splice variants regulate different sets of genes.
Another approach to check if different isoforms perform different roles is to evaluate if they can repress different sets of genes. For this purpose, I will use the complemented lines developed in "Aim 1" to perform chromatin immunoprecipitation experiments followed by qPCR to test different gene regulation.

Aim 3. Verify redundant functions of *JAZ10* with other *JAZ* genes.
To fully understand the roles of *JAZ10* in the regulation of jasmonate-signaling pathway, it is necessary to study the interactions between *JAZ10* and other members of the JAZ family. I will phenotypically characterize crosses of the *jaz10-1* mutant with other *JAZ* knockout mutants available. Furthermore, RNA-seq will aid in the understanding of the complexity of the JAZ-JAZ interactions leading to jasmonate responses.

II. BACKGROUND AND SIGNIFICANCE:

II.A) Plants utilize hormones to alter development and respond to environmental stimuli:

In nature, plants are constantly exposed to a variety of environmental conditions. Light fluctuations, nutrient and water availability, herbivory/pathogen attack, day/night cycles and temperature changes are just a few of the many external stimuli affecting plants. In order to thrive in an ever-changing environment, plants must sense and adapt to their surroundings. However, due to their sessile nature, plants cannot simply escape a detrimental condition. They need to reconfigure their development quickly and efficiently so as to survive.

Plant hormones are master regulators of plant development. These substances are vital for survival, being involved in virtually every stage of a plant's life cycle [1]. For this reason, plants constantly adjust balances, sensitivity and crosstalks of hormones as a sophisticated system in response to environmental stimuli [2]. Under certain adverse conditions, plants make use of specific hormone pathways to activate the necessary response to survive: Abscisic acid, for example, plays a pivotal role in drought stress tolerance [3]. Auxin is involved in the shade avoidance response, a process where plants elongate the stems to compete for light with other individuals [4]. Ethylene levels may be altered to induce salt stress tolerance [5]. One hormone, Jasmonic acid (JA), plays an indispensable role in promoting resistance to many biotic stresses and is fundamentally involved with herbivory, pathogen and mechanical stress responses. It is not surprising therefore, that JA is constantly referred to as "the wound hormone" [6].

II.B) Jasmonates are key regulators of plant growth and defense.

Jasmonic acid (JA, Figure 1), its structurally related precursors, conjugated forms and other derivatives comprise a family of oxylipins collectively referred to as jasmonates, named for their initial identification as the main constituent of the jasmine scent [7]. Jasmonates were later found to be ubiquitously occurring throughout the plant kingdom [8] but gained attention when it was observed that they are potent plant growth regulators and inducers of leaf senescence [9, 10]. Subsequent research showed that jasmonates are also required in processes such as seed germination, photomorphogenesis, carbon partitioning, mechanotransduction, sexual reproduction and fruit development [11], unquestionably achieving the status of plant hormones.

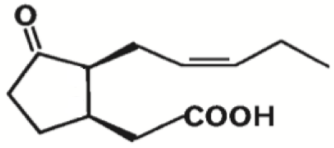

Figure 1. Chemical structure of (3R,7S)-jasmonic acid.

A major breakthrough in the field of JA research occurred when Farmer & Ryan observed that methyl-JA (MeJA) was a powerful inducer of plant proteins involved in insect herbivory defense in tomato [12]. It was the first of many subsequent studies proving one of the most striking roles of jasmonates: their function in wounding defense. They are now well characterized for their role as mediators of defense against arthropod herbivores by promoting, for example, the production of toxic secondary compounds, the formation of morphological structures that hinder insect mobility (trichomes) and the synthesis of proteins that alter leaf digestibility [11-13]. Treatments of plants with exogenous JA results in major transcriptome reprogramming, including alteration in expression of many genes involved in wound stress [14-16]. For this reason it is not surprising that plants impaired in the synthesis or perception of JA are severely damaged upon arthropod herbivory [13, 15, 17, 18]. JA-dependent stress responses not only include defense against arthropods but also necrotrophic pathogens, UV-radiation, ozone and other abiotic stresses [11].

Plant defenses are produced at the cost of photoassimilates, which are also the source of energy for growth processes. As a result, plants are in a constant dilemma regarding how much energy should be invested in defenses in detriment of growth or vice-versa [19]. The fact that jasmonates play a crucial role in both growth and defense processes indicates that these molecules act as regulators of resource allocation, suggesting that the biosynthesis and signaling of JA evolved as an instrument to optimize plant fitness in a rapidly changing environment [20, 21]. Thus, the understanding of the molecular mechanisms behind the synthesis and signaling of jasmonates is a key step to comprehend how plants develop and respond to surrounding signals.

II.C) The jasmonate-biosynthesis and signaling pathways.

More than forty years after the discovery that jasmonates are the main constituent of the jasmine scent [7], knowledge of the biosynthetic pathway of JA is now well established. The overall chemistry for JA synthesis, the octadecanoid pathway, was first proposed by Vick & Zimmerman [22]. Subsequent work confirmed their findings, adding details to the pathway such as the sub-cellular localization of the enzymes and how the synthesis is regulated. Briefly, the synthesis of JA is initiated by an environmental cue (such as insect wounding) that triggers the release of α-linolenic acid from plastid membrane lipids [23]. A series of enzymatic reactions [23, 24] convert this polyunsaturated fatty acid into (9S-13S)-12-oxo-phytodienoic acid (OPDA). OPDA is then imported into the peroxisome [25], where it is converted into the substrate used for cycles of β-oxidation that finally yields (3R,7S)-jasmonic acid (Figure 1) [9, 22, 26]. JA is further metabolized into a wide range of conjugates/derivatives through various enzymatic transformations, many of which have no known biological function. One relevant reaction is performed by JAR1 (Jasmonate-Resistant1), which conjugates JA to the amino acid isoleucine, generating (+)-7-*iso*-Jasmonoyl-L-isoleucine (JA-Ile), the endogenous bioactive form of the hormone [27, 28]. An important aspect of JA-Ile synthesis is its strong positive regulation by JA signaling: Many of the genes encoding enzymes of the biosynthetic pathway are strongly induced by JA treatment or mechanical wounding such that the endogenous levels of JAs increase significantly in a matter of few minutes upon stress [18, 29]. The accumulation of JAs leads to stronger activation of the JA-signaling pathway, creating a positive feedback loop. This induced JA accumulation is not observed in JA-insensitive mutants, hence these are impaired in insect herbivory defense [18, 29].

