STRUCTURE-FUNCTION ANALYSIS OF THE JASMONATE RECEPTOR IN ARABIDOPSIS

By

John C. Withers

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Plant Biology

2012
ABSTRACT

STRUCTURE-FUNCTION ANALYSIS OF THE JASMONATE RECEPTOR IN ARABIDOPSIS

By

John C. Withers

The plant hormone jasmonate (JA) plays an important role in regulating growth, development and defense against herbivores and microbial pathogens. A key step in JA signaling is perception of jasmonoyl-isoleucine (JA-Ile) by a co-receptor complex consisting of the F-box protein CORONATINE INSENSITIVE 1 (COI1) and Jasmonate Zim-Domain (JAZ) transcriptional repressors. COI1 is a component of an E3 ubiquitin ligase. The assembly of the COI1-JAZ receptor complex results in ubiquitination and proteasome-mediated degradation of JAZ repressors, which in the absence of JA-Ile bind to and repress transcription factors (e.g., MYC2) that control JA-dependent gene expression. Degradation of JAZ proteins results in transcriptional reprogramming that directs the cellular response to defense and JA-regulated developmental processes.

Specific protein domains from each component of the JA receptor contribute to hormone perception. COI1 contains two conserved domains, an N-terminal F-box domain and a C-terminal leucine-rich repeat (LRR) domain, and it is the LRR domain that confers ligand and substrate specificity. All JAZ proteins share a conserved Jas motif near the C-terminus that is necessary for ligand-dependent binding to COI1. To
gain an in-depth understanding of the COI1-JAZ interaction, I conducted a comprehensive structure-function analysis of the LRR\textsuperscript{COI1} domain and the Jas motif of JAZ9. This analysis revealed amino acids that are critical for the ligand-dependent formation of the COI1-JAZ co-receptor, physical interaction between JAZ9 and a major JA-response transcription factor, MYC2, and nuclear targeting and repressor function of JAZ9 in Arabidopsis. The results from experiments described here contribute to the ongoing understanding of jasmonate perception and signal transduction and provide evidence for a novel transcription factor-dependent mechanism for nuclear import of transcriptional repressors in plants.
ACKNOWLEDGMENTS

Throughout the course of my dissertation research at Michigan State University, I have been fortunate to work with many talented individuals who have enhanced my training as a plant biologist. I would like to thank my advisor, Dr. Sheng Yang He, for his continuous support and intellectually stimulating discussion that have always encouraged me to look for the novelty of a situation that may help to take it to the next level. I will take this lesson with me in science and in life. I also thank the members of my graduate committee, Dr. Brad Day, Dr. Gregg Howe and Dr. Jonathan Walton for always being open to discussions, offering advice, and for helping to shape the course of my research project. Also special thanks to Gregg, because as one of the few people in the He lab studying JA-related questions, I was always grateful for him taking time out of his day for in-depth discussions related to my experiments. I would like to thank all previous and past He lab and Howe lab members that I had a chance to interact with and learn from. I would also like to thank Dr. Melinda Frame at the Center for Advanced Microscopy at Michigan State University for advice on microscopy. Thanks to Dr. John Scott-Craig for taking the time for a final critical read-through of my dissertation and for offering many useful suggestions.

Aside from those who helped guide my research, I have had continuous support from my friends and family. I could not have done it without them. Thanks to Jonathan Davis, a critical scientist and great friend, who stayed up late into the night on many occasions to talk about science and life. Special thanks to my Mom for teaching me to “scrub seashells” when I was a child, thereby imparting to me an appreciation for the extraordinary detail of the natural world. I started down this road to discovery a long
time ago. Finally, I would like to say special thanks to my wife, Saunia Withers, who has supported me from the beginning. When we met, neither of us were practicing scientists in the way we are now. Among other things in life, we have been traveling this path of science together and she has been a constant source of mental and emotional support, intellectual stimulation, advice, and love; it means the world to me.
## TABLE OF CONTENTS

### LIST OF TABLES

- .......................................................................................................................... x

### LIST OF FIGURES

- .......................................................................................................................... xi

### Chapter 1  Role of jasmonic acid in growth, development and defense .......................................................... 1
  1.1  Abstract ........................................................................................................ 2
  1.2  Plant hormones: perception and signaling .................................................... 4
  1.3  Jasmonic acid biosynthesis ........................................................................ 10
  1.4  JA/COR perception and signal transduction ............................................... 11
    1.4.1  The F-Box Protein CORONATINE INSENSITIVE 1 (COI1) ................ 16
    1.4.2  Jasmonate ZIM-Domain (JAZ) transcriptional repressors ............ 18
    1.4.3  Transcriptional regulation of JA response genes .......................... 23
  1.5  Jasmonic acid signaling: a balance between growth and defense signaling pathways in plants ........................................................................ 27
    1.5.1  Regulation of growth and developmental processes ......................... 27
    1.5.2  Jasmonic acid in defense against herbivory ..................................... 29
    1.5.3  Jasmonic acid in abiotic stress responses ........................................ 31
    1.5.4  Jasmonic acid in plant microbe interactions ..................................... 32
    1.5.5  *Pseudomonas syringae* manipulates JA signaling to increase host susceptibility .................................................................. 33
  1.6  Specific aims .............................................................................................. 35
  1.7  Literature cited ............................................................................................ 36

### Chapter 2  Structure-function analysis of the COI1 F-box protein for the formation of the jasmonate co-receptor complex .......................................................... 49
  2.1  Abstract ...................................................................................................... 50
  2.2  Introduction ................................................................................................. 52
  2.3  Materials and Methods ............................................................................... 55
  2.4  Results ...................................................................................................... . 63
    2.4.1  Homology modeling of the COI1 LRR domain defines a putative set of amino acids that may contribute to the formation of the JA co-receptor ....... 63
    2.4.2  Critical amino acids in the ligand binding pocket of COI1 .......... 70
    2.4.3  Differential effects of mutations in the ligand binding pocket on COI1-JAZ interactions in the presence of JA-Ile vs. coronatine ...... 74
    2.4.4  COI1 amino acids contributing to JAZ peptide binding ............... 74
    2.4.5  Charged amino acids contribute to a co-factor (IP5) binding pocket 79
    2.4.6  Functional analysis of mutants that disrupt COI1-JAZ interaction .... 79
    2.4.7  COI1-LRR mutations that may confer hypersensitivity to JA-Ile or coronatine in Y2H assay ............................................................... 85
  2.5  Discussion .................................................................................................. 96
  2.6  Literature cited .......................................................................................... 102
Chapter 3  Transcription factor-dependent nuclear import of transcriptional repressors in jasmonate hormone signaling ............................................. 108
  3.1 Abstract .................................................................................................... 109
  3.2 Introduction ............................................................................................... 110
  3.3 Materials and Methods ............................................................................. 113
  3.4 Results ..................................................................................................... 118
    3.4.1 Constitutive expression of different JAZΔJas proteins results in disparate JA-insensitive phenotypes ......................................................... 118
    3.4.2 Role of MYC2 interaction in nuclear targeting of JAZ1 and JAZ9 ... 119
    3.4.3 Arginine$^{\text{Jas}17}$ is critical for JAZ9 nuclear localization and interaction with MYC2 ..................................................................... 129
    3.4.4 R$^{\text{Jas}17}$ is required for JAZ9 to repress JA responses in planta ...... 138
  3.5 Discussion ................................................................................................ 148
  3.6 Literature cited .......................................................................................... 154

Chapter 4  Conclusions and future directions ............................................................. 162
  4.1 Abstract .................................................................................................... 163
  4.2 Conclusions and future directions ............................................................ 164
  4.3 Mutational analysis of COI1 ...................................................................... 165
  4.4 Mutational analysis of JAZ ........................................................................ 167
  4.5 Practical implications of my research ........................................................ 169
  4.6 Conclusion ................................................................................................ 171
  4.7 Literature cited .......................................................................................... 173
LIST OF TABLES

Table 1  COI1 amino acids chosen for site-directed mutagenesis  ............................ 65
LIST OF FIGURES

Figure 1.1 Sites of hormone perception in the plant cell .................................................. 5
Figure 1.2 Jasmonic acid biosynthesis occurs through enzymatic reactions in the chloroplast and peroxisome ................................................................. 12
Figure 1.3 Production of methyl-jasmonate and jasmonoyl-L-isoleucine ..................... 13
Figure 1.4 The JAZ proteins of Arabidopsis share a conserved Jas domain ............. 21
Figure 1.5 Model of transcriptional regulation of JA-response genes ....................... 25
Figure 2.1 Y2H assay to test the effect of alanine substitutions of COI1-LRR amino acids on interaction with JAZ proteins ..................................................... 68
Figure 2.2 Y2H analysis of the effect of COI1-LRR alanine substitution mutants generated based on the crystal structure of the JA co-receptor ............... 71
Figure 2.3 Point mutations in the ligand binding pocket of COI1 disrupt coronatine-dependent COI1-JAZ interaction ............................................................ 72
Figure 2.4 COI1-LRR alanine substitutions that affect JA-Ile-dependent interaction with JAZ9 ................................................................. 75
Figure 2.5 Model of COI1 in complex with ASK1 and a JAZ1 degron peptide .... 76
Figure 2.6 COI1 amino acids involved in JAZ degron binding ............................... 77
Figure 2.7 COI1 amino acids involved in coordination of the IP5 cofactor ......... 80
Figure 2.8 Root growth inhibition assay ................................................................. 83
Figure 2.9 Anthocyanin production in transgenic seedlings expressing COI1 mutants ................................................................. 86
Figure 2.10 Representative examples of silique production in transgenic Arabidopsis expressing COI1-LRR mutants ............................................................ 87
Figure 2.11 COI1-K147A enhances interaction with JAZ9 ........................................ 90
Figure 2.12 Quantitative Y2H assay testing COI1-K147A interaction with JAZ9 .... 92
Figure 2.13 COI1-K147S also has enhanced interaction with JAZ proteins ........... 94
Figure 3.1  Phenotypic differences in Arabidopsis Col-0 plants expressing JAZ1 or JAZ9 lacking a functional Jas domain ........................................... 120

Figure 3.2  Deletion of the Jas domain differentially effects interaction with MYC2 and sub-cellular localization of JAZ1 and JAZ9 ......................... 122

Figure 3.3  Subcellular localization of COI1 and NINJA ........................................... 125

Figure 3.4  Nuclear localization of JAZ is influenced by interaction with MYC2 ...... 127

Figure 3.5  JAZ9 interacts with the N terminus of MYC2 ........................................ 130

Figure 3.6  Arginine$^{\text{Jas}17}$ in the Jas domain is critical for nuclear localization of JAZ9 ............................................................................................................ 131

Figure 3.7  Localization of YFP:JAZ9 and its mutants in transgenic Arabidopsis seedlings ........................................................................................................... 134

Figure 3.8  Arginine$^{\text{Jas}17}$ is critical for JAZ9 interaction, stability, and colocalization with MYC2 ........................................................................ 136

Figure 3.9  Forced targeting of JAZ9$^{\Delta \text{Jas}}$ to the nucleus is not sufficient to confer JA-insensitivity ........................................................................................................ 140

Figure 3.10  Transgenic expression of YFP:JAZ9-RK$^{\text{Jas}6,7\text{AA}}$ causes insensitivity to rosette growth inhibition by methyl-JA treatment .................. 142

Figure 3.11  Transgenic expression of YFP:JAZ9-RK$^{\text{Jas}6,7}$AA causes JA-insensitivity .................................................................................. 143

Figure 3.12  Root growth inhibition assays for all lines containing JAZ9 alanine substitutions ..................................................................................... 145

Figure 3.13  Transgenic expression of JAZ9-RK$^{\text{Jas}6,7}$AA confers resistance to $P.\ syringae$ pv $tomato$ DC3000 ........................................................................... 146
Chapter 1

The role of jasmonic acid in growth, development and defense
1.1 Abstract

Plant hormones are a diverse collection of small organic compounds that are produced in many tissues and cell-types. Hormone signaling leads to large-scale changes in gene expression that translates environmental signals to phenotypic responses allowing plants to rapidly adapt to challenges from abiotic and biotic stress factors while maintaining normal growth and development. Plant hormones are perceived through physical binding of the hormone to its receptor in target cells or tissue types, and two major biochemical strategies are used to transduce perception of a hormone signal into a cellular response: protein phosphorylation and targeted proteolysis. In particular, the ubiquitin-proteasome pathway for targeted destruction of regulatory proteins plays a crucial role in many aspects of growth, development and defense that are influenced by hormone signal transduction.

Ubiquitin is covalently attached to target proteins by E3 ubiquitin ligases and regulates subcellular localization or function of target proteins. In contrast, poly-ubiquitination results in degradation of the target proteins by the 26S proteasome. Although several classes of E3 ubiquitin ligases have been identified, the SCF-type E3 ubiquitin ligases have emerged as central components of plant hormone perception and signaling. One extensively studied example is the receptor for jasmonic acid (JA), a plant hormone that contributes to both growth and defense-related processes. JA is perceived by a co-receptor complex that includes CORONATINE INSENSITIVE 1 (COI1), the F-box component of an SCF E3 ubiquitin ligase, and members of the Jasmonate ZIM Domain (JAZ) family of transcriptional repressors. The biologically active form of JA promotes formation of the SCF$^{COI1}$ – JAZ receptor complex, which in
turn, poly-ubiquitinates JAZ proteins targeting them for degradation through the 26S proteasome. Degradation of JAZ proteins results in transcriptional reprogramming leading to activation of defense responses.
1.2 Plant hormones: perception and signaling

Throughout their lifecycles, plants perceive environmental signals and translate them into adaptive phenotypic responses. Hormone signal transduction cascades are often central to these integrative responses, leading to large-scale changes in gene expression and effectively reprogramming cellular activity to cope with changes in the environment. For plants, survival to maturity and reproductive success (i.e., fruit set and viable seed production) is often dependent on the ability to rapidly adapt to challenges from abiotic and biotic stress factors while maintaining normal growth and development.

Hormones that regulate cellular responses in plants are a diverse collection of small organic compounds that are produced in many tissues and cell-types. Currently the list of well-characterized plant hormones involved in growth and/or defense includes auxin (IAA), cytokinins (CK), brassinosteroids (BR), gibberellic acid (GA), abscisic acid (ABA), ethylene (ET), jasmonic acid (JA), salicylic acid (SA), and a recently defined class of growth promoting molecules, strigolactones (SL) (Davies, 2010). When hormone biosynthesis is induced by various developmental and environmental stimuli, perception of the signal occurs through physical binding of the hormone to its receptor in target cells or tissue types. Plant hormone receptors can be found at the plasma membrane (PM), the endoplasmic reticulum (ER), in the cytoplasm or within the nucleus. Two major biochemical strategies are used to transduce perception of a signal into a cellular response: regulation of phosphorylation events and targeted proteolysis (Fig. 1.1).
Figure 1.1. Sites of hormone perception in the plant cell.
Figure 1.1 (cont’d). Sites of hormone perception in the plant cell. Hormone receptors can be found at the plasma membrane and endoplasmic reticulum (ER), in the cytoplasm, and in the nucleus. Brassinosteroids are recognized by the extracellular domain of the membrane-bound kinases BRI1-BAK1, which form heterodimer complexes and transduce the signal through phosphorylation cascades. Abscisic acid is perceived by soluble PYR/PYL receptors in the cytoplasm, which then inhibit protein phosphatase activity. Ethylene and cytokinins bind to ER localized histidine kinases that function as two-component phospho-relay systems affecting response regulators in the nucleus. Jasmonoyl-isoleucine (JA-Ile), auxin, and gibberellin are perceived by specific SKIP/CULLIN/F-BOX (SCF) E3 ubiquitin ligases in the nucleus that direct targeted degradation of transcriptional repressors. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.
Several plant hormones including brassinosteroids (BR), cytokinins (CK) and ethylene (ET) are perceived at the plasma membrane or ER. Brassinosteroids are perceived through the activity of the heterodimer complex of BRASSINOSTEROID INSENSITIVE 1 (BRI1) receptor-like kinase and BRI1-ASSOCIATED KINASE (BAK1) and signal transduction occurs through the activation of MAP kinase signaling cascades leading to activation of transcription factors in the nucleus (Nam and Li, 2002; Stepanova and Alonso, 2009; Clouse, 2011). Cytokinins bind to the recognition domain of three ER-localized histidine kinases, ARABIDOPSIS HISTIDINE KINASE (AHK2 and AHK3) and CYTOKININ RESPONSE 1/AHK4. Through auto- and trans-phosphorylation these kinases activate ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER proteins (AHPs) that move into the nucleus and activate transcription factors and other response regulators (Inoue et al., 2001; Argueso et al., 2010; Wulfetange et al., 2011). The gaseous hormone ethylene is also perceived at the ER by members of the ETR1 family of histidine kinase-like proteins that activate MAP kinase signaling and transcriptional responses in the nucleus (Grefen et al., 2008; Stepanova and Alonso, 2009).

Perception of hormone signals also occurs within the cytoplasm and cell nucleus. For example, recognition of the stress hormone abscisic acid (ABA) occurs in the cytoplasm through binding to the PYR/PYL family of START domain-containing proteins. ABA binding inhibits the activity of PP2C phosphatases that in turn represses the activity of SnRK kinases to relieve repression of key transcription factors (Cutler et al., 2010). Defense hormone signaling mediated by SA also occurs within the cytoplasm. An increase in cytoplasmic SA levels leads to nuclear translocation of the
transcriptional co-activator NPR1; the degradation of NPR1 regulates a delicate balance of defense gene activation and induction of systemic acquired resistance (Dong, 2004). SA binds to the recently identified receptors NPR3 and NPR4, substrate adaptors of a CUL3 E3 ubiquitin ligase that targets NPR1 for degradation through the 26S proteasome (Fu et al., 2012). Perception of auxin (IAA), gibberellic acid (GA) and jasmonic acid (JA) occurs in the nucleus where the hormones bind to protein components that confer ligand and substrate specificity of the SKP1(AtASK1)-Cullin1(Cul1)-F-box protein (SCF) E3 ubiquitin ligase and signal through targeted proteolysis of transcriptional repressor proteins (Tan et al., 2007; Sheard et al., 2010; Sun, 2011). Identification of receptors for the classical plant hormones and determination of down-stream hormone signaling mechanisms has provided evidence that utilization of the ubiquitin proteasome pathway for targeted destruction of regulatory proteins plays a crucial role in many aspects of growth, development and defense.

The ubiquitin proteasome system (UPS) is involved in diverse aspects of cellular responses and plays a major role in the activation of many hormone-based responses (Howe, 2010; Kelley and Estelle, 2012). Central to the cellular function of the UPS is ubiquitin, a small protein that is covalently attached to target proteins, typically at an exposed lysine amino acid side chain (Pickart, 2001; Pickart and Eddins, 2004). While conjugation of a single ubiquitin to a target protein can regulate its subcellular localization or function, poly-ubiquitination leads to recognition of the target proteins by the 26S proteasome, a high molecular weight, multi-subunit protease (Voges et al., 1999; Pickart, 2001; Mukhopadhyay and Riezman, 2007). The conjugation of ubiquitin to target proteins occurs through the series of reactions carried
out by three enzymes, designated E1, E2 and E3. First, the E1 ubiquitin activating
enzyme binds and trans-esterifies ubiquitin to an E2 ubiquitin conjugating enzyme, the
second enzyme in the cascade. The E2-ubiquitin intermediate complex is then brought
into close proximity to the target protein by association with the E3 ubiquitin ligase, a
larger protein complex that catalyzes the transfer of ubiquitin to the target protein. The
transfer mechanism is repeated until a poly-ubiquitin chain is generated and recognized
by the 26S proteasome that degrades the target protein.

At least three types of E3 ubiquitin ligases are found in all eukaryotes, including
RING, HECT and U-box E3s, which specify the protein domain responsible for
associating with the E2 conjugating enzymes. In Arabidopsis, RING E3 ligases are
encoded by a gene family consisting of more than 400 members (Freemont, 2000).
RING E3s promote the interaction between E2 conjugating enzymes and substrate
recruitment adaptors through interaction with scaffold proteins known as cullins (CUL),
which include the Anaphase Promoting Complex (APC-E3), the Broad-complex,
Tramtrack, Bric-a-Brac complex (BTB-CUL3-E3) and the Skp-Cullin-F-box (SCF E3)
(Moon et al., 2004). The SCF-type E3 ubiquitin ligases have emerged as central
components of plant hormone perception and signaling with the most extensively
studied examples being the receptors for auxin and JA. A major focus of the research
described in this thesis is an effort to better understand the structural requirements for
JA binding to a co-receptor complex that includes the F-box protein CORONATINE
INSENSITIVE 1 (COI1) and members of the Jasmonate ZIM Domain (JAZ) family of
transcriptional repressors.
1.3 Jasmonic acid biosynthesis

Jasmonic acid (JA), a major plant hormone involved in regulating the balance between growth and defense, belongs to a class of lipid-derived molecules collectively termed jasmonates (JAs) (Wasternack and Hause, 2002; Koo and Howe, 2009; Kazan and Manners, 2011; Yang et al., 2012). The initial steps of JA biosynthesis occur in the chloroplast and involve the release of membrane lipids via the lipoxygenase pathway. Evidence for involvement of phospholipase activity in JA biosynthesis stems from research in both tomato (Solanum lycopersicon) and Arabidopsis thaliana. In tomato, phospholipase A2 (PLA2) activity is stimulated upon wounding (Narváez-Vásquez et al., 1999) and the Arabidopsis DELAYED ANTHER DEHISCENCE 1 (DAD1) phospholipase has been linked to JA biosynthesis and floral organ development (Ishiguro et al., 2001). This first biosynthetic step in the chloroplast results in release and accumulation of linolenic acid (18:3), that is further converted to 12-oxo-phytodienoic acid (OPDA) through the activity of a series of enzymes including lipoxygenases (13-LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC) (Ziegler et al., 2000; Ishiguro et al., 2001).

