JASMONATE BIOSYNTHESIS AND SIGNALING IN THE BALANCE OF PLANT GROWTH AND DEFENSE

By

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JASMONATE BIOSYNTHESIS AND SIGNALING IN THE BALANCE OF PLANT GROWTH AND DEFENSE

Abstract

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Jasmonic acid (JA) is a plant hormone that controls many aspects of growth, development, and defense. As a defense hormone JA is rapidly synthesized in response to wounding, or attack from herbivores and pathogens, triggering a gene induction cascade that leads to the deployment of defense compounds. The activation of defenses by JA is accompanied by a potent repression of growth. This effect is thought to involve the diversion of resources from growth to defense. In this dissertation I investigated mechanisms regulating the plant wound response. In particular I performed genetic and biochemical studies on the JA-biosynthesis enzyme OPR3, which is implicated in the control of wound-induced JA biosynthesis. I found that OPR3 is chemically altered in wounded plants, and de-phosphorylation of an active site tyrosine hypothesized to control OPR3 activity is not necessary for OPR3 control in vivo. I also addressed the question of how JA signaling controls the balance between plant growth and defense. I performed a successful forward genetic screen to identify mutants that are insensitive to wound-induced growth inhibition. Seven mutants were identified which, unlike the wild-type, continue to grow during chronic wounding treatment. Four of these mutants maintain resistance to an insect herbivore and a necrotrophic pathogen suggesting that growth inhibition and the activation of defense responses can be uncoupled. I characterized one of the wound-insensitive mutants in detail. I show that the F-box protein, FBK51, is required for wound-induced growth inhibition, but
not herbivore or pathogen resistance. I demonstrate that FBK51 interacts with the growth-promoting transcription factors VOZ1 and VOZ2, and the interaction leads to VOZ degradation. Transcriptional profiling of the wound response in *fbk51* mutants suggests that a JA-independent pathway involving FBK51 may alter patterns of secondary metabolite accumulation and repression of photosynthesis. In summary, in this dissertation I address the question of how wounding triggers the rapid synthesis of JA, and how the wound response regulates the balance between plant growth and defense.
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To the people who most inspire me and enrich my life: Cindy, Edwin, Harper, and Katie
CHAPTER ONE

General introduction

By the middle of the 21st century the world population is projected to rise to nearly ten billion. To meet the food, feed, fiber, and fuel needs for the next half century and beyond, agricultural production must double despite constraints involving the limited availability of arable land and water, and the problems of increasing soil salinity, and changing climates (Fedoroff, Battisti et al. 2010). In the face of these challenges a more complete understanding of the molecular basis of key crop traits is urgently needed. Growth, fertility, senescence, and fruit ripening are all regulated in part by the plant hormone jasmonate (JA) (Ueda and Kato 1980; Dathe, Rönsch et al. 1981; Creelman, Tierney et al. 1992; McConn and Browse 1996; Pérez, Sanz et al. 1997). Also, JA is an essential mediator of induced defenses and is rapidly synthesized in response to biotic and abiotic stresses (Chung, Koo et al. 2008; Howe and Jander 2008; Browse 2009). Induced defenses confer pest and pathogen resistance, but at a metabolic cost. The construction of defensive structures and the synthesis of defensive compounds involves the diversion of resources away from growth and reproduction (Baldwin 1998; Cipollini, Purrington et al. 2003; Havko, Major et al. 2016). Although the JA-mediated defense response has received considerable attention over the last two decades, major questions remain related to how JA signaling is activated by environmental cues, and how JA manages the balance between growth and defense. Unraveling the network of signals and responses involved in plant defense will certainly yield insights related to growth potential, and the basis for innate plant resistance to insects and pathogens. These regulatory networks will likely present important targets for transgenic crop engineering and molecular breeding programs.

The first JA described was the methyl ester of jasmonic acid, MeJA, isolated in 1962 as an essential oil of the jasmine flower (Demole, Lederer et al. 1962). Nearly twenty years later the
first physiological roles for JAs were discovered when MeJA was found to promote senescence in wormwood and inhibit the growth of broad bean (Ueda and Kato 1980; Dathe, Rönsch et al. 1981). JA garnered attention as a defense hormone in 1990, when Farmer and Ryan demonstrated that airborne MeJA induces expression of defensive protease inhibitors involved in resistance to caterpillar herbivory (Farmer and Ryan 1990). Subsequently, JA was shown to be essential for insect defense by McConn and Browse, who observed dramatically increased susceptibility to the fungal gnat Bradyisia impatiens in an Arabidopsis triple mutant deficient in a fatty acid precursor of JA. Resistance to the gnats could be mostly restored with exogenous application of MeJA (McConn, Creelman et al. 1997).

Early studies on the role of JA in plant defense revealed that the leaves of healthy, unwounded plants accumulate low levels of JA. Herbivory and wounding trigger JA biosynthesis leading to the deployment of induced defenses (Creelman, Tierney et al. 1992; Farmer and Ryan 1992; Albrecht, Kehlen et al. 1993; Li, Li et al. 2002). JA-induced defenses are accompanied by a potent inhibition of growth (Yan, Stolz et al. 2007; Zhang and Turner 2008). The strong repression of growth by JA signaling and the wound response was thought to be related to the allocation of limited resources toward defense that would otherwise contribute to growth or reproductive development (Herms and Mattson 1992; Leone, Keller et al. 2014). This budgeting of resources between growth and defense has been described as the ‘dilemma of plants’ because growth must be sufficient for effective competition with neighbors and for reproduction, while defense must be sufficient to protect existing tissues (Herms and Mattson 1992). As a strong activator of induced defenses and a potent growth inhibitor JA plays an important role in managing the dilemma of growth versus defense.

Many aspects of JA biology including JA biosynthesis and perception are well understood. The pathway for JA biosynthesis was first proposed by Vick and Zimmerman in 1983 (Vick and Zimmerman 1983). Subsequently, biochemical and genetic studies have confirmed their
model (Wasternack 2007; Wasternack and Hause 2013). JA biosynthesis begins in the chloroplast with the release of α-linolenic acid (18:3) from membrane glycerolipids by the action of a lipase. A triple mutant deficient in fatty acid desaturation, fad3/fad7/fad8 accumulates negligible levels of α-linolenic acid and is incompetent in JA production (Farmer and Ryan 1992; McConn and Browse 1996). The free acid is oxygenated by 13-lipoxygenase, producing 13-hydroperoxylinolenic acid (Chauvin, Caldelari et al. 2012). In the first committed step of the pathway the hydroperoxy fatty acid is rapidly dehydrated to the unstable 12,13-epoxyoctadecatrienoic acid, by allene oxide synthase (AOS) (Howe, Lee et al. 2000). This intermediate is rapidly cyclized into (9S,13S)-12-oxo-phytadienoic acid (OPDA) by allene oxide cyclase (AOC). The AOC enzyme determines the stereochemistry of OPDA, and prevents spontaneous conversions to α-ketols, racemic OPDA, and other products (Lee, Nioche et al. 2008). The cyclopentenone ring of OPDA is reduced by one of three isozymes of the peroxisomal OPDA reductase, OPR3, to form 3-oxo-2(2"-[A]-pentenyl)cyclopentane-1-octanoic acid (OPC-8:0) (Schaller, Biesgen et al. 2000; Stintzi and Browse 2000). OPC-8:0 undergoes three rounds of β-oxidation to produce JA which is finally converted to the active form, jasmonoyl-isoleucine by JAR1 (Fig. 1) (Schneider, Kienow et al. 2005; Koo, Chung et al. 2006)

Despite decades of progress toward understanding JA biosynthesis and perception, it is still unknown how environmental signals stimulate the production of JA in response to a threat. Wound-induced JA biosynthesis begins very rapidly in response to wounding; JA begins to accumulate within thirty seconds of wounding in damaged leaves, and as early as 120 seconds in distal leaves sharing direct vascular connections with wounded tissues. The speed of the response suggests that an intracellular cellular signaling cascade post-translationally activates constitutively expressed JA-biosynthesis enzymes (Chung, Koo et al. 2008; Koo, Gao et al. 2009). Although no definite connections have been established between JA biosynthesis enzymes and
signaling pathways, several signaling modules are implicated in the activation of JA synthesis. For example, in Arabidopsis wounding rapidly activates mitogen-activated protein kinases MPK4 and MPK6, and MPK4 kinase activity correlates with JA biosynthesis (Schweighofer, Kazanaviciute et al. 2007). Although the biochemical steps to JA synthesis are well characterized, the point of regulation in the pathway has remained undiscovered. The short time scale of JA accumulation suggests that a JA-biosynthesis enzyme is post-translationally activated. Several lines of evidence point to OPR3 as the key regulated step. The OPR3 substrate, OPDA is present in the tissues of resting plants. Wounding causes a systemic drop in OPDA levels, concomitant with the accumulation of JA. The crystal structure of OPR3 from tomato suggested a potential mechanism of post-translational regulation- Tomato OPR3 crystallized as a self-inhibited homodimer in which the active site of each monomer was blocked by a loop domain from the second monomer (Breithaupt, Kurzbauer et al. 2006). A hydrogen bonding network at the interface of the loop and the active site involved a sulfate ion from the crystallization liquor and an active site tyrosine residue. The position of the sulfate is proposed to mimic phosphorylation of the tyrosine (Breithaupt, Kurzbauer et al. 2006; Koo, Gao et al. 2009). This finding is the basis for the hypothesis that the tyrosine phosphorylation occurs \textit{in vivo} to stabilize the inactive dimeric form of OPR3, and that JA biosynthesis can be triggered by dephosphorylation (Breithaupt, Kurzbauer et al. 2006).

For decades it was known that JA activates responses to biotic and abiotic stresses by stimulating transcriptional reprogramming (Creelman, Tierney et al. 1992; Herde, Atzorn et al. 1996; Howe, Lightner et al. 1996). However, the mechanistic details of JA-mediated transcriptional regulation remained elusive. An insight came from the identification of a mutant line resistant to the bacterial phytotoxin coronatine: \textit{coronatine insensitive 1 (coi1)}. The effects of coronatine treatment were found to be similar to the effects of JA treatment, and \textit{coi1} displayed phenotypes similar to lines deficient in JA biosynthesis, including male-sterility, and sensitivity to
insect and pathogen attack. The coi1 mutant was also resistant to exogenous JA (Feys, Benedetti et al. 1994). Map-based cloning revealed that COI1 encodes an F-box protein, a specificity module in SCF E3 ubiquitin ligase complexes. This suggested a mechanism of JA perception in which JA promotes degradation of transcriptional repressors through the activation of an SCF^{COI1} ubiquitin ligase complex. (Xie, Feys et al. 1998). The missing repressors, Jasmonate-Zim domain proteins (JAZ) were identified independently by three groups in 2007 (Chini, Fonseca et al. 2007; Thines, Katsir et al. 2007; Yan, Stolz et al. 2007). With the receptor module identified the structural requirements for the active JA hormone could be tested. COI1 interacts strongly with JAZ proteins in the presence of the isoleucine conjugate of jasmonic acid: jasmonyl-L-isoleucine, (JA-Ile), and coronatine, but not in the presence of other jasmonates. JAZ repressors are subsequently targeted for degradation by the SCF^{COI1} complex relaxing repression of JA-responsive transcription factors including MYC2 (Thines, Katsir et al. 2007).

The critical role for JA signaling in plant defense and development is well-established, and the key steps in JA biosynthesis and perception are well-understood. What remains to be uncovered are the molecular signals upstream of the activation of JA biosynthesis, and the details of downstream metabolic and regulatory reprogramming triggered by JA to shift the plant from growth to defense mode. In this dissertation I report my investigations of both of these aspects of JA signaling. In chapter three I present experimental evidence that OPR3, the JA biosynthesis enzyme implicated in the control of the pathway, is post-translationally modified in response to wounding, but that blocking the proposed regulatory tyrosine dephosphorylation does not enhance OPR3 activity in vivo. In chapter four I show that growth and defense are uncoupled in a newly identified jasmonate hypersensitive mutant. This result is part of a growing body of evidence that the balance between growth and defense is more complicated than a simple diversion of resources between the two processes. I present a successful forward genetic screen for mutants that are insensitive to wound-induced growth inhibition, without compromising
pathogen and herbivore resistance. This screen reveals four distinct mutant lines with uncoupled growth and defense. In chapter five I show that one of the genes identified in my screen encodes an F-box protein, FBK51, which is required for wound-induced growth inhibition. FBK51 interacts with two redundant transcription factors, VOZ1 and VOZ2 which promote plant growth and reproduction. This research helps to further our understanding of the dynamic control of the growth defense balance and the role of JA signaling in the process.
Figure 1. Pathway and compartmentalization for jasmonic acid biosynthesis. A The pathway begins in the chloroplast with the release of trienoic fatty acids from plastid membranes followed by oxygenation and cyclization to (9S,13S)-12-oxo-cis-10,15-phytodienoic acid (Cis-OPDA). B Cis-OPDA is exported to the peroxisome for reduction, followed by β-oxidation to produce jasmonic acid.
References:


CHAPTER TWO
CONTROL OF CARBON ASSIMILATION AND PARTITIONING BY JASMONATE: AN
ACCOUNTING of GROWTH-DEFENSE TRADEOFFS

Abstract: Plant growth is often constrained by the limited availability of resources in the microenvironment. Despite the continuous threat of attack from insect herbivores and pathogens, investment in defense represents a lost opportunity to expand photosynthetic capacity in leaves and absorption of nutrients and water by roots. To mitigate the metabolic expenditure on defense, plants have evolved inducible defense strategies. The plant hormone jasmonate (JA) is a key regulator of many inducible defenses. Synthesis of JA in response to perceived danger leads to the deployment of a variety of defensive structures and compounds, along with a potent inhibition of growth. Genetic studies have established an important role for JA in mediating tradeoffs between growth and defense. However, several gaps remain in understanding of how JA signaling inhibits growth, either through direct transcriptional control of JA-response genes or crosstalk with other signaling pathways. Here, we highlight recent progress in uncovering the role of JA in controlling growth-defense balance and its relationship to resource acquisition and allocation. We also discuss tradeoffs in the context of the ability of JA to promote increased leaf mass per area (LMA), which is a key indicator of leaf construction costs and leaf life span.
Introduction:

This chapter was published as a literature review in the journal: *Plants*. I co-authored this with Ian Major, who also performed Leaf mass/area measurements. Jeremy Jewell, Elham Attaran, John Browse and Gregg Howe were also contributing authors.

As the primary photosynthetic organ in plants, leaves are the major source of reduced carbon skeletons that fuel the biosynthesis of energy-rich macromolecules. However, green leaves in general and protein-rich mesophyll cells in particular, are also a nutritional wellspring for arthropod herbivores and pathogens whose life cycles depend on plants as a food source. Plants effectively counter these biotic threats through constitutive or induced synthesis of myriad defense compounds, as well as development of specialized cells in which these compounds are produced and stored (Howe and Jander 2008). The expression of defense traits may exact allocation costs that negatively impact plant growth, particularly when resources such as light and nitrogen are limiting in the environment. Diversion of resources to defense at the expense of construction of new leaf tissue, which otherwise return revenue in the form of photosynthate over the lifetime of the organ, also imposes so-called opportunity costs that are compounded as plants age (Coley, Bryant et al. 1985; Herms and Mattson 1992). These direct costs of defense may also have indirect ecological costs, including reduced ability to compete with neighboring plants or increased susceptibility to enemies that are not targeted by the induced defense (Stamp 2003). The allocation of limited resources to plant growth and defense has therefore been described as a “dilemma” because growth rates must be sufficient to compete with neighboring plants for light capture while not neglecting investment in defense against plant-eating organisms (Herms and Mattson 1992).

Among the biochemical components that must be budgeted between growth and defense are carbon skeletons, ATP and reducing equivalents from photosynthesis, as well as assimilated
nitrogen, phosphorous, sulfur, and trace nutrients. Investment of these resources into growth increases the area of resource-acquiring tissues (i.e. photosynthetic structures and roots) and thus accelerates growth capacity. Conversely, investment in defense reduces the ability of herbivores and pathogens to destroy tissues that are needed for resource acquisition (Herms and Mattson 1992). To mitigate the cost of defense on reproductive fitness, plants have evolved inducible defenses, which are deployed upon perception of attack (Karban and Baldwin 1997). The plant hormone jasmonate (JA) has become recognized as a chief mediator of inducible defenses. JA is synthesized within minutes of the perception of a threat and exerts transcriptional control over thousands of genes to effect resistance to herbivory and disease (Glauser, Dubugnon et al. 2009; Koo, Gao et al. 2009; Campos, Kang et al. 2014).

The expression of defense traits in response to increased JA levels is accompanied by a potent inhibition of growth (Yan, Stolz et al. 2007; Zhang and Turner 2008). This has been demonstrated through the use of mutants that lack the ability to produce or perceive JA; such mutants are not only susceptible to biotic aggressors but also fail to respond to stress cues with growth arrest. In the last decade, much progress has been made toward understanding the core components of JA signaling and the way in which they are integrated into the wider hormone response network (Browse 2009; Wasternack and Hause 2013; Huot, Yao et al. 2014). Despite these advances, however, our understanding of how JA signaling exerts control over growth, either through crosstalk with other growth-signaling pathways or as a consequence of resource allocation to defense (Figure 1), is still in its infancy. In the simplest scenario, carbon and energy diverted from growth would provide for a stoichiometric increase in defense compounds. However, as discussed below, there is a net loss in dry-weight gain within a day or so of the induction of defense signaling, suggesting a more complex scenario. Here, we discuss current opinions regarding the role of JA in governing metabolic tradeoffs between growth and defense, with particular emphasis on how JA modulates resource acquisition and allocation to achieve growth-defense balance.
Jasmonate is Central to the Growth-Defense Tradeoff:

Jasmonate was first identified more than half a century ago as a component of the essential oil of jasmine flowers (Demole, Lederer et al. 1962). Decades later, it was recognized that JA serves a physiological role in growth inhibition of a wide variety of tissues in both monocot and dicot plants (Aldridge, Galt et al. 1971; Dathe, Ronsch et al. 1981; Ueda and Kato 1982; Corbineau, Rudnicki et al. 1988; Nojiri, Sugimori et al. 1996). The ability of exogenous JA to inhibit root elongation in a dose-dependent manner is now widely used as a simple quantitative bioassay of JA sensitivity (Staswick, Su et al. 1992; Feys, Benedetti et al. 1994; Lorenzo, Chico et al. 2004). A role for JA in defense was recognized by Farmer and Ryan, who demonstrated that airborne JA (JA methyl ester) induces the expression of proteinase inhibitors that contribute to resistance to herbivory (Farmer and Ryan 1990). Pioneering work in Zenk’s laboratory further showed that JA is a potent elicitor of defense-related secondary metabolites in suspension-cultured plant cells (Gundlach, Muller et al. 1992). Early genetic studies established that JA biosynthesis is essential for insect defense in both tomato and Arabidopsis (Howe, Lightner et al. 1996; McConn, Creelman et al. 1997). Subsequently, numerous studies have confirmed the central role of JA in plant defense against a variety of herbivores and necrotrophic pathogens (Kessler and Baldwin 2001; Glazebrook 2005; Browse 2009; Campos, Kang et al. 2014).