Although the biosynthesis of JAs is reasonably well understood, the signaling pathway is yet to be fully elucidated. Several genetic screens helped dissect how JA is sensed in plant cells and initial findings focused on a mutant named *coronatine insensitive1* (or *coi1*) [30, 31]. The Arabidopsis mutant *coi1* is insensitive to the bacterial phytotoxin coronatine, a molecular mimic of JA-Ile, and thus is also insensitive to and defective in JA responses. A map-based cloning approach showed that *COI1* encodes an F-box protein that is part of an E3-ubiquitin ligase complex known as Skp/Cullin/F-box (SCF^{COI1}). This complex functions by targeting proteins for removal by ubiquitination and further degradation by the 26S proteasome [31]. COI1 was later found to be part of the JA receptor complex in Arabidopsis [32, 33], acting in a degradation-signaling process remarkably similar to other plant hormone receptors such as gibberellin and auxin [34]. Similar genetic screens and transcriptome analysis led to the identification of several transcription factors (TFs) involved in the induction of specific JA-responsive genes such as MYC2, MYC3, MYC4, MYB21 and ERF1. Plants carrying mutations in these genes are altered in particular JA-related phenotypes such as defective expression of a group of JA-related genes, suggesting that specific TFs regulate certain sets of JA responses [35-37].

With the identification of two components of JA-sensing (COI1 and the JA-related TFs), it became clear that a link was missing in the core-signaling pathway: What are the proteins targeted for degradation by the SCF^{COI1} complex and how is their removal related to the activation of the JA-related TFs? Extensive genetic screens and transcriptome experiments were performed to find regulators downstream of COI1, COI1 interacting proteins or candidate genes among JA-responsive genes, but all to no avail [18, 38].

II.D) The JAZ proteins.

Three independent groups using different approaches showed that a family of uncharacterized proteins, all containing the zinc-finger inflorescence meristem (ZIM)-domain, is crucial to JA responses [39-41]. Several members of this family were identified among genes rapidly upregulated in JA-deficient plants upon treatment with JA, named Jasmonate ZIM-domain (or JAZ) [40]. Many of the JAZ proteins physically associate with COI1 in a JA-Ile dependent manner [42, 43] being further targeted for degradation by the 26S proteasome, strongly suggesting that these proteins were the missing targets for the SCF^{COI1}complex [40]. Together with the findings that JAZ proteins also interact with the TFs involved with JA-responses such as MYC2, MYC3 and MYC4 [35, 39] and that truncated forms of JAZ proteins confer a dominant JA-insensitive phenotype, it became clear that the JAZ acts as negative regulators of JA-responses [29, 40].

JAZ are plant-specific proteins present in phylogenetically diverse plant species [39, 44]. Twelve JAZ members are present in the Arabidopsis genome (*JAZ1*-*JAZ12* – Figure 2 [45]) and homology search led to the identification of three domains: a weakly conserved C-terminal domain 1 (D1), the ZIM domain [46] and the N-terminal Jas domain. D1 is the least characterized and appears to be involved in JA crosstalk with other plant hormone regulators [47]. The ZIM domain is directly involved with the repression mechanism as it is necessary for homo- and heteromerization between the JAZ [42, 48] and also essential for interaction with the Novel Interactor of JAZ (NINJA). NINJA is a protein that contains an ETHYLENE RESPONSIVE FACTOR-associated amphiphilic repressor (EAR)-motif, a hallmark of transcriptional repressors. The EAR-motif in NINJA is necessary for the recruitment of the co-repressor TOPLESS (and TOPLESS-related proteins – all referred to as TPL), a Groucho/Tup-1/TLE like protein [49]. Although the mode of action for TPL is unknown, data obtained in animal and fungi systems shows that the Groucho/Tup-1/TLE family carries out repression by altering chromatin structure or by interacting with the Mediator complex [50]. Since these co-repressor proteins lack a DNA-binding ability, the specificity of repression is probably achieved through interaction with other proteins, such as the NINJA/JAZ complex, which interact with TFs that contain DNA-binding domains. Finally, the Jas motif is a feature of the family, only present in JAZ proteins. It is a protein-protein interaction surface necessary for

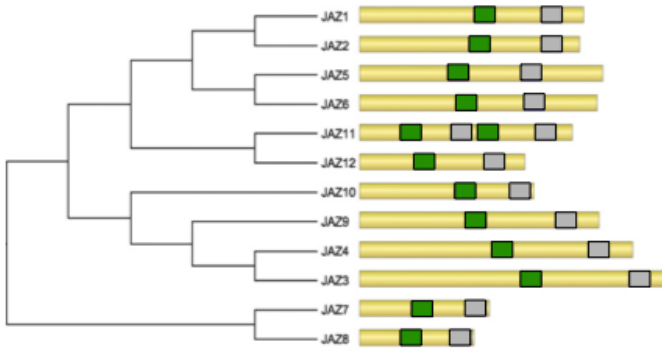

Figure 2. The Arabidopsis JAZ family members. Phylogeny of the JAZ family members in Arabidopsis, based on full amino-acid sequences. Green and gray bars represent the ZIM and Jas domains respectively. Figure as shown in (45), with alterations.

association with the multitude of TFs involved with JA responses [39, 43, 51, 52]. It also contains the JAZ degron, a minimal amino acid structure necessary for hormone binding. The degron in the Jas domain forms the protein structure that binds COI1 in the presence of the JA-Ile [33], further leading the JAZ for degradation. For this reason, truncated proteins lacking the Jas domain are stable in the presence of JA-Ile [39, 41].

A canonical model for the JA signaling is shown in Figure 3. In a non-induced state, where levels of JA-Ile are low, the JAZ proteins repress the activity of TFs involved with JA-responses by recruiting the NINJA/TOPLESS complex that probably acts by altering chromatin status or by interaction with the Mediator complex. The presence of an environmental signal such as mechanical wounding leads to a burst of JA-Ile in the system. The hormone works as a molecular glue that allows the physical interaction of JAZ with the SCF^{COI1} complex, which ubiquitinates the JAZ proteins, targeting them for degradation by the 26S proteasome. Degradation of the JAZ releases TFs from repression, activating the expression of JA-responsive genes. The JAZ proteins are amongst the genes rapidly induced by JA treatment [40], suggesting a mechanism of negative feedback regulation to attenuate the JA-signaling pathway, returning it back to a repressed state (see below).

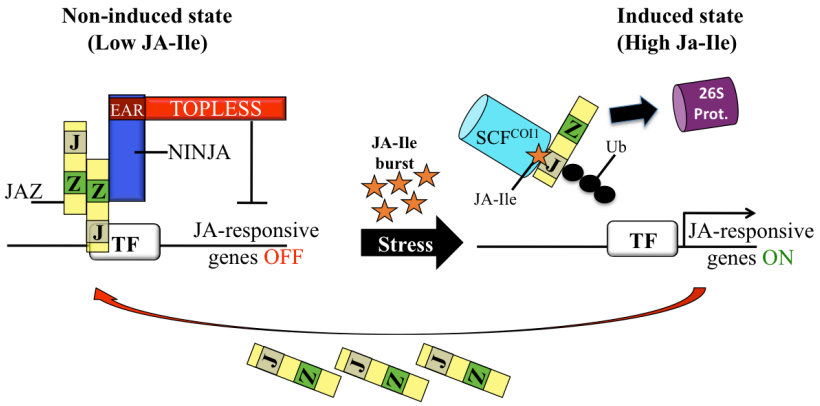

Figure 3. Overview of the JA signaling pathway. An environmental stimulus (such as a wounding) causes a burst of JA-Ile in the system, leading to COI1-JAZ association and JAZ degradation. Transcription factor (TF) released from repression alter the expression of JA-related genes such as the JAZ proteins. See text for details. Z= Zim domain, J= Jas domain.