While the first steps in JA biosynthesis occur in the chloroplast, subsequent steps occur in the peroxisome. The metabolite OPDA is transported into the peroxisome, likely through the activity of an ATP-binding cassette transporter, PXA1 (Footitt et al., 2007). In the peroxisome, OPDA is first converted to 3-oxo-2-(cis-2’-penetenyl)-cyclopentane-1-octanoyl acid (OPC-8:0) through the activity of OPDA reductase (OPR3), then activated by OPC-8:0 CoA ligase (OPCL1) (Koo et al., 2006), and finally subjected to a series of β-oxidation reactions carried out by members of the acyl-CoA
oxidase (ACX) family of enzymes (Schilmiller et al., 2007b). A summary of the JA biosynthetic reactions in the chloroplast and peroxisome is presented in Figure 1.2.

Upon hydrolysis of the CoA moiety, jasmonic acid is released into the cytoplasm where it undergoes further enzymatic conversions including, but not limited to, methylation by JA methyl transferase (JMT) to produce the volatile signaling molecule methyl-jasmonate (MeJA). Additionally, some JA is converted to JA-isoleucine (JA-Ile) by the enzyme JAR1, and minute amounts of other amino acid conjugates have been found (Farmer and Ryan, 1990; Staswick and Tiryaki, 2004; Suza and Staswick, 2008; Suza et al., 2010b). In Arabidopsis, evidence for two naturally occurring stereoisomers of JA-Ile has been reported; the highly bioactive isomer (3R, 7S)-JA-Ile [i.e. (+)-7-iso-JA-Ile], and (3R, 7R)-JA-Ile [i.e. (-)-JA-Ile] that is biochemically less active (Wasternack, 2007; Fonseca et al., 2009) (Fig. 1.3). However, in tomato, only (+)-7-iso-JA-Ile has been detected in wounded leaves (Suza et al., 2010a).

1.4 JA/COR perception and signal transduction

Recent research has elucidated a core JA signaling cascade. Perception of JA-Ile occurs in the nucleus through formation of a co-receptor complex consisting of COI1 and JAZ repressors (Katsir et al., 2008a; Browse, 2009). Formation of the COI1-JAZ receptor complex in the presence of JA-Ile, or the bacterial phytotoxin coronatine (COR), a structural mimic of JA-Ile, results in degradation of JAZ proteins that bind to
Figure 1.2. Jasmonic acid biosynthesis occurs through enzymatic reactions in the chloroplast and peroxisome.
Figure 1.2 (cont’d). Jasmonic acid biosynthesis occurs through enzymatic reactions in the chloroplast and peroxisome. In the first steps of JA biosynthesis, α-linolenic acid released from chloroplast membranes through the activity of phospholipases (PLA/DAD1) is converted to 12-oxo-phytodienoic acid (OPDA). The enzymes involved in ODPA synthesis include lipoxygenases (13-LOX) that synthesize 13-hydroperoxy linolenic acid (13-HPOT), allene oxide synthase (AOS) and allene oxide cyclase (AOC). In the peroxisome, OPDA is first reduced to 3-oxo-2-(cis-2’-peneteryl)-cyclopentane-1-octanoyl acid (OPC-8:0) by OPDA reductase (OPR3), activated by OPC-8:0 CoA ligase (OPCL1) and subjected to a series of β-oxidation reactions mediated in part by members of the acyl-CoA oxidase (ACX) family of enzymes. The final product, jasmonic acid (JA), is released into the cytoplasm for further metabolism.
Figure 1.3. Production of methyl-jasmonate and jasmonoyl-L-isoleucine.
Figure 1.3 (cont’d). Production of methyl-jasmonate and jasmonoyl-L-isoleucine.

Jasmonic acid can be methylated by the enzyme JA-methyl transferase (JMT). This process can be reversed through the activity of methyl jasmonate esterase (MJE). In addition to methylation, the enzyme JAR1 synthesizes JA-isoleucine (JA-Ile). The biologically active form of the hormone is (+)-7-iso-JA-Ile.
and repress the function of JA-responsive transcription factors (TFs) (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007; Fernandez-Calvo et al., 2011). The final outcome of this signaling cascade includes significant transcriptional reprogramming, and typically shifts the balance from growth to defense-related cellular processes. This shift is primarily accomplished through inhibition of genes involved in cell cycle progression and photosynthesis, and activation of defense-related genes (Goossens et al., 2003; Uppalapati et al., 2005; Pauwels et al., 2008; Zhang and Turner, 2008).

1.4.1 The F-box protein CORONATINE INSENSITIVE 1 (COI1)

The initial observation that the bacterial phytotoxin coronatine (COR) and methyl jasmonate exert similar effects on plant growth prompted a genetic screen for Arabidopsis mutants that were resistant to growth inhibition upon treatment with either compound. From this screen, multiple independent alleles of coi1 were isolated that were found to confer pleiotropic phenotypic effects. In addition to insensitivity to COR or MeJA treatment, Arabidopsis coi1 mutants are also male sterile, deficient in JA-induced gene expression, lacking in production of anthocyanins upon treatment with JA, and resistant to infection by a COR-producing strain of Pseudomonas syringae (Feys et al., 1994). The subsequent cloning of COI1 revealed that it encodes a member of the F-box protein family with one of the closest homologs being Arabidopsis TIR1 (Xie et al., 1998; Tan et al., 2007). The F-Box domain at the N-terminus of COI1 facilitates interaction with AtASK1 or AtASK2 to form the SKIP1-CULLIN1-F-box (SCF) E3 ubiquitin ligase complex,
providing a link between COI1 and the ubiquitin proteasome system (Xu et al., 2002). COI1 also contains a C-terminal Leucine Rich Repeat (LRR) domain that confers specificity for both ligand recognition and recruitment of JAZ proteins to the co-receptor complex (Xie et al., 1998; Sheard et al., 2010). Until recently, little was known about the contribution of individual amino acids in the LRR domain of COI1 to ligand perception and recruitment of JAZ proteins. Arabidopsis COI1 shares approximately 33% sequence identity with the auxin receptor TIR1 (Tan et al., 2007) and structural comparisons of these two F-box proteins have been useful in initial investigations into the contribution of specific LRR domain residues to interactions among components of the JA receptor. Specifically, basic residues in COI1 hypothesized to have a role in binding an inositol phosphate co-factor were predicted based on conservation with amino acids in TIR1 that contribute to coordination of inositol-hexakisphosphate (IP6). Recently, the LRR domain of COI1 was shown to coordinate inositol-pentakisphosphate (IP5) as a co-factor that is essential for formation of the receptor complex (Sheard et al., 2010). The contribution of these conserved residues to IP5 binding in COI1 were subsequently confirmed experimentally and mutations of these residues were shown to have an effect on JA signaling in planta (Mosblech et al., 2011). As a portion of the research described here, a series of LRR domain amino acids that were initially hypothesized to play a role in ligand perception, JAZ1 binding, and/or IP5 coordination were shown to disrupt coronatine-dependent COI1-JAZ interactions have also been reported recently (Sheard et al., 2010).
1.4.2 Jasmonate ZIM-Domain (JAZ) transcriptional repressors

In addition to the characterization of COI1, recent advances in the field of JA signal transduction have elucidated the involvement of the JASMONATE ZIM DOMAIN (JAZ) family of transcriptional repressors as key components of this signaling pathway. Initially several JAZ genes were identified based on transcript profiling experiments using the JA biosynthetic mutant opr3 treated with MeJA. In these experiments, the expression of seven previously uncharacterized genes was induced in stamens in response to MeJA. These genes were subsequently shown to contain a conserved ZIM motif that had been previously characterized in a plant-specific transcription factor gene family (Shikata et al., 2004; Thines et al., 2007). Further bioinformatic analysis revealed that Arabidopsis has 12 JAZ proteins that share two conserved functional motifs: the ZIM motif in the central part of the protein and the Jas motif at the C-terminus (Thines et al., 2007; Katsir et al., 2008a). Interestingly, although the JAZ proteins are localized in the nucleus (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007), they possess neither an identifiable nuclear localization signal nor a DNA binding motif, indicating that these proteins might enter the nucleus and function through dimerization with other components of the JA signal transduction pathway. The hypothesis that JAZ proteins exert their repressive effect on transcription by physically interacting with JA-responsive transcription factors and recruiting a larger transcriptional co-repression complex has been confirmed through the research efforts of several independent groups (Chung et al., 2009; Pauwels and Goossens, 2011; Kazan and Manners, 2012).

The JAZ proteins all contain two conserved functional motifs: the ZIM motif near the central portion of the protein and the Jas motif located at the C-terminus. The first
Arabidopsis ZIM (Zinc-finger protein expressed in Inflorescence Meristem) gene was identified as a novel protein during studies on the initiation and development of the inflorescence meristem (Nishii et al., 2000). The ZIM gene contains a region of DNA sequence encoding a zinc finger DNA-binding motif similar to the human GATA-1 transcription factor, suggesting that ZIM could bind directly to DNA. Other ZIM-like (ZML) homologs in plants contain a novel motif named the ZIM domain in addition to the GATA zinc finger domain (Reyes et al., 2004; Shikata et al., 2004; Vanholme et al., 2007). Among the members of the Arabidopsis ZIM domain transcription factor family are the PEAPOD (PPD1 and PPD2) proteins, which lack a GATA DNA binding domain (White, 2006) and are phylogenetically related to JAZ proteins (Katsir et al., 2008a). Recently 18 members of the ZIM family including PPD and JAZ proteins have been grouped into a separate clade designated TIFY proteins, all members of which contain a conserved TIFY(X)G amino acid motif (Vanholme et al., 2007). The TIFY motif in Arabidopsis JAZ proteins mediates homo- and heteromeric interactions among JAZ repressors and interactions with the adapters/co-repressors NOVEL INTERACTOR OF JAZ (NINJA) and TOPLESS (TPL) (Chini et al., 2009; Chung and Howe, 2009; Pauwels et al., 2010).

The JAZ proteins also share a conserved Jas motif near the C-terminus that is 27 amino acids in length and is necessary for interaction with the LRR domain of COI1 (Chini et al., 2007; Thines et al., 2007; Chung et al., 2008; Katsir et al., 2008b; Melotto et al., 2008c; Sheard et al., 2010). The Jas motif is characterized by the consensus sequence SLX₂FX₂KRX₂RX₅PY (Yan et al., 2007). A short peptide of 21 amino acids within the Jas motif defines the minimal “degron” peptide from JAZ1 that is sufficient for
ligand-dependent formation of COI1-JAZ1 receptor complexes (Sheard et al., 2010). All JAZ proteins share a high degree of conservation within this region of the Jas motif. Based on the structural requirements for formation of COI1 receptor complexes with JAZ1, JAZ6, and JAZ8 (Sheard et al., 2010; Shyu et al., 2012), differences in the chemical nature of these amino acids sequences may be linked to differing strengths of interaction between COI1 and individual JAZ proteins (Fig. 1.4).

In addition to contributing to the formation of the JA receptor complex, the Jas motif mediates interactions with MYC transcription factors that regulate gene expression in response to an increase in JA-Ile concentration. Yeast two-hybrid experiments indicate that basic amino acids in the Jas motif of JAZ1 and JAZ9 are critical for ligand-dependent interaction with COI1 yet are not required for interaction with MYC2, indicating the possibility of distinct structural contributions of this motif to interactions with different protein components of the JA signal transduction machinery (Melotto et al., 2008c). The Jas motif also plays an important role in induction of the JA response as it is required for induced degradation of JAZ proteins in the presence of JA-Ile or COR. Several studies have demonstrated that JAZ variants that lack a functional Jas motif, including JAZ1, JAZ3, and JAZ10, are resistant to degradation upon an increase in JA or coronatine levels and thus exhibit dominant JA-insensitive phenotypes (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007; Chung et al., 2008; Melotto et al., 2008c; Chung and Howe, 2009; Chung et al., 2010).
Figure 1.4. The JAZ proteins of Arabidopsis share a highly conserved Jas domain.

A

B

DLPI ARRASL HRFL EKRKDRXVST SPYQ

JAZ12  DLPI ARRHSL QRFL EKRKRDLVKNKPY
JAZ11  DVPI ARRRL QRFF EKRHRFVHTKPYS
JAZ10  DLPI ARRKSL QRFL EKRKERLVSSTSPYY
JAZ3   ALPLARKASL ARFL EKRKVERTSVSPYC
JAZ4   GLPQTRKASL ARFL EKRKERVINVSPYY
JAZ9   SVPQARKASL ARFL EKRKERTLMSAMPYK
JAZ1   ELPI ARRASL HRFL EKRKDRVTSKAPYQ
JAZ2   ELPI ARRASL HRFL EKRKDRTSKAPYQ
JAZ5   VERI ARRASL HRF AKRKRDAVA RAPYQ
JAZ6   VERI ARRASL HRF AKRKRDAVA RAPYQ
JAZ7   QKAMS KR- SLHSFL QKRSRLR IQATSPYH
JAZ8   P KASMKK- SLQSFL QKRKRIRI QATSPYH
Figure 1.4 (cont’d). The JAZ proteins of Arabidopsis share a highly conserved Jas domain. (A) Phylogenetic relationship among members of the JAZ family of transcriptional repressors in Arabidopsis. The twelve JAZ proteins group into five distinct clades based on similarity of their amino acid sequences. Sequence alignments and generation of the phylogenetic tree was performed using ClustalW included in DNA STAR Laser Gene 8 sequence analysis software package. (B) Amino acid sequence alignment of the Jas domain. The degree of sequence conservation is indicated by the heat map above the consensus sequence (blue = little similarity; red = conserved). The Jas degron as defined by the crystal structure of JAZ1-COR/JA-Ile-COI1 complex is delineated with a double red line above the consensus sequence. Sequence variations that may contribute to the strength of ligand-dependent interaction with COI1 are outlined in red.
1.4.3 Transcriptional regulation of JA response genes

In healthy, unwounded tissues with low concentrations of bioactive JA-Ile, JA-responsive genes are typically repressed by JAZ transcriptional repressors. The repressors interact with NINJA to recruit TPL proteins into a multimeric transcriptional co-repression complex that binds to JA-responsive transcription factors (TFs) (Pauwels et al., 2010). Upon wounding, herbivory and/or pathogen attack, bioactive JA-Ile is synthesized by JAR1 and perceived in the nucleus by a co-receptor complex consisting of COI1 and JAZ proteins (Xie et al., 1998; Xu et al., 2002; Chini et al., 2007; Thines et al., 2007; Yan et al., 2007; Fonseca et al., 2009; Sheard et al., 2010). Upon formation of the JA-Ile co-receptor complex, JAZ proteins are poly-ubiquitinated and degraded through the proteasome, thereby releasing target transcription factors from their repressed state and inducing JA-responsive gene expression (Fig. 1.5). Recently, evidence has been presented that recruitment of the Mediator complex, specifically the MED25 and MED16 sub-units, that links the JA-responsive transcription factors to the general RNA polymerase II complex, is a requirement for JA responses (Cevik et al., 2012; Chen et al., 2012; Wathugala et al., 2012).

The JAZ proteins interact with several classes of transcription factors that control downstream physiological responses. Among the best Arabidopsis TFs targeted by JAZ are members of the basic-helix-loop-helix (b-HLH) family, including MYC2, MYC3, and MYC4, that are key regulatory components of diverse aspects of JA-mediated physiological responses (Abe et al., 2003; Lorenzo et al., 2004; Chini et al., 2007; Dombrecht et al., 2007; Melotto et al., 2008c; Cheng et al., 2011; Fernandez-Calvo et al., 2011; Niu et al., 2011). Other b-HLH transcription factors targeted by JAZ proteins
are TRANSPARENT TESTA 8 (TT8), GLABRA 3 (GL3) and ENHANCER of GLABRA 3, which are involved in trichome development and production of anthocyanins (Qi et al., 2011). JAZ proteins also interact with two R2R3 MYB transcription factors, MYB21 and MYB24, which contribute to regulation of reproductive development (Song et al., 2011). JAZ proteins also interact with other signal transduction-related transcription factors including EIN3 and EIL1, which are involved in ethylene signaling, and DELLA repressors, which are involved in GA signaling (Hou et al., 2010; Zhu et al., 2011b; Yang et al., 2012).

In many of these JAZ-TF interactions a requirement for the Jas domain has been demonstrated. How the Jas domain mediates both the interaction with target TFs as well as ligand-mediated formation of SCF$^{\text{COI1}}$-JAZ co-receptor complexes in the nucleus is a point of considerable interest in structure-function approaches to understanding JA signaling. The contribution of individual amino acids in the Jas motif to specific protein-protein interactions has received little attention to date. Recently, evidence has been presented two that basic amino acids in the Jas domain are required for interaction with COI1 and MYC2 and mutagenesis of these important residues in JAZ1 results in dominant JA-insensitivity in Arabidopsis plants overexpressing these JAZ1 variants (Melotto et al., 2008c).
Figure 1.5. Model of transcriptional regulation of JA-response genes.
Figure 1.5 (cont’d). Model of transcriptional regulation of JA-response genes. (A) At resting state JA-response genes are repressed by a protein complex consisting of a JAZ protein bound to a transcription factor such as MYC2. The TIFY domain of JAZ proteins interacts with NINJA that has an EAR motif known to recruit TOPLESS. TOPLESS is responsible for recruiting factors that may alter the state of chromatin structure to repress transcription. (B) Upon increase of biologically active ligands such as JA-Ile or COR, a high affinity co-receptor complex is formed between COI1 and JAZ proteins. JAZ proteins are poly-ubiquitinated and degraded through the 26S proteasome relieving the repression of JA-responsive transcription factors.
1.5 Jasmonic acid signaling: a balance between growth and defense signaling pathways in plants

1.5.1 Jasmonic acid in regulation of growth and developmental processes

Since the discovery of jasmonates and their important role in plants, genetic approaches using mutagenesis and phenotypic screening have been extremely useful in elucidating many of their functional roles. Throughout growth and development JA signaling regulates reproductive development, flowering time and many growth responses involving other hormones. During production of flowers in Arabidopsis, proteins involved in jasmonate biosynthesis including specific lipoxygenases (LOX2, LOX3, and LOX4) and 12-OXO-PHYTODIENOIC ACID REDUCTASE 3 (OPR3), JA-Ile perception (COI1 and JAZ), and tissue-specific gene expression of transcription factors (MYB21 and MYB24) regulate male fertility in Arabidopsis by contributing to the fine tuning of anther elongation, pollen viability, and timing of anther dehiscence (Feys et al., 1994; Sanders et al., 2000; Stintzi and Browse, 2000; Mandaokar et al., 2006; Caldelari et al., 2011; Song et al., 2011). While jasmonates also play a similar role in affecting reproductive development in plant species other than Arabidopsis, the specific influences of JAs may differ. In tomato (Solanum lycopersicon), the JA perception mutant jasmonate insensitive 1 (jai1), which is homologous to AtCOI1, is defective in female reproductive development (Li et al., 2001; Li et al., 2004). In Arabidopsis, jasmonate and gibberellic acid signaling are integrated to balance the expression of MYB TFs involved in stamen development (Cheng et al., 2009). In rice (Oryza sativa),
RNA interference-mediated silencing of OsCOI1 also results in defects in reproductive development such as infertile flowers and the lack of viable seed production, indicating that the JA signaling pathway is also critical for reproduction in monocots (Ye et al., 2012).

Jasmonate signaling is tightly linked with light and other hormone signaling pathways to control growth responses such as photomorphogenesis. The Arabidopsis protein JAR1, which is responsible for synthesizing the bioactive JA-Ile and other JA-amino acid conjugates, affects sensitivity to far red (FR) light-induced hypocotyl elongation associated with the shade avoidance response (Staswick et al., 1992; Staswick et al., 2002). In Arabidopsis, COI1 and JAZ1 are required for full sensitivity to FR light responses in seedlings (Robson et al., 2010). In fact, JAZ1 is among a set of early auxin-induced genes, linking JA and auxin signaling in the control of growth responses (Grunewald et al., 2009). The interaction between JA signaling and light responses extends to GA signaling: recent evidence suggests gene expression in response to light signaling involves a competition between JAZ and DELLA transcriptional repressors for interaction with PHYTOCHROME INTERACTING FACTOR (PIF) TFs (Yang et al., 2012). Furthermore, studies of Arabidopsis MYC2 have shown that it can bind cis elements in light-regulated promoters and function as a negative regulator of photomorphogenesis in response to blue and far-red light (Yadav et al., 2005).

Other growth responses that are influenced by JA signaling include touch-induced developmental responses (thigmomorphogenesis). Jasmonate signaling in response to repetitive touch stimulation requires functional JAR1 and COI1 alleles and
promotes delayed flowering and reduced rosette size (Chehab et al., 2012). Elevated levels of JA also inhibit growth of both the aerial portions and primary root in Arabidopsis. Negative crosstalk between JA and GA pathways is likely involved in growth inhibition of both above and below ground portions of the plant as demonstrated by increased hypocotyl and root growth inhibition in della quadruple mutants in response to JA treatment (Hou et al., 2010). Recently, interaction between the DELLA protein RGL3 and JAZ proteins has been demonstrated along with a requirement for COI1 and MYC2 in JA-induced RGL3 expression that enhances JA responses (Wild et al., 2012). JA-mediated inhibition of root growth also involves auxin signaling. AtMYC2 has been shown to repress the expression of PLETHORA, a key TF regulating activity and maintenance of the root apical meristem (Chen et al., 2011).

Although jasmonates play an important part in plant growth and reproduction, this hormone has been most extensively studied for its role in plant defense responses that are activated by environmental stress factors including tissue damage from herbivorous insects, mechanical wounding, pathogen attack, drought, and UV irradiation (Browse, 2005; Browse and Howe, 2008).

### 1.5.2 Jasmonic acid in defense against herbivory

Damage to plant tissue from herbivory or mechanical wounding results in rapid increases in JA biosynthesis, although the very early events in initiation of stress-induced JA biosynthesis are still under investigation (Chung et al., 2008; Suza and Staswick, 2008; Koo and Howe, 2009; Erb et al., 2012). Additionally, mutants that are
blocked in JA biosynthesis or perception exhibit increased susceptibility to insect herbivory (Kessler et al., 2004; Chung et al., 2008; Koo et al., 2011). Grafting studies in tomato have indicated that acyl-coA oxidase, which catalyzes the first enzymatic reaction in the beta-oxidation of JA precursors in the peroxisome, is required for induced expression of protease inhibitor defense proteins and the induction of systemic responses to tissue damage by insects (Li et al., 2005). Additionally, a specific member of the acyl-coA oxidase family in Arabidopsis, ACX1, is required for resistance to insect herbivores, and the compromised resistance of the mutant can be restored by exogenous application of JA (Schilmiller et al., 2007a). Along with the requirement of earlier steps in the JA biosynthetic pathway for resistance to herbivory, viral induced gene silencing of \textit{NaJAR4} in tobacco (\textit{Nicotiana attenuata}; \textit{Na}) showed that the synthesis of biologically active JA-Ile is also required for defense against caterpillar feeding (Kang et al., 2006).