The dual roles of JA in growth inhibition and defense have mostly been studied as independent phenomena but, in light of the tradeoffs mentioned above, there is a need to understand how these two processes are integrated. Recent work is beginning to uncover mechanisms that tip the growth-defense balance through crosstalk between JA signaling and other hormone response pathways (Kazan and Manners 2012; Ballare 2014; Huot, Yao et al. 2014). A particularly important node of crosstalk is signal antagonism between JA and the growth-promoting, gibberellin (GA) pathway. The core JA signaling module includes the JAZ transcriptional repressors that inhibit JA
responses by blocking the activity of transcription factors, such as MYC2. The bioactive form of JA, jasmonoyl-L-isoleucine (JA-Ile), promotes the interaction between JAZ proteins and the Coronatine Insensitive (COI1)-containing Skp1/Cullin/F-box (SCF$^{COI1}$) E3 ubiquitin ligase complex, leading to the ubiquitination and 26S proteasome-dependent degradation of JAZs (Chini, Fonseca et al. 2007; Thines, Katsir et al. 2007; Sheard, Tan et al. 2010). Mutants lacking a functional COI1 are completely compromised in all known jasmonate regulated processes and do not respond to the effects of exogenously applied JA (Xie, Feys et al. 1998; Li, Zhao et al. 2004; Paschold, Halitschke et al. 2007). The JAZ proteins modulate growth responses, at least in part, through direct interaction with the DELLA repressors of the GA pathway. Current evidence indicates that JAZ-DELLA interactions competitively inhibit the ability of each repressor to exert repression on cognate transcription factors (Hou, Lee et al. 2010; Wild, Daviere et al. 2012; Yang, Yao et al. 2012). Thus, degradation of DELLA proteins by the GID1-GA-SCF$^{SLY1}$ complex frees JAZ and results in greater inhibition on transcription factors (e.g., MYC2) that promote JA responses. Likewise, JAZ-DELLA interaction interferes with the ability of DELLA to repress the activity of growth-promoting PIF (Phytochrome-Interacting Factor) transcription factors.

In addition to the crosstalk between the JA and GA pathways, other hormones have received attention for modulating the effect of JA on growth. Evidence suggests that part of the growth repression arm of JA signaling involves antagonism between JA and the growth-promoting brassinosteroids. A leaky mutation in DWARF4 (DWF4), a gene encoding a key enzyme in brassinosteroid biosynthesis, partially restores the sensitivity of a hypomorphic coi1-2 allele to the growth inhibitory effects of exogenous JA (Ren, Han et al. 2009). Additionally, JA treatment represses DWF4 transcript accumulation in a COI1-dependant manner. Together these results suggest that the growth repression limb of the JA pathway could function in part by blocking brassinosteroid production and downstream activation of cell expansion, a possibility supported by the identification of DWF4 over-expressing Arabidopsis lines in which root sensitivity to JA is
decreased (Kim, Fujioka et al. 2013). Growth inhibition by JA may also involve repression of the cytokinin signaling pathway, which is a major positive regulator of cell division activity (Ueda and Kato 1982; Schaller, Street et al. 2014).

**Growth Inhibition: Cell Elongation or Cell Division?**

Observations of JA-treated plants suggest that JA signaling interferes with processes that promote growth through both cell division and cell elongation. In Arabidopsis leaves, wound-induced JA biosynthesis resulted in a 50% decrease in the calculated number of palisade mesophyll cells per leaf without noticeable effects on the cell size, suggesting that JA inhibits leaf expansion primarily through cell cycle arrest (Zhang and Turner 2008). This finding was supported by decreased activity of a cell-cycle reporter, *CycB1;2pro-GUS*, and is consistent with earlier work with tobacco suspension cultured cells in which exogenous JA arrested the cell cycle in the G2 phase (Swiatek, Lenjou et al. 2002; Swiatek, Azmi et al. 2004). An independent study of Arabidopsis leaves at various developmental stages also showed that JA-induced growth inhibition is associated with a strong reduction in cell number. However, delayed cell expansion also contributed to growth inhibition in this experimental system (Noir, Bomer et al. 2013).

A number of studies have demonstrated JA-dependent inhibition of Arabidopsis hypocotyl elongation (Robson, Okamoto et al. 2010; Chen, Sun et al. 2011; Leone, Keller et al. 2014; Chen, Chen et al. 2015) and rice internode elongation (Yang, Yao et al. 2012). In studies where cell length was determined, the JA-dependent reduction in cell length could account for differences in internode elongation (Yang, Yao et al. 2012). Given that elongation of Arabidopsis hypocotyls results primarily from cell elongation rather than division (Szekeres, Nemeth et al. 1996; Smalle, Haegman et al. 1997; Cowling and Harberd 1999), it is likely that hypocotyl growth inhibition by JA is caused by a reduction in cell elongation as well.
Primary root growth inhibition appears to result from a combined reduction in both cell division and cell elongation. In Arabidopsis and rice, exogenous JA inhibits root growth via decreased cell elongation and cell division, although at low JA concentrations decreased cell elongation appears to be primarily responsible for growth inhibition (Chen, Sun et al. 2011; Toda, Tanaka et al. 2013). Repeated wounding of Arabidopsis cotyledons triggered a JA-dependent shoot-to-root signal that is also reported to reduce root growth by decreasing cell elongation and cell division in the root differentiation zone (Gasperini, Chetelat et al. 2015). Interestingly, loss-of-function mutations in the gene encoding the JA co-repressor NINJA results in reduced cell elongation in the differentiation zone, but not reduced cell division (Acosta, Gasperini et al. 2013). In summary, JA-dependent growth inhibition appears to occur through repression of both cell division and elongation, although the extent to which each plays a role appears to depend on the tissue type.

**Modulation of Carbon Assimilation by Jasmonate:**

Central to understanding the mechanistic basis of growth-defense tradeoffs is the question of how changes in endogenous JA levels modulate carbon assimilation and subsequent partitioning to various metabolic sinks. The effects of arthropod herbivore attack, simulated herbivory, and exogenous JA on photosynthesis have been examined in a variety of experimental systems, with mixed results. This variability likely reflects differences in damage type and intensity, location and type of tissue damaged, plant developmental stage, and environmental conditions (Délano Frier, Sánchez Hernández et al. 2012). A preponderance of studies report that treatments associated with activation of JA signaling reduce photosynthesis (Délano Frier, Sánchez Hernández et al. 2012). One interpretation of downregulation of growth and photosynthetic capacity in herbivore-damaged leaves is that these responses are part of the plant’s defense strategy to limit the availability of food for herbivores (Hermsmeier, Schittko et al. 2001).
However, robust plant defense responses also depend on a continuous supply of photosynthetic products to fuel local and systemic biosynthesis of energetically demanding defense compounds. Reduction in photosynthetic capacity by silencing of Rubisco activase in wild tobacco, for example, attenuated JA-mediated resistance to herbivory (Mitra and Baldwin 2008). Evidence for positive or compensatory effects further suggests that foliage loss and induced expression of defenses can drive increased photosynthesis in remaining and new leaves, perhaps through altered source-sink relationships (Thomson, Cunningham et al. 2003; Halitschke, Hamilton et al. 2011). These observations are in general agreement with metabolic labeling studies showing that wounding and exogenous JA can stimulate carbon partitioning to belowground tissues (Babst, Ferrieri et al. 2005; Schwachtje, Minchin et al. 2006; Ferrieri, Agtuca et al. 2013). Zangerl and coworkers (Zangerl and Rutledge 1996) reported a positive correlation between herbivore-induced furanocoumarin synthesis and the amount of leaf area having a reduced photosynthetic efficiency, suggesting that the production of this defense compound is relatively insensitive to herbivore-induced reduction in photosynthetic efficiency. This study also introduced the use of chlorophyll fluorescence imaging to spatially map the photosynthetic efficiency of whole leaves subjected to herbivory. This approach demonstrated decreases in photosynthesis in damaged tissue at the insect bite zone, which may result from water loss, as well as in undamaged regions of the leaf located a considerable distance from the wound site (Zangerl and Rutledge 1996; Délano Frier, Sánchez Hernández et al. 2012).

Many plants downregulate the expression of photosynthetic genes and their corresponding proteins in response to herbivory and JA treatment (Reymond, Bodenhausen et al. 2004; Giri, Wunsche et al. 2006; Mitra and Baldwin 2008; Bilgin, Zavala et al. 2010; Attaran, Major et al. 2014). Given that photosynthesis-related transcripts are highly abundant in leaves, this transcriptional reprogramming may help to provide the metabolic building blocks needed to redirect the cell’s biosynthetic capacity from growth to defense without short-term negative effects.
A recent study by Attaran et al (2014) showed that treatment of Arabidopsis with coronatine (COR), a potent agonist of the JA receptor, triggered a strong reduction of photosynthesis-associated gene expression without an accompanying decrease in photochemical efficiency. This experimental system allows for strong and rapid activation of the JA receptor without the complicating effects of tissue damage that accompany herbivory or simulated herbivory. The reported effects of JA on multiple aspects of photosynthesis make it an important consideration for understanding the physiological basis of growth-defense tradeoffs.

**Jasmonate Promotes Increased Leaf Mass per Area (LMA):**

Whereas the effects of JA on photosynthesis have been well-examined, less is understood about how JA modulates the partitioning of fixed carbon to drive various aspects of growth, including leaf area expansion, leaf thickening, petiole elongation, and the construction of new leaves. Modeling of photosynthetic rate and carbon partitioning in relation to growth showed that carbon is partitioned to leaf area expansion and biomass at different rates, and that the relationship between leaf area and biomass is important to consider since leaf area determines light interception (Weraduwage, Chen et al. 2015). In particular, it was shown that partitioning of carbon to leaf thickness impacts growth more strongly than photosynthesis. Although it is known that activation of the JA defense response reduces leaf expansion (McConn, Creelman et al. 1997; Attaran, Major et al. 2014) and biomass accumulation (Yan, Stolz et al. 2007; Zhang and Turner 2008; Leone, Keller et al. 2014), we are not aware of studies that examined how JA affects growth rates of both leaf area and biomass.

Our early experiments showed that COR slows leaf area growth almost immediately after treatment (Figure 2A) (Attaran, Major et al. 2014). This effect was even more evident by an
apparent complete arrest of rosette diameter expansion (Figure 2B). This rapid effect partly reflects an arrest of petiole elongation and is consistent with the ability of JA to suppress cell elongation through inhibition of PIF transcription factors (Yang, Yao et al. 2012). Leaf area and rosette expansion were arrested for several days before increasing again, though at an apparent reduced rate. This resumption in expansion appeared to be due to leaves that emerged after treatment, and which perhaps are less sensitive to the COR treatment. By comparison, dry mass accumulation remained similar to mock-treated plants for at least one day after treatment (Figure 2C). The absence of a corresponding, immediate reduction in dry mass accumulation is consistent with our previous conclusion that JA does not have an immediate repressive effect on photosynthesis in Arabidopsis leaves (Attaran, Major et al. 2014). These findings are consistent with the idea that reduced leaf area growth in response to JA results in opportunity costs as a consequence of lower total light interception and carbon assimilation on a per plant basis. However, that JA-elicited plants appear to “pay back” such costs in ways that enhance fitness in the presence of herbivores (Baldwin 1998) suggests that a more in-depth analysis of the effects of JA on photosynthetic and non-photosynthetic leaf traits is warranted.

The effects of JA on altered partitioning of resources to leaf area and biomass can be further examined from the ratio of dry mass to leaf area (Leaf Mass per Area, LMA). LMA is helpful to describe the resources invested in a given area of leaf and may change as a consequence of variability in leaf thickness, leaf density, or both (Poorter, Niinemets et al. 2009). LMA has been widely used in studies of plant ecology and agronomy to describe different plant strategies for optimizing fitness to particular environments, as well as for analysis of key chemical, physiological, and structural traits that together define the leaf economics spectrum (Wright, Reich et al. 2004). In general, plants with low LMA tend to acquire resources rapidly and have higher growth rates, whereas plants with high LMA tend to conserve resources and have lower growth rates, but have higher fitness in stressful environments (Poorter, Niinemets et al. 2009). With their
increased resource investment, high-LMA leaves tend to be more durable, and in terms of defense, are less preferred by herbivores to leaves with low LMA (Poorter, Niinemets et al. 2009). Thus, it would appear that leaf traits associated with increased LMA have striking similarity to known effects of JA on leaf development and function.

In the experiment described in Figure 2, LMA was significantly increased the day after treatment with COR, and remained significantly higher for the duration of the experiment (Figure 2D). Following day 1, the average difference in LMA between mock- and COR-treated leaves at each time point remained remarkably constant at approximately 3 g/m$^2$, perhaps as a consequence of negative feedback loops that rapidly restrain JA responses (Campos, Kang et al. 2014). The higher LMA of COR-treated leaves could reflect a concerted change in strategy from rapid leaf area-based growth to protection of energy acquiring-foliage through investment of resources in leaf defense and structural integrity. Thus, the opportunity costs associated with JA-mediated inhibition of leaf area growth may be counterbalanced by other features associated with high-LMA leaves, including decreased respiration and increased leaf life span (Poorter, Niinemets et al. 2009). These areas deserve future attention to better understand the physiological determinants of JA-mediated growth suppression and its relationship to solar energy capture, conversion, and deposition. Studies employing mutants that are impaired in growth-defense balance may provide additional insight into this question. In this context, it is interesting to note that light perception mutants (e.g., phyB) exhibiting rapid growth and reduced defense have low LMA, whereas mutants with reduced responsiveness to GA grow slowly and have increased LMA (Moreno, Tao et al. 2009; Poorter, Niinemets et al. 2009; Hou, Lee et al. 2010). These observations support the emerging paradigm that crosstalk between the JA and GA signaling pathways plays a major role in the control of plant growth-defense balance, and further suggest that consideration of LMA may be useful to explain the complexities of growth-defense balance.
Auditing the Growth-Defense Budget:

A comprehensive, quantitative understanding of the metabolic costs associated with partitioning of carbon into growth and defense pathways is still lacking. Such efforts should recognize that the growth-defense dichotomy oversimplifies the complexities of carbon budgeting because many other physiological processes, including reproduction, respiration, exudation, and volatile emission also depend on photoassimilates (Weraduwage, Chen et al. 2015). Proximate analysis provides one approach to estimate the relative cost of producing defense metabolites from precursors in primary metabolism (De Vries, Brunsting et al. 1974; Gershenzon 1994; Gershenzon 1994). Generally, more reduced secondary metabolites such as terpenoids have a higher metabolic cost than carbohydrate and phenolic metabolites. For example, production of one gram of the monoterpene camphor is calculated to cost 3.1 g glucose equivalents based on the requirements of acetyl-CoA, ATP and NADPH, whereas 1 g of phenol glycoside costs approximately 1.5 to 2 g glucose equivalents (Gershenzon 1994). Cost estimates should account not only for the synthesis of a particular compound, but also for its transport, storage and maintenance. Glucosinolate production in Arabidopsis provides an example for which there is sufficient biochemical knowledge to undertake such an analysis (Halkier and Gershenzon 2006). Flux balance modeling studies, for example, suggest that production of glucosinolates in Arabidopsis would increase the photosynthetic requirement by 15% (Bekaert, Edger et al. 2012).

Nitrogen is a small proportion of plant dry biomass but is vitally important for plant growth and photosynthesis, and is often a limiting factor in natural environments. Thus, JA-dependent changes in nitrogen assimilation or allocation could be expected to have significant impacts on plant growth. Available data indicate that growth versus defense tradeoffs involve not only the reallocation of reduced carbon but also substantial redistribution of nitrogen (Herms and Mattson 1992; Stamp 2003). Recent $^{15}$N flux studies have leveraged nitrogen allocation to RuBisCO and
small N-containing defense metabolites as a proxy for growth-defense tradeoffs (Ullmann-Zeunert, Muck et al. 2012; Ullmann-Zeunert, Stanton et al. 2013). In young rosette leaves of *Nicotiana attenuata*, simulated herbivory reduced nitrogen investment in RuBisCO and total soluble proteins by 89% (Ullmann-Zeunert, Stanton et al. 2013). Importantly, nitrogen investment in RuBisCO and total soluble protein was only reduced by 47% in transgenic plants with partially impaired JA biosynthesis. Thus, diversion of nitrogen in response to JA signaling may be a significant driver of growth inhibition.

Calorimetry offers a potentially informative yet simple approach for estimating the biochemical cost of defense. Although not as informative as proximate analysis in assessing the contribution of individual compound classes to tissue construction costs, the two methods generally agree for a given compound (Williams, Percival et al. 1987; Griffin 1994). Moreover, calorimetric estimates can be applied to whole plants. Although this approach has been used to assess altered carbon partitioning to lipid biosynthesis in Arabidopsis leaves (Sanjaya, Miller et al. 2013), to our knowledge the method has not been applied to understanding growth-defense metabolic tradeoffs elicited by JA.

If increased LMA represents a significant component of JA-mediated changes in growth-defense balance as suggested by our preliminary studies (Figure 2), it may be possible to use this trait as a starting point for quantitative analysis of the growth-defense budget. Variation in LMA as a function of genotype and environment often reflects differences in leaf anatomical traits (Poorter, Niinemets et al. 2009). Such features include mesophyll tissue volume, cellular volume occupied by the central vacuole and other organelles, volume of air spaces between cells, and overall leaf thickness. A second and complementary approach to explain variation in LMA is to deconstruct the trait into the chemical constituents of leaf biomass, including structural and non-structural carbohydrates, lignin, proteins, lipids, organic acids, minerals, and secondary metabolites (Poorter, Niinemets et al. 2009). In-depth knowledge of central carbon metabolism
and partitioning in Arabidopsis leaves is expected to facilitate this approach (Stitt and Hurry 2002; Weraduwage, Chen et al. 2015), as is knowledge of JA-regulated secondary metabolism in Arabidopsis leaves (Attaran, Major et al. 2014).

Genetic Dissection of Growth-Defense Antagonism:

Over the last twenty years, genetic investigations have revealed key steps in the synthesis and perception of JA in a variety of plant species (Browse 2009; Wasternack and Hause 2013). Studies employing these mutants support the general conclusion that stress-induced JA biosynthesis leads to activation of various transcriptional modules that simultaneously attenuate vegetative growth and heighten defense against insect herbivores and necrotrophic pathogens. More recently, molecular genetic investigations are beginning to untangle regulatory circuits through which JA governs growth and defense. The recent identification of mutants that disrupt either the growth or the defense arm of the JA response pathway suggests that the two processes involve interconnected but distinct regulatory networks.

Hu and coworkers (Hu, Zhou et al. 2013) used an RNA interference (RNAi)-based screen to identify JAV1 as negative regulator of the defense limb of JA signaling. Silencing of JAV1 dramatically reduced plant susceptibility to the necrotropic fungus Botrytis cinera and the lepidopteran herbivore Spodoptera exigua. Similar to JAZ repressors, JAV1 was degraded by a pathway that depends both on COI1 and the 26S proteasome. Unlike JAZs, however, JAV1 does not interact directly with COI1 in the presence of COR. The E3 ubiquitin ligase responsible for targeting JAV1 for destruction remains to be identified. Interestingly, JAV1-silenced plants had no obvious growth phenotypes despite their enhanced defense response (Hu, Zhou et al. 2013). These findings provide genetic evidence for distinct growth and defense branches of JA response pathway.
Additional evidence that JA-mediated growth and defense responses can be genetically separated comes from recent studies on the interaction between the JA and light signaling pathways that control shade avoidance responses. The shade avoidance syndrome is characterized by rapid growth of stems and petioles, which allows neighboring plants to compete for light (Casal 2013). Full sunlight is characterized by a high ratio of red (R) to far-red (FR) light that maintains the phytochrome B (phyB) photoreceptor in its active Pfr form. In this state, phyB represses vegetative growth by inhibiting the activity of PIF transcription factors. Because canopy leaves preferentially absorb red light, light that is transmitted or reflected from neighboring plants has a decreased R:FR ratio. Perception of this shade signal by phyB converts the photoreceptor to its inactive (Pr) form, thereby relieving restraint on PIFs. The ensuing transcriptional responses promote the biosynthesis of auxin, which together with other growth hormones, promotes elongation growth (Casal 2013). Recent studies have revealed that inactivation of phyB by shade light also suppresses JA-triggered defense responses by a mechanism that involves depletion of DELLA proteins, enhanced stability of JAZ repressors, and accelerated degradation of MYCs (Moreno, Tao et al. 2009; Cargnel, Demkura et al. 2014; Chico, Fernandez-Barbero et al. 2014; Leone, Keller et al. 2014). This mechanism of growth-defense balance provides a compelling example of how crosstalk at the DELLA-JAZ interface controls a key aspect of plant phenotypic plasticity, and opens the way to identifying specific family members that mediate these interactions. JAZ10, for example, which encodes several alternative splice variants that strongly repress MYC2 (Yan, Stolz et al. 2007; Chung and Howe 2009; Chung, Cooke et al. 2010; Moreno, Shyu et al. 2013), plays a central role in repressing JA-mediated defense responses during the shade avoidance response (Moreno, Tao et al. 2009; Cargnel, Demkura et al. 2014; Chico, Fernandez-Barbero et al. 2014; Leone, Keller et al. 2014).