Several questions are yet to be answered concerning the JAZ proteins. First, although twelve *JAZ* genes are present in the Arabidopsis genome, almost no information is known about the specific roles of each. The fact that most loss-of-function *jaz* mutants do not show any obvious defects in JA-related responses [39, 40] strongly suggests that *JAZ* family members share some level of functional redundancy. However, although part of the same family, these genes show more differences than similarities. The different *JAZ* genes are differentially induced by wound or insect feeding [29]. Different JAZ proteins associate with distinctive sets of TFs [51] and show different levels of interaction with COI1 [44, 53]. They also show diverse levels of homo/heteromerization [42, 48], and even use different strategies to perform repression [53, 54]. For those reasons, it is also tempting to speculate that individual members of the JAZ family do exert unique functions during jasmonate signaling.

It is also unclear whether different JAZ proteins inhibit the expression of different sets of hormone-responsive genes. Besides the association with different sets of TFs, it appears that

different JAZ employ distinct domain architectures to recruit co-repressors to related TFs. Generally, the ZIM domain is involved with NINJA interaction and the EAR-motif in NINJA further recruits TPL [49]. However, some JAZ such as JAZ5 and JAZ8 contain a cryptic EAR motif in their C- and N-terminal region respectively, directly associating with TPL [53]. Some JAZ can directly associate with chromatin remodelers such as histone deacetylases, to possibly repress TF function [52]. Repression mechanisms may vary depending on the TF [55]. It is already known that JAZ proteins compete for the DNA binding domain of some TFs, suggesting that in the absence of JA-Ile, JAZ proteins repress by inhibiting the TF DNA binding activity [47, 52, 56]. For other TFs such as MYC2, the JAZ proteins bind to a region other than the DNA binding domain [35, 57] taken together with the fact that TPL probably acts as a chromatin remodeler, suggests a model where the whole repressive complex is bound to the DNA [49] (as in Figure 3). The differences in hormone-dependent degradation, repression domains used by different JAZ, repression partners and modes of action from TF to TF probably arise as a mechanism for plants to finely adjust specific JA-responses and control different sets of JA-responsive genes. These facts strongly suggest that the specificity and diversity of the JA responses are partially determined by different JAZ proteins

Finally, it is still puzzling why some JAZ proteins are stable even in the presence of the bioactive hormone [44, 53]. Artificially truncated JAZs lacking the COI1 interacting domain (Jas) are resistant to JA-mediated degradation and confer dominant low-sensitivity to exogenous JA [39-41]. Naturally occurring JAZ proteins lacking this domain also confer the same phenotype if overexpressed [42]. Moreover, the Howe lab has recently showed that JAZ8 lacks the known JAZ degron and, for this reason, is stable even at high physiological concentrations of JA-Ile [53]. It is possible that the presence of JAZ members having a wide range of stabilities could provide a mechanism to modulate the amplitude and duration of JA responses. Two primary regulatory loops are present in the JA signaling pathway: First, the biosynthesis of JA is positively regulated by itself (a positive feedback loop already described in section II.C) [18, 29]. Second, the *JAZ* genes are strongly and rapidly induced by JA treatment (Figure 3), creating a negative feedback loop possibly involved with the return of TFs to a repressed state. The combination of these two regulatory feedback loops prevents the overrun of JA responses that could possibly damage plants [53]. However, other mechanisms are necessary to further return the system to a non-induced state. Research in the Howe lab has already demonstrated that catabolization of the bioactive JA-Ile to a less active 12OH-JA-Ile is likely to be a mechanism involved with the attenuation of JA-responses [58]. However, effective desensitization is likely to involve other means and thus, it is believed that JAZ proteins that are not degraded in the presence of JA-Ile are involved in hormone desensitization.

II.E) The role of *JAZ10* in the regulation of jasmonate signaling.

The Arabidopsis *JAZ10* gene (AT5G13220) is an interesting focus of study among the JAZ family. It is quickly and highly expressed upon exogenous JA treatment or wounding [39, 41, 59], it is so far the only *JAZ* gene where a loss of function mutation leads to an obvious phenotype (JA-hypersensitivity) [59, 60] and, most importantly, it produces alternative splice isoforms that differentially associates with COI1 in the presence of JA-Ile [42].

Alternative splicing is a post-transcriptional mechanism that changes the structure of transcripts and the proteome of organisms. The process increases the magnitude of a gene function and thus biological complexity by generating proteins with different roles deriving from the same transcript. Little is known about the mechanisms of alternative splicing in plants but

stressful conditions can have dramatic effects on it [61, 62]. The *JAZ10* gene produces four mRNA isoforms (*JAZ10.1* to *JAZ10.4*) that are transcribed into three different proteins (Figure 4). *JAZ10.1* produces a protein containing an intact Jas motif, which readily interacts with COI1 in a hormone-dependent manner. *JAZ10.2* and *JAZ10.3* are generated respectively through intron retention and alternative splice donor variants that are translated into the same protein (JAZ10.2/3) containing a truncated Jas motif lacking 7 amino acids from its C-terminus. This protein variant weakly interacts with COI1. Finally, JAZ10.4 is produced by use of an alternative splice donor variant in the third exon that creates a translational frameshift, generating a protein with a different C-terminus that fully lacks the Jas domain and does not interact with COI1, hence being stable in the presence of JA-Ile [42, 44].

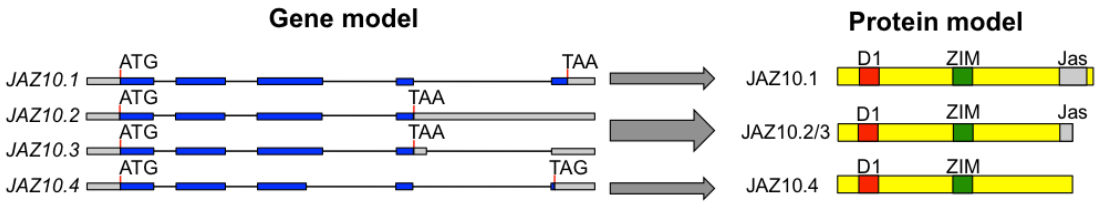

Figure 4. *JAZ10* gene and protein models. The *JAZ10* pre-mRNA is spliced into four different variants that are transcribed into three different proteins varying in their C-terminal (see text for details). The three protein isoforms associate at different levels with COI1 in the presence of JA-Ile. In the gene model, thin lines represent introns, UTRs and coding sequence are displayed as grey and blue bars respectively.