Perception of JA-Ile is also required for defense against insect herbivores, as mutations in tomato \textit{SlJAI1} and Arabidopsis \textit{AtCOI1} confer increased susceptibility to insect feeding (Chen et al., 2005; Mewis et al., 2005), and transcriptional responses to wounding and herbivory show a requirement for \textit{AtCOI1} (Devoto et al., 2005). Perception of JA in monocots is also required for resistance to herbivory. \textit{OsCOI1} RNAi lines are more susceptible to feeding by chewing insects that damage leaf tissue but not to phloem feeding insects that pierce the leaf tissue (Ye et al., 2012). Many of the \textit{JAZ} genes in Arabidopsis are induced in response to herbivory and wounding (Chung et al., 2008), and mutations of the JA-responsive transcription factor genes \textit{MYC3} and \textit{MYC4}
results in increased susceptibility to herbivory. The myc3/myc4 double mutant has the most severe effect (Fernandez-Calvo et al., 2011).

1.5.3 Jasmonic acid in abiotic stress responses

In crop species, protection from abiotic stress factors such as drought and salinity are of great concern. Research aimed at studying the role that jasmonates play during abiotic stress responses has indicated that induction of JA signaling helps to protect against some specific environmental challenges. During induction of responses to abiotic stresses, JA and ABA signaling are apparently linked. A basic helix-loop-helix (b-HLH) TF from rice (Oryza sativa; OsbHLH148) was up-regulated by exogenous methyl-JA (MeJA) or ABA, a process dependent on OsCOI1 and OsJAZ1 genes, and enhanced drought tolerance when overexpressed in rice plants (Seo et al., 2011). The jasmonate signaling pathway helps protect grape plants (Vitis sp.) from salt stress. Dose-dependent increases in transcript levels of grape homologs of JAZ1, JAZ2 and JAZ3 from V. rupestris and V. riparia are detected when plants are exposed to salt (NaCl) treatment, and application of JA relieves salt-induced growth inhibition of V. riparia, a plant that is hypersensitive to salt stress (Ismail et al., 2012). In soybean (Glycine soja), expression of a JAZ homolog, GsTIFY10, increases upon exposure to bicarbonate and salt stress, and over-expression of GsTIFY10 in Arabidopsis enhances the plant’s tolerance to these abiotic factors (Zhu et al., 2011a). In addition to the examples of JA-mediated drought and salinity tolerance, ultraviolet (UV) irradiation induces expression of JA-responsive genes, most likely through stimulation of early events in the JA
biosynthesis pathway (Conconi et al., 1996). Taken together, many lines of evidence indicate that the protective effect of JA against abiotic stress is common among plants.

1.5.4 Jasmonic acid in plant microbe interactions

In addition to mediating responses to insect herbivory, wounding and abiotic stress, JA signaling is also important for the plant immune response to microbial pathogens. While wild type Arabidopsis plants are resistant to many necrotrophic fungal pathogens including *Alternaria brassicicola* and *Botrytis cinerea*, mutations in *COI1* result in increased susceptibility to both pathogens (Thomma et al., 1998; Glazebrook, 2005). Interestingly, the transcription factor AtMYC2 appears to repress defense responses to fungal pathogens. Arabidopsis *jin1 (myc2)* mutants are more resistant to *B. cinerea* than wild type plants and show increased expression of pathogen responsive genes such as the plant defensin *PDF1.2* (Lorenzo et al., 2004). The requirement of JA-specific signal transduction components for resistance to microbial pathogens is supported by much research. However, JA-dependent defense against necrotrophic pathogens is also tightly linked with ethylene (ET) signaling through the activity of ethylene response factor (ERF) TFs that bind to the GCC box, a *cis*-element present in the promoter of several JA-inducible defense genes (Dong, 1998; Ohme-Takagi et al., 2000; Lorenzo and Solano, 2005; Broekaert et al., 2006; van Loon et al., 2006; Kachroo and Kachroo, 2007; Verhage et al., 2011; Yu et al., 2011). Recently, several members of the ERF family in Arabidopsis, including ERF1, ERF2, ERF5, and ERF6, were shown to be linked to activation of JA-dependent defenses, leading to
increased resistance to *B. cinerea* (McGrath et al., 2005; Moffat et al., 2012). Also, the activity of ETHYLENE INSENSITIVE 3 (EIN3) and EIN3-LIKE1 (EIL1), which control expression of ERF1, is repressed through direct interaction with JAZ1 (Zhu et al., 2011b).

While the integrated JA/ET pathways are critical for defense responses to necrotrophic pathogens, induction of JA signaling results in inhibition of the salicylic acid (SA) pathway, which is involved in defense against biotrophic and hemi-biotrophic pathogens and in systemic acquired resistance (SAR) (Durrant and Dong, 2004; Browse, 2005; Glazebrook, 2005; Mandaokar et al., 2006; Browse and Howe, 2008; Leon-Reyes et al., 2009; Leon-Reyes et al., 2010).

### 1.5.5 *Pseudomonas syringae* manipulates JA signaling to increase host susceptibility

Through the course of co-evolution of plants and microbes, successful plant pathogens have acquired the ability to suppress plant immune responses. The compatible interaction between Arabidopsis and the bacterial pathogen *Pseudomonas syringae* has been extensively used as a model system to probe molecular and biochemical aspects of plant-microbe interactions including pathogen manipulation of host signaling systems. *P. syringae* pv *tomato* DC3000 (*Pst* DC3000) is considered a hemi-biotrophic plant pathogen. The natural infection cycle of *Pst* begins with inocula present on infected seeds or plant tissues and debris. During the growing season *Pst* first exhibits epiphytic growth on the leaf surface (Hirano and Upper, 2000) and enters the plant through
surface wounds and natural openings such as stomata. Endophytic growth within the plant eventually leads to disease. In an elegant example of phytohormone mimicry, several pathovars (pvs) of \textit{P. syringae}, including pvs. \textit{glycinea}, \textit{atropurpurea}, \textit{morsprunorum}, \textit{maculicola} and \textit{tomato}, produce the non-host-specific phytotoxin coronatine (COR) (Bender et al., 1999).

Coronatine is a structural mimic of JA-Ile that increases the susceptibility of the host plant. COR contributes to suppression of host defenses both during bacterial entry through stomata as well as in the apoplast of infected tissue (Bender et al., 1999; Brooks et al., 2005; Melotto et al., 2008a; Zeng et al., 2012). In accordance with a requirement of COR to overcome host defense responses, the COR-deficient \textit{Pst} strain DC3118 cannot re-open stomata and has dramatically reduced bacterial populations in Arabidopsis plants, especially when inoculated onto the plant surface (Melotto et al., 2006).

COR consists of molecular moieties derived from two distinct biosynthetic pathways. Coronafacic acid (CFA) is produced through polyketide biosynthesis and is linked by an amide bond to coronamic acid (CMA), a cyclized derivative of isoleucine (Ichihara et al., 1977; Parry and Mafoti, 1986; Mitchell et al., 1994). At the molecular level, COR is a structural mimic of JA-Ile (3R,7S-JA-L-Ile), the most biologically active isomer of JA-Ile (Staswick, 2008; Fonseca et al., 2009). COR played an important role in the discovery and characterization the JAZ family of transcriptional repressors and the ligand-dependent formation of the jasmonate receptor complex (Katsir et al., 2008c; Melotto et al., 2008b; Fonseca et al., 2009; Sheard et al., 2010). The established role of COR/JA signaling in plant-\textit{P. syringae} interactions makes \textit{Pst} DC3000 pathogenesis an
excellent functional assay for the study of JA signaling. Throughout the research described here, *Pst* DC3000 infections and application of purified COR are used to experimentally probe the response of the JA signaling mutants that were generated and as a ligand to induce COI1-JAZ receptor complex formation.

1.6 Specific aims

The major goal of the research described in this thesis is to achieve a better understanding of the role of specific amino acids involved in the Jas domain of JAZ9 and the LRR domain of COI1 in the formation of the co-receptor complex for JA-Ile and COR. Site-directed mutagenesis was used to generate a comprehensive series of point mutations in the Jas domain of JAZ9 and the LRR domain of COI1. Specific amino acids were identified within the JAZ9 Jas motif and COI1 LRR domain that are necessary for nuclear localization of JAZ repressors, and for interactions between JAZ9 and COI1 and between JAZ9 and a major JA-responsive transcription factor, MYC2. In addition, JAZ9 and COI1 constructs containing selected point mutations were introduced into Arabidopsis for functional analyses including their effects on plant defense mechanisms. These results provide molecular insights into the formation of a key plant hormone receptor involved in regulating plant defense and development.
LITERATURE CITED
1.6 Literature Cited


Chapter 2

Structure-function analysis of the COI1 F-box protein for the formation of the jasmonate co-receptor complex

2.1 Abstract

The F-box protein CORONATINE INSENSITIVE 1 (COI1) is an integral component of the jasmonate (JA) co-receptor in Arabidopsis thaliana (At) along with members of the Jasmonate Zim-Domain (JAZ) family of transcriptional repressors. Biologically active ligands of the JA co-receptor include (+)-7-iso- jasmonoyl-L-isoleucine (JA-Ile) and the bacterial phytotoxin coronatine (COR), a potent structural mimic of JA-Ile. COI1 contains two conserved domains, an N-terminal F-box domain and a C-terminal leucine-rich repeat (LRR) domain. The F-box domain allows COI1 to be assembled into a Skip (AtASK1)-Cullin1-F-box (SCF) E3 ubiquitin ligase complex (SCFCOI1) through direct association with ASK1, whereas the LRR domain confers specificity through recognition of the substrate JAZ repressors. I conducted a comprehensive structure-function analysis of the LRR\textsuperscript{COI1} domain, guided initially by the crystal structure of the auxin receptor protein TIR1, an F-box protein that shares approximately 34% sequence identity with COI1. In total, 43 alanine substitution mutations were made at amino acid positions that are conserved between TIR1 and COI1 and are important for TIR interaction with auxin and other components of the TIR1 receptor complex. Yeast two-hybrid (Y2H) assays were used to test the ability of the resulting COI1 mutants to interact with JAZ1, JAZ3 and JAZ9 in the presence of COR. This analysis revealed amino acids that are critical for the ligand-dependent formation of the COI1-JAZ co-receptor. During the course of this research, the crystal structure of the COI1 protein was solved by Dr. Ning Zheng’s laboratory (Sheard et al., 2010). When mapped to the COI1 structure, many of these residues corresponded to those
that are involved in JA-Ile/COR binding, recruitment of target JAZ proteins, and/or coordination of an inositol phosphate co-factor, thus providing one line of experimental support for the functional relevance of the COI1 crystal structure. Three COI1 mutants that lost ligand-dependent interactions with JAZ proteins in Y2H assays were further analyzed by functional complementation of the null *coi1-1* allele and were found to be loss-of-function mutants *in planta.*
2.2 Introduction

The ubiquitin-proteasome system (UPS) is an important signaling mechanism used by eukaryotic organisms to regulate cellular processes through targeted protein degradation. Polyubiquitination of target proteins occurs through the activity of E3 ubiquitin ligases and typically results in ATP-dependent degradation through the 26S proteasome. Although several types of multi-subunit E3 ligases that control myriad cellular responses have been characterized in plants, the important role of SKIP/CULLIN/F-box (SCF) E3 ligases in plant hormone perception and signaling has only recently become apparent. SCF E3 ubiquitin ligases are composed of four major subunits, including three that are common to all SCF complexes: SKIP1 (AtASK1 and AtASK2 in Arabidopsis), CULLIN1 (CUL1) and the ring finger protein RBX1. The ASK proteins serve as adapters which bind to F-box proteins that confer substrate-specific recognition. Once the ASK1/F-box complex is formed, this complex then associates with the N terminal domain of the scaffold protein CUL1 that also binds to RBX1. RBX1 recruits the E2 ubiquitin conjugating enzyme and positions it in close proximity to the target proteins for transfer of ubiquitin proteins.

In the plant kingdom the F-box proteins comprise one of the largest gene families, and they have been recruited for diverse regulatory mechanisms. F-box domain containing loci exist in all plants, with approximately 700 predicted in Arabidopsis thaliana (Gagne et al., 2002; Hua et al., 2011). F-box proteins have roles in diverse cellular processes including reproductive development and flowering time, circadian rhythms, leaf senescence and phytochrome signaling (Dieterle et al., 2001; Kim et al., 2008; Nelson et al., 2000; Ni et al., 2004; Samach et al., 1999; Woo et al.,
Signal transduction of many of the known plant hormones are also regulated by F-box proteins, either functioning as a component of the hormone receptor itself or during subsequent steps of the signaling pathway. Classic examples of F-box protein components that function in hormone perception include the receptor complexes for auxin (indole-3-acetic acid; IAA) and jasmonic acid (JA). Auxin binds directly to a ligand-binding pocket in the LRR domain of SCF$^{\text{TIR1}}$ and AUX/IAA transcriptional repressors are recruited for ubiquitination (Tan et al., 2007). In addition to ligand binding and recruitment of target proteins, TIR1 also has a positively charged binding pocket for coordination of an inositol hexakisphosphate (IP6) co-factor (Tan et al., 2007). Similar to the mechanism of auxin perception, the biologically active form of jasmonic acid, (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile) (Fonseca et al., 2009), binds in the LRR domain of SCF$^{\text{COI1}}$ forming a co-receptor complex with members of the Jasmonate ZIM domain (JAZ) transcriptional repressors (Katsir et al., 2008b; Sheard et al., 2010).

COI1 was originally discovered in a genetic screen for coronatine insensitive mutants of Arabidopsis (Feys et al., 1994) and was subsequently determined to encode an F-box/LRR domain-containing protein (Xie et al., 1998). COI1 forms a complex with AtCUL1, AtRbx1, and ASK1/ASK2, i.e., the SCF$^{\text{COI1}}$ ubiquitin ligase complex, and is involved in recruiting JAZ transcriptional repressors of the JA response pathway and targeting them for degradation by the 26S proteasome (Xu et al., 2002). COI1 recruitment of JAZ repressors requires active ligand JA-Ile or coronatine, a bacterial toxin that structurally mimics JA-Ile (Chini et al., 2007; Katsir et al., 2008b; Sheard et al., 2010).
In other words, JAZ transcriptional repressors are key components of a high affinity JA-Ile/COR co-receptor. In Arabidopsis, the JAZ transcriptional repressors comprise a family of at least 12 members, all of which share two conserved functional domains: the ZIM domain located at the middle of the protein and the Jas domain near the C-terminus (Katsir et al., 2008a). The ZIM domain is important for mediating homo- and heteromeric protein-protein interactions among the JAZ repressors and recruitment of a multi-subunit transcriptional corepression complex involving NINJA (NOVEL INTERACTOR OF JAZ) and/or TOPLESS or TOPLESS-like (TPL) proteins (Chini et al., 2009; Chung and Howe, 2009; Pauwels et al., 2010). The C terminal Jas domain is necessary for ligand-dependent interaction with the LRR domain of COI1 (Chini et al., 2007; Chung et al., 2008; Melotto et al., 2008; Sheard et al., 2010; Thines et al., 2007). In addition, the Jas domain mediates the interaction of the JAZ proteins with the basic helix-loop-helix proteins AtMYC2, AtMYC3 and AtMYC4 (Chini et al., 2007), which are transcription factors that regulate JA-mediated physiological responses (Abe et al., 2003; Dombrecht et al., 2007; Laurie-Berry et al., 2006; Lorenzo et al., 2004).

Signal transduction initiated through perception of bioactive JAs begins with the formation of the COI1-JAZ co-receptor, in the presence of JA-Ile or COR. The ligand-dependent interaction between COI1 and JAZ repressors occurs between the Jas domain of JAZ repressors and the LRR domain of COI1 (Katsir et al., 2008b; Melotto et al., 2008). In a previous study, it was found that two conserved, positively charged residues within the C-terminal Jas domain of JAZ1 and JAZ9 are crucial for JA-Ile/COR-dependent formation of JAZ-COI1 complexes (Katsir et al., 2008b; Melotto et al., 2008).
In contrast, it was not known which residues in the LRR domain of COI1 are important for ligand-dependent formation of the COI1-JAZ co-receptor. A major focus of my thesis research was to determine the structural contributions of specific amino acids in the COI1-LRR domain to the function of COI1 as a part of the JA receptor.

Initially, I adopted a homology-driven approach to determining the contribution of individual LRR domain amino acids to the formation of the COI-JAZ complex. Guided by the information obtained from the crystal structure of the auxin receptor TIR1 (Tan et al., 2007), I generated a series of individual alanine substitutions at key positions within the COI1 LRR domain. I hypothesized that because of the high degree of sequence conservation between TIR1 and COI1, some of the critical amino acids contributing to the function of COI1 would fall into the same or similar positions as the homologous TIR1 amino acids that have functional contributions to receptor complex formation.

2.3 Materials and Methods

Generation of gene constructs and site-directed mutagenesis of AtCOI1

The coding sequences (CDS) of AtCOI1, AtJAZ1, AtJAZ3 and AtJAZ9 were amplified by reverse transcription of total mRNA extract from Arabidopsis Col-0 leaf tissue using oligo-dT primers followed by first strand cDNA synthesis. From the cDNA preparation, the COI1 CDS was further amplified with Pfull proofreading DNA polymerase (Agilent Technologies, Santa Clara, CA) using gene-specific primers. For COI1, the CDS was
then amplified from the total cDNA using forward and reverse gene specific primers
designed to incorporate a 5' Xmal and 3' Xhol restriction enzyme recognition
sequences. For the JAZ CDSs, gene specific primers designed to incorporate 5' EcoRI
and 3' Xhol restriction enzyme sequences were used. The resulting DNA fragments
were sub-cloned into pCR2.1 TOPO (Life Technologies, Grand Island, NY) and
transformed into E. coli DH5α chemically competent cells. Bacterial colonies were
screened for plasmids containing inserts of interest by colony PCR using one gene-
specific primer and one vector-specific primer. Colonies containing positive clones were
cultured and plasmid extractions using a Wizard Plus SV DNA purification mini-prep kit
(Promega, Madison, WI). Plasmids were sequenced using M13 forward and reverse
primers in combination with gene specific primers to confirm that no additional
mutations were introduced during PCR.

To obtain alanine substitution mutants of COI1, primers for generating alanine
codons at appropriate sites were designed using QuickChangeII primer design software
(www.genomics.agilent.com) and specific mutations were introduced into the coding
sequence by PCR using the QuickChangeII site directed mutagenesis kit (Agilent
Technologies, Santa Clara, CA). Full length sequences for each mutant CDS were
obtained using M13 forward and reverse primers to sequence the 5’ and 3’ ends and
also gene specific sequencing primers to ensure sequence coverage on both DNA
strands.
Generation of yeast two-hybrid (Y2H) constructs

The coding sequences of the JAZ genes as well as the wild type and the alanine substitution mutants of COI1 were released from plasmid pCR2.1 by digestion with appropriate restriction enzymes and the resulting fragments separated by agarose gel electrophoresis. The DNA fragments were purified using a Qiagen gel extraction kit (Qiagen, Valencia, CA). The JAZ coding sequences were ligated into the multi-cloning site of the Y2H vector pB42AD (Clontech, Mountain View, CA) to generate N-terminal fusions to the B42 transcriptional activation domain. The WT and site-directed mutants of COI1 were ligated into the multi-cloning site of the Y2H vector pGilda (Clontech, Mountainview, CA) to generate C-terminal fusions to the LexA DNA binding domain. The ligated DNA was transformed into E. coli DH5 alpha chemically competent cells and selected on LB plates with ampicillin. Positive clones were identified by colony PCR and cultured for plasmid extractions. Plasmids were sequenced using vector-specific primers to confirm that the inserts were in the proper reading frame.

Y2H assays

For the yeast two-hybrid assays, pGilda:COI1 (or mutant COI1s) were co-transformed into yeast strain EGY48 carrying the p8Op:LacZ reporter plasmid along with pB42AD:JAZ1, :JAZ3, :JAZ4 or :JAZ9. Yeast transformation reactions were selected on plates containing SD minimal media (BD Biosciences, San Jose, CA) supplemented with -uracil (U)/-tryptophan (W)/-histidine (H) amino acid drop out solution. Following selection colonies were cultured overnight in liquid SD-UWH drop out media. The
overnight cultures were harvested, washed 2X in sterile water, adjusted to OD$_{600}$ = 0.2 and 10 µl of each culture was spotted onto agar plates containing SD galactose/raffinose-UWH media supplemented with X-gal (80 µg/ml) and coronatine (10 µM for Y2H assays with COI1 and JAZ3 or JAZ9 and 50 µM for Y2H assays with COI1 and JAZ1 or JAZ4). Y2H plates were incubated at 30 °C for 5-7 days and positive interactions/colonies were identified by development of blue color.

Liquid Y2H assays were conducted using either the o-nitrophenyl β-D-galactopyranoside (ONPG, Sigma, St. Louis, MO) as a colorometric substrate for β-galactosidase activity or the β-Glo chemiluminescent Assay System (Promega, Madison, WI) following the manufacturer’s protocol. For both types of liquid assays, yeast clones were cultured in minimal SD-UWH media overnight and cells were harvested by centrifugation at 3,500 rpm for 10 minutes and washed 2X in sterile water. The cells were then resuspended to OD$_{600}$ = 0.2 in SD galactose/raffinose-UWH media and cultured for 18 hours before proceeding with the assays. Liquid ONPG assays were conducted according to the protocol described in the Yeast Protocol Handbook (Clontech, Mountain View, CA).