Although phyB-JA crosstalk appears to be hardwired to ensure mutually exclusive expression of either growth- and defense-related transcriptional programs, there are emerging
examples of how genetics can be used to tease apart the physiological basis of these tradeoffs. One example comes from studies with the sav3 mutant that lacks the ability to synthesize auxin during the shade avoidance response (Tao, Ferrer et al. 2008). sav3 mutant plants lack shade-triggered growth responses but remain sensitive to the attenuating effects of low R:FR on defense responses, indicating that repression of defense does not result simply from diversion of resources to growth (Moreno, Tao et al. 2009). Conversely, a phyB jaz10 double mutant was shown to retain a constitutive shade avoidance growth response (due to genetic inactivation of phyB) and simultaneously express higher levels of JA-induced secondary metabolites (Leone, Keller et al. 2014). These collective studies provide clues as to how growth-defense tradeoffs may be genetically manipulated.

8. Conclusions and Future Perspectives:

The attention that JA signaling has received in the last several decades has placed the hormone as a key regulator of plants response to attack. Now, JA is receiving renewed consideration as a growth regulator. The elucidation of the JAZ-DELLA antagonism has confirmed a model in which JA mediated defense works in a dynamic balance with growth promotion. Although this and other recent discoveries begin to frame the counterpoise of JA signaling with plant growth, a tremendous effort is required to understand the relationship between JA signaling, deployed defenses, and reductions in components of growth including leaf expansion, dry-weight gain and carbon partitioning. Available results indicate that the shift from growth to defense in response to JA signaling is not a simple reallocation of resources and instead involves a rapid and substantial decrease in the rate of dry-matter accumulation. The positive effect of JA signaling on LMA, however, suggests that the hormone may influence leaf architecture via increased leaf thickness and/or leaf density, potentially extending the lifetime photosynthetic output of challenged plants.
These insights are critically important for an understanding of the survival and fitness of plants in their environment and may reveal opportunities to optimize plant growth, especially in protected agricultural contexts.
Figure 1. Jasmonate induces the re-budgeting of resources from tissue expansion to the production of defense compounds. (A) Plant growth is achieved using carbon skeletons, ATP, and NADPH from photosynthesis. (B) During jasmonate mediated defense plant antifeedant compounds are synthesized de novo from carbon skeletons, ATP, and NADPH that could otherwise contribute to plant growth. (C) In the absence of attack defense compounds are produced in growing plants at a low basal level. (D) Jasmonate triggers the accumulation of defense compounds accompanied by an arrest of tissue expansion. In defended leaf tissue cell size is similar to undefended, but the leaf mass per area is significantly increased.
**Figure 2.** Simplified model depicting interactions between the JA, GA, and phyB signaling pathways. Points of positive and negative regulation are indicated by arrows and perpendicular lines, respectively. bHLH-TFs, basic helix-loop-helix transcription factors that bind G-box cis-regulatory elements typically located in the promoter region of response genes. In full sunlight, PIF transcription factors are inhibited by the active conformer of phyB. See text for details.
Figure 3. Jasmonate-mediated suppression of leaf area and biomass growth in *Arabidopsis thaliana* plants. Soil-grown Columbia-0 plants treated with mock (grey) or with 5 µM coronatine (COR, black) solutions (Attaran, Major et al. 2014) were measured for leaf area (A), rosette diameter (B), and dry weight (C) at indicated times after treatment. Data are the mean increase in growth (n = 12 plants) relative to the day of treatment (day 0) from two independent experiments (diamonds and squares distinguish experiments) and lines are second-order polynomial regressions of combined data from both experiments. Projected leaf area and rosette diameter were determined from overhead images, and dry weight was determined from rosettes (without roots) freeze-dried in a lyophilizer. In D, leaf mass per area (LMA) was calculated from dry weight / leaf area from one experiment in A and C (square points). Error bars are standard
deviation and asterisks indicate $P<0.05$ between mock and COR treatment from a Student's t-Test.
References:


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CHAPTER THREE
AN INVESTIGATION OF THE POST-TRANSLATIONAL REGULATION OF OPR3 IN THE
CONTROL OF JASMONATE BIOSYNTHESIS

Abstract:

Plant survival depends on dynamic responses to diverse environmental threats. The phytohormone jasmonate (JA) plays a key role in initiating stress responses and is indispensable for resistance against herbivore and pathogen attack. Tissue damage triggers the rapid synthesis of JA, which exerts transcriptional control over thousands of genes to reprogram the plant for defense. Considerable progress has been made in uncovering the details of JA biosynthesis. All enzymes necessary for the production of JA in Arabidopsis have been identified and are known to be expressed in the tissues of resting plants, pointing to the likelihood that JA biosynthesis is controlled at the level of post-translational activation of a JA biosynthetic enzyme. Recently, measurements of JA precursors revealed that wounding leads to a systemic depletion of one particular precursor: OPDA. In wounded Arabidopsis OPDA levels drop concomitantly with the accumulation of JA. This observation potentially implicates the OPDA-reductase, OPR3 as the key regulated enzyme in wound-induced JA biosynthesis. A crystal structure of the OPR3 protein from tomato reveals a self-inhibited homodimer that may represent an in vivo mechanism of regulation by control of the dimerization. Here I present a biochemical and genetic investigation of the hypothesis that OPR3 is the point of regulation for JA biosynthesis through post-translational modification.
**Introduction:**

Jeremy Jewell contributed the transgenic opr3 complemented lines used for analysis in this chapter. Matt Salie and Gerhard Munske performed LCMS.

The plant hormone jasmonate (JA) is an important signaling molecule that controls development and responses to environmental stresses. Herbivory and wounding stimulate the rapid production of JA followed by JA-induced defenses, both in the wounded tissue and throughout the plant (Creelman, Tiemey et al. 1992; Farmer and Ryan 1992; Li, Li et al. 2002). Inducible defenses permit a restrained investment of metabolic resources when plants are under minimal threat (Baldwin and Callahan 1993). Continuous induction of defenses in *Nicotiana attenuata* by the exogenous application of MeJA decreased plant size and the lifetime seed production of plants grown under conditions with low frequency of herbivore attack (Baldwin 1998). During defense JA triggers a remodeling of plant secondary metabolism resulting in the production of antifeedant compounds and defense-related proteins (Farmer and Ryan 1990; Bodnaryk 1994; Koiwa, Bressan et al. 1997; Constabel and Ryan 1998; Howe and Jander 2008). Many valuable secondary metabolites including, nicotine, the anti-malarial drug artemisinin, and anti-cancer agent vinblastine, also act as JA-inducible defense compounds in plants (Facchini 2001; Yu, Li et al. 2012; Wasternack and Hause 2013).

In addition to its role in defense JA also controls plant reproductive development. One of the most striking phenotypes of Arabidopsis mutants deficient in JA signaling is male-sterility. Early studies of a fatty acid desaturation triple mutant Arabidopsis *fad3,fad7,fad8* uncovered the requirement for linoleic acid for male-fertility. The anthers of the triple mutant were delayed in
opening and pollen deposition, and the pollen that was produced was found to be inviable. Application of JA restored fertility in fad3, fad7, fad8, suggesting that linoleic acid is required as a jasmonate precursor. Subsequent JA biosynthesis mutants identified in Arabidopsis were also found to be male sterile (McConn and Browse 1996; Stintzi and Browse 2000).

The JA-mediated defense response has been extensively studied. The advent of microarray-based transcript profiling allowed for the identification of the genes under the control of exogenously applied JA. Additionally, mutants deficient in JA synthesis and perception have been used to study the JA-dependent transcriptional response of plants challenged by pathogens, herbivores and mechanical wounding (Reymond, Weber et al. 2000; De Vos, Van Oosten et al. 2005; Devoto, Ellis et al. 2005). These studies show that patterns of JA-dependent transcriptional reprogramming vary depending on the elicitor (Reymond, Bodenhausen et al. 2004). It remains unclear how different herbivores and pathogens stimulate unique JA-dependent gene expression patterns. Fine-tuning of the defense response is thought to involve gene-for-gene interactions between the host and attacker. These interactions involve resistance genes (R genes), the products of which act as receptors for attacker-specific chemical signatures (Nombela, Williamson et al. 2003). Insect salivary secretions, containing fatty acid amino acid conjugates, and lipid-remodeling enzymes are well-known elicitors of defense responses (Giri, Wünsche et al. 2006).

For years JA was thought to antagonize photosynthetic processes. Microarray-based transcription profiling experiments revealed that JA treatment led to transcriptional repression of the photosynthetic machinery. For example JA-treatment repressed transcription of genes encoding the components of the photosystems (Jung, Lyou et al. 2007; Wasternack 2007). More recently, measurements of the photochemical efficiency of JA-treated plants have shown that photochemistry is mostly unaffected by exogenous application of an agonist of the JA signaling pathway (Attaran, Major et al. 2014). It could be that the attenuation of growth by JA involves a
reduction in the rate of de novo synthesis new photosynthetic structures, which were being produced to fill expanding leaf area. As many jasmonate-dependent defenses are induced rather than constitutive, there is a period of vulnerability between the detection of a threat and the deployment of full-strength response. Therefore, plants need to minimize the latency of induced defenses. Accordingly, as a defense mediator, jasmonate begins to accumulate within thirty seconds of wounding in damaged leaves, and as early as 120 seconds in distal leaves sharing direct vascular connections with wounded tissues (Koo, Gao et al. 2009; Chauvin, Caldelari et al. 2012; Mousavi, Chauvin et al. 2013). Although the pathway to JA biosynthesis is well characterized the signaling steps that initiate JA production are not understood.

Thirty years have passed since the pathway for JA synthesis was proposed by Vick and Zimmerman (Vick and Zimmerman 1983). Subsequently, tremendous strides have been taken toward an integrated understanding of JA production and action within the cell. Despite this work it remains unknown how JA synthesis is activated in response to biotic and abiotic threats.

Jasmonate is synthesized via a branch of the oxylipin pathway, which begins in the chloroplast and ends in the peroxisome (Browse 2009; Wasternack and Hause 2013). The polyunsaturated α-linolenic (18:3) acid is released from plastidial membrane galactolipids by the action of a lipase (Ishiguro, Kawai-Oda et al. 2001; Ellinger, Stingl et al. 2010). A 13-lipoxygenase peroxidates the α-linolenic acid to 13(S)-hydroperoxyoctadecatrienoic acid (13-HPOT) (Chauvin, Caldelari et al. 2012). 13-HPOT is the substrate for the first committed step of the JA branch of the oxylipin pathway, and is converted to the 12,13(S)-epoxyoctadecatrienoic acid (12,13(S)-EOT) by allene oxide synthase (AOS) (Lee, Nioche et al. 2008). The 12,13(S)-EOT is unstable and can decompose into α and γ ketols, or form racemic 12-oxophytodienoic acid. 12,13(S)-EOT involved in jasmonate synthesis is enzymatically converted to enantiomerically pure cis-(+)-12-oxophytodienoic acid ((9S,13S)-OPDA) by allene oxide cyclase (AOC) . The cis stereo
configuration determined by the AOC is required for the production of biologically active cis-JA-Ile (Wasternack 2007). (9S,13S)-OPDA is transported to the peroxisome for further processing. OPDA translocation is mediated by the COMATOSE transporter (CTS1 or alternately PXA1/PED3) (Theodoulou, Job et al. 2005). Ion trapping may also allow entry of OPDA into the peroxisome. A single isoform of the OPDA reductase, OPR3, can accept ((9S,13S)-OPDA) to catalyze the conversion of cis-OPDA to 3-oxo-2-(2[Z]-pentenyl)-cyclopentan-1-octanoic acid (OPC-8) (Schaller, Biesgen et al. 2000; Breithaupt, Kurzbauer et al. 2006; Chehab, Kim et al. 2011; Han, Malone et al. 2011). Interestingly, cis-OPDA accumulates in unwounded Arabidopsis tissues, but is systemically depleted in tissues distant from the wound site, concomitantly with JA-Ile accumulation. This observation implicates mobilization of the OPDA pool in regulation of the pathway. Downstream, OPC-8 is activated to a coenzyme a thioester by an acyl activating enzyme (Schneider, Kienow et al. 2005; Koo, Chung et al. 2006). The OPC-8 esters undergo three rounds of β-oxidation involving acyl-CoA oxidase (ACX), multifunctional protein (MFP), and L-3-ketoacyl-CoA thiolase (KAT), reducing the carboxy-containing chain by six carbon units. (+)-7-iso-JA ((3R,7S)-JA) (JA) is released by an unidentified thioesterase. The free JA is readily conjugated to isoleucine to produce the receptor active JA-Ile (Staswick, Tiryaki et al. 2002; Wasternack 2007).

JA-Ile perception involves the F-box protein COI1 and the JAZ proteins. In the absence of JA-Ile JAZ actively repress transcription factors, including MYC2. JA-Ile or the bacterial phytotoxin coronatine promote the recruitment of JAZ to an SCF\textsuperscript{COI1} E3 ubiquitin ligase complex. JAZ are subsequently ubiquitinated and targeted for degradation via the 26S proteasome, relieving repression of JA responsive (Thines, Katsir et al. 2007; Sheard, Tan et al. 2010).

JA is an extremely well-studied plant hormone. Knowledge regarding JA enzymatic synthesis, perception and its role as a transcriptional regulator is extensive. Still, little is known
about how environmental signals are decoded and transduced to activate JA biosynthesis. Because JA is synthesized so rapidly after wounding, and because JA biosynthesis enzymes are constitutively expressed, it is likely that the JA synthesis pathway is activated through post-translational modification of JA biosynthesis enzymes (Koo, Gao et al. 2009). The OPDA reductase has been proposed to be a likely point of regulation. Measurements of JA precursors revealed that OPDA is present in resting tissue, but then rapidly depleted in response to wounding (Koo, Gao et al. 2009). Considering the evidence concerning OPDA depletion, the OPR3 catalyzed conversion of OPDA to OPC 8:0 by OPR3 may contain the ‘molecular switch’ that controls wound-induced systemic JA synthesis. This speculation was bolstered by a recent report of the crystal structure of the OPR3 from tomato, which suggested a remarkable mechanism of autoinhibition (LeOPR3). LeOPR3 crystallized as self-inhibited homodimer in which a flexible loop region, L6, from each monomer was inserted into the active site of the other (Fig 1). Specifically, a glutamate residue (E291) from L6 is interacts with residues within the substrate binding pocket of the second protomer, blocking substrate access to the catalytic residues and the flavin mononucleotide co-factor. The dimer was also stabilized by hydrogen bonding interactions involving a sulfate ion from the crystallization liquor in close proximity to tyrosine 364 of the active site. The sulfate ion formed a hydrogen-bonding network with arginine residues from both protomers within the substrate binding pocket, supported also by the hydrogen bonding actions of a glutamic acid residue 291. The orientation of the sulfate ion near the tyrosine has been suggested to mimic an in vitro phosphorylation (Breithaupt, Kurzbauer et al. 2006; Koo, Gao et al. 2009). This hypothetical phosphorylation could potentially serve as a reversible mechanism of negative regulation in which phosphorylated OPR3 remains as an inhibited dimer until released by a phosphatase. Indeed, the monomeric LeOPR3 harboring a substitution of the L6 glutamate with the oppositely charged lysine (E291K) was shown to correlate with a higher oxidoreductase activity in vitro. In analytical ultracentrifugation experiments recombinant LeOPR3 E291K
exhibited a sedimentation equilibrium expected for the predicted mass of monomeric OPR3, while sedimentation of the wild-type LeOPR3 was consistent with a dimer. The mutant protein turned over a substrate analog, trans-hex-2-enal 6-fold faster than the WT, as measured by spectroscopic monitoring of NAD(P)H oxidation, indicating that the dimeric protein has reduced oxidoreductase activity (Breithaupt, Kurzbauer et al. 2006). Although dynamics of JA precursors, the OPR3 crystal structure, and the in vitro measurements of OPR3 activity point to the hypothesis that OPR3 phosphorylation governs JA biosynthesis, there is still no in vivo evidence supporting this hypothesis. Here I present my investigation of the post-translational regulation of OPR3 in the regulation of JA biosynthesis.

Results:

Bioinformatics reveals a conservation of Y364 and E291 in all known cis-OPDA reductases

The crystal structure of LeOPR3 suggests that tyrosine 364 and glutamic acid 291 are important for the proposed inhibitory dimerization of OPR3. Data from Arabidopsis, tomato, wheat, rice, and maize suggest that plants in general exhibit rapid synthesis of JA in the wound response. It may be that mechanisms leading to the activation of JA biosynthesis are broadly conserved. Homologues of the tomato and Arabidopsis OPR3 have been identified in rice and maize. OsOPR7 and the redundant ZmOPR7 and ZmOPR8 encode enzymes that are required for JA biosynthesis in their respective species (Tani, Sobajima et al. 2008; Yan, Christensen et al. 2012). To test for the conservation of Y365 and E291 a MUSCLE alignment was performed between all five known OPR enzymes that contribute to JA-Ile biosynthesis (Edgar 2004). There is considerable sequence conservation among the five known OPRs. Percent identity ranged from 70% between the Arabidopsis OPR3 and the maize homologue OPR7. Maize paralogues were 94% identical. Percent identity was 76% between tomato and Arabidopsis OPR3 amino acid
sequences. Both tyrosine 364 and glutamic acid 291 from the tomato sequence are conserved in all five species paralogues aligned including the monocots Zea maize and Oryza sativa (Fig 2).

**Complementation of opr3 sterility with amino acid substituted OPR3 variants.**

The suggestion of a regulatory dimerization of OPR3 is well supported *in vitro*, however there is no evidence that the suggested dimerization is relevant *in vivo*. Based on the conservation of two key residues it is possible that Y364, (conserved as Y365 in Arabidopsis) and E291 are important residues for the regulation of OPR activity *in vivo*. To address this I tested the efficiency by which a transgene carrying OPR3, and OPR3 variants with the E291K substitution and phospho-mimic and non-phosphorylatable amino acid substitutions at tyrosine 364 can complement the male-sterile phenotype of the opr3 mutation in Arabidopsis. opr3 mutants harbor a transgene within the second exon of the OPR3 coding sequence (Fig. 3) and lack sufficient JA production for fertility. Substitution of phospho-site residues with negatively charged amino acids aspartate and glutamate has been used as successful strategy for mimicking the presence the negatively charged moiety of phosphorylated tyrosine residues (Dissmeyer and Schnittger 2011). Additionally, substitution with the non-phosphorylatable phenylalanine blocks the regulatory effects of phosphorylation (Zhang and McCormick 2007; Kurepa, Li et al. 2014). In this case a tyrosine to phenylalanine substitution prevents phosphorylation by removal of the phenolic hydroxyl group required for a phosphoester bond while substantially retaining the overall size of the side-chain. Transgenes carrying the wild-type and variant OPR3 tyrosine 364 to aspartate (Y364D), tyrosine 364 to glutamate (Y364E) and tyrosine 364 to phenylalanine substitutions were transformed into the opr3 mutant background by Jeremy Jewell. I selected T1 transgenic lines on glufosinate-containing soil and scored each T1 plant for complementation of the male-sterility phenotype of the parental opr3 line.
The transgene driving expression of the wild-type protein complemented the male sterile phenotype of the opr3 mutant more frequently than any OPR3 variant (Fig 1). 53% of T1 plants harboring the pOPR3-OPR3 transgene were fertile (26/49), compared with 30% for plants with the pOPR3-OPR3 (Y364F) transgene (22/74). The phosphomimics, pOPR3-OPR3 (Y364D) and pOPR3-OPR3 (Y364E) complemented the sterility phenotype at 3% and 36% respectively (1/30 and 9/16). Sterile lines were completely infertile, with no seeds produced; fertile lines showed partially restored fertility (Fig 5). As with the parental line, stamen filaments in sterile lines were delayed in elongation and anthers were indehiscent (Fig 6).