Gene expression analysis (Figure 5, RNA-seq data from A. Koo, unpublished results) showed that upon wounding, JAZ10.1 is the main mRNA isoform spliced from the *JAZ10* pre-mRNA gene, then followed by JAZ10.3 as the second most abundant form. JAZ10.2 and JAZ10.4 are present at relative low levels when compared to the other two isoforms. Besides this distinct expression levels, several mechanistic differences are found among the *JAZ10* splice variants. They share different protein partners, as evidenced by different levels of interaction with other JAZ proteins [42]. Furthemore, overexpression of the different isoforms gives rise to plants with different phenotypes. Plants overexpressing *JAZ10.1* are similar to wild-type (WT) but overexpression of *JAZ10.2/3* and *JAZ10.4* show several phenotypes typical of JA-insensitive plants such as reduced levels of JA-synthesis upon wounding and reduced wound-induced growth inhibition [41, 42]. Overexpression of *JAZ10.4*, but not *JAZ10.2/3* leads to male sterility, another phenotype typical of strong JA synthesis/perception mutants [42]. These facts raise the possibility that the different splice variants of JAZ10 perform different roles in the JA-signaling pathway. The three isoforms could be used as elements that respond differently to hormone signals, creating a refined way to regulate JA responses. Moreover, data obtained in the Howe Lab (J.E. Moreno, unpublished results) shows that JAZ10.4 uses the D1

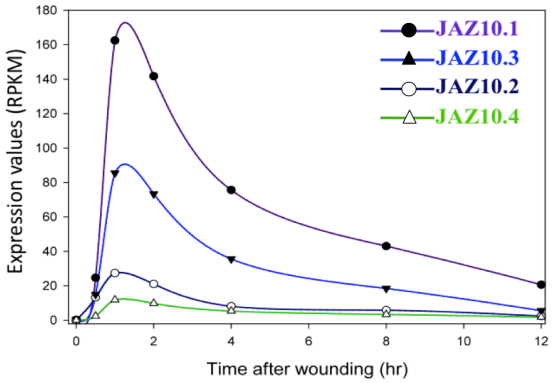

Figure 5. JAZ10.1 is the main transcript produced from the *JAZ10* gene. Whole transcriptome sequencing (RNA-seq) from wounded rosettes showing the expression values (RPKM) of each splice isoform generated from the *JAZ10* gene.

domain to bind transcription factors. Although this domain is also present in the other splice variants, results show that removal of D1 does not affect interaction of JAZ10.1 to MYC2, suggesting that *JAZ10* splice variants use different domain architectures to bind TFs.

This proposal outlines my intention to analyze the role of *JAZ10* in the regulation of JA signaling. Combining the obvious phenotype of the *JAZ10* knockout mutant and the splice isoforms of *JAZ10*, I propose that this gene is a model to study a multitude of processes involved in the JA signaling pathway.

III. RESEARCH DESIGN AND PRELIMINARY DATA.

Aim 1. Examine the specific roles of *JAZ10* splice isoforms in the jasmonate signaling.

Rationale: In the presence of the bioactive hormone, the three different protein isoforms generated from the *JAZ10* gene differentially associate with COI1 and show diverse levels of JA-Ile stability [42, 44]. Preliminary studies from the Howe lab strongly support the idea that *JAZ10.1*, *JAZ10.2/3* and *JAZ10.4* perform different functions. Besides their different expression levels, overexpression of the each splice variant gives rise to plants with different phenotypes. The different isoforms associate differently with other JAZ proteins [42] and they are capable of interacting with TFs using alternative domain architectures (J.E. Moreno unpublished). Therefore, **I hypothesize that the different splice variants of JAZ10 do exert differential roles in the JA signaling.** The fact that *JAZ10* expression is highly induced by wounding or JA-treatment [29] suggest a model where, upon stress, stable forms of JAZ10 accumulate in the system, further leading to JA-desensitization. This premise is supported by the observation that a knockout mutant for *JAZ10* (*jaz10-1*) shows a JA-hypersensitivity phenotype [41, 59, 60]. I here propose to corroborate this hypothesis by taking two different approaches: A) Transforming the *jaz10-1* mutant with each of the *JAZ10* splice variants fused to the *JAZ10* endogenous promoter and evaluate the level of complementation of the phenotype and; B) observing the phenotype of plants silenced in specific *JAZ10* splice isoforms.

Experimental approach:

1.A) Complementation of *jaz10-1*: *jaz10-1* is a *JAZ10* knockout mutant that shows JA-hypersensitivity phenotypes such as strong growth inhibition upon exogenous JA application and higher susceptibility to pathogen attack [41, 59, 60]. I will transform *jaz10-1* with the coding sequence of each splice isoform (*JAZ10.1*, *JAZ10.2/3* or *JAZ10.4*) fused to the promoter of *JAZ10* [59] and evaluate at what level each splice isoform complements the phenotype of the mutant. Other phenotypes that can also be altered, such as such as wound-induced growth inhibition and defense against insect feeding will also be evaluated in each line. As a control, a *jaz10-1* line transformed with the *JAZ10* genomic version (*JAZ10G*) will also be created (Figure 6). This construct contains all the exons and introns of *JAZ10* and likely will produce all the splice variants at the same endogenous levels of WT (Col-0) plants.

Figure 6. Promoter-HA-fusion constructs used in the proposed experiments. The promoter of *JAZ10* (*JAZ10*p) is fused to the coding sequence of each splice variant, containing an HA tag in the N-terminal. The genomic version of *JAZ10* (*JAZ10G*) will be used as a control.

Since no specific antibody for JAZ10 is available, the constructs described also carry an HA tag after the transcription start site, making if possible to monitor protein levels in the transgenic lines (see scheme in Figure 6). This will enable the verification of the JAZ10 protein dynamics upon wounding, JA-treatment and insect feeding (i.e. stresses that induce a JA-Ile burst). I will determine if the JAZ10.1 isoform signal (predicted to be very labile since it readily associates with COI1) is detectable after a burst of JA-Ile or if JAZ10.2/3 and JAZ10.4 signals increase after wounding, suggesting their participation in desensitization processes.

Finally, using the *JAZ10G* transgenic line, I will also verify if different variants are present at similar levels in different plant tissues. In a recent publication it was observed that overexpression of *JAZ10.4* but not *JAZ10G* leads to male sterility [44]. One possible explanation for this result is that *JAZ10.4* is not expressed in the flowers, suggesting that some splice variants may be expressed in a tissue specific manner.