**Plant transformation constructs**

The promoter of COI1 (PCOI1; a genomic DNA fragment 1,800 bp upstream of the COI1 start codon) and the wild type COI1 CDS had been previously cloned into the pENTR-D TOPO Gateway entry vector in the He laboratory (prepared by Dr. Jian Yao).
Several coi1 point mutants were selected based on the yeast two hybrid assays for further functional analysis in planta. A restriction enzyme digest strategy was designed to create PCOI1:coi1:pENTR-D TOPO constructs containing coi1 point mutants. A 5' EcoRI restriction enzyme recognition sequence and a 3' BamHI recognition sequence already present in the CDS of COI1 were chosen to digest coi1 fragments containing the mutations and the PCOI1:COI1:pENTR-D constructs. The resulting fragments were gel purified and the ligated using T4 DNA ligase (New England Biolabs, Ipswich, MA). The constructs were then sequenced to confirm that the mutant CDSs were in the proper reading frame.

The PCOI1:COI1 and PCOI1:coi1 DNA sequences were then recombined into the plant expression vector pGWB516 (Nakagawa et al., 2007) using LR ClonaseII (Life Technologies, Grand Island, NY). The pGWB516 vector encodes resistance to hygromycin and permits addition of a 4x c-MYC epitope tag for detection of transgenic protein expressions in transformed plants. Full-length sequence for both DNA strands of each construct were obtained in order to confirm the 3' fusion to the epitope tag and to ensure no additional mutations were introduced during re-amplification. Confirmed constructs were introduced into Agrobacterium tumefaciens (GV3101) by electroporation.

**Transformation of Arabidopsis coi1-1 mutants**

Segregating coi1-1 populations were planted in standard Arabidopsis potting medium and germinated in short day (12 h light/12 h dark) conditions. Because homozygous
coi1-1 plants are male sterile, heterozygous plants were used for transformation. Three-week-old plants were genotyped to identify heterozygous COI1/coi1-1 individuals according to the protocol described by Xie et al. (1998). The heterozygous plants were then moved to long day (16 h light/8 h dark; 100 µE light intensity) conditions to induce flowering. Once inflorescences began to develop the plants were transformed using the standard floral dip protocol for Agrobacterium tumefaciens-mediated Arabidopsis transformation (Clough and Bent, 1998).

Selection of transgenic Arabidopsis plants

First-generation transgenic seeds were surface sterilized and germinated on MS media containing 1% sucrose and 50 µg/ml hygromycin to select for seedlings containing the PCOI1:COI1:4xMYC or PCOI1:COI1:4xMYC transgenes. All hygromycin resistant seedlings were transplanted into Arabidopsis potting medium and moved to a long day chamber for selection and propagation of the appropriate parental genotypes. For transgenic plants containing gene constructs of COI1 mutants that did not interact with JAZ proteins and were predicted to confer JA-insensitive phenotypes, heterozygous COI1/coi1-1 parent genotypes were identified using the genotyping protocol cited above in order to maintain viable lines.

For lines containing the wild type pCOI1:COI1:4xMYC construct, or those point mutants that did not disrupt the COI1-JAZ interaction, homozygous coi1-1 parental genotypes were selected. For subsequent generations, all transgenic lines were selected based on hygromycin resistance before proceeding with the experimental
procedures described below. The third generation was used for identification of individuals that were homozygous for the transgene based on segregation analysis of hygromycin resistance in 50 seedlings per line.

**Protein expression analysis**

All lines that were identified from the screening procedure described above were analyzed for expression of the fusion protein by western blotting. Seedlings were selected based on hygromycin resistance and four 4-6 day old seedlings from each line were transferred into liquid MS media in 12-well tissue culture plates and grown for another 6-8 days. Seedlings were harvested from the tissue culture plates 10-12 days after germination, blotted on paper towels to dry, and frozen in liquid nitrogen. Frozen seedlings were ground to a fine power and total protein extracts were prepared in plant protein extraction buffer (PPEB) (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% Triton X-100; 0.1% SDS; 1 mM EDTA, 1 mM DTT; 1% protease inhibitor cocktail (Sigma, St. Louis, MO). The extracts were diluted at 1:5 in PPEB without DTT and protein concentration was measured using the RC/DC protein assay kit with BSA as the standard (BioRad, Hercules, CA). Each sample was adjusted to an equal concentration based on the lowest protein quantity detected, usually 5 µg/µl in PPEB. After samples were adjusted to equal concentration, 2x SDS loading buffer (sterile water; 120 mM Tris-HCl, pH 6.8; 10% glycerol; 4% SDS; 5% beta-mercaptoethanol and 0.05% bromophenol blue) was added and the samples were incubated at 95 °C for 10 minutes.
For each sample, 20 µg of the total protein extraction was resolved by SDS polyacrylamide gel electrophoresis and transferred to PVDF membranes for western blot analysis. Membranes were blocked for one hour with 5% low fat dry milk dissolved in tris-buffered saline (TBS), probed for one hour at room temperature or over-night at 4 °C, with rabbit polyclonal anti-c-MYC primary antibodies (Clontech, Mountainview, CA) at a concentration of 1:5,000, and washed two times with TBS-Tween 20 (TBST) and once with TBS before addition of a goat anti-rabbit secondary antibody conjugated to horse radish peroxidase (HRP), at a concentration of 1:30,000. Membranes were incubated in the secondary antibody for one hour at room temperature and washed four times with TBST. HRP activity from the secondary antibody was with the SuperSignal WestPico chemiluminescent detection kit (Thermo Scientific, Waltham, MA) following the manufacturer’s directions. The membranes were then exposed to X-ray film for appropriate lengths of time to visualize the signal.

**Root growth inhibition assays**

Arabidopsis Col gl1, coi1-1 and transgenic pGWB516:PCOI1:coi1: seedlings were surface-sterilized, cold-stratified, and germinated on ½ strength MS agar media containing 50 µg/ml hygromycin. Four days after germination, seedlings were transferred to MS agar plates with either no treatment or plates with 10 µM methyl jasmonate (Sigma, Hercules, CA) added to the media. Seedlings were grown for 10-12 days in long day light conditions (16 hr light : 8 hr dark; 100 µE light intensity). Images
of seedlings on the plates were collected by scanning and root lengths were measured using ImageJ software (http://rsbweb.nih.gov/ij/).

**Anthocyanin measurements**

Anthocyanin content was determined using a previously published protocol (Deikman and Hammer, 1995). Briefly, seedlings were grown for 14 days in MS media with or without 10 µM methyl-JA. Eight seedlings per genotype per treatment were harvested and fresh weight was recorded. Seedlings were placed in 1 ml of 1-propanol:HCl:H₂O (18:1:81), vortexed for 10 seconds, incubated at 100 °C for 3 - 5 mins, and left overnight at room temperature in the dark. One milliliter of supernatant was collected and sample absorbances were measured at 535 nm and 650 nm. Anthocyanin levels were reported as (A₅₃₅–A₆₅₀) g⁻¹ fresh weight.

**2.4 Results**

**2.4.1 Homology of the COI1 and TIR1 LRR domains defines a set of amino acids that may contribute to the formation of the JA receptor.**

When the crystal structure of the auxin receptor TIR1 was solved, the amino acids contributing to formation of the ligand binding pocket, binding to the AUX/IAA peptide, and coordination of an IP6 cofactor were annotated in the context of an amino
acid sequence alignment of TIR1, four other Auxin F-box Proteins (AFBs) and COI1 (Tan et al., 2007). The significant sequence homology between TIR1 and the JA receptor COI1 prompted an initial hypothesis that functional surfaces in the LRR domain of COI1 would be positioned in similar locations in the three dimensional protein fold, and that key differences in these conserved residues would confer specificity to the differential recognition of the respective ligands (i.e., auxin vs. JA-Ile/COR) and target transcriptional repressors (i.e., AUX/IAAs vs. JAZs). Based on this hypothesis, 32 amino acids were initially chosen for site-directed mutagenesis to determine their individual contributions to the formation of the COI1-JAZ receptor complex. The specific COI1 amino acids that were chosen, their effects on the COR-dependent COI1- JAZ9 interaction in Y2H assays, and a comparison to the corresponding TIR1 residues and their contribution to the function of the auxin receptor are listed in Table 1. We found that 56% (18 out of 32) of the alanine substitution mutants had altered levels of interaction between COI1 and JAZ9 in Y2H assays (Fig. 2.1). Similar although not identical effects were observed for COI1 interactions with JAZ1, JAZ3, and JAZ4. For example, alanine substitutions at R85, E173, or W467 disrupted the interaction between COI1 and JAZ1 or JAZ4, but not interaction with JAZ3 or JAZ9 (Fig. 2.1). Three alanine substitutions, at E355, G357 and Q491, only disrupted COI1 interaction with JAZ4 (Fig. 2.1). Taken together the results of the initial Y2H screen indicate that the interaction between COI1 and individual JAZ proteins may have differences.
### Table 1. COI1 amino acids chosen for site-directed mutagenesis

<table>
<thead>
<tr>
<th>COI1 Amino Acids</th>
<th>JAZ9 Interaction&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Homologous TIR1 Amino Acid&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Role of TIR1 Amino Acid&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine 54&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+++</td>
<td>Lysine</td>
<td>Not known</td>
</tr>
<tr>
<td>Serine 77&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+++</td>
<td>Serine</td>
<td>Not known</td>
</tr>
<tr>
<td>Lysine 79&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+++</td>
<td>Glutamate</td>
<td>Not known</td>
</tr>
<tr>
<td>Lysine 81</td>
<td>+++</td>
<td>Lysine</td>
<td>IP6 coordination</td>
</tr>
<tr>
<td>Arginine 85</td>
<td>+++</td>
<td>Histidine</td>
<td>Auxin binding, IP6 coordination</td>
</tr>
<tr>
<td>Methionine 88</td>
<td>No</td>
<td>Aspartate</td>
<td>Aux/IAA binding</td>
</tr>
<tr>
<td>Phenylalanine 89</td>
<td>No</td>
<td>Phenylalanine</td>
<td>Auxin and AUX/IAA binding</td>
</tr>
<tr>
<td>Leucine 91</td>
<td>No</td>
<td>Leucine</td>
<td>Aux/IAA binding</td>
</tr>
<tr>
<td>Histidine 118&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+++</td>
<td>Arginine</td>
<td>Not known</td>
</tr>
<tr>
<td>Arginine 121</td>
<td>No</td>
<td>Arginine</td>
<td>IP6 coordination</td>
</tr>
<tr>
<td>Lysine 144&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+++</td>
<td>Valine</td>
<td>Not known</td>
</tr>
<tr>
<td>Lysine 147</td>
<td>++++</td>
<td>Serine</td>
<td>Aux/IAA binding</td>
</tr>
<tr>
<td>Glutamate 173</td>
<td>++</td>
<td>Glutamate</td>
<td>Aux/IAA binding</td>
</tr>
<tr>
<td>Methionine 201&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+++</td>
<td>Cysteine</td>
<td>Not known</td>
</tr>
<tr>
<td>Leucine 301&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+++</td>
<td>Serine</td>
<td>Not known</td>
</tr>
<tr>
<td>Tyrosine 302&lt;sup&gt;c&lt;/sup&gt;</td>
<td>No</td>
<td>Tyrosine</td>
<td>Not known</td>
</tr>
<tr>
<td>Arginine 326&lt;sup&gt;c&lt;/sup&gt;</td>
<td>No</td>
<td>Leucine</td>
<td>Not known</td>
</tr>
<tr>
<td>Arginine 348</td>
<td>No</td>
<td>Arginine</td>
<td>IP6 coordination</td>
</tr>
<tr>
<td>Arginine 351</td>
<td>No</td>
<td>Proline</td>
<td>AUX/IAA binding</td>
</tr>
<tr>
<td>------------</td>
<td>----</td>
<td>---------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Aspartate 354</td>
<td>No</td>
<td>Glutamate</td>
<td>AUX/IAA binding</td>
</tr>
<tr>
<td>Glutamate 355</td>
<td>+++</td>
<td>Proline</td>
<td>AUX/IAA binding</td>
</tr>
<tr>
<td>Glutamine 356</td>
<td>+++</td>
<td>Phenylalanine</td>
<td>AUX/IAA binding</td>
</tr>
<tr>
<td>Glycine 357</td>
<td>++</td>
<td>Valine</td>
<td>AUX/IAA binding</td>
</tr>
<tr>
<td>Tyrosine 386</td>
<td>No</td>
<td>Phenylalanine</td>
<td>Auxin and AUX/IAA binding</td>
</tr>
<tr>
<td>Aspartate 407</td>
<td>No</td>
<td>Arginine</td>
<td>IP6 coordination</td>
</tr>
<tr>
<td>Arginine 409</td>
<td>No</td>
<td>Arginine</td>
<td>Auxin and AUX/IAA binding</td>
</tr>
<tr>
<td>Leucine 410</td>
<td>No</td>
<td>Leucine</td>
<td>Auxin binding</td>
</tr>
<tr>
<td>Valine 411</td>
<td>No</td>
<td>Cysteine</td>
<td>Auxin and AUX/IAA binding</td>
</tr>
<tr>
<td>Leucine 412</td>
<td>No</td>
<td>Isoleucine</td>
<td>Aux/IAA binding</td>
</tr>
<tr>
<td>Leucine 413</td>
<td>No</td>
<td>Isoleucine</td>
<td>Aux/IAA binding</td>
</tr>
<tr>
<td>Arginine 415</td>
<td>++</td>
<td>Proline</td>
<td>AUX/IAA binding</td>
</tr>
<tr>
<td>Arginine 440</td>
<td>++</td>
<td>Arginine</td>
<td>IP6 coordination</td>
</tr>
<tr>
<td>Phenylalanine 443</td>
<td>No</td>
<td>Leucine</td>
<td>Auxin binding</td>
</tr>
<tr>
<td>Tyrosine 444&lt;sup&gt;c&lt;/sup&gt;</td>
<td>No</td>
<td>None</td>
<td>Not known</td>
</tr>
<tr>
<td>Tryptophan 467</td>
<td>+++</td>
<td>Methionine</td>
<td>IP6 coordination</td>
</tr>
<tr>
<td>Leucine 469</td>
<td>No</td>
<td>Serine</td>
<td>Auxin binding</td>
</tr>
<tr>
<td>Leucine 470</td>
<td>No</td>
<td>Valine</td>
<td>Auxin binding</td>
</tr>
<tr>
<td>Tyrosine 472</td>
<td>+++</td>
<td>Phenylalanine</td>
<td>AUX/IAA binding</td>
</tr>
<tr>
<td>Glutamine 491</td>
<td>+++</td>
<td>Arginine</td>
<td>IP6 coordination</td>
</tr>
</tbody>
</table>
Table 1. (cont’d)

<table>
<thead>
<tr>
<th>Lysine 492</th>
<th>+++</th>
<th>Lysine</th>
<th>IP6 coordination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine 496</td>
<td>+++</td>
<td>Arginine</td>
<td>Auxin and AUX/IAA binding</td>
</tr>
<tr>
<td>Arginine 516</td>
<td>No</td>
<td>Arginine</td>
<td>IP6 coordination</td>
</tr>
</tbody>
</table>

a) Y2H assays were conducted with 10 µM coronatine included in the medium. The strength of interaction for COI1-JAZ9 is (+++). The strength of coi1-JAZ9 interactions are scored relative to COI1-JAZ9 and are indicated by “+” symbols.

b) Amino acid alignment of COI1 with TIR1 and structural annotations of TIR1 were reported by Tan et al. (2007).

c) Additional amino acids selected for mutagenesis based on data from the crystal structure of the COI1-JAZ1 degron peptide in complex with coronatine or JA-Ile.
Figure 2.1. Effect of alanine substitutions in COI1-LRR on the interaction with JAZ proteins.

<table>
<thead>
<tr>
<th>Alanine Substitution Mutants of COI1-LRR</th>
</tr>
</thead>
<tbody>
<tr>
<td>81 R M F L R K 147 E R R D E 351 354 355</td>
</tr>
<tr>
<td>JAZ1</td>
</tr>
<tr>
<td>JAZ3</td>
</tr>
<tr>
<td>JAZ4</td>
</tr>
<tr>
<td>JAZ9</td>
</tr>
<tr>
<td>356 Q G Y D R L 411 V L L R R F 440 443</td>
</tr>
<tr>
<td>JAZ1</td>
</tr>
<tr>
<td>JAZ3</td>
</tr>
<tr>
<td>JAZ4</td>
</tr>
<tr>
<td>JAZ9</td>
</tr>
<tr>
<td>467 W L L Y Q K R R 496 R 516 COI1 BD AD</td>
</tr>
<tr>
<td>JAZ1</td>
</tr>
<tr>
<td>JAZ3</td>
</tr>
<tr>
<td>JAZ4</td>
</tr>
<tr>
<td>JAZ9</td>
</tr>
</tbody>
</table>
Figure 2.1 (cont’d). Effect of alanine substitutions in COI1-LRR on the interaction with JAZ proteins. JAZ proteins were expressed as translational fusions to the B42 transcriptional activation domain (AD) and COI1 mutants were fused to the LexA DNA binding domain (BD). The gene constructs were co-transformed into the yeast strain EGY48 carrying the p8OpLacZ reporter plasmid. Positive interactions between COI1 variants and JAZ proteins are indicated by blue color. Assay plates were supplemented with varying concentrations of coronatine as a ligand to facilitate interactions (50 µM for JAZ1-COI1 and JAZ4-COI1, 10 µM for JAZ3-COI1 and JAZ9-COI1).
During my initial characterization of the 32 COI1 alanine substitution mutants, the crystal structure of COI1 was solved in the laboratory of Ning Zheng at the University of Washington. In collaboration with the Zheng lab and based on their COI1 structural data, I generated ten additional alanine substitution mutants in order to help validate the contribution of amino acids that had potential to be functionally important for receptor complex formation (Table 1). Of these ten additional mutants, six did not disrupt interaction with any JAZ protein tested. Only three, Y302A, R326A and Y444A, disrupted interaction with all JAZ proteins tested (Fig. 2.2). A fourth amino acid substitution, L301A, disrupted the COR-dependent interaction with JAZ1 but not with JAZ3 or JAZ9 (Fig. 2.2).

2.4.2 Critical amino acids in the ligand binding pocket of COI1.

Based on the structural similarity between COI1 and TIR1, we mutagenized nine COI1 amino acids that were predicted to be located in the putative ligand binding pocket and therefore might contribute to the recognition of JA-Ile and COR. Of these nine residues, only three were conserved between COI1 and TIR1; phenylalanine 89, arginine 409 and leucine 410. When mutated F89A and R409A disrupted the COI1 interaction between all JAZs tested (Fig. 2.3).
Figure 2.2. Y2H analysis of the effect of COI1-LRR alanine substitution mutants generated based on the crystal structure of the JA co-receptor. JAZ proteins were expressed as translational fusions to the B42 transcriptional activation domain (AD), and COI1 mutants were fused to the LexA DNA binding domain (BD). The gene constructs were co-transformed into the yeast strain EGY48 carrying the p8OpLacZ reporter plasmid. Positive interactions between COI1 variants and JAZ proteins are indicated by blue color. Assay plates were supplemented with varying concentrations of coronatine as a ligand to facilitate interactions (50 µM for JAZ1-COI1, 10 µM for JAZ3-COI1 and JAZ9-COI1) and 80 µg/ml X-Gal.
Figure 2.3. Point mutations in the ligand binding pocket of COI1 disrupt coronatine-dependent COI1-JAZ interaction.
Figure 2.3 (cont’d). **Point mutations in the ligand binding pocket of COI1 disrupt coronatine-dependent COI1-JAZ interaction.** Top panel: Amino acids in the ligand-binding pocket of COI1, including an overlay of JA-Ile (orange) and coronatine (green) and a portion of the JAZ1 degron peptide (red). This ligand-binding pocket was generated using PyMol software and structural coordinates from PDB:3OGK and PDB:3OGL. Bottom panel: Y2H analysis of JAZ1, JAZ3, and JAZ9 interaction with COI1 variants. Positive interactions are indicated by blue colony color. COI1-JAZ1 assay plates were supplemented with 50 µM coronatine, COI1-JAZ3 and COI1-JAZ9 plates were supplemented with 10 µM coronatine (+COR). All plates contained 80 µg/ml X-Gal.
2.4.3 Differential effects of mutations in the ligand binding pocket on COI1-JAZ interactions in the presence of JA-Ile vs. coronatine.

To further investigate the contribution of individual amino acids in the ligand binding pocket of COI1, liquid Y2H assays were used to determine whether any point mutations differentially disrupted interaction with only one of the biologically relevant ligands of the receptor complex (i.e., JA-Ile or coronatine). Alanine substitutions at two amino acid positions, R85 and R496, completely disrupted JA-Ile-dependent COI1-JAZ9 interactions, but only marginally reduced coronatine-dependent interactions (Fig. 2.4). Mutation of R409 reduced both JA-Ile and coronatine-dependent interaction between COI1 and JAZ9, and all other amino acids tested in this assay completely disrupted COI1-JAZ9 interaction in the presence of either ligand (Fig. 2.4).

2.4.4 COI1 amino acids contributing to JAZ peptide binding.

The crystal structure of the COI1-JAZ1 receptor complex revealed that the JAZ degron peptide binds to the top surface of the LRR domain of COI1 and lies over the top of the ligand, effectively locking it into place (Fig. 2.5). Of the 18 COI1-LRR amino acids that were predicted to contribute to the formation of a binding surface for JAZ proteins (Table 1), I found only eight to be essential for JAZ interaction (Fig. 2.6).
Figure 2.4. COI1-LRR alanine substitutions that affect JA-Ile-dependent interaction with JAZ9. Results from of liquid Y2H assays using a luminescence β-galactosidase system are represented as relative light units (RLU) that indicate the degree of interaction between COI1 mutants and JAZ9 in the presence of either 1 µM coronatine (blue bars), 10 µM JA-Ile (red bars), or 1% DMSO negative control (green bars). Error bars represent the standard deviation among three independent biological replications.
Figure 2.5. Surface model of COI1 in complex with ASK1 and a JAZ1 degron peptide. (A, B) The ligand binding pocket of COI1. JA-Ile (gray) packs tightly against the interior surface and is coordinated by amino acids in the binding pocket (green). The surface where the JAZ degron interacts is shown in blue. (C, D) Side and top view of COI1 in complex with the JAZ1 degron peptide. (C) The Arabidopsis ASK1 protein (grey) interacts with the F-Box domain of COI1 (tan) and serves as an adapter for the formation of the SCF$^{COI1}$ E3 ubiquitin ligase. The JAZ1 degron peptide (red) binds to the top surface of the LRR domain of COI1 and covers the ligand binding pocket. Surface modeling was conducted using PyMol software and molecular coordinates from PDB:3OGL.
Figure 2.6. COI1 amino acids involved in JAZ degron binding.
Figure 2.6 (cont’d). **COI1 amino acids involved in JAZ degron binding.** Top panel: A model of the COI1 amino acids contributing to the recruitment of a JAZ1 degron peptide. JA-Ile (orange) and coronatine (green) are also shown in this model. This model was generated using PyMol software and structural coordinates from PDB:3OGK and PDB:3OGL. Bottom panel: Y2H analysis of JAZ1, JAZ3, and JAZ9 interaction with COI1 mutants. Positive interactions are indicated by blue colony color. COI1-JAZ1 assay plates were supplemented with 50 µM coronatine, COI1-JAZ3 and COI1-JAZ9 plates were supplemented with 10 µM coronatine (+COR).
2.4.5 Charged amino acids contribute to a co-factor (IP5) binding pocket.