Detection of OPR3 modifications by electrophoretic mobility in SDS-PAGE

The low efficiency of complementation of the opr3 mutant phenotype by the Y364F variant, compared with the wild-type was not consistent with the model of OPR3 inhibition through the reversible phosphorylation of tyrosine 364. Additionally, the E291K mutant which was shown to be dimeric and exhibited enhanced catalytic activity in vitro, was also not more efficient in restoring fertility that the WT protein. I therefore took a top-down approach to probe OPR3 protein for any evidence of wound-specific modification. Many post-translational protein modifications can cause a shift in a protein's electrophoretic mobility in SDS-PAGE. Phosphorylation, glycosylation, hydroxylation, methylation, and ubiquitination, have all been demonstrated to cause a mobility shift in SDS-PAGE (Carruthers, Parker et al. 2015). I used western blotting to probe for differences in the electrophoretic mobility of epitope-tagged OPR3 protein from extracts of wounded plants and controls flash frozen 10 minutes after wounding with forceps. Independent experiments were carried out on lines expressing transgenic OPR3 fused at the N-terminus to the hemagglutinin (HA) epitope under the control of the strong CaMV 35S promoter. A second experimental system used the native OPR3 promoter driving the expression of OPR3 fused to a 9XCmyc epitope. A
subtle reduction in electrophoretic mobility was observed in both systems when epitope-tagged OPR3 protein was extracted from wounded plants. This effect was small, and could only be observed when OPR3 protein was near the limit of detection at which small, closely spaced bands could be resolved (Fig. 8 A & B).

Detection of OPR3 modification by 2D Gel analysis

Following the detection of a wound-dependent mobility shift I used 2-dimensional gel electrophoresis coupled with western blotting to monitor wound-dependent changes in the isoelectric point of OPR3 protein. Two-dimensional gel electrophoresis is a powerful method that involves protein separation based on both charge and apparent mass. Extracts of wounded and unwounded plants expressing the pOPR3-myc9xOPR3 transgene were separated by isoelectric focusing from pH 3-10 and then by SDS-PAGE, and transferred to a PVDF membrane. 10 ug of Cmyc9OPR3 produced heterologously in E. coli BL21 was incubated in lysates of wounded and unwounded plants extracted with kinase and protease inhibitors. After 10 minutes at room-temperature the mixtures were boiled in 2D-loading buffer and separated by isoelectric focusing and SDS-PAGE. Western blots of the abundant bacterially expressed OPR3 gave distinct patterns of separation in IEF with two distinct spots being present in the extracts of wounded plants whereas only a single spot was present in the unwounded sample (Fig. 8).

GeLC-MS- detection of OPR3 peptides

The observed difference in the electrophoretic mobility and charge pattern of epitope labeled OPR3 extracted from wounded and unwounded plants support the hypothesis that OPR3
may be the target of a wound-regulated protein modification pathway. I used Gel-electrophoresis Liquid Chromatography Mass Spectrometry (GeLC-MS) to probe OPR3 protein for post-translational modifications. LC-MS conditions were first optimized using in-gel digests of bacterially-expressed Cmyc9xOPR3 from E. coli BL21. 9 OPR3 peptides were detected from a trypsin digestion of the recombinant protein with an expectation scores >0.05. These peptides contained 255 of 391 residues from OPR3, giving coverage of 65%. Acetic anhydride protection of lysine residues during the trypsin digestion produced an additional five peptides, which were a spectral match to predicted OPR3 peptides increasing the coverage to 310 of 391 residues, or 79% (Fig. 10). These techniques were applied to samples from wounded and unwounded plants. Despite optimization, only five peptides were detected from plant-expressed OPR3. Two ions were detected with mass spectral patterns consistent with acylated OPR3 peptides, However, these may be an artifact of sample workup.

**Immunoprecipitation and analysis of OPR3**

To increase the coverage of OPR3 for analysis, I isolated OPR3 from wounded and unwounded Arabidopsis by immunoprecipitation. Purified proteins were analyzed by Collision-Induced-Dissociation (CID) using an Orbitrap mass spectrometer. In this analysis coverage was 47% for unwounded plants and 51% for wounded plants (Table 2). Several methionine oxidations and cysteine carbamylation were observed; however, these are likely artifacts of sample work up. There was significant non-overlap in the coverage obtained by GeLC-MS and the IP-MS. The combined coverage from mass spectrometric analysis of OPR3 was 61%.

**Materials and methods**
Plant materials and growth conditions:

Seeds of opr3, the parental ecotype Wassilewskija, and Transgenic myc9OPR3, transgenic 35S-HA-OPR3 were sown on Sunshine mix potting soil, stratified for 3 days at 4° C and germinated on soil with Glufosinate. Plants were grown at 22° C under a 16 hour photoperiod with 100 to 120 μmol photons/m²/S with 8 hours of darkness. Transgenic pOPR3::cmyc9-OPR3 and 35S::OPR3 in the Wassilewskija genetic background were a gift from Jeremy Jewell. Scoring of fertility was based on silique seed filling. Sterile plants produced no seeds.

Western Blotting:

Three-to-four week old plants were used for electrophoretic mobility shift experiments. Wounded plants were crushed by forceps on the apical 30% of the leaf. Five minutes later whole rosettes were collected and flash frozen in liquid nitrogen. Frozen tissues were disrupted with a mortar and pestle, and were not allowed to thaw. Ground leaf tissue was added to Lysis buffer: 50 mM HEPES, 10% glycerol, 2 mM EDTA, 0.5% Triton X-100, protease inhibitor cocktail (Roche) and kinase inhibitor cocktails (Roche) and vortexed vigorously. Proteins were run on 4-20% polyacrylamide gels (Bio-rad) and transferred to PVDF membranes in transfer buffer (25 mM Tris, 190 mM glycine, 20 % methanol, pH 8.3) at 90 volts for 60 minutes. Membranes were blocked overnight at 4° C in TBS-T with 5% dried milk. Primary antibodies were diluted 1:5000 in 1% dried milk TBS-T and incubated for 1 hour at room temperature. Secondary antibody incubations were performed with 1:10,000 dilutions of secondary antibodies in 1 % milk in TBS-T at room temperature for 1 hour, followed by development. Secondary anti HA antibody conjugated was developed using one-step NBT/BCIP blotting reagent (Thermo). The secondary antibody used to
detect the anti-Cmyc antibody was fused to horshradish peroxidase. Images were developed using the luminol reagent (Santa Cruz).

2D Gel electrophoresis

Protein extracts were prepared using an ammonium acetate/methanol precipitation procedure (Agrawal and Thelen 2005). This is necessary to minimize carbohydrate and small metabolite contaminants that interfere with isoelectric focusing. 0.5 g of Arabidopsis leaves were harvested directly into liquid nitrogen and ground in liquid nitrogen in a mortar and pestle. Pulverized tissue was added to 10 mL of homogenization media (50% Phenol, 0.45 M Sucrose, 5 mM EDTA, 0.2% [v/v] 2-mercaptoethanol, 50 mM Tris-HCL, pH 8.8). Room temperature homogenate was agitated for 30 min on an orbital shaker at 4° C. Samples were then spun down at 5000 x g for 15 min at 4° C. The phenol phase was removed, and the proteins were precipitated out of the aqueous phase in 5 volumes of ice-cold 0.1 M ammonium acetate in 100% methanol at -20 C overnight. Precipitated proteins were collected with a centrifugation at 5000 x g for 10 minutes, followed by 2 washes in 0.1 M ammonium acetate 100% methanol. Another 2 washers were performed using ice-cold 80% acetone, followed by a wash in 70% ethanol. The pellet was dried at 37° C for 5 minutes before suspending in 125 μl of isoelectric focusing buffer sample buffer (0.8 M Urea, 2 M thiourea, 65 mM DTT, 4% CHAPS (w/v). Immobiline Drystrip gels (GE) were re-hydrated in DeStreak Rehydration buffer (GE) overnight. Samples were loaded using the sample cup method.

Immunoprecipitation:

Tissues were collected as described for western blotting. 20 μL Protein Agarose beads (Sigma) were washed 3 times in 1 mM HEPES and mixed with 1 μg High affinity Anti-HA Rat monoclonal
antibody (Roche). The lysate-antibody-beads mixture was incubated overnight on a rotator at 4°C. Protein was boiled off of the beads in 6X SDS Sample Buffer (375 mM Tris HCL, 9% SDS, 50% Glycerol, 0.03% Bromophenol blue, 5% β-mercaptoethanol. Samples were run on a 4-20% SDS-PAGE gel and stained with commassie-blue or transferred to a PVDF membrane using a bio-rad transfer apparatus, 90 volts, 60 minutes.

**Discussion:**

The experiments presented here provide some support for the existence of a wound-induced modification of the OPR3 enzyme, however the identity of the modification and whether it posseses *in vivo* regulatory function are still open questions. Furthermore, these results cast doubt on the proposed role of tyrosine 364 phosphorylation in the regulation of OPR3 activity in Arabidopsis.

We took advantage of the male-sterility phenotype of opr3 mutants to test the *in vivo* activity of OPR3 variants. In Arabidopsis a mutation in the opr3 locus blocks JA production and causes a male-sterility phenotype which can be rescued by the application of exogenous JA (Stintzi and Browse 2000). OPR3 variants harboring amino acid substitutions that block, or mimic the proposed tyrosine 364 phosphorylation should complement male sterility with an efficiency that correlates with *in vivo* Cis-OPDA reductase activity- a modified OPR3 enzyme with enhanced activity should contribute sufficient OPDA reduction for fertility in higher proportion of lines compared to the wild-type variant. On the otherhand, lines harboring a constituitively inactivated OPR3 variant should rarely complement the sterility phenotype. The observations presented here suggest the wild-type OPR3 is more active in vivo than the Y364F variant that lacks the capacity for the proposed inhibitory regulation. It is possible that there is a structural requirement for tyrosine 365 in addition to the role of that residue in regulation. Furthermore substitution of
glutamate 291 with lysine was shown to inhibit the observed dimer of the tomato OPR3, and displayed enhanced oxidoreductase activity in vitro. Contrary to the model of inhibition by dimerization, the E291K line was less efficient than the wild type in rescuing opr3 fertility. A tyrosine 364 phosphomimic Y364D was highly inefficient in complementing fertility, consistent with proposed interaction of a hydrogen bond acceptor at tyrosine 364 with positively charged sidechains on L6, but this result is overshadowed by the failure of the Y364F. It should be noted that it is possible that separate mechanisms exist to induce JA biosynthesis in flowers and leaves.

The wound-induced change in the electrophoretic mobility of OPR3 supports the possibility that OPR3 may be regulated by modifications on residues other than Y364. The crystal structure of OPR1, which is prevented from reducing the biologically active CIS-OPDA suggests that the substrate binding cavity can be closed. In OPR1 tyrosine-246 and tyrosine-78 act as gatekeeping residues that restrict the space near the FMN cofactor, which specifically prevents activity against (9S,13S)-OPDA. In the OPR3 crystal structure loop 3 turned away from the substrate channel by a hinging L3 Histidine 244, permitting activity on the (9S,13S)-OPDA (Breithaupt, Kurzbauer et al. 2006). It is possible that phosphorylation or other modifications to OPR3 at or near the L3 induces L3 into a closed lid conformation, blocking enzyme activity. Although more experiments are required, we have provided some evidence that OPR3 is chemically modified, but the identity of the modification and the biological relevance remain unknown.

The biochemical approach to investigating the regulation of OPR3 can be complemented with future reverse genetic studies to pursue the hypothesis that OPR3 harbors the molecular switch that activates JA biosynthesis. The existence of a wound-dependent modification of OPR3 implies the existence of signaling cascade that enters the peroxisome. The role of the peroxisome as a hub for signaling cascades in innate immunity is well-known in mammals (Dixit, Boulant et al. 2010), but such processes have only been suggested in plants. Potentially, a targeted reverse
genetic approach could be taken in which protein modifying enzymes with known or predicted peroxisomal localization are genetically removed. Additionally, components of wound-activated signaling pathways can be tested for components that may cross the peroxisome membrane. Mitogen activated protein kinases (MAPKs) are well-known transducers of environmental stress signals and, and may be involved in the transmission of molecular messages to jasmonate-mediated stress response mechanisms. The Arabidopsis MAPKs MPK4 and MPK6 have been shown to be rapidly activated by wounding. MPK4 is a known positive regulator of JA and ethylene signaling. MPK4 is required for undiminished accumulation of jasmonic acid and jasmonate-responsive transcripts after wounding. The negative regulator of MPK4, AP2C1 is also required for normal wound-induced accumulation of JA. Loss-of-function ap2c1 mutants hyper accumulate JA wound stressed tissues (Ichimura, Mizoguchi et al. 2000; Liu and Zhang 2004; Gomi, Ogawa et al. 2005; Schweighofer, Kazanaviciute et al. 2007). Studies of the effects of altered MPK4, AP2C, activity on phosphorylation states, or the identification of peroxisome-associated kinase/phosphates targets could represent a missing link in the transduction of the wound signal to JA biosynthesis.

Finally, it must be recognized that the hypothesis that OPR3 is the key point of post-translational control in jasmonate signaling could be wrong. There are also arguments for different points of regulation JA biosynthesis. For example, a single lipoxygenase, LOX6, is necessary for the rapid, systemic JA accumulation. LOX6 is expressed along the xylem in a cell niche that may be sensitive to changes in water pressure changes induced by a distant wound. A ‘squeeze cell hypothesis’ has been proposed by Farmer et al. in which changes in xylem pressure, accompanied by membrane depolarization may activate calcium fluxes into xylem-adjacent cells. Interestingly Arabidopsis LOX proteins have putative calcium-binding domains. Calcium fluxes may activate oxylipin production by activating LOX enzymes.
Although I obtained some indications that OPR3 is the target of wound-induced post-translational modification, it was not possible to get clear cut results that indicate regulation of OPR3 activity. Therefore, I focused on a mutant screen designed to isolate genes controlling the response to wounding. The screen turned up seven mutants required for wound-induced growth inhibition.
Figures:

**Figure 1.** Proposed model of tyrosine 364 phosphorylation in the regulation of OPR3 activity. 

A In resting tissues OPR3 forms a self-inhibited homodimer. Phosphorylated tyrosine 364 interacts through hydrogen bonding with arginine 294 of a second protomer.

B Dephosphorylation releases the dimer allowing substrate access to the active site.

**Figure 2.** Multiple sequence alignment of five plant OPR enzymes with characterized roles in JA biosynthesis. The conserved L6 Glutamate residue is highlighted in blue and the conserved active-site tyrosine is highlighted in red.
Figure 3. Genomic structure of the OPR3 locus. Normal function of the opr3 locus is disrupted by the T-DNA insertion in the second exon.
Figure 4. Complementation of opr3 sterility by OPR3 variant transgenes. Complementation of opr3 was observed in 26/49 of T1 plants harboring the pOPR3-OPR3 transgene. 22/74 plants with the pOPR3-OPR3<sup>Y364F</sup> transgene had restored fertility 1 of 30 lines harboring the phosphomimic pOPR3-OPR3<sup>Y365D</sup> was complemented and 9/16 lines harboring the pOPR3-OPR3<sup>Y364E</sup> lines were complemented.
**Figure 5.** Complementation of the male-sterile phenotype of *opr3* by the pOPR3-c-myc9OPR3 transgene. *opr3* mutants (right) produce no seeds. Transgenic complementation of *opr3* transgene restores seed production.

**Figure 6.** Flower phenotypes of *opr3* plants (right) and complemented pOPR3-OPR3 (left).
Figure 7. Western blotting of OPR3 from extracts of wounded and unwounded plants. **A.** Western blot of HA-OPR3 under the control of the strong 35S promoter. Extracts from wounded plants contained a band with slightly reduced electrophoretic mobility. **B.** Western blot of c-myc9X-OPR3 expressed under the control of the native OPR3 promoter.

Figure 8. Western blot of cmyc9OPR3 from Arabidopsis extracts separated by 2D-GE
Figure 9. Optimization of OPR3 peptide detection with GeLC-MS. Detection of underlined residues was achieved for bacterially expressed OPR3. Acetic anhydride protection of lysine residues extended the detection to include residues in red. The acetic anhydride treatment and optimized MS method improved OPR3 coverage by 54 residues.

<table>
<thead>
<tr>
<th>Peptide:</th>
<th>Expectation value</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPR3 Unwounded</td>
<td></td>
</tr>
<tr>
<td>VVEDYCLSALNAIR</td>
<td>0.025</td>
</tr>
<tr>
<td>ALNGVPAALAEYQAR</td>
<td>0.0081</td>
</tr>
<tr>
<td>FLKQVVEGVSAGSKVGR</td>
<td>0.0001</td>
</tr>
<tr>
<td>FLKQVVEGVSAGSKVGR + 2 Acetylations</td>
<td>3.80E-12</td>
</tr>
<tr>
<td>AGFDGIEIHGAHYLIDQFLKGDIND + Acetylation</td>
<td>3.30E-05</td>
</tr>
<tr>
<td>MAYNTFMSSSGFNKELGMQAQVQGADLVYGR</td>
<td>3.80E-06</td>
</tr>
<tr>
<td>OPR3 Wounded</td>
<td></td>
</tr>
<tr>
<td>FKDGKLKYNK + 2 Acetylations</td>
<td>0.007</td>
</tr>
<tr>
<td>FLKQVVEGVSAGSKVGR</td>
<td>0.046</td>
</tr>
<tr>
<td>MAYNTFMSSSGFNKELGMQAQVQGADLVYGR</td>
<td>3.70E-03</td>
</tr>
</tbody>
</table>

Table 1. Peptides detected by GeLC-MS from wounded and unwounded plants. Acetylations may have been an artifact of sample preprations.
Figure 10. OPR3 immunoprecipitation. A Coomassie-stained gel of immunoprecipitated HA-OPR3 from wounded and unwounded 35S::HA-OPR3 lines. The IgG was non-specifically detected at a higher molecular weight than the HA-OPR3 B. B. Western blot of Immunoprecipitated material confirms the presence of HA-OPR3.
Table 2. Peptides detected by immunoprecipitation/CID MS.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Coverage</th>
<th>Number of peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPR3 Unwounded</td>
<td>47%</td>
<td>20</td>
</tr>
<tr>
<td>OPR3 Wounded</td>
<td>51%</td>
<td>17</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peptide</th>
<th>Modifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPR3 Unwounded</td>
<td>ALESEIPR ALNPGPAALAEYYAQR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ASHAIYYPQPGISSTNKPISENR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DGINDRTDQYGGSIANR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FDLHRVVLAPMTR</td>
<td>M1(Oxidation)</td>
</tr>
<tr>
<td></td>
<td>FKIDGELNK</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FKIDGELNKYNR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LAYLFVQPR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LFIANPDLVSR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MAYNTFSSGFFNKK</td>
<td>M1(Oxidation); M8(Oxidation)</td>
</tr>
<tr>
<td></td>
<td>MGRFDLSHR</td>
<td>M1(Oxidation)</td>
</tr>
<tr>
<td></td>
<td>QGSDEEEAKLMI</td>
<td>M11(Oxidation)</td>
</tr>
<tr>
<td></td>
<td>QVEGVSAGK</td>
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</tr>
<tr>
<td></td>
<td>TDQYGGSIANR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>YVEDYCLSLNAR</td>
<td>C6(Carbamidomethyl)</td>
</tr>
<tr>
<td></td>
<td>YHAYGQTESGR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>YHAYGQTESGRQRGSDEEEAKLMK</td>
<td>M22(Oxidation)</td>
</tr>
</tbody>
</table>

| OPR3 Wounded    | ALESEIPR ALNPGPAALAEYYAQR    |                       |
|                 | ASHAIYYPQPGISSTNKPISENR      |                       |
|                 | DGINDRTDQYGGSIANR            |                       |
|                 | FDLHRVVLAPMTR               | M12(Oxidation)        |
|                 | FKIDGELNK                   |                       |
|                 | FKIDGELNKYNR                |                       |
|                 | LAYLFVQPR                   |                       |
|                 | LFIANPDLVSR                 |                       |
|                 | MAYNTFSSGFFNKK              | M1(Oxidation); M8(Oxidation) |
|                 | MGRFDLSHR                  | M1(Oxidation)         |
|                 | MGRFDLSHR                  |                       |
|                 | MGRFDLSHRVRVLAPMTR          | M1(Oxidation); M15(Oxidation) |
|                 | QGSDEEEAKLMI               | M11(Oxidation)        |
|                 | QVEEWAHK                   |                       |
|                 | QVEGVSAGK                  |                       |
|                 | TDQYGGSIANR                |                       |
|                 | VLPDSGHVK                  |                       |
|                 | YVEDYCLSLNAR                | C6(Carbamidomethyl)   |
|                 | YHAYGQTESGR                |                       |
|                 | YHAYGQTESGRQRGSDEEEAKLMK   | M22(Oxidation)        |
References:


CHAPTER FOUR
A FORWARD GENETIC SCREEN FOR MUTANTS RESISTANT TO WOUND-INDUCED GROWTH INHIBITION

Abstract:

The plant hormone jasmonate (JA) promotes defense and inhibits growth. I found that a recently described mutant, *JASMONATE HYPERSENSITIVE2*, which displays enhanced sensitivity to growth inhibition by exogenous JA treatment and wounding, is deficient in resistance to an insect herbivore. This result is inconstant with the phenotypes of previously characterized JA-hypersensitive mutants and suggests that the co-regulation of growth and defense by JA can be uncoupled. This discovery spurred my investigation of genes that control growth inhibition during the plant response to wounding. I screened a mutant population for plants that continued growth during chronic wounding, with a particular interest in mutants that were not also compromised in defense. Seven mutants were isolated with robust growth despite the chronic wounding treatment, four of which are also resistant to insect and pathogen attack. The four well-defended, wound-insensitive lines displayed a variety of morphological phenotypes. Two were morphologically similar to the wild-type, one line displayed long petioles and elongated leaves, and another line produced more leaves than the wild-type. Further analysis of these mutants will be useful for understanding the biochemical and regulatory features of plant growth/defense antagonism.
**Introduction:**

Plant growth and reproduction is accomplished despite a gauntlet of ever-changing environmental stresses and biotic threats. To thrive, plants continuously adjust their investment of limited resources between growth, reproduction and stress responses through intricate networks of environmental sensors and regulatory pathways (Huot, Yao et al. 2014). These pathways evolved in nature under selective pressure from a wide variety of competition stresses as well as diverse threats from herbivores and pathogens (Rausher 2001). However, crops are grown in an artificial environment where the investment of crop protections may reduce stress from competition, herbivores and microbes. Also, efficient crop rotation and the application of fertilizers can mitigate nutrient stress. Crop protections and fertilizer are costly inputs, but are necessary to optimize the productivity of cultivated species. A more detailed understanding of the cues and signals that control the balance of plant growth and defense is needed to inform future breeding and molecular engineering efforts to optimize the balance of growth and immunity with the goal of reducing agricultural input cost while maintaining productivity and pest resistance.