1.B) Silencing *JAZ10* splice variants: As a second independent approach, artificial microRNAs (amiRNA) will be used to silence specific *JAZ10* splice variants in WT plants. The amiRNA technique uses artificially developed 21nt sequences that are incorporated into the RNA-induced silencing complex for further cleavage of the targeted mRNA. One benefit of the amiRNA technique is that extensive base pairing with targets is required for its function, greatly increasing specificity and allowing silencing of splice variants [63]. In order to target specific splice variants, vectors for amiRNA will be developed based on unique splice junctions present in each of the *JAZ10* mRNA isoforms. However, since some splice variants cannot be uniquely targeted without also targeting the *JAZ10* pre-mRNA, it will be necessary to silence two or more splice isoforms at the same time. Two splice isoforms can be targeted individually: *JAZ10.3* and *JAZ10.4*. The possibility of silencing only *JAZ10.3* will enable me to evaluate the importance of two mRNAs (*JAZ10.2* and *JAZ10.3*) coding for the same protein. In addition, multiple groups of variants are targeted: *JAZ10.1* and *JAZ10.4* share a common splice junction and can be targeted together, in a similar manner as *JAZ10.1*, *JAZ10.2* and *JAZ10.3* together. The amiRNA lines will be developed in the *jaz10-1* complemented with *JAZ10G* (described in section 1.A). Since all the JAZ10 splice isoforms in this line are tagged with HA, silencing efficiency can be evaluated by checking alteration in the protein levels of each splice variant. Silenced lines will be analyzed for the same phenotypes already described for the complemented lines (section 1.A).

The silencing of specific isoforms will permit the evaluation of loss-of-function phenotypes for specific splice variants of *JAZ10*. For example, silencing only *JAZ10.3* will allow the analysis of the role of the other splice variants that results in the same protein, *JAZ10.2*. On the other hand, removing only *JAZ10.4* will permit me to evaluate the contribution of this variant or the *JAZ10.2/3* isoforms on the predictable desensitization process.

Predicted outcomes: Three independent results obtained with the proposed experiments could strongly support the hypothesis that *JAZ10* splice variant perform singular roles: 1) each isoform show different phenotypes in the complementation/silencing experiment. I expect that JAZ10.4 and, at some level, JAZ10.2/3 are involved with desensitization mechanisms. For this reason, they will be the only isoforms that complement the JA-hypersensitivity phenotype of *jaz10-1*. Furthermore, silencing of those splice variants will lead to plants phenocopying *jaz10-1* JA-hypersensitivity; 2) Protein dynamics will be different for each variant. JAZ10.1 is a more labile protein and will be rapidly degraded upon stressful conditions. On the other hand, JAZ10.2/3 and JAZ10.4 will accumulate for further hormone desensitization; 3) Different tissues will show

different levels of JAZ10 protein isoforms. Specific tissues can control their level of JA-sensitivity by altering the levels of labile/stable JAZ10 protein isoforms.

The lines described for the complementation and silencing experiment are already under development. Preliminary data obtained from the *jaz10-1* carrying *JAZ10G* show that this transgene is able to complement the *jaz10-1* hypersensitive phenotype in root growth assays (Figure 7A). Furthermore, I observed that all JAZ10 protein isoforms could be detected in protein blots from seedlings of this line (Figure 7B), confirming that this transgene is expressing all the splice variants. From these complemented lines I observed that the JAZ10.2/3 and JAZ10.4 proteins appears to be the predominant JAZ10 forms obtained in seedlings, as the band signal corresponding to JAZ10.1 is relatively weaker when compared to JAZ10.2/3 and JAZ10.4. This result indicates an initial process of JA-desensitization since these plants were grown on stressful conditions that were probably triggering the production of JA (high seedling density).

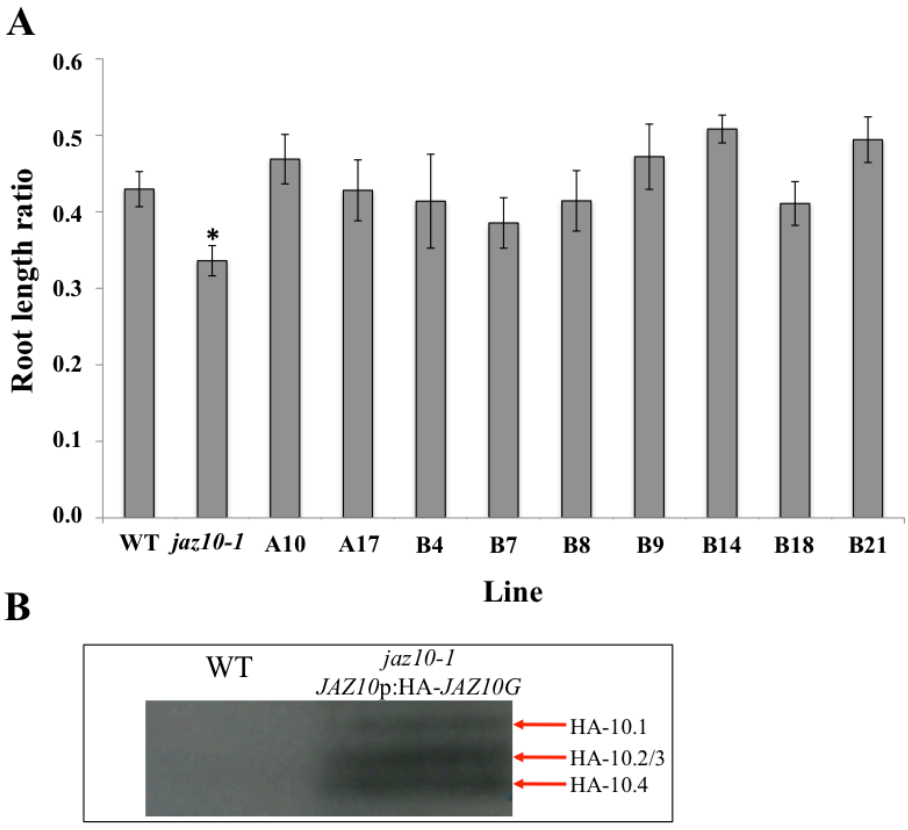

Figure 7. *JAZ10P:*JAZ10G complements the phenotype of *jaz10-1*. A) Seedlings of Col-0 (WT), *jaz10-1* and *jaz10-1* lines (T2) carrying the *JAZ10P*:HA-JAZ10G transgene were grown for 10 days on MS media with or without 10μM MeJA. The root length ratio was calculated by dividing the average of root 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 show the means ± SE of >18 seedlings. Asterisk denotes significant difference (Student's T-test, $p<0.05$, all compared to Col-0). **B)** Protein blot (α-HA) of JAZ10 isoforms in seedlings of Col-0 and *jaz10-1* (J10P:HA-J10G) complemented lines (grown on MS-media without MeJA), where it is possible to observe three bands with predicted size of HA-JAZ10 splice variants.

Potential pitfalls and solutions: One possible challenge is that the level of expression of the splice variants in the complementation lines can be different from what is normally found in WT plants. Since the coding sequence of each splice variant is being directly fused to the *JAZ10* promoter, it is expected that level of expression of the splice variants will be higher than what is normally found in WT plants, given that there is no control of splicing anymore. For this reason the *JAZ10G* will be taken as a control. Only phenotypes also observed in the *JAZ10G* lines can be counted as true physiological functions of the splice variant. For example, if the line complemented with *JAZ10.4* reverts the phenotype of *jaz10-1* back to WT, a similar phenotype also needs to be observed in *JAZ10G*, where the level of expression of *JAZ10.4* is similar to a WT plant.