Ten amino acids predicted to have an important role in coordinating the inositol phosphate co-factor were chosen for mutagenesis (Table 1). The inositol phosphate co-factor for TIR1 was identified as IP6 (Tan et al., 2007), while in the COI1 structure, IP5 appears to be the co-factor (Sheard et al., 2010). The results of my Y2H analysis indicated five alanine substitutions in the potential IP5-binding pocket had no effect on interaction with JAZ9 and five alanine substitutions (R121, R348, D407, R409 and R516), disrupted COI1-JAZ9 interaction (Fig. 2.7). Differential effects on COI1-JAZ interactions were also observed for some mutations. The R85A mutation only disrupted interaction with JAZ1, not JAZ3 or JAZ9 (Fig. 2.7). Additionally, R121A disrupted interaction with JAZ1 and JAZ9, but not JAZ3 (Fig. 2.7).

2.4.6 Functional analysis of mutants that disrupt COI1-JAZ interaction.

Y2H analysis of the LRR domain of COI1 revealed a number of amino acid residues that are important for the formation of the JA receptor complex in the presence of coronatine or JA-Ile. To test the physiological relevance of mutations in the ligand binding pocket and at the JAZ peptide binding surface in planta, I selected three COI1 alanine substitutions for functional complementation tests in the coi1-1 mutant background. For a ligand binding pocket mutation, Y444A was selected because it disrupted interaction with all JAZs tested (Fig. 2.3). For mutations of the JAZ peptide binding surface, Y302A and R326A were chosen as they also disrupted interaction with all JAZs tested (Fig. 2.6).
Figure 2.7. COI1 amino acids involved in coordination of the IP5 cofactor.
Figure 2.7 (cont’d). COI1 amino acids involved in coordination of the IP5 cofactor. Top panel: A model of the amino acids contributing to the formation of an IP5-binding pocket. The model was generated using PyMol software and structural coordinates from PDB:3OGK and PDB:3OGL. Bottom panel: Y2H analysis of JAZ1, JAZ3, and JAZ9 interaction with COI1 mutants. Positive interactions are indicated by blue colony color. COI1-JAZ1 assay plates were supplemented with 50 µM coronatine, COI1-JAZ3 and COI1-JAZ9 plates were supplemented with 10 µM coronatine (+COR).
Transgenic plants expressing these gene constructs under control of the promoter for COI1 were generated. To determine the responses of the transgenic plants to exogenous application of JA, root growth assays were conducted on MS agar medium supplemented with 10 µM methyl JA (MeJA).

Seedlings of independent transgenic Arabidopsis lines carrying each of the indicated mutations in the coi1-1 genetic background, Col gl1 (parent of coi1-1), and coi1-1 mutant were grown for 14 days. As expected, Col gl1 seedlings were sensitive to MeJA treatment, as indicated by inhibition of root growth compared to seedlings grown on media containing no treatment (Fig. 2.8A). All three alanine substitutions that were transformed into coi1-1 mutants failed to restore sensitivity to MeJA treatment to the level of Col gl1 (Fig. 2.8A). Of the three COI1 mutants introduced into Arabidopsis, Y444A appeared to be most defective, with root growth that was not statistically different from that of coi1-1 seedlings. Y302A was partially defective (Fig. 2.8A). Western blot analysis showed that all lines were expressing COI1 proteins (Fig. 2.8B).

Since strong coi1 alleles (e.g., coi1-1) are known to confer pleiotropic JA phenotypes, I examined other JA-dependent physiological responses such as the production of anthocyanins and fertility. To determine if transgenic seedlings were affected in anthocyanin biosynthesis, seedlings were treated with MeJA for 14 days as described for the root growth assays and total anthocyanin content was measured in eight seedlings per genotype per treatment. All seedlings that showed sensitivity to MeJA treatment as indicated by inhibition of root growth (Fig. 2.8A) produced anthocyanins (Fig. 2.9).
Figure 2.8. Root growth inhibition assay.

A

![Bar graph showing mean root length (mm) for different treatments.]

B

![Western blot analysis showing MYC protein expression under different conditions.]

α MYC

CBB
Figure 2.8 (cont’d). Root growth inhibition assay. (A) Mean root lengths of Col gl1, transgenic coi1-1 mutants expressing alanine substitution mutants in the LRR domain of COI1, and the coi1-1 mutant. Statistically significant differences as determined by one-way ANOVA followed by Tukey’s HSD test are indicated by different lower case letters. (B) Western blot analysis of transgenic Arabidopsis seedlings expressing the indicated gene constructs in a population that was segregating the coi1-1 allele. NT = mock treatment; S = seedlings that were sensitive to MeJA because of the presence of a COI1 allele; I = JA-insensitive, homozygous coi1-1 seedlings expressing the indicated gene constructs. Transgenic fusion proteins were detected in total protein extracts using rabbit polyclonal antibodies to the c-MYC epitope tag (α MYC). PVDF membranes were stained with Coomassie brilliant blue (CBB) as loading control.
Interestingly, transgenic seedlings expressing the Y302A or R326A fusion proteins that were partially insensitive to MeJA in root growth assays still produced elevated levels of anthocyanins, indicating that these seedlings were not deficient in all aspects of JA signaling responses (Fig. 2.9). Of the three mutations tested, only COI1-Y444A lines had completely disrupted anthocyanin biosynthesis (Fig. 2.9). These results are in agreement with root growth assay results, suggesting that Y444A is a completely nonfunctional allele, whereas the alleles in Y302A and R326A are partially functional.

To examine the effect of mutations on fertility, eight seedlings per genotype were transplanted to Arabidopsis potting medium and grown to maturity under long day conditions. After six weeks, inflorescence stems were examined for the ability to produce siliques. Col gl1 plants and a transgenic coi1-1 line carrying a PCOI1:COI1:4xMYC fusion construct were included as positive controls and all plants from these lines produced siliques as expected (Fig. 2.10). Plants that were expressing COI1-Y302A and COI1-R326A produced few or no siliques on any inflorescence stems, while no siliques were produced on plants expressing the COI1-Y444A construct, similar to the coi1-1 mutants (Fig. 2.10).

2.4.7 COI1-LRR mutations that enhance COR or JA-Ile-dependent interactions between COI1 and JAZ proteins.

During investigation of the effects of mutations in the COI1-LRR domain, one particular alanine substitution, K147A, not only did not disrupt coronatine-dependent COI1-JAZ
Figure 2.9. Anthocyanin production in transgenic seedlings expressing COI1 mutants. Eight seedlings were selected from each genotype and treatment. Seedlings were grown on MS medium with no treatment (NT). Since the transgenic lines were segregating the transgene, some seedlings that were grown on plates supplemented with 10 µM MeJA were sensitive to MeJA (JA-S) and a quarter of the population were insensitive to MeJA (JA-I).
Figure 2.10. Representative examples of silique production in transgenic Arabidopsis expressing COI1-LRR mutants.

A

<table>
<thead>
<tr>
<th></th>
<th>COI1</th>
<th>Y302A</th>
<th>Y302A</th>
<th>R326A</th>
<th>R326A</th>
<th>Y444A</th>
<th>Y444A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-gl</td>
<td>L3</td>
<td>L6</td>
<td>L12</td>
<td>L1</td>
<td>L8</td>
<td>L12</td>
<td>L18</td>
</tr>
</tbody>
</table>

B

Col gl1
PCOI1:COI1
PCOI1:Y302A L6
PCOI1:Y302A L12
PCOI1:Y444A L12
PCOI1:Y444A L18
coi1-1
Figure 2.10 (cont’d). Representative examples of silique production in transgenic Arabidopsis expressing COI1-LRR mutants. (A) Pictures of inflorescence stems of Arabidopsis Col-0 and transgenic plants expressing PCOI1:coi1:4xMYC constructs. Inflorescence stems from eight individuals from each genotype were assessed for silique production. Scale bar = 3 cm. (B) Quantification of silique production. Siliques were counted from eight individuals from each of the lines.
interaction, but actually appeared to enhance the COI1-JAZ9 interaction in Y2H assays (Fig. 2.1). The interaction between COI1-K147A and JAZ9 was evident within 24 hours of spotting yeast onto inducing media and was clearly stronger than the COI1-JAZ9 interaction after 48 hours of incubation. By the 96 hour time point, the color development of COI1-K147A and JAZ9 and COI1 and JAZ9 reached similar levels (Fig. 2.11). For a more quantitative assay, liquid Y2H experiments were conducted, and in agreement with the initial findings from plate assays, the interaction between COI1-K147A and JAZ9 was approximately 1.5 times stronger than was observed between wild-type COI1 and JAZ9 (Fig. 2.12).

Additional K147 mutagenesis was conducted to determine if any other amino acid substitutions at this position could have an effect on interaction with JAZ proteins. Of all 19 substitutions that were generated, K147S also enhanced the interaction with JAZ9, so this COI1 variant was tested against all JAZs that interact with COI1. In all cases COI1-K147S showed a stronger signal in the Y2H assays, suggesting enhanced interaction with JAZ proteins (Fig. 2.13). All COI1 substitution mutations at K147 were also tested for JAZ interaction in the absence of coronatine, and there was no indication that receptor complex formation occurred without a ligand.
Figure 2.11. COI1-K147A enhances interaction with JAZ9.

A

<table>
<thead>
<tr>
<th>COR</th>
<th>+</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K147A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time (hr)</td>
<td>24</td>
<td>48</td>
<td>72</td>
<td>96</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

JAZ9 + COI1

55 kD

95 kD

α-HA

α-LexA
Figure 2.11 (cont’d). **COI1-K147A enhances interaction with JAZ9.** (A) Y2H assay showing the interaction between JAZ9 and COI1 or COI1-K147A. Saturated yeast cultures were adjusted to $\text{OD}_{600\text{nm}} = 0.2$ and $10 \mu\text{l}$ from each culture was spotted on SD-galactose/raffinose –UWH media supplemented with $30 \mu\text{M}$ coronatine (COR). Negative controls were AD:JAZ9 with empty vector expressing LexA DNA binding domain (BD) and BD:COI1 with empty vector expressing transcription activation domain (AD). (B) Western blot analysis of yeast cultures to determine the expression level of proteins in each clone. Cell cultures were adjusted to $\text{OD}_{600\text{nm}} = 0.2$ in inducing media (SD-gal/raf-UWH) and incubated for 6 hours at $30 ^\circ\text{C}$. Cells were harvested and total protein was extracted and probed for HA:JAZ9 and LexA:COI1 fusion proteins or HA and LexA epitopes expressed from the empty vector controls.
Figure 2.12. Quantitative Y2H assay of the COI1-K147A interaction with JAZ9.

A

![Bar graph showing β-Gal activity (U min⁻¹ OD₆₀₀⁻¹) for COI1, COI1-K147A, BD, and AD constructs.]

B

![Western blots for α HA and α LexA showing protein expression for lanes 1 to 4.]
Figure 2.12 (cont’d). Quantitative Y2H assay of the COI1-K147A interaction with JAZ9. (A) Liquid Y2H assay showing the interaction between JAZ9 and COI1 or COI1-K147S. Saturated yeast cultures were adjusted to $\text{OD}_{600} = 0.2$ and induced overnight in SD-galactose/raffinose –UWH media supplemented with 5 µM coronatine. The interaction between each pair of proteins is reported as units of $\beta$-galactosidase activity /$\text{OD}_{600\text{nm}}$/minute. Negative controls were AD:JAZ9 with empty vector expressing LexA DNA binding domain (BD) and BD:COI1 with empty vector expressing transcription activation domain (AD). (B) Western blot analysis of yeast cultures demonstrating the level of expressed proteins in each clone. Samples were collected from each culture after overnight induction. Total protein was extracted and probed for HA:JAZ9 and LexA:COI1 fusion proteins or HA and LexA epitopes expressed from the empty vector controls.
Figure 2.13. COI1-K147S also has enhanced interaction with JAZ proteins.

Y2H assay showing the interaction between COI1 or COI1-K147S and all JAZs that are known to interact with COI1. Saturated yeast cultures were adjusted to OD$_{600nm}$ = 0.2 and 10 µl from each culture was spotted on SD-galactose/raffinose –UWH media supplemented with 30 µM coronatine. Negative controls were AD:JAZ9 with empty vector expressing LexA DNA binding domain (BD) and BD:COI1 with empty vector expressing transcription activation domain (AD).
2.5 Discussion

Several types of multi-subunit E3 ligases have been characterized in plants; however, SKIP/CULLIN/F-box (SCF) E3 ligases have emerged as important regulators of plant hormone signaling either functioning as a component of the hormone receptor itself, as exemplified by the well characterized examples of auxin and JA-Ile perception, or during subsequent steps of the signaling pathway. Over the last decade, structural information pertaining to E3 ubiquitin ligases in plants has become available and has greatly enhanced structure-function analyses of plant hormone receptors.

A critical component of the SCF ubiquitin ligase is the F-box protein subunit that confers specificity for the recognition of ligands and protein targets that regulate many cellular processes. All members of the F-box protein family share a conserved amino acid motif near the N terminus of the protein that was first classified in the human cyclin F protein (Bai et al., 1996). F-box proteins have been identified or are predicted to be encoded in the genomes of all model eukaryotes including 11 proteins in *Saccharomyces cerevisiae*, 326 in *Caenorhabditis elegans*, 22 in *Drosophila melanogaster*, and at least 38 in *Homo sapiens* (Kipreos and Pagano, 2000). In plants, the F-box protein family has greatly expanded during the course of evolution. In *Arabidopsis thaliana* approximately 700 loci are predicted to contain F-box motifs (Gagne et al., 2002; Hua et al., 2011) yet to date only a small number of them have been thoroughly investigated.

Recent evidence indicates that plants have recruited F-box proteins as key components of hormone signal transduction pathways regulating cellular responses.
through induction of changes in gene expression (Kelley and Estelle, 2012; Shan et al., 2012). The receptor complexes for auxin and JA incorporate F-box proteins for recognition of these signaling molecules. Important to the focus of this study, the F-box protein component of the auxin receptor, TIR1, and the JA receptor, COI1, share a significant degree of homology at the amino acid level and similarity in their overall signal transduction mechanism (Sheard et al., 2010; Tan et al., 2007). In this work, contributions of individual COI1 amino acids in the ligand binding pocket, peptide recruitment surface and IP5 cofactor binding pocket of the JA receptor COI1 have been characterized.

In the initial investigation of the ligand binding pocket of COI1, nine amino acids were identified that are involved in receptor complex formation. Of these nine residues, seven are essential for COI1 interaction with JAZ1, JAZ3, and JAZ9 (Fig. 2.3). In particular, the essential amino acid Y444 is predicted to participate in a complex network of hydrogen bonds with JA-Ile or coronatine and the JAZ degron peptide (Fig. 2.3). Substitution of COI1-Y444 with alanine disrupted the formation of the receptor complex in the presence of JA-Ile or coronatine (Fig. 2.4). Furthermore, COI1-Y444A was unable to restore JA sensitivity and JA-related physiological responses when transgenically expressed in the Arabidopsis coi1-1 mutant background under control of the COI1 promoter (Fig. 2.8, Fig. 2.9 and Fig. 2.10). In spite of the significant homology between COI1 and TIR1, analysis of COI1-Y444, which does not align with any amino acids in the equivalent position in TIR1 (Tan et al., 2007), provides one example of a key structural difference in the ligand binding pocket that confers specificity to recognition of JA-Ile, coronatine, and recruitment of the JAZ repressor proteins.
The crystal structure of the JA receptor is based on a complex between COI1-JAZ1, which is only one of the 12 JAZ proteins in Arabidopsis (Sheard et al., 2010). Because plants have multiple JAZ proteins that contain differing sequences in the COI1-interacting Jas domain, it is not clear whether formation of COI1 complexes with different JAZ repressors would exhibit different ligand-binding specificity resulting in different biological responses. Different ligand affinities between COI1 and different JAZ proteins have been seen in experiments using JA-Ile or COR as ligand (Melotto et al., 2008; Katsir et al., 2008; Sheard et al., 2010; this study). My analysis of COI1-LRR domain amino acids in the JA-Ile/coronatine binding pocket revealed two amino acid mutations that differentially affect COI1 interaction with different JAZ proteins: V411A disrupts interaction with only JAZ9 and R496A disrupts interaction with only JAZ1 (Fig. 2.3). The identification of specific COI1 mutants with differential interactions with different members of the JAZ repressor family may provide a molecular tool for future investigation of the possibly distinct physiological roles of individual JAZ proteins. Interestingly, COI1-R496A is completely defective in JA-Ile-dependent interaction with JAZ9, although it still interacts with JAZ9 in the presence of coronatine (Fig. 2.4). These results suggest the possibility that the ligand binding pocket of COI1 is amenable to manipulations that could discriminate between ligands (e.g., endogenous hormone JA-Ile vs. pathogen virulence factor COR). Further mutagenesis of the ligand-binding pocket may yield a COI1 variant that could exclude coronatine but still recognize JA-Ile. Although at this point all evidence points to coronatine as being a more efficient agonist of the JA receptor than JA-Ile itself, such a COI1 variant could prove useful in protecting
crop species that are susceptible to COR-producing pathovars of *Pseudomonas syringae*.

Along with amino acids within the ligand binding pocket of COI1, residues that were predicted to play a role in the specific recognition of JAZ proteins, based on homology with TIR1 residues, were also investigated. Eight residues were identified that disrupt COI1-JAZ interaction in Y2H assays (Fig. 2.6). F89 was initially predicted to be involved in both ligand binding and peptide recognition based on homology with the TIR1 structure; however, the COI1 crystal structure (Sheard et al., 2010) suggests that this residue is critical for hydrophobic interactions with the ligand (Fig. 2.3). Of the remaining seven amino acids, four mediate hydrophobic interactions with the JAZ1 degron peptide (Sheard et al., 2010) and, in Y2H assays, mutations of these residues either completely disrupted or greatly reduced the COI1 interaction with JAZ1, JAZ3, and JAZ9 (Fig. 2.6). Three charged amino acids near the JAZ-binding surface are also important for COI1 interaction with JAZ proteins, indicating that they likely play a role in stabilizing JAZ protein binding through specific hydrogen bond formation. One of these amino acids, COI1-Y302, is predicted to form a hydrogen bond with a serine residue at position 9 in the JAZ1 degron peptide, an interaction that could help to anchor the JAZ protein in the receptor complex and/or provide a structural conformation in the JAZ peptide to position the C terminus for attachment of the polyubiquitin chain (Fig. 2.6). Another charged amino acid, COI1-R496, is predicted to form a hydrogen bond with the carboxyl moiety of the backbone of isoleucine at position 4 in the JAZ1 degron peptide (Fig. 2.6). The third charged side chain of COI1-R326 was not predicted to form any
hydrogen bonds with either the JAZ1 degron or the ligands, but most likely contributes to the stabilization of the LRR domain in the correct conformation.

The COI1-Y302A and COI1-R326A mutations were selected for functional analysis in planta because they interrupted interaction with all JAZs tested. When expressed in the coi1-1 mutant background, COI1-Y302A lines appeared to be more sensitive to MeJA treatment than plants expressing COI1-R326A and were significantly more sensitive to MeJA than COI1-Y444A plants. Also, transgenic lines expressing COI1-Y302A and COI1-R326A were still able to produce anthocyanins in response to prolonged exposure to MeJA, whereas COI1-Y444A plants that showed no significant root growth inhibition compared to coi1-1 mutants did not produce anthocyanins in response to JA treatment (Fig. 2.9). Additionally, although the reproductive capability of COI1-Y302A and COI1-R326A lines was drastically reduced, some plants did eventually produce a few siliques late in inflorescence development (Fig. 2.10). These in planta functional assay results suggest that COI1-Y302A and COI1-R326A are partially functional, whereas COI1-Y444A is nonfunctional even though the Y2H assays clearly showed that all three mutants are defective in interaction with JAZ proteins. It remains possible that COI1-Y302A and COI1-R326A still interact with some other JAZs that were not tested in my study, leading to the subtle JA sensitivity observed. Therefore, it would be interesting in the future to test possible interaction of these COI1 mutants with other JAZs in addition to JAZ1, JAZ3, JAZ4 and JAZ9. Alternatively, perhaps COI1-Y302A and COI1-R326A could still interact with JAZs in response to alternative ligands other than JA-Ile or COR.
Both the auxin receptor TIR1 and the JA receptor COI1 incorporate an inositol phosphate (IP) cofactor (i.e., TIR1 binds IP6 and COI1 binds IP5) that is essential for the formation of the receptor complex (Sheard et al., 2010; Tan et al., 2007). The amino acids of TIR1 and COI1 that form a charged binding pocket for the IP cofactor are mostly conserved between these two proteins (Table 1). The predicted IP5 binding pocket is created largely by arginine residues and one aspartate, and all were found to be essential for COI1-JAZ1 interactions. Interestingly, COI1-R85A does not disrupt interaction with JAZ3 or JAZ9 and COI1-R121A does not disrupt COI1-JAZ3 interaction, indicating that not all of these residues are essential for COI1 interaction with some JAZs that are known to be strong COI1-interactors, such as JAZ3 and JAZ9. Several months after the crystal structure of the COI1-JAZ1 was solved, it was shown that these same amino acids are essential for JA sensitivity in Arabidopsis (Mosblech et al., 2011). In this work, the authors demonstrated that manipulation of IP5 levels in yeast IP6 biosynthetic mutants can also enhance the sensitivity of the COI1-JAZ9 interaction, possibly implicating IP5 as a limiting factor for this signaling system.