The hormone jasmonate (JA) is central to the growth-defense balance. Stress-induced JA signaling activates physiological changes that promote defenses and impairs growth (Yang, Yao et al. 2012; Havko, Major et al. 2016). These defensive changes include the strengthening of the cell wall, the production of trichomes, and the accumulation of defensive metabolites and proteins (Farmer and Ryan 1990; Martin, Tholl et al. 2002; Li, Zhao et al. 2004; Howe and Jander 2008; Denness, McKenna et al. 2011; Qi, Huang et al. 2014). It is still an open question whether growth inhibition by JA-signaling results from the simple diversion of resources required for expansion,
or through active repression of growth processes, although the two are not mutually exclusive (Havko, Major et al. 2016). In silico models suggest that the induction of defenses is indeed costly. For example, the production of only one class of JA-induced metabolite, glucosinolates, requires 15% of a growing plant’s photosynthetic input (Bekaert, Edger et al. 2012). Although the simple diversion model is attractive, evidence exists in support of the direct repression of growth by JA. Wound-induced endogenous JAs and exogenous JA application arrests the growth of Arabidopsis and tobacco cells in the G2 phase of mitosis and causes the repression of the cell cycle regulator CyclinB1;1 (Swiatek, Lenjou et al. 2002; Zhang and Turner 2008). Further study of the metabolic and regulatory reorganization triggered by JA signaling is required to understand the antagonism between plant growth and defense.

Mutant analysis has proved to be exceptionally powerful for advancing our understanding of JA signaling. An Arabidopsis mutant insensitive to the JA mimic coronatine provided early insights into the mechanism of JA perception, and many other JA-deficient or insensitive mutants have helped to identify key enzymes, and transcriptional regulators in involved in the pathway (Xie, Feys et al. 1998; Browse 2009). Recently, a JA-hypersensitive mutant was discovered that may help to elucidate the consequences of JA-mediated metabolic reprogramming. An Arabidopsis line, jasmonate hypersensitive2 (jah2) with dramatic hypersensitivity to growth inhibition by exogenous JA and JA-Ile was found to contain a leaky mutation in GLUTATHIONE SYNTHETASE2. In jah2 glutathione levels were reduced by 29% and the glutathione precursor $\gamma$-glutamylcysteine ($\gamma$-EC) hyper-accumulated nearly 300-fold relative to the wild type. The concentration of exogenous JA and JA-Ile required for a 50% inhibition of root growth was ~100 fold lower in jah2 than Col-0. Growth of vegetative tissues was also severely reduced in soil grown jah2, and crossing jah2 to the JA deficient aos mutant restored normal growth under these conditions. A careful study of jah2 revealed that the hyper-accumulation of $\gamma$-EC, and not glutathione depletion is responsible for the JA hypersensitivity. A double mutant of jah2 and
hypomorphic allele of \textit{GLUTATHIONE SYNTHETASE1}, accumulate low levels of both glutathione and \(\gamma\)-EC, but was not hypersensitive to JA (Wei, Rowe et al. 2015). An explanation of the action of \(\gamma\)-EC on JA sensitivity may lead to an improved understanding of the growth-defense balance. In this chapter I show that although \textit{jah2} is hypersensitive to JA with respect to growth inhibition, it is deficient in resistance to an insect herbivore, providing compelling evidence that the dual regulation of growth and defense can be uncoupled. With this new knowledge I undertook a genetic screen aimed at identifying other mutations that uncouple growth and defense.

**Results:**

**Wound-induced growth inhibition is enhanced in \textit{jah2}.

Growth and defense are co-regulated by JA. Mutants that are hypersensitive to growth inhibition by exogenous JA typically exhibit enhanced induction of JA-mediated defenses, and are hyper-resistant to herbivores and pathogens (Ellis, Karafyllidis et al. 2002; Bonaventure, Gfeller et al. 2007). The recently identified \textit{jasmonate hypersensitive 2} (\textit{jah2}) mutant of Arabidopsis is hypersensitive to growth inhibition by exogenous MeJA. To investigate whether wound-induced endogenous JA-dependent growth inhibition is also enhanced in \textit{jah2}, I wounded two-week-old plants across the axial 30\% of a single leaf with padded forceps daily for seven days. Relative to Col-0, \textit{jah2} were hypersensitive to wound-induced growth inhibition (Fig 1A). Wounded Col-0 were 31\% smaller than unwounded controls (Fig 1B) (2.76 cm ± 0.05 wounded, versus 4.01 cm ± 0.07 P < 0.001, T test). Wounded \textit{jah2} were 40\% smaller than unwounded \textit{jah2} controls (2.18 cm ± 0.06 wounded, versus 3.78 cm ± 0.12 P = .037 t-test). The 42\% reduction in rosette diameter observed with \textit{jah2} approaches a significant difference compared with the 31\% difference observed in Col-0 P = 0.06 T test.
Resistance to a necrotrophic pathogen is normal but resistance to a leaf chewing herbivore is reduced in jah2.

Several JA-hypersensitive mutants have been identified. In these cases, both growth inhibition and defense are amplified, producing small plants with enhanced resistance to attack. Based on these observations the JA hypersensitive jah2 mutants should be expected to demonstrate enhanced resistance to herbivores and necrotrophic pathogens. To test this hypothesis I measured the resistance of jah2 to the necrotroph Botrytis cinerea (B. cinerea), and the leaf-chewing herbivore Trichoplusia ni (T. ni). No difference was observed between Col-0 and jah2 with respect to B. cinerea resistance (Fig 1B). However, when Col-0 and jah2 mutants were challenged with T. ni larvae, jah2 mutants unexpectedly supported enhanced larval growth, indicating a defect in resistance to herbivore feeding (Fig 1C). The T. ni larvae reared for nine days on jah2 were 32% heavier than larvae reared on Col-0 (4.53 mg Col-0, versus 7.77 jah2 P = 0.036). The defect in herbivore resistance observed in jah2 was intermediate to aos, which lacks JA biosynthesis, and blocks JA-induced defenses. The inconsistency of jah2 phenotypes with respect to growth and defense may suggest that the two processes are regulated through separate branches of the JA signaling cascade or uncoupled by disruption of some aspect of the plant’s biochemistry due to hyper-accumulation of \( \gamma \)-EC.

Forward genetic screen to identify additional mutants with uncoupled growth and defense.

The herbivore sensitivity of the JA-hypersensitive jah2 mutant is inconsistent with the expectations implied by current models of the plant growth/defense balance in which the simple diversion of resources to defense slows growth by limiting the availability of resources for growth.
During the defense response, carbon skeletons, nutrients, ATP and NAD(P)H are thought to be diverted from the construction of new tissues toward the deployment of defensive secondary metabolites, defensive proteins, and defensive structures (Herms and Mattson 1992; Leone, Keller et al. 2014). In all previously studied mutations with constitutive or hyperactive JA responses plant growth is restricted and defense responses are enhanced (Ellis, Karafyllidis et al. 2002; Bonaventure, Gfeller et al. 2007). The observation that growth inhibition and defense activation are not found to be in lock step in the jah2 mutant may suggest growth and defense can be uncoupled.

The question of how the co-regulation of growth and defense can be uncoupled merits further inquiry. Forward genetic screening is a powerful tool to identify novel components of the regulation of growth inhibition during the wound response. I designed a forward genetic screen aimed at the identification of mutants that do not show reduced growth in response to wounding. The identification of plants with enhanced growth during the wounding treatment required minimal plant-to-plant variation. Also, replicate planting was required to ensure that observed phenotypes were repeatable. Owing to these requirements a chemically mutagenized M2 population was not suitable. The Salk Unimutant collection served as an excellent tool for this application. Thousands of T-DNA insertional mutants have been sequence-indexed, confirmed for homozygosity of the insertion and made publically available. (O’Malley and Ecker 2010). I grew 8,467 independent unimutant lines planted in triplicate along with wild-type controls. Growth conditions were carefully controlled to minimize variation. Plants were grown for two weeks, then wounded across a single leaf tip daily for one week with forceps as described for the wound assay performed on jah2. Mutants were selected based on robust growth during the course of the wounding treatment. Twelve mutants were selected for rescreening, with seven showing a reproducible phenotype in repeated experiments (Table 1). Of these wound-insensitive lines, four were morphologically normal, resembling the wild-type when unwounded. Two mutants showed a long-petiole and leaf
phenotype, similar to shade avoidance. Also, a single mutant was isolated with shortened plastochron and enhanced rosette leaf number.

**A plastochron length mutant is resistant to wound-induced stunting.**

Arabidopsis rosette leaves emerge from the apical meristem under developmentally controlled intervals. The duration between the formations of two leaf primordia is the defined as the plastochron. Several genetic factors have been identified that influence the rate of leaf initiation including *ALTERED MERISTEM1*, and the microRNA156, and SQUAMOSA PROMOTER BINDING PROTEIN-LIKE system (Chaudhury, Letham et al. 1993). Additionally, mutations in the photoreceptor *phyB*, or the *serrate* (*se*) genes produce new leaves less frequently than Col-0 (Prigge and Wagner 2001). I isolated a mutant with a shortened plastochron, which developed a greater number of rosette leaves than Col-0, and displayed a robust growth phenotype during wounding (Fig 2A). SALK_019255 contains an insertion in At3g59010-*PECTIN METHYLESTERASE35* (*PME35*). SALK_019255 has been previously described as *pme35*, although the leaf number phenotype may exist at a separate locus from the At3g59010 insertion (Hongo, Sato et al. 2012). At three weeks-post-germination, I observed a larger number of rosette leaves in SALK_019255 compared with Col-0 (Fig 2B) (10.67 ± 0.29 for Col-0 versus 14.66 ± 0.29 for SALK_019255 P = 0.0010 t-test). This mutant displayed resistance to wound-induced growth inhibition. The rosette diameter of wounded Col-0 was reduced by 30% ± 5.4 relative to unwounded controls, compared with 7% ± 3.8 for SALK_019255 (Fig 2C) Col-0 wounded versus Col-0 unwounded P < 0.05, Col-0 wounded versus SALK_019255 wounded (P > 0.05 by t-test). The resistance of SALK_019255 to wound-induced stunting is similar to the *aos* mutant, which is deficient in JA signaling. Wounded *aos* were 12% ± 8 smaller than unwounded controls. No difference was observed in the resistance of SALK_019255 to *Botrytis* or *T. ni* attack (Fig 2D, 2E).
These results show that a leaf number mutant is resistant to wound-induced growth inhibition, but is not sensitive to attack.

**Growth is enhanced and defense is compromised in two large rosette, long petiole mutants.**

Two wound-resistant mutants were isolated that exhibit a large rosette phenotype, characterized by elongated leaves and petioles. These phenotypes resemble the response of plants to shade-adaptation and are observed in mutations in light signaling pathways. For example a mutation in *phyB*, causes a constitutive shade-adaptation phenotype, known to involve modulation of GA signaling and PIF transcription factor activity. Shade-adaptation is associated with the direct repression of defense through the stabilization of the repressors of JA signaling: JAZ proteins. I observed robust growth of SALK_020615 and SALK_148815 during the wound response. These lines contain T-DNA insertions in At1g76470-CINNAMOYL-CoA REDUCTASE2 (CCR2), and At3g58360- RESTRICTED TEV MOVEMENT3 (RTM3) respectively (Chisholm, Parra et al. 2001; Peng, Hudson et al. 2008). In addition to wound-resistance these lines displayed elongated petioles and large rosette diameter with and without wounding treatment (Figure 3A). The petiole length of unwounded SALK_020615 and SALK_148815 was 43% and 73% longer than unwounded Col-0. Wounded SALK_020615 and SALK_148815 displayed a reduced sensitivity to wound-induced growth inhibition. The relative difference in rosette diameter between wounded and unwounded plants was less than half that of Col-0 (Fig 3C) (30% difference Col-0 ± 0.5 versus 12% ± 4 and 6% ± 5 SALK_148815 and SALK_020615 respectively P < .01). To test whether the growth-defense tradeoff was uncoupled in SALK_148815 and SALK_020615, I performed herbivory and pathogen resistance assays. Both mutants performed similarly to Col-
0 with respect to herbivore resistance (Fig 3D). The average weight of caterpillars reared on Col-0 was 2.6 mg. The weight of caterpillars reared on both long-petiole mutant lines was similar 2.4 mg, and 2.3 mg for SALK_020615 and SALK_148815 respectively $P = 0.31$ and $P = 0.11$ T test. However, like previously identified long-petiole mutants, both lines were compromised in pathogen resistance. The lesion area caused by *Botrytis* infection spread to an average of 0.056 cm$^2$ on the leaves of Col-0 four days after infection. On SALK_020615 and SALK_148815 the lesion area was 0.23 cm$^2$ and 0.103 cm$^2$ respectively $P > 0.01$ in both cases T test (Fig 3E).

**Isolation of morphologically normal plants with wound insensitivity.**

Four lines were isolated with resistance to wound-induced growth inhibition that exhibited no other obvious morphological phenotypes: SALK_018772, SALK_017915, SALK_123000 and SALK_003021 (SALK_003021 is discussed in chapter 5) (Fig 4A-C). The magnitude of growth inhibition was reduced relative to Col-0 by approximately 30%, 65% and 55% in SALK_018772 SALK_017915, SALK_123000 respectively (Fig 4C). SALK_018772- contains a T-DNA insertion in At1g28410, a gene of unknown function. SALK_017915 contains T-DNA within in At2g25310, a predicted protein-coding gene with a putative carbohydrate-binging domain and predicted targeting to the endomembrane system, and SALK_123000 contains an insertion in At3g09400 which encodes a protein phosphatase: **POL-LIKE 3 (PLL3)**, a homologue of the POLTERGEIST phosphatase (Yu, Miller et al. 2003; Berardini, Reiser et al. 2015). Both *pll3* and SALK_017915 display normal resistance to insect and pathogen attack (Fig 4E-F). The Average weight of *T. ni* larvae reared on Col-0 was 2.6 mg, similar to the 2.4 mg, 2.1 mg and 2.8 mg average larval weights for SALK_018772, SALK_017915, and SALK_123000 respectively $P < 0.1$ t-test. However, SALK_018772 is highly sensitive to *Botrytis*. (Fig 4F). Four days after infection the average botrytis lesion area on leaves of SALK_28410 was more than five times greater than
on the leaves of Col-0 (P = 0.0022 t-test) and comparable to the JA-synthesis mutant aos. The observation that the loss of wound-induced growth inhibition may, or may not be associated with compromised defense suggests that the two processes are interacting, and may be uncoupled to different extents.

**Materials and methods:**

**Plant growth conditions**

Plants were grown at 22°C on Jiffy 7 peat pellets (Jiffy) under a 10 hour photoperiod. Light intensity was between, 100 and 120 μmol photons/m²/s. The wild-type Arabidopsis, Col-0, stock CS70000 was originally received from the Arabidopsis Biological Resource Center (ABRC). jah2 mutants were a gift from Paul Staswick.

**Wounding assay**

Three-week old plants were wounded across the axial 30% of a single leaf using a hemostat wrapped in floral tape to provide an even crushing of the leaf tissue. Wounding was performed daily at the middle of the photoperiod. I pinched a new leaf each day proceeding clockwise from the previously wounded leaf. Images of plants were taken with a digital camera next to a ruler and the rosette diameter of each plant was measured using ImageJ.

**Defense analysis**

Caterpillar feeding assays were performed based on previous protocols (Schilmiller, Koo et al. 2007). *Trichoplusia ni* eggs were acquired from Benzon research and placed at 30°C for 48 hours to promote hatching. Vigorous larvae were moved to the rosette leaves of plants using a paintbrush. Larvae fed on plants for 10 days, after which time all larvae from a single plant were
weighed together and the average larval weight for that plant was calculated by dividing the total weight by the number of larvae. Four such measurements were made for each genotype.

Botrytis cinerea challenge was performed based on (Rowe and Kliebenstein 2007). Spores of Botrytis cinerea pv. B.C. grape were suspended at 5000 spores/ml in 50% organic grape juice (Honest Kids juice). Single 4-μl droplets were placed on excised leaves. Leaves were sealed in pitri plates with 10 ml sterile deionized water absorbed into filter paper (whatman). Digital pictures were taken of the advancing lesion and ImageJ was used to quantify the lesion area.

Discussion:

Several mutants are known to exhibit hypersensitivity to JA or constitutive JA activation. A mutation in the cellulose synthase cev1 causes constitutive activation of JA signaling characterized by small growth phenotype, enhanced accumulation of JA-responsive mRNAs and the hyper-accumulation of JA-induced secondary metabolites (Ellis, Karafyllidis et al. 2002). Also, a point mutation in the calcium channel fou2 confers JA-hypersensitivity characterized by enhanced accumulation of JA-regulated transcripts and repressed growth (Bonaventure, Gfeller et al. 2007; Bonaventure, Gfeller et al. 2007). Both cev1 and fou2 display enhanced resistance to herbivores and pathogens, which is consistent with the co-regulation of growth and defense by JA. In the recently identified jah2 mutant γ-EC accumulation is somehow sufficiently disruptive to the plant’s metabolism to cause a hypersensitivity to growth inhibition by exogenous JA (Wei, Rowe et al. 2015). In this chapter I show that the jah2 mutation unexpectedly weakens, rather than enhances the plant’s herbivore resistance. Although it is still not known how cev1 and fou2 fit into the JA signaling network, it is likely that both of these mutations affect processes that are upstream of JA biosynthesis and perception. The dysregulation of growth and defense in jah2
may involve a disruption of a proposed ‘metabolic shift’ downstream of JA perception. In jah2 resources diverted from growth processes may be inefficiently converted to defense compounds, explaining the herbivore sensitivity phenotype. Future experiments can test these possibilities through the metabolic profiling of wounded or JA-treated jah2. The discovery that JA-dependent growth and defense are not regulated in lock-step in jah2 spurred the search for mutants with uncoupled growth and defense responses.