The two strategies proposed here to identify the roles of *JAZ10* splice isoforms mutually exclude potential pitfalls of one another. The phenotypes obtained for *jaz10-1* plants complemented with a splice variant should be correlated to phenotypes of plants silenced in that same isoform. For example, although unlikely amiRNAs can cause silencing of other genes, a process called transitivity [63]. One way to control transitivity is to compare the phenotype of silenced plants for a splice variant with *jaz10-1* plants complemented for that same splice isoform and verify if the phenotypes are symmetric.

Aim 2. Evaluate if different *JAZ10* splice variants regulate different sets of genes.

Rationale: The scope of action of JAs ranges from growth processes such as seed germination and flower development to defensive processes such as insect herbivory and pathogen resistance. Due to this broad range of action, it is unclear how the specificity, diversity and duration of JA responses are determined throughout the plant. Although recent data is helping us understand how the hormone responses are triggered, our knowledge so far still cannot explain how the varieties of these responses are specifically regulated. Transcription is a major regulatory step in the activation of JA responses and many TFs involved with expression of different sets of JA-responsive genes are already known [35, 51, 56]. However, the profound transcriptional reprogramming involves not only TFs, but also the complex interplay between TFs and JAZ proteins. These interactions may determine how specificity of response is achieved. The fact that different JAZ proteins associate with different sets of TFs [35, 38, 51, 57] strongly suggests that specific JAZ proteins may repress singular sets of JA-related genes as a way to achieve specificity. Moreover, since the different splice variants of JAZ10 are differentially degraded in the presence of the hormone and that the different isoforms can use different domains to bind TFs (J.E. Moreno, unpublished preliminary data) leads to a **hypothesis that the different JAZ10 splice isoforms can be used as distinctive repressive units that regulate singular sets of JA-responsive genes.** A well established technique to detect the association of proteins with specific genomic sequences, chromatin immunoprecititation (ChIP), will be used to check if the different splice variants are bound to TFs and associated with promoter regions of different genes.

Experimental approach:

2) Chromatin immunoprecipitation with JAZ10 splice variants: The HA-tagged lines described in "Aim 1.A" will be used to perform ChIP experiments. Two experimental approaches will be employed. First, seedlings of the *jaz10-1* complemented lines will be grown on MS-media plates and harvested at 14 days. Crosslinking of the JAZ-TF-DNA complex will be performed by vacuum-infiltrating the tissue with formaldehyde following steps that isolate the

chromatin. To enrich the sample for DNA fragments bound to JAZ10-TFs, an immunoprecipitation step will be performed using an anti-HA antibody followed by purification with special magnetic beads that reduce non-specific binding and increase signal-to-noise ratio. After purification, the crosslinks are reversed by heat and purified DNA is ready to be analyzed.

MYC2, MYC3 and MYC4 are TFs associated with activation of many JA-responses [35] and data from the Howe lab already have shown that MYC2 can interact with all JAZ10 splice isoforms [44]. The consensus DNA binding site for these three TFs is a motif known as the G-box (CACGTG). Using the Arabidopsis Gene Regulatory Information Server (AGRIS [64]) G-boxes were found in the promoter region of several JA-responsive genes and developed qPCR primers regions around these motifs. Some of the genes are already known to be targets for MYC2 such as *JAZ1*, *JAZ3* and *LOX3* [47]. DNA samples isolated from ChIP experiments using each splice variant complemented line will be submitted to analysis by qPCR to evaluate enrichment of promoter signals [47].

To fully understand the breadth of genes associated with each of the JAZ10 splice variants, DNA samples isolated from each line will be submitted for next-generation sequencing (ChIP-seq). Reads obtained from ChIP-seq will be aligned to the Arabidopsis genome sequence, allowing the analysis of all sets of genes that are under the regulation of each splice variants. Furthermore, the data obtained in ChIP-seq can be used to compare results between different splice variant and to speculate about the contribution of each isoform in repression of specific genes.

The second experimental approach involves the same lines and similar methodology of ChIP (tissue fixation, immunoprecitipation, isolation of DNA, etc.), but includes treatments that cause a burst of JAs in the system, such as MeJA treatment and wounding. These treatments will likely cause variation in the ratio of each splice variant present in the system (when using the *JAZ10p:*HA-*JAZ10G*) and possibly generate different patterns of promoters associated with each JAZ10 isoform. This will allow further analysis of the roles of each splice variant. For example, if stable isoforms are involved in desensitization mechanisms, they will be associated with more promoters after treatments. These samples will also be submitted for next-generation sequencing.

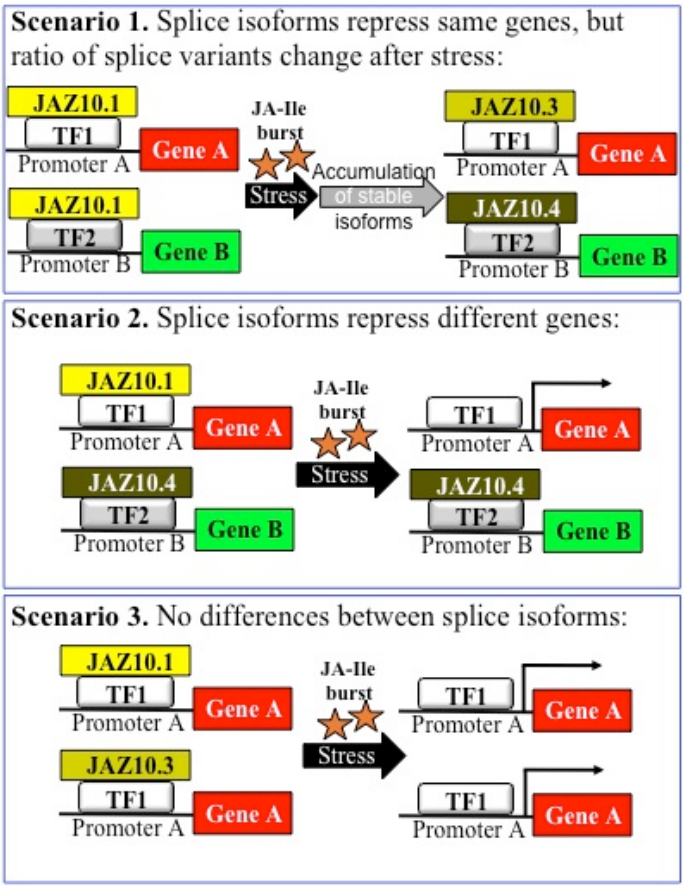

Figure 8. Three possible scenarios for ChIP results. See text for details.

Finally, as a confirmation that splice isoforms do regulate the different sets of genes, the level of expression of candidate genes obtained on all ChIP experiments will be evaluated through qPCR.