A surprising result from the work described here was the enhanced interaction between COI1 and JAZ interaction as a result of the COI1-K147A and COI1-K147S mutations (Fig. 2.11, Fig. 2.12 and Fig. 2.13). If mutagenesis of this particular amino acid were to confer hypersensitive perception of endogenous JA-Ile, transgenic lines expressing this construct would be expected have increased resistance to herbivorous insects and necrotrophic fungal pathogens. However, they might also be more susceptible to COR-producing pathovars of *P. syringae*. This line of investigation is currently ongoing.
In total, the contribution of 42 amino acids to the formation of COI1-JAZ receptor complexes has been investigated in this study. Approximately 55% of the alanine substitutions that were generated had an effect on ligand-dependent receptor complex formation as demonstrated by Y2H experiments. Many of the COI1 variants that were generated were shown to differentially affect COI1 interaction with different JAZ proteins. Using these variants as tools to investigate the function of individual JAZ proteins in planta I hope to further contribute to the understanding of the role of individual JAZ proteins in JA signaling.
LITERATURE CITED
2.6 Literature Cited


Chapter 3

Transcription factor-dependent nuclear import of transcriptional repressors in jasmonate hormone signaling

1 The materials and experimental results presented in Figure 3.1, Phenotypic differences in Arabidopsis Col-0 plants expressing JAZ1 or JAZ9 lacking a functional Jas domain and Figure 3.5, JAZ9 interacts with the N terminus of MYC2 were generously provided by Dr. Jian Yao.
3.1 Abstract

The plant hormone jasmonate (JA) plays an important role in regulating growth, development and immunity. A key step in JA signaling is ligand-dependent assembly of a co-receptor complex consisting of the F-box protein COI1 and JAZ transcriptional repressors. Assembly of this receptor complex results in proteasome-mediated degradation of JAZ repressors which at resting state bind to and repress the MYC transcription factors. Although the JA receptor complex is believed to function within the nucleus, how this receptor complex enters the nucleus and, more generally, the cell biology of jasmonate signaling are not well understood. In this study, we conducted mutational analysis of the C-termini (containing the conserved Jas motif) of two JAZ repressors, JAZ1 and JAZ9. These analyses unexpectedly revealed distinct subcellular localization patterns of JAZ1ΔJas and JAZ9ΔJas, which were associated with differential interaction of JAZ1ΔJas and JAZ9ΔJas with MYC2 and differential repressor activity \textit{in vivo}. Importantly, physical interaction with MYC2 appears to play an active role in the nuclear targeting of JAZ1 and JAZ9, and the nuclear localization of JAZ9 was compromised in \textit{myc2} mutant plants. We identified a highly conserved arginine residue in the Jas motif that is critical for coupling MYC2 interaction with nuclear entry of JAZ9 and JAZ9 repressor function \textit{in vivo}. Our results suggest a model for explaining why some JAZΔJas proteins, but not others, confer constitutive JA-insensitivity when overexpressed in plants. Results also provide evidence for a novel transcription factor-dependent mechanism for nuclear import of cognate transcriptional repressors in plants.
3.2 Introduction

Plants, being sessile organisms subjected to ever-changing environmental stresses, have evolved complex mechanisms to properly balance their growth and development with appropriate defense responses. One of the major plant hormones involved in regulating the balance between growth and defense belongs to a class of lipid-derived molecules, collectively termed jasmonates (JAs) (Yang et al., 2012). In healthy plants, JA signaling plays a role in reproductive development, photomorphogenesis and other growth responses (Feys et al., 1994; Li et al., 2004; Mandaokar et al., 2006; Robson et al., 2010; Song et al., 2011). Defense responses that are regulated by JA signaling are activated by environmental stress factors including tissue damage from herbivorous insects, pathogen attack, drought, and UV irradiation (Browse, 2009a; Browse and Howe, 2008; Conconi et al., 1996; Glazebrook, 2005; Kim et al., 2009; Seo et al., 2011). Induction of the JA response pathway results in significant transcriptional reprogramming and typically shifts the balance from growth to defense-related cellular processes, through inhibition of gene expression involved in cell cycle progression and photosynthesis, and activation of defense-related genes (Goossens et al., 2003; Pauwels et al., 2008; Uppalapati et al., 2005; Zhang and Turner, 2008).

In the past five years, exciting progress has been made in the understanding of JA signaling and how this stress hormone influences plant growth, development and defense. The bioactive JA ligand, (3R,7S)-jasmonoyl-L-isoleucine (JA-Ile), is perceived by a receptor complex consisting of CORONATINE-INSENSITIVE1 (COI1), the F-box subunit of the SCF$^{COI1}$ ubiquitin ligase, and members of the JASMONATE-ZIM
DOMAIN (JAZ) family of transcriptional repressors (Chini et al., 2007; Fonseca et al., 2009b; Sheard et al., 2010; Thines et al., 2007; Xie et al., 1998; Xu et al., 2002; Yan et al., 2007). In an example of phytohormone mimicry, several strains of the plant pathogen *Pseudomonas syringae* have developed the ability to produce coronatine (COR), a structural mimic of JA-Ile that increases susceptibility of the host plant (Katsir et al., 2008b; Melotto et al., 2006; Thilmony et al., 2006; Zhao et al., 2003). Arabidopsis has at least 12 JAZ proteins, which share two conserved functional motifs: the ZIM motif in the central part of the protein and the Jas motif at the C-terminus (Katsir et al., 2008a). The ZIM motif mediates homo- and heteromeric interactions among JAZ repressors and JAZ interactions with the adapters/co-repressors NINJA and TOPELESS proteins (Chini et al., 2009b; Chung and Howe, 2009; Pauwels et al., 2010). The C-terminal Jas motif is necessary for interaction with the LRR domain of COI1 (Chini et al., 2007; Chung et al., 2008; Melotto et al., 2008; Sheard et al., 2010; Thines et al., 2007). A short peptide of 21 amino acids within the Jas motif defines the minimal “degron” that is sufficient for ligand-dependent formation of COI1-JAZ1 receptor complexes (Sheard et al., 2010). In addition, the Jas motif mediates interaction between JAZ proteins and their target transcription factors (TFs) that control downstream physiological responses (Chini et al., 2007). Among the most well characterized TFs targeted by JAZ are members of a basic-helix-loop-helix family, including MYC2, 3, and 4, that are key regulatory components of diverse aspects of JA-mediated physiological responses (Abe et al., 2003; Cheng et al., 2011; Chini et al., 2007; Dombrecht et al., 2007; Fernandez-Calvo et al., 2011; Lorenzo et al., 2004; Melotto et al., 2008).
The core JA signaling components, including a multimeric transcriptional co-repression complex consisting of JAZ, NINJA and TOPLESS proteins bound to JA-responsive TFs and the COI1-JAZ receptor complex, are believed to function in the nucleus (Pauwels et al., 2010). However, direct evidence of nuclear localization (and underlying nuclear targeting mechanisms) for many of the key JA signaling components is lacking and, more generally, the cell biology of jasmonate signaling is poorly understood. Previous studies have shown that JAZ1 variants (JAZ1ΔJas) lacking the entire Jas motif or carrying alanine substitutions at specific arginine residues in the Jas motif are unable to interact with COI1 in a ligand-dependent manner and are therefore resistant to SCF\(^\text{COI1}\)-dependent degradation through the proteasome (Melotto et al., 2008; Thines et al., 2007). As a result, transgenic Arabidopsis plants that express these JAZ1 variants exhibit JA insensitivity (Chung et al., 2008; Melotto et al., 2008; Thines et al., 2007). A similar JA-insensitive phenotype was observed for JAZ3 (JAZ3ΔJas) and JAZ10 (JAZ10ΔJas) (Chini et al., 2007; Chung et al., 2010; Chung and Howe, 2009; Yan et al., 2007). However, it has also been shown that the Jas motif is required for JAZ3, but not JAZ1 or JAZ10, interaction with TFs such as MYC2, which should be a key step in TF-specific transcriptional repression (Chini et al., 2009b; Chung and Howe, 2009). Therefore, although transgenic overexpression of JAZΔJas proteins has been used as the primary means in the discovery of the repressor function of JAZ proteins, how JAZΔJas proteins repress JA signaling has remained enigmatic (Browse, 2009b). We report here that, in contrast to plants expressing JAZ1ΔJas, plants expressing JAZ9ΔJas do not exhibit obvious JA-insensitive phenotypes. This observation led us to conduct a series of experiments to understand the apparently different effects of the Jas
motifs of JAZ1 and JAZ9 on JA signaling. Our results provide evidence for the existence of a novel TF-dependent mechanism for nuclear import of cognate transcriptional repressors in plants, and show that nuclear entry of JAZ1 and JAZ9 and their interaction with MYC2 in the nucleus are coupled and are both required for repression of JA signaling.

3.3 Materials and Methods

Chemicals and reagents used

Methyl-jasmonate, coronatine, complete protease inhibitor cocktail for plant cell and tissue extracts, antibiotics, and acetosyringone (Sigma, St. Loois, MO), 26S proteasome inhibitor MG132 (Cayman Chemical Co., Ann Arbor, MI), rabbit polyclonal antibody to GFP (Ab290, AbCam, Cambridge, MA), HRP-conjugated goat anti-rabbit antibody (Pierce, Rockford, IL).

Site-directed mutagenesis

The DNA sequences of selected amino acid residues in the Jas domain of JAZ9 (At1G70700) were mutated to alanine codons using the Quick-Change II site-directed mutagenesis kit (Agilent, Santa Clara, CA). Mutagenesis was confirmed by DNA sequencing, and western blot was performed to confirm the production of the mutant proteins when coexpressed with COI1 (At2G39940) or MYC2 (At1G32640) in yeast-two-hybrid assays.
Generation of plant expression constructs

The coding sequences of JAZ9 and selected mutants were cloned into pENTR4 (Invitrogen, Grand Island, NY), and transferred by LR recombination into the binary expression vector pEarleyGate 104 (Earley et al., 2006) to generate a YFP:JAZ9 fusion. The correct reading frame was confirmed by sequencing. All constructs were introduced into Agrobacterium tumefaciens (GV3101). GV3101 clones containing the gene fusion constructs were selected on LB medium containing rifampicin, kanamycin and gentamycin antibiotics.

Transient gene expression in N. tabacum

Single Agrobacterium colonies containing JAZ9 constructs were selected and cultured over-night at 30 °C in LB medium containing appropriate antibiotics. Agrobacterium tumefaciens (GV3101) cells were harvested from overnight cultures by centrifugation at 3,500 rpm for ten minutes, washed in sterile water and resuspended to OD600nm = 0.2 in infiltration buffer (10 mM MES pH 5.8; 10 mM MgCl2; 0.2 % sucrose; 300 µM acetylsheringone). Cultures were syringe-inoculated into mature leaf tissue of Nicotiana tabacum. Infiltrated plants were returned to previous growing conditions and expression was analyzed by confocal microscopy at 24-30 hours after inoculation. Fusion protein expression was analyzed by western blot.

Generation of transgenic Arabidopsis

Arabidopsis Col-0 plants were transformed using the standard floral dip protocol (Clough and Bent, 1998). Ten to fifteen BASTA resistant T1 seedlings were identified, then transplanted to separate pots and grown to maturity. The T2 generation seedlings
were again selected on MS-agar plates containing 10 mg/L BASTA, and screened for gene expression level by RT-PCR (using YFP- and JAZ9-specific forward and reverse primers, respectively) and western blot using a polyclonal antibody against GFP.

**Confocal microscopy**

Transiently expressed fusion proteins and all transgenic Arabidopsis lines were analyzed using a Zeiss 510 Meta ConfoCor3 laser scanning confocal microscope. Microscopic examination of all plant tissue samples was conducted using an EC Plan-Neofluar 40x/1.30 Oil objective with 2X and 4X digital zoom applied with AIM software (Zeiss). For detection of YFP, samples were excited with an argon laser at 10% strength, 514nm; the optical pathway included an HFT 458/514 beam splitter, BP520-555IR band pass filter and NFT 515 and 595 long pass filters. For detection of CFP fusion proteins, samples were excited with an argon laser at 30-40% strength, 458nm; the optical pathway included an HFT 458/514 beam splitter, BP465-510IR band pass filter and NFT 515 and 595 long pass filters. For detection of mCherry fusion proteins, samples were excited with a HeNe 543 laser at 30%. The optical pathway included HFT 488/543 beam splitter, NFT 635 and NFT 545 long pass filters, and a BP 560-615 band pass filter. For colocalization experiments, dual channel, sequential imaging was used to capture images.

**Yeast-two-hybrid (Y2H)**

The GAL4 system (Clontech, Mountainview, CA) was used for testing interaction of MYC2 with JAZ1\(\Delta\)Jas or JAZ9\(\Delta\)Jas, and also interaction of truncated MYC2 with JAZ9. Cotransformed yeast were grown on SD glucose (-LW) for selective propagation of
cultures and on SD glucose (-LWHA) for determining protein interactions. LexA system (Clontech, Mountainview, CA) was used for all other Y2H experiments. The coding sequence of COI1 was cloned into the Y2H bait vector pGILDA (Clontech, Mountainview, CA) using XmaI and XhoI restriction enzyme recognition sequences previously added to the 5’ and 3’ end of the COI1 gene, respectively, creating a LexA:COI1 fusion. The coding sequence of JAZ9, and site-directed mutants, were cloned into the Y2H prey vector pB42AD (Clontech), creating AD:JAZ9 fusions. Individual COI1 constructs were co-transformed with JAZ9 or JAZ9 mutant constructs into yeast (S. cerevisiae) strain EGY48 (p8opLacZ) using the frozen-EZ yeast transformation II kit (Zymo Research, Irvine, CA). Transformants were selected on SD-glucose medium (BD Biosciences, San Jose, CA) supplemented with –Ura/-Trp/-His drop-out solution (BD Biosciences).

To detect the interaction between COI1 and JAZ9 or JAZ9 mutant proteins, yeast transformants that had been selected in SD-Glu –U/W/H medium were resuspended in sterile water, and 10 µl of each suspension was spotted onto inducing media (SD-Galactose/Raffinose –U/W/H; BD Biosciences) supplemented with 80 µg ml⁻¹ X-Gal and 10 µM coronatine (Sigma). The Y2H assays were incubated in the dark at 30°C; pictures were taken after approximately 48 hours. Induced clones were analyzed to confirm protein expression by western blotting using epitope-specific antibodies. Interaction between JAZ9, JAZ9 mutants, and MYC2 was conducted as described above; however, for these Y2H assays, JAZ9 and mutants were fused to the DNA binding domain in pGILDA, and MYC2 was fused to the transcriptional activation domain pB42AD to eliminate auto-activation of the LacZ reporter gene.
Growth inhibition assays

Arabidopsis wild-type Col-0, heterozygous coi1-30 plants and transgenic seedlings expressing YFP:JAZ9 (or mutant variations) under the CaMV 35S promoter were surface sterilized, cold-stratified and germinated on MS agar media containing no treatment or 10 µM methyl jasmonate. Seedlings were grown for 10-12 days in long day light conditions (100 µE light intensity; 16L:8D). Pictures of seedlings were taken and plates were then scanned to high-resolution images. Root lengths were analyzed using ImageJ software (http://rsbweb.nih.gov/ij/).

Bacterial infection assays

For plant growth, all seedlings were surface sterilized, cold-stratified and germinated on plates containing MS agar with appropriate selection reagents in the media. For selection of coi1-30 mutants, seeds from the segregating population were germinated on media supplemented with 50 µM methyl jasmonate, grown for five to seven days, and then JA-insensitive mutants were transplanted into pots containing standard Arabidopsis potting soil. Transgenic Arabidopsis expressing the YFP:JAZ9 (or JAZ9 mutant variations) were selected for the presence of the transgene by selection on media supplemented with 10 mg/L glufosinate ammonium (BASTA) and then transplanted to soil. Wild-type Col-0 seedlings were germinated on blank MS agar media; all seedlings were germinated and transplanted simultaneously. After transplanting, the seedlings were grown under short day light conditions (100 µE light intensity; 12L:12D) for approximately five weeks. Syringe-inoculation of Pseudomonas syringae pv. tomato DC3000 was conducted using the standard laboratory protocol.
(Katagiri et al., 2002). Symptom development and bacterial multiplication were analyzed three days post-inoculation.

3.4 Results

3.4.1 Constitutive Expression of Different JAZΔJas Proteins Results in Disparate JA-insensitive Phenotypes.

Several previous studies have reported the effects of ectopically expressing JAZΔJas proteins on JA signaling. Specifically, overexpression of JAZ1, JAZ3 and JAZ10 variants lacking the C-terminal Jas motif led to JA insensitivity, providing key evidence that JAZ proteins are transcriptional repressors (Chini et al., 2007; Chung and Howe, 2009; Melotto et al., 2008; Thines et al., 2007; Yan et al., 2007). During investigation of the role of JAZ9 in JA signaling, we introduced an HA-tagged JAZ9 variant lacking the entire Jas motif (35S:3xHA:JAZ9ΔJas) into Arabidopsis, expecting a phenotypic effect on JA signaling similar to that in plants overexpressing JAZ1ΔJas (Melotto et al., 2008; Thines et al., 2007). Surprisingly, lines expressing this construct did not exhibit JA insensitivity as determined by root growth inhibition assays, in contrast to lines transformed with 35S:3xHA:JAZ1ΔJas (Fig. 3.1A and B). One explanation for the lack of phenotype in 35S:3xHA:JAZ9ΔJas lines is that JAZ9ΔJas maintains the ability to interact with COI1 in vivo, resulting in JAZ9ΔJas degradation in response to JA stimulus. However, in vivo degradation assays performed on 10-day-old transgenic seedlings revealed that both 3xHA:JAZ1ΔJas and 3xHA:JAZ9ΔJas were highly stable in
comparison to the wild-type JAZ1 and JAZ9 fusion proteins (Fig. 3.1C). This finding is in agreement with previous yeast-two-hybrid (Y2H) results indicating that JAZ1ΔJas and JAZ9ΔJas do not interact with COI1 in the presence of COR (Melotto et al., 2008). Therefore, the observed phenotypic difference cannot be explained by differential stability of JAZ1ΔJas and JAZ9ΔJas.

3.4.2 Role of MYC2 Interaction in Nuclear Targeting of JAZ1 and JAZ9

We next compared the ability of JAZ1ΔJas and JAZ9ΔJas mutants to interact with MYC2 in the Y2H system. Here, we found a difference between JAZ1ΔJas and JAZ9ΔJas. Whereas JAZ1ΔJas retained the ability to interact with MYC2, albeit to a lesser extent than the wild-type JAZ1, JAZ9ΔJas did not interact with MYC2 (Fig. 3.2A). In addition, we found a difference in the subcellular localization patterns of YFP:JAZ1ΔJas and YFP:JAZ9ΔJas in Agrobacterium-mediated transient expression experiments in Nicotiana tabacum. Both YFP:JAZ1 and YFP:JAZ9 localized to the nucleus, mostly in undefined subnuclear bodies, and YFP:JAZ1ΔJas partially retained the ability to enter the nucleus (Fig. 3.2B). In contrast, YFP:JAZ9ΔJas largely lost nuclear localization and was found in the cytoplasm, mainly in distinct punctuate structures (Fig. 3.2B).
Figure 3.1. Phenotypic differences in Arabidopsis Col-0 plants expressing JAZ1 or JAZ9 lacking a functional Jas domain.
Figure 3.1 (cont’d). Phenotypic differences in Arabidopsis Col-0 plants expressing JAZ1 or JAZ9 lacking a functional Jas domain. (A, B) Twelve-day-old seedlings grown on MS media (-) or MS supplemented with 10 µM MeJA (+). Images were collected by scanning growth plates (A), and root growth was quantified using ImageJ software (B). Data are the means from 15 plants; error bars represent SD. Letters on columns indicate statistically significant differences (p < 0.01, Tukey’s HSD test). (C) Western blot analysis of the stability of JAZ1 and JAZ9 in transgenic Arabidopsis plants after one-hour treatment with coronatine (COR) or methyl JA (MeJA) treatment. 3xHA:JAZ fusion proteins were detected by western blot using a monoclonal antibody against the HA tag; CBB = Coomassie brilliant blue staining of PVDF membranes. The materials and data shown here were generated by Dr. Jian Yao.
Figure 3.2. Deletion of the Jas domain differentially effects interaction with MYC2 and sub-cellular localization of JAZ1 and JAZ9.
Figure 3.2 (cont’d). Deletion of the Jas domain differentially effects interaction with MYC2 and sub-cellular localization of JAZ1 and JAZ9. (A) MYC2 interaction with JAZ1 and JAZ9 variants in yeast-two-hybrid assays. Yeast cultures co-transformed with JAZ:BD and MYC2:AD (left panel) or GAL4AD (right panel) spotted on -LWHA (top panel) and -LW (bottom panel) drop-out media. Protein interactions in yeast colonies grown on selective media (top panel) are indicated by colony growth. (B) Overlay of bright-field and fluorescent images of YFP:JAZ1 and YFP:JAZ9 variants transiently expressed in *N. tabacum* epidermal cells. Inserts are portions of images enlarged to show nuclei. Scale bar = 10 µm.
These results establish a correlation between the ability of JAZ proteins to interact with MYC2 and correctly localize to the nucleus. We also determined the subcellular localization of COI1 and NINJA using our transient expression system and found that both YFP:COI1 and YFP:NINJA localize diffusely within the nucleus (Fig. 3.3). NINJA localization was consistent with previously reported localization results (Pauwels et al., 2010). The correlation between the ability of JAZ proteins to interact with MYC2 and correctly localize to the nucleus prompted us to examine the possibility that MYC2 might play an active role in targeting JAZ repressors to the nucleus. MYC2 was transiently coexpressed together with either JAZ1 or JAZ9 in *N. tabacum* leaves. Both YFP:JAZ1 and YFP:JAZ9 colocalized with CFP:MYC2 in subnuclear bodies (Fig. 3.4A). Interestingly, coexpression of YFP:JAZ1ΔJas with CFP:MYC2 altered the localization of this truncated JAZ from a predominately cytosolic location (Fig. 3.2) to the nucleus (Fig. 3.4A). In contrast, coexpression of YFP:JAZ9ΔJas with CFP:MYC2 did not significantly alter the cytoplasmic location of YFP:JAZ9ΔJas (Fig. 3.4A). Thus, overexpression of CFP:MYC2 was sufficient to drive complete nuclear localization of YFP:JAZ1ΔJas (which retains interaction with MYC2), but not YFP:JAZ9ΔJas (which does not interact with MYC2).