I conducted a genetic screen for mutants that are insensitive to wound-induced growth inhibition. The wounding treatment involved daily crushing of a single leaf, leaving the majority of the rosette uninjured, a treatment which is known to cause rosette-wide growth arrest through the propagation of a mobile signal from the wound site to distal tissues. Several mobile signals are involved in the transmission the wound signal to distal tissues. Both membrane depolarization and a burst of reactive oxygen species are required for wound responses in undamaged distal tissues (Miller, Schlauch et al. 2009; Mousavi, Chauvin et al. 2013). Yet, it is unknown how these signals are decoded to activate JA biosynthesis (Glauser, Dubugnon et al. 2009; Koo, Gao et al. 2009). Furthermore, the downstream effects of activating JA signaling are not well understood. The screen described here is capable of isolating mutations that block both the upstream activation of JA-biosynthesis, and also key regulators of the JA-mediated shift from growth-mode to defense.

Seven mutants were discovered which display robust growth despite chronic wounding. These mutations belonged to three phenotypic classes, which may provide a clue regarding the mechanistic role of each mutated gene.

A mutation in a pectin methylesterase gene, pme35 was found to be associated with the accelerated development of leaves. In pme35 plants a larger leaf number may dilute the propagating wound signal and diminish the effect of the wound stimulus in the undamaged leaves. The recent characterization of the electrical signals that are required for the activation of JA
biosynthesis in distal tissues showed membrane depolarization signals follow parastiches- direct vascular connections. Parastiches are established during development such that as new leaves emerge, leaf n shares a connection with leaf n ± 5 and n ± 8, and additional contact parastiches may forming in proximal leaves (Mousavi, Chauvin et al. 2013). The larger number of total leaves in pme35 at the time of wounding will mean that a larger number of total leaves will not have a direct vascular connection to the wounded site. This effect might result in a weaker wound response on a per-leaf basis, which might explain the resistance of pme35 to wound-induced growth inhibition. The systemic activation of defenses is thought to prepare the distal tissues for attack. In the case of T. ni assay the localized defense response may be sufficiently strong and rapid to provide wild-type levels of resistance against caterpillars. Future experiments comparing the transcriptional activation of wound-responsive genes in leaves with a direct vascular connection to the wounded site in pme35 in individual leaves, and on a whole-rosette basis may help to test this explanation. These hypotheses may also be tested by repeating the wounding and defense assays on additional mutants with increased leaf number.

Two mutants were isolated with constitutive ‘big rosette’ phenotypes. The elongated petioles and leaf morphology of these mutants resemble the response to shade-adaptation, which is also observed in mutants with disrupted light signaling pathways. For example, a mutation in phyB, causes a constitutive etiolated phenotype, which is known to involve modulation of auxin, gibberellin (GA), brassinosteroid signaling, and PIF transcription factor activity (Devlin, Halliday et al. 1996; Casal 2012). Shade adaptation represents a stress in which the plant must prioritize growth over all else to position leaves under sufficient illumination for survival (Leone, Keller et al. 2014). In the dark plants attenuate defenses, possibly to divert resources to growth, through the direct repression of JA signaling. The antagonism between dark adaptation and defense is one of the best-understood aspects of the growth/defense balance. Light quality is monitored by the photoreceptor phyB. Under low quality light PHYB assumes an inactive form, which promotes the
stabilization of a repressor of JA signaling, JAZ10. Mutational or dark-induced inactivation of phyB also enhances JAZ protein action by promoting the degradation of DELLA repressors. The JAZ proteins physically interact with DELLA proteins. Under low light-stress GA synthesis is promoted, leading to the GID1/SLY1-mediated degradation of DELLA. DELLA depletion releases JAZ to interact with and inhibit JA-responsive transcription factors including MYC2 (Moreno, Tao et al. 2009; Cerrudo, Keller et al. 2012). Consequentially, phyB mutants, multiple della mutants and dark grown plants accumulate lower levels of defensive metabolites and are sensitive to attack (Cerrudo, Keller et al. 2012). Both of the etiolated mutants isolated here are resistant to wound-induced stunting and hypersensitive to pathogen attack. These phenotypes are consistent with repression of both growth and defense branches of JA-signaling. An analysis of JAZ protein accumulation and a careful diagnosis of the light sensing and GA signaling pathways can test the hypothesis that these mutants are involved with light signaling.

Four mutants with no obvious morphological differences compared to the parental Col-0 resisted wound-induced growth inhibition. Of these, three were resistant to both B. cinerea and T. ni. SALK_018772, that is mutated in a gene encoding a protein of unknown function contains a mutation that blocks wound-induced stunting and compromises resistance to B. cinerea. This result is interesting, but analysis of a second allele is required to confirm the causal relationship between the mutation in At1g28410 and any observed phenotypes.

The wound-insensitive phenotype of SALK_123000 which contains an insertion in At3g09400/PLL3 was of interest because PLL3 encodes a protein phosphatase with a predicted type 1 peroxisome targeting signal (Reumann 2004). An unknown peroxisome-localized protein phosphatase has been implicated in the activation of wound-induced JA biosynthesis. JA is present at low levels in the leaves of unwounded Arabidopsis (Creelman, Tierney et al. 1992; Koo, Gao et al. 2009). Wounding stimulated the rapid production of JA de novo accompanied by the depletion of the of the JA precursor, OPDA. It has been suggested that the OPDA reductase,
OPR3, a peroxisome-localized enzyme required for JA biosynthesis is under tight regulatory control by protein phosphorylation (Breithaupt, Kurzbauer et al. 2006; Koo, Gao et al. 2009). To test the hypothesis that PLL3 might be the missing OPR3 phosphatase involved in activating wound-induced JA synthesis, I measured wound induced JA and JA-Ile levels in pll3. No deficiency was detected in wound-induced JA biosynthesis in pll3. The cause of the wound-insensitive phenotype in three of the otherwise morphologically normal SALK_018772, SALK_123000, and SALK_017915C is still an open question. Further analysis of these interesting mutants may help to elucidate mechanisms in the growth/defense balance.
**Figures:**

**Figure 1.** Growth/defense phenotypes of jah2 mutants.  
A. Picture of wounded and unwounded Col-0 and jah2. Three week old jah2 are significantly more sensitive to wounding than Col-0.  
B. Measurement of the rosette diameter of wounded Col-0 and jah2 relative to unwounded controls.  
C Botrytis resistance assay. jah2 has normal resistance to the necrotrophic fungal pathogen *Botrytis cinerea*  
D. *T. ni* resistance assay. jah2 is sensitive to attack from the leaf chewing herbivore *T. ni.*
Table 1. Frequency and types of phenotypes accompanying wound-insensitivity observed in 8467 SALK unimutant lines. Plants grown in triplicate were selected with the most dramatic, repeatable phenotypes. Phenotypes divided into morphological characteristics. Morphologically normal plants resembled unwounded controls, and the unwounded wild type. Long petiole, and extra leaves were also observed in lines that exhibited robust growth through chronic wounding. Also a leaf number phenotype was observed in a wound-resistant line.

<table>
<thead>
<tr>
<th>Total lines screened</th>
<th>8467</th>
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<tbody>
<tr>
<td>Growth pattern:</td>
<td></td>
</tr>
<tr>
<td>No. of isolates</td>
<td></td>
</tr>
<tr>
<td>Morphologically normal</td>
<td>4</td>
</tr>
<tr>
<td>Long petioles</td>
<td>2</td>
</tr>
<tr>
<td>Leaf number</td>
<td>1</td>
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</tbody>
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Figure 2. SALK_019255 mutants grow extra leaves, and are resistant to wound-induced growth-inhibition, but have normal resistance to herbivory and a necrotropic pathogen. A. Photographs of Col-0, SALK_019255 and pme-2. B. Leaf number at 4 weeks. SALK_019255 mutants develop more rosette leaves than Col-0 at four weeks. C. Wounding assay. SALK_019255 are resistant to
wound-induced growth inhibition D T. ni resistance assay. SALK_019255 is not compromised in resistance to attack from the leaf chewing herbivore T. ni. E. Botrytis resistance assay. SALK_019255 has normal resistance to the necrotrophic fungal pathogen Botrytis cinerea.

Figure 3. Constitutively shade-adapted mutants are resistant to wound-induced growth-inhibition, and are compromised in pathogen defense. A. Photographs of Col-0, SALK_020615 and SALK_148815 showing the large rosette phenotypes. B. Petiole length. SALK_020615 and
SALK_148815 lines display longer petioles than Col-0 C. Wounding assay. SALK_020615 and SALK_148815 are resistant to wound-induced growth inhibition D. T. ni resistance assay. SALK_020615 and SALK_148815 are resistant to attack from the leaf chewing herbivore T. ni. E. Botrytis resistance assay. SALK_020615 and SALK_148815 are compromised in defense against the necrotrophic fungal pathogen Botrytis cinerea.
Figure 4. Morphologically normal plants resistant to wound-induced growth inhibition. A-C. Photographs of wounded and control SALK_018772, SALK_017915, and SALK_123000. D. Wounding assay. These three mutant lines are resistant to wound-induced growth inhibition. E T. ni resistance assay. All three wound-insensitive lines with normal morphology are resistant to attack from the leaf chewing herbivore T. ni. F. Botrytis resistance assay. At1g84260 is required for resistance to botrytis. Botrytis resistance is normal in SALK_017915, and SALK_123000.

Figure S1. Wound-induced JA-Ile accumulation in SALK_123000. Wounded SALK_123000 accumulated similar levels of JA-Ile compared with Col-0 60 minutes-post wound. Plants were pinched along the axial 30% of the leaf and collected into liquid nitrogen 60 minutes later. Measurements were performed using a LCMS-TOF.
References:


CHAPTER FIVE

THE F-BOX PROTEIN FBK51 IS REQUIRED FOR GROWTH INHIBITION DURING THE WOUND RESPONSE IN ARABIDOPSIS

Abstract:

The life of a plant involves balancing growth, defense, and reproduction. To maximize the total lifetime investment in growth and reproduction plants have evolved defenses that are not deployed until an attack is perceived. The hormone Jasmonate (JA) is a key signal that activates many of these induced defenses. JA production is stimulated by wounding or the detection of the molecular signatures of pests and pathogens, and leads to global changes in gene expression which promote the construction of defensive structures and anti-pest compounds. JA is also a potent growth inhibitor. Growth inhibition by JA was thought to involve the simple diversion of resources including nutrients and photosynthate from growth to defense, coupling the two processes. However, recent evidence is suggesting that plants are capable of deploying effective defenses without sacrificing growth. In this chapter I show that an F-box protein, FBK51, is required for growth inhibition during the defense response, but is not involved with defense. Plants with mutated fbk51 are resistant to wound-induced stunting, but have normal resistance to an insect herbivore and a necrotrophic pathogen. I show that FBK51 interacts with components of an E3 Ubiquitin ligase complex, and targets two redundant growth-promoting transcription factors, VOZ1 and VOZ2 for degradation. Loss of fbk51 function alters the transcriptional response to wounding, leading to altered regulation of genes involved in photosynthesis, secondary metabolism, and hormone responses.
Introduction:

Jeremy Jewell performed the non-targeted yeast-2-hybrid screen in which VOZ were identified as putative substrates of FBK51. Jeremy also generated the voz1/voz2/fbk51-1 triple mutant and FBK51 over-expression lines.

Plant survival and propagation requires the precise coordination of growth, defense, and reproduction. These three processes are governed by regulatory networks that integrate information from environmental cues including attack from plant-consuming organisms, nutrient availability, light quality, and inter-plant competition. For decades prevailing models have described an antagonism between development and defense involving the allocation of limited resources to one process at the expense of the others. The assumptions of these zero-sum game models are now being challenged and contradicted by an increasing body of evidence that the regulation of growth and defense can be uncoupled under some circumstances. An improved understanding of the plant growth-defense balance may aid future efforts to breed and engineer crops with strong innate defense and robust growth.

Several models have been fundamental to our understanding of the principles and patterns of plant growth and defense against herbivores. Optimal defense hypothesis (OD), carbon nutrient balance (CNB), and the growth-defense balance hypothesis (GDBH) all generally propose that the availability of resources in the environment shape the type and efficacy of defenses that plants can deploy against herbivores, with any allocation to defenses having a net negative effect on growth and reproduction via the diversion of resources away from the production of new leaf area, roots, and progeny (Herms and Mattson 1992; Gershenzon 1994; Stamp 2003). These models were built upon the observation that plants produce a wide variety of metabolites separate from primary metabolism, many of which seem to exist as an armory of
anti-herbivore and anti-microbe toxins. Recent objections to simplistic growth defense balance models challenge the assumption that all inputs including energy, carbon, and other resources are necessary limiting for plant growth and fitness under all conditions (Herms Mattson Pieterse (cell and development biology). An updated understanding of growth-defense balance rejects the implication that there are no processes at the plant's disposal to utilize non-limiting assets for defense. Instead a complicated balancing of resource acquisition and allocation coordinates growth and defense in response to ever-changing environmental conditions. If, for example, nitrogen is limiting for growth it may be possible for the plant to deploy carbonaceous compounds e.g. terpenoids, phenylpropanoids without a significant investment of nitrogen (Kliebenstein 2016). Although there are several examples of constitutive or enhanced defenses correlating with reduced plant size (Ellis, Karafyllidis et al. 2002; Bonaventure, Gfeller et al. 2007), the precise relationship between defense and stature has not been comprehensively attributed to resource allocation or direct regulation. However, recent studies are beginning to suggest that regulatory control rather than simplistic allocation accounts for development-defense antagonism in several plant stress responses.

Plants under the shade of their neighbors display a prioritization of growth over defense. Arabidopsis responds to shading with accelerated elongation of stems and petioles to place leaves in well-illuminated positions above the leaves of competitors (Ballare 1999; Tao, Ferrer et al. 2008). The photoreceptor PHYB monitors light quality in this system. Photosynthetic tissues absorb red light, and allow far-red light to pass through. Low red to far-red ratios stimulate competition stress responses. As the ratio of red to far-red light decreases PHYB transitions from the red-activated Pfr form to the inactive Pr form. Active PHYB translocates to the nucleus to suppress the shade response through interactions with Phytochrome-Interacting Factors (PIFs). Shade, mutations in PHYB, and supplemental far-red light all induce the morphological shade avoidance response, which is accompanied by the attenuation of defenses (Morelli and Ruberti
Plants grown in low light or in light supplemented with far-red light are susceptible to herbivores and pathogens (Griebel and Zeier 2008; Moreno, Tao et al. 2009; Leone, Keller et al. 2014). Under standard growth-defense balance models defense attenuation during shade avoidance is attributed to the allocation of resources from defense to tissue expansion. The recent identification of an Arabidopsis mutant lacking the shade response has allowed this hypothesis to be tested. Shading triggers the production of auxin from L-tryptophan via a pathway requiring a shade-induced aminotransferase, SAV3. A knockout of sav3 prevents the morphological shade response; sav3 grown under low red:far-red resembles wild type plants under high red:far-red (Tao, Ferrer et al. 2008). Interestingly, sav3 grown in the shade, or with supplemental far-red light remain sensitive to attack indicating that regulatory control of defense, not resource allocation accounts for attenuated defense. This cross talk between light quality and defense signaling has received considerable attention and is known to involve repression of the defense-promoting Jasmonate (JA) signaling pathway. JA repressor proteins (JAZ) are stabilized and released from sequestration under low red:far-red ratios (Leone, Keller et al. 2014).

In addition to the shade-avoidance system the wound response elicits one of the best-studied examples of plant growth-defense antagonism. Wounding activates rapid signals that relay messages throughout the organism. Propagating membrane depolarizations, a reactive oxygen species burst and calcium transients are all well-defined early signaling steps that promote physiological changes associated with the deployment of induced defenses (Wildon, Thain et al. 1992; Torres, Jones et al. 2005; Qi, Stephens et al. 2006; Galletti, Denoux et al. 2008; Miller, Schlauch et al. 2009; Arimura and Maffei 2010; Mousavi, Chauvin et al. 2013). Wounded plants undergo extensive transcriptional and metabolic reprogramming (Reymond, Weber et al. 2000; Strassner, Schaller et al. 2002). JA is a key second messenger in the wound response. JA is synthesized within seconds of wounding and controls the expression of thousands of genes via its co-receptor COI1/JAZ. Gene expression profiling experiments using mutants deficient in JA
Synthesis or perception have suggested that approximately half of wound-regulated genes are under the control of the canonical JA signaling pathway (Reymond, Weber et al. 2000; Strassner, Schaller et al. 2002). JA induces the synthesis of secondary metabolites, defensive proteins and controls pathways in primary plant metabolism to provide the entry-point compounds for secondary metabolic pathways (De Geyter, Gholami et al.; Gómez, Ferrieri et al. 2010). Many JA-regulated secondary metabolites are medicinally and commercially valuable. The antimalarial artemisinin, and the cancer drugs vinblastine, and taxol are all made in plants or cultured plant cells using JA to promote their synthesis (Facchini 2001; Yu, Li et al. 2012; Wasternack and Hause 2013). But, the induction of defenses via JA signaling comes at a cost. The wound response and subsequent activation of defenses correlates with the direct repression of growth (Zhang and Turner 2008). Although the magnitude of the effect varies between studies, Arabidopsis rosettes subject to crushing damage are significantly reduced in size compared with controls. In plants lacking JA signaling the effect is significantly reduced, suggesting that the wound response involves JA-dependent and JA-independent repression of growth-process including cell proliferation, elongation, nutrient acquisition and photosynthesis (Yan, Stolz et al. 2007).

The potent activation of defense-related metabolism and inhibition of growth by JA during the wound response has been taken as evidence that metabolic diversion is the prime cause for the growth defense balance, but a recent study call this model into question. A quintuple mutant lacking 5 JAZ proteins (JAZq) exhibits constitutive JA responses including inhibited growth, accumulation of anthocyanins, and hyper-resistance to herbivores. A suppressor screen of the growth phenotype of JAZq reveals that a mutation in PHYB restores the size of JAZq to that of the wild-type, while maintaining the enhanced herbivore resistance (Campos, Yoshida et al. 2016). The observation of well-defended plants with growth traits similar to unstressed plants is a further evidence for the uncoupling of growth and defense and suggests that the network of stress responses governing growth and defense can be untangled.
Increasing evidence that growth-defense antagonism involves direct regulatory control rather than simple resource diversion implies that the two processes may be uncoupled. In chapter four I have reported a forward genetic screen to identify genes required for wound-induced growth inhibition. Here I describe the characterization of a gene, which contributes to stunting. FBK51 encodes an F-box protein, part of an E3 ubiquitin ligase complex that promotes the degradation of pro-growth phytochrome-interacting non-PIF transcription factors, VOZ1 and VOZ2. Transcriptional profiling of the wound response in the fbk51 mutant background suggests a role for FBK51 in fine-tuning the JA pathway, photosynthesis, and the deployment of defensive proteins in response to attack.

Results:

FBK51 is required for wound-induced growth inhibition.

The balance between growth and defense is vital for plant survival. The elicitation of defenses by mechanical wounding is well-established as a stimulus that activates plant defenses while inhibiting the expansion of leaves (Zhang and Turner 2008). I previously identified the T-DNA line SALK_003021 (fbk51-1) as insensitive to wound-induced stunting. SALK_003021 interrupts the 5’ UTR of At1g14330 (fbk51-1). A second allele, WiscDSLox364H10 (fbk51-2), contains a T-DNA insertion within the only exon of At1g14330.