Predicted outcomes: One of the three following scenarios will likely be

obtained through the ChIP experiment (Figure 8). 1) The different isoforms are associated with the same promoters, but the ratio between the splice variants is different before and after wounding/MeJA treatment, leading to changes in the level of enrichment obtained with each isoform. This scenario is in agreement with the hypothesis that different isoforms are involved with desensitization mechanisms. 2) The different JAZ10 splice variants may bind different transcription factors and ChIP samples obtained for each complemented line will be enriched in different promoters. This scenario is in agreement with the hypothesis that different isoforms regulate different sets of genes and, as scenario 1, strongly suggest different splice isoforms have different roles in JA-signaling. 3) Finally, it is also possible that all JAZ10 variants are associated with the same promoters, and there is no ratio change upon wounding/MeJA treatment, a scenario that argues against the hypothesis of splice variants performing different roles and the involvement of stable isoforms in desensitization mechanisms.

Potential pitfalls and solutions: Although being an established method in many model organisms, ChIP is not a straightforward technique. One potential pitfall for performing ChIP with JAZ proteins concern the model of action of JAZ repression, as already described, it is not clear if the JAZ-TF complexes are bound to DNA or not. The fact that JAZ10 possibly represses through TPL [53] and that this protein possibly acts by recruiting chromatin remodeling enzymes or by association with the Mediator complex suggests that, at some point, the TPL-JAZ-TF complex need to be associated with the DNA. The Howe lab is already performing electrophoretic mobility shift assays to evaluate the mechanism underlying JAZ10 repression.

It is also unclear what the levels of each JAZ10 splice variants normally found in plant cells actually are. Since the lines that will be used for ChIP experiments express JAZ10 isoforms under the *JAZ10* native promoter, it is possible that these proteins are present in a magnitude that hinders the immunoprecipitation step. This is unlikely to pose a problem for our ChIP experiments as results already obtained in "Aim 1" (Figure 7) suggest that all splice variants can be easily detected from small amounts of plant tissue. Alternatively, one can perform ChIP experiments with splice isoforms expressed under the constitutive 35S promoter (lines also under development). Furthermore, although it is already shown that formaldehyde can fix protein-protein complexes associated to the DNA [65], different agents can be used to improve protein-protein (JAZ-TF) crosslinking, such as dimethyl adipimidate [66].

It is relevant to point out that even if Aim 1 is unsuccessful (if it cannot be shown that different splice variants perform different functions), it would still be very promising to perform the ChIP experiments with JAZ10. As mentioned earlier, the function of specific JAZ proteins is still unknown and evaluating which genes are regulated by JAZ10 will greatly improve our knowledge of how specificity of the JA-response is achieved.

Aim 3. Verify redundant functions of *JAZ10* with other *JAZ* genes.

Rationale: After the identification of COI1 as a key component of the JA-signaling pathway [30], extensive genetic screens failed to identify the proteins targeted by the SCF^{COI1} complex. Part of this difficulty can be attributed to the functional redundancy of the *JAZ* family [44]. Twelve *JAZ* genes are found in the Arabidopsis genome (Figure 2) and many are likely to have overlapping roles with each other, explaining why almost all single JAZ knockout mutants show no observable phenotype [39, 40]. It is also evident that JAZ proteins can interact with each other, forming homo and heteromeric complexes [42, 48] that appear to be involved in the repression of JA-responsive genes [42]. The formation of these complexes not only increases the

complexity of the regulation of JA responses, but also supports the idea that functions of *JAZ* genes are shared with other members of the family. This functional redundancy between the *JAZ* family members thus suggests that **certain roles of *JAZ10* overlap with other *JAZ* genes.** For this reason, certain functions of this gene will only be observed by removing *JAZ10* and other *JAZ* genes from the biological system. Making use of the JA-hypersensitive phenotype of the *JAZ10* knockout mutant *jaz10-1* [59, 60], crosses will be performed with other *JAZ* knockout mutants to create higher-order mutants. Phenotypic characterization of these mutants and whole transcriptome sequencing analysis will allow the study not only of roles shared by *JAZ10* and other *JAZ* genes, but also to speculate about functions of other *JAZ* genes and provide a glimpse of how the specificity and diversity of JA responses are determined throughout the plant

Experimental approach:

3.A) Phenotypic characterization of *jaz10-1* x JAZ knockout mutants: Several *jaz* knockout mutants have been described in the literature [39, 40, 60]. Our research group has acquired many of these mutants and started performing some crosses to obtain higher order mutants (Y. Yoshida, unpublished results). Since many of these mutants are from collections of T-DNA insertion lines, confirmation of genotype in multiple mutants can be easily scored by PCR. Phenotypic characterization will focus on numerous JA-hypersensitive phenotypes (many of which have already being described for *jaz10-1* [59, 60]) such as root growth on MS media containing MeJA, pathogen and insect resistance, anthocyanin accumulation, trichome density and growth inhibition upon wounding. As controls, all phenotypes will also be compared to Col-0 (WT) and in the single mutants.

3.B) Whole transcriptome analysis of higher-order mutants: To fully comprehend the sets of JA-responses regulated by *JAZ10* together with other *JAZ* family members, higher-order mutants with noticeable phenotypes (see "predicted outcome" below) will be submitted for whole transcriptome sequencing (RNA-seq). RNA will be extracted from seedlings grown on MS plates with or without exogenous JA (MeJA) and submitted for high-throughput sequencing. All steps of RNA extraction, preparation of cDNA library, ligation of sequencing adaptors, mapping of RNA-seq reads to annotated genes and sequence analysis will be performed as described previously [62] and following the MSU Research Technology Support Facility (RTSF) protocols. Sequences will be obtained using the Illumina Hi-Seq 2000 Plataform.

Predicted outcomes: One of the aspects that set *JAZ10* apart from other *JAZ* family members is its clear JA-hypersensitive phenotype. This phenotype can be used as a platform to uncover shared roles of *JAZ10* with other *JAZ* genes. Preliminary data already obtained with phenotypic characterizations of some high order mutants can be used to exemplify the expected outcome of this proposed methodology. Seedlings of WT (Col-0), *jaz1*, *jaz10* and *jaz1 jaz10* (double mutants) grown on MS media containing MeJA show stunted root growth, when compared to seedlings grown on MS media only (no MeJA). No phenotype is observable for *jaz1* single mutant (growth inhibition as WT), but *jaz10* is hypersensitive to JA (the roots are more stunted than WT, as already described [60]). Moreover, the *jaz1 jaz10* double mutant shows enhancement of the *jaz10* phenotype (Figure 9A and B), not only unveiling the role of the *JAZ1* gene as a regulator of root responses to JA, but also showing that these two genes (*JAZ1* and *JAZ10*) present overlap of functions.