MYC2 has been shown to interact with JAZs via its N-terminus (Fernandez-Calvo et al., 2011). Protein sequence analysis using ProteinPredict software, which is based on the LOCtree and PredictNLS algorithms (Nair et al., 2003; Nair and Rost, 2005), showed that the C-terminal half of MYC2 contains a monopartite nuclear localization signal (NLS), KRPKKRGRK_{433-441}. 

124
Figure 3.3. Subcellular localization of COI1 and NINJA. Overlay of bright-field and fluorescent images of YFP:COI1 and YFP:NINJA transiently expressed in N. tabacum epidermal cells. YFP signal was detected inside the nucleus. Images were taken 24-30 hours post-inoculation using laser scanning confocal microscopy. Scale bar = 10 µm.
We examined the localization of the N-terminal (1-333) and C-terminal (207-624) halves of MYC2 and the effects of the N-terminal and C-terminal halves of MYC2 on the localization of JAZ9. Consistent with the presence of a NLS in the C-terminus, the C-terminal half of MYC2 was localized exclusively in the nucleus. In contrast, the N-terminal half was localized partially in the cytoplasm and partially in the nucleus. YFP-JAZ9 nuclear localization was not affected when coexpressed with the C-terminus of MYC2, which does not interact with JAZ9 in Y2H (Fig. 3.4B and Fig. 3.5). However, the cytoplasmically localized N-terminus of MYC2, which interacts with JAZ9, trapped a portion of YFP:JAZ9 in the cytoplasm (Fig. 3.4B and Fig. 3.5). This result further indicates that physical interaction with MYC2 affects JAZ9 localization in the cell.

We next asked the question about whether endogenous MYC2 is necessary for nuclear targeting of full-length JAZ9. For this purpose, we transformed the 35S:YFP:JAZ9 construct into the Arabidopsis jin1-9 (myc2) mutant (Anderson et al., 2004). Confocal microscopy of the resulting lines revealed significant mis-localization of YFP:JAZ9 to the cytoplasm, in addition to the expected nuclear localization (Fig. 3.4C); this localization pattern contrasted with that of YFP:JAZ9 in wild-type Col-0 plants, where YFP:JAZ9 signal was located entirely in the nucleus. These results provide genetic evidence that MYC2 is required for proper nuclear localization of JAZ9.
Figure 3.4. Nuclear localization of JAZ is influenced by interaction with MYC2.
Figure 3.4 (cont’d). Nuclear localization of JAZ is influenced by interaction with MYC2. Overlay of bright-field and fluorescent images of (A) YFP:JAZ and CFP:MYC2, or (B) YFP:JAZ9 and CFP:MYC2 or truncated derivatives of CFP:MYC2, transiently coexpressed in *N. tabacum* epidermal cells. Insets are CFP channel alone (top) and YFP channel alone (bottom). Scale bar = 10 µm. Inserts are portions of images enlarged to shown nuclei. (C) Fluorescent images of leaf epidermal cells expressing YFP:JAZ9 in transgenic Col-0 or *jin1-9 (myc2)* mutants. Scale bar = 50 µm. (D) Full-length YFP:JAZ9 protein was detected from each line by western blot using a polyclonal antibody against GFP; CBB = Coomassie brilliant blue staining of PVDF membrane.
3.4.3 Arginine\textsubscript{Jas17} is Critical for JAZ9 Nuclear Localization and Interaction with MYC2.

Having established an active role for MYC2 in nuclear targeting of JAZ9, we next sought to identify specific residues within the Jas motif that are required for this process. The minimal JAZ1 degron was previously identified in the crystal structure of the COI1-JAZ1 co-receptor complex (Sheard et al., 2010). Because different JAZs are of different lengths, the specific amino acid positions within the conserved Jas motif vary greatly among different JAZs. We use a simplified nomenclature to describe amino acid positions within the Jas motif (Fig. 3.6A). The Jas motif contains clusters of basic amino acid residues that resemble the classic mono- and bipartite NLS (Lange et al., 2007; Stewart, 2007; Wagstaff and Jans, 2009). In addition, the highly conserved KRK(E/D)RX\textsubscript{6}PY sequence in the C-terminal end of the Jas motif resembles a non-classical NLS (Lee et al., 2006; Stewart, 2007), and has been implicated as a nuclear localization signal for JAZ1 (Grunewald et al., 2009). However, alternative splice variants of JAZ10 that lack the PY motif retain the ability to enter the nucleus (Chung et al., 2010; Chung and Howe, 2009). To determine which, if any, of the motifs are involved in nuclear import of JAZ9, we transiently expressed the wild-type JAZ9 and the RK\textsuperscript{Jas6,7}AA or KRK\textsuperscript{Jas16-18}AAA mutants as YFP fusions in \textit{N. tabacum} leaf cells and analyzed their subcellular localization by confocal microscopy. YFP:JAZ9-RK\textsuperscript{Jas6,7}AA retained the ability to accumulate in the nucleus and to form subnuclear bodies, whereas YFP:JAZ9-KRK\textsuperscript{Jas16-18}AAA exhibited a cytoplasmic localization (Fig. 3.6B).
Figure 3.5. **JAZ9 interacts with the N-terminus of MYC2.** (A) Diagram of MYC2 and truncated derivatives. AD = transcriptional activation domain, BHLH = basic helix loop helix region. (B) Yeast two hybrid assay testing the interaction between JAZ9 and specific regions of MYC2. Yeast cultures cotransformed with MYC2:AD derivatives, and either JAZ9:BD (top panel), or GAL4BD (bottom panel) were spotted on –LWH media. Protein interactions are indicated by colony growth. Material and data were generated by Dr. Jian Yao.
Figure 3.6. Arginine$^{\text{Jas17}}$ in the Jas domain is critical for nuclear localization of JAZ9.

A

| Jas | E | L | P | I | A | R | R | A | S | L | H | R | F | L | E | K | R | K | D | R | V |
|     | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10| 11| 12| 13| 14| 15| 16| 17| 18| 19| 20| 21|   |
| S V | P | Q | A | R | K | A | S | L | A | R | F | L | E | K | R | K | E | R | L |

JAZ9 Degron (aa: 218-238)

B

<table>
<thead>
<tr>
<th></th>
<th>YFP:JAZ9</th>
</tr>
</thead>
<tbody>
<tr>
<td>YFP</td>
<td>WT</td>
</tr>
<tr>
<td>K Jas16 A</td>
<td></td>
</tr>
<tr>
<td>R Jas17 A</td>
<td></td>
</tr>
<tr>
<td>K Jas18 A</td>
<td></td>
</tr>
<tr>
<td>KRK Jas16-18 AAK</td>
<td></td>
</tr>
<tr>
<td>KRK Jas16-18 ARA</td>
<td></td>
</tr>
<tr>
<td>KRK Jas16-18 AAA</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.6 (cont’d). Arginine$^{Jas17}$ in the Jas domain is critical for nuclear localization of JAZ9. (A) Amino acid sequences of the Jas degron from JAZ1 and JAZ9. Jas1 is the first amino acid in sequence. Subsequent amino acids are numbered sequentially. (B) Bright-field and fluorescent image overlay of YFP:JAZ9 variants transiently expressed in $N. tabacum$ epidermal cells. Single, double or triple alanine substitutions in the Jas motif of JAZ9 are indicated in each panel. Inserts are portions of images enlarged to shown nuclei. Scale bar = 10 µm.
These nuclear and cytoplasmic localization patterns were further confirmed in transgenic Arabidopsis expressing the same YFP:JAZ9 fusions (Fig. 3.7), thus implicating KRK as a critical element for nuclear localization of JAZ9.

To further delineate the KRK^{Jas16-18} motif in nuclear localization of JAZ9, we next analyzed single, double and triple alanine mutations of KRK^{Jas16-18}. Single alanine point mutations of K^{Jas16} or K^{Jas18} had no effect on nuclear localization, as evidenced by the formation of subnuclear bodies (Fig. 3.6B). However, R^{Jas17}A completely excluded the fusion protein from the nucleus; all double and triple amino acid substitutions that included the R^{Jas17}A mutation had the same effect (Fig. 3.6B). Moreover, with only R^{Jas17} present in the KRK motif (i.e., KRK^{Jas16-18} ARA), the fusion protein became properly localized to subnuclear bodies (Fig. 3.6B). The results from these experiments revealed a key role of R^{Jas17} in the correct nuclear localization of YFP:JAZ9.

The identification of R^{Jas17} as a critical determinant of JAZ9 nuclear localization provided us with another opportunity to test the hypothesis that JAZ9 nuclear localization is coupled to the physical interaction with MYC2. Results from Y2H assays revealed that KRK^{Jas16-18} triple alanine mutations disrupted interaction with MYC2. However, JAZ9-KRK^{Jas16-18} ARA had normal interaction with MYC2.
Figure 3.7. Localization of YFP:JAZ9 and its mutants in epidermal cells of transgenic Arabidopsis seedlings. Fluorescent images of YFP:JAZ9 carrying specific alanine substitutions in the Jas motif expressed in transgenic Arabidopsis seedlings. T2 generation transgenic seedlings were used for microscopy. Scale bar = 50 µm.
Therefore, within the KRK sequence, $R^{Jas_{17}}$ was necessary and sufficient for mediating interaction with MYC2 (Fig. 3.8A).

Next, we examined the ability of various mutants to colocalize with CFP:MYC2 upon transient expression in *N. tabacum*. Again, we observed a strong correlation between nuclear import of YFP:JAZ9 and the ability of these fusion proteins to interact with MYC2 and to colocalize with CFP:MYC2. Specifically, YFP:JAZ9-RK$^{Jas_{6,7}}$ AA, which interacted with MYC2 in Y2H assays, and was colocalized with CFP:MYC2 in subnuclear bodies, similar to YFP:JAZ9 and CFP:MYC2 (Fig. 3.8B). Single lysine mutations in the KRK$^{Jas_{16-18}}$ motif had no effect on nuclear import or colocalization with CFP:MYC2. However, any variation of YFP:JAZ9 lacking the critical amino acid $R^{Jas_{17}}$ was excluded from the nucleus, while the CFP:MYC2 signal remained nuclear. Furthermore, within the KRK sequence $R^{Jas_{17}}$ was sufficient for JAZ9 colocalization with CFP:MYC2 to subnuclear bodies (Fig. 3.8B). Consistent with findings reported in a previous study (Melotto et al., 2008), we found that RK$^{Jas_{6,7}}$ are not required for JAZ9 interaction with MYC2 (Fig. 3.8A). This result can now be explained by the fact that, of the basic amino acids in the Jas motif, only $R^{Jas_{17}}$ is critical for JAZ9 interaction with MYC2.
Figure 3.8. Arginine\textsuperscript{Jas17} is critical for JAZ9 interaction, stability, and colocalization with MYC2.

<table>
<thead>
<tr>
<th>A</th>
<th>Alanine Substitutions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>JAZ9</strong></td>
<td><strong>RK</strong></td>
</tr>
<tr>
<td><strong>WT</strong></td>
<td><strong>Jas6,7</strong></td>
</tr>
<tr>
<td><strong>AD</strong></td>
<td><strong>AA</strong></td>
</tr>
<tr>
<td><strong>BD</strong></td>
<td><strong>ARA</strong></td>
</tr>
</tbody>
</table>

| B | | | | |
|---|---|---|---|
| **YFP** | **YFP:JAZ9** | **CFP** | **CFP:MYC2** |
| **WT** | | | |
| **RK** | | | |
| **Jas6,7** | | | |
| **Jas16** | | | |
| **Jas17** | | | |
| **Jas18** | | | |
| **K** | **KRK** | **AAK** | **KRK** |
| **Jas16-18** | **ARA** | **AAA** | |

| C | | | | |
|---|---|---|---|
| **YFP** | **WT** | **RK** | **KRK** |
| **Jas6,7** | **AA** | **Jas17** | **Jas16-18** |
| **Jas18** | **A** | **ARA** | **AAA** |

<table>
<thead>
<tr>
<th>+ COR</th>
<th>0</th>
<th>6</th>
<th>0</th>
<th>60</th>
<th>0</th>
<th>60</th>
<th>0</th>
<th>6</th>
<th>Time (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>JAZ9</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CBB</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.8 (cont’d). Arginine\textsuperscript{Jas17} is critical for JAZ9 interaction, stability, and colocalization with MYC2. (A) Yeast-two-hybrid assay. Yeast cultures cotransformed with AD:MYC2 and BD:JAZ9 variants (top panel), or BD:COI1 and AD:JAZ9 variants (bottom panel), were spotted on –UWH drop-out media supplemented with X-Gal (and 10µM COR for COI1 interaction). Blue color indicates protein interaction. (B) Colocalization of YFP:JAZ9 variants and CFP:MYC2 transiently expressed in \textit{N. tabacum} epidermal cells. Top row: fluorescent images of YFP:JAZ9, CFP:MYC2 and vector controls. Middle and bottom rows: dual channel overlay of YFP:JAZ9 carrying the indicated alanine substitutions coexpressed with CFP:MYC2. Scale bar = 5 µm. (C) \textit{In vivo} JAZ protein degradation assay. Stability of YFP:JAZ9 variants expressed in Arabidopsis was determined by western blot one hour after 1 µM COR or ethanol treatment. CBB = Coomassie brilliant blue staining of PVDF membrane.
We also determined the ability of JAZ9 mutants to interact with COI1 in Y2H experiments. Consistent with previous results (Melotto et al., 2008), JAZ9-RK\textsuperscript{Jas6,7}AA did not interact with COI1, and again, we found that the R\textsuperscript{Jas17}A mutation disrupted interaction (Fig. 3.8A). These results indicated that alanine substitutions of both JAZ9-RK\textsuperscript{Jas6,7} and -R\textsuperscript{Jas17} would result in increased stability. As expected, \textit{in vivo} protein degradation experiments in transgenic Arabidopsis seedlings showed that whereas YFP:JAZ9 and YFP:JAZ9-KRK\textsuperscript{Jas16-18}ARA are completely degraded within 1 hour after treatment with coronatine, YFP:JAZ9-RK\textsuperscript{Jas6,7}AA was resistant to coronatine-mediated degradation (Fig. 3.8C). This finding is consistent with the ability of JAZ9 and JAZ9-KRK\textsuperscript{Jas16-18}ARA, but not JAZ9-RK\textsuperscript{Jas6,7}AA, to interact with COI1 (Fig. 3.8C) (Melotto et al., 2008).

3.4.4 R\textsuperscript{Jas17}A Is Required for JAZ9 to Repress JA Responses \textit{in planta}.

Our results suggest a model in which the repressor function of JAZ proteins \textit{in planta} requires two inseparable R\textsuperscript{Jas17}A-mediated processes: i) MYC2-assisted nuclear import of JAZ proteins, and ii) physical interaction with MYC2. If this model is correct, we expect that: i) directing JAZ9\textsubscript{\Delta}Jas (SCF\textsubscript{COI1} degradation-resistant, but not MYC2-interacting) to the nucleus may not be sufficient to confer JA insensitivity, but ii) JAZ9-
RK^{Jas6,7}AA (SCF^{COI1} degradation-resistant, MYC2-interacting, and nuclear-localized) should confer JA insensitivity when transgenically expressed in wild-type plants. To test these predictions, we fused $\text{JAZ9}\Delta\text{Jas}$ to mCherry (mCH) and VirD2NLS, a well-characterized nuclear localization signal from the 
Agrobacterium tumefaciens
VirD2 protein (Citovsky et al., 2006; Lee et al., 2008) and expressed these constructs in Col-0 plants. As expected, mCH:$\text{JAZ9}\Delta\text{Jas}:\text{NLS}$ gained the ability to enter the nucleus (Fig. 3.9A). However, Col-0 plants expressing the $35\text{S}:\text{mCH}:\text{JAZ9}\Delta\text{Jas}:\text{NLS}$ transgene remained sensitive to JA, as indicated by inhibition of root elongation in seedlings grown on MS agar supplemented with 10 µM methyl-JA (MeJA; Fig. 3.9B and C). This result demonstrates that redirecting $\text{JAZ9}\Delta\text{Jas}$ to the nucleus with a classical NLS is not sufficient to repress JA signaling. In contrast, transgenic Col-0 seedlings expressing the $\text{YFP}:\text{JAZ9}-\text{RK}^{\text{Jas6,7}}\text{AA}$ transgene were partially insensitive to JA (26% root growth inhibition, compared to 62%-65% root growth inhibition for Col-0), and did not show growth inhibition of the rosettes when treated with methyl-JA (Fig. 3.10, Fig. 3.11A and B, and Fig. 3-12). Transgenic expression of $\text{YFP}:\text{JAZ9}-\text{JR}^{\text{Jas17}}\text{A}$ and $\text{YFP}:\text{JAZ9}-\text{KR}^{\text{Jas16-18}}\text{AAA}$ did not affect root growth sensitivity to JA or growth inhibition of the rosettes (Fig. 3.11A and B; and Fig. 3.12).

We also performed disease assays to evaluate whether these plants are altered in response to the coronatine-producing strain 
Pseudomonas syringae pv tomato (Pst)
DC3000. We found typical symptom development on wild-type Col-0 and transgenic plants expressing YFP, YFP:JAZ9, or YFP:JAZ9-KR$^{\text{Jas16-18}}\text{AAA}$ (Fig. 3.13A).
Figure 3.9. Forced targeting of JAZ9ΔJas to the nucleus is not sufficient to confer JA-insensitivity.
**Figure 3.9 (cont’d).** Forced targeting of JAZ9ΔJas to the nucleus is not sufficient to confer JA-insensitivity. (A) Bright-field and fluorescent image overlay of mCherry (mCH), mCH:JAZ9, mCH:JAZ9ΔJas, and mCH:JAZ9ΔJas-NLS stably expressed in transgenic Arabidopsis Col-0 plants. mCH:JAZ9ΔJas-NLS is contains a VirD2-NLS at the C-terminal end of JAZ9ΔJas. (B) Pictures of ten-day-old transgenic Arabidopsis seedlings grown on 10 μM MeJA. Scale bar = 10 mm. (C) Quantification of root length. Growth plates were scanned to capture images, and root lengths were measured using ImageJ software. Data are the means from 15 plants; error bars represent SD. Letters on columns indicate statistically significant differences (p < 0.01, Tukey’s HSD test). mCH constructs and T1 seeds were provided by Dr. Jian Yao.
Figure 3.10. Transgenic expression of YFP:JAZ9-RK\textsuperscript{Jas6,7} AA causes insensitivity to rosette growth inhibition by methyl-JA treatment. Pictures of 14-day-old seedlings grown on 10 µM MeJA. YFP:JAZ9-RK\textsuperscript{Jas6,7} AA (Jas6,7) and coi1-30 were insensitive to MeJA treatment as indicated by green cotyledons and leaves compared to Col-0, YFP, YFP:JAZ9, and YFP:JAZ9-KRK\textsuperscript{Jas16-18} AAA (Jas16-18) seedlings. Scale bar = 10 mm.
Figure 3.11. Transgenic expression of YFP:JAZ9-RK$^{Jas6,7}$ AA causes JA-insensitivity.
Figure 3.11 (cont’d). Transgenic expression of YFP:JAZ9-RK$^{\text{Jas6,7 AA}}$ causes JA-insensitivity. (A) Pictures of ten-day-old Arabidopsis seedlings grown on 10 μM MeJA. Scale bar = 10 mm. (B) Quantification of root growth. Seedlings were grown in the absence or presence of 10 μM MeJA for 10 days, and root length was quantified using ImageJ software. Data are the means from 15 plants; error bars represent SD. Letters on columns indicate statistically significant differences (p < 0.01, Tukey’s HSD test).
Figure 3.12. Root growth inhibition assays for all lines containing JAZ9 alanine substitutions. Quantification of root growth during treatment with methyl-JA (MeJA). Seedlings were grown in the absence or presence of 1 µM and 10 µM MeJA for 12 days, and root length was quantified using ImageJ software. Col-0 and coi1-30 were included as controls. All JAZ9 variants are expressing YFP fusions, and included RK^{Jas6,7}AA (RK-AA), R^{Jas17}A (R-A), KRK^{Jas16-18}ARA (KRK-ARA) and KRK^{Jas16-18}AAA (KRK-AAA). Data are the means from 15 plants; error bars represent SD. Letters on columns indicate statistically significant differences (p < 0.01, Tukey’s HSD test was conducted on the 1µM and 10 µM data sets independently).
Figure 3.13. Transgenic expression of JAZ9-RK$_{\text{Jas6,7}}$AA confers resistance to \textit{P. syringae pv tomato} DC3000.
Figure 3.13 (cont’d). Transgenic expression of JAZ9-RK$^{\text{Jas6,7 AA}}$ confers resistance to *P. syringae pv tomato* DC3000. (A) Symptom development on leaves three days post-inoculation with 1x $10^6$ colony forming units (CFU)/ml *Pst* DC3000. Scale bar = 1.5 cm. Five-week-old plants were syringe-infiltrated with *Pst* DC3000. (B) Bacterial enumeration was conducted three days post-inoculation. Data are the mean cfus counted from 4 individual leaves per genotype; error bars represent SD. Letters on columns indicate statistically significant differences (p < 0.01, Tukey’s HSD test).
In contrast, plants overexpressing YFP:JAZ9-RK\textsuperscript{Jas6,7}AA showed significant resistance to \textit{Pst DC3000}, comparable to that seen in the \textit{coi1-30} mutant (Fig. 3.13A). Bacterial counts were as much as 100 times lower than that in Col-0, and leaves retained a disease-free appearance (Fig. 3.13B). These findings indicate that the ability of JAZ9 to strongly repress JA responses \textit{in planta} requires nuclear localization, MYC2 interaction, and resistance to SCF\textsuperscript{COI1}-dependent degradation.

### 3.5 Discussion

JA is an important hormone that regulates diverse physiological processes in plants, ranging from growth and development to immunity against biotic and abiotic stresses. Recent studies from many laboratories have contributed to several significant advances in the dissection of the COI1-JAZ-MYC core signaling module and molecular connections to different downstream cellular pathways (Browse, 2009a; Chico et al., 2008; Chini et al., 2009a; Chung et al., 2009; Fonseca et al., 2009a; Kazan and Manners, 2012; Pauwels and Goossens, 2011; Staswick, 2008). Although the COI1-JAZ-MYC signaling module is thought to perceive ligands and functions inside the nucleus, direct evidence supporting this model and the underlying mechanisms for the nuclear entry of various signaling components have remained largely enigmatic. In this study, we addressed this important issue and show that COI1, JAZ1, JAZ9, MYC2, and NINJA are constitutively localized in the nucleus. In particular, our results revealed a TF-dependent mechanism for the nuclear entry of JAZ9 and provide a potentially unified model for explaining why some JAZ\textDeltaJas proteins, but not others, confer constitutive JA-
insensitivity when overexpressed in plants, a puzzle that has remained unresolved since
the discovery of JAZ repressors.

All JAZ proteins studied to date have been shown to be localized in the nucleus
and contain a highly conserved Jas motif at the C-terminus (Chung and Howe, 2009;
Grunewald et al., 2009; Shyu et al., 2012; Thines et al., 2007; Yan et al., 2007). How
the Jas motif mediates nuclear import, however, is not understood. The Jas motif
contains clusters of basic amino acids conserved among the JAZ proteins (RK$^{\text{Jas6,7}}$
and KRKE$^{\text{Jas16-20}}$), separated by eight amino acids in JAZ9, which superficially
resembles a putative bipartite NLS. Importantly, Grunewald and colleagues (2009)
showed that this stretch of basic amino acids, plus five additional non-basic amino acids
in the Jas motif of JAZ1, was capable of driving GFP into the nucleus. Unexpectedly,
our results show that none of the basic amino acids, except for R$^{\text{Jas17}}$, in the Jas motif
of JAZ9, is required for nuclear localization (Fig. 3.6B). Because clustered, basic amino
acids are essential for the function of an NLS (Conti et al., 1998; Lange et al., 2007), our
results cast doubt on the idea that the Jas motif functions as an NLS per se. Instead,
we found that the requirement of R$^{\text{Jas17}}$ for JAZ nuclear entry is correlated with its
requirement for JAZ9 interaction with MYC2. First, overexpression of nuclear-localized
CFP:MYC2 in N. benthamiana cells is sufficient to drive complete nuclear localization of
YFP:JAZ1$\Delta$Jas (which retains interaction with MYC2), but not YFP:JAZ9$\Delta$Jas (which
does not interact with MYC2) (Fig. 3.4A). Second, CFP:MYC2 lacking its C-terminal
NLS (KRPKKRGRK$^{433-441}$) not only was partially localized in the cytoplasm, but also
trapped a substantial portion of YFP:JAZ9 in the cytoplasm (Fig. 3.4B and Fig. 3.5). Third, the nuclear localization of YFP:JAZ9 is compromised in the myc2 mutant plants (Fig. 3.4C). Altogether, these results suggest that MYC2, but not JAZ1 or JAZ9, plays an active role in determining the nuclear localization of both MYC2 and JAZ in the cell. We therefore propose that the Jas motif (in particular, $R^{\text{Jas17}}$) is necessary for the nuclear entry of JAZ9 because it is essential for physical interaction with MYC2. This proposal is further supported by protein sequence analysis using ProteinPredict software, which shows a monopartite NLS, KRPKKRGRK$_{433-441}$, in the C-terminal half of MYC2, but failed to identify a predictable NLS in either JAZ1 or JAZ9.

It seems likely that the TF-dependent mechanism for nuclear import would be generally applicable to other JAZ repressors, because interaction with MYC2 and related TFs is a common property of all characterized JAZ repressors. Furthermore, while all of our results are consistent with the physical interaction with MYC2 (and presumably other TFs) being a major factor in directing the nuclear entry of JAZs, it is possible that other MYC2-dependent processes also influence this process. For example, MYC2 may positively regulate the expression of the components of the nuclear import machinery as an additional mechanism of assisting the nuclear entry of JAZs. However, examination of publicly available Arabidopsis gene expression databases (Dombrecht et al., 2007; Srinivasasainagendra et al., 2008) did not reveal obvious MYC2-dependent regulation of the expression of such genes.

Although transgenic overexpression of JAZΔJas proteins has been used as the primary means in the discovery of the repressor function of JAZ proteins because these
derivatives are resistant to SCF$^{\text{COI1}}$-dependent degradation and confer JA-insensitive phenotypes (Chini et al., 2007; Chung et al., 2010; Chung and Howe, 2009; Melotto et al., 2008), the mechanism by which JAZΔJas confers the dominant repressor function is still unresolved (Browse, 2009b; Chini et al., 2009b; Chini et al., 2007; Thines et al., 2007). Our results now provide an explanation for why some, but not other JAZΔJas proteins, confer constitutive JA-insensitivity when overexpressed in plants, and suggest a model in which the ability of a JAZΔJas to confer JA insensitivity is linked to three features: TF-interacting, nuclear-localized, and resistant to SCF$^{\text{COI1}}$-dependent degradation. First, we found that JAZ9ΔJas failed to repress JA signaling (Fig. 3.1). This was in contrast to strong JA insensitivity caused by overexpression of JAZ1ΔJas (Melotto et al., 2008; Thines et al., 2007). By investigating the capability of JAZ1ΔJas and JAZ9ΔJas to interact with MYC2, we have determined that interaction with MYC2 is a key feature that is associated with eliciting JA-insensitivity, as JAZ1ΔJas, but not JAZ9ΔJas, is still able to interact with MYC2 (Fig. 3.2A). Second, confocal microscopic examination revealed that YFP:JAZ9 is colocalized with CFP-MYC2 in the nucleus, whereas those Jas motif mutants that do not confer JA insensitivity, despite being resistant to degradation in response to coronatine, were not found to be co-localized with CFP-MYC2, but instead localized mostly in the cytosol (Fig. 3.6, Fig 3.7, Fig. 3.8). Third, we were able to identify two COI1-interacting residues, RK$^{\text{Jas6,7}}$, that when mutated to alanine, created a stable JAZ9 derivative that is nondegradable, still interacts with MYC2, and is co-localized with MYC2 in the nucleus (Fig. 3.6, Fig. 3.7, Fig. 3.8). This JAZ9 derivative now exerts JA insensitive phenotypes when
transgenically overexpressed (Fig. 3.10, Fig. 3.11, Fig. 3.12, Fig. 3.13). On the other hand, we found that simply directing JAZ9ΔJas (resistant to SCF\(^{\text{COI1}}\)-dependent degradation, but not interacting with MYC2) into the nucleus by fusion to a NLS was not sufficient to create dominant jasmonate insensitive phenotypes (Fig. 3.9). The MYC2 interaction-based model of repression not only could explain the lack of dominant-negative effect of JAZ9ΔJas, but also the ability of JAZ1ΔJas and JAZ10ΔJas to confer JA-insensitivity because both of them still interact with MYC2, indicating more than one MYC2-interacting region in these particular JAZs (Chung and Howe, 2009; Zhang and Turner, 2008). However, this model could not yet explain the ability of JAZ3ΔJas to confer JA-insensitive phenotypes because the Jas motif is required for JAZ3 interaction with MYC2, but JAZ3ΔJas confers JA insensitivity (Chini et al., 2009b; Chini et al., 2007). It is possible that TFs other than MYC2 are biologically relevant targets of JAZ3, or that JAZ3ΔJas confers JA-insensitivity through heterodimerization with other nuclear-localized and MYC2-interacting JAZ repressors, as proposed previously (Chini et al., 2009b).

To our knowledge, TF-dependent nuclear import of cognate transcriptional repressors has not been reported in plants or other eukaryotic systems. However, Pfeiffer and colleagues recently reported that phytochrome-interacting factor (PIF) TFs facilitate the nuclear import of a light receptor protein, phytochrome B, as a critical step in regulating light signaling (Pfeiffer et al., 2012). Thus, at least two major signaling systems in plants, light signaling and JA signaling, utilize the TF-dependent nuclear import mechanism. It is possible that plants have evolved this mechanism to enable immediate targeting of TFs for repression by cognate repressors, possibly even as they
are synthesized in the cytoplasm. This mechanism may be important if the TOPLESS transcriptional repression complex is rate-limiting in the nucleus because of its involvement in many signaling processes (Pauwels et al., 2010). TF-dependent import of JAZs could efficiently target the NINJA/TOPLESS transcriptional repression complex to only TF-bound JAZs, but not “nonproductive” TF-free JAZs, which could occur if JAZs were imported independently. Regardless, the TF-dependent nuclear import mechanism may be needed to ensure maximal and immediate repression of JA signaling to minimize unnecessary growth inhibition and other undesirable side effects that are known to accompany the activation of JA signaling (Yang et al., 2012).
LITERATURE CITED
3.6 Literature Cited


and jasmonoyl isoleucine-dependent interactions with the COI1 F-box protein. Plant J 55, 979-988.


Chapter 4

Conclusions and future directions
4.1 Abstract

Detailed structural knowledge of hormone receptors is central to our understanding of hormone biology. Structure/function-based approaches to understanding the role of hormone perception in plants have been useful for elucidating many important components of the receptor complexes and signal transduction pathways. The results of mutational analysis of COI1 and JAZ9 described in this thesis have led to many interesting observations pertaining to the contribution of individual amino acids in COI1 and JAZ9 that are critical for formation of the JA receptor complex and its function.

In total, 43 amino acid substitutions were generated in the LRR domain of COI1, which confers ligand binding and substrate specificity. Essential amino acids were identified in the putative ligand binding pocket, JAZ binding surface and co-factor binding pocket. Three mutations that disrupted ligand-dependent COI1-JAZ interaction in yeast-two-hybrid experiments were chosen for further functional analysis in planta. The role of specific amino acids in the Jas motif of JAZ9 was also investigated, and revealed a remarkable correlation between JAZ9 interaction with the transcription factor MYC2 and nuclear localization. Additionally, one amino acid was identified, JAZ9-R^{Jas17}A, that was critical for interaction with MYC2 and nuclear localization. In this chapter, suggestions are made for continuation of the research described in this thesis, future directions and potential applications.
4.2 Conclusions and future directions

Jasmonic acid (JA) is an important plant hormone regulating many physiological aspect of plant growth, development and defense. The most well-characterized aspects of JA signaling include its role in wounding (Koo and Howe, 2009), defense responses to challenges from herbivorous insects (Erb et al., 2012), and in concert with ethylene contributes to defense against necrotrophic pathogens (Glazebrook, 2005). Upon perception of developmental cues, herbivory or wounding, or pathogen attack, the biologically active form of the hormone JA-isoleucine (JA-Ile) is synthesized via biochemical activity of enzymes localized in the chloroplast, peroxisome and cytoplasm (Gfeller et al., 2010). In Arabidopsis, there is evidence for two stereoisomers of JA-Ile; one of these, (+)-7-iso-JA-Ile, is biologically active (Fonseca et al., 2009). Induction of JA responses is known to suppress salicylic acid (SA) signaling, and as such, actually suppresses defense responses to biotrophic or hemibiotrophic pathogens such as Pseudomonas syringae. Several pathovars of P. syringae have produce a structural mimic of JA-Ile, coronatine (COR), which is a potent agonist of the JA receptor complex (Katsir et al., 2008; Sheard et al., 2010) and increases host susceptibility during infection.

Central to our understanding of hormone biology is obtaining detailed structural knowledge of the receptor. Recently, the crystal structure of the JA receptor has been solved (Sheard et al., 2010) and this new insight of JA perception has shed light on the mechanistic aspects of not only JA recognition and signaling, but also suggests a structural explanation for the observation that COR is a more potent agonist of JA
receptor complex formation. In the ligand binding pocket, JA-Ile and COR are packed in between the aromatic side chains of a phenylalanine and a tyrosine through hydrophobic interactions (Sheard et al., 2010). The cyclopentanone ring of JA-Ile contains two points of stereochemistry at carbons 3 and 7, and is therefore more flexible than the rigid cyclohexene ring of COR. This difference in chemical structures of the two ligands has been proposed as a structural explanation for the higher binding affinity of COR compared to JA-Ile (Fonseca et al., 2009; Sheard et al., 2010). My research on the roles of individual amino acids in the LRR domain of COI1 and the Jas motif of JAZ9 contributed to the ongoing understanding of formation of the COI1-JAZ co-receptor complex, nuclear localization of JAZ repressors, and differential phenotypic responses resulting from transgenic expression of JAZ repressors lacking the Jas domain.

4.3 Mutational analysis of COI1

During the course of my thesis research, a total of 43 amino acid substitutions were generated in the LRR domain of COI1, which confers ligand binding and substrate specificity. Five amino acids in the putative ligand binding pocket, F89, Y386, Y444, L469, and L470, were found to be essential for interaction between COI1 and all JAZ proteins tested in Y2H experiments. Two amino acids, R409 and R496, differentially affected ligand interactions with JAZ1, JAZ3 and JAZ9. I also identified six amino acids, M88, L91, Y302, R326, L412 and L413 that are essential for COI1 interaction with the JAZ degron peptide. Only one amino acid mutation, R351, had a differential effect that disrupted COI1 interaction with JAZ1 and JAZ9 but not JAZ3.
Three mutations that disrupted ligand-dependent COI1-JAZ interaction in Y2H were chosen for further functional analysis in planta. Y302A and R326A disrupt JAZ binding, and Y444A disrupted ligand binding. Of these, only Y444A resulted in apparently complete interruption of examined JA responses, as demonstrated by insensitivity to JA-mediated root growth inhibition, lack of JA-induced anthocyanin production, and reproductive defects. Y302A and R326A had partial effects on these JA responses. Additional experiments using the transgenic plant material generated in this research should be performed, such as ligand binding assays for COI1-Y444A, to confirm that this mutation affects binding. Furthermore, pull-downs using purified JAZ proteins and protein extracts from COI1-Y302A and COI1-R326A plants should be conducted to test if interaction with all, or only some, JAZ proteins has been disrupted.

Several amino acids that are located near the inositol-phosphate binding pocket were identified, that when mutated to alanine, disrupted COI1-JAZ interaction in Y2H. Of these, the basic amino acids R121, R348, R409 and R351 and the acidic amino acid D407 all contribute to COI1-JAZ interaction. Interestingly, several of these amino acids have recently been reported to affect JA signaling responses in planta when mutated and transgenically expressed in Arabidopsis (Mosblech et al., 2011). Taken together, these results suggest the importance of inositol-phosphate binding for the formation of the COI1-JAZ co-receptor, a situation mirroring the auxin receptor (Mosblech et al., 2011; Sheard et al., 2010; Tan et al., 2007).
One interesting observation from the Y2H experiments was that COI1-K147A and COI1-K147S appear to enhance COI1 interaction with all JAZs tested. Currently, transgenic Arabidopsis lines expressing these COI1 mutants are being screened for enhanced sensitivity to JA or COR. Future experiments using these lines will include quantitative PCR (qPCR) to determine if JA-response genes are turned on earlier or more strongly when seedlings are treated with JA-Ile or COR. If lines are identified that have enhanced sensitivity, these plants will be challenged with biotic stresses such as insect feeding and necrotrophic pathogen infection to determine if these mutations in COI1 can confer increased resistance to these challenges. Alternatively, *P. syringae* infection assays could be used to probe response to bacterial pathogens. In this case, increased susceptibility would be expected if the transgenic lines are hypersensitive to COR.

4.4 Mutational analysis of JAZ

In my experiments on the role of specific amino acids in the Jas motif in COI1-JAZ9 interactions, I found a remarkable correlation between JAZ9 interaction with the transcription factor MYC2 and nuclear localization. Specifically, the JAZ9-R\(^{\text{Jas17}}\) A mutant protein could not interact with MYC2 and was also excluded from the nucleus. In fact, any double or triple alanine substitutions that contained R\(^{\text{Jas17}}\) A had the same effect on MYC2 interaction and nuclear localization. Interestingly, when R\(^{\text{Jas17}}\) was present in the KRK\(^{\text{Jas16-18}}\) ARA double mutant, the interaction with MYC2 and nuclear
localization was restored, suggesting that $R^{Jas17}$ is necessary and sufficient in this context to couple MYC2 interaction and nuclear localization of JAZ9. This arginine residue is completely conserved among JAZ proteins, and therefore I hypothesize that it has the same function in other JAZ proteins. To test this hypothesis, $R^{Jas17}$ from all JAZ proteins could be mutated to alanine, and then the sub cellular localization of the mutants could be analyzed to determine the effect of this mutation on nuclear localization. Additionally, these mutants could be tested for the ability to interact directly with MYC2.

Genetic evidence for a role of MYC2 in nuclear localization of JAZ9 was provided by experiments using Col-0 and $jin1-9/myc2$ transgenic lines expressing a $35S:YFP:JAZ9$ gene construct. I found that YFP:JAZ9 was partially excluded from the nucleus when expressed in $jin1-9$, but was nuclear localized in Col-0 plants. To determine if localization of other JAZ proteins are also affected by the $jin1-9$ mutation, I am currently preparing $35S:YFP:JAZ1$ transgenic lines in both Col-0 and $jin1-9$ genetic backgrounds. The observation that the wild type JAZ9 protein was localized in both the nucleus and cytoplasm suggests that other MYC transcription factors, such as MYC3 and MYC4, might also contribute to nuclear localization of JAZ proteins. It would be interesting to determine if nuclear localization of JAZ proteins is completely abolished in a $myc2/myc3/myc4$ genetic background. Altogether, my results suggest a “piggy-back” mechanism where JAZ proteins enter the nucleus by interacting with their target transcription factors, and the future experiments that are planned will help to determine how wide-spread this mechanism is among the JAZ proteins.
4.5 Practical implications of my research

A potentially interesting finding from the research described in this thesis is that single alanine substitutions at two amino acid positions, R85 and R496, result in the disruption of JA-Ile-dependent COI1-JAZ9 interaction in Y2H experiments, but not COR-dependent interaction, indicating the possibility that a receptor complex could be designed that would exclude one of the two known ligands of the JA receptor. A COI1 mutant that excludes COR, but retains an ability to bind to JA-Ile could conceivably protect crop species that are susceptible to COR-producing pathovars of *P. syringae*. In this respect, all currently existing coi1 mutants should be tested for interactions using both ligands. To test the possibility that interaction between coi1 and other JAZ proteins may confer differential recognition of either JA-Ile or COR, the coi1 mutants reported in this thesis should also be tested for interaction with all JAZ proteins in the presence of both ligands.

In addition to probing the COI1-JAZ complex for ability to discriminate between JA-Ile and COR, there is also the possibility that chemicals that antagonize receptor formation could be identified. Recently, we have initiated a collaboration with Dr. Hong Gao Yan in the Biochemistry and Molecular Biology department at MSU, to model the ligand binding pocket of the COI1-JAZ co-receptor in an effort to identify compounds that may bind to COI11 and inhibit JAZ binding. In a similar approach, the COI1 ligand binding site including the JAZ peptide is being modeled with predicted mutations that might open up the binding pocket to alternative artificial ligands. If successful, such recombinant JA receptors may be further engineered for targeted protein degradation regulated by artificial ligands independent of JA-Ile or COR. In particular, a minimal JAZ
degron could be fused to any protein of interest to facilitate targeted degradation by application of an alternative ligand. Such a fusion might have biotechnology applications.

A project has been initiated to determine the smallest piece of the Jas motif that can bind to COI1, accept polyubiquitination, and target a protein of interest for degradation through the proteasome. Four constructs have been designed to identify the JAZ fragment that would maintain these characteristics. Using the coding sequence of JAZ9 as a template, the C-terminal 50 amino acids (CT50), which begin at the start of the Jas motif, were chosen as a starting fragment. Work by Dr. Jian Yao in the He laboratory has demonstrated that this fragment is sufficient for receptor complex formation and degradation. This stretch of amino acids includes the region homologous to the JAZ1 degron found in the crystal structure of COI1, and also two additional lysine residues, K245 and K246, that may serve as ubiquitination sites. A 28 amino acid fragment, beginning at the same position and excluding the extreme C-terminus, has been included to determine if the JAZ9 fragment containing the lysine residues could facilitate binding and degradation. Additionally, a 21 amino acid fragment was amplified that removes the K245 and K246 residues to determine if the lysine residues within the currently defined JAZ degron can accept ubiquitination. Based on the structure of the JAZ1 degron, K217 appears to be free of interactions with other amino acids in the receptor complex and positioned in a manner that suggests a potential ubiquitin target site. As a negative control, the CT50 fragment was mutagenized to change the first 5 amino acids in the sequence to alanine. A similar modification of the JAZ1 degron disrupts receptor complex formation (Sheard et al., 2010). Additionally, single, double,
triple and quadruple lysine to alanine substitutions were generated at K233, K235, K245 and K246 in JAZ9 to determine if any C-terminal residues can accept ubiquitin. These C-terminal fragments and lysine mutations of JAZ9 have been transferred into Y2H vectors to test for ability to interact with COI1 and have also been tagged with YFP to determine if the YFP:JAZ degron fusion can be degraded by COR treatment.

4.6 Conclusion

Structure-function approaches to understanding the role of hormone perception in plants have elucidated many important components of the receptor complexes and signal transduction pathways. Since the discovery of jasmonates and their important role in plant development and defense many research groups around the world have contributed to the understanding of this signaling pathway. During my thesis research, many major advances in our understanding of JA signaling have been made, and it has been exciting to witness, and to be a part of, the fast-paced progress in this field. Through modification of individual amino acids in both COI1 and JAZ protein components of the receptor complex, my research has contributed to our over-all understanding of the structural requirements for perception of JA-Ile and COR. In the future, I expect that our knowledge of jasmonate signaling will be further enhanced by studies focusing on cell-biological aspects of jasmonate signaling components, such as how tissue specific expression of individual components of this pathway affect the numerous responses to JA and COR. Further, as more components of all hormone response pathways are elucidated, our understanding of the immensely complex
interactions between and among plant hormones, and how these interactions integrate growth, developmental and defense responses throughout the life of a plant will continue to grow.
4.6 Literature Cited