I tested the sensitivity of fbk51-1, fbk51-2, and a transgenic complementation line to wound-induced growth inhibition. Three-week-old plants were pinched daily across a single leaf tip. After one week wild-type plants subject to the wound treatment were 31% smaller than unwounded controls (rosette diameter unwounded 3.38 cm ± 0.12 cm versus wounded 2.31 cm ± 0.11). The wounding treatment repressed the growth of fbk51-1 to a lesser degree. Wounded fbk51-1 were 15% smaller than unwounded controls (3.47 cm ± SE 0.16 and 2.92 cm ± cm 0.087).
Wounded fbk51-2 plants were also 15% smaller than unwounded controls (3.04 cm ± SE 0.08 versus 3.66 cm ± 0.0154). The magnitude of the response to wounding was restored in a complementation line in which fbk51-1 mutants harbored a transgene expressing FBK51 under the control of the CMV35S promoter. Wounded complemented plants were 33% smaller than controls (3.20 cm ± SE 0.14 versus 2.21 cm ± SE 0.06 cm). Mutants deficient in JA signaling are also known to lack a stunting response to wounding. The aos mutant, deficient in JA biosynthesis was also resistant to stunting. The rosette diameter of wounded aos was reduced by 9% relative to unwounded aos (3.78 cm ± 0.12 versus 3.46 cm ± 0.15). The fbk51 mutations did

**JA biosynthesis is not inhibited in fbk51**

Wound-induced endogenous JA has been found to play an important role in the growth-inhibition response to wounding. JA accumulates to low-picomolar concentrations in the tissues of unwounded Arabidopsis. Mechanical wounding stimulates a rapid burst of JA-Ile synthesis, which peaks within one hour. JA-Ile induces transcriptional reprogramming through its co-receptor SCF<sup>Col</sup>/JAZ, which correlates with growth arrest. Growth-inhibition by wounding is reduced in Arabidopsis mutants deficient in JA biosynthesis or perception, such as aos (Yan, Stolz et al. 2007; Zhang and Turner 2008). To test for deficiencies in wound-induced JA biosynthesis in the fbk51 mutant lines I measured JA-Ile accumulation in wounded and unwounded plants using high-performance liquid chromatography-mass spectrometry. JA-Ile was undetectable in unwounded tissues of Col-0 or fbk51 lines. 60 minutes after wounding JA-Ile levels had increased to ~1 nmol/gFW in Col-0, fbk51-1, and fbk51-2, indicating that a defect in JA-Ile biosynthesis is not responsible for the resistance of fbk51 mutants to wound-induced growth inhibition.

**FBK51 is not required for defense against an insect herbivore or a necrotrophic pathogen.**
Historically, the balance between growth and defense has been described as a budgeting of resources between tissue expansion and secondary metabolism, implying that sustained growth during effective defense is not possible (Herms and Mattson 1992). Mutants lacking functional JA signaling pathways continue to grow following wounding stress, but are sensitive to herbivores and necrotrophic pathogens. I tested the resistance of fbk51-1 and fbk51-2 to the larva of the lepidopteran herbivore Tricuplusia ni (T. ni). Larval weight can be used to measure the feeding success of the herbivore, with plants deficient in defenses supporting enhanced larval growth (Schilmiller, Koo et al. 2007). Five T. ni larvae were placed on the rosette leaves of 4-week old fbk51 mutants and controls and allowed to feed for one week, after which time larval weight was recorded. The weight of larvae reared on fbk51 mutants was indistinguishable from wild type (fbk-1: 15.8 mg ± SE 2.9 fbk-2 17.8 mg ± SE 0.4) indicating the mutants maintain intact herbivore resistance. In contrast, insects reared on the JA-deficient aos were more than two-fold heavier than insects reared on the Col-0 (average weight aos: 38.9 mg ± SE 1.62 versus Col-0: 17.8 mg ± SE 2.0).

The growth-inhibiting responses induced by JA were thought to be tightly coupled to defense through the diversion of metabolic resources from expansion (Rowe, Walley et al. 2010). The resistance of fbk51 mutants to T. ni feeding suggests that the two processes can be uncoupled. To test the resistance of fbk51 mutants against a necrotrophic pathogen I challenged fbk51 and controls with Botrytis cinerea (B. cinerea). B. cinerea is a necrotropic phytopathogen that forms a circular necrotic lesion that spreads as it consumes host tissue. Lesion growth on excised wild-type leaves was compared between fbk51 mutants and wild-type. At 96 hours post-infection B. cinerea formed lesions on Col-0 with an average area of 770 mm² ± se 78 mm². Lesion growth for both alleles of fbk51 was similar to or less than the wild type: fbk51-1 621 mm²
± se 47 mm², fbk51-2 656 ± se 21 mm² indicating that fbk51 possesses normal resistance to B. cinerea.

**FBK51 over-expression restricts rosette growth but does not affect defense.**

Both fbk51-1 and fbk51-2 lines display resistance to wound-induced growth inhibition. It follows that FBK51 may function as a growth inhibitor in the wound response. Over-expression of FBK51 was used to confirm that FBK51 activity represses leaf growth. The strong CMV35S promoter was used to over-express FBK51 mRNA in the fbk51-1 background. In over-expression lines FBK51 transcript levels ranged from five to 48-times higher than Col-0. Higher FBK51 levels correlated with rosette growth inhibition. The line with the highest FBK51 transcript levels was 'line 15' with a 48.8-fold more FBK51 transcript than Col-0. Line 15 was 37% smaller than the Col-0 as measured by rosette diameter. Lines with lower FBK accumulation were less restricted in growth. Lines 'i', and '19' accumulated 14 fold more FBK51 transcript than Col-0, and were 31% and 30% smaller than Col-0 as measured by rosette diameter. Lines 'a', and 'c', accumulated six and five fold more FBK51 transcript relative to Col-0 and were 23% and 13% smaller than Col-0 by rosette diameter. The restriction of growth in FBK51 over-expression lines clearly demonstrates that FBK51 is a growth inhibitor *in vivo*. If FBK51 functions specifically in the growth branch of the wound-response than the FBK51 over-expression lines should exhibit normal resistance to herbivory. I challenged FBK51 over-expression lines and controls with the leaf-chewing herbivore *T. ni*. Larval growth was similar for *T. ni* reared on FBK51 over-expression lines, and Col-0. These results demonstrate the specificity of FBK51 as a regulator of growth, but no detected role in defense. Further, the growth restriction effected by FBK is independent of alterations to defense.
FBK51 encodes an F-box protein with C-terminal Kelch repeats.

FBK51 has been previously identified as an F-box protein harboring carboxy (C)-terminal Kelch repeats. The F-box family of proteins is one of the largest in the plant kingdom, with over 700 encoded by the Arabidopsis genome. Characterized by their amino (N)-terminal consensus, F-box proteins form a component of Skp1-Cullin-F-box E3 ubiquitin ligases complexes through interaction with a Skp1 protein via the F-box domain. Recruitment of specific protein substrates to SCF complexes is facilitated by diverse protein-protein interaction domains in the C-terminus of the F-box protein. FBK51 contains an F-box from amino acids 94-138 and three C-terminal Kelch repeats, from residues 180-223, 273-320, and 239-284, and (Marchler-Bauer A et al 2004, 2009, 2011, 2015). F-box proteins with C-terminal Kelch repeats (FBKs) are part of a large subfamily; 103 putative FBKs have been identified in the Arabidopsis genome. FBK51 was shown to interact with at least 17 of 19 Arabidopsis Skp1 proteins, and localize to the nucleus of tobacco epidermal cells, although no functional characterization has been reported (Schumann, Navarro-Quezada et al. 2011).

FBK51 interacts with VOZ1 and VOZ2 transcription factors in yeast, and in planta.

I hypothesized that FBK51 functions as a component of an E3 ubiquitin ligase, which targets growth-promoting proteins for ubiquitin/proteasome-dependent degradation. In a search for putative substrates Jeremy Jewell performed a non-targeted yeast 2-hybrid (Y2H) screen, which revealed that FBK51 interacts with VOZ1 in yeast cells. VOZ1 is a Zinc-Finger transcription factor that functions redundantly with VOZ2 to promote rosette growth and the transition to reproductive phase in Arabidopsis (Yasui, Mukougawa et al. 2012; Celesnik, Ali et al. 2013). I cloned VOZ1 and VOZ2 and confirmed through a targeted Y2H experiment that FBK51 interacts
with both VOZ1 and VOZ2 in the Y2H system. To test for interactions between FBK51 and VOZ1/VOZ2 in planta I used bimolecular fluorescence complementation (BiFC). With BiFC two non-fluorescent halves of a fluorescent protein, such as YFP are brought together by a protein/protein interaction resulting in reconstitution of fluorescence. I expressed a translational fusion of the N-terminal half of enhanced YFP (eYFP) with FBK51, and the C-terminus of eYFP to VOZ1, VOZ2 and Arabidopsis SKP1. A weak but clear signal was observed indicating the low abundance of FBK51/VOZ complexes, consistent with a role for this interaction in promoting VOZ degradation. In contrast, a strong fluorescent signal was observed in plants co-expressing FBK-nEFYP, and SKP1-cEYFP, which is expected to form stable complexes. Because the interaction between FBK51 and VOZ is hypothesized to result in VOZ degradation via the 26S proteasome, I tested the effects of the proteasome inhibitor MG-132 on the BiFC signal. Signal strength was greatly enhanced by MG-132, suggesting that VOZ is the substrate of SCF$^{FBK51}$ mediated-degradation.

To confirm the hypothesis that VOZ/FBK51 interaction results in VOZ degradation, I used western blotting to test the accumulation of VOZ2 protein when co-expressed with FBK51 in the N. benthamina transient expression system. FBK51-GFP and VOZ2-HA fusions were transiently expressed in tobacco leaves. Six hours before harvesting of tissues 100 μM MG-132, or vehicle was infiltrated into leaves transiently expressing FBK51 and VOZ2. Leaf discs from three separate samples were pooled and proteins were extracted for Western blotting. Co-expression of FBK51-GFP with VOZ2-HA prevents significant VOZ2-HA accumulation. However MG-132 treatment promotes the accumulation of VOZ2 protein.

The $fbk51-2$ mutation stabilizes VOZ2 in vivo.
To test the model that FBK51 targets VOZ for destruction in vivo, I performed Western blots for VOZ2 protein in the fbk51-2 and Col-0 backgrounds. Plants were wounded and tissues were harvested one, two, and six hours after wounding. VOZ2 protein was detectable in extracts from wounded fbk51-2 plants, but was not detectable in Col-0, or the voz1/2 double mutant.

Transcript profiling reveals a role for FBK51 in specific aspects of the wound response.

To further elucidate the role of FBK51 in the wound response, I used RNA sequencing to compare the transcriptional wound response of Col-0 and fbk51-2. Plants grown in a 10-hour photoperiod for 3 weeks were wounded across three rosette leaves. Samples were collected before wounding, and from wounded plants and unwounded controls at two hours and twenty-four hours after wounding. In Col-0 1868 genes were altered in expression >two-fold two hours after wounding compared with the unwounded controls. Most differentially expressed genes are up-regulated in the wound response. In Col-0 1546 genes were up regulated >two-fold, and 324 were repressed >two-fold. In the fbk51-2 mutant 1473 genes were up-regulated >two-fold after two hours, and 117 were down by >two-fold. By 24 hours post-wound the transcriptional response had significantly subsided; 465 and 201 genes were up-regulated in Col-0 and fbk51-2 respectively, and 76 and 28 genes were >two-fold repressed in Col-0 and fbk51-2 respectively.

Gene ontology enrichment of the subset of genes induced by the wound response reveals processes regulated by wounding. ‘Response to stimulus,’ ‘response to stress’, response to ‘jasmonic acid stimulus’ and ‘response to biotic stimulus’ were among the processes significantly enriched in the list of genes up-regulated two hours after wounding in both Col-0 and fbk51-2. Consistent with numerous reports of wound-induced secondary metabolite accumulation, genes associated with ‘secondary metabolism’ were also enriched in the list of wound responsive genes (Campos, Yoshida et al. 2016). In Col-0 29 genes associated with phenylpropanoid biosynthesis
were induced, whereas 19 were enriched in \textit{fbk51-2} two hours after wounding. Of the ten phenylpropanoid-related genes uniquely up-regulated in wounded Col-0 versus unwounded the average fold change was 42, compared with the 1.5 average fold difference between wounded and unwounded \textit{fbk51-2}. The list of genes associated with phenylpropanoid synthesis found to be uniquely up-regulated in Col-0 includes the transcription factor \textit{MYB114} (267 fold upregulated in Col-0, not significantly changed in \textit{fbk51-2}), which is known to promote the synthesis of phenylpropanoid metabolites.

A striking difference between the expression profiles of Col-0 and \textit{fbk51-2} at two hours post-wound is that many genes related to photosynthesis were repressed to a greater extent in Col-0 than \textit{fbk51-2}. JA signaling is known to cause repression of genes associated with photosynthesis, but chlorophyll fluorescence measurements of plants treated with the potent JA-mimic coronatine have revealed that JA has little effect on photochemical efficiency. In contrast, stimuli that damage the plant, including mechanical wounding and herbivore attack have been shown to decrease photosynthetic efficiency and CO$_2$ fixation (Chang, Ball et al. 2004; Quilliam, Swarbrick et al. 2006). JA-independent signals including damage-associated molecular patterns or perturbations to leaf water balance may contribute to reduction in photochemical efficiency, in an \textit{fbk51}-dependant manner. In JA deficient plants JA-independent inhibition of photosynthesis may account for the size difference between wounded and unwounded plants. There were 19 genes with an assigned GO term related to photosynthesis that were repressed more than two-fold by wounding in Col-0, but not \textit{fbk51-2}. The average fold repression in Col-0 for the 19 was 9.3 ± 0.99 se, compared with 1.48 ± 0.08 Among the genes repressed in Col-0 was the photosystem II D1 and D2 genes (17.6 and 13.6 fold repressed in Col-0 respectively), a cytochrome b6-f complex subunit gene (repressed 7.51 fold in Col-0) and the photosystem II CP47 reaction center gene (repressed 7.8 fold in Col-0). The implication that photosynthesis is repressed in response to plant-damaging stresses like insect chewing and wounding but not
exogenous JA merits further study, and may suggest that wound-induced growth inhibition is results from several independent pathways.

**Discussion:**

As sessile organisms, plants must continuously monitor and adapt to a variety of environmental stresses. The plasticity of plant development, and metabolism allows for a fine-tuning of growth rate, morphology, and composition as resource availability and biotic threat levels change. Plants subject to wounding stress generally arrest their growth (Yan, Stolz et al. 2007; Zhang and Turner 2008), and plants under competition for light capture accelerate growth while attenuating defenses (Griebel and Zeier 2008; Leone, Keller et al. 2014). The mechanisms that control the balance between growth and defense are not well understood, but evidence is pointing to an ever-more complex network of interacting regulatory pathways that govern the two processes.

Previous studies of the wound response in Arabidopsis have revealed that wounding induces the production of the defense hormone JA, which is responsible for the differential regulation of thousands of genes. *De novo* JA activates the expression of genes related to the synthesis of defensive secondary metabolites (Creelman, Tierney et al. 1992; Gundlach, Müller et al. 1992; Bodnaryk 1994). Consequentially, JA-biosynthesis is required for the accumulation of many anti-herbivore and anti-pathogen molecules (Bekaert, Edger et al. 2012). In addition to promoting defense-gene expression, JA was also found to represses genes related to photosynthesis (Bilgin, Zavala et al. 2010). The dual regulation of secondary metabolism and photosynthesis was the basis for the hypothesis that JA signaling simply depletes the resources for growth by committing these to defense, and by slowing the production of new carbon skeletons, ATP, and reductant through repression of photosynthesis (Havko, Major et al. 2016). This model of growth/defense antagonism through resource diversion and depletion has been
supported by the observation that mutations with constitutive activation of induced defenses are slow growing and display hyper-resistance to herbivore and pathogen attack (Bonaventure, Gfeller et al. 2007). However, new evidence is complicating the story and suggesting that pathways contributing to defense can be uncoupled from growth inhibition.

In a recent study, Arabidopsis were treated with the potent JA mimic coronatine and chlorophyll florescence measurements were used to monitor the effects on photochemistry. Surprisingly, only a modest delay in the activation of photochemistry at the beginning of the photoperiod was observed and photochemical efficiency was otherwise unchanged (Attaran, Zeier et al. 2009). However, substantial reductions of photochemistry have been reported when defense responses were elicited by stimuli that damage the plant, including wounding and arthropod feeding (Chang, Ball et al. 2004; Délano Frier 2012). Together these results suggest that JA signaling is one component in a complex wound response that involves both JA-dependent and independent pathways to achieve the observed wound-induced deployment of defenses and inhibition of growth. In chapter four I have described a mutant screen to identify new genes required for wound-induced growth inhibition. In this chapter I have shown that the F-box protein FBK51 is involved in a previously unknown JA-independent pathway, which contributes to growth inhibition during the wound-response.

The work presented here demonstrates that FBK51 targets the growth-promoting VOZ1 and VOZ2 transcription factors for degradation via the 26S proteasome. Previous studies have established that VOZ proteins are involved in environmental stress responses, and are targeted by stress for degradation via the 26S proteasome (Nakai, Nakahira et al. 2013; Yasui and Kohchi 2014). Furthermore, experiments show VOZ proteins are continually degraded in a phyB, and 26S proteasome-dependent manner. A VOZ targeting F-box protein has been proposed to facilitate both the stress-induced degradation of VOZ, and to modulate the rate of VOZ turnover in the plant (Nakai, Nakahira et al. 2013; Yasui and Kohchi 2014). I show that the unknown F-box
protein is FBK51. Although the role of VOZ transcription factors in growth has not been characterized it is possible that FBK51-dependent VOZ degradation contributes to JA-independent repression of photochemistry during the wound response. RNA sequencing of wounded fbk51-2 mutants and Col-0 reveals that the repression of photosynthesis-related transcripts is potentiated by FBK51. It is possible that both JA-signaling and an unidentified JA-independent pathway involving FBK and VOZ1/2 converge to repress genes involved in photosynthesis, with the JA-independent pathway specifically affecting photochemistry near the time of the wound. Future studies employing chlorophyll fluorescence to measure the effects of wounding on the photochemistry of fbk51 mutants, Col-0 and voz1/2 double mutants are needed to address these questions.

Defense signaling is also linked with the initiation of reproductive phase in Arabidopsis. Plants under biotic stress delay the onset of flowering. This may be to divert resource allocation to defenses rather than the construction of reproductive organs. Additionally, delayed reproduction during defense might prevent the destruction of reproductive organs. JA-signaling mutants are early flowering, and the induction of defenses by exogenous application of JA delays the flowering of the plant (Zhai, Zhang et al. 2015). Like JA mutants, fbk51 is early flowering, and over-expression of FBK51 delays the onset of flowering. Furthermore, VOZ1 and VOZ2 have well-characterized roles in regulating the initiation of reproductive phase in Arabidopsis through the repression of the repressor of flowering, FT (Yasui and Kohchi 2014). The results here suggest that the FBK51-mediated VOZ degradation is part of a pathway that integrates environmental signals to coordinate both growth and reproduction.

Understanding of the relationship between growth and defense is evolving quickly. Previously, it has been unclear whether the balance of resources between growth and defense inextricably couples the two. Very recently, evidence is suggesting this is not the case. In chapter 4 I show that in jah2, a mutant that hyper-accumulates y-glutamylcysteine JA-dependent growth
is enhanced, but defense against an insect herbivore is weakened (Wei, Rowe et al. 2015). This motivated my search for mutants which are capable of growing during chronic wounding treatment, but do not compromise defense. In addition to my identification of wound-insensitive mutants in chapter four, a recent report has been published demonstrating that disruption of the light signaling phyB pathway can block the growth inhibition by JA. A quintuple mutant of JAZ repressor, JAZq shows constitutive JA responses including hyper-resistance to attack, accumulation of anthocyanins, and show growth. A suppressor screen of the small plant phenotype revealed that a mutation in phyB mostly restores the growth rate of JAZq to that of the wild-type. Restoration of growth in the phyB/JAZq mutant did not compromise the heightened resistance phenotypes (Campos, Yoshida et al. 2016). My identification of stunting insensitive mutants, and the work with JAZq/phyB show that the growth defense balance is not a simple diversion of resources between the two processes. This revelation opens the door for new strategies to optimize plant growth and defense.