One phenotype was obtained by removing five *JAZ* genes from plants (*jaz1 jaz3 jaz4 jaz9 jaz10* quintuple mutants), where root growth was inhibited even in absence of any exogenous hormone treatment (Figure 10). This result can be explained by the fact that removal of these JAZ proteins releases from repression the TFs involved in root growth, causing JA-responses to be triggered constitutively. *JAZ3*, *JAZ4* and *JAZ9* form a clade of JAZ members (Figure 2) in which removal from plants did not result in observable phenotype (even triple mutants grown on MeJA plates present root growth inhibition similar to WT (data not shown)). Thus, the characterization of the quintuple mutant also shows the role of this clade of JAZ members in JA root responses.

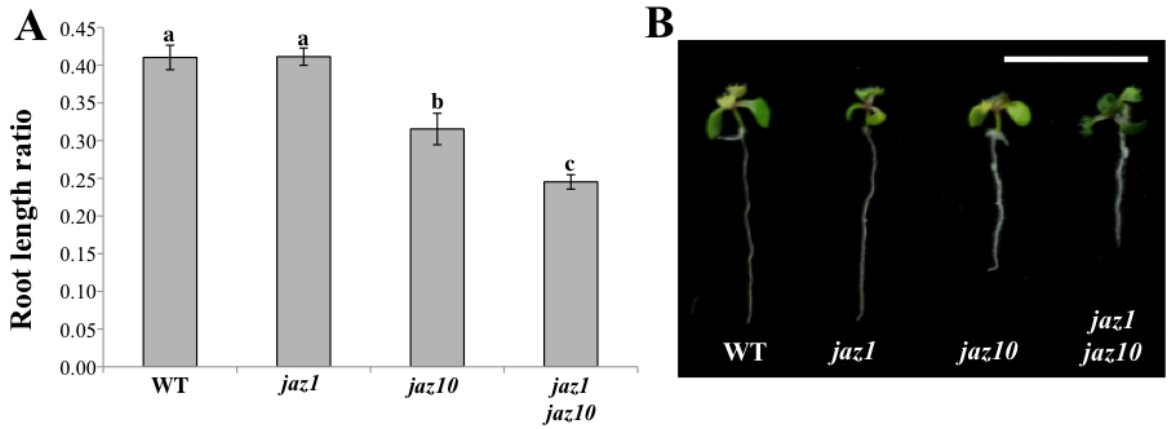

Figure 9. The *jaz1* mutation enhances the phenotype of *jaz10*. A) Seedlings of WT, *jaz1*, *jaz10* and *jaz1 jaz10* double mutant were grown for eight days on MS media with or without 10μM MeJA. The root length ratio was calculated by dividing the average of root length of seedlings grown on MeJA-containing medium by the average root length of seedlings of the same genotype grown in the absence of MeJA. Data show the means ± SE of >15 seedlings. Letters denotes significant differences between genotypes (Student's T-test, $p<0.05$). **B)** Enhancement of *jaz10* phenotype in the *jaz1 jaz10* double mutant (bar = 1cm).

The initial phenotypic characterization of higher-order mutants has already improved our understanding of the complexity of the regulation of specific JA-responses and not only aids the functional characterization of *JAZ10*, but also shares functions with other *JAZ* members. RNA-seq data will show which sets of genes exhibit altered expression in the quintuple mutant shown in Figure 9. This experiment can help in understand which gene sets are involved in root JA-responses and
help to elucidate the complexity of the JA-signaling is achieved throughout the plant.

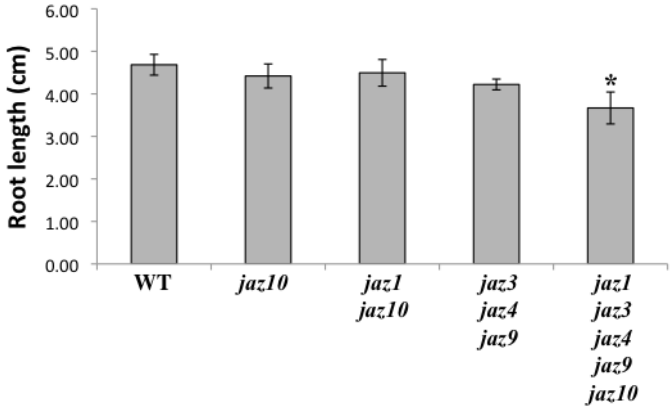

Figure 10. The *jaz1 jaz3 jaz4 jaz9 jaz10* quintuple mutant shows constitutive JA sensitive activation. The *jaz1 jaz3 jaz4 jaz9 jaz10* quintuple mutant show defects in root growth even grown in absence of MeJA in the media (seedlings grown for 8 days on MS media).

Potential pitfalls and solutions: a potential pitfall with the proposed methodology is a possible absence of

phenotype on the mutants created. Although already observed that some double mutants display phenotypes of interest, as for the addictive phenotype of the *jaz1jaz10* double mutant (Figure 9), it remains possible that other crosses with other *JAZ* knockouts will show no observable phenotype. The creation of higher-order mutants even when no phenotype is observed is a possible solution for this weakness. For example, even though the triple mutant *jaz3jaz4jaz9* showed no phenotype (Figure 10), the creation of a higher-order mutant that includes these three mutations and also more *JAZ* mutants (*jaz1jaz3jaz4jaz9jaz10*) made it possible to evaluate some roles for these three genes.

IV. RESEARCH SUMMARY

The present proposal aims to explore the role of *JAZ10* on the jasmonate-signaling pathway. Initial efforts will focus on understanding if the different splice isoforms of this gene can perform different repressive roles and regulate different sets of genes. Completion of "Aim 1" can provide new insights on the different functions of specific JAZ proteins and explore the involvement of JAZ proteins in the underlying mechanism of JA-desensitization. Work on "Aim 2" will complement results of "Aim 1", expanding our understanding of how different JAZ proteins can be involved with how the specificity, diversity and durations of JA responses throughout the plant. Moreover, the approach of evaluating roles of *JAZ10* shared with other *JAZ* family members in "Aim 3" can provide further insights into the complexity of the JAZ-JAZ interactions and advance our understanding into the unknown functions of specific JAZ family members. Understanding the function of a specific JAZ protein is a key step to comprehend how a hormone with such diverse functions can trigger responses so specific throughout the plant.

V. TENTATIVE TIMETABLE

	2012			2013			2014		
	Spring	Summer	Fall	Spring	Summer	Fall	Spring	Summer	Fall
Aim 1. Complementation of *jaz10-1*	X	X	X						
Aim 1. amiRNA silencing of splice variants	X	X	X						
Aim 2. ChIP experiments			X	X	X	X			
Aim 3. Crosses and characterization of high order mutants					X	X	X	X	
Aim 3. Whole transcriptome analysis of high order mutants						X	X	X	
Literature review and preparation of PhD thesis	X	X	X	X	X	X	X	X	X
Thesis defense.									X

VI. POTENTIAL FUNDING SOURCES

One potential funding source for the present proposal is the Network and Regulation Cluster in the Division of Molecular and Cellular Biosciences (MCB) from The National Science Foundation (NSF). This cluster supports research proposals "addressing questions about how cells integrate environmental signals with their internal genetic and metabolic programs to regulate physiology, development or behavior".

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