Methods:

Plant Material and growth conditions

Plants were grown on Jiffy 7 peat pellets (wounding experiments, defense assays, JA-Ile accumulation measurements, RNA sequencing) or Sunshine Mix LC1 potting soil. The photoperiod was either 8 h (wounding experiments, flowering time measurements), 10 h (defense assays) or 16 h (flowering time). The light intensity was monitored and was kept between, 100 and 120 μmol photons/m2/s. Arabidopsis Col-0, stock CS70000 was originally received from the Arabidopsis Biological Resource Center (ABRC). The fbk51-1 allele SALK was identified from a pool of confirmed homozygous lines obtained from ABRC stock #CS27943 SALK_003021. The fbk51-2 allele was obtained from ABRC WiscDSLox364H10. The aos mutant was provided by Wassim Chehab. FBK51 over-expression lines were generated by Jeremy Jewell. Gene specific
and insert specific primers were used for PCR genotyping of fbk51-1 and fbk51-2 these primers are as follows: fbk51-1 gene specific forward primer: 5’-3’ TTG AAC ACG GAA ATT AGC CTG, gene specific reverse primer: 5’-3’ TTC TCC GTA CAG ATT CCG ATG. For fbk51-2 the gene specific forward primer was 5’-3’ TTC TGA CTT TCT CCA CCA AAC and the gene specific reverse primer was TCT TGT CAG CTT TTG GAA TGG. Insert-specific primers were 5’-3’ TGG AAC AAC ACT CAA CCC TAT CTC GG and AAC GTC CGC AAT GTG TTA TAA GTT GTC For fbk51-1 and fbk51-2 respectively.

**Wounding assay**

Wounding assays were blinded. Plant labels were replaced with coded labels by a lab assistant labels so that I did not know which lines I was wounding. Three week old plants were wounded across the axial 30% of a single leaf using a hemostat wrapped in floral tape to provide an even crushing of the leaf tissue. Wounding was performed daily at the middle of the photoperiod. I pinched a new leaf each day proceeding clockwise from the previously wounded leaf. Images of plants were taken with a digital camera next to a ruler and the rosette diameter of each plant was measured using ImageJ.

**JA-Ile Measurements**

Four-week-old plants were wounded across the axial 30% of the leaves on half of the rosette. Rosettes were quickly collected into pre-weighed tubes and flash frozen in liquid nitrogen to halt metabolism. Extraction and quantification of wound-induced endogenous JA-Ile was performed as previously described (Heitz, Widemann et al. 2012). [13C6]JA-Ile was added to a concentration of 5 pmol/gFW in the frozen samples as an internal standard. Extracts were separated on a C18 column (1.7 μM, 2.1 × 3 × 50 mm) using an Acquity ultraperformance liquid
chromatography system (Waters). An Agilent 6200 quadrupole time-of-flight mass spectrometer (Waters) was used in an electrospray negative mode to detect JA-Ile (322) and [13C6]JA-Ile (328).

Defense analysis

Caterpillar feeding assays were performed based on previous protocols (Schilmiller, Koo et al. 2007). *Trichoplusia ni* eggs were acquired from Benzon research and placed at 30°C for 48 hours to promote hatching. Vigorous larvae were moved to the rosette leaves of plants using a paintbrush. Larvae fed on plants for 10 days, after which time all larvae from a single plant were weighed together and the average larval weight for that plant was calculated by dividing the total weight by the number of larvae. Four such measurements were made for each genotype.

*Botrytis cinerea* challenge was performed based on (Rowe and Kliebenstein 2007). Spores of *Botrytis cinerea* pv. B.C. grape were suspended at 5000 spores/ml in 50% organic grape juice (Honest Kids juice). Single 4-μl droplets were placed on excised leaves. Leaves were sealed in petri plates with 10 ml sterile deionized water absorbed into filter paper (whatman). Digital pictures were taken of the advancing lesion and ImageJ was used to quantify the lesion area.

Analysis of *FBK51* over-expression

Lines over-expressing *FBK51* under the control of the strong CaMV 35S promoter were produced and *FBK51* transcripts were quantified by Jeremy Jewell. I measured the rosette area for these lines at four weeks post-germination using a digital image as described for the wounding assay. Flowering time measurements were made by recording the number of days from germination until the emergence of a bolt 1 cm from the rosette.

Yeast-two hybrid assay
I cloned the coding sequence of \textit{FBK51} into the Y2H prey vector pAD-GAL4, resulting in a fusion of \textit{FBK-GAL4 AD}. This construct was transformed into \textit{Saccharomyces cerevisiae} YRG2 using the one-step transformation protocol (Chen, Yang et al. 1992). I selected transformants on SD medium lacking tryptophan (-Trp) (Clonetech). I cloned the coding sequences of \textit{AtSKP1}, \textit{VOZ1} and \textit{VOZ2} into the Y2H bait vector pBD, constructing fusions of these with GAL4 BD. These constructs were transformed into yeast containing pAD-FBK-GAL4 AD. Co-transformants were selected on –Trp/-Leu (clonetech). Interactions between FBK51 and SKP1, VOZ1, VOZ2 were determined by growth of co-transformants on –Trp/-Leu/-His, and by MEL1 α-galactosidase activity against 5-bromo-4-chloro-3-indolyl alpha-D-galactopyranoside.

\textbf{Transient expression in \textit{Nicotiana benthamiana} BiFC/Western blotting}

BiFC constructs were made using the pSITE System (Martin and Goodin). \textit{FBK51}, \textit{VOZ1}, \textit{VOZ2}, and \textit{SKP1} were cloned from cDNA into the pENTR vector. pENTR FBK51 was recombined with pSITE nEYFP N1 to form an FBK51 fusion to the N terminal half of eFYP. Fusions of cEYFP with VOZ1, VOZ2, and SKP1 were made by recombining the corresponding pENTR clones with pSITE cEYFP N1. Constructs were transformed into Agrobacterium tumefaciens (gv3101) and selected on LB plates using spectinomycin, gentamicin, and rifampicin. Transient expression of BiFC constructs was performed by co-infiltration of Agrobacterium strains into \textit{Nicotiana benthamina}. After infiltration, plants were placed in continuous light at 28°C for five days. On the day of imaging plants were infiltrated with infiltration buffer (10 mm MgCl2, 10 mm MES, pH 5.6) with and without 100 µM MG-132. Six hours after treatment leaves were imaged with a Leica TCS SP5 confocal microscope.

\textbf{Western Blotting}
Three week old plants were wounded on three rosette leaves with forceps. Whole rosettes were harvested into liquid nitrogen, and disrupted in a ball mill. Ground leaf tissue was added to lysis buffer (50 mM HEPES, 10% glycerol, 2 mM EDTA, 0.5% Triton X-100, protease inhibitor cocktail (Roche)). Protein extracts were quantified using a protein quantification method based on the Bradford method. 12 µg of protein was on 4-20% polyacrylamide gels (Bio-rad) and transferred to PVDF membranes in transfer buffer (25 mM Tris 190 mM glycine 20% methanol pH 8.3) at 90 volts for 60 minutes. Membranes were blocked overnight at 4°C in TBS-T with 5% dried milk. The primary anti HA antibody was diluted 1:5000 in 1% dried milk (great value) TBS-T and incubated for 1 hour at room temperature. Secondary antibody incubations were performed with a 1:5000 dilution of the secondary antibodies in 1% milk in TBS-T at room temperature for 1 hour, followed by development using the luminol reagent (Santa Cruz).

Gene expression profiling

Three week old Arabidopsis were wounded on three rosette leaves and collected into liquid nitrogen, then disrupted using a ball mill as described for Western blotting. Three independent biological replicates were collected for each genotype and treatment. RNA was extracted using an RNeasy Plant mini extraction kit (QIAGEN). RNA integrity was analyzed using a ABI 3730 bioanalyzer (Thermo). mRNAs were purified and converted into barcoded libraries using the TruSeq RNA library prep kit (Illumina). Library fragment size was verified again using the ABI 3730 bioanalyzer. Samples were sequenced at the Washington State University Spokane genomic core on an Illumina HiSeq 2500 using 100 bp single-end reads. Two technical replicates were sequenced for each biological replicate. On average 45.496 million reads per sample passed quality control standards with a Q score higher than 30. Reads were mapped to TAIR10 gene models using the software Kallisto (Bray et al. 2015). Differential gene expression analysis was done using the RobiNA software suite to perform edgeR analysis (Robinson et al. 2010) using
the Benjamini & Hochberg’s FDR correction (Benhamini & Hochberg 1995). Gene ontology (GO) categories over-represented in the list of differentially expressed genes were identified using the AgriGO program (Du et al. 2010).
Figures:

**Figure 1.** Robust growth of wounded *fbk51* mutants. **A.** Photograph of wounded and unwounded plants **B.** Rosette diameter of four-week old plants grown on peat pellets. Wounded plants were pinched across the axial 30% of a single leaf with padded forceps for 1 week.
Figure 2. Wound-induced JA-Ile accumulation of JA-Ile in four-week-old Col-0 fbk51-1, fbk51-2, an FBK51 complementation line and aos 60 minutes post-wound. Maximal JA-Ile accumulation occurs 60 minutes after the wound. Wound-induced JA-Ile accumulation is not deficient in fbk-1 or fbk-2. No JA-Ile was detected in unwounded plants.
**Figure 3.** Genomic structure of *FBK51*. FBK51 Encodes a single exon gene with an N-terminal f-box and c-terminal Kelch repeats. A T-DNA insertion (not to scale) interrupts the 5' UTR of *fbk51*-1. The coding sequence of *fbk51*-2 is disrupted by T-DNA insertion in the second Kelch repeat.
A

Caterpillar feeding assay

Genotype

Col  fbk51-1  fbk51-2  aos

Caterpillar weight (mg)

B

Col-0  fbk51-1  fbk51-2  aos
Figure 4. Defense phenotypes of fbk51 mutants. A. *T. ni* weight after one week of feeding on Col-0, fbk51-1 fbk51-2 and aos. B. Photograph of *t. ni* larvae C. Resistance of Col-0, fbk51-1 fbk51-2 and aos to the spread of a *Botrytis cinerea* necrotic lesion. Excised leaves were incubated on wetted filter paper in humid petri dishes.
Figure 5. Restriction of rosette growth by FBK51 over-expression. A. Relative transcript levels of FBK51 normalized to PP2C. B. Growth inhibition correlates with relative transcript abundance of FBK51. C FBK51 over-expression does not affect herbivore resistance D Images of unstressed Arabidopsis: Col-0, fbk51-1, fbk51-2 and aos are larger than lines expressing 35S::FBK51
Figure 6. Interaction of FBK51 with a SKP1 and VOZ1/VOZ2. A. Yeast-2 hybrid assay between pDEST-GAL4AD-FBK51 and pDESTGAL4-BDSKP1,VOZ1,VOZ2. Yeast grown on SD (-Leu/-His) medium containing X-gal (top) and on SD (-Leu/-Trp/-His) bottom B. Model of FBK51 interactions with SKP1 in the SCF^{fbk} Ubiquitin ligase complex. FBK51 interacts with SKP1 via the N-terminal F-box, and with VOZ1 and VOZ2 via the C-terminal domains.
Figure 7. *voz1/voz2* double mutants are small and insensitive to wounding. **A.** Rosette diameter of four-week old plants. Wounded plants were pinched across a single leaf daily for one week. Wounded Col-0 are 31% smaller than controls. Wounded *voz1/voz2* are 14% smaller than unwounded controls **B.** Pictures of wounded and unwounded Col-0 and *voz1/2.*
Figure 8. Time-to-flowering phenotype of voz1/voz2 is epistatic to fbk51-1 mutants and over-expression lines; FBK51 OE are delayed in flowering A. The delayed flowering phenotype of the voz1/2 double mutant is epistatic to the early flowering phenotype of fbk51-1. B. Flowering is delayed in FBK51 over-expression lines
Figure 9. FBK51 interacts with VOZ in planta. A. BiFC of FBK51-nEYFP and VOZ1-cEYFP. A weak fluorescent signal (left) is enhanced by infiltration with 100 μM MG-132 6 hours prior to visualization. B. BiFC of FBK51 FBK51-nEYFP and VOZ2-cEYFP with MG-132 (right) and without (left).
Figure 10. FBK51 interacts with SKP1 in planta. A. BiFC of FBK51-nEYFP and SKP1-cEYFP. B-C BiFC controls
Figure 11. FBK51 promotes degradation of VOZ2 A. Accumulation of VOZ2 in the *N. benthamiana* transient co-expression required the proteasome inhibitor MG-132. Wounding leads to the degradation of VOZ2 in Col-0, but not in *fbk51-2*. VOZ2 protein is seen as a faint band above the dominant, nonspecific RBCL band.
Figure 12. Wounding triggers global reprogramming of gene expression. Horizontal volcano plots of differentially expressed genes in Col-0 and fbk51-2 2 hours A-B, and 24 hours C-D after wounding.
Figure 13. Wounding promotes global reprogramming of gene expression A-B Summary of the effects of wounding treatment on differential gene expression. C Summary of major gene ontology terms enriched in the wound-regulated genes
References:


Chapter Six

Summary and discussion

In this final chapter I will briefly outline the main contributions presented in this dissertation, and how these results contribute to our developing understanding of the biology of jasmonate signaling and the wound response.

Chapter two: summary and discussion

As plants grow they expand their capacity to perform photosynthesis and absorb nutrients. However, new growth will be wasted if it is consumed by herbivores or pathogens. Plants protect their tissues from these threats by deploying a wide variety of defense strategies. Growth and defense represent an important investment by the plant, as the available resources required for both are limited by what is readily available in the immediate microenvironment. Over-defended plants may miss the opportunity to maximize resource acquisition, and under-defended plants may be killed. To manage the balance between growth and defense plants have evolved inducible defenses, which are not produced until a threat is detected. Jasmonate (JA) is a key regulator of inducible defenses. JA synthesis is triggered in response to attack and promotes the activation of defenses while inhibiting growth. In chapter two of this dissertation I discuss the current progress in understanding the role of JA in managing the growth/defense balance.

Both JA synthesis and perception have been well understood for nearly a decade. More recently, new discoveries are uncovering the complex network of regulatory pathways into which JA signaling is wired. In Chapter two of this dissertation my co-authors and I contextualize JA signaling within a network of hormone signaling pathways that control a variety of plant processes.
For example, the recent discovery that JA signaling interacts with the GA pathway through competitive interaction between JAZ and DELLA transcriptional repressors revealed a new mechanism by which JA influences plant growth patterns. The literature now suggests that JA also interacts with several other hormone pathways to influence cell elongation and division. In the light of these discoveries accounting for JA-mediated growth inhibition by simple allocation of resources to defense seems dramatically incomplete. Chapter two of this dissertation highlights the need to reevaluate the role of JA signaling in the growth defense balance, and work toward a more complete accounting of the true cost of JA-induced defenses. Because JA controls many moving parts of plant physiology and metabolism it may be possible to uncouple the mechanisms through which JA inhibits growth, from the activation of effective defenses against herbivores and pathogens.

Chapter three: Summary and discussion

Despite an extensive understanding of JA biosynthesis and perception, and a rapidly growing understanding of the network of signals that JA signaling is wired into, the mechanisms that activate JA biosynthesis in response to environmental stress are still not well understood. I investigated a model of the regulation of JA biosynthesis through post-translational control of a JA biosynthesis enzyme, 12-oxophytodienoate reductase 3 (OPR3). The tomato OPR3 homolog was shown to crystallize as a self-inhibited homodimer, in which the active sites of each monomer are blocked by a peptide loop from the second monomer. A sulfate ion positioned conspicuously near a tyrosine within the active site is proposed to mimic an in vivo phosphorylation. Working with transgenic lines produced by Jeremy Jewell, I showed that a phenylalanine substitution of the tyrosine residue does not enhance the in vitro function of OPR3 protein expressed from a transgene, despite preventing the proposed mechanism of OPR3 inhibition. However, I showed,
using Western blotting, that epitope labeled OPR3 extracted from wounded plants has an altered electrophoretic mobility; a signature of post-translational modification. Despite my efforts to identify wound-induced modifications, none were found. The results of my work are consistent with the hypothesis that OPR3 is involved in the activation of JA biosynthesis in response to wounding. Future studies will be required to positively identify the mechanisms regulating the accumulation of JA signaling in response to wounding.

The eventual discovery of the regulation of wound-induced JA synthesis is crucial to an integrated understanding of JA biology. Because plant growth and the deployment of secondary metabolism is controlled by JA signaling, knowledge regarding the activation of JA signaling may allow the engineering of crops through fine-tuning the regulation of JA synthesis in an agricultural context, which is very different from the environmental context in which plant defense pathways evolved.

**Chapter four: Summary and discussion**

In Chapter four I describe my forward genetic screen to identify genes that are required for growth-arrest during the wound response. Previous work showed that chronic wounding causes a potent repression of plant growth, and that the effect was largely JA-dependent. The growth-defense balance model suggests that resource allocation to defense contributes to the observed growth inhibition, and implies that growth and defense are inexorably linked such that maintaining growth rates during defense requires compromising the potency of defenses. I found that a jasmonate-hypersensitive mutant, jah2, which displays a heightened sensitivity to growth inhibition by JA, is more sensitive to pathogen attack than the wild-type. This finding suggests that the defense response can be disconnected from growth inhibition under some conditions. To
identify new genes that are necessary for wound-induced growth inhibition I designed and performed a forward genetic screen for mutants that do not stop growing during chronic wounding treatment using replicate planting of homozygous T-DNA mutants in carefully controlled growth conditions. Seven mutants were identified which are insensitive to wound-induced stunting. The resistance to stunting was accompanied by a variety of additional morphological phenotypes. Two stunting-resistant mutant lines also had constitutive petiole elongation phenotypes. Also, a mutant with enhanced leaf production was found to resist wound induced-stunting. I also discovered four mutants with unaltered rosette morphology that displayed enhanced growth during wounding. The long-petiole mutants and one of the 'morphologically normal' mutants were found to be sensitive to herbivore attack. Further study of these mutants may provide key insights into the process governing the growth/defense balance.

Chapter five: Summary and discussion

In Chapter five I characterized one of the wound-insensitive mutants from the screen described in Chapter four. I found that FBK51 gene encodes an F-box protein that is required for wound-induced stunting. In two separate alleles of fbk51, fbk51-1, and fbk51-2 effects of chronic wounding on plant growth were reduced, but resistance to an insect herbivore and a necrotrophic pathogen remained intact. These results are part of a growing body of evidence that that the co-regulation of growth and defense can be uncoupled. During the writing of this dissertation a report was published describing the restoration of mostly-normal growth in a quintuple mutant of JAZ repressors (JAZq). JAZq exhibits constitutive activation of JA responses, is slow growing and hyper-resistant to herbivore attack. A mutation in the photoreceptor phyB rescues the growth rate of JAZq without abolishing the enhanced herbivore resistance. This recent report and the work presented in this dissertation contest the model that growth and defense are necessarily coupled.
through the simple allocation of resources between the two processes. Instead plants balance growth and defense in a subler way, which can allow both growth and effective defense simultaneously.

I characterized the molecular function of FBK51. As an F-box protein FBK51 interacts with components of E3 ubiquitin ligase complexes. F-box proteins recruit substrates to these complexes for poly-ubiquitination and degradation. Using a non-targeted yeast 2-hybrid screen (Y2H) Jeremy Jewell identified a putative substrate of FBK51: a growth promoting transcription factor, VOZ1. I confirmed that FBK51 interacts with VOZ1, and its redundant paralogue VOZ2 using a targeted Y2H experiment. I also showed that the interaction between FBK51 and VOZ1, and VOZ2 occurs in planta and leads to VOZ destabilization. Preliminary Western blots suggest that VOZ2 is degraded in vivo in response to wounding, but is stabilized in the fbk51-2. Transcription profiling suggests that FBK51 may influence photosynthesis or secondary metabolism in the wound response. With this dissertation I have helped to reframe the role of JA signaling in the defense response. JA signaling should be seen in the context of a network of interacting hormone responses, independent signaling pathways to dynamically alter the balance of growth, defense and reproduction during the life of the plant.
References:


