THE ROLE OF JAZ PROTEINS IN THE REGULATION OF PLANT GROWTH AND DEFENSE

By

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ABSTRACT

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When challenged with environmental stress, plants devote a significant proportion of their biosynthetic capacity to the production of secondary metabolites and other defense-related strategies. Increased production of defensive compounds is associated with diversion of resources (e.g., carbon) from primary growth, thereby limiting plant biomass accretion. This growth-defense antagonism has a profound impact on plant biology and ecological relationships. However, the molecular mechanisms controlling tradeoffs between growth and defense are still poorly understood. The plant signaling molecule jasmonate (JA) is a key regulator of resource allocation as it reprograms transcriptional networks that appear to redirect resources from primary metabolism and growth to secondary metabolism and defense. This molecular "switch" is mediated in part by JAZ repressor proteins that, in the absence of JA, bind to and inhibit the action of JA-related transcription factors (TFs). Stress-induced production of JA promotes the formation of COI1-JAZ co-receptor complexes that targets JAZ proteins for rapid destruction by the E3 ubiquitin ligase SCF^{COI1}, thereby releasing TFs from inhibition. Here, I use two approaches to show that JAZ proteins have a major role in balancing resource allocation between growth and defense in the model plant *Arabidopsis thaliana*. First, I demonstrate that alternative splice variants of JAZ10 that are stable in the presence of high levels of JA function to attenuate JA responses, thereby prioritizing growth over defense. Second, I developed a *jaz quintuple* mutant (*jazQ*) that lacks five of the 13 JAZ genes and show that this mutant constitutively produces defense compounds but grows slowly. The *jazQ* mutant was then employed as the

parental line in a genetic suppressor screen aimed at uncoupling the growth-defense antagonism. Characterization of one suppressor mutant showed that loss of function of the red light receptor phytochrome B (phyB) rescues the slow growth of *jazQ* without significantly affecting defense traits. These findings suggest that growth-defense antagonism may not be dictated by limited metabolic resources but rather by hard-wired transcriptional programs that exert control over resource partitioning in dynamic environments. In the long term, the findings described in this dissertation may inform efforts to increase food production and security while reducing the use of pesticides that are detrimental to the environment and human health.

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CHAPTER ONE

Literature review: Jasmonate-triggered plant immunity

Work presented in this chapter has been published:

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Abstract

The plant hormone jasmonate (JA) exerts exquisite control over the production of chemical defense compounds that confer resistance to a remarkable spectrum of plant-associated organisms, ranging from microbial pathogens to vertebrate herbivores. The underlying mechanism of JA-triggered immunity (JATI) can be conceptualized as a multi-stage signal transduction cascade involving: i) pattern recognition receptors (PRRs) that couple the perception of danger signals to rapid synthesis of bioactive JA; ii) an evolutionarily conserved JA signaling module that links fluctuating JA levels to changes in the abundance of transcriptional repressor proteins; and iii) activation (de-repression) of transcription factors that orchestrate the expression of myriad chemical and morphological defense traits. Multiple negative feedback loops act in concert to restrain the duration and amplitude of defense responses, presumably to mitigate the metabolic cost and negative impact of JATI on plant fitness. The convergence of diverse plant- and non-plant-derived signals on the core JA module indicates that JATI is a general response to perceived danger. However, the modular structure of JATI may accommodate attacker-specific immune responses through evolutionary innovation of PRRs (inputs) and chemical defense traits (outputs). The efficacy of JATI as a defense strategy is highlighted by its capacity to shape natural populations of plant attackers, as well as the propensity of plant-associated organisms to subvert or otherwise manipulate JA signaling. As both a cellular hub for integrating informational cues from the environment and a common target of pathogen effectors, the core JA module provides a focal point for understanding immune system networks and the evolution of chemical diversity throughout the plant kingdom.

Introduction

Plants are a source of nutrition for a vast biota in terrestrial environments. Selective pressure imposed by pathogens and herbivores has shaped the evolution of an astonishing array of specialized plant defense compounds that exert direct toxic, anti-nutritional, or repellant effects on plant consumers. Other defensive compounds work indirectly by attracting natural enemies of plant-associated organisms. Strategies to deploy these protective chemical shields and associated morphological structures may be constitutive or inducible. It is thought that natural selection, at least in some plant species, favored the evolution of induced defenses because they have lower resource allocation costs than constitutive resistance traits (Baldwin 1998; Herms and Mattson 1992; Thaler et al. 2012). A key feature of many induced defense traits is their expression in tissues distal to the site of infection or attack. The combined effect of local and systemic defense responses provides broad-spectrum resistance against subsequent biotic attacks, and constitutes a form of induced immunity (Fu and Dong 2013; Howe and Jander 2008; Jones and Dangl 2006).

Intensive research efforts to understand the molecular mechanisms and evolutionary ecology of induced immunity have focused on the question of how plants recognize a foreign threat. Significant insight into this question has come from molecular genetic analyses of plant-pathogen interactions. Pattern-triggered immunity (PTI) confers basal resistance and is mediated by cell surface-localized pattern recognition receptors (PRRs) that bind conserved foreign molecules, known collectively as microbial/pathogen-associated molecular patterns (MAMPs) (Chisholm et al. 2006; Dodds and Rathjen 2010; Jones and Dangl 2006). A second layer of induced resistance, referred to as effector-triggered immunity (ETI), relies on polymorphic intracellular resistance (R) proteins to detect effector molecules that plant attackers deliver into host cells to counteract defense. ETI responses often include localized host cell death and are

qualitatively similar, though typically more robust and faster, than PTI responses (Dodds and Rathjen 2010). Major conceptual contributions of the PTI/ETI paradigm include the distinction between plant defense responses triggered by conserved patterns *versus* effectors, and a model of how these branches of immunity influence the evolution of plant-pathogen associations (Jones and Dangl 2006). The PTI/ETI model has also influenced current views of how plants recognize attack by arthropod herbivores, which constitute the majority of plant-consuming species on Earth (Erb et al. 2012). Accordingly, eliciting compounds produced by arthropod herbivores have been dubbed herbivore-associated molecular patterns (HAMPs) (Felton and Tumlison 2008; Mithofer and Boland 2008).

In addition to cell surveillance systems that recognize foreign threats in the form of MAMPs/HAMPs and effectors, it has long been known that plant-derived (i.e., self) signals are also potent elicitors of local and systemic defense responses (Bergey et al. 1996; Green and Ryan 1972; Heil et al. 2012; Huffaker et al. 2006, 2011; Krol et al. 2010; Mousavi et al. 2013). These endogenous elicitors are produced in response to general cellular injury and may be classified as damage-associated molecular patterns (DAMPs). Because DAMPs are generated in response to diverse types of tissue injury, their role in cellular recognition of pathogen attack has traditionally been ignored. However, the recent identification of DAMP receptors and associated signal transduction components (Brutus et al. 2010; Choi et al 2014; Mousavi et al., 2013; Yamaguchi et al. 2006, 2010) is shaping a broader view of how plant cells perceive and respond to injurious threats (Boller and Felix 2009; De Lorenzo et al. 2011; Heil 2009; Koo and Howe, 2009). The diversity of conserved patterns that trigger local and systemic defense reactions supports the concept that cellular perception of “danger”, regardless of its source, is a unifying

principle of induced immunity in plants and animals (Boller and Felix 2009; Koo and Howe 2009; Lotze et al. 2007; Matzinger 2002).

A second major question surrounding induced immunity concerns the extent to which cellular recognition of a threat is translated into a host response that neutralizes the attacking pathogen or herbivore. Indeed, genome-wide transcriptome studies indicate a significant degree of overlap in molecular responses triggered by different MAMPs/HAMPs/DAMPs and effectors (Bidart-Bouzat and Kliebenstein 2011; Caillaud et al. 2013; Gouhier-Darimont et al. 2013; Kim et al. 2014; Navarro et al. 2004; Reymond et al. 2004; Reymond et al. 2004; Tao et al. 2003; Thilmony et al. 2006; Tsuda et al. 2008, 2009; Wise et al. 2007; Zhurov et al. 2014). There is also evidence to indicate that PTI and ETI converge on similar downstream signaling components, including MAP kinase pathways, ROS production, and calcium-dependent signaling events (Romeis and Herde 2014; Sato et al. 2010). Although quantitative differences in the timing and strength of induction is likely to shape the outcome of specific plant-attacker associations (De Vos et al. 2005; Katagiri and Tsuda 2010; Tao et al. 2003; Wise et al. 2007), most evidence indicates that specific danger signals trigger general host defense responses that are effective against broad classes of pathogens and herbivores (Erb et al. 2012).

The central role of small-molecule hormones in controlling the expression of chemical and morphological defense traits provides an impetus for describing induced immunity from the perspective of phytohormone networks (Erb et al. 2012; Pieterse et al. 2009; Reymond and Farmer 1998). It is now evident that diverse danger signals converge on the immune-promoting effects of two major defense hormones, jasmonic acid (JA) and salicylic acid (SA). A wealth of genetic evidence indicates that JA-triggered immunity (JATI) and SA-triggered immunity (SATI) contribute to plant resistance to many, if not most, pathogens and arthropod herbivores

studied to date. JATI and SATI interact and further crosstalk with other plant hormone pathways to activate the most effective responses to the particular type of attacking organism, in a way that finely balance tradeoffs between defense, growth, and reproductive success. Here, we focus on recent advances in understanding how JA and its cognate receptor system control a bewildering array of defense responses across the plant kingdom. We describe JATI as a multistage process in which a highly conserved core JA module links a variety of PRR-based recognition systems (inputs) to the expression of specific defense traits (outputs). Based on these considerations, we propose that the regulatory structure of JATI has potential to create new specificities of host resistance through evolutionary innovation of input and output modules. We also highlight the various ways in which plant-associated organisms manipulate JATI to their own advantage. These new mechanistic insights will help to explain how JATI shapes patterns of chemical diversity and species interaction in the plant kingdom, and how these relationships affect genome evolution to modulate phenotypic plasticity. Our focus on JA is not intended to minimize the role of other signals in coordinating plant defense responses, or to distract from the important endeavor of understanding the complexities of phytohormone networks and their relationship to induced immunity (Ballaré 2014; Kazan and Manners 2012; Kim et al. 2014; Mukhtar et al. 2011; Pieterse et al. 2009;). Nevertheless, we subscribe to the view that an accurate, and ultimately predictive, understanding of interconnected signaling networks depends on knowledge of how individual signals are produced and perceived at the molecular level. Readers are referred to several excellent review articles for a comprehensive discussion of JA-mediated signal transduction, its interaction with other signaling pathways, and the function of JA in development processes (Ballaré 2011; Browse 209; Huot et al., 2014; Kazan and Manners 2008,

2013; Kombrink 2012; Meldau et al. 2012; Moreno and Ballaré 2014; Pauwels and Goossens 2011; Robert-Seilianiantz et al. 2011; Shyu and Brutnell 2015; Wasternack and Hause 2013)

JATI confers broad-spectrum resistance in dicots and monocots

The central role of JA as an activating signal for induced immunity is grounded in three general observations: First, biotic attack and other forms of tissue injury result in the rapid synthesis of JA and its receptor-active derivative, jasmonoyl-L-isoleucine (JA-Ile). Stress-induced accumulation of JA-Ile occurs in both above- and below-ground tissues and, depending on the eliciting signal and tissue type, is a systemic response (Chauvin et al. 2013; Fragoso et al. 2014; Grebner et al. 2013; Koo et al. 2009; Mousavi et al. 2013; Schilmiller and Howe 2005). Second, JA promotes the expression of virtually all major classes of secondary metabolites and proteins that have established roles in defense, including alkaloids, terpenoids, phenylpropanoids, amino acid derivatives, anti-nutritional proteins, and some pathogenesis-related (PR) proteins (Browse and Howe 2008; De Geyter et al. 2012; De Vleesschauwer et al. 2013; Farmer and Ryan 1990; Gonzales-Vigil et al. 2011; Mohan et al. 2006; Van Loon et al. 2006). The JA pathway also promotes the development of morphological structures, including glandular trichomes, resin ducts, and nectaries that produce a rich variety of compounds serving direct and indirect roles in defense (Dicke and Baldwin 2010; Hudgins et al. 2004; Li et al. 2004; Peiffer et al. 2009; Qi et al. 2011; Radhika et al. 2010; Traw and Bergelson 2003; Van Poecke and Dicke 2002; Yoshida et al. 2009). Finally, studies employing JA mutants have demonstrated the crucial role of this hormone in plant protection against diverse biota (Browse and Howe 2008). Among the plant-associated organisms whose fitness is curtailed by JATI are necrotrophic and (hemi)biotrophic pathogens, mutualistic fungi, nematodes, leafhoppers, beetles, caterpillars, thrips, spider mites, fungus gnats,

slugs, crustaceans, and some vertebrate herbivores (Table 1.1). Indeed, it is reasonable to think that the number of plant-eating species affected by JATI may exceed the total number of plant species on Earth.

Much of our knowledge about the protective effects of JATI comes from studies on a limited number of dicot species, including Arabidopsis, tomato, and tobacco. These studies have led to the generalization that tissue-consuming insect herbivores and necrotrophic pathogens are particularly sensitive to JATI, whereas biotrophic organisms that obtain nutrients from living host tissues succumb to the effects of SATI (Cailldaud et al. 2013; Glazebrook 2005; Pieterse et al. 2009). There is every indication that JA promotes resistance of monocot and gymnosperm species to a wide range of pathogens and herbivores (Hudgins et al. 2004; Schmelz et al. 2013; Shyu and Brutnell 2015; Yan et al. 2012; Ye et al. 2012; Zulak and Bohlmann 2010). In contrast to the biotroph/necrotroph dichotomy that has emerged from studies with Arabidopsis, it is noteworthy that JA is required for induced immunity of rice to (hemi)biotrophic parasites, including the root knot nematode (*Meloidogyne graminicola*) and *Xanthomonas oryzae* (Nahar et al. 2011; De Vleesschauwer et al. 2013). This conclusion is consistent with the ability of JA to elicit expression of many *PR* genes and defensive secondary metabolites in rice and maize (Mitsuhara et al. 2008; Schmelz et al. 2011; Yamane 2013). Given these findings, together with the high endogenous levels of free SA in rice leaves (Silverman et al. 1995), the precise role of SA as a signal for induced immunity in monocots awaits for further clarification (De Vleesschauwer et al. 2013).

The serendipitous discovery that JA mutants maintained in artificial growth environments succumb to attack by unsuspected pathogens and herbivores vividly demonstrates the robust protection afforded by JATI (Browse and Howe 2008). Elegant field studies have em-

Table 1.1. Examples in which there is genetic evidence for JA-mediated plant resistance to pathogens and herbivores.

ORGANISM	HOST PLANT	REFERENCE
Pathogenic bacteria		
Erwinia carotovora	*Arabidopsis thaliana* (Brassicaceae)	Norman-Setterblad et al. 2000
Xanthomonas oryzae	*Oryza sativa* (Poaceae)	Yamada et al. 2012
Necrotrophic fungi / oomycetes		
Alternaria brassicicola	*A. thaliana* (Brassicaceae)	Thomma et al. 1998
Botrytis cinerea	*Zea mays* (Poaceae)	Vijayan et al. 1998
Pythium spp	*Solanum lycopersicum* (Solanaceae)	Staswick et al. 1998; Yan et al. 2012 This study
Nematodes		
Meloidogyne graminicola	*O. sativa* (Poaceae)	Nahar et al. 2011
Mollusks		
Arion lusitanicus	*A. thaliana* (Brassicaceae)	Falk et al. 2013
Crustaceans		
Porcellio scaber	*A. thaliana* (Brassicaceae)	Farmer and Dubugnon 2009
Armadillidium vulgare	*O. sativa* (Poaceae)	
Cell content feeders		
Tetranychus urticae (Acari)	*S. lycopersicum* (Solanaceae)	Li et al. 2004
	A. thaliana (Brassicaceae)	Zhurov et al. 2014
Frankliniella occidentalis	*Solanum lycopersicum* (Solanaceae)	Li et al. 2002
(Thysanoptera)	*A. thaliana* (Brassicaceae)	Abe et al. 2009
Piercing-sucking insects		
Myzus persicae (Hemiptera)	*A. thaliana* (Brassicaceae)	Ellis et al. 2002
Empoasca sp. (Hemiptera)	*Nicotiana attenuata* (Solanaceae)	Kessler et al. 2004
Leafminer insects		
Scaptomyza flava (Diptera)	*A. thaliana* (Brassicaceae)	Whiteman et al. 2011
Leaf / root chewing insects		
Manduca sexta (Lepidoptera)	*N. attenuata* (Solanaceae)	Howe et al. 1996; Kessler et al. 2004; Campos et al. 2009
Spodoptera frugiperda	*S. lycopersicum* (Solanaceae)	
(Lepidoptera)	*A. thaliana* (Brassicaceae)	McConn et al. 1997
Bradysia impatiens (Diptera)	*Zea mays* (Poaceae)	Yan et al. 2012
Spodoptera exigua (Lepidoptera)		
Vertebrate herbivores		
Eurotestudo boettgeri	*A. thaliana* (Brassicaceae)	Mafli et al. 2012

ployed natural and synthetic genetic variation to demonstrate this phenomenon in natural habitats and have established the ecological importance of JATI in shaping herbivore community composition (Kallenbach et al. 2012; Kessler et al. 2004; Thaler et al. 2001; Züst et al. 2012). We employed this unbiased "ask the plant" approach to query the biological role of the JATI in mediating interaction of cultivated tomato (*S. lycopersicum*) with potential biotic attackers in the field. Previous studies at this field site showed that glandular trichomes, whose development on tomato leaves controlled in part by the JA pathway (Boughton et al 2005; Li et al 2004; Peiffer et al 2009), provide an important layer of anti-insect defense (Kang et al 2010a, 2010b, 2014). Replicated field trials showed that a tomato mutant (*jai1-1*) lacking the JA-Ile receptor suffered 100% mortality from root rot disease caused by the oomycete pathogen *Pythium* (Figure 1.1). Similar results have been reported for JA mutants of Arabidopsis and maize (Staswick et al 1998; Vijayan et al 1998; Yan et al. 2012). These collective studies provide a compelling demonstration of the efficacy of JATI in protecting diverse plants against the same soil-borne pathogen.

Core JA signaling module

The signal transduction events that couple perception of danger signals at the cell surface to the expression of JA-responsive defense genes relies on an evolutionarily conserved core apparatus to synthesize and perceive JA-Ile (Figure 1.2) (Chico et al. 2008; Katsir et al. 2008a). A crucial feature of JA-Ile as a trigger for defense gene expression is its rapid and reversible accumulation in vegetative tissues that are frequently targeted for attack. Unstressed leaves of Arabidopsis, for example, contain extremely low or undetectable amounts of bioactive JA (Glauser et al. 2008; Koo and Howe 2009). JA synthesis is initiated in plastids from the pre-existing C18 precursor li-

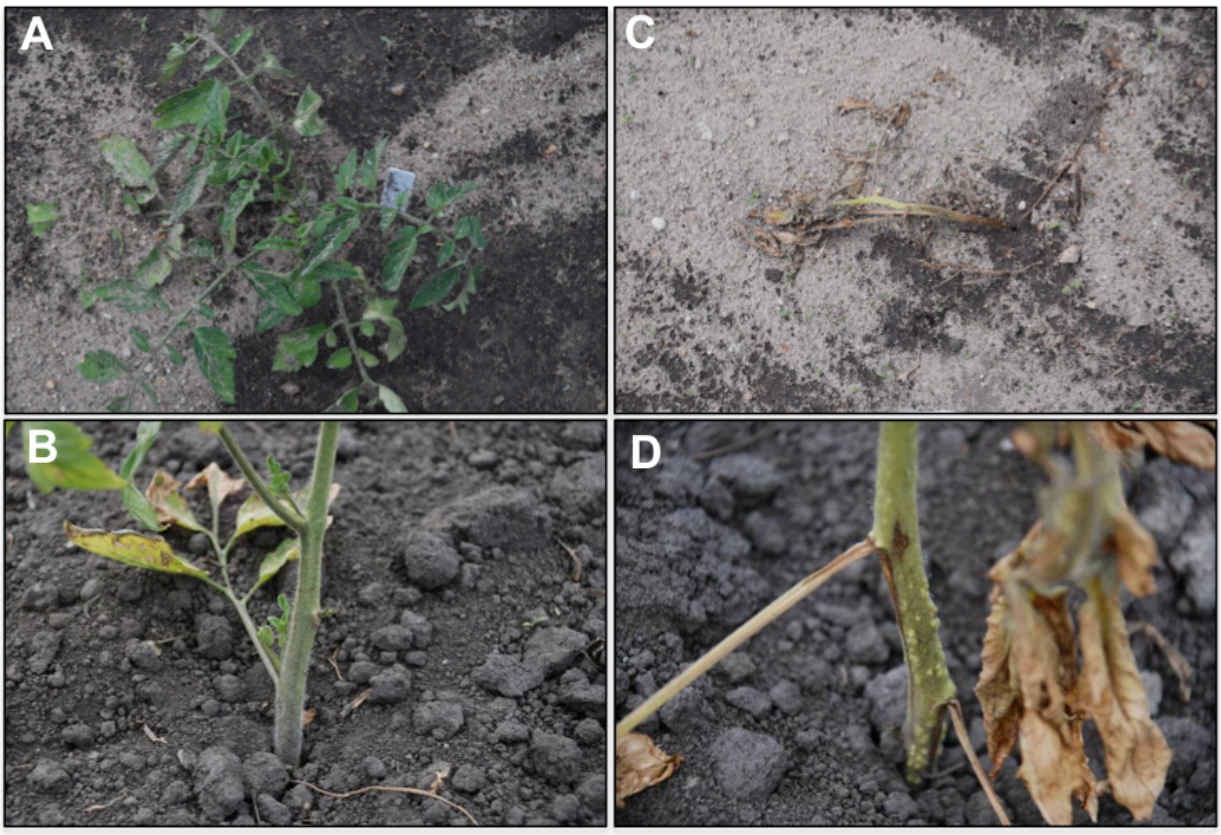

Figure 1.1. **Jasmonate perception by the COI1 receptor system is essential for resistance of cultivated tomato to the oomycete pathogen *Pythium*.**

(A and B) Wild-type (cv Castlemart) and **(C and D)** *jai1-1* mutant plants grown for three weeks in a growth chamber without visible signs of disease were transplanted to a field plot at Michigan State University, East Lansing, MI. Two weeks after transplanting, all *jai1-1* plants (n = 30) died from a disease that was diagnosed as *Pythium* stem/root rot by the MSU Diagnostics Lab. Sequencing of PCR products derived from 5.8S ribosomal genes and internal transcribed spacer region in infected tomato tissue confirmed the presence of *Pythium ultimum*. Of several hundred wild-type (*Jai1*/*Jai1*) plants grown side-by-side at the same field site, none showed symptoms of the disease. The figure shows photographs of representative wild-type and *jai1-1* mutant plants two weeks after transplantation. Identical results were obtained in three independent trials performed at the same site.

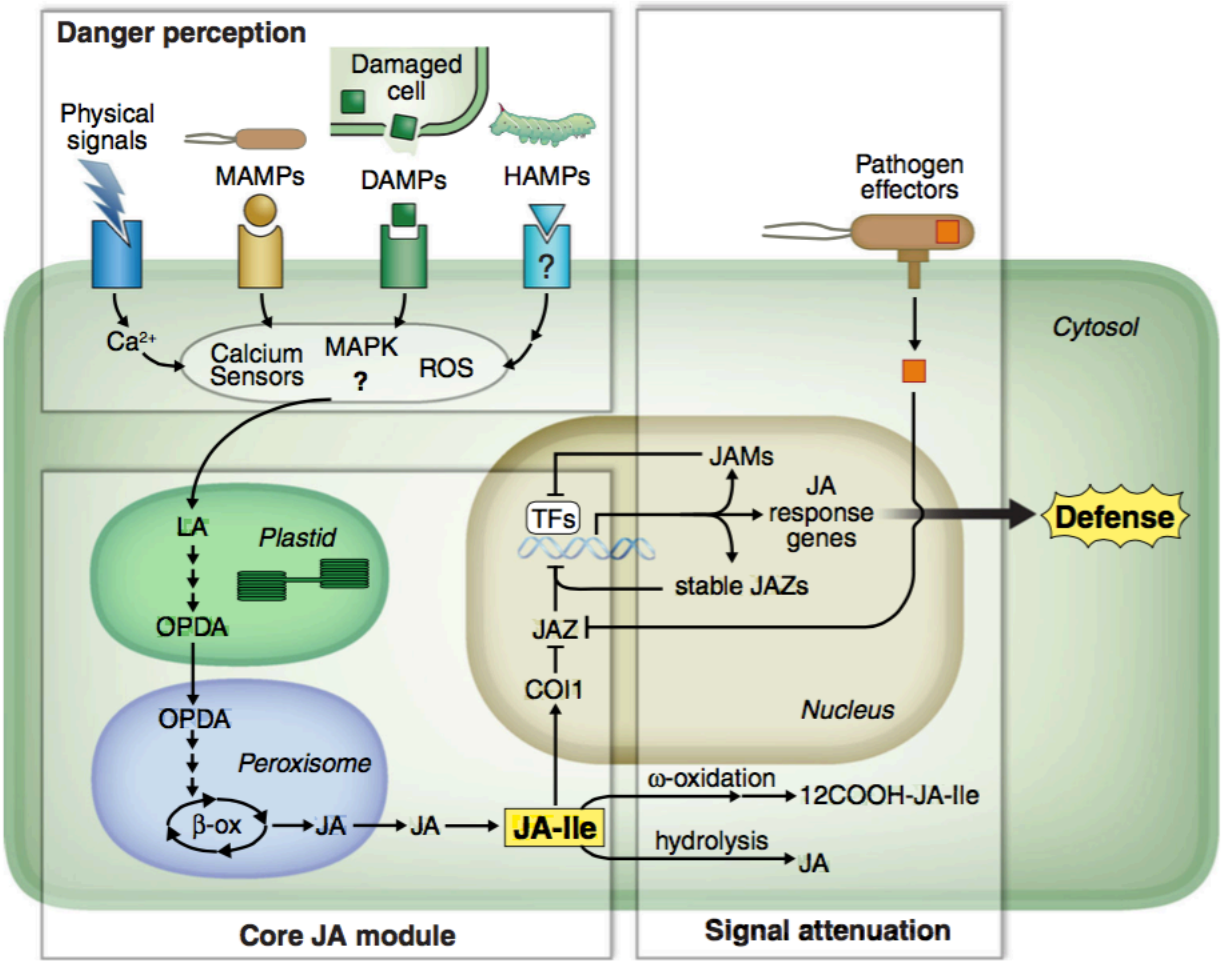

Figure 1.2. Model of jasmonate-triggered plant immunity (JATI).

Danger signals (MAMPs/HAMPs) derived from attacking organisms and damaged plant cells (DAMPs) are recognized by pattern recognition receptors (PRRs) at the cell surface. PRR activation is coupled to intracellular signaling systems involving MAP kinase pathways (MAPK), Calcium ion-sensing proteins, and reactive oxygen species (ROS), among others. How these signaling events are connected to activation of the core JA signal module, which includes JA biosynthesis from its precursor linolenic acid (LA), is largely unknown (?). Plastidic and peroxisomal enzymes convert LA to jasmonic acid (JA), which is the substrate for synthesis of JA-Ile in the cytosol. Within the nucleus, JA-Ile promotes JAZ-COI1 interaction and targets JAZs for proteolytic degradation by the ubiquitin-proteasome system. Removal of JAZ alleviates TFs from repression, thereby activating the expression of JA-responsive genes and the production of defense-related chemicals and morphological structures (Defense). Several mechanisms to attenuate signaling through the core module have been elucidated, including catabolism of JA-Ile via ω-oxidation and hydrolysis, *de novo* synthesis of JAZ repressors that are stable in the presence of JA-Ile, and accumulation of JAM TFs that negatively regulate transcription. Pathogen-derived effectors target the core JA signal module to disrupt hormonal balance and induced immune responses. Abbreviations: Microbe-associated molecular patterns

Figure 1.2 (cont'd). (MAMPs); Herbivore-associated molecular patterns (HAMPs); Damage-associated molecular patterns (DAMPs), Mitogen-activated protein kinase (MAPK); Reactive oxygen species (ROS), 12-oxo-phytodienoic acid (OPDA), β-oxidation (β-ox), jasmonoyl-L-isoleucine (JA-Ile), JASMONATE-ZIM domain (JAZ), JA-related transcription factor (TF), JASMONATE-ASSOCIATED MYC2-LIKE (JAM), 12-carboxy-JA-Ile (12COOH-JA-Ile).

nolenic acid (LA). LA is converted in the plastid to a cyclic 12-oxo-phytodienoic acid (OPDA) intermediate, which is then transported to the peroxisome for subsequent reduction and β-oxidation steps that give rise to JA (Howe and Schilmiller 2002; Schaller and Stintzi 2009; Wasternack and Hause 2013). JA is conjugated to Ile in the cytosol to produce JA-Ile (Kang et al. 2006; Staswick and Tiryaki 2004). As the receptor-active form of the hormone (Fonseca et al. 2009; Katsir et al. 2008b; Sheard et al. 2010; Staswick 2004; Thines et al. 2007), JA-Ile presumably diffuses into the nucleus where it is perceived by its receptor. Many genes required for JA-Ile biosynthesis are coordinately upregulated by the JA signaling pathway (Browse 2009; Koo et al. 2006; Wasternack and Hause 2013). Although this observation suggests a positive feedback loop to amplify JA responses, the existence of JA-inducible negative feedback loops (see below) highlights the complexity of processes involved in JA-Ile homeostasis.

JA-Ile controls defense gene expression by promoting the destruction of JAZ (JAsmonate ZIM-domain) transcriptional repressors via the ubiquitin-26S proteasome system (Chini et al. 2007; Thines et al. 2007; Yan et al. 2007). JAZ proteins are defined by two highly conserved sequence motifs referred to as ZIM (or TIFY) and Jas (Browse 2009; Chung et al. 2009; Yan et al. 2007). In the absence of stress, low levels of JA-Ile permit JAZ proteins bind to and repress TFs in the nucleus. The basic helix-loop-helix TF MYC2 and its closely related paralogs MYC3 and MYC4 are the most extensively studied JAZ-interacting TFs having a direct role in JATI (Fernández-Calvo et al. 2011; Kazan and Manners 2013; Schweizer et al. 2013). Nuclear localization of JAZ repressors is dependent of physical association with MYC2 (Withers et al. 2012). Within the nucleus, the repressive function of some JAZ requires the NINJA (Novel Interactor of JAZ) protein to mediate interaction with corepressors such as TOPLESS (TPL) (Pauwels et al 2010; Acosta et al 2013). NINJA contains a so-called EAR (ERF-associated

amphiphilic repression) motif that binds TPL and TPL-related corepressors (Pauwels et al. 2010; Szemenyei et al. 2008). Other JAZ proteins, (*e.g.*, JAZ8), contain an EAR motif to allow direct recruitment of TPL and repression of JA responses independently of NINJA (Shyu et al. 2012). A rapidly expanding list of JAZ- and MYC2-interacting regulatory proteins that participate in other hormone-response pathways indicate that the JAZ-TF interactome is a cellular hub for integrating diverse environmental and developmental signals (Ballaré 2014; Hou et al. 2010; Hu et al. 2013a; Kazan and Manners 2013; Nakata et al. 2013; Pauwels et al. 2010; Qi et al. 2011, 2014; Song et al. 2011, 2014; Toda et al. 2013; Yang et al. 2012b; Zhu et al. 2011).

In response to perception of signals that trigger JA-Ile synthesis, JA-Ile promotes direct binding of JAZ repressors to the F-box protein COI1 (CORONATINE INSENSITIVE1), which is the specificity determinant of the E3 ubiquitin ligase SCF^{COI1} (Katsir et al. 2008b; Melotto et al. 2008; Sheard et al. 2010; Thines et al. 2007; Xie et al. 1998). Ubiquitin-dependent degradation of JAZ proteins relieves repression of TFs, thereby allowing the expression of JA-response genes (Figure 1.2). The timing, amplitude, and duration of JA responses appears to be is controlled primarily by the intracellular levels of JA-Ile (Koo et al., 2009; Wasternack and Hause 2013). Moreover, the speed with which danger signals are transmitted to gene activation via the core JA pathway may be remarkably fast. Crush-type wounds inflicted to Arabidopsis leaves, for example, result in increased JA-Ile levels within minutes of tissue damage, with increased accumulation of primary JA-response transcripts observed within 5 min of wounding (Chauvin et al. 2013; Chung et al. 2008). Mechanical tissue damage also triggers rapid systemic responses, including JA-Ile accumulation, degradation of JAZ proteins, activation of JA-response genes, and induced resistance (Acosta et al 2013; Green and Ryan 1972; Koo et al. 2009; Mousavi et al. 2013; Zhang and Turner 2008).

Activation of the core JA module

MAMPs, HAMPs, and DAMPs – Available evidences indicate that JA/JA-Ile synthesis is controlled at the post-transcriptional level via activation of pre-existing JA biosynthetic enzymes (Wasternack and Hause 2013). Although the precise mechanism of activation remains to be determined, a wide range of endogenous (DAMPS) and foreign (MAMP/HAMP) signals have been implicated in the process (Figure 1.2; Table 1.2). Analysis of phytohormone production and defense gene expression in response to elicitors such as flagellin, elongation factor-Tu (EF-Tu), and chitin, for which the cognate PRRs have been identified, indicate that these conserved bacterial and fungal patterns activate multiple branches of induced immunity, including JATI (Kim et al. 2014). Several HAMPs, including fatty acid-amino acid conjugates (FACs), also amplify JA responses (McCloud and Baldwin 1997; Schmelz et al. 2003, 2007). Elicitation of JA-mediated defense responses by DAMPs, including the 18-amino-acid peptide systemin and cell wall-derived oligogalacturonides (OGs), is consistent with the ability of these compounds to stimulate JA synthesis (Doares et al. 1995; Lee and Howe 2003). Likewise, endogenous peptide elicitors from Arabidopsis (AtPep1) and maize (ZmPep3) exert potent stimulatory effects on JATI (Table 1.2) (Huffaker et al. 2006, 2013). Identification of plant receptors for OGs and AtPep1 marks a major advance in efforts to understand the contribution of DAMPs to plant immunity (De Lorenzo et al., 2011; Krol et al., 2010; Yamaguchi et al., 2006;). Equally exciting is the recent discovery of the receptor for extracellular ATP, which exhibits properties of a danger signal released by damaged cells (Choi et al. 2014; Song et al. 2006).

Ca^{2+} signaling, ROS and MAPKs - It is generally accepted that JA synthesis is initiated in the plastid by stress-induced activation of lipases that release fatty acid precursors of JA (Bergey et

Table 1.2. Selected examples of danger signals and effectors that modulate JA-mediated plant defense responses.

SIGNAL	MECHANISM OF PERCEPTION / ACTION	REFERENCES
DAMPs		
AtPep1	LRR-RK receptors PEPR1 and PEPR2. Activates JA- and SA-dependent innate immune responses.	Huffaker et al. 2006; Yamaguchi et al. 2006
Systemin	Receptor unknown (presumed LRR-RK). Elicits JA synthesis and production of defense compounds.	Pearce et al. 1991
ZmPep3	Unknown receptor. Activates JA synthesis and production of defense compounds.	Huffaker et al. 2013
Oligogalacturonides	WAK1 receptor. Activates JA synthesis and production of defense compounds.	Doares et al. 1995 Brutus et al. 2010
Extracellular ATP	DORN1 receptor. Activates transcriptional responses that are similar to wound responses.	Choi et al. 2014
MAMPs/HAMPs		
Flagellin (bacterial pathogens)	LRR-RK receptor FLS2. Activates the JA and other branches of induced immunity.	Chinchilla et al. 2006; Kim et al. 2014
Elongation factor-Tu (bacterial pathogens)	LRR-RK receptor EFR. Activates the JA and other sectors of induced immunity.	Zipfel et al. 2006; Kim et al. 2014
Chitin (fungal pathogens)	LysM-RK receptor CERK1. Predominately activates the JA sector of induced immunity.	Wan et al. 2008; Kim et al. 2014
Volictin and other fatty acid-amino acid conjugates (Lepidopteran herbivores)	Unknown receptor. Released from insect oral secretions to stimulate JATI.	Alborn et al. 1997; Halitschke et al. 2001
Inceptin (Lepidopteran herbivores)	Unknown receptor. Activates JA accumulation and associated defense responses.	Schmelz et al. 2007
Physical signals		
Electrical potentials (Mechanical tissue damage)	Glutamate-like receptors mediate systemic JA responses.	Mousavi et al. 2013
Microbial effectors		
Coronatine (*Pseudomonas syringae*)	JA-Ile analog that promotes formation of COI-JAZ co-receptor complexes and JAZ degradation.	Katsir et al. 2008b; Sheard et al. 2010
HopZ1a (*Pseudomonas syringae*)	Putative acetytransferase that promotes COI1-dependent JAZ degradation.	Jiang et al. 2013
HopX1 (*Pseudomonas syringae*)	A cysteine protease that promotes COI1-independent JAZ degradation.	Gimenez-Ibanez et al. 2014
MiSSP7 (*Laccaria bicolor*)	An effector from a mutualistic fungus that binds to and protects JAZ6 from JA/COI1-induced degradation.	Plett et al. 2014b
HaRxL44 (Downy mildew)	Promotes degradation of Mediator subunit 19a to activate JA responses and suppress SATI.	Caillaud et al. 2013

al. 1996; Hyun et al. 2008; Wasternack and Hause 2013). Alternatively, there is evidence to suggest that tissue damage may stimulate JA synthesis from an existing pool of OPDA (Koo et al. 2009). Regardless of the precise mechanism involved, a major gap in our understanding of JATI concerns the molecular events that link perception of MAMP/HAMP/DAMPs by PRRs to accumulation of JA-Ile (Figure 1.2). Among the intracellular signals implicated in this process are calcium ions, reactive oxygen species (ROS), and mitogen-activated protein (MAP) kinase cascades.

Calcium ions have long been recognized as ubiquitous second messengers in signal transduction pathways. The involvement of Ca^{2+} in JATI is supported by studies showing that cytosolic Ca^{2+} levels increase in response to herbivore feeding and treatment with exogenous MAMP/HAMP/DAMPs (Arimura and Maffei 2010; Jeter et al. 2004; Maffei et al. 2004, 2006). Changes in membrane polarization caused by wounding and insect attack also increase the level of cytosolic Ca^{2+} (Maffei et al. 2006). Ca^{2+} fluxes and associated Ca^{2+}-binding proteins, including calmodulin and Ca^{2+}-dependent protein kinases (CDPKs), exert control during the activation of JA-response genes (Bonaventure et al. 2007; Boudsocq et al. 2010; Levy et al. 2005; Romeis and Herde 2014; Yang et al. 2012a). Dynamic changes in cytosolic Ca^{2+} levels during plant-attacker interactions are tightly linked to the production of reactive oxygen species (ROS), including hydrogen peroxide (Arimura and Maffei 2010). Alterations in cellular redox status are associated with local and systemic JATI, and have been linked to the activity of the respiratory burst oxidase homolog D (RBOHD) (Miller et al. 2009; Orozco-Cárdenas et al. 2001). Direct phosphorylation of RBOHD by the PRR-associated kinase BIK1 provides a mechanism to integrate MAMP perception with calcium-based regulation of immune function (Kadota et al. 2014; Li et al. 2014).

MAP kinase signaling cascades serve a prominent role in the early steps of induced immunity (Asai et al. 2002; Schweighofer et al. 2007; Seo et al. 2007; Wu and Baldwin 2010; Zhang and Klessig 2001). Plants silenced in the expression of specific MAPKs showed reduced JA biosynthesis and decreased expression of JA-related defense genes, suggesting that these kinases control an early step in the activation of JA synthesis (Kandoth et al. 2007; Wu et al. 2007). The manner in which MAPK cascades are linked to a specific step in the JA biosynthesis pathway, however, remains unknown.

Long-distance electrical and glutamate-like receptors - One of the most fascinating and least understood areas of plant signaling concerns the mechanism by which mechanical tissue injury, including that elicited by chewing insects, results in rapid systemic changes in defense gene expression (Koo and Howe 2009). Studies in Arabidopsis, for example, show that a signal generated at the site of leaf injury travels rapidly (2-3 cm/min) to trigger JA-Ile synthesis and associated JA responses in undamaged leaves (Glauser et al. 2008; Koo et al. 2009). Despite the importance and wide-spread occurrence of this phenomenon, the molecular and genetic basis of rapid systemic JATI signaling has being uncertain. A recent study by Mousavi et al. (2013) built on previous work showing that tissue damage results in changes in electrical activity and membrane depolarization, which are associated with activation of JA responses in systemic tissues (Mousavi et al., 2013; Wildon et al. 1992; Zimmermann et al. 2009). A screen for mutants that exhibit reduced wound-triggered changes in eletric potential showed that members of the GLUTAMATE RECEPTOR-LIKE (GLR) family of ion channel proteins are required for the response; *glr3.3* and *glr3.6* mutants are deficient in electrical activity in wounded leaves and show reduced expression of JA-responsive genes in distal undamaged leaves (Mousavi et al.

2013). Systemic depolarization events were triggered in a GLR3.3/GRL3.6-dependent manner by caterpillar feeding but not by caterpillar walking in the leaf (Mousavi et al. 2013; Salvador-Recatalà et al. 2014). The ability of mechanical leaf wounding and insect chewing to elicit comparable changes in electrical activity indicates that insect-derived factors are not required for this response. Current evidence thus indicates that insect feeding generates long-distance electrical signals through the action of GRLs, and that decoding of this signal in systemic responding leaves results in JA-Ile synthesis, JA-Ile perception via the COI1-JAZ co-receptor system and activation of defense gene expression. The JATI-eliciting electrical signal thus has all the hallmarks of a DAMP (Figure 1.2) The mechanism by which the propagating signal is perceived and subsequently linked to JA biosynthesis remains to be determined. However, there is evidence to suggest that calcium ions may be involved in propagating and/or interpreting the signal in responding target cells (Felle and Zimmermann 2007; Maffei et al. 2006; Qi et al. 2006) and a role for RBOHD-dependent ROS production was excluded (Mousavi et al. 2013).

Negative Regulation of JATI

Although JATI confers effective resistance to a broad spectrum of pathogens and herbivores, hyperactivation of the pathway can negatively affect plant growth and fitness (See Chapter 3). Many specialized defense compounds, for example, are toxic to the plant that produces them (Baldwin and Callahan 1993; Gog et al. 2005). In addition, increased allocation of limited metabolic resources to defense compounds may reduce the extent to which these resources can be used to fuel plant growth and reproduction (Agrawal 1999; Baldwin 1998; Herms and Mattson 1992; Yan et al. 2007; Zhang and Turner 2008). JATI may, therefore, provide a cost-saving strategy to coordinate the timing of chemical defense production with perceived threats

from the environment. Until recently, relatively little attention has been paid to understanding mechanisms that restrain defense signaling pathways or desensitize plant cells to the presence of eliciting signals. As described below, molecular studies have elucidated several JA-induced negative feedback loops within the core JA signaling module.

Catabolism of JA-Ile – The dependence of JATI on intracellular accumulation of JA-Ile suggests that turnover of the hormone could provide a mechanism to attenuate JA responses. Initial support for this hypothesis came from studies showing that various oxidized and conjugated derivatives of JA-Ile accumulate in wounded leaves (Glauser et al. 2008; Miersch et al. 2008; Paschold et al. 2008). Recent studies have employed genetic approaches to elucidate two metabolic routes for JA-Ile catabolism, referred to here as the JA-Ile ω-oxidation and hydrolysis pathways (Figure 1.2). The latter pathway is catalyzed by aminohydrolases that cleave JA-Ile to JA and Ile (Bhosale et al. 2013; Widemann et al. 2013; Woldemariam et al. 2012;). This reaction is readily reversible by the JA-conjugating enzyme JAR1 (Staswick and Tiryaki 2004), suggesting that the relative level of conjugating and aminohydrolase activity is an important factor in the control of JA-Ile homeostasis. In contrast to JA-Ile hydrolysis, the ω-oxidation pathway provides a mechanism for permanent inactivation of JA-Ile. This pathway involves at least two members of the CYP94 family of cytochromes P450 (CYP94B3 and CYP94C1) that oxidize the ω-carbon of JA-Ile to produce 12OH-JA-Ile, which is further oxidized to 12COOH-JA-Ile (Heitz et al. 2012; Kitaoka et al. 2011; Koo et al. 2011). 12OH-JA-Ile is less active than JA-Ile in promoting COI1 binding to JAZ proteins (Koo et al. 2011). The fact that 12OH-JA-Ile retains some activity in COI1-JAZ interaction assays, however, suggests that CYP94-mediated

oxidation of 12OH-JA-Ile to 12COOH-JA-Ile, or conjugation of 12OH-JA-Ile to other small molecules (Gidda et al. 2003; Kitaoka et al. 2014), is required for complete inactivation of JA-Ile.

Consistent with a role in negative feedback regulation of JATI, genes encoding enzymes in both the ω-oxidation and hydrolysis pathways are rapidly induced in response to wounding, herbivory, and JA treatment (Bhosale et al. 2013; Heitz et al. 2012; Kitaoka et al. 2011; Koo et al. 2011; Widemann et al. 2013; Woldemariam et al. 2012). Remarkably, Bhosale et al. (2013) found that the *IIL6* gene encoding a JA-Ile aminohydrolase in Arabidopsis is co-expressed with other JA-response genes in plants grown under tightly controlled growth conditions in which stress treatments were not intentionally imposed. This finding highlights the exquisite sensitivity of JA-associated surveillance and response systems, and suggests a broader role for JA signaling in modulating phenotypic plasticity in response to subtle changes in the environment. It can be anticipated that future research will uncover mechanisms by which JA responses are integrated with various environmental perturbations, including changes in light, water status, nutrient availability, soil microbe communities and wind/touch (*e.g.* Chehab et al. 2012).

Stable JAZ proteins – A hallmark of most *JAZ* genes is rapid and strong expression in response to exogenous JA or stress-induced accumulation of endogenous JA (Chung et al. 2008; Chini et al. 2007; Thines et al. 2007; Yan et al. 2007). This pattern of expression suggests that *de novo* synthesis of JAZ proteins is part of a negative feedback system to desensitize cells to the presence of the hormone. Such a mechanism of feedback control, however, would depend on the existence of JAZ proteins that are relatively stable in presence of JA-Ile. Whereas initial studies demonstrated that some JAZ proteins (e.g., JAZ1) are rapidly degraded ($t_{1/2} < 2$ min) in the presence of JA-Ile (Chini et al. 2007; Grunewald et al. 2009; Pauwels et al. 2010; Thines et al.

2007), recent studies have advanced the concept that other JAZs exhibit a wide range of stability, which could allow fine-tuning of TF activity in response to fluctuating JA-Ile levels (Chung and Howe 2009; Chung et al. 2009, 2010; Shyu et al. 2012).

The conserved Jas motif of JAZ proteins contains a degradation signal (degron) that binds COI1 in a JA-Ile-dependent manner (Katsir et al. 2008b; Melotto et al. 2008; Sheard et al. 2010; Yan et al. 2007). Point mutations within the degron disrupt JAZ-COI1 interaction without affecting JAZ binding to TFs, thereby stabilizing and enhancing the activity of the repressor (Melotto et al. 2008; Withers et al. 2012). Natural sequence variation within degron also affects JAZ stability and associated physiological outputs of JATI (Shyu et al. 2012). JAZ8, for example, contains a non-canonical degron that evidently does not interact with COI1 in the presence of JA-Ile. As a consequence, JAZ8 maintains the ability to interact with target TFs and repress transcription through recruitment of a co-repressor complex. Stress-induced expression of JAZ8 may thus provide a mechanism to desensitize cells to the presence of JA-Ile (Shyu et al. 2012).

JAZ repressors are also stabilized by alternative splicing (AS) events that remove or modify the Jas motif and its associated degron. AS of *JAZ10* pre-mRNA produces several splice variants that differentially interact with COI1 in the presence of JA-Ile. These isoforms of JAZ10 exhibit a range of stability in JA-stimulated cells and, when overexpressed *in planta*, attenuate JA signal outputs to varying degrees (Chung and Howe 2009; Chung et al. 2010; Moreno et al. 2013). A direct role for *JAZ10* AS in negative feedback control of JA signaling is supported by the JA-hypersensitive phenotype of *jaz10* null mutants, as well as the ability of specific JAZ10 splice variants to complement the hypersensitive phenotype of *jaz10* mutants (Cerrudo et al. 2012; Demianski et al. 2012; Moreno et al. 2013; Yan et al. 2007) (See Chapter 2). The AS event responsible for generating the stable JAZ10.3 isoform involves retention of an intron whose

location within the Jas motif results in truncation of the C-terminal end of the motif. Interestingly, this intron is present in most *JAZ* genes from phylogenetically diverse land plants, suggesting that this conserved AS event provides a general mechanism to desensitize cells to the presence of high JA-Ile levels (Chung et al. 2010). It remains to be determined how stable JAZ repressors are removed from cells in order to reset full sensitivity of the JA response.

Transcriptional JAMming – A third mechanism to negatively regulate JA responses involves a phylogenetic clade of bHLH-type proteins (JAM1/bHLH017, JAM2/bHLH013, JAM3/bHLH003) that is closely related to the positively acting MYC2 TF and its functional paralogs, MYC3, and MYC4 (Fonseca et al. 2014; Nakata et al. 2013; Sasaki-Sekimoto et al. 2013; Song et al. 2013). JAM proteins compete with MYC2 for binding to *cis*-acting G-box elements within the promoters of JA-responsive genes. However, because they lack the conserved activation domain found in MYC2/3/4, JAMs function as transcriptional repressors rather than activators. JAM TFs also interact directly with JAZ proteins, which may serve to increase the strength of transcriptional repression through recruitment of the co-repressors NINJA and TOPLESS (Fonseca et al. 2014; Song et al. 2013). Similar to other negative feedback loops, the expression of *JAM1* is strongly upregulated by JA treatment and associated stress responses (Fonseca et al. 2014; Nakata et al. 2013; Sasaki-Sekimoto et al. 2013; Song et al. 2013).

Other modes of negative regulation – The multiple negative feedback loops described above likely act in concert to restrain the amplitude and duration of JATI after the response is initiated. It should be noted, however, that the onset of JATI could be actively suppressed by other signals

when the benefit of growth outweighs the cost of defense. A compelling example is repression of JATI during the shade avoidance response in which changes in light quality, as perceived by the photoreceptor phytochrome B, modulates the stability of MYC TFs and JAZs to prioritize elongation growth over defense (Ballaré 2014; Cerrudo et al. 2012; Chico et al. 2014; Moreno et al. 2009; Izzaguirre et al. 2013). Recent studies have also provided insight into the mechanisms by which JATI is suppressed by the growth-related hormones gibberellic acid (Hou et al. 2010; Yang et al. 2012b) and ethylene (Kim et al. 2014; Song et al. 2014), as well as other transcriptional regulators whose mode of action is just beginning to be understood (Hu et al. 2013b).

Manipulation of JATI by plant-associated organisms

The efficacy of any given immune system is often reflected by the extent to which host-associated organisms evolved to evade that response. Consistent with its role in re-directing primary and secondary metabolism, perhaps it is not surprising that plant pathogens and herbivores evolved strategies to manipulate (activate or suppress) JATI. Current views on this topic are influenced by the notion that JATI and SATI are often mutually antagonistic (Caillaud et al. 2013; Kunkel and Brooks, 2002; Robert-Seilaniantz et al. 2011; Thaler et al. 2012). Studies performed with Arabidopsis, for example, have led to the generalization that increased activity of the JA sector of immunity enhances the virulence of biotrophic pathogens that are sensitive to SATI, whereas expression of SATI favors the performance of insect herbivores and necrotropic pathogens that are more sensitive to JATI. Compelling evidence for JATI-SATI antagonism comes from studies showing that many plant-associated organisms use effector-based strategies to create JA-SA imbalances that suppress JATI (Table 1.2).

An important emerging paradigm in plant-herbivore interactions is the ability of herbivores to activate the SA pathway and thereby reduce the effectiveness of JATI as a basal defense (Hogenhout and Bos 2011; Walling, 2008). For example, phloem feeding by silverleaf whitefly (*Bemisia tabaci*) results in increased expression of SA-related defense genes and concomitant repression of JATI (Zarate et al. 2007; Zhang et al. 2013). Similarly, insect egg-associated effectors trigger SA accumulation and JATI suppression in host tissues surrounding the egg, thus favoring the survival of newly hatched larvae (Bruessow et al. 2010; Reymond 2013). Secretion of SA into the locomotion mucus (slime trail) by some molluskan herbivores (Kästner et al. 2014), or excretion of SA into honeydew by some aphid species (Schwartzberg and Tumlinson 2013), may reflect additional mechanisms to suppress JATI. The Coleopteran herbivore *Leptinotarsa decemlineata* (Colorado potato beetle) employs an alternative but no less effective strategy to hijack JATI (Chung et al. 2013). Symbiotic bacteria in the oral secretion of the beetle activate SA-dependent responses and repress local and systemic JATI. That this phenomenon also occurs in a root-feeding insect herbivore (*Diabrotica* virgifera, western corn rootworm) of maize suggests that host defense suppression by symbiotic bacteria may be a general feeding strategy adopted by insect herbivores (Barr et al. 2010).

Studies of the Arabidopsis-*Pseudomonas syringae* strain DC3000 (*Pst* DC3000) pathosystem have provided considerable insight into how bacterial pathogens manipulate JA-SA antagonism to their own advantage. In this system, immunity to *Pst* DC3000 is mediated in large part by SATI. Interestingly, *Pst* DC3000 uses multiple effectors to activate JA responses through targeted destruction of JAZ proteins, which in turn suppresses SATI (Figure 1.2). One well-studied effector is the polyketide coronatine (COR) that acts as a potent agonist of the COI1-JAZ co-receptor system (Bender et al. 1993; Katsir et al. 2008b; Sheard et al. 2010). COR-induced

degradation of JAZ repressors strongly upregulates the expression of JA-responsive defense genes and downregulates growth-related genes, and impairs multiple aspects of SATI (Attaran et al. 2014; Brooks et al. 2005; Melotto et al. 2006; Uppalapatti et al. 2007; Zhao et al. 2003; Zheng et al. 2012;). Suppression of SATI by COR is mediated in part by NAC-type TFs that concomitantly repress the expression of the key SA biosynthetic enzyme ICS1 and activate expression of a methyltransferase (BSMT1) that converts SA to volatile MeSA (Attaran et al. 2009, 2014; Zheng et al. 2012). That release of MeSA is also observed in other plant-enemy interactions (Dempsey et al., 2011) suggests that JA-induced disposal of SA through volatilization of MeSA may be a general mechanism to antagonize SATI by stresses that trigger JA signaling.

Pseudomonas syringae strains produce at least two type III secreted protein effectors that also promote degradation of JAZ proteins to increase pathogenicity. HopZ1a is a putative acetyltransferase that modifies JAZ proteins to stimulate their degradation in a COI1-dependent manner (Jiang et al. 2013). HopX1 is a cysteine protease that destroys JAZs independently of COI1 (Gimenez-Ibanez et al. 2014). Interestingly, HopX1 is produced by a strain of *P. syringae* that does not synthesize COR, indicating that distinct mechanisms to activate JA signaling through proteolytic destruction of JAZs have arisen independently in the evolution of this pathogen (Gimenez-Ibanez et al. 2014). These findings are consistent with results of large-scale protein-protein interaction screens showing that JAZs are targets of effectors from both *P. syringae* and the obligate biotrophic oomycete *Hyaloperonospora arabidopsidis* (Mukhtar et al. 2011). In contrast to other biotrophic organisms, colonization of host tissues by some mutualistic ectomycorrhizal fungi is inhibited by JATI (Plett et al. 2014a). A recent study showed that the ectomycorrhizal fungus *Laccaria bicolor* produces an effector (MiSSP7) that binds to and

stabilizes a host JAZ protein to repress JA responses that presumably inhibit establishment of the symbiosis (Plett et al. 2014b). These collective studies highlight the COI1-JAZ co-receptor system as a central hub of plant immunity and portend the discovery of additional effectors from other plant-associated organisms that target the core JA module.

Summary and future perspectives

Recent research on many fronts has tremendously advanced our understanding of the mechanism of JA signaling and its relationship to induced plant immunity. These efforts have coalesced around a simple model (Figure 1.2) to explain how fluctuating levels of a small-molecule hormone (JA-Ile) exert transcriptional control over complex morphological and chemical defense traits. We suggest that the modular structure of JATI allows the conserved core JA module to link different combinations of PRR-based recognition systems (inputs) and defense traits (outputs) to create new specificities of host resistance. Indeed, there is a good evidence that JATI is a significant driving force in shaping plant-animal associations in natural environments (Kallenbach et al. 2012; Züst et al. 2012). This conceptual framework provides a foundation for studies aimed at understanding the underlying mechanisms by which recognition-response systems give rise to phenotypic plasticity, and for revealing how interactions between the environment and the genome have spawned highly diverse, idiosyncratic defense traits in the plant kingdom. Meeting this challenge will require integrative approaches spanning the ecosystems-to-gene continuum, as applied to experimental systems that offer both genomic and ecological resources.

With the exception of a few model plants, remarkably little is known about the identity of JA-regulated compounds that provide resistance against specific attackers. It is currently unclear,

for example, whether JA/COI1-mediated resistance of Arabidopsis, tomato, and maize to soil-borne *Phythium* spp (Table 1.1) involves similar or divergent suites of defense traits. Major differences in specialized defense chemistry between these species, however, suggests that different plants use a conserved core JA module to deploy different suites of chemical defense against the same broad host-range attacker. Similarly, there is evidence that tomato and Arabidopsis use distinct JA-regulated defense chemistry for protection against the two-spotted *Tetranychus urticae* (spider mite) and *Trichoplusia ni* (cabbage looper) (Herde and Howe 2014; Li et al. 2002, 2004; Zhurov et al. 2014). The modular architecture of JATI thus appears to support the evolution, in different host plants, of independent chemical solutions to the same pathogen or herbivore, which may contribute to the diversity and sporadic distribution of secondary metabolites in higher plants (Fraekel 1959). On the other hand, there are several examples of similar defense compounds that evolved independently in diverse plant families (Berenbaum and Zangerl 2008). Modern omics-based technologies offer tremendous potential to beeter understand the evolution of constitutive and induced defense compounds by elucidating gene-pathway-metabolite relationships in diverse group of plants (Berenbaum and Zangerl 2008; Kliebenstein 2012). Insight into the evolutionary forces that drive the diversity of chemical defenses also will benefit from a better understanding of how these defense systems are matched by equally complex counter-defenses in plant attackers (Herde and Howe 2014).

It is becoming increasingly evident that the JA/COI1/JAZ/TF module is a convergence point for direct crosstalk with other signaling pathways that control growth and development (Ballaré 2014; Erb et al. 2012; Huot et al. 2014). It appears these crosstalks occur primarily through direct interaction between nuclear factors that regulate transcription, including the Mediator complex (Caillaud et al. 2013; Kidd et al. 2011). Future research aimed at

understanding changes in chromatin structure, epigenetic modification, and *cis*-regulatory codes (Zou et al. 2011) that direct TF-DNA interactions is expected to provide new insight into how transcriptional networks control complex JATI outputs, including transgenerational immunity (Rasmann et al., 2012). Various negative feedback loops act in concert to restrain JATI outputs, but whether these control mechanisms constitute an adaptive response to balance tradeoffs between JATI and growth, or perhaps other forms of immunity, remains to be determined. Knowledge of how proteins in different signaling pathways functionally interact to regulate growth-defense antagonism has potential practical application in the development of crop varieties that are both high yielding and stress tolerant (Shyu and Brutnell 2015). These efforts may be aided by mathematical models to predict how environmental inputs are integrated within phytohormone networks to generate specific physiological outcomes (Middleton et al. 2012).

A significant gap in our understanding of JATI is how recognition of a danger signal at the cell surface activates JA biosynthesis. By analogy to stress-responsive regulation of ethylene biosynthesis (Liu and Zhang 2004), identification of direct targets of the relevant MAPK cascades may provide important clues. Attention should also be given to the hypothesis that JA biosynthesis is controlled by calcium-dependent signaling events that are coupled PRR activation (Romeis and Herde 2014). Further analyses of how GLRs generate and/or propagate long-distance electrical signals will undoubtedly yield important new insights as well. A systems-level understanding of JA-Ile homeostasis, including pathways by which JAs are transported within and between cells, is ultimately needed to understand how specific TFs are controlled by thresholds and time-dependent signatures of the hormone. Finally, it should be noted that although there is molecular evidence that plant resistance to insect herbivore is mediated by

PRRs (Abuqamar et al. 2008; Prince et al. 2014; Truitt et al. 2004; Yang et al. 2011), HAMP receptors remain to be identified in any plant.

One of the most exciting recent advances in the field of induced immunity is evidence that the core JA module is a common target of effectors from multiple plant-associated microbes (Table 1.2). This finding is consistent with the idea that different pathogens independently evolved virulence effectors that converge on common host targets within the PTI network (Jones and Dangl 2006; Mukhtar et al. 2011). Only time will tell whether the current list of COI1/JAZ-targeting effectors is complete or, more likely, will continue to expand as effector repertoires from diverse plant-associated microbes, insects, and nematodes are systematically scrutinized (Boller and He 2009; Elzinga and Jander 2013; Hogenhout and Bos 2011; Kandoth and Mitchum 2013). The strong selection pressure imposed by JATI on arthropod herbivores and necrotrophic pathogens, together with evidence that these organisms actively suppress JA-based defenses, suggests the existence of novel mechanisms by which plant- associated organisms disrupt JATI. Interdisciplinary approaches aimed at understanding how the JA module promotes broad-spectrum immunity through the control of specialized metabolism, and how this branch of immunity is subverted by plant attackers, offer tremendous potential to help solve pressing problems facing the world (Plant Science Research Summit 2013). From a biotechnological perspective, for example, these efforts may inform synthetic approaches to harness specialized biochemical pathways for metabolic engineering of new chemistries for a variety of plant-based products, including pigments, fragrances, flavors, pesticides and pharmaceuticals. Given the current pace of discovery and technological tools available, exciting new discoveries may be just around the corner.

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CHAPTER TWO

Alternative splicing in *JAZ10* regulates jasmonate signaling in *Arabidopsis thaliana*

Contributions:

The constructs used to study JAZ10 alternative splicing dynamics described in this chapter (*JAZ10p:HA-JAZ10g*, *JAZ10p:HA-JAZ10.1*, *JAZ10p:HA-JAZ10.3* and *JAZ10p:HA-JAZ10.4*) were developed previously by Dr. Javier E. Moreno and Lalita C. Patel.

Abstract

Jasmonates (JAs) are essential orchestrators of plant development, controlling a myriad of growth and defense processes. A fundamental step in the activation of the JA pathway involves the JA-dependent degradation of JASMONATE ZIM-domain (JAZ) transcriptional repressors through the activity of the E_3 ubiquitin-ligase SCF^{COI1}. It is now becoming evident that events of alternative splicing in the JAZ repressors expand the repertoire of regulatory proteins modulating JA responses. Alternative splicing in the *Arabidopsis thaliana JAZ10* gene generates three protein isoforms with remarkable differences in their degree of stability upon JA-mediated degradation, but whose biological relevance is still poorly understood. We here show that two stable splice variants of *JAZ10* (*JAZ10.3* and *JAZ10.4*) are involved in attenuation of JA signaling, acting as dominant repressors to regulate the amplitude and duration of the JA responses. Complementation experiments performed with the *jaz10-1* mutant indicate that, upon induction, JAZ10.3 and JAZ10.4 are retained in plant cells for longer periods of time to downregulate JA-related processes such as the expression of defense-related genes and the JA-mediated inhibition of root elongation. This JA-desensitization is mainly performed by JAZ10.3, the most abundant protein produced from *JAZ10.* Structural analysis showed that JAZ10.3 stability is caused by a single amino acid disruption in its degron sequence. The observation that JAZ10.3-like genes are found in phylogenetically diverse plant species highlights that these stable repressors are essential for proper regulation of the JA pathway. Moreover, the observation that JAZ10 functions are not shared with other JA-stable JAZ genes highlights a degree of functional specificity among the JAZ family members. In conclusion, our results illustrate how plants utilize specific sets of JAZ repressors to precisely regulate the JA signaling pathway and optimize plant fitness under different environmental conditions.

Introduction

Jasmonates (JAs) are a class of fatty-acid derived hormones controlling diverse aspects of plant physiology. Besides its widely studied ability to promote plant defense against a plethora of environmental stresses (Campos et al. 2014; Howe and Jander 2008; Wasternack and Hause 2013), JAs also play a role in growth processes such as cell differentiation and division, seed germination, root and shoot growth, flower formation, senescence, among many others (Browse et al. 2009; Wasternack et al. 2013; Wasternack and Hause 2013). The idea that these lipid-derived molecules evolved as essential modulators of developmental plasticity is supported by its ubiquitously occurrence throughout the plant kingdom and its extensive crosstalk with other plants hormones to mediate virtually every aspect of plant biology (Ballaré 2011; Erb et al. 2012, Hamberg and Gardner 1992; Song et al. 2014; Yang et al. 2012). Thus, it is not surprising that elaborate genetic networks have evolved to regulate the JA responses in order to optimize plant fitness under different environmental conditions.

A major regulatory step in activation of JA responses involves the removal of the JASMONATE ZIM-domain (JAZ) proteins, transcriptional repressors that negatively regulate the hormone responses by binding to and inhibiting transcription factors such as MYC2 (Chini et al. 2007; Fernández-Calvo et al. 2011; Thines et al. 2007; Yan et al. 2007). Upon elicitation by environmental signals, a burst in the endogenous levels of the bioactive form of the JA, jasmonoyl-isoleucine (JA-Ile), promotes the association of the JAZ proteins with the CORONATINE INSENSITIVE1 (COI1) component of the SCF^{COI1} ubiquitin E3 ligase complex, leading to ubiquitination and degradation of the JAZ by the 26S proteasome, and further release of transcription factors (TFs) from repression to activate the JA responses (Chung and Howe 2009; Melotto et al. 2008; Xie et al. 1998). Biochemical and structural studies demonstrate that

the formation of the COI1-JAZ complex is dependent on the conserved Jas motif, located at the C-terminal end of the JAZ proteins (Katsir et al. 2008; Melotto et al. 2008; Sheard et al. 2010). A short 21 amino-acid sequence within this motif defines the "degron", the minimal peptide necessary and sufficient for COI1-JAZ physical interaction. The degron peptide adopts a bipartite loop/α-helix structure that physically traps the JA-Ile molecule inside the COI1 ligand-binding pocket to form the JA co-receptor complex (Sheard et al. 2010). Accordingly, artificially truncated JAZ variants lacking the degron sequence are unable to interact with COI1 in a JA-Ile dependent manner, therefore being resistant to degradation through the 26S proteasome (Chini et al. 2007; Thines et al. 2007; Yan et al. 2007). Plants ectopically expressing these JA-stable JAZ repressors exhibit decreased sensitivity to exogenous JA, increased susceptibility to insect feeding, improper development of reproductive organs and reduced production of secondary metabolites (Browse 2009; Chung et al. 2008; Thines et al. 2007; Yamada et al. 2012; Yan et al. 2007).

The finding that similar modifications in the JAZ degron sequence also occur naturally indicate the existence of a complex layer of regulatory mechanisms modulating JA responsiveness, whose role and contribution to plant phenotypic plasticity are still poorly understood. It was recently demonstrated that a conserved clade of JAZ proteins lacks the canonical degron sequence and weakly associate with COI1 in the presence of JA-Ile (Shyu et al. 2012; Thireault et al. 2015). Due to their stability against JA-mediated degradation, JAZ8 and JAZ13 are more stable in JA-elicited cells and are capable of repressing JA responses in the presence of high concentrations of the hormone. The occurrence of these inherently stable JAZs in phylogenetically distinct plant species implies that these degron-altered JA-stable repressors

are fundamental for proper function of the JA pathway (Pirrello et al. 2014; Shyu et al. 2012; Thireault et al. 2015; Wang et al. 2014).

Alternative splicing is a fundamental process underlying the increased cellular and functional complexity in eukaryotes. As a molecular mechanism to generate multiple mature mRNAs from a single gene, alternative splicing is the main mechanism expanding proteome diversity in complex genomes (Ben-Dov et al. 2008; Graveley 2001). Recent analysis in numerous species indicates that alternative splicing in plants is a more prevalent process than previously thought (Li et al. 2014; Mandadi and Scholthof 2015; Marquez et al. 2012; Shen et al. 2014; Thatcher et al. 2014). In *Arabidopsis thaliana*, for example, 60% of the intron-containing genes are alternatively spliced (Filichkin et al. 2010; Marquez et al. 2012). The application of high-throughput sequencing and studies using different organs, developmental stages and external conditions continues to reveal new splice variants in plant genomes (Mandadi and Scholthof 2015; Syed et al. 2012). However, examples demonstrating the function and biological relevance of different splice isoforms are still scarce. Analysis of phylogenetically diverse species indicates that alternative splicing is a common feature of the JAZ gene family (Chung et al. 2010; Pirrello et al. 2014). The importance of this process as a post-transcriptional mechanism to control JA responses is evidenced by the recurrence of splicing events that modify the Jas domain and affect JA-mediated COI1 interaction (Chung and Howe 2009; Chung et al. 2010; Moreno et al. 2013; Pirrello et al. 2014; Yan et al. 2007). The structure of numerous *JAZ* genes in different plant species includes a conserved Jas intron whose retention through alternative splicing generates proteins with a truncated Jas domain; these splice isoforms interact weakly with COI1 in the presence of JA-Ile (Chung and Howe 2009; Chung et al. 2010). Other alternative splicing events create JAZ isoforms that lack the entire JAZ degron and thus are

unable to interact with COI1 in the presence of the hormone (Chung and Howe 2009; Moreno et al. 2013; Yan et al. 2007). Together, these events generate a repertoire of JAZ variants that can possibly respond differently to dynamic range of JA concentrations. This hypothesis remains to be tested and uncovering the role of this repertoire of repressors may improve our understanding of the regulatory mechanisms controlling the JA signaling pathway and how hormone responses are finely tuned in response to changing environmental conditions.

At least nine of the 13 JAZ genes in *Arabidopsis* are subject to alternative splicing (Chung et al. 2010). *JAZ10* is the best example of how this post-transcriptional mechanism increases the functional diversity in this family. The *JAZ10* pre-mRNA is spliced to produce three protein isoforms that differ in their stability due to the varying length of their Jas domain (Figure 2.1A): JAZ10.1 carries a full-length Jas domain, strongly interacts with COI1 and, as a consequence, is rapidly degraded in the presence of low concentrations of JA-Ile (Chung and Howe 2009; Shyu et al. 2012). JAZ10.3 is generated through an intron retention event that results in a partial truncation of the Jas domain; this isoform weakly interacts with COI1 and is stable in JA-elicited plant cells (Chung and Howe 2009; Yan et al. 2007). Finally, utilization of an alternative splice donor in third exon of *JAZ10* leads to a frame-shift mutation that removes the entire Jas domain, creating a highly stable isoform (JAZ10.4) that does not interact with COI1 (Chung and Howe 2009; Moreno et al. 2013; Yan et al. 2007). Although the biochemical features of JAZ10.3 and JAZ10.4 suggest a role for these proteins in attenuation of JA responses in sensitized cells (Chung and Howe 2009; Moreno et al. 2013), the biological relevance of *JAZ10* alternative splicing remains largely unknown.

Here we describe the wound- and JA-induced accumulation of alternatively spliced *JAZ10* transcripts and their corresponding protein isoforms. We show that, although JAZ10.1 is

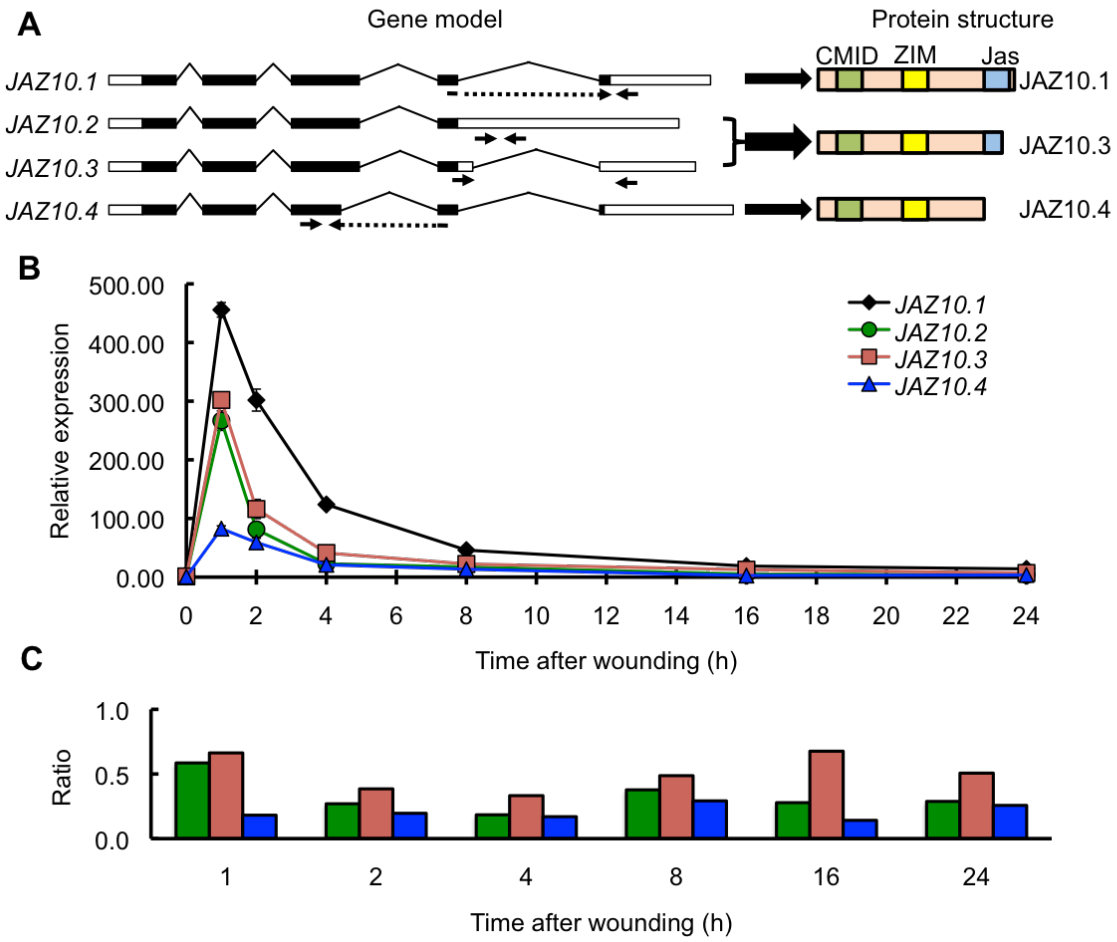

Figure 2.1. The *Arabidopsis thaliana JAZ10* gene is subjected to alternative splicing.

(A) Schematic diagram of alternative spliced *JAZ10* transcripts and their corresponding protein isoforms. White and black bars in the gene models represent UTRs and coding sequences, respectively. Splice variant-specific primers used for qRT-PCR experiment are denoted as arrows below gene models. Dotted lines indicate primers that span a splice junction. The cryptic MYC2-interacting domain (CMID), ZIM and Jas domains are represented, respectively, by green, yellow and blue boxes in the protein structure.
(B) Expression of *JAZ10* splice variants in response to mechanical wounding. Wounded leaves were collected at various time points after wounding. Data was normalized to JAZ10.1 levels at the 0 h time point (relative expression). As controls, leaves were harvested immediately before the start of the experiment ("0"). Bars denote mean ± S.D. of three biological replicates with three technical replicates each.
(C) Ratio of *JAZ10.2*, *JAZ10.3* and *JAZ10.4* over *JAZ10.1* showed by green, red and blue bars, respectively. Values were obtained using the expression data shown in (B).

the most abundant transcript produced from splicing of the JAZ10 pre-mRNA, JAZ10.3 is the most abundant protein isoform to accumulate in response to elicitation. We also describe a genetic complementation system to assess the relative contribution of each JAZ alternatively splicing variant in various JA-mediated physiological responses, and we use this assay to demonstrate that other stable JAZ repressors are not functionally equivalent to JAZ10. Finally, we provide insight into the molecular mechanism by which the abundant JAZ10.3 isoform represses JA signaling, and propose that this mechanism of alternative splicing-induced JAZ stabilization is generally conserved throughout the plant kingdom.

Results

Expression dynamics of *JAZ10* splice variants in response to wounding

As is the case for most *JAZ* genes in Arabidopsis, *JAZ10* is transcriptionally activated in response to tissue damage and other stimuli that trigger the biosynthesis of JA-Ile (Chung et al. 2008; Demianski et al. 2012; Mousavi et al. 2013; Yan et al. 2007). We used quantitative Real-Time PCR (qRT-PCR) to determine the expression pattern and relative abundance of each of the alternatively spliced *JAZ10* transcripts in local (damaged) and systemic (undamaged) leaves of wounded plants (Figures 2.1 and 2.2). Wounding induces a rapid but transient increase in all four *JAZ10* transcripts in locally damaged leaves. The level of each transcript peaked one hour after wounding and, at this point, was at least 100-fold greater than that in unwounded leaves (Figure 2.1B). *JAZ10.1* was consistently the most abundant (~50% of total *JAZ10*-derived mRNA) of the alternatively spliced transcript at each time point analyzed. The level and time-dependent pattern of accumulation of *JAZ10.2* and *JAZ10.3* were remarkably similar to each other, and together accounted for 40-45% of total *JAZ10* transcripts. *JAZ10.4* was the least abundant transcript,

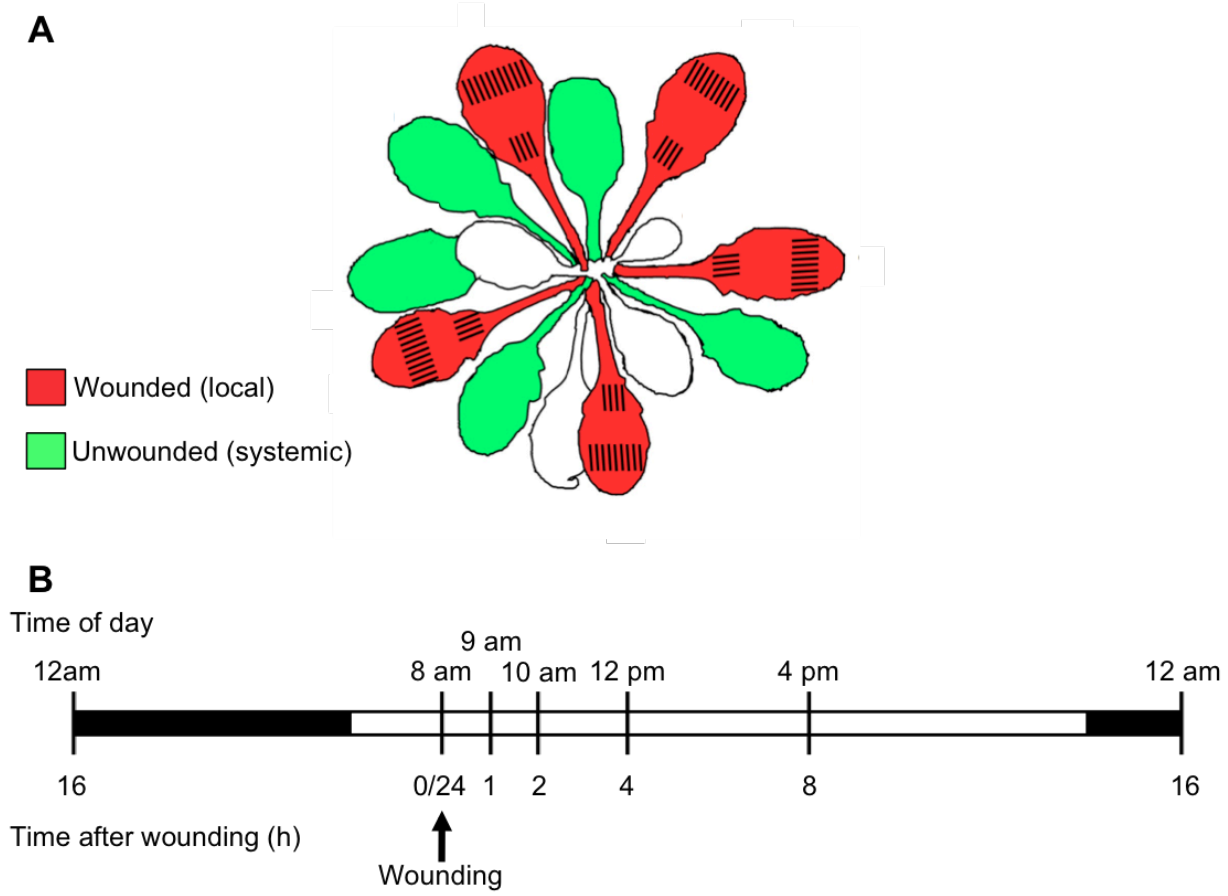

Figure 2.2. Schematic representation of the wounding experiment.

(A) Four week-old *Arabidopsis* plants were mechanically wounded with a hemostat. Wounded leaves (red) were selected based on their position in the stem. Non-wounded (systemic) leaves (green) were also harvested.
(B) Timeline for the wounding experiment. Time of day and time when tissue was collected (and wounding performed) are depicted. White and black bars in the timeline indicate light and dark periods, respectively. Three plants were pooled for each time point and the experiment was repeated three independent times with similar results.

accounting for only 5 to 10% of total *JAZ10* mRNA. Consistent with the general pattern of expression shown in Figure 2.1B, the relative proportion of the four *JAZ10* transcripts remained constant during the time course (Figure 2.1C), suggesting that the alternative splicing of *JAZ10* pre-mRNA is likely a non-regulated process. The temporal dynamics and relative abundance of *JAZ10* transcripts in undamaged leaves of wounded plants (systemic response) was remarkably similar to the local response, with the exception that the absolute level of *JAZ10* mRNA is systemic leaves was ~10-fold less than that in wounded leaves (Figure 2.3)

To further test the hypothesis that alternative splicing of *JAZ10* pre-mRNA is a non-regulated process, we quantified the relative expression level of splice variants in flowers and roots of a transgenic line (*35S:JAZ10G*) that expresses a genomic copy of *JAZ10* from the constitutive 35S promoter of Cauliflower Mosaic Virus (CaMV) (Chung et al., 2010). In good agreement with the analysis of wounded leaves, the results showed that in both roots and flowers, *JAZ10.1* and *JAZ10.4* were the most and least abundant, respectively, *JAZ10* transcript, whereas the combined level of *JAZ10.2* and *JAZ10.3* was comparable to *JAZ10.1* (Figure 2.4). Collectively, these results indicate that, regardless of the level of pre-mRNA expression, tissue type and mode of induction, alternative splicing of *JAZ10* pre-mRNA results in the production of four splice variants whose relative proportion remain constant.

Dynamics of JAZ10 protein splice variant accumulation in response to wounding

JAZ10 splice variants differ from each other in the length of the Jas domain, which harbors the COI1-interacting degron sequence that mediates JA-dependent degradation of JAZ proteins (Chung and Howe 2009; Moreno et al. 2013). A second major factor influencing JAZ10 protein accumulation is JA-dependent transcriptional activation of the *JAZ10* gene. Moreover, since

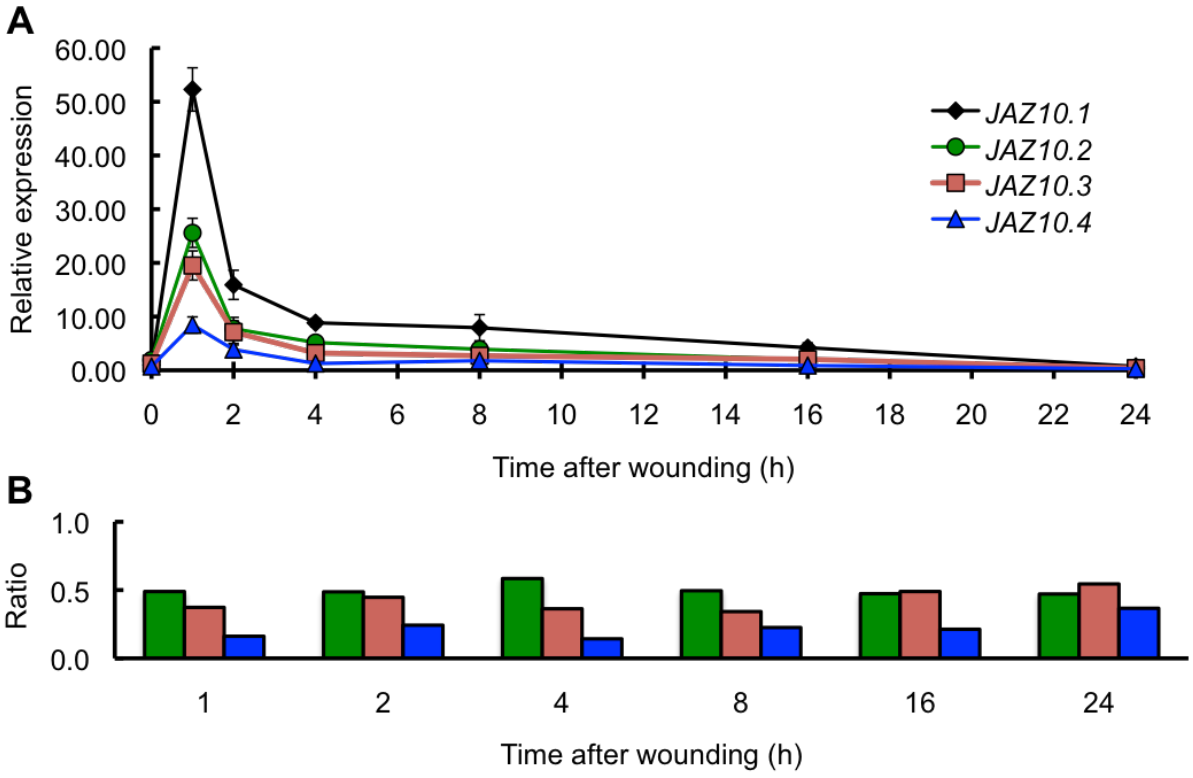

Figure 2.3. Wounding systemic expression of *JAZ10* transcripts.

(A) Unwounded (systemic) leaves were harvested for RNA extraction at the indicated times after wounding. Transcript levels were quantified by qRT-PCR as described above. Bars denote mean ± S.D. of two biological replicates with three technical replicates each.
(B) Ratio of *JAZ10.2*, *JAZ10.3* and *JAZ10.4* over *JAZ10.1* showed by green, red and blue bars, respectively. Values were obtained using the expression data shown in (A).

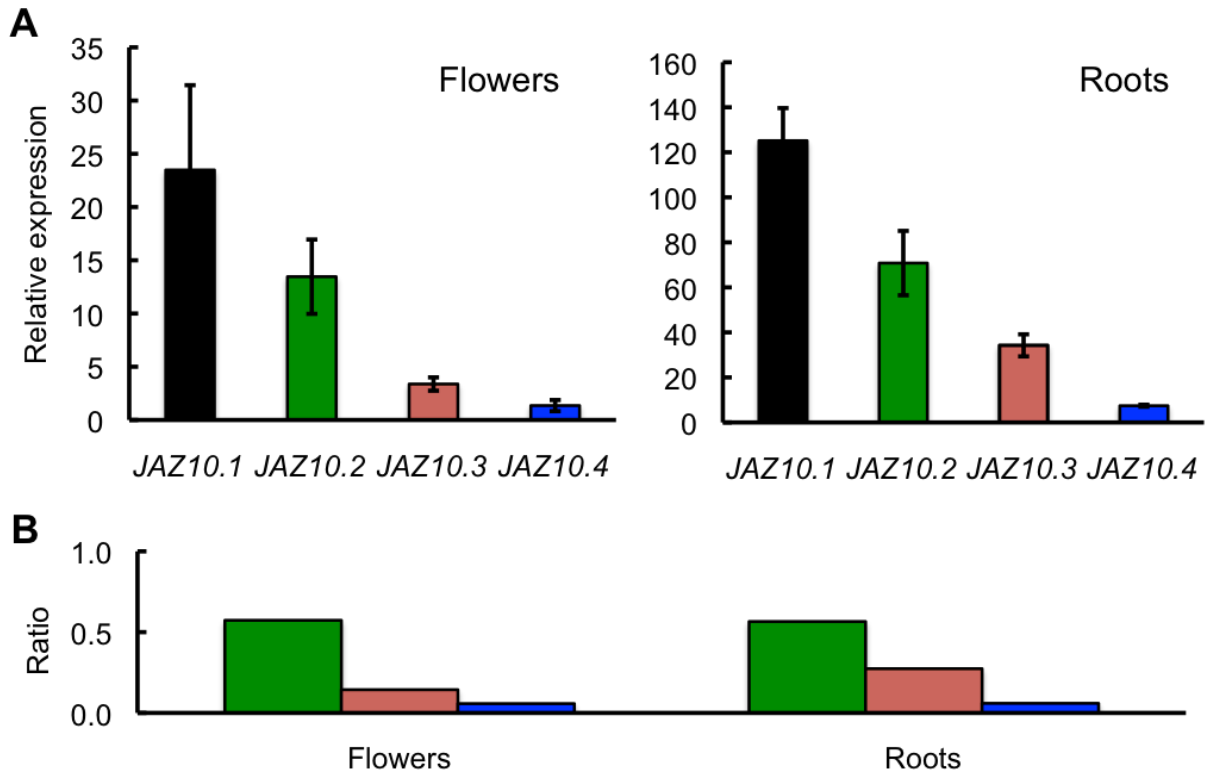

Figure 2.4. Quantification of *JAZ10* transcripts in flowers and roots of *35S:JAZ10G* transgenic plants.

(A) Relative expression of *JAZ10* splice variants in flowers and roots of transgenic plants overexpressing the JAZ10 genomic sequence from the *35S* promoter (*35S:JAZ10G*). Data was normalized to reference genes and bars denote mean ± S.D. of three biological replicates with three technical replicates each.
(B) Ratio of *JAZ10.2*, *JAZ10.3* and *JAZ10.4* over *JAZ10.1* in different tissues is showed by green, red and blue bars, respectively. Values were obtained using the expression data shown in (A).

alternative splicing can lead to selective degradation of transcripts through non sense-mediated mRNA decay (Wang and Brendel 2006), it is unclear whether all alternatively spliced JAZ10 transcripts are indeed translated. To clarify these questions, we fused a 2.0 kb *JAZ10* promoter fragment (*JAZ10p*), which is sufficient to confer JA-inducibility to a reporter gene (Moreno et al. 2013; Sehr et al. 2010), to the genomic sequence of *JAZ10* (*JAZ10g* – Figure 2.5A). Placement of a hemagglutinin (HA) epitope tag at the N-terminus of the protein allowed detection of all JAZ10 protein splice isoforms that share same N-termini but differ in the length of the C-terminus (Figure 2.5B). The resulting *JAZ10p:HA-JAZ10g* transgene was transformed into the *jaz10-1* mutant (Demianski et al. 2012) for production of stable transgenic lines. Immunoblot experiments performed with protein extracts from wounded leaves of such line showed that that all three JAZ10 protein isoforms accumulate within 1h of mechanical wounding, with protein levels peaking at the 2h time point (Figure 2.6A). Although *JAZ10.1* was the most abundant transcript in wounded leaves (Figure 2.1B), we found that the corresponding JAZ10.1 protein was significantly less abundant than JAZ10.3 throughout the time course. At the 16-h time point, for example, the JA-stable isoforms JAZ10.3 and JAZ10.4 were both detected whereas JAZ10.1 was not. All three variants were undetectable at the 24 h time point, indicating the existence of mechanisms to efficiently remove even those isoforms (e.g. JAZ10.3) that are stable in the presence of JA.

To further assess the expression dynamics of JAZ10 splice variants in wounded leaves, we also expressed individual HA-tagged *JAZ10* cDNAs (*JAZ10.1, JAZ10.3* or *JAZ10.4*) from the native *JAZ10* promoter in the *jaz10-1* mutant background (Figure 2.5A). Wounding experiments performed with the resulting in *JAZ10p:HA-JAZ10.1*, *JAZ10p:HA-JAZ10.3* and *JAZ10p:HA-JAZ10.4* lines showed a similar trend as observed in the *JAZ10p:*HA-*JAZ10g*. For example,

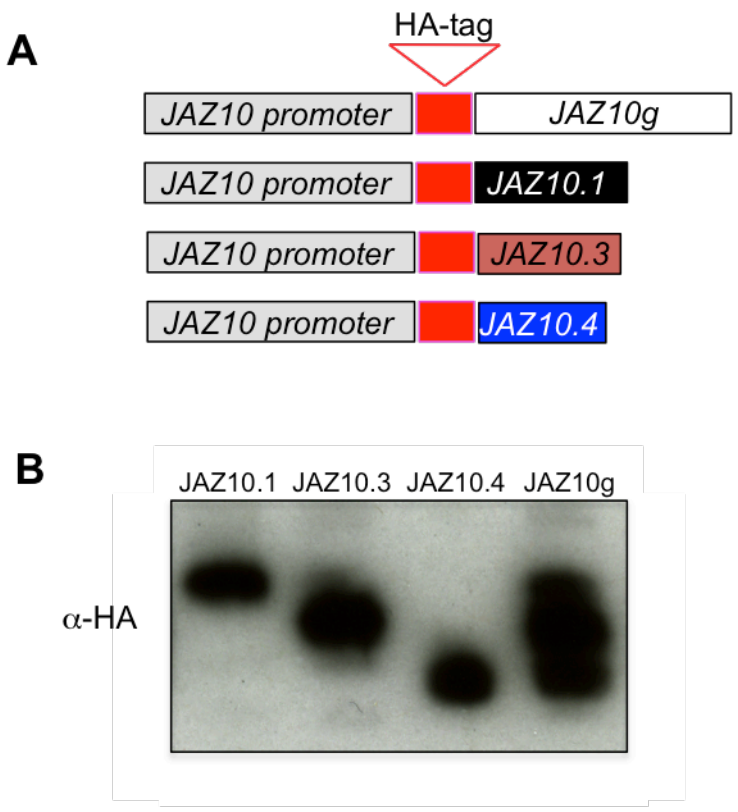

Figure 2.5. Constructs used to study protein dynamics of *JAZ10* splice variants *in planta*.

(A) Schematic representation of the constructs used to study protein dynamics. A 2 kb region comprising the *JAZ10* promoter was fused to the genomic sequence of *JAZ10* (*JAZ10g*) or the cDNA of *JAZ10.1*, *JAZ10.3* and *JAZ10.4*. The hemagglutinin (HA)-tag sequence used for immunoblot detection is shown in red. Constructs were used to transform the *jaz10-1* mutant (see Methods).
(B) Tricine-SDS-PAGE gels (Schägger, 2006) were used to resolve JAZ10 protein splice variants. Samples were obtained from leaf tissue of *jaz10-1* plants transformed with constructs in (A), one hour after mechanical wounding. JAZ10 splice variants were detected with an α-HA antibody.

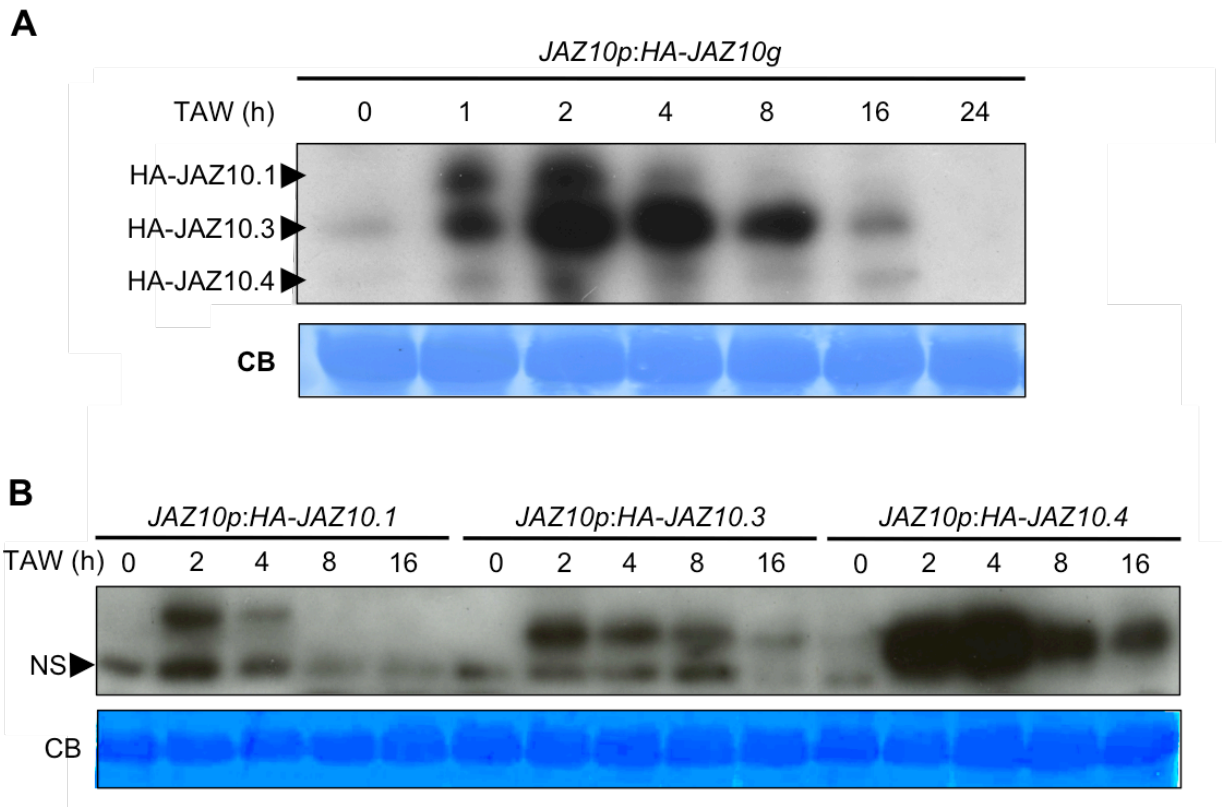

Figure 2.6. Dynamics of JAZ10 splice variant accumulation in response to mechanical wounding.

(A) Accumulation of JAZ10 protein variants in response to mechanical wounding. Rosette leaves of *JAZ10p:HA-JAZ10g* in the *jaz10-1* background were mechanically wounded with a hemostat. Wounded leaves were harvested for protein extraction at the indicated time after wounding (TAW). As a control, leaves were harvested immediately prior to wounding (0). A Coomassie blue-stained membrane (CB) is shown as a loading control.
(B) Wound induced accumulation of JAZ10 protein in lines expressing individual splice isoforms (*JAZ10p*:HA-*JAZ10.1*, *JAZ10p*:HA-*JAZ10.3* and *JAZ10p*:HA-*JAZ10.4*). A Coomassie blue-stained membrane (CB) is shown as a loading control. TAW, Time after wounding; NS, Non-specific band.

JAZ10.1 peaked at 2 h post wounding and then declined to undetectable levels at the 8 h time point (Figure 2.6B). Conversely, the JA-stable variants JAZ10.3 and JAZ10.4 were also rapidly induced, but were retained for the duration (16 h) of the time course. The high level of wound-induced JAZ10.4 accumulation in *JAZ10p:HA-JAZ10.4* relative to that in the *JAZ10p:HA-JAZ10g* lines likely reflects the absence, in the formed line, of alternative splicing control mechanisms that limit the production of JAZ10.4-encoding mRNAs, as observed for the endogenous JAZ10 gene (Figure 2.6A) (Moreno et al. 2013).

Taken together these results indicate that, upon induction by mechanical wounding, all three proteins splice variants of JAZ10 are rapidly (<1h) and transiently produced. However, differences in protein stability dictated by the COI-interacting Jas motif differentially affect the stability of each isoform, thus leading to the accumulation of the more stable isoforms. The combined effect of transcript abundance and protein stability make JAZ10.3 the most abundant of the JAZ10 splice variant protein in wounded leaves.

Stable isoforms of JAZ10 complement the JA-hypersensitive phenotype of *jaz10-1*

Overstimulation of the JA signaling pathway is associated with fitness costs, including inhibition of growth processes (Baldwin 1998; Yan et al. 2007; Yang et al. 2012; Zhang and Turner 2008). Thus, it is reasonable to hypothesize that mechanisms may exist to desensitize cells to JA or to and restrain the duration and amplitude of JA responses. The JA-hypersensitive phenotype of the *jaz10-1* mutant further suggests that one or more JAZ10 splice variants may be involved in attenuation of JA responses. To test this hypothesis we first compared the root growth phenotype of the above-described *JAZ10p:HA-JAZ10g* to *jaz10-1* (Demianski et al. 2012). When grown for eight days on Murashige and Skoog (MS) medium supplemented with

20 μM of methyl-JA (MeJA), the root length of independent *JAZ10p*:HA-*JAZ10g* transgenic lines was significantly longer than that of *jaz10-1* seedlings but comparable to WT seedlings, indicating that *JAZ10p:HA-JAZ10g* complements the JA-hypersensitivity of *jaz10-1* roots (Figure 2.7A). To evaluate which splice variant is responsible for this effect, we next tested the ability of each individual JAZ10 splice variant to complement the JA-hypersensitive root growth phenotype of *jaz10-1*. We found that the *JAZ10p:HA-JAZ10.3* and *JAZ10p:HA-JAZ10.4* transgenes but not *JAZ10p*:HA-*JAZ10.1*, reduce the sensitivity of *jaz10-1* roots to exogenous MeJA (Figure 2.7A). This finding supports the hypothesis that JA-induced expression of the stable JAZ10.3 and JAZ10.4 isoforms play a role in attenuation of JA responses, and further indicate that the JA-hypersensitive phenotype of *jaz10-1* is caused by the elimination of these two splice variants.

We used immunoblot analysis to determine whether the ability of JAZ10 splice variants to attenuate JA-induced root growth inhibition correlates with the accumulation of the splice variants in JA-elicited plants (Figure 2.7B). For this purpose, total protein extracted from eight day-old seedlings grown either in the presence or absence of MeJA was subjected to western blot analysis with an anti-HA antibody. The results showed that seedlings grown continuously in the presence of MeJA accumulate the stable JAZ10.3 and JAZ10.4 protein variants but do not accumulate detectable levels of JA-labile isoform JAZ10.1. The absence of JAZ10.1 signal in the immunoblots is likely a consequence of the strength of interaction of this variant with COI1 and the prolonged exposure of seedlings (8 d) to MeJA treatment. Together, these results establish a causal relationship between the accumulation of specific JAZ10 splice variants and the attenuation of JA responsiveness.

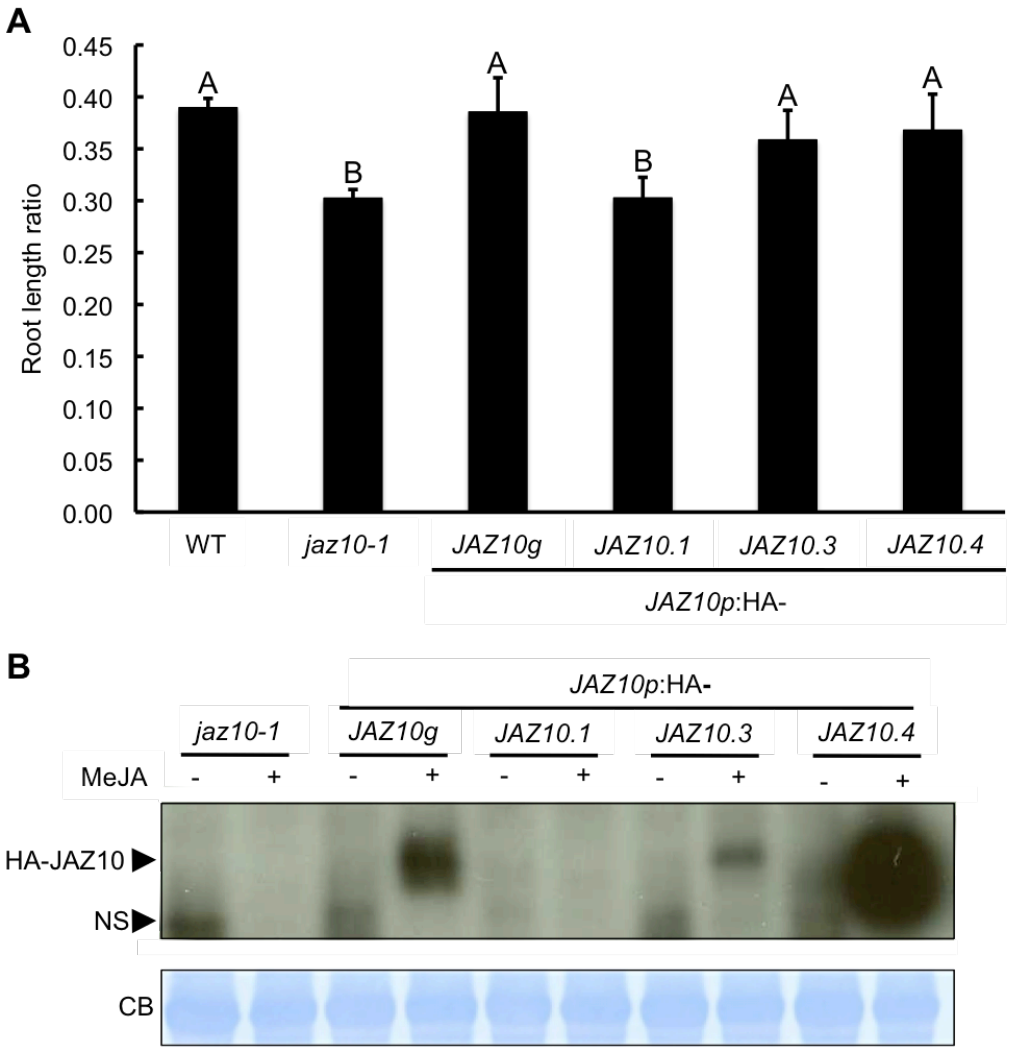

Figure 2.7. Stable splice variants of JAZ10 complement the JA-hypersensitive root growth phenotype of *jaz10-1*.

(A) MeJA-induced root growth inhibition assay of wild type (WT), *jaz10-1*, and *jaz10-1* lines transformed with the indicated transgenes. Root length was measured in eight-day old seedlings grown in MS medium supplemented or not with 20μM MeJA. The root length ratio was calculated by dividing the average root length of seedlings grown on medium containing MeJA by the average root length of the same genotype grown in MS medium lacking MeJA. Data show the mean ± S.E. of at least ten seedlings for WT and *jaz10-1* and >40 seedlings per transgenic line. Letters denote a statistical difference in comparison to WT control (Tukey's HSD, *p*-value <0.05). A minimum of seven independent lines were used to obtain the average shown for each transgenic line.
(B) Accumulation of JAZ10 protein isoforms in seedlings of WT, *jaz10-1* and *jaz10-1* transgenic lines eight days after grown on the MS medium lacking (-) or containing (+) of 20μM MeJA. Coomassie blue (CB) stained membrane is shown as a loading control. NS, non-specific band.

We used qRT-PCR to investigate the role of JAZ10 splice variants in attenuating the wound-induced expression of primary JA-response genes *LIPOXYGENASE3* (*LOX3*) and *12-OXO-PHYTODIENOIC ACID REDUCTASE3* (*OPR3*) in leaves of adult plants (Chung et al. 2008). Initial comparisons between WT and *jaz10-1* plants showed that the latter genotype hyper-accumulates *LOX3* and *OPR3* transcripts in wounded leaves (Figure 2.8). This finding is consistent with previous studies of a *jaz10* mutant generated by RNAi silencing (Yan et al. 2007). Next we analyzed *LOX3* and *OPR3* expression in the same transgenic lines used for the root growth assays. The results showed that *JAZ10p:HA-JAZ10g* and *JAZ10p:HA-JAZ10.3* restore wound-induced marker gene expression to levels seen in WT plants. Wound-induced expression of both *LOX3* and *OPR3* in *JAZ10p:HA-JAZ10.4* plants was significantly lower than in WT, which may reflect the high levels of wound-induced JAZ10.4 protein accumulation in these lines (Figure 2.6B). On the other hand, no complementation was observed in the transgenic (*JAZ10p*:HA-*JAZ10.1*) expressing the labile JAZ10.1 protein isoform (Figure 2.8). These findings support the hypothesis that the up-regulation in *LOX3* and *OPR3* transcript levels in *jaz10-1* is caused by the absence of the stable JAZ10 repressors.

To further test the idea that wound-induced production of stabilized variants of JAZ10 is sufficient to attenuate JA responses, we mutated a key Arg residue (R171 in JAZ10.1) within the degron sequence that mediates ligand-dependent interaction of JAZs to COI1 (Melotto et al. 2008; Sheard et al. 2010; Withers et al. 2012). Yeast two-hybrid (Y2H) assays evidence that the R171A mutation disrupted JAZ10.1-COI1 interaction, with the two proteins failing to interact even in the presence of high concentrations of the JA-Ile analog coronatine (Figure 2.9). To determine whether this stabilized form of JAZ10.1 can complement the JA-hypersensitive phenotype of *jaz10-1*, we performed root growth inhibition assay with a *jaz10-1* line

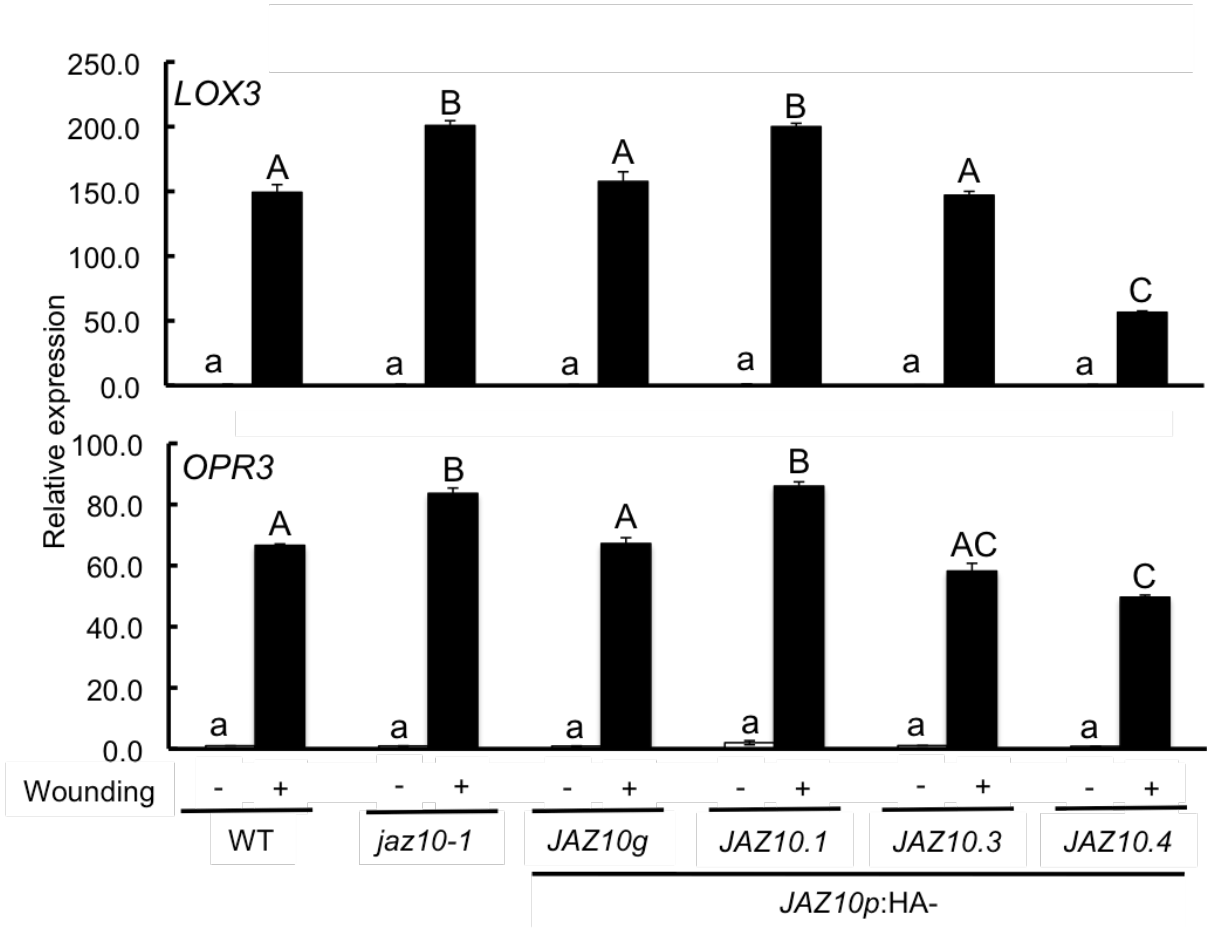

Figure 2.8. Stable splice variants of JAZ10 complement a transcriptional phenotype of *jaz10-1*.

Transcript levels of two biosynthetic genes (*LOX3* and *OPR3*) quantified by qRT-PCR using RNA extracted from leaves before (-) and two hours after (+) mechanical wounding. Data represents expression relative to WT unwounded control. Bars denote mean ± S.E. of three biological replicates with three technical replicates each. Letters denote a statistical difference according to Tukey's HSD (p-value<0.05).

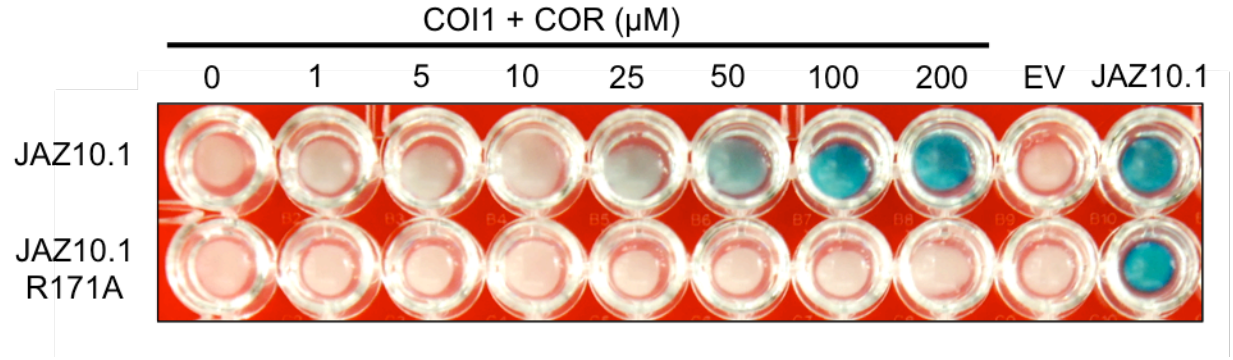

Figure 2.9. R171A mutation disrupts JAZ10.1-COI1 interaction.

Yeast two-hybrid analysis of coronatine (COR)-mediated JAZ10.1-COI1 interaction. Yeast strains were co-transformed with JAZ10.1 or JAZ10.1 R171A and COI1 and plated on medium containing different concentrations of COR. As negative and positive controls for protein interaction, yeast cells were also transformed with empty vector (EV) and JAZ10.1 respectively.

(*JAZ10p:HA-JAZ10.1*^{R171A}) that expresses HA-JAZ10.1^{R171A} under the control of the *JAZ10* promoter. Among eight independent *JAZ10p:HA-JAZ10.1*^{R171A} lines tested (T2 generation), all exhibited significantly reduced sensitivity to JA (Figure 2.10A). Western blot analysis of protein extracts from two representative lines (H2 and H4) showed that the JA insensitivity is associated with induced accumulation of JAZ10.1^{R171A}. In contrast, and consistent with the results showed in Figure 2.7B, the labile WT form JAZ10.1 was not detected in *JAZ10p:HA-JAZ10.1* grown continuously either in the presence or absence of MeJA (Figure 2.10B). These results demonstrate that JA-induced expression of stable JAZ10 protein variants is sufficient to dampen the plant's sensitivity to JA.

JAZ8 does not functionally complement stable isoforms of JAZ10

The *Arabidopsis* JAZ8 protein has also been described as a stable JAZ. Unlike stable isoforms of JAZ10, however, the stability of JAZ8 results from a non-canonical degron loop region that fails to interact with COI1 in the presence of JA-Ile (Shyu et al. 2012). The domain architecture of JAZ8 and JAZ10 is also distinct with respect to the mechanism by which the two proteins recruit the co-repressor TOPLESS (TPL). The biological significance of these differences, however, remains unknown. We therefore designed an experiment to test whether JAZ8 and JAZ10 are functionally equivalent. Specifically, we transformed the *jaz10-1* mutant with a HA-tagged derivative of JAZ8 expressed under the control of the *JAZ10* promoter, and then tested the resulting *JAZ10p:HA-JAZ8* lines for JA-induced root growth inhibition. Among seven independent transgenic lines (T2 generation) analyzed, none complemented the JA-hypersensitive phenotype of *jaz10-1* (Figure 2.11A). Western blot analysis performed with seedlings from representative *JAZ10p:HA-JAZ8* lines (K13 and K15) showed that, despite the

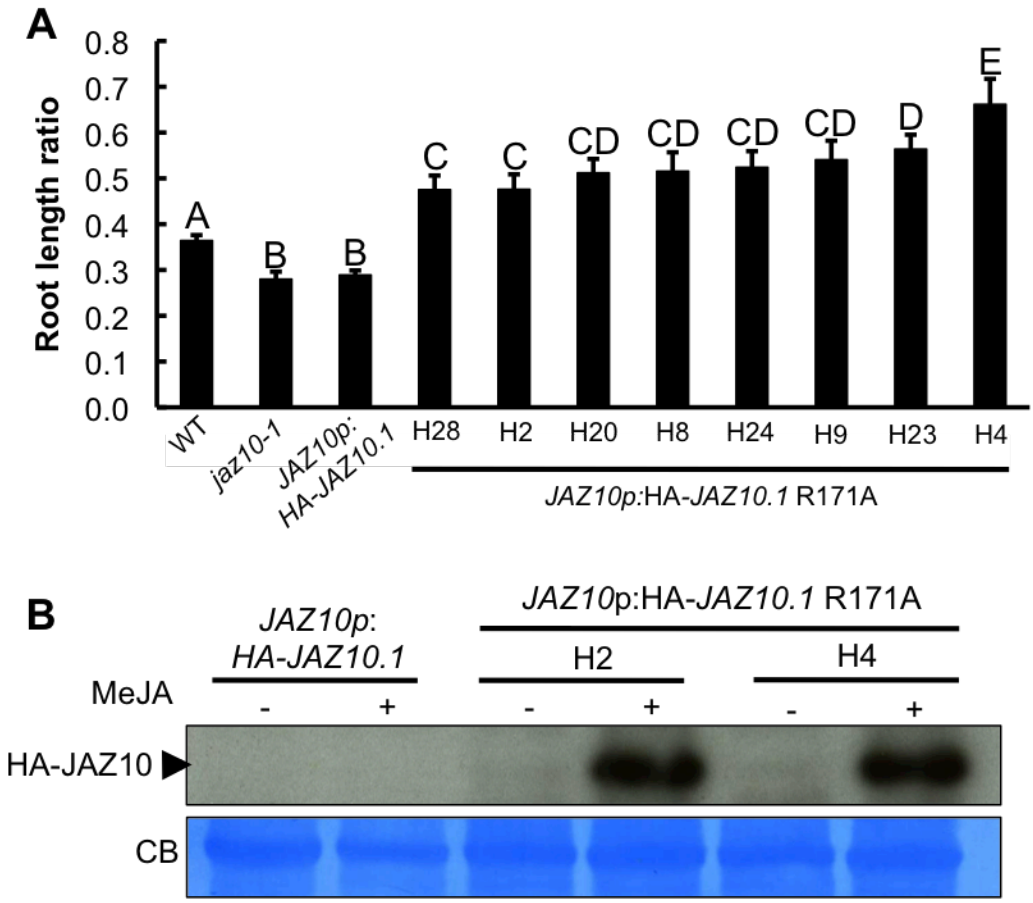

Figure 2.10. A stabilized form of JAZ10.1 complements the JA-hypersensitive phenotype of *jaz10-1*.

(A) Root elongation assay on WT, *jaz10-1* and *jaz10-1* lines transformed with *JAZ10p:HA-JAZ10.1* or its mutant form, R171A. Eight independent *JAZ10p:HA-JAZ10.1* R171A lines (T2 generation) were used for the experiment. Root length was measured in eight-day old seedlings grown in MS medium supplemented or not with 20 μM MeJA. The root length ratio was calculated by dividing the average root length of seedlings grown on medium containing MeJA by the average root length of the same genotype grown in MS medium lacking MeJA. Data show the mean ± S.E. of at least ten seedlings for each genotype. Letters denote a statistical difference in comparison to WT control (Tukey's HSD, p-value<0.05).
(B) Accumulation of JAZ10.1 protein in *JAZ10p:HA-JAZ10.1* R171A seedlings grown form eight days after on MS medium in the absence (-) or presence (+) of 20 μM MeJA. Two independent R171A lines (H2 and H4) were tested. Coomassie blue-stained membrane (CB) is shown as a loading control.

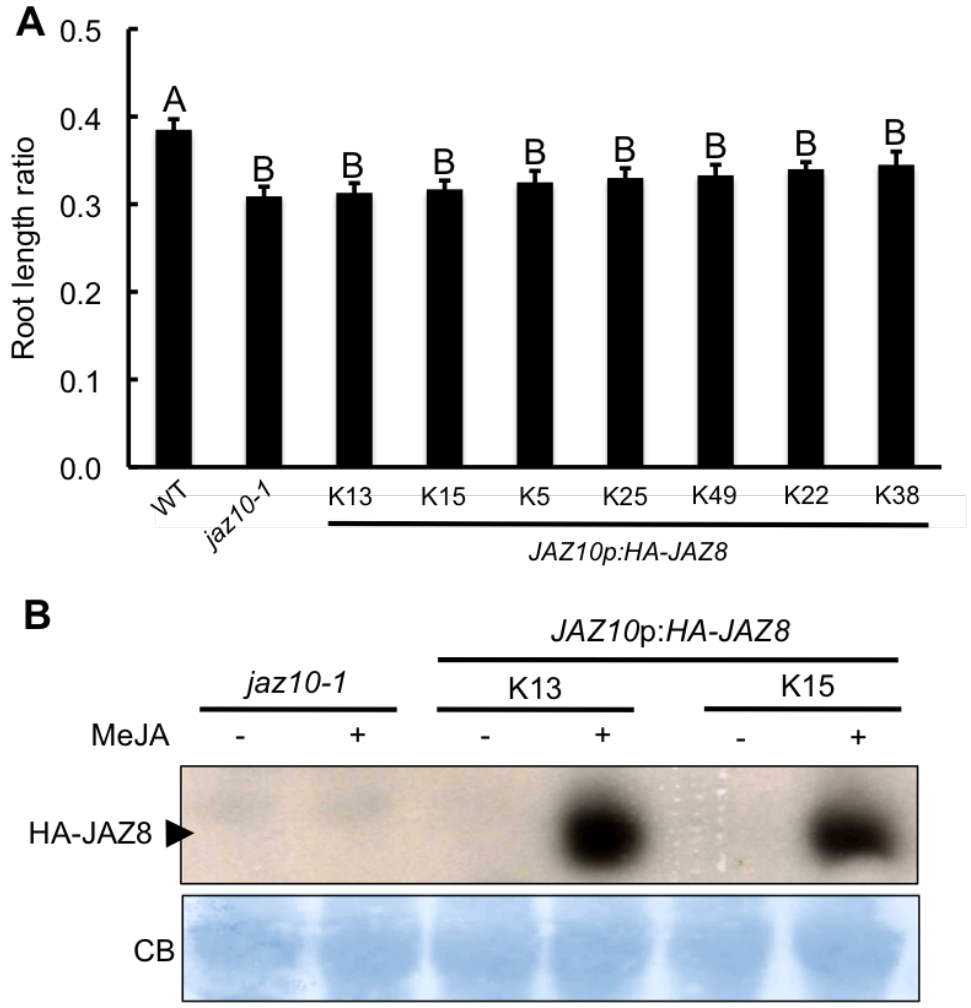

Figure 2.11. JAZ8 does not complement the JA-hypersensitive root growth phenotype of *jaz10-1*.

(A) Root growth inhibition with WT, *jaz10-1* and eight independent *jaz10-1* lines transformed with *JAZ10p:HA-JAZ8*. Root length was measured in eight-day old seedlings grown in MS medium supplemented or not with 20 μM MeJA. The root length ratio was calculated by dividing the average root length of seedlings grown on medium containing MeJA by the average root length of the same genotype grown in MS medium lacking MeJA. Data show the mean ± S.E. of at least ten seedlings for each genotype. Letters denote a statistical difference in comparison to WT control (Tukey's HSD, p-value<0.05).
(B) Accumulation of JAZ8 protein in eight day-old seedlings of *jaz10-1* and two *jaz10-1* transgenic lines (K13 and K15) carrying *JAZ10p:HA-JAZ8* grown on the MS media in the absence (-) or presence (+) of 20 μM MeJA. Coomassie blue-stained membrane (CB) is shown as a loading control.

absence of complementation, JAZ8 protein accumulated in a JA-dependent manner (Figure 2.11B). We conclude that JAZ8 does not functionally complement stable isoforms of JAZ10.

Increased stability of JAZ10.3 is caused by alternative splicing-induced truncation of the Jas motif helix

Given our result showing that JAZ10.3 plays a central role in attenuating JA responses, we next turned our attention to the mechanisms by which the intron retention event responsible for production of JAZ10.3, which is truncated at R185, weakens the interaction of this splice variant with COI1 (Figure 2.12A). Structural studies have revealed that the Jas motif harbors a bipartite JAZ degron sequence consisting of an N-terminal hormone-binding loop followed by an amphipatic α-helix that docks the JAZ proteins on the surface of COI1 (Sheard et al. 2010). Because JAZ10.3 contains an intact hormone-binding loop (Figure 2.12A), we hypothesized that the weak COI1-JAZ10.3 interaction results from modification of the C-terminus of the Jas motif α-helix. In structural studies of the JAZ1 degron, Sheard et al. (2010) noted that hydrophobic residues near the N-terminal end of the Jas motif helix cluster on one face of the helix to form a hydrophobic interface with COI1. The potential contribution of the C-terminal end of the helix, however, was not resolved in the x-ray crystal structure. Helical wheel plots of the JAZ1 and JAZ10 Jas motif helix reveals that V220 and L179 in JAZ1 and JAZ10, respectively, are embedded together with two highly conserved Leu residues on one face of the helix (Figure 2.12B). Because JAZ10.3 lacks L186 (i.e., the protein is truncated after R185), we hypothesized that extending the C-terminus of JAZ10.3 could restore the JAZ10.3-COI1 interaction.

To test this idea, we used site-directed mutagenesis to add a single Leu residue (corresponding to L186) to the C-terminus of JA10.3. The resulting protein (JAZ10.3^{+L186}) was

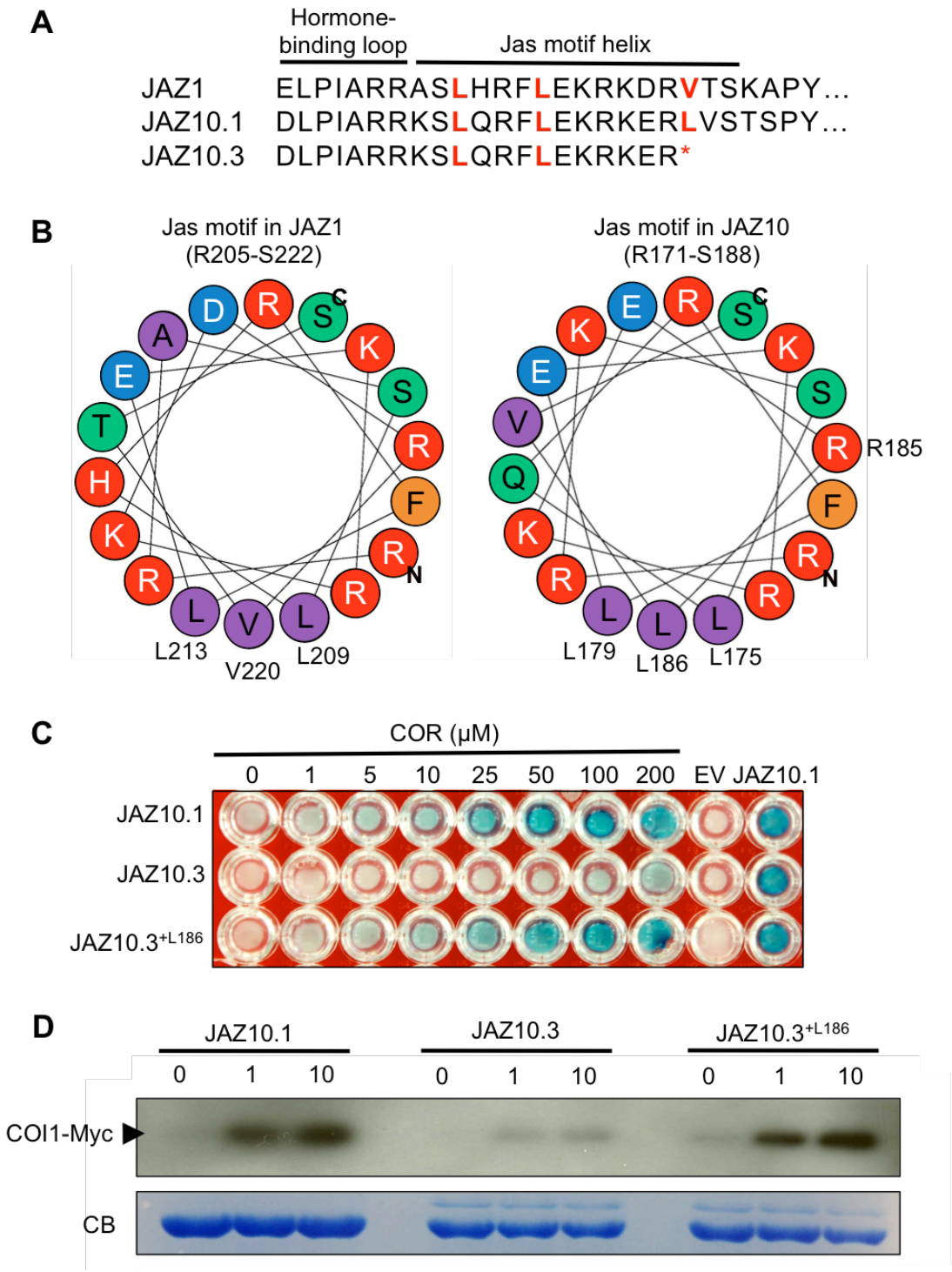

Figure 2.12. JAZ10.3-COI1 interaction is restored by addition of Leu186 to the C-terminal end of JAZ10.3

(A) Amino acid sequence of JAZ1, JAZ10.1 and JAZ10.3 C-terminus. The hormone-binding loop and Jas motif helix are indicated (Sheard et al. 2010). Leu and Val residues that group together to form one face of the helix are highlighted in red.

Figure 2.12 (cont'd). (B) Helical wheel plots of Jas motif helix from JAZ1 and JAZ10. Residues were color-coded based on their physical properties. The three non-polar residues forming a face in the helix (L209, L213 and V220 in JAZ1 and L175, L179 and L186 in JAZ10.3) as well as the last amino acid in JAZ10.3 (R185) are indicated.
(C) Coronatine (COR)-mediated JAZ-COI1 interaction in Y2H assays. Yeast strains were co-transformed with COI1 and JAZ10.1, JAZ10.3 or JAZ10.3+^{L186}. Yeast colonies were plated on media containing different concentrations of COR, as indicated. As negative and positive controls for protein interaction, yeast was also transformed with empty vector (EV) and JAZ10.1 respectively.
(D) In vitro pull-down assays performed with JAZ10.1, JAZ10.3 and JAZ10.3+^{L186}. Assays were performed in the presence of difference concentrations of coronatine (COR). Coomassie Blue-stained gel (CB) is shown as a loading control.

then evaluated in Y2H assays for ligand-dependent interaction with COI1. The results showed that, in comparison to the full-length JAZ10.1 isoform, COR stimulated very weak interaction of JAZ10.3 with COI1, as previously described (Chung and Howe 2009) (Figure 2.12C). Strikingly, however, the addition of L186 in JAZ10^{+L186} restored this interaction to a level comparable to that of JAZ10.1. In vitro pull-down experiments confirmed that the addition of L186 to JAZ10.3 fully restores ligand-dependent interaction with COI1 (Figure 2.12D).

To evaluate whether the strong interaction of JAZ10.3+L186 with COI1 depends on particular biochemical features of the C-terminal amino acid, we constructed a complete series of JAZ10.3 variants in which L186 was substituted with the remaining 19 individual amino acid residues. The resulting set of JAZ10.3 derivatives was tested in Y2H system for interaction with COI1 in the presence of COR. The results showed that the addition of positively charged amino acids and most amino acids containing a non-polar or polar uncharged side chain was sufficient to restore JAZ10.3-COI1 association in the presence of COR (Figure 2.13). However, substitution of L186 with negatively charged or bulky aromatic side chains did not restore interaction with COI1.

To evaluate the relevance of L186 in the function of JAZ10.3, we tested the ability of JAZ10.3^{+L186} (expressed from the *JAZ10* promoter) to complement the JA-hypersensitive phenotype of *jaz10-1*. None of the T2 progeny from eight independent *JAZ10p:HA-JAZ10.3*$^{+L186}$ transgenic lines showed altered sensitivity to JA in comparison to the parental *jaz10-1* mutant (Figure 2.14A). By contrast, a *jaz10-1* line expressing *JAZ10p:HA-JAZ10.3* showed a WT-level of sensitivity to the hormone. In agreement with these findings, Western blot analysis showed that whereas HA-JAZ10.3 accumulates in a JA-dependent manner in *JAZ10p:HA-JAZ10.3* seedlings, JAZ10.3^{+L186} does not (Figure 2.14B). These collective findings demonstrate that the

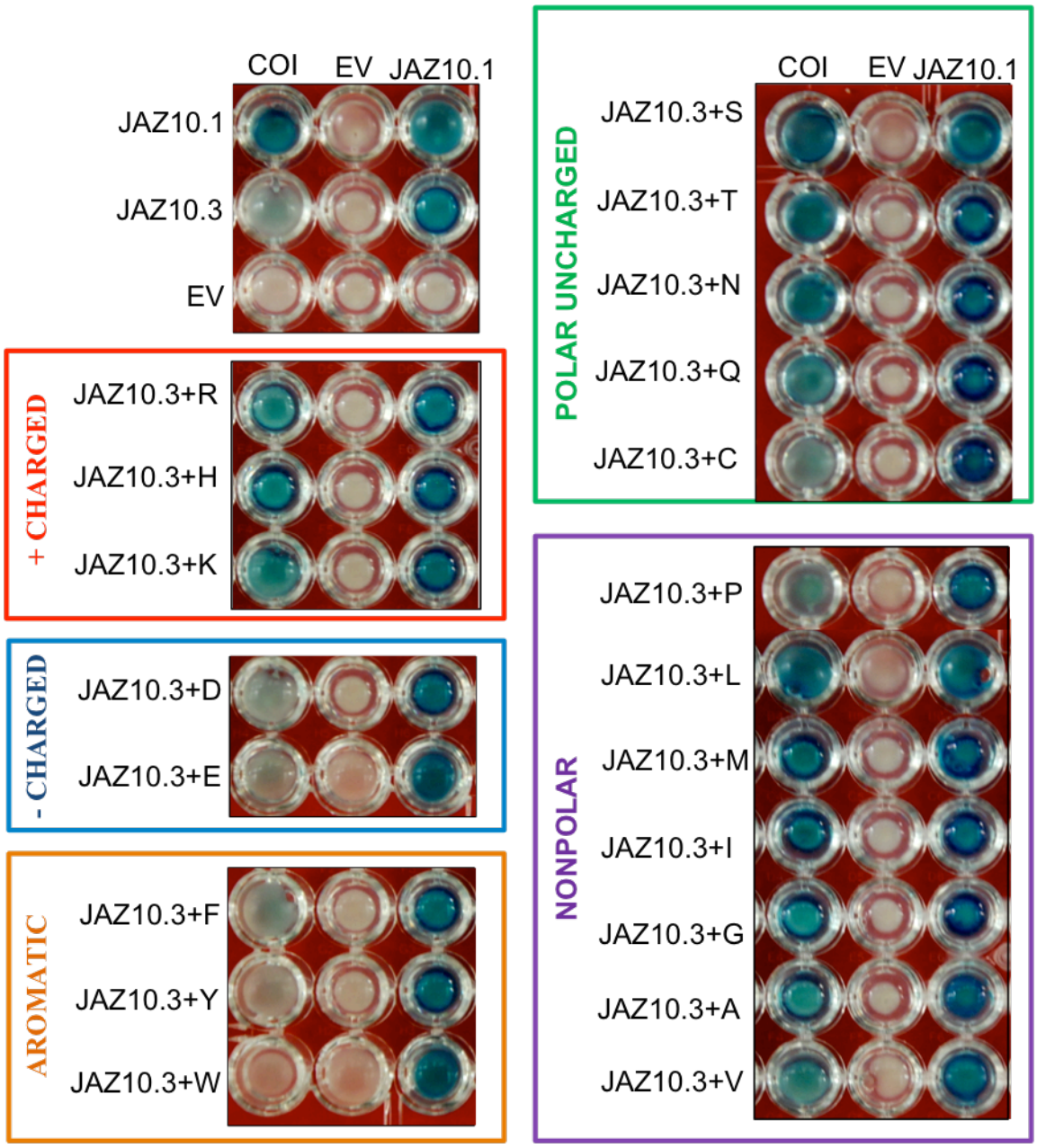

Figure 2.13. Complementation of JAZ10.3-COI1 interaction is dependent on the physical properties of amino acid (aa) residues.

Y2H analysis of COI1 interaction with variants of JAZ10.3 in which an extra amino acid residue was added to its C-terminus end. Yeast strains were co-transformed with COI1 and JAZ10.1, JAZ10.3 or a JAZ10.3 derivative. Yeast was also transformed with empty vector (EV) and JAZ10.1 As a negative and positive controls for protein interaction, respectively. Coronatine (COR - 100 μM) was used to test for COI interaction.

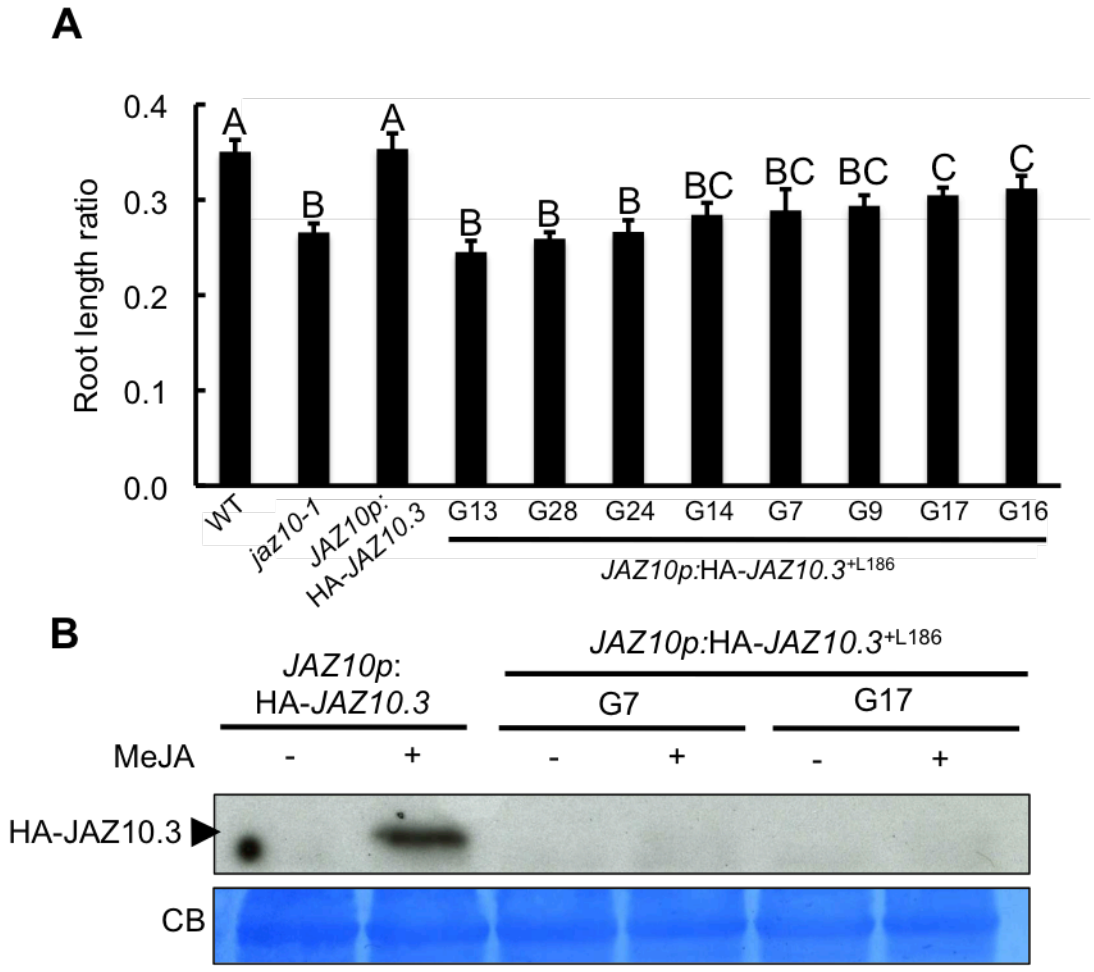

Figure 2.14. JA-stability of JAZ10.3 is caused by a single amino acid truncation in the C-terminal end of its degron.

(A) Root elongation assay Root elongation assay on WT, *jaz10-1* and *jaz10-1* lines transformed with *JAZ10p:HA-JAZ10.3* or *JAZ10p:HA-JAZ10.3*$^{+L186}$. Eight independent *JAZ10p:*HA-*JAZ10.3*+^{L186} lines transgenic lines are shown. Root length was measured in eight-day old seedlings grown in MS medium supplemented or not with 20 μM MeJA. The root length ratio was calculated by dividing the average root length of seedlings grown on medium containing MeJA by the average root length of the same genotype grown in MS medium lacking MeJA. Data show the mean ± S.E. of at least ten seedlings for each genotype. Letters denote a statistical difference in comparison to WT control (Tukey's HSD, p-value<0.05).
(B) Accumulation of HA-JAZ10.3 protein in seedlings of *jaz10-1* carrying the *JAZ10p:*HA-*JAZ10.3* or *JAZ10p:*HA-*JAZ10.3*+L^{Jas21} transgenes. Two *JAZ10p:*HA-*JAZ10.3*+L^{Jas21} lines (G7 and G17) are shown. Seedlings were grown for eight days in MS media in the absence (-) or presence (+) of 20 μM MeJA. Coomassie blue-stained membrane (CB) is shown as a loading control.

removal of L186 via alternative splicing plays a major role in the stability and repressive function of JAZ10.3.

JAZ10.3-like JAZ genes are widespread in the plant kingdom

Sequence analysis on the JAZ genes of evolutionary diverse plant species highlighted the frequent occurrence of an intron splitting the Jas domain into a two submotifs organization (Chung et al. 2010). Remarkably, the retention of this conserved intron through alternative splicing can lead to the formation of JAZ variants lacking the last amino acid in their degron sequence in a similar fashion as observed for the JAZ10.3, leading us to speculate that the formation of this type of repressors is conserved in the plant kingdom. To gain additional insights into evolutionary and functional significance of the JAZ10.3-like genes, we searched the Phytozome genome database for the presence of JAZ-related genes lacking only the last amino acid in their degron sequence. Our analysis showed that the phylogenetically diverse plant species such as rice (*Oryza sativa*), orange (*Citrus sinensis*), cucumber (*Cucumis sativus*), rubber tree (*Hevea brasiliensis*) and others can produce a JAZ10.3-like protein, where their C-terminal ends in a stop codon truncating the degron in the last amino acid (Table 2.1). This list is just a glimpse of how widely spread this phenomenon is, since the sequencing of new plant genomes and the frequent identification of new splice variants will likely increase the number of examples found. Indeed, it has been experimentally demonstrated that three other *Arabidopsis* JAZ genes (*JAZ2*, *JAZ6* and *JAZ11*) produce splice isoforms similar to JAZ10.3, generating JA-stable JAZ repressors (Chung et al. 2010). These results indicate that the presence of a JA-stable JAZ generated by the absence of the last amino acid in their degron sequence is a common strategy that evolved the plant kingdom to finely regulate the amplitude and duration of the JA responses.

Table 2.1. Examples of JAZ genes missing the last amino acid in the JAZ degron in different plant species[a].

SPECIES	FAMILY	LOCUS NAME	TIFY SEQUENCE	JAZ DEGRON SEQUENCE
Oryza sativa	Poaceae	Os03g27900	TIVYGG	VMPIARKASLQRFLQKRKQK*
Brachypodium distachyon[b]	Poaceae	Bradi3g10820.2	TIFYNG	DLPIARKASLHRFLEKRKDR*
Citrus sinensis	Rutaceae	orange1.1g030695	TIFYNG	DLPIARRKSLQRFLEKRKER*
Arabidopsis thaliana[b]	Brassicaceae	At1g74950 (JAZ2)	TIFYGG	ELPIARRASLHRFLEKRKDR*
		At1g72450 (JAZ6)	TIFFGG	VERIARRASLHRFFAKRKDR*
		At5g13220 (JAZ10)	TIFYNG	DLPIARRKSLQRFLEKRKER*
		At3g43440 (JAZ11)	TIIFGG	DVPIARRRSLQRFFEKRRHR*
Capsela rubellata	Brassicaceae	Carubv10002439	TIFYNG	DLPIARRKSLLRFLEKRKER*
Brassica rapa	Brassicaceae	Bra023399	TIFYNG	DLPIARRKSLQRFLEKRKER*
Malus domestica	Rosaceae	MDP0000757701	TIFYAG	AVPQARKASLARFLEKRKER*
		MDP0000891920	TIFYAG	AVPQARKASLARFLEKRKER*
Cucumis sativus	Cucurbitaceae	Cucsa.095580.1	TIFYNE	DLPLARKRSLHRFLEKRKER*
Linum usitatissimum	Linaceae	Lus10002576	TIFYNG	ADLPIARRKSLQRFLEKRKER*
Hevea brasiliensis[c]	Euphorbiaceae	HbJAZ_1660	TIFYAG	DLPIARRASLHRFLEKRKDR*
		HbJAZ_29511	TIFYNG	DLPIARRKSLQRFLEKRKER*

[a]- JAZ genes were searched on Phytozome for sequences containing the TIFY (PF06200) and Jas (PF09425) motifs.
[b]- Chung et al. 2010.
[c]- Pirrello et al. 2014.

Discussion

Splice variants of JAZ10 regulate the amplitude of JA responses

Alternative splicing is a widespread mechanism that increases protein diversity and gene function in eukaryotes, providing additional layers of regulation in biological networks. It is becoming increasingly evident that alternative splicing is a prevalent process in plants (Li et al. 2014; Mandadi and Scholthof 2015; Marquez et al. 2012), but examples demonstrating the biological relevance of alternative splicing in plant growth and development is scarce. We focus our work on the JAZ family of repressors and more specifically on *Arabidopsis JAZ10,* which is subject to alternative splicing to generate three protein isoforms that differentially interact with COI1 in the presence of the bioactive JA (Chung and Howe 2009; Chung et al. 2010; Moreno et al. 2013). In an effort to understand the function of these splice isoforms, we first evaluated *JAZ10* gene expression upon induction by mechanical wounding. Transcripts for all alternatively spliced *JAZ10* transcripts were rapidly (<1h) and strongly stimulated by leaf injury, in agreement with the observation that this gene is a robust marker for activation of the JA pathway (Mousavi et al. 2013; Yan et al. 2007). On the other hand, even though splicing can be regulated in a tissue specific manner and dependent on developmental and environmental cues (Posé et al. 2013; Reddy et al. 2013; Staiger and Brown 2013), we found that *JAZ10.1* was consistently the most abundant transcript in all wounded and wounded tissues. Moreover, the relative proportions of the alternatively spliced transcripts derived from *JAZ10* pre-mRNA tended to remain constant in all tissue types and induction conditions studied. These results suggest that the relative abundance of alternative spliced JAZ10 mRNAs is dictated by the strength of splice sites and that the spliceosome components that guide JAZ10 pre-mRNA splicing occur in all tissues.

Alterations in the reading frame caused by alternative splicing can lead to the formation of premature termination codons (PTCs), which trigger the process of non-sense mediated mRNA decay (Wang and Brendel 2006). Even though PTCs are formed in *JAZ10.2* and *JAZ10.3*, immunoblot experiments indicated that all *JAZ10* transcripts are indeed translated, ruling out the possibility that this form of gene silencing controls *JAZ10* expression. Our results indicate that differences in the strength with which JAZ10 proteins variants interact with COI1 is a much more critical factor in the control of JAZ10 protein levels in stimulated cells. Because mechanical wounding causes a rapid and massive rise in JA-Ile levels (Koo et al. 2011), JAZ repressors that strongly associate with COI1 in the presence of the JA-Ile will be rapidly targeted for proteasome-mediated degradation (Thines et al. 2007; Shyu et al. 2012). Consistent with this idea, we found that JAZ10.1, which interacts strongly with COI1 (Chung and Howe 2009), is the most abundant *JAZ10* transcript, but the most unstable protein isoform in wounded leaves. Conversely, the enhanced stability of JAZ10.3 and JAZ10.4 in JA-stimulated cells is a direct consequence of alternative spliced-truncation of the COI-interacting Jas motif.

Plant hormones are potent modulators of plant fitness, controlling virtually every aspect of plant growth and development (Fonseca et al. 2014b; Nemhauser et al. 2006). Thus, it is not surprising that plants have evolved complex strategies to regulate hormone biosynthesis and signaling. Among the mechanisms that control the amplitude and duration of JA responses are the catabolism of JA-Ile (Heitz et al. 2012; Koo et al. 2011), the formation of transcriptional regulators that compete with JA-related TFs for DNA binding (Fonseca et al. 2014a; Song et al. 2013) and the formation of stable JAZ repressors (Chung and Howe 2009; Moreno et al. 2013; Shyu et al. 2012). The observation that the JAZ10.3 is the most abundant JAZ10 isoform in induced cells provided initial evidence that this protein plays a major role in attenuation of JA

responses. Complementation experiments performed with the *jaz10-1* null mutant provided definitive evidence for this. These experiments also indicate that JAZ10.4, although present in JA- stimulated cells in relative low levels, also functions as a dominant repressor of JA signaling. Our data further suggest any environmental stress capable of activating JA synthesis will lead to *de novo* synthesis of all three JAZ10 splice isoforms. Under sustained or chronic stress, however, only the stable JAZ10.3 and JAZ10.4 repressor will accumulate. Direct interaction of these splice variants with TFs such as MYC will then dampen JA responses. The multiple JA-hypersensitive phenotypes observed in *jaz10-1* are therefore a consequence of the prolonged activity of the JA pathway caused by the absence of these stable JAZ10 repressors.

An important question that remains to be addressed is how JAZ10.3 and JAZ10.4 are further removed JA-stimulated cells such that the plants regain its ability to respond robustly to subsequent stress events that trigger JA production. The existence of a mechanism to remove these stable JAZ repressors is supported by the complete absence of detectable JAZ10.3 and JAZ10.4 protein signal 24hrs after induction of the system (Figure 2.6A). It is possible that a JA derivative other than JA-Ile acts as a ligand to promote association of these proteins with COI1, although evidence for this hypothesis is currently lacking (Heitz et al. 2012; Koo et al. 2011). Alternatively, stable JAZ repressors may be targeted for degradation through the action of E3 ligases other than SCF^{COI1}. Finally, the light-dark cycle may also influence the stability of these proteins, as no protein was detected in the 24 h-time point, which is the only where samples were collected in the absence of light (Figure 2.2B). Indeed, there is evidence to indicate that JA responses are controlled by the circadian clock (Goodspeed et al. 2012).

Integrity of the JAZ degron is necessary for COI1 interaction

JAZ proteins contain a degron sequence within the Jas motif that interacts with COI1 in the presence of JA-Ile (Katsir et al. 2008; Melotto et al. 2008; Sheard et al. 2010). It is now evident that natural sequence variation in the degron, together with various mechanisms to modify the degron sequence generate a repertoire of JAZ variants that differentially interact with COI1; as a consequence, plant cells contain multiple JAZ repressors with a wide spectrum of stability at a given concentration of JA-Ile (Chung et al. 2010; Moreno et al. 2013; Shyu et al. 2012). In the case of JAZ10, we introduced a R171A point mutation that impaired ligand-dependent COI1 binding and thus stabilized JAZ10.1. Similar Ala substitutions were previously shown to impede COI1 interaction with JAZ1 and JAZ9 without affecting JAZ interaction with MYC2 (Melotto et al. 2008; Withers et al. 2012). Experiments with JAZ10.1^{R171A} confirmed that this variant behaves as a strong dominant repressor and is capable of reducing the sensitivity of *jaz10-1* to JA. The stabilized JAZ10.1^{R171A} protein may be a useful tool for further studies of JAZ10 function.

A major challenge in the JA field is to determine whether individual JAZ proteins perform different functions. We thus addressed the question of whether JAZ8, another well-characterized stable JAZ repressor (Shyu et al. 2012), is functionally equivalent to JAZ10. Our strategy was to express JAZ8 under the control of the *JAZ10* promoter. This experiment was performed in the *jaz10-1* null genetic background in order to evaluate whether JAZ8 can complement the function of JAZ10 in repressing JA responses. Interestingly, although JAZ8 overexpression can lead to JA insensitivity in roots (Shyu et al. 2012) and that JAZ8 protein was produced in response to JA stimulation, this stable repressor was unable to complement the JA-hypersensitivity of *jaz10-1*. JAZ8 and JAZ10 interact with similar sets of TFs and dimerize with

similar JAZ proteins (Chung et al. 2009; Qi et al. 2011). However, their mechanism of repression of the JA signaling pathway is distinct. JAZ10 interacts with the adaptor protein NINJA to indirectly recruit the co-repressor TOPLESS (TPL), whereas JAZ8 directly interacts with TPL through an EAR motif located at the N-terminus of JAZ8 (Moreno et al. 2013; Pauwels et al. 2010; Shyu et al. 2012). The demonstration that NINJA is indispensable for repression of JA signaling in roots (Acosta et al. 2013) may explain why JAZ8 could not complement the *jaz10-1* root phenotype. Taken together, these observations provide direct genetic evidence for functional specificity among the JAZ family members.

We also investigated the mechanism by which alternatively spliced-mediated truncation of JAZ10 increases the stability and repressive activity of JAZ10.3. We found that extension of the JAZ10.3 C-terminus by addition of L186 not only restore ligand-dependent interaction with COI1, but also eliminates the repressive function of the protein through destabilization. Structural studies demonstrate that the C-terminal end of the JAZ degron forms an α-helix that serves as a low-affinity anchor that, in the presence of JA-Ile, docks the JAZ protein on COI1 (Sheard et al. 2010). Our results support a model in which alternative splicing-mediated truncation of JAZ10.3 at R185 removes a key part of the helix (L186) that is required for COI1 interaction, thus stabilizing the protein in the presence of JA. It is also possible that the structure of the helix may be affected by absence of L186

It is becoming evident that variation in the JAZ degron sequence is a widespread mechanism to generate stable JAZ repressors that are required for appropriate restraining of JA signaling. *JAZ* genes in diverse plant species are subject to alternative splicing events that modify the degron to create a spectrum of repressors that differentially interact with COI1 (Chung et al. 2009). Furthermore, the observation that diverse plant species use alternative

splicing to generate stable JAZ10.3-like repressors highlight the importance of alternative splicing as a fundamental regulatory feature that evolved concomitantly with the appearance of the JA pathway in land plants.

Stable JAZ repressor modulate resource allocation

To thrive in an ever-changing environment, plants need to constantly modify their growth and development to respond to external signals. However limitations in resource availability may create tradeoffs between growth- and defense- related processes (Herms and Mattson 1992; Huot et al. 2014). Plants appear to use JA as one mechanism to regulate this type of resource allocation. Complex regulatory networks controlling JA biosynthesis and signaling are interconnected with other hormone signaling pathways to presumably optimize plant fitness in changing environments (Ballaré 2011; Erb et al 2012; Yang et al 2012). Misregulation of the JA pathway leads to an imbalance in resource allocation (Leone et al. 2014; Yan et al. 2007). For example, strong JA-induced inhibition of root growth, increased expression of JA-related genes and insensitivity to FR-light observed in the *jaz10-1* mutant ((Leone et al. 2014) highlight how the absence of JAZ10 repressors lead to over-activation of defense processes and downregulation of growth. In WT plants, JAZ10.3 and JAZ10.4 (and other stable JAZ proteins) are part of an essential regulatory feedback loop that is activated upon induction of the JA pathway to directly control the activity of TFs. This level of feedback control presumably evolved as a mechanism to rewires protein-protein interaction networks that serve to fine tune metabolic pathways and the balance between growth and defense processes.

Methods

Plant material and growth conditions

Arabidopsis thaliana plants were grown in soil at 20 +/- 1°C under long-day conditions (16h light, 120 μE $m^{-2}.s^{-1}$). Columbia-0 was used as the wild-type (WT) genetic background for all experiments. The *jaz10-1* mutant (SAIL_92_D08; Sehr et al. 2010) was obtained from the Arabidopsis Biological Resource Center. The *35S:JAZ10G* line was described previously (Chung et al. 2010). JA-mediated root growth inhibition assays were performed as previously described (Moreno et al. 2013; Shyu et al. 2012). Unless otherwise noted, all experiments were independently repeated at least three times.

Wounding time-course experiment

Mechanical wounds were inflicted to leaves of four week-old soil-grown plants with a hemostat as previously described (Koo et al. 2009) (Figure 2.2). Wounded leaves were selected on the basis of their position in the stem (Mousavi et al. 2013). Leaf tissue was harvested and immediately frozen in liquid nitrogen prior extraction of RNA or protein (see below). To reduce plant-to-plant variation, leaves from three plants were pooled for analysis of each time point. Five unwounded systemic leaves were collected an pooled per plant (Figure 2.2).

RNA extraction and qRT-PCR

Plant tissue was ground to a fine powder and used for RNA extraction with a RNeasy kit (Qiagen) followed by on-column DNase treatment (Qiagen) according to the manufacturer's instructions. cDNA was reverse transcribed from 1 μg total RNA with random primers and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, ABI). qRT-PCR was

performed on an ABI 7500 qPCR instrument (ABI), using Power SYBR Green (ABI). Reactions consisted of 2 μL of cDNA template (0.5ng/μL), 1 μL forward and reverse primers (5μM) (Table 2.2), 5 μL of Power SYBR master mix and 2 μL of nuclease-free water. Reactions were incubated under the following conditions: 50°C for 2min, 95°C for 10min and 40 cycles consisting of 95°C for 15s and 60°C for 60s. Dissociation curves confirmed primer specificity. No-template controls were included for each primer set to confirm the absence of contamination or primer dimers. Transcript levels for reference genes *SERINE/THREONINE PROTEIN PHOSPHATASE 2a* (*PP2a*) and *YELLOW-LEAF-SPECIFIC GENE8* (*YLS8*) (Vandesompele et al. 2002) were used to normalize gene expression of all studied genes. All reactions were performed with a minimum of two technical replicates per RNA sample.

Protein extraction and immunoblots analysis

Protein was extracted from frozen ground tissue by the addition of 1 mL of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 50 μM MG132, 1 μM phenylmethylsulfonyl fluoride, pH8.0 and one tablet of MiniProtean cocktail (Roche) per 10 mL of lysis buffer) per gram of tissue powder. Samples were gently rocked at 4°C for 10 min and then centrifuged at 14,000g for 15 min at 4°C. The resulting supernatant was transferred to a new tube and used for protein quantification. Protein samples were resolved on 10% polyacrylamide gels or on 14% Tricine-SDS-PAGE gels, prepared as described by Schägger (2006). Immunoblots were carried out using an anti-HA antibody (Covance) as previously described (Moreno et al. 2013).

Table 2.2. List of PCR primers used in this chapter.

TARGET	NAME	PRIMER SEQUENCE	NOTES
Quantification of transcript levels by qRT-PCR			
PP2a	PP2a_qPCR_Fw PP2a_qPCR_Rv	5'-AAGCAGCGTAATCGGTAGG-3' 5'-GCACAGCAATCGGGTATAAAG 3'	Described in Attaran et al., 2014
YLS8	YLS8_qPCR_Fw YLS8_qPCR_Rv	5'-CTCTCAAGGACA AGCAGGAGTTCATT-3' 5'-CGGTATTTGGTG GAGTAATCTTTTGG-3'	Described in Attaran et al., 2014
JAZ10.1	JAZ10.1_qPCR_Fw	5'-GAAGCGCAAGGAGAGATTAG-3'	
JAZ10.3	JAZ10.3_qPCR_Fw	5'-AAGGAGAGGTAAT GATTCTTCAACAAT-3'	
JAZ10.1/3	JAZ10.1/3_qPCR_Rv	5'-AGCCAAATCCAAAAACGAACA-3'	Same Rv primer is used to amplify JAZ10.1 and JAZ10.3
JAZ10.2	JAZ10.2_qPCR_Fw JAZ10.2 qPCR Rv	5'-CCCCCAAATAATT AAAGAAAGGTTTTT-3' 5'-AAGCATGTGCGTTGTTGAACA-3'	
JAZ10.4	JAZ10.4 qPCR Fw JAZ10.4_qPCR_Rv	5'-GCTAATGAAGCAG CATCTAAGAAAGA-3' 5'-GCGATGGGAAGATCGAAAGA-3'	
OPR3	OPR3_qPCR_Fw OPR3_qPCR_Rv	5'-GTTACAAGGTGTT AATGGCTCAAAGC-3' 5'-ATCACTCCCTTGCCTTCCAGAC-3'	
LOX3	LOX3_qPCR_Fw LOX3_qPCR_Rv	5'-CCTAGACCGGAT TAATGCGCTAGAC-3' 5'-GACCGATGTTTTGGACCATGGGG-3'	
Generation of transgenic lines			
JAZ10 promoter	JAZ10pro NotI GW_Fw JAZ10pro NotI GW_Fw	5'-CACCGCGGCCGCGA CTTTGGCGAGCAAACC-3' 5'-GCGGCCGCCTTCTTTG ATCTTATTAGAAAGTG-3'	Describe in Moreno et al., 2013
JAZ10g and JAZ10 CDS	JAZ10 HA NotI GW_Fw	5'-CACCATGTACCCTTATGATGTGCCA GATTATGCCTCTTCGAAAGCTAC-3'	Describe in Moreno et al., 2013; Fw primer used to amplify JAZ10g and all JAZ10 SV
HA-JAZ10g	JAZ10g_Rv	5'-GTTATAATTTTCTT TACCATATACTAAA-3'	
HA-JAZ10.1	JAZ10.1_Rv	5'-TTAGGCCGATGTCGGATAGTAAGG-3'	
HA-JAZ10.3	JAZ10.3_Rv	5'-TTACCTCTCCTTGCGCTTCTCGAG-3'	
HA-JAZ10.4	JAZ10.4_Rv	5'-CTAATCTCTCCTTGC GCTTCTCGAGAAAACG-3'	
HA-JAZ8	JAZ8_Fw JAZ8_Rv	5'-CCACGCGGCCGCATGTACCCTT ATGATGTGCCAGATTATGCCTCT-3' 5'-TTATCGTCGTGAATGGTAC-3'	
Site-directed mutagenesis (Y2H and transgenic plants)			
JAZ10.1-R^{Jas6}A	JAZ10.1 R^{Jas6}A_Fw JAZ10.1 R^{Jas6}A_Rv	5'-GATCTTCCCATCGCAGC GAGAAAGTCACTGCAACGT-3' 5'-ACGTTGCAGTGACTTTC TCGCTGCGATGGGAAGATC-3'	Same primer pair is used to perform mutagenesis in all used vectors

Table 2.2 (cont'd).

TARGET	NAME	PRIMER SEQUENCE	NOTES
Site-directed mutagenesis (Y2H and transgenic plants)			
JAZ10.3+L	JAZ10.3+L_Fw	5'-GCAAGGAGAGATTA TAAGGCCGACTCGAGAAG-3'	
	JAZ10.3+L_Rv	5'-CTTCTCGAGTCGGCC TTATAATCTCTCCTTGC-3'	
JAZ10.3+A	JAZ10.3+A_Fw	5'-GCAAGGAGAGAGCGT AAGGCCGACTCGAGAAG-3'	
	JAZ10.3+A_Rv	5'-CTTCTCGAGTCGGCC TTACGCTCTCTCCTTGC-3'	
JAZ10.3+V	JAZ10.3+V_Fw	5'-GCAAGGAGAGAGTGTA AGGCCGACTCGAGAAG-3'	
	JAZ10.3+V_Rv	5'-CTTCTCGAGTCGGCC TTACACTCTCTCCTTGC-3'	
JAZ10.3+I	JAZ10.3+I_Fw	5'-GCAAGGAGAGAATCTA AGGCCGACTCGAGAAG-3'	
	JAZ10.3+I_Rv	5'-CTTCTCGAGTCGGCCT TAGATTCTCTCCTTGC-3'	
JAZ10.3+P	JAZ10.3+P_Fw	5'-GCAAGGAGAGACCCTA AGGCCGACTCGAGAAG-3'	
	JAZ10.3+P_Rv	5'-CTTCTCGAGTCGGCCT TAGGGTCTCTCCTTGC-3'	
JAZ10.3+M	JAZ10.3+M_Fw	5'-GCAAGGAGAGAATGTA AGGCCGACTCGAGAAG-3'	
	JAZ10.3+M_Rv	5'-CTTCTCGAGTCGGCCT TACATTCTCTCCTTGC-3'	
JAZ10.3+F	JAZ10.3+F_Fw	5'-GCAAGGAGAGATTCTA AGGCCGACTCGAGAAG-3'	
	JAZ10.3+F_Rv	5'-CTTCTCGAGTCGGCCT TAGAATCTCTCCTTGC-3'	
JAZ10.3+W	JAZ10.3+W_Fw	5'-GCAAGGAGAGATGGTA AGGCCGACTCGAGAAG-3'	
	JAZ10.3+W_Rv	5'-CTTCTCGAGTCGGCCT TACCATCTCTCCTTGC-3'	
JAZ10.3+G	JAZ10.3+G_Fw	5'-GCAAGGAGAGAGGGTA AGGCCGACTCGAGAAG-3'	
	JAZ10.3+G_Rv	5'-CTTCTCGAGTCGGCCT TACCCTCTCTCCTTGC-3'	
JAZ10.3+S	JAZ10.3+S_Fw	5'-GCAAGGAGAGATCCTA AGGCCGACTCGAGAAG-3'	
	JAZ10.3+S_Rv	5'-CTTCTCGAGTCGGCCT TAGGATCTCTCCTTGC-3'	
JAZ10.3+T	JAZ10.3+T_Fw	5'-GCAAGGAGAGAACCTA AGGCCGACTCGAGAAG-3'	
	JAZ10.3+T_Rv	5'-CTTCTCGAGTCGGCCT TAGGTTCTCTCCTTGC-3'	
JAZ10.3+C	JAZ10.3+C_Fw	5'-GCAAGGAGAGATGCTA AGGCCGACTCGAGAAG-3'	
	JAZ10.3+C_Rv	5'-CTTCTCGAGTCGGCCT TAGGATCTCTCCTTGC-3'	
JAZ10.3+N	JAZ10.3+N_Fw	5'-GCAAGGAGAGAAACTA AGGCCGACTCGAGAAG-3'	
	JAZ10.3+N_Rv	5'-CTTCTCGAGTCGGCCT TAGTTTCTCTCCTTGC-3'	
JAZ10.3+Q	JAZ10.3+Q_Fw	5'-GCAAGGAGAGACAGTA AGGCCGACTCGAGAAG-3'	
	JAZ10.3+Q_Rv	5'-CTTCTCGAGTCGGCCT TACTGTCTCTCCTTGC-3'	

Table 2.2 (cont'd).

TARGET	NAME	PRIMER SEQUENCE	NOTES
		Site-directed mutagenesis (Y2H and transgenic plants)	
JAZ10.3+Y	JAZ10.3+Y_Fw	5'-GCAAGGAGAGATACTA AGGCCGACTCGAGAAG-3'	
	JAZ10.3+Y_Rv	5'-CTTCTCGAGTCGGCCT TAGTATCTCTCCTTGC-3'	
JAZ10.3+D	JAZ10.3+D_Fw	5'-GCAAGGAGAGAGACTA AGGCCGACTCGAGAAG-3'	
	JAZ10.3+D_Rv	5'-CTTCTCGAGTCGGCCT TAGTCTCTCTCCTTGC-3'	
JAZ10.3+E	JAZ10.3+E_Fw	5'-GCAAGGAGAGAGAGTA AGGCCGACTCGAGAAG-3'	
	JAZ10.3+E_Rv	5'-CTTCTCGAGTCGGCCT TACTCTCTCTCCTTGC-3'	
JAZ10.3+K	JAZ10.3+K_Fw	5'-GCAAGGAGAGAAAGTA AGGCCGACTCGAGAAG-3'	
	JAZ10.3+K_Rv	5'-CTTCTCGAGTCGGCCT TACTTTCTCTCCTTGC-3'	
JAZ10.3+R	JAZ10.3+R_Fw	5'-GCAAGGAGAGAAGGTA AGGCCGACTCGAGAAG-3'	
	JAZ10.3+R_Rv	5'-CTTCTCGAGTCGGCCT TACCTTCTCTCCTTGC-3'	
JAZ10.3+H	JAZ10.3+H_Fw	5'-GCAAGGAGAGACACTA AGGCCGACTCGAGAAG-3'	
	JAZ10.3+H_Rv	5'-CTTCTCGAGTCGGCCT TACTGTCTCTCCTTGC-3'	
pRMG-nMAL JAZ10.3+L^{Jas21}	pRMG-nMAL JAZ10.3+L^{Jas21}_F	5'-GCAAGGAGAGGTTAGT CGAGCACCACCACCAC-3'	
	pRMG-nMAL JAZ10.3+L^{Jas21}_Rv	5'-GTGGTGGTGGTGCTC GACTAACCTCTCCTTGC-3'	
JAZ10p:HA-*JAZ10.3*+L^{Jas21}	*JAZ10p*:HA-*JAZ10.3*+L^{Jas21}_Fw	5'-GCAAGGAGAGGTTATA AAAGGGTGGGCGCGCC-3'	
	JAZ10p:HA-*JAZ10.3*+L^{Jas21}_Rv	5'-GGCGCGCCCACCCTT TTATAACCTCTCCTTGC-3'	

Transgene constructs

KAPA HIFI Polymerase (Kapa Biosystems) was used for all PCR reactions performed for cloning purposes, as specified by the manufacturer. Primer sets used are listed in Table 2.2. WT lines expressing the *JAZ10* genomic sequence (*JAZ10G*) under the control of the CaMV 35S promoter were previously described by Chung et al. (2010). Expression of *JAZ10g*, *JAZ10* splice variants and *JAZ8* under the control of the native *JAZ10* native promoter was performed as previously described (Moreno et al. 2013). Briefly, a 2.0-kb fragment of the *JAZ10* promoter (*JAZ10p*; Sehr et al., 2010) was PCR amplified from an *XhoI*-predigested bacterial artificial chromosome (clone T31B5) and cloned into pEntr-D-Topo to generate pEntr-*JAZ10p*. Primer sets used to amplify *JAZ10g* (from genomic DNA) and the full-length cDNA for *JAZ10.1*, *JAZ10.3*, *JAZ10.4* and *JAZ8* where designed to add a hemagglutinin (HA)-epitope tag on the N-terminus of the proteins. The resulting amplicons were cloned into pEntr-D-Topo and named accordingly (e.g. pEntr-*HA-JAZ10g*). The *JAZ10* promoter was then released from pEntr-J*AZ10p* using *NotI* and ligated into *NotI*-linearized pEntr-*HA-JAZ10* or pEntr-*HA-JAZ8* vectors. A LR-clonase reaction (Invitrogen) was used to transfer the final constructs into the pGWB401 destination vector (Nakagawa et al. 2007), which was then used to transform *Agrobacterium tumefaciens* (strain C58C1). Transgenic *Arabidopsis* plants were obtained using the floral dip method (Clough and Bent 1998). Seedlings of the transformed lines (T1) were screened on half-strength Murashige and Skoog (MS) agar plates supplemented with 0.8% sucrose (w/w) and kanamycin (50μg/mL). At least 24 independent T1 lines were transferred to soil. T2 plants carrying a single T-DNA insertion were selected on the basis of the segregation ratios of Kan-resistant to Kan sensitive plants. These lines were further propagated for the identification of homozygous T3 lines.

Yeast two-hybrid (Y2H) analysis

Y2H assays were performed with the Matchmaker LexA system (Clontech). Yeast strain EGY48 was used for co-transformation with pGILDA and pB42AD vectors containing the cDNAs for *COI1*, *JAZ10.1* and *JAZ10.3* as described in Chung and Howe (2008). Yeast transformant were grown in 3 mL SD-glucose medium (Clontech) supplemented with –Ura/-His/-Trp dropout solution, to an OD_{600} of 1.0. Cells were recovered by centrifugation at 5000 rpm for 4min and resuspended in 150 μL of distilled water. A total of 15 μl of cell suspension was added to SD-gal/raf (-Ura/-His/-Trp) inducing medium containing 200 μg/mL of X-gal. In test for JAZ-COI1 interaction, coronatine (Sigma-Aldrich) was added to the medium before cells were plated. Pictures were taken 48h after incubation of plates at 30^{o}C.

In vitro pull-down assays

Pull-down assays were performed using pRMG-nMAL vectors carrying the cDNA for *JAZ10.1* and *JAZ10.3* (Chung et al, 2010) or mutant derivatives of these proteins (see below). MBP-JAZ-His_6 fusion proteins were expressed and purified as previously described (Chung et al. 2010; Shyu et al. 2012). Leaf extracts from an *Arabidopsis* transgenic line expressing Myc-tagged COI1 were used as a source of COI1 protein (Melotto et al. 2008) and coronatine (Sigma-Aldrich) was used as a ligand to evaluate JAZ-COI1 interaction. Immunoprecipitated protein was separated on a 10% SDS-PAGE gel, transferred to polyvinylidene fluoride (PVDF) membrane and probed with anti-c-Myc antibody (Roche) as previously described (Chung et al. 2010).

Site-directed mutagenesis

Site directed mutagenesis was performed on JAZ10.1 and JAZ10.3 cDNAs in pB42AD, pRMG-nMAL or pGWB401 vectors, which served as templates to generate the JAZ10.1-R^{Jas6}A and all the JAZ10.3 constructs containing an added amino acid residue at its C-terminal end. PCR reactions were performed using Pfu Turbo DNA polymerase and primers described in Table 2.2. The presence of the desired mutation was confirmed by DNA sequencing.

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CHAPTER THREE

Rewiring of jasmonate and phytochrome B transcriptional networks simultaneously activate plant growth and defense

Contributions:

The *jazQ* and *jazQ phyB* mutants utilized in this chapter were developed by Dr. Yuki Yoshida, who also performed the hypocotyl elongation assay in monochromatic chambers that lead to identification of the mutation in the *PHYB* gene in *sjq11*. Dalton Oliveira performed the screening for suppressors and enhancers in the EMS-mutagenized population of *jazQ*. Dr. Georg Jander was responsible for quantification of glucosinolates.

Abstract

In order to thrive in the face of stressful environmental conditions, plants invest resources into the production of defensive traits. These defense responses are energetically demanding, imposing on plants a "dilemma" to commit limited metabolic resources to growth- or defense-related processes. The regulation of resource allocation tradeoffs has a profound impact on plant biology and ecological relationships, but the molecular mechanisms behind it are still poorly understood. Here, we use a genetic approach to show that transcriptional rewiring of the jasmonate (JA) and phytochrome B (phyB) signaling pathways can uncouple the growth-defense tradeoffs and describe a novel genotype in which both processes are concomitantly upregulated. We show that a high-order mutant (*jazQ*) constitutively activated in the JA pathway exhibits constitutive expression of defensive traits, including increased resistance to herbivores attack. As a tradeoff, *jazQ* plants have reduced stature and other slow-growth phenotypes. Through the use of a genetic suppressor screen, we show that mutation of the gene encoding the red light receptor phyB rescues the slow growth of *jazQ* without significantly affecting defense traits. We provide molecular evidence that uncoupling of growth-defense antagonism in *jazQ phyB* results from simultaneous activation of MYC2 and PIF transcription factors that promote the expression of defense and growth-related genes, respectively. Our findings suggest that growth-defense antagonism may not be dictated by constraints on metabolic resources but rather by hard-wired regulatory programs that exert control over resource partitioning in dynamic environments. Our results suggest a novel approach for biotechnological efforts to produce crop variaties with improved growth and enhanced pest resistance.

Introduction

Plants exhibit a remarkable degree of developmental plasticity that allows them to cope with the rapid and ever-changing environmental circumstances they experience as consequence of their sessile nature. In response to biotic stress, plants utilize sophisticated developmental programs to mount defenses against the attacker. Although essential for survival, these defense responses are resource demanding and may constrain growth processes (Herms and Mattson 1992; Huot et al. 2014). These growth-defense tradeoffs may have profound impacts on plant physiology and ecology. Empirical evidence from natural plant populations show, for example, that exposure to various environmental cues (such as the presence of herbivores or plant competitors) can lead to tradeoffs in the evolution of growth or defense traits, such that one is usually favored in at the expense of the other (Agrawal et al. 2012; Züst et al. 2012). Economically important crops have been bred to maximize growth and yield, which may constrain the expression of defense traits and thus necessitate the application of pesticides and fungicides (Herms and Mattson 1992; Strange and Scott 2005). In this sense, knowledge of the molecular mechanisms underlying tradeoffs in resource allocation may facilitate the development of crop varieties that combine high yield with increased pest resistance. At the present, however, these mechanisms remain poorly understood.

Jasmonate (JA) is a lipid-derived plant hormones that regulates responses to a multitude of biotic and abiotic stresses (Campos et al. 2014; Dombrowski 2008; Goodspeed et al. 2012; Li et al. 2004; Howe and Jander 2008; Wasternack and Hause 2013). JA also controls a wide variety of growth processes, including cell division and expansion, cell differentiation, flower development and senescence (Browse 2008; Li et al. 2004; Pauwels et al. 2008; Wasternack et al. 2013, Yan et al. 2007). Given its dual function in the control of growth and defense, JA plays

pivotal role in determining how limited resources are allocated to specific metabolic pathways. Increasing evidence indicates that JA-regulated reprogramming of gene expression serves to redirect resource allocation from primary metabolism and growth to secondary metabolism and defense (Attaran et al. 2014; Baldwin 1998; Campos et al. 2014; Huot et al. 2014). This molecular "switch" involves the action of JASMONATE ZIM-domain (JAZ) family of proteins that bind to and inhibit transcription factors (TFs) such as MYC2 to promote defense responses. Stress-induced increases in JA levels promote JAZ degradation via the ubiquitin-proteasome system, allowing MYC2 and related TFs to transcribe genes that confer resistance to herbivore and pathogen attack (Chini et al. 2007; Thines et al. 2007). This model of induced resistance predicts that genetic removal of one or more JAZ repressors will constitutively activate defense responses and likely affect the growth-defense equilibrium. However, apparent genetic redundancy among the 13 members of the JAZ family in Arabidopsis has hindered rigorous testing of this hypothesis (Demianski et al. 2012; Thines et al. 2007; Thireault et al. 2015).

Consistent with its role as in the modulation of growth-defense tradeoffs, the JA signaling pathway is tightly integrated within a larger, highly complex regulatory network that orchestrates hormonal control of plant growth and development. Various components of the JA signaling pathway, for example, mediate crosstalk with signal transduction pathways for gibberellins (Yang et al. 2012), brassinosteroids (Campos et al. 2009) and other defense hormones such as ethylene (Lorenzo et al. 2002) and salicylic acid (Thaler et al. 2012). JA also interacts with signaling pathways associated with light (Moreno et al. 2009), pathogen perception (Campos et al. 2014), temperature sensing (Hu et al. 2013) and many others (Wasternack and Hause 2013). Dissection of the molecular basis of these interactions is providing exciting new insight into how plants finely tune resource allocation in response to changing environmental conditions. In this

context, recent evidence highlights the interaction of JA and light signaling as a key node for regulation of growth-defense tradeoffs (Ballaré 2014, Leone et al. 2014).

Phytochromes (phys) are the principal plant photoreceptors for perception of red and far red light and for detecting neighboring plants that complete for photon capture (Casal 2012). Through their ability to sense changes in the red to far-red (R:FR) ratio of sunlight caused by plant overcrowding and shade, the phy receptors modulate the activity of PIF TFs that promote cell extension-type growth; the resulting growth processes allow plants to better compete for light. These so-called shade avoidance responses include stem and hypocotyl elongation, petiole and leaf extension, increased apical dominance and early flowering (Smith and Whitelam 1997). However, activation of growth processes during shade avoidance responses impairs the plant's ability to mount robust defense responses to pest and pathogen attack (Cerrudo et al. 2012; Moreno et al. 2009). There is increasing evidence to indicate that repression of defense during shade avoidance responses involves the active suppression of the JA signaling pathway, perhaps through increased activity of JAZ repressors (Cerrudo et al. 2012; de Wit et al. 2013; Leone et al. 2014; Moreno et al. 2009; Robson 2010). The observation that phy-impaired mutants display downregulation of JA-dependent defense responses (Cheng et al. 2013; Zhai et al. 2007), and that JA-induced growth inhibition is associated with suppression of PIF TFs (Yang et al. 2012) suggest a binary model where in which phy-mediated growth and JA-mediated defense signaling pathways reciprocally antagonize each other (Ballaré 2014; Moreno et al. 2009). From an ecological perspective, antagonistic coupling of these two pathways may provide a mechanism to appropriately allocate limited resources, thus optimizing plant fitness in dynamic environments.

Here, we describe a genetic approach to uncouple signal antagonism between the JA and phyB pathways, which allowed us to identify unique Arabidopsis genotype in which growth and

defense processes are concomitantly upregulated. This was achieved through initial construction of a *jaz quintuple* (*jazQ*) mutant that exhibits constitutive JA-dependent defense responses and slow growth in the absence of JA treatment. We subsequently employed *jazQ* as a starting point for a genetic suppressor screen aimed at identifying plants that regain growth while maintaining robust defense phenotypes. Characterization of one such suppressor line (*sjq11*) identified the causal mutation as a non-sense mutation in the *PHYB* gene, which was confirmed by genetic reconstitution of a *jazQ phyB* sextuple mutant that incorporates the *phyB-9* reference allele. Genome-wide transcript profiling revealed that genes normally repressed by phyB and JAZ are concomitantly upregulated in *jazQ phyB* plants and also suggest that the unique combination of *jazQ* and *phyB* activates new regulatory circuits that are silent in the individual *jazQ* and *phyB* parental lines. These results suggest that the growth-defense antagonism is not dictated by constrains on metabolic resources but rather reflect the circuitry of transcriptional programs that have evolved to optimize resource partitioning in dynamic environments.

Results

The *jazQ quintuple* mutant shows hypersensitivity to exogenous JA and constitutive activation of defense responses

Genetic redundancy among the *JAZ* gene family has hindered efforts to discern the biological relevance of the JAZ proteins as repressors of JA responses. To help overcome this problem, we used transfer-DNA (T-DNA) insertion mutants to construct a *jaz quintuple* mutant (*jazQ*) that is defective in *JAZ1*, *JAZ3*, *JAZ4*, *JAZ9* and *JAZ10* (Figure 3.1A-B and Methods). These particular members of the *JAZ* family were selected on the basis of their chromosomal location, phylogenetic relationship within the *JAZ* family and interaction with common transcriptional re-

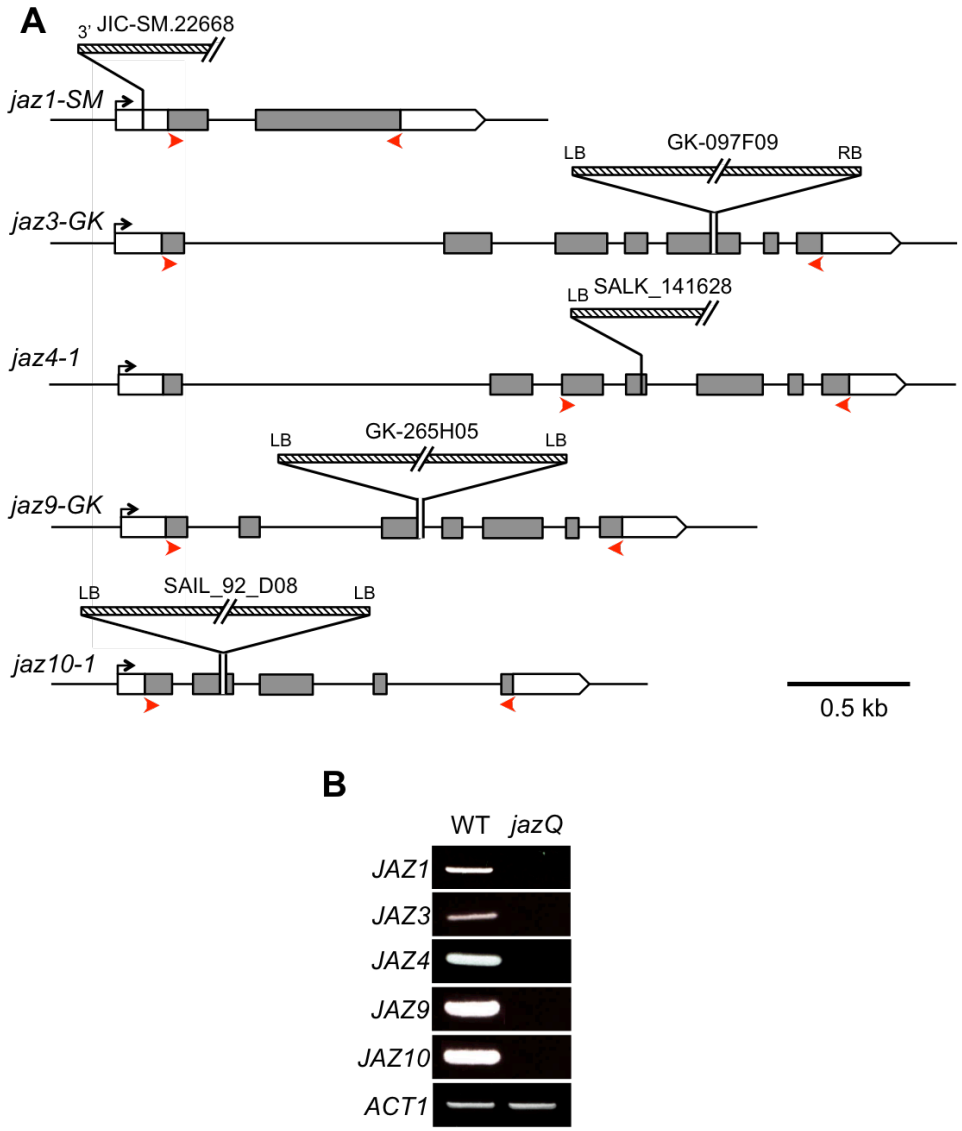


Figure 3.1. The *jaz quintuple* (*jazQ*) mutant is defective in *JAZ1*, *JAZ3*, *JAZ4*, *JAZ9* and *JAZ10*.

(A) T-DNA lines used for construction of the *jazQ* mutant. Genomic organization of each *JAZ* gene is depicted by white and grey boxes, representing UTRs and exons, respectively. The identity and position of the T-DNA insertion is shown.
(B) RT-PCR analysis of *JAZ* gene expression in WT and *jazQ*. RNA was obtained from seedlings grown for eight days on plates containing 25 µM MeJA. Red arrows in (A) indicate the position of the primers used for the experiment. The *ACTIN1* gene (*ACT1* - AT2G37620) was used as a positive control.

gulators (Katsir et al. 2008; Yang et al. 2012). To test whether simultaneous disruption of these five *JAZ* genes affects plant sensitivity to JA, we grew *jazQ* seedlings on solid Murashige and Skoog (MS) medium supplemented with 25 μM of methyl JA (MeJA). As shown in Figure 3.2, *jazQ* seedlings exhibit severe JA-induced inhibition of root and shoot growth in comparison to WT. Quantitative analysis showed that JA-induced root growth inhibition was much more pronounced in *jazQ* than in the *jaz10-1* single mutant, which is known to be JA hypersensitive (Demianski et al. 2013) or a *jaz3/4/9* triple mutant (Figure 3.2B). Interestingly, we found that *jazQ* seedlings develop shorter roots in the absence of exogenous JA (Figure 3.2A-B), suggesting that JA responses may be constitutively activated in this mutant. These results indicate that genetic removal of five *JAZ* genes results in strong hypersensitivity to JA.

We also observed that *jazQ* plants grown in absence of exogenous JA accumulate anthocyanin pigments in leaf petioles (Figure 3.2D). Quantitative analysis showed that anthocyanin levels in *jazQ* petioles are nearly five-fold higher than that in WT (Figures 3.3A-B). To determine whether *jazQ* plants over-accumulate other JA-regulated metabolites, we also measured the level of glucosinolates that perform a major role in anti-insect defense (Schweizer et al. 2013). The results showed that *jazQ* significantly enhance the levels of both aliphatic and indole glucosinolates (Figure 3.3C).

To evaluate the biological relevance of these findings in plant defense against insect herbivory, we performed insect feeding assays with the generalist herbivore *Trichoplusia ni* (Cabbage looper). Neonate larvae reared for 10 days on adult *jazQ* plants gained significantly less weight than larvae feeding on WT (Figure 3.4A-B). The decreased mass of the *T. ni* caterpillars grown on *jazQ* plants was associated with the decreased consumption of leaf tissue

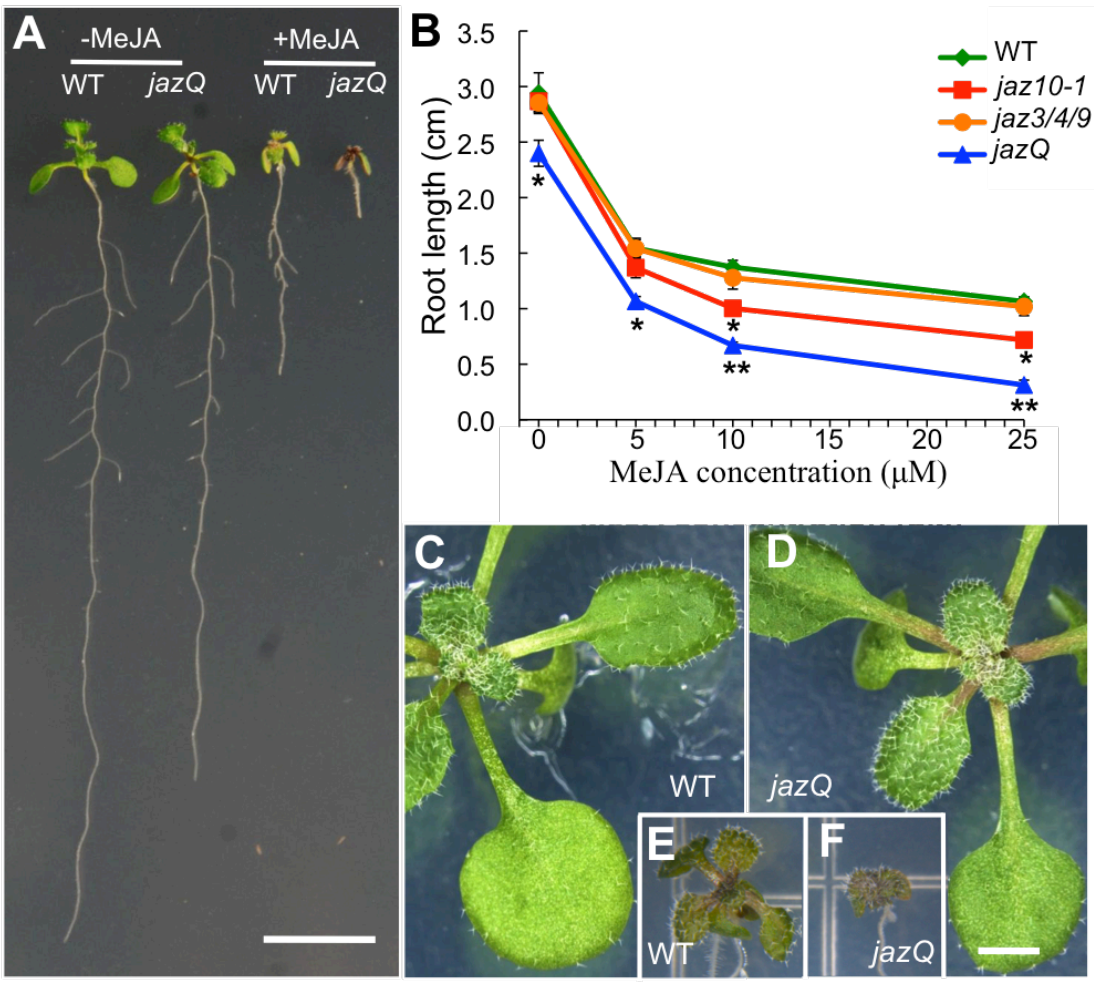


Figure 3.2. *jazQ* is hypersensitive to exogenous JA.

(A) *jazQ* seedlings are highly senstitive to JA. The photograph show wild-type (WT) and *jazQ* seedlings grown for eight days in MS medium supplemented or not with 25 µM of MeJA. Scale bar = 1 cm.
(B) Root length of WT, *jaz10-1*, *jaz3/4/9* triple and *jazQ* mutant seedlings grown for eight days on MS medium supplemented with 5, 10 or 25 µM MeJA. Seedlings were also grown in MS medium not supplemented with MeJA (indicated as 0 µM) as a control. Data show the mean ± SE (n>12). Asterisks represent statistical difference according to Tukey HSD test (*p*-value < 0.05).
(C-F) Shoot phenotype of WT (C and E) and *jazQ* (D and F) seedlings grown for 12 d on MS plates without (C and D) or with 25 µM MeJA (E and F). Scale bar = 0.2 cm.

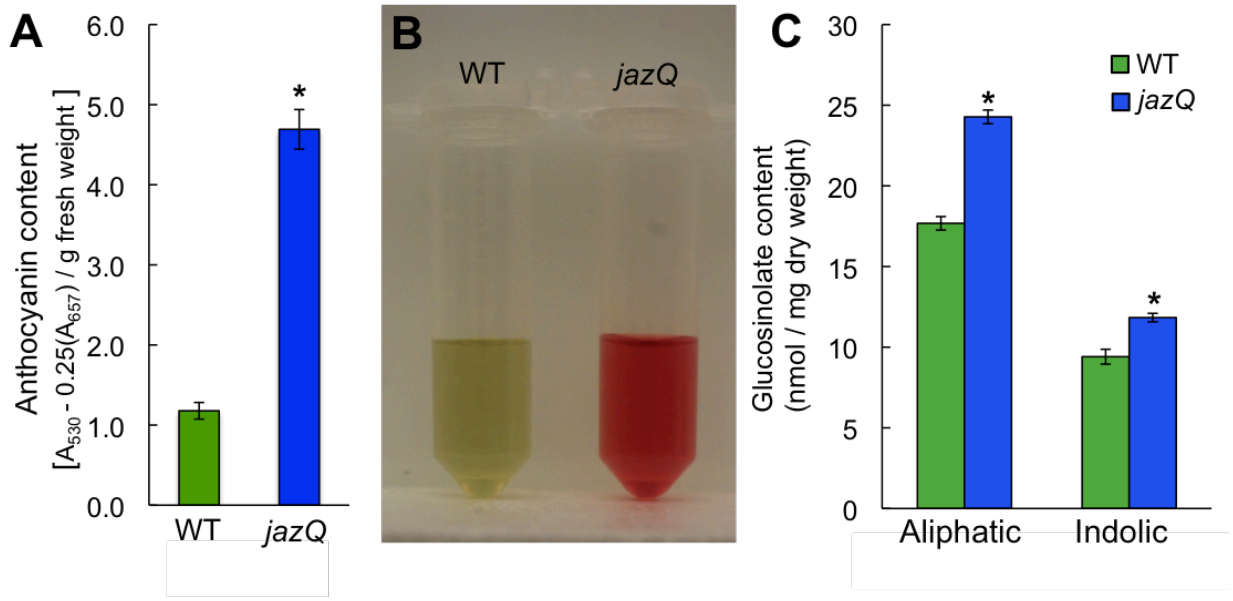


Figure 3.3. Constitutive accumulation of secondary metabolites in *jazQ*.

(A-B) Anthocyanin content in WT and *jazQ* petioles. Pigments were extracted from excised petioles from ten plants. Data represent the mean ± SE. Asterisks (*) represent statistical differences according to Student's T-test (*p*-value < 0.05). A photograph of representative pigment extracts obtained for anthocyanin quantification is show in (B).
(C) Quantification of indole and aliphatic glucosinolates in WT and *jazQ* seedlings. Samples were extracted from seedlings grown on MS media plates for eight days. Data show the mean ± SE. Ten samples (consisting of 50 seedlings each) were used per genotype. Asterisks (*) represent statistical differences according to Student's T-test (*p*-value < 0.05).

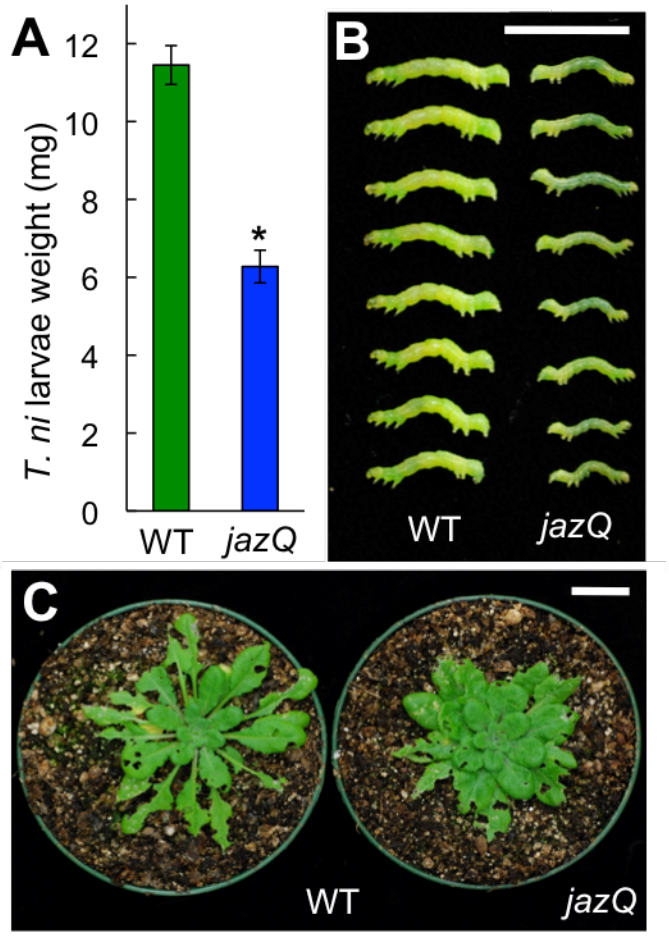


Figure 3.4. *jazQ* exhibits increased resistance to insect herbivory.

(A) *Trichoplusia ni* weight after feeding on WT and *jazQ* plants. Neonate caterpillars were reared on genotypes for 10 days. Data represents the mean ± SE (n=12). Asterisks (*) represent statistical differences according to Student's T-test (p-value < 0.05).
(B) Photograph of representative *T. ni* larvae recovered from WT and *jazQ*. Scale bar = 1 cm.
(C) Photograph of seven-weeks-old WT and *jazQ* plants at the end of feeding assay. Scale bar = 2 cm.

on this genotype (Figure 3.4C). These collective results demonstrate a role for JAZ1/3/4/9/10 in the repression of multiple JA-regulated defense processes.

The *jazQ* mutation impedes plant growth

In addition to constitutive expression of defense-related traits, soil-grown *jazQ* plants exhibited several phenotypes indicative of slow growth. The rosette size of *jazQ* plants prior to flowering was significantly less than that of WT, which was quantitatively assessed as a reduction in petiole length, leaf area, number of rosette leaves 21 days after seed sowing and rosette dry weight (Figure 3.5A-E). *jazQ* plants were also delayed in the time to bolting (Figure 3.5F-H), although the number of rosette leaves at the time of bolting was not different between *jazQ* and WT (Figure 3.5I). These findings indicate that the mutant is not impaired in flower meristem formation *per se* but rather that the genetic removal of multiple JAZ repressors in *jazQ* results in slow growth of vegetative tissues.

JA-response genes are constitutively upregulated in *jazQ*

Our data suggest that resource allocation in *jazQ* is shifted toward defense at the cost of growth. Because JAZ proteins function as transcriptional repressors, we used RNA sequencing (RNA-seq) to gain additional insight into the growth and defense phenotypes of *jazQ*. Sequencing of transcripts from WT and *jazQ* seedlings grown in the absence of exogenous JA indentified 1098 genes (p-value < 0.05 according to DESeq statistical package, Anders and Huber 2010, see methods) that were differentially expressed in *jazQ*. Gene onthology (GO) analysis of the 597 genes upregulated in *jazQ* showed that many of these genes are associated with secondary metabolic pathways (Table 3.1), including the biosynthesis of glucosinolates, phenylpropanoids

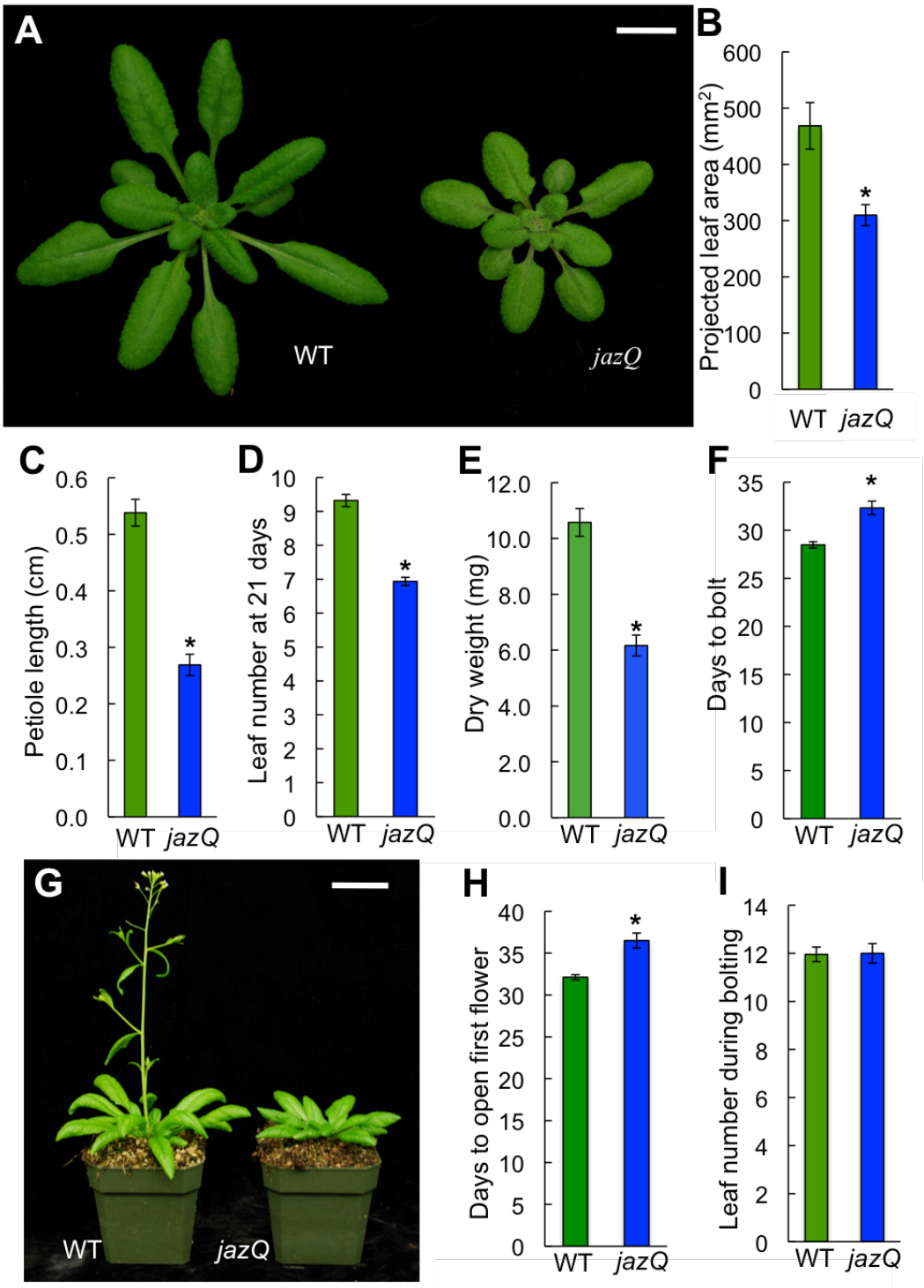


Figure 3.5. Growth processes are hindered in *jazQ*.

(A) Photograph of 21 days-old WT and *jazQ* plants. Scale bar = 1 cm.
(B) Projected leaf area of WT and *jazQ*. Data was obtained from 21 days-old rosettes as described in methods. Data show the mean ± SE (n>20).

Figure 3.5 (cont'd). (C) Petiole length of WT and *jazQ*. Petiole length was measured on the third true leaf of 21 days-old plants. Data show the mean ± SE (n=10).
(D) Leaf number of WT and *jazQ* at 21 days. Data show the mean ± SE (n>10).
(E) Dry weight of WT and *jazQ*. Dry weight was measured by freeze-drying the excised rosette of plants grown on soil for a period of 21 days. Data show the mean ± SE (n=10).
(F) Bolting time in WT and *jazQ*. Data shows the mean ± SE (n>12).
(G) Photograph of 30 days-old WT and *jazQ* plants. Scale bar = 2 cm.
(H) Number of days to open the first flower in WT and *jazQ*. Data represents the mean ± SE (n>12).
(I) Number of rosette leaves at the time of bolting. Data was obtained by counting the number of leaves on the day that a floral meristem was observed. Data shows the mean ± SE (n>12). For all data shown, asterisks (*) represent statistical differences according to Student's T-test (p-value < 0.05).

Table 3.1. List of selected gene onthology (GO) biological processes upregulated in *jazQ*.

GO-ID	DESCRIPTION	p-VALUE
GO:0019748	Secondary metabolic process	3.23E-27
GO:0019760	Glucosinolate metabolic process	6.39E-22
GO:0009753	Response to jasmonic acid stimulus	2.47E-14
GO:0009611	Response to wounding	1.30E-10
GO:0006952	Defense response	2.12E-10
GO:0009694	Jasmonic acid metabolic process	3.73E-10
GO:0009698	Phenylpropanoid metabolic process	2.74E-08
GO:0010683	Tricyclic triterpenoid metabolic process	2.08E-06
GO:0051554	Flavonol metabolic process	1.19E-04

and triterpenoids. Indeed, we found that the vast majority of genes involved in glucosinolates biosynthesis and breakdown are upregulated in *jazQ*, relative to WT (Figure 3.6). Inside these categories are found transcripts for many well-characterized JA-responsive genes, including *ALLENE OXIDE SYNTHASE* (*AOS*), *ALLENE OXIDE CYCLASE* (*AOC*), *OPC-8:0 COA LIGASE1* (*OPCL1*), *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*) and *THIOGLUCOSIDE GLUCOHYDROLASE2* (*TGG2)* (Chung et al. 2008), corroborating the hypothesis that JA responses are constitutively active in this mutant. Consistent with this observation, the GO categories "Defense response", "Jasmonic acid metabolic process" and "Response to wounding" were significantly enriched in *jazQ* (Table 3.1).

Identification of suppressors and enhancers of *jazQ*

The constitutive JA-response phenotype of *jazQ* suggested that the genetic ablation of multiple JAZ repressors causes a shift in resource allocation from growth to defense, consistent with the general theory of growth-defense antagonism. *jazQ* therefore provided a new genetic tool to investigate the molecular components involved in growth-defense tradeoffs. For this purpose we mutagenized *jazQ* seeds with EMS and screened approximately 30,000 soil-grown M2 plants for individuals in which the growth-defense antagonism is "uncoupled". Specifically, we looked for mutants that retained constitutive anthocyanin accumulation but whose slow-growth phenotype was suppressed (e.g., reversion to WT-like growth stature). A total of 34 such lines were identified and categorized as Class I mutants (Figure 3.7 and Table 3.2). An example of such line is *sjq11* (suppressor of *jazQ* 11; Figure 3.9), which was selected for detailed characterization as described below.

Table 3.2. List of suppressors (*sjq*) and enhancers (*ejq*) isolated from a M2 population of EMS-mutagenized *jazQ* seeds.

NAME	SUPPRESSED *jazQ* PHENOTYPES	ENHANCED *jazQ* PHENOTYPES	ADDITIONAL PHENOTYPES
sjq1	Anthocyanin accumulation in young seedlings.		
sjq2	Late flowering.		
sjq3	Late flowering, short petiole.		
sjq4	Late flowering.		
sjq5	Late flowering.	Dwarf.	Increased trichome density.
sjq6	Short petiole.		
sjq7	Late flowering.		
sjq8	Short petiole, late flowering.		
sjq9	Anthocyanin accumulation.		Wide, round leaves.
sjq10	Anthocyanin accumulation.		
sjq11	Short petiole		
ejq12		Short petiole.	
ejq13		Anthocyanin accumulation.	Lanceolate leaf shape.
ejq14		Anthocyanin accumulation, short petiole, late flowering.	Flat leaves.
ejq15	Anthocyanin accumulation.	Anthocyanin accumulation.	
ejq16		Anthocyanin accumulation, dwarf	
sjq17	Short petiole.		Increased leaf number at flowering, large siliques.
sjq18	Late flowering.		
sjq19	Short petiole.		
ejq20		Short petiole (much shorter).	
sjq21	Anthocyanin accumulation.		Dentate leaf shape.
sjq22	Short petiole, late flowering.		
sjq23	Short petiole, anthocyanin accumulation.	Late flowering.	
sjq24	Anthocyanin accumulation.		Leaf shape (lanceolate, dentate), smaller plant stature, Increased trichome density.
sjq25	Short petiole, late flowering, anthocyanin accumulation (late).		
ejq26		Anthocyanin accumulation, short petiole.	
sjq27	Short petiole, late flowering, anthocyanin accumulation (late).		
ejq28		Dwarf, anthocyanin accumulation.	
sjq29	Anthocyanin accumulation in young seedlings.		
sjq30	Short petiole.		Curled leaves.
ejq31		Anthocyanin accumulation, small rosette, late flowering.	Increased trichome density.
sjq32	Short petiole.	Short petiole, late flowering.	
sjq33	Late flowering, short petiole (early).		

Table 3.2 (cont'd).

NAME	SUPPRESSED *jazQ* PHENOTYPES	ENHANCED *jazQ* PHENOTYPES	ADDITIONAL PHENOTYPES
sjq34	Late flowering.	Short petiole, late flowering.	Curled leaves.
sjq35	Short petiole.	Anthocyanin accumulation, late flowering.	Flat leaves.
ejq36		Anthocyanin accumulation, short petiole.	Flat leaves.
ejq37	Short petiole, late flowering.	Anthocyanin accumulation, short petiole.	
sjq38	Anthocyanin accumulation, short petiole, late flowering.		Dentate leaf shape, curled leaves.
sjq39	Late flowering, anthocyanin accumulation, short petiole.		Curled leaves.
sjq40	Short petiole, late flowering.		
sjq41	Anthocyanin accumulation, short petiole, late flowering.		Seeds have greenish color, large wide leaves.
sjq42	Anthocyanin accumulation; however, M3 plants accumulate some anthocyanins, have increased trichome density.	Short petiole, late flowering.	
ejq43		Anth accumulation, short petiole.	Increased trichome density.
ejq44		Short petiole, anthocyanin accumulation, late flowering.	
ejq45		Short petiole, anthocyanin accumulation, late flowering.	
sjq46	Short petiole, late flowering.		
sjq47	Anthocyanin accumulation.	Short petiole, late flowering.	Dentate leaf shape; small, flat leaves.
sjq48	Short petiole.	Short petiole, late flowering.	Curled leaves.
ejq49		Short petiole, anthocyanin accumulation, late flowering.	
sjq50	Short petiole, late flowering.		
sjq51	Short petiole, late flowering.		
sjq52	Short petiole.	Late flowering.	
ejq53		Anthocyanin accumulation.	
sjq54	Short petiole, late flowering.		Some chlorosis; variable phenotypes.
sjq55	Short petiole, anthocyanin accumulation, late flowering.		
ejq56		Anthocyanin accumulation.	Wavy leaves; highly variable phenotypes.
ejq57		Anthocyanin accumulation, late flowering.	
sjq58	Short petiole.		
ejq59	Short petiole, late flowering.	Enhanced jazQ phenotypes.	Flat leaves.
sjq60	Short petiole.		
sjq61	Short petiole, late flowering.	Short petiole.	
ejq62		Anthocyanin accumulation.	
ejq63	Late flowering.	Anthocyanin accumulation, short petiole.	Lanceolate leaf shape, altered leaf angle, pale-green leaves.

Table 3.2 (cont'd).

NAME	SUPPRESSED *jazQ* PHENOTYPES	ENHANCED *jazQ* PHENOTYPES	ADDITIONAL PHENOTYPES
ejq64		Short petiole, late flowering.	Ovate leaf shape, flat leaves.
sjq65	Late flowering, short petiole.		
sjq66	Anthocyanin accumulation, short petiole, late flowering.		
sjq67	Late flowering.		Wide leaves.
sjq68	Short petiole.	Late flowering.	Flat leaves, delayed leaf growth.
sjq69	Short petiole.		Curled leaves.
sjq70	Short petiole.		
sjq71	Short petiole, anthocyanin accumulation.	Late flowering.	Curled leaves.
sjq72	Short petiole.		
sjq73	Short petiole.		

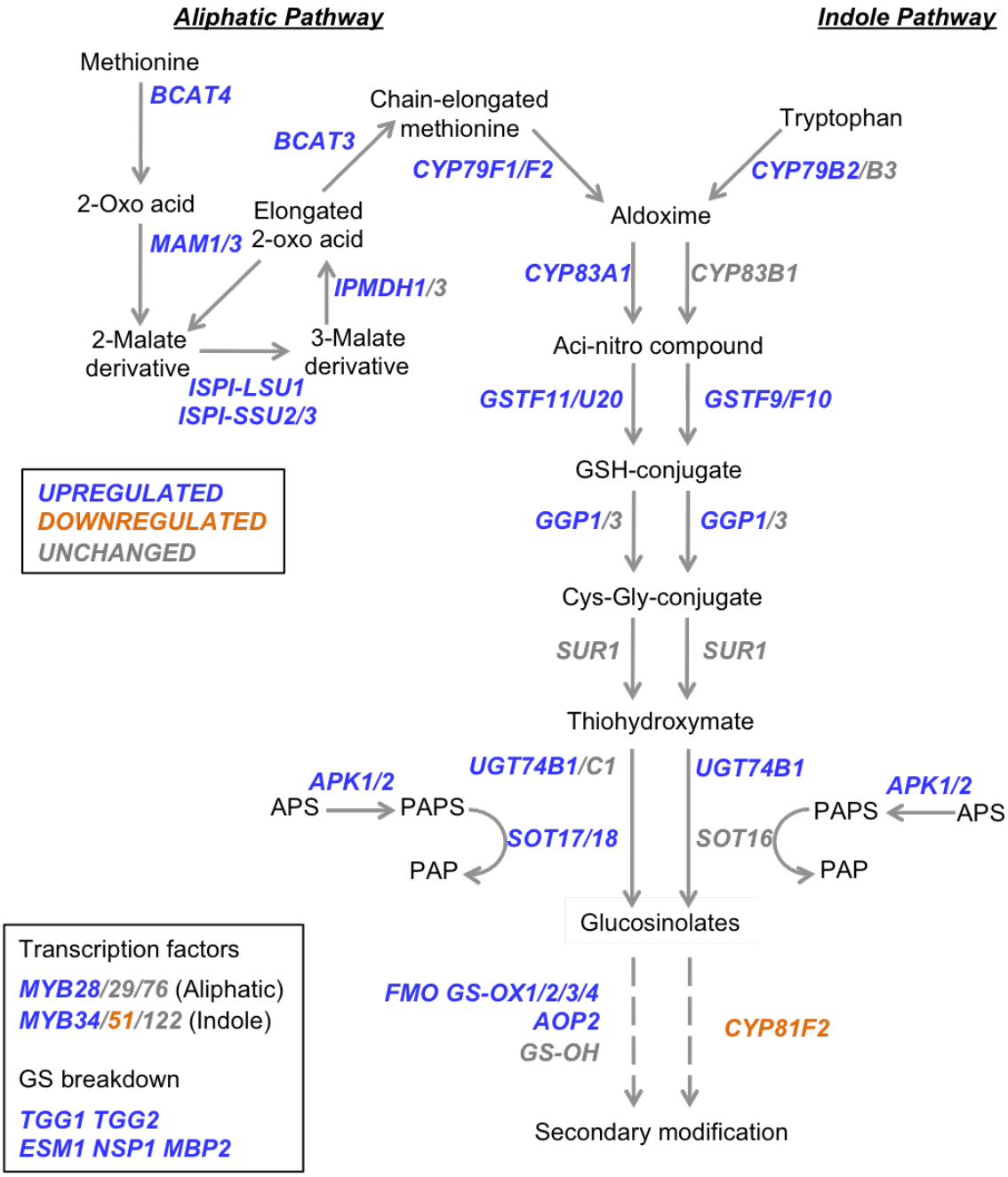


Figure 3.6. Genes involved in glucosinolate biosynthesis and breakdown are upregulated in *jazQ*.

Full transcriptome-sequencing in WT and *jazQ* seedlings show that the majority of the genes involved with GS biosynthesis, including the transcription factors associated with GS production), and GS breakdown are upregulated in *jazQ*.

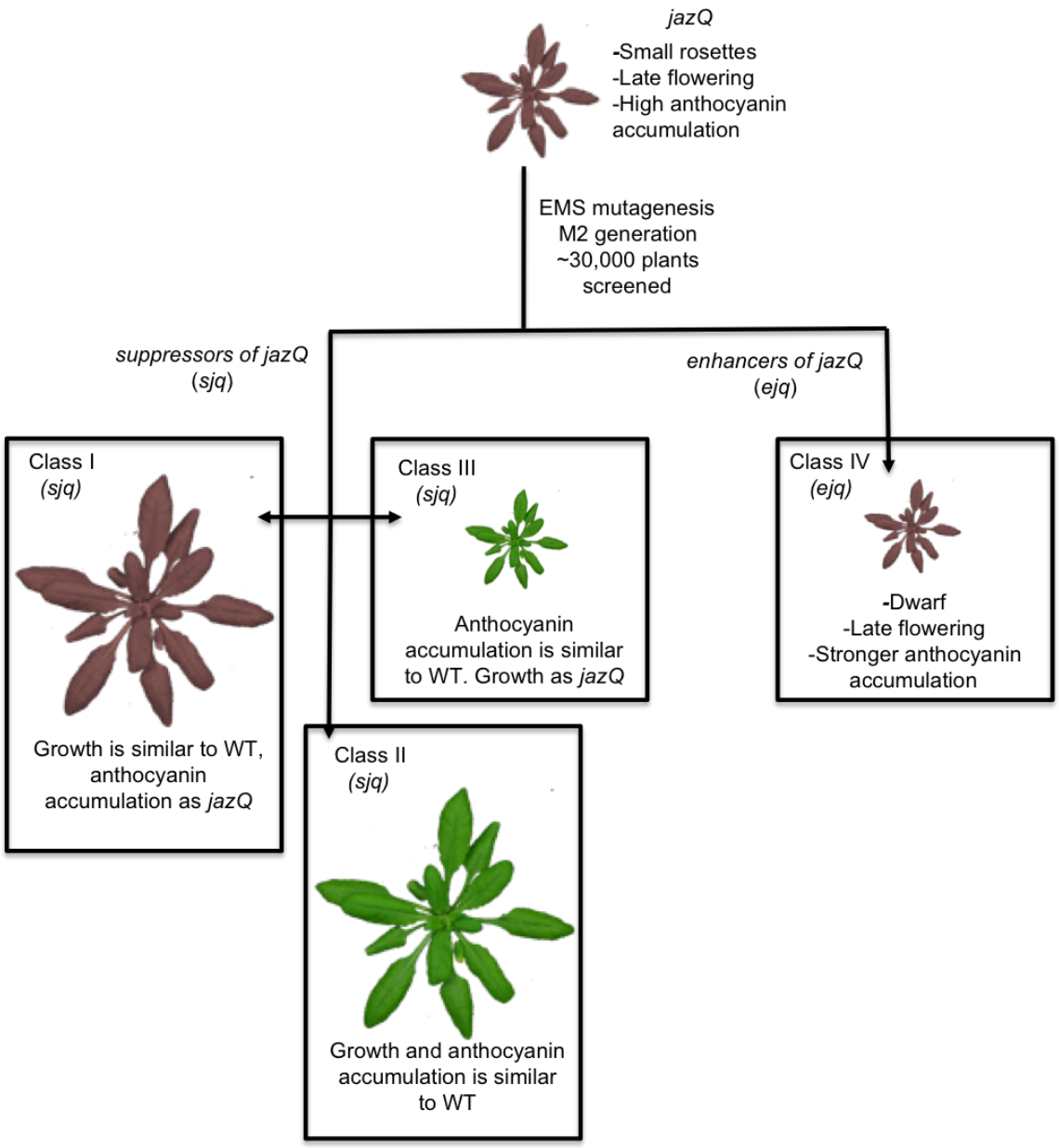


Figure 3.7. Isolation of enhancers and suppressor mutants from a population of EMS-mutagenized *jazQ* M2 plants.

Approximately 30,000 plants were screened in the M2 generation and four phenotypic classes of mutants were identified: Classes I to III (*suppressors of jazQ – sjq*) suppress one of *jazQ* phenotypes. Class I mutants have a WT-like growth pattern but maintain anthocyanin accumulation in the petiole as *jazQ*. Class II mutants grow and accumulate anthocyanins as WT whereas class III mutants grow as *jazQ* but accumulate anthocyanins as WT. Class IV (*enhancers of jazQ – ejq*) mutants show enhancement of a *jazQ* phenotype. False purple coloration was added to plants to facilitate description.

We also identified a distinct group of mutants, in which the anthocyanin accumulation phenotype was suppressed concomitantly or not with suppression of slow-growth (respectively Classes II and III - Figure 3.7). Among the lines where both anthocyanin accumulation and slow-growth was suppressed (Class II), are included two male sterile plants *sjq10* and *sjq66* (Figure 3.8A), which resemble *Arabidopsis* mutants defective in JA biosynthesis or signaling (Wasternack et al. 2013; Xie et al. 1998). Root inhibition assays showed that *sjq10* is fully sensitive to exogenous MeJA, whereas *sjq66* is strongly insensitive to the hormone (data not shown). Subsequent DNA sequencing of candidate genes identified a C→T non-sense mutation in the codon 56 of *AOS* gene in *sjq10* and a C→T missense mutation in the codon 86 of the *COI1* gene of *sjq66* (Figure 3.8B). These results suggest that the growth and defense phenotypes of *jazQ* are dependent on functional JA biosynthesis and signaling pathways.

Finally, we also identified several mutants in which phenotypes of *jazQ* are enhanced (Class IV – Figure 3.7). These *enhancers of jazQ* (*ejq*) lines exhibited severe dwarfism, increased anthocyanin content and/or delayed flowering. A total of 22 *ejq* mutants were identified, but there were not further investigated.

sjq11 carries a nonsense mutation in the *PHYTOCHROME B* gene

Phenotypes observed in the *sjq11* M2 plants, including long petioles, early flowering time (i.e. days to bolting), were confirmed in the M3 generation (Figures 3.10A-B). Root growth inhibition assays further showed that *sjq11* maintains hypersensitivity to JA and also exhibits the constitutive short root phenotype of parental *jazQ* mutant (Figure 3.10C). During the course of these experiments, we observed that *sjq11* seedlings grown in constant white light have phenotypes reminiscent of photomorphogenic mutants, including elongated hypocotyls and pale

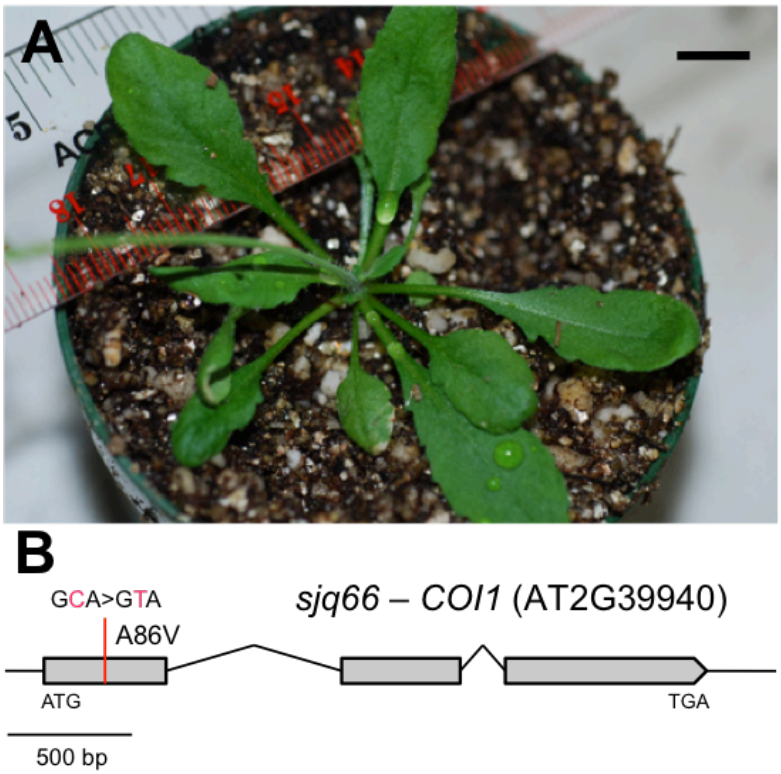


Figure 3.8. *sjq66* carries a mutation in the *CORONATINE INSENSITIVE1* gene.

(A) Photograph of five weeks-old *sjq66* plants. Scale bar = 1 cm.
(B) Schematic representation of the *CORONATINE INSENSITIVE1* (*COI1*) gene. Genomic DNA was extracted from *sjq66* and used for sequence analysis. The cytosine to thymine transition mutation in the *COI1* gene of *sjq66* is illustrated.

Figure 3.9. *sjq11* suppresses the slow-growth phenotype of *jazQ* but not its anthocyanin accumulation in petioles.

Photograph of five week-old WT, *jazQ* and *sjq11* plants. Scale bar = 1 cm.

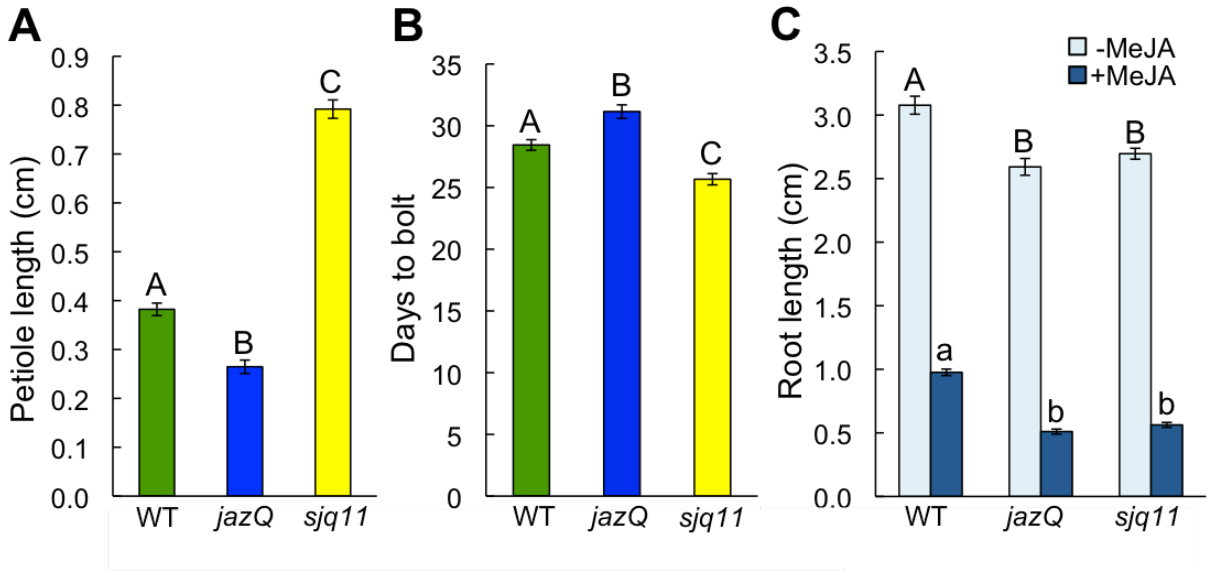


Figure 3.10. *sjq11* shows improved growth and retains hypersensitivity to JA treatment.

(A) Petiole length of WT, *jazQ* and *sjq11*. Petiole length was measured on the third true leaf of 21 days-old plants. Data show the mean ± SE (n=10).
(B) Number of days to bolt in WT, *jazQ* and *sjq11*. Data shows the mean ± SE (n>15).
(C) Root length of WT, *jazQ* and *sjq11* grown on MS medium supplemented or not with 20 µM MeJA. Data show the mean ± SE (n>12). For all data shown, letters indicate statistical differences according to Tukey HSD-test (p-value < 0.05).

green cotyledons (Figure 3.11A-B) (Muramoto et al. 1999; Reed et al. 1994). To test the hypothesis that *sjq11* is impaired in light signaling, we compared the hypocotyl response of *sjq11* to the well-characterized photoreceptor mutants grown in monochromatic light chambers that provide specific wavelengths of light (See methods). We found that *sjq11* is as insensitive to red light as determined a *phyB* null mutant (*phyB-9*) (Figure 3.12). This hypocotyl response phenotype was specific to red light, suggesting that *sjq11* is defective in the phyB red light signaling pathway. Indeed, sequencing of *PHYB* gene (AT2G18790) in *sjq11* revealed a C→T transition (Figure 3.13) that creates a stop codon in the chromophore-binding domain of the protein. Allelism tests performed with the *phyB-9* mutant showed that the F1 plants obtained from a cross between *sjq11* and *phyB-9* display long hypocotyls under white light (Figure 3.14). These findings demonstrate that *sjq11* harbors a null mutation in the *PHYB* gene.

***jazQ phyB* is upregulated in growth and defense parameters.**

To address the possibility that spurious EMS-induced mutations contribute to phenotypes of *sjq11*, we reconstituted a pure *jazQ phyB* line through a cross between *jazQ* and *phyB-9*, followed by selection of a mutant that is homozygous for *jazQ* and *phyB*. As was observed in *sjq11*, the resulting *jazQ phyB* sextuple mutant had a larger rosette diameter and petioles with high anthocyanin content (Figure 3.16A-C). The larger rosette diameter of *jazQ phyB* was attributed in part to longer petioles, which is a hallmark of *phyB* mutants (Figure 3.16A). However, the projected leaf area of *jazQ phyB* was also greater than that of *jazQ*, WT and *phyB* plants as well (Figure 3.16B). Despite large differences in rosette diameter and projected leaf area between *jazQ* and *phyB*, the rosette dry mass of these two lines was not significantly different (Figure 3.16C), presumably because of differences in specific leaf area (leaf area/lead

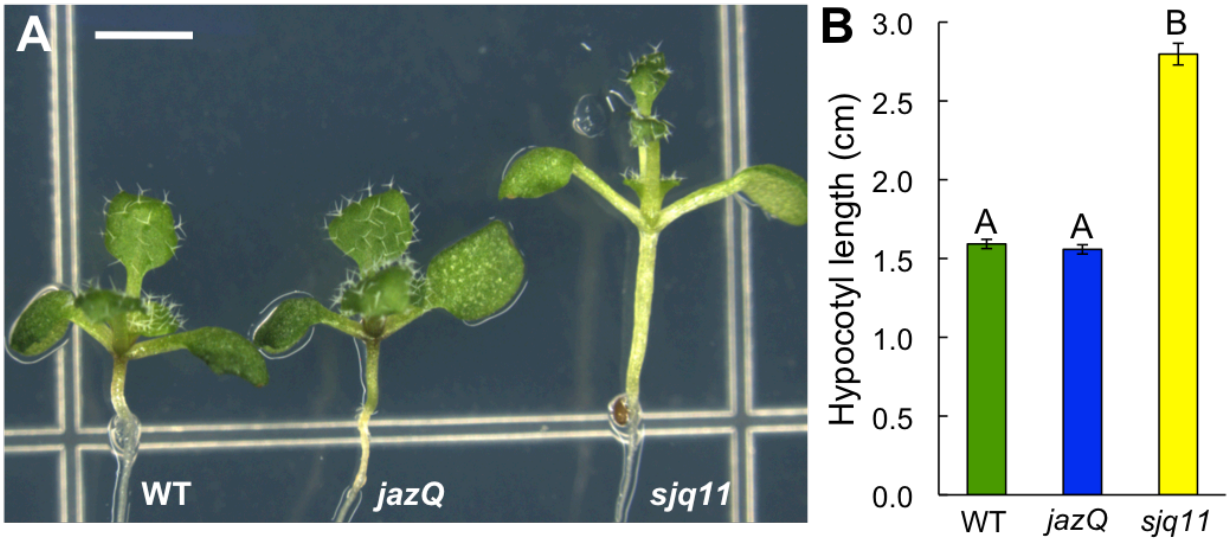


Figure 3.11. *sjq11* seedlings develop long hypocotyls under white light.

(A) Photograph of WT, *jazQ* and *sjq11* seedlings grown on MS medium for eight days, under continuous white light. Representative seedlings of each genotype are shown. Scale bar = 0.2 cm. **(B)** Hypocotyl length of WT, *jazQ* and *sjq11*. Hypocotyls were measured on seedlings grown on MS medium for eight days, under continuous white light. Data shows the mean ± SE (n>20). Letters represent statistical differences according to Tukey HSD-test (p-value < 0.05).

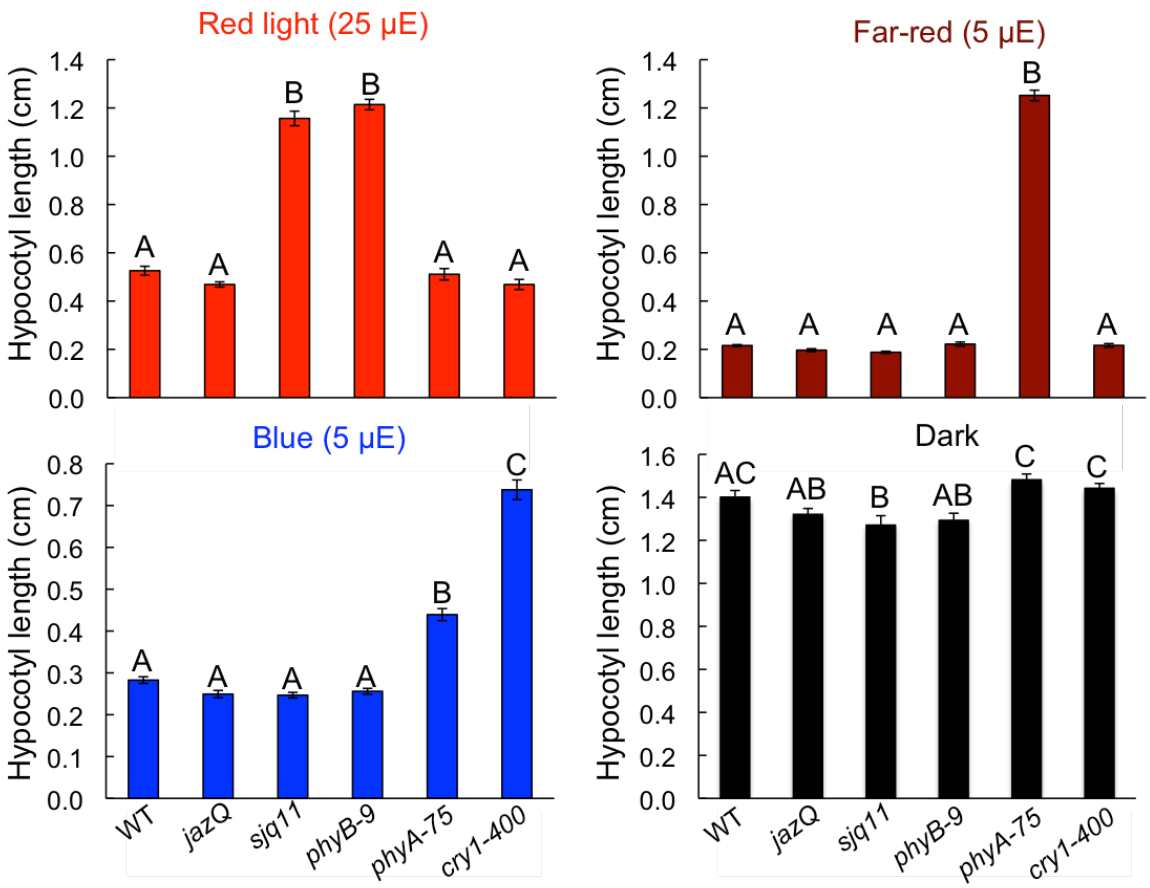


Figure 3.12. *sjq11* is impaired in red light perception.

Hypocotyl elongation in response to different light wavelengths. Seedlings of WT, *jazQ*, *sjq11* and mutants impaired in red (*phyB-9*) far-red (*phyA-75*) or blue (*cry1-400*) light perception were grown for three days on MS medium in monochromatic light. Seedlings were also grown in the dark as a control. Data represent the mean ± SE (n>20). Letters indicate statistical differences according to Tukey HSD-test (p-value < 0.05).

Figure 3.13. *sjq11* harbors a mutation in the *PHYTOCHROME B* (*phyB*) gene.

Schematic representation of the *phyB* gene in *sjq11*. Genomic DNA was extracted from *sjq11* and used for sequence analysis. Red letters indicate the cytosine to thymine transition that leads to a nonsense mutation (R322*).

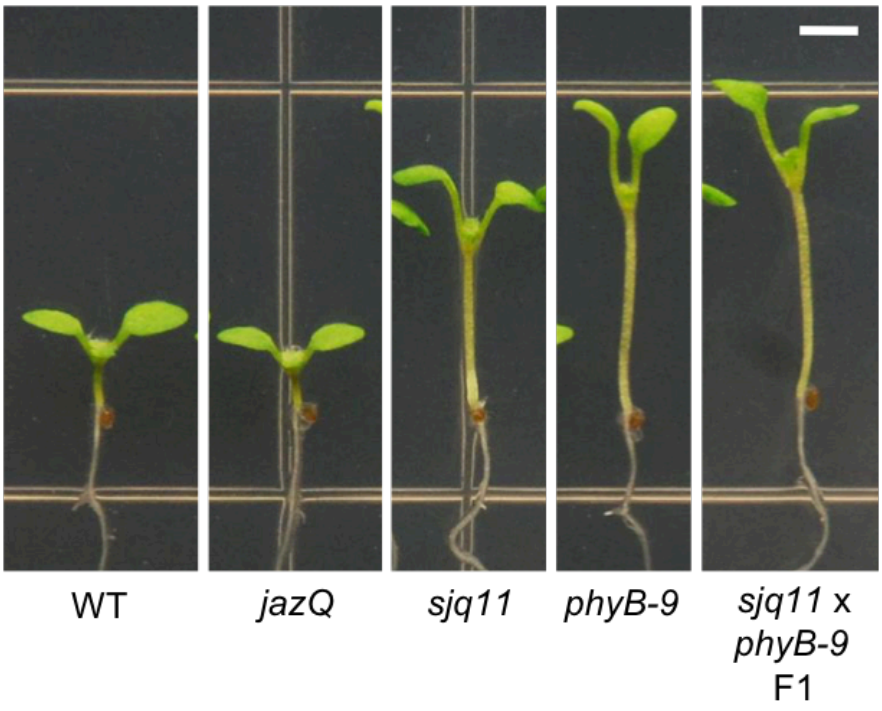


Figure 3.14. Genetic non-complementation of log hypocotyl phenotype in *sjq11* and *phyB-9*.

Photograph of representative seedlings of WT, *jazQ*, *sjq11*, *phyB-9* and the F1 generation obtained from a cross between *sjq11* and *phyB-9*. Seedlings were grown for three days under constant white light. Scale bar = 0.2cm.

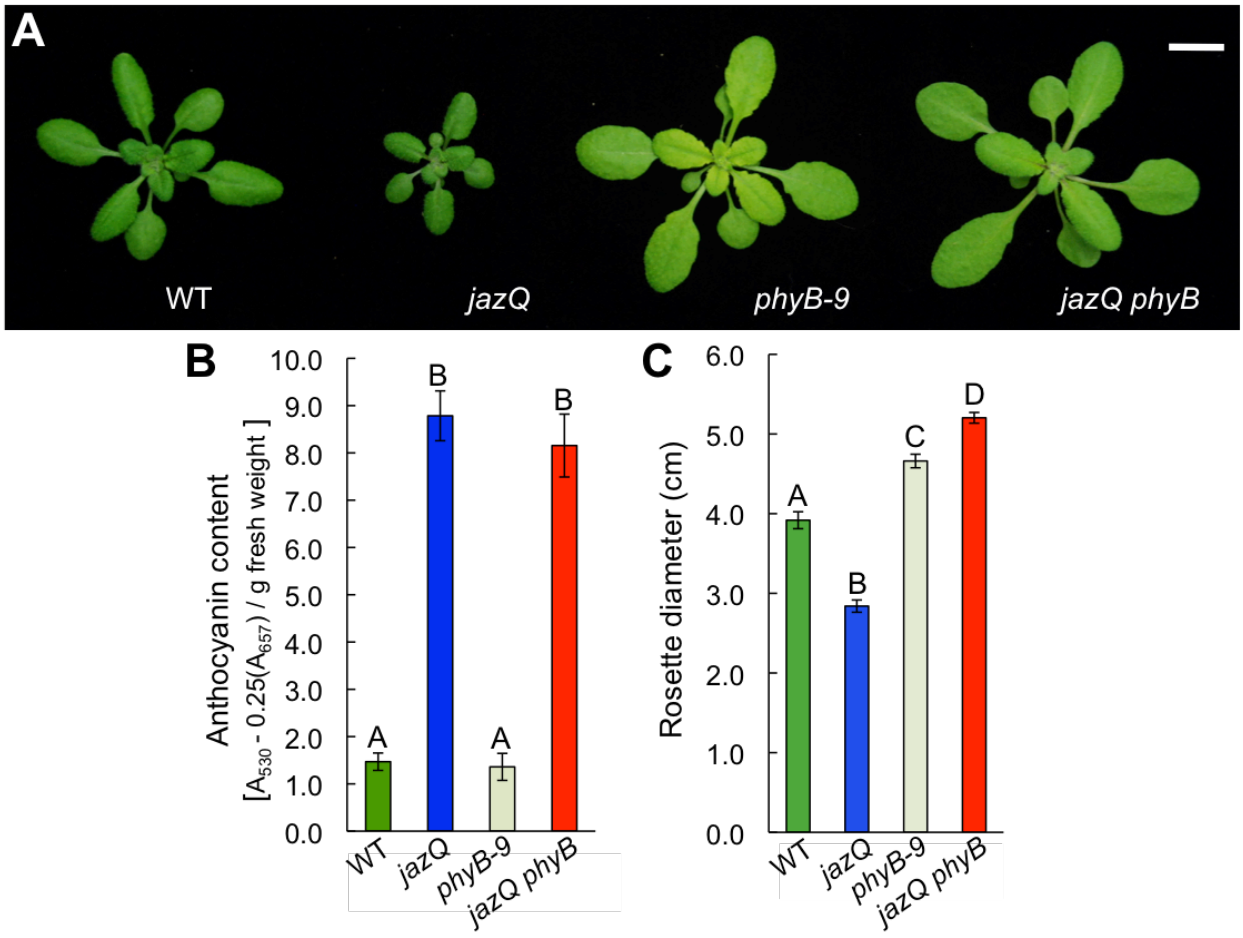


Figure 3.15. *jazQ phyB* plants combine the stronger anthocyanin accumulation of *jazQ* with the large rosette size of *phyB-9*.

(A) Photograph of four weeks-old WT, *jazQ*, *phyB-9* and *jazQ phyB* plants. Scale bar = 1 cm.
(B) Anthocyanin content in WT, *jazQ*, *phyB-9* and *jazQ phyB*. Pigments were extracted from leaf petioles of 21 days-old plants. Data represent the mean ± SE (n>10).
(C) Rosette diameter of WT, *jazQ*, *phyB-9* and *jazQ phyB*. Data was obtained through image analysis of four week-old plants using ImageJ. Data show the mean ± SE (n>20). For all data shown, letters indicate statistical differences according to Tukey HSD-test (p-value < 0.05).

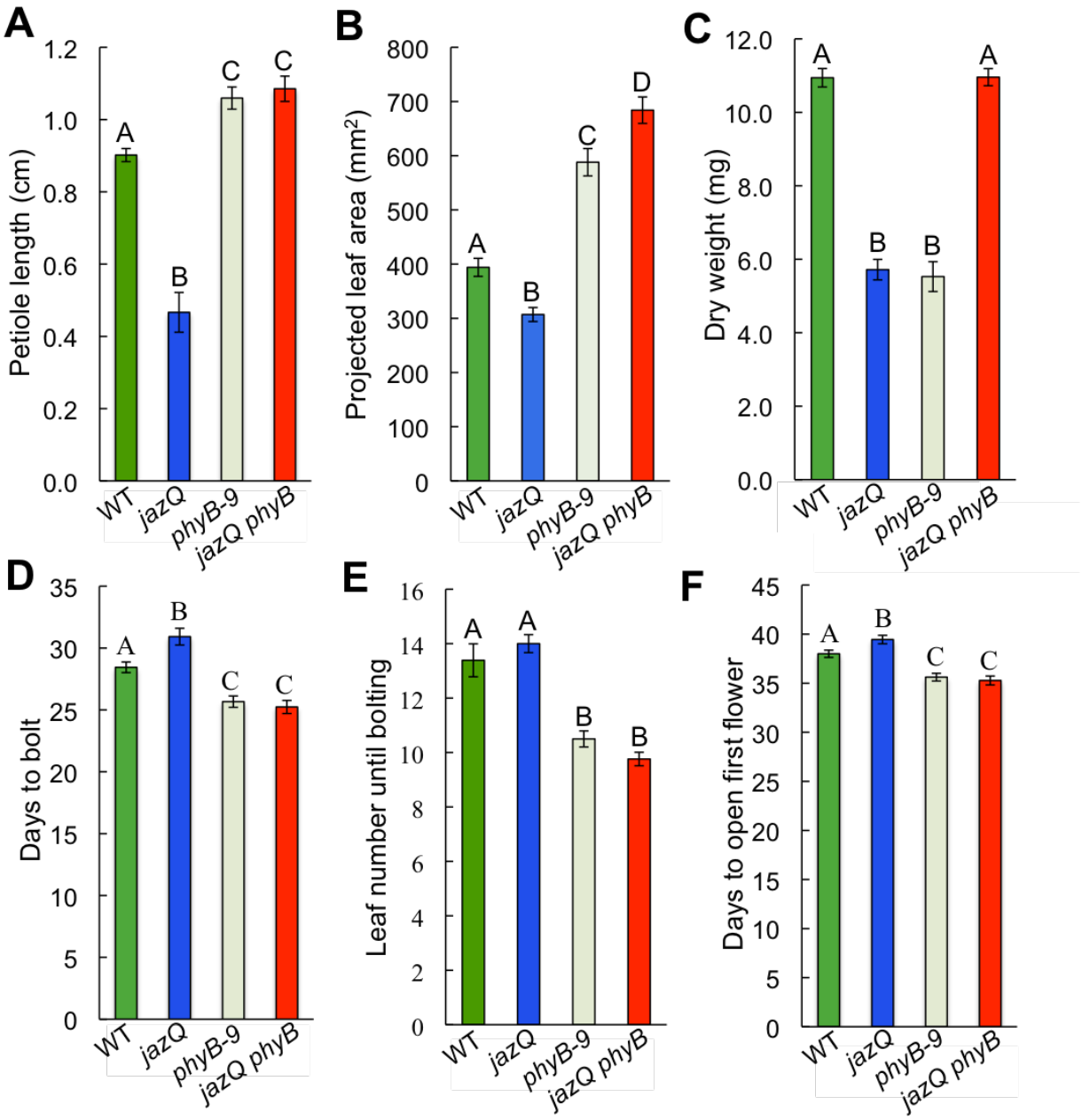


Figure 3.16. Growth parameters are improved in *jazQ phyB*.

(A) Petioles length of WT, *jazQ*, *phyB-9* and *jazQ phyB* plants. Petiole length was measured on the third true leaf of 21 days-old plants. Data represent the mean ± SE (n=10).
(B) Projected leaf area of WT, *jazQ*, *phyB-9* and *jazQ phyB*. Data was obtained from 21 days-old rosettes as described in methods. Data show the mean ± SE (n>20).

Figure 3.16 (cont'd). (C) Dry weight of WT, *jazQ*, *phyB-9* and *jazQ phyB*. Dry weight was measured by freeze-drying the excised rosette of plants grown on soil for a period of 21 days. Data represents the mean (n=10) ± SE. For all data shown, letters indicate statistical differences according to Tukey HSD-test (*p*-value < 0.05).
(D) Bolting time in WT, *jazQ*, *phyB-9* and *jazQ phyB*. Data shows the mean ± SE (n>12).
(E) Number of leaves at time of bolting in WT, *jazQ*, *phyB-9* and *jazQ phyB*. Data was obtained by counting the number of leaves on the day that a floral meristem was observed. Data shows the mean ± SE (n>12).
(F) Number of days to open the first flower in WT, *jazQ*, *phyB-9* and *jazQ phyB*. Data represents the mean ± SE (n>12).

dry mass). The dry weight of *jazQ phyB* rosettes was comparable to that of WT and, remarkably, nearly twice that of either *jazQ* or *phyB-9*. As in the case for the *phyB-9* mutant (Reed et al. 1993), *jazQ phyB* showed accelerated flowering as determined by measurements of time to bolting and time to opening of the first flower (Figure 3.16D-F). We conclude that loss-of-function of phyB suppresses numerous aspects of the slow-growth phenotype of *jazQ*.

We next evaluated JA- and defense-related traits in *jazQ phyB*. Root growth inhibition assays showed that, as observed for *sjq11*, *jazQ phyB* seedlings retain both the hypersensitivy to exogenous JA and the constitutive short root phenotype of *jazQ* (Figure 3.17A). The root length of *phyB* seedlings was similar to that of WT both in the presence and absence of JA. We also found that *jazQ phyB* was similar to the *jazQ* parental line in having small but significant increases in the content of indole and aliphatic glucosinolates, as compared to WT and *phyB-9* (Figure 3.17B). Insect feeding assays performed with *T. ni* larvae provided additional evidence that the robust JA-mediated defense responses exhibited by *jazQ* are maintained in *jazQ phyB*. *T. ni* weight gain on *jazQ phyB* was similar to that on *jazQ* and less than half of that observed on WT plants (Figure 3.18A-B). There also appeared to be more leaf damage on WT than *jazQ* and *jazQ phyB* rosette leaves after 10 days of feeding (Figure 3.18C). We also found that the *phyB-9* mutant is extremely susceptible to insect herbivory, consistent with previous studies (Moreno et al. 2009). Feeding trials involving *phyB-9* plants had to be terminated early (i.e., within five days of challenge) because of near-complete consumption of *phyB-9* leaves, which was accompanied by high weight gain of *T. ni* larvae reared on this mutant relative to other genotypes (Figure 3.19A-C). This result can be correlated with the lower levels of indole GS in this mutant (Figure 3.17B) since these compounds are known to play a fundamental role in defense against herbivores (Hopkins et al. 2009; Schweizer et al. 2013).

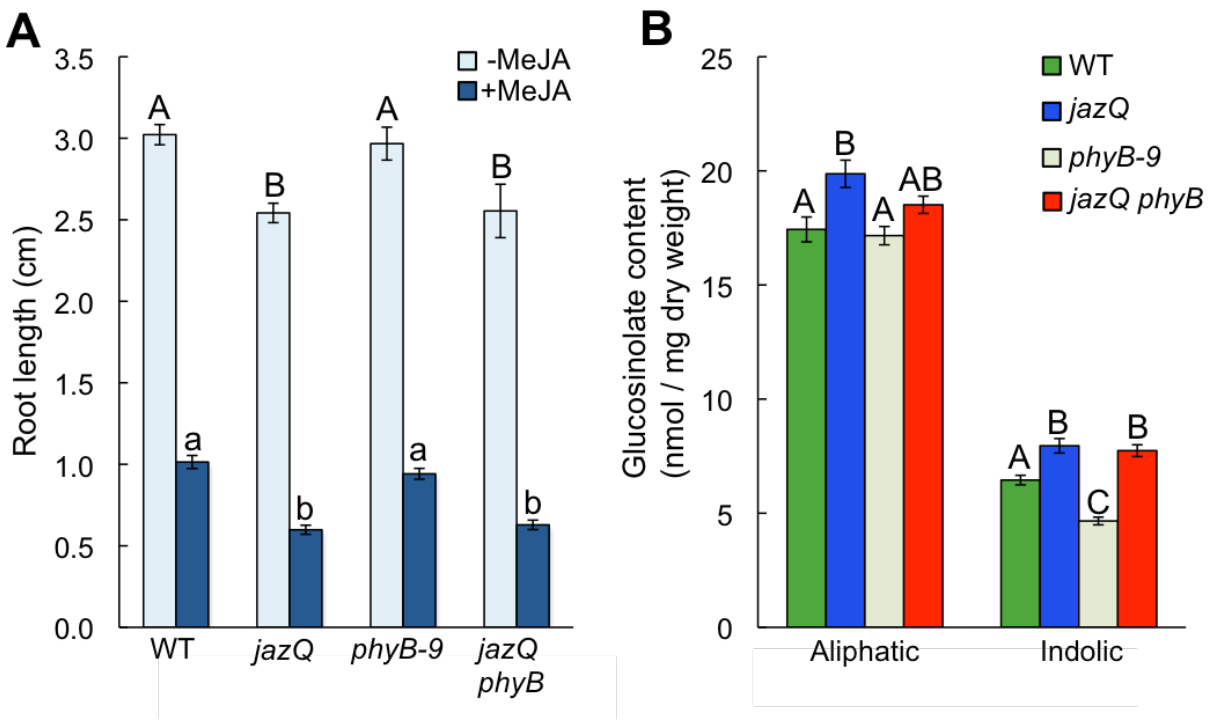


Figure 3.17. *jazQ phyB* is hypersensitive to JA and accumulates more glucosinolates than WT.

(A) Root length of WT, *jazQ*, *phyB-9* and *jazQ phyB* seedlings grown for eight days in MS medium supplemented or not with 20 µM MeJA. Data show the mean ± SE (n>12). Letters indicate statistical differences according to Tukey HSD-test (*p*-value < 0.05).
(B) Glucosinolate content of WT, *jazQ*, *phyB-9* and *jazQ phyB*. Data show the mean ± SE of ten samples per genotype. Letters indicate statistical differences according to Tukey HSD-test (*p*-value < 0.05).

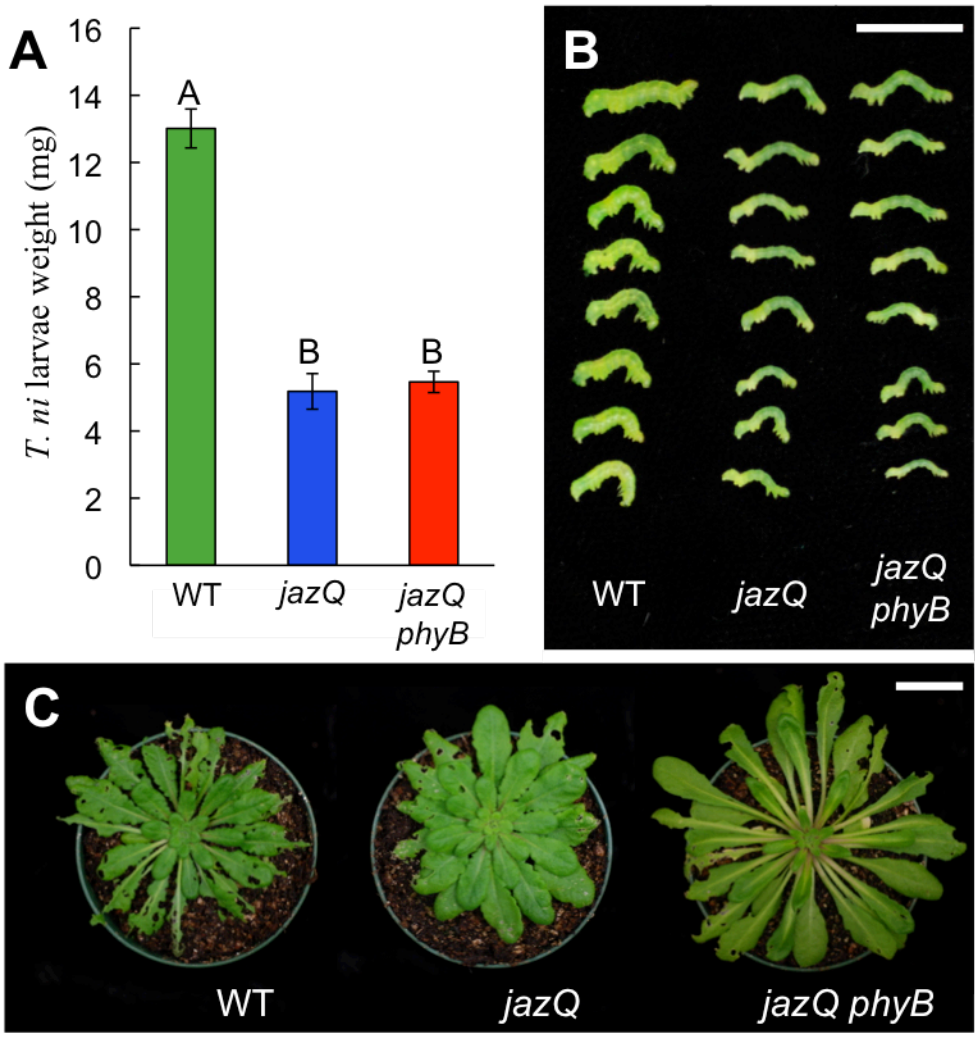


Figure 3.18. *jazQ phyB* is more resistant to insect herbivory.

(A) *Trichoplusia ni* weight after feeding on WT, *jazQ* and *jazQ phyB* plants. Neonate caterpillars were reared on genotypes for 10 days. Data represent the mean ± SE (n=12). Letters indicate statistical differences according to Tukey HSD-test (p-value < 0.05).
(B) Photograph of representative *T. ni* larvae recovered from WT, *jazQ* and *jaz phyB* after feeding period. Scale bar = 1 cm.
(C) Photograph of seven-weeks-old WT, *jazQ* and *jazQ phyB* plants at the end of feeding assay. Scale bar = 3 cm.

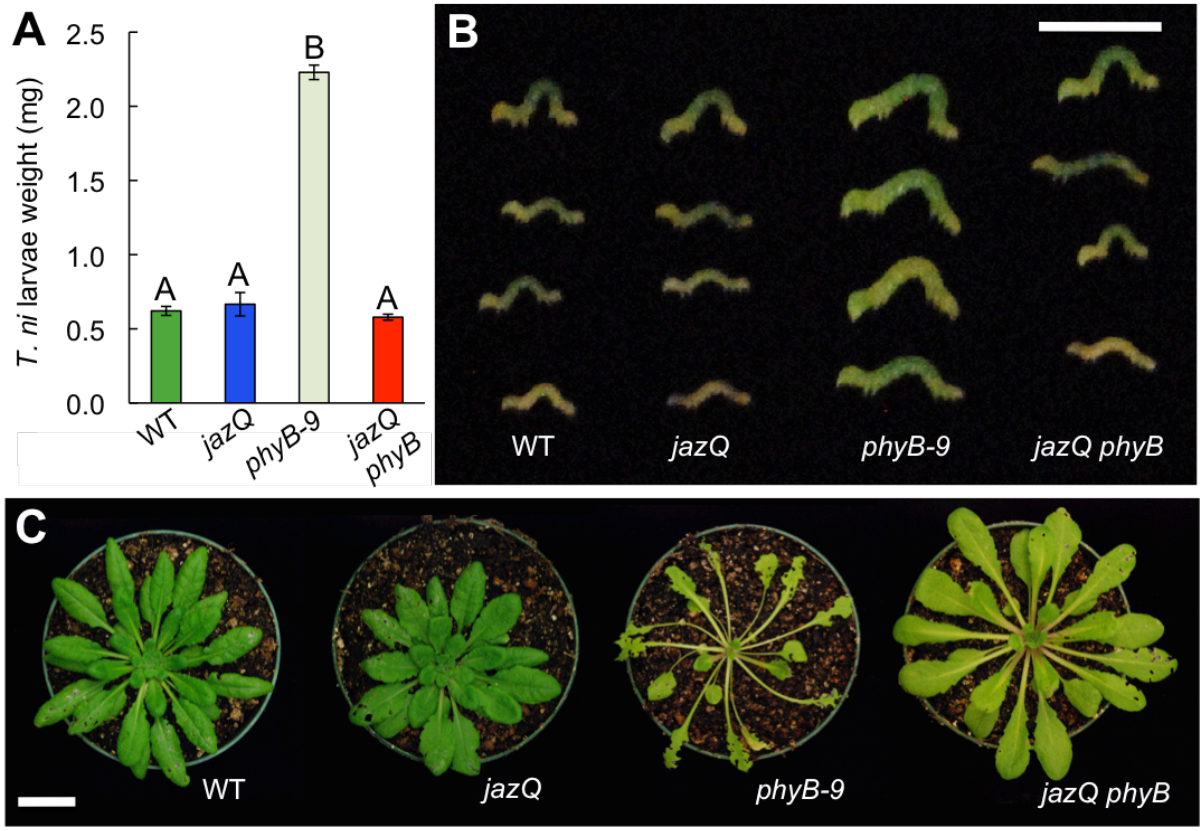
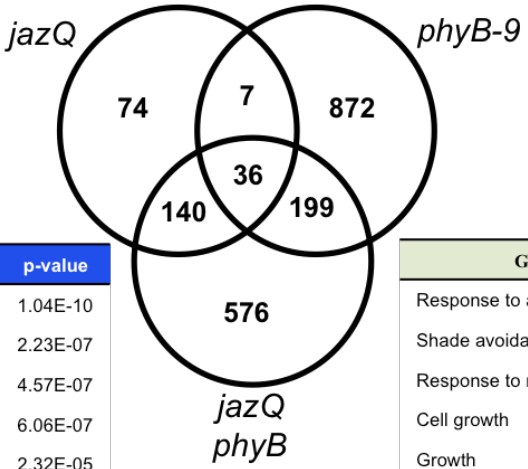


Figure 3.19. *phyB-9* plants are extremely susceptible to insect herbivory.

(A) *Trichoplusia ni* weight after feeding on WT, *jazQ*, *phyB-9* and *jazQ phyB* plants. Neonate caterpillars were reared on genotypes for five days. The experiment was halted at this time due to full consumption of leaf material in the *phyB-9* plants (C). Data represent the mean ± SE (WT, *jazQ* and *jazQ phyB* n=4; *phyB-9* n=12). Letters indicate statistical differences according to Tukey HSD-test (p-value < 0.05).
(B) Photograph of representative *T. ni* larvae recovered from WT, *jazQ*, *phyB-9* and *jaz phyB* after feeding period. Scale bar = 1 cm.
(C) Photograph of six-weeks-old WT, *jazQ, phyB-9* and *jazQ phyB* plants at the end of feeding assay. Scale bar = 3 cm.

Rewiring of transcriptional networks upregulate growth and defense in *jazQ phyB*

Results described above suggest two different patterns of resource allocation in the parental *jazQ* and *phyB-9* mutants. The removal of five *JAZ* genes leads to constitutive activation of JA-mediated defense responses but hindered growth in *jazQ*. On the other hand, impaired red light perception results in increased growth parameters at the cost of defense in *phyB-9*. The combination of *jazQ* and *phyB-9* appears to create a genetic background (*jazQ phyB*) in which both robust defense and growth are maintained. Given the direct role of JAZs and phyB in transcriptional control (Chen and Chory 2011; Jiao et al. 2007; Pauwels et al. 2008), we used RNA-seq to test the hypothesis that the uncoupling of growth-defense antagonism in *jazQ phyB* results from genome-wide re-programming of gene expression. Analysis of RNA-seq data from WT and mutant seedlings grown under identical conditions showed that the overall gene expression pattern of *jazQ phyB* represents the additive effect of defense processes that are activated in *jazQ* and the growth processes that are transcriptionally activated in *phyB-9* (Figure 3.20). A comparison of GO categories that are upregulated in both *jazQ* and *jazQ phyB*, for instance, identified defense-associated processes such as "Secondary metabolism", "JA biosynthesis" and "Response to wounding" (Figure 3.20, blue sector). As shown in Figure 3.21, genes involved with GS biosynthesis provide an example of a process that is upregulated both in *jazQ* and *jazQ phyB*. These results are consistent with the increased secondary metabolite content and enhanced resistance of *jazQ* and *jazQ phyB* (Figure 3.17 to 3.19). In agreement with the well-described antagonism between JA and the defense hormone salicylic acid (SA) (Robert-Seilaniantz et al. 2011), we found that SA responses are downregulated in *jazQ* and *jazQ phyB* (Figure 3.22, blue sector).



GO-Description	p-value
Secondary metabolic process	1.04E-10
Tricyclic triterpenoid metabolic process	2.23E-07
Jasmonic acid biosynthetic process	4.57E-07
Glucosinolate metabolic process	6.06E-07
Response to stress	2.32E-05
Response to wounding	9.67E-03
Phenylpropanoid biosynthetic process	2.16E-02

GO-Description	p-value
Response to auxin stimulus	6.71E-07
Shade avoidance	2.40E-05
Response to red or far red light	2.50E-05
Cell growth	6.02E-04
Growth	1.50E-03
Cell wall organization or biogenesis	3.46E-03
Cell maturation	3.98E-03

GO-Description	p-value
Cell wall organization or biogenesis	2.28E-06
Auxin polar transport	2.39E-04
Cell morphogenesis	1.21E-03
Regulation of cell size	2.11E-03
Cell growth	3.14E-03
Cell development	1.80E-02
Plant-type cell wall loosening	4.92E-02

Figure 3.20. The combination of *phyB-9* and *jazQ* leads to additive transcriptional effects in *jazQ phyB*.

Venn diagram showing number of upregulated genes in *jazQ*, *phyB-9* and *jazQ phyB* when compared to WT (Col-0). Gene ontology (GO) analysis indicates that *jazQ phyB* reflects the additive upregulated defense processes of *jazQ* (blue region) and the growth processes of *phyB* (pale-green region). The combination of *jazQ* and *phyB* mutations also leads to a reprogramming of processes that are specific to *jazQ phyB* (red region). Differentially expressed genes were called on the basis of a *p*-value <0.05 using the statistical package DESeq.

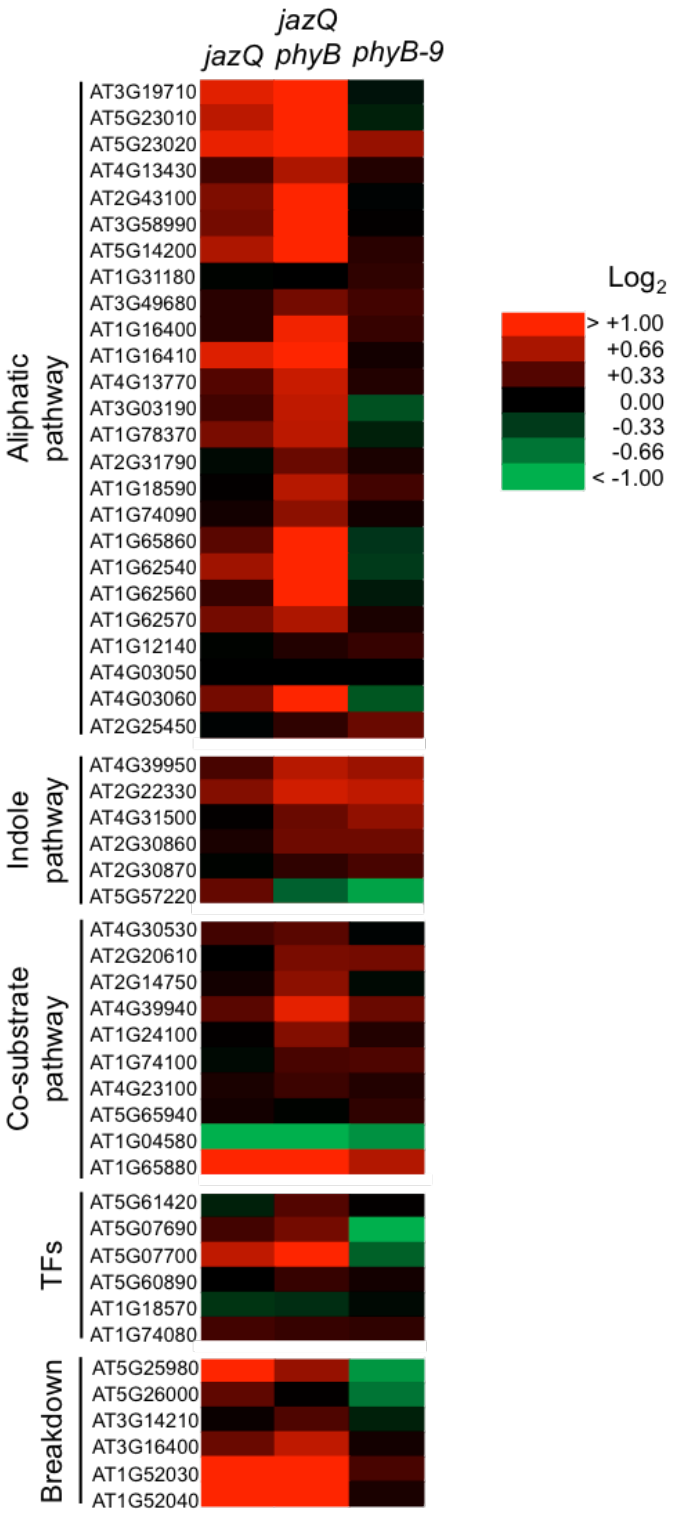
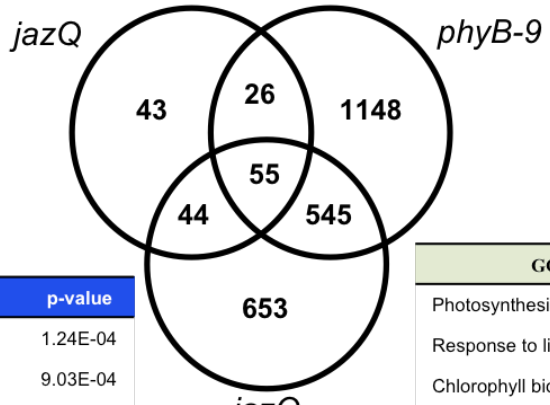


Figure 3.21. Genes associated with glucosinolate biosynthesis are upregulated in *jazQ* and *jazQ phyB* but partially downregulated in *phyB-9*.

Figure 3.21 (cont'd). Heat map showing the expression levels of genes involved in GS biosynthesis in *jazQ*, *phyB-9* and *jazQ phyB*. Genes were organized according to Sonderby et al. (2010). Values obtained by RNA-Seq represent fold changes (Log_2) over WT.



GO-Description	p-value
Response to chitin	1.24E-04
Regulation of transcription	9.03E-04
Response to ethylene stimulus	1.20E-03
Response to salicylic acid stimulus	1.10E-02
Response to gibberellin stimulus	4.03E-02

GO-Description	p-value
Photosynthesis	4.23E-54
Response to light stimulus	5.75E-11
Chlorophyll biosynthetic process	2.42E-05
Plastid organization	1.84E-04
Hexose metabolic process	3.34E-04
Carbon fixation	3.36E-04
Glucose catabolic process	4.50E-03

GO-Description	p-value
Response to temperature stimulus	6.55E-09
Starch catabolic process	6.45E-08
Chloroplast organization	9.27E-05
Response to oxidative stress	5.27E-04
Response to radiation	2.10E-03
RNA metabolic process	1.12E-02
Protein folding	1.60E-02

Figure 3.22. The combination of *phyB-9* and *jazQ* leads to additive and synergistic transcriptional reprogramming in *jazQ phyB*.

Venn diagram showing the number of downregulated genes in *jazQ*, *phyB-9* and *jazQ phyB* when compared to WT (Col-0). GO analysis was performed with differentially expressed genes and called on the basis of *p*-value of <0.05 using the statistical package DESeq.

A comparison of transcript profiles in *phyB-9* and *jazQ phyB* revealed that these two genotypes share upregulated GO categories related to growth, including "Response to auxin stimulus", "Shade avoidance", "Response to red or far-red light" and "Cell growth" (Figure 3.17, pale-green sector). Among the specific upregulated genes are members of the expansin family, including the *EXPA3* (AT2G37640), *EXPA5* (AT3G29030) and *EXPA14* (AT5G56320), which are involved with cell enlargement and growth (Cosgrove et al. 2000). We also observed that genes encoding TFs belonging to the PHYTOCHROME-INTERACTING FACTOR (PIF) family such as *PIF3-LIKE1* (*PIL1*, AT2G46970) and *REDUCED PHYTOCHROME SIGNALING1* (*REP1*, AT1G02340) are also strongly upregulated in *phyB-9* and *jazQ phyB*. These TFs are required for growth promotion during shade avoidance responses, which is mediated in large part by light conditions (low R:FR ratio) that decreases the activity of phyB (Lorrain et al. 2008).

Analysis of gene expression profile in *jazQ phyB* also revealed a large number of differentially regulated genes that were unique to this genotype (Figure 3.20 red zone). Among the genes that were uniquely upregulated in *jazQ phyB* were members of the expansin, extensin, pectinase and cellulase families involved in cell wall organization. The GO term "Cell wall organization or biogenesis" was also upregulated in *jazQ phyB* and included *POLTERGEIST LIKE1* (AT2G35350), *GLABRA3* (AT5G41315) and *ROTUNDIFOLIA3* (AT4G36380) that are involved in the control of meristem size and organ formation (Bancos et al. 2002; Cho and Cosgrove 2000).

Overexpression of *PHYTOCHROME INTERACTING FACTOR4* in *jazQ* partially recapitulates the *jazQ phyB* phenotype

A central component of the phytochromes transcriptional network are the basic helix-loop-helix (bHLH) PHYTOCHROME INTERACTING FACTORs (PIFs), TFs that promote a wide range of growth processes, including hypocotyl elongation, cell expansion, chloroplast differentiation and flowering (Chen and Chori 2011; Lorrain et al. 2008; Lucas and Prat 2014). Under light conditions indicative of shade or plant crowding (low R:FR ratios), however, phyB activity is reduced, thus allowing PIF to activate the expression of growth-related genes involved in shade avoidance responses (Leivar and Quail 2011; Lorrain et al. 2008; Lucas and Prat 2014; Park et al. 2012). PIF proteins also accumulate in phyB mutants, which display shade avoidance-like growth phenotypes (Park et al. 2004; Park et al. 2012; Soy et al. 2012). The observation that PIF overexpression partially restores JA-induced growth inhibition, together with the ability of JAZs to negatively regulate PIF stability (Yang et al. 2012), lead us to test the hypothesis that the enhanced growth stature of *jazQ phyB* involves increased activity of PIF4.

To test this, we analyzed the effects of PIF4 overexpression on the growth and defense phenotypes of *jazQ*. In two independent transgenic lines analyzed (#1-2 and #3-1), we found that *jazQ* plants expressing *PIF4* under the CaMV *35S* promoter (*35S:PIF4 jazQ*) have rosettes that are comparable in size to WT plants (Figure 3.23A-B). Petioles of both lines, however, accumulated anthocyanins to similar levels observed in *jazQ* (Figure 3.23C). Furthermore, insect feeding assays showed that *T. ni* larvae reared on *35S:PIF4 jazQ* plants gain less weight than larvae reared on WT, similar to the resistance observed for *jazQ* (Figure 3.24A-B). Based on these findings, we conclude that overexpression of PIF4 in *jazQ* background rescues at least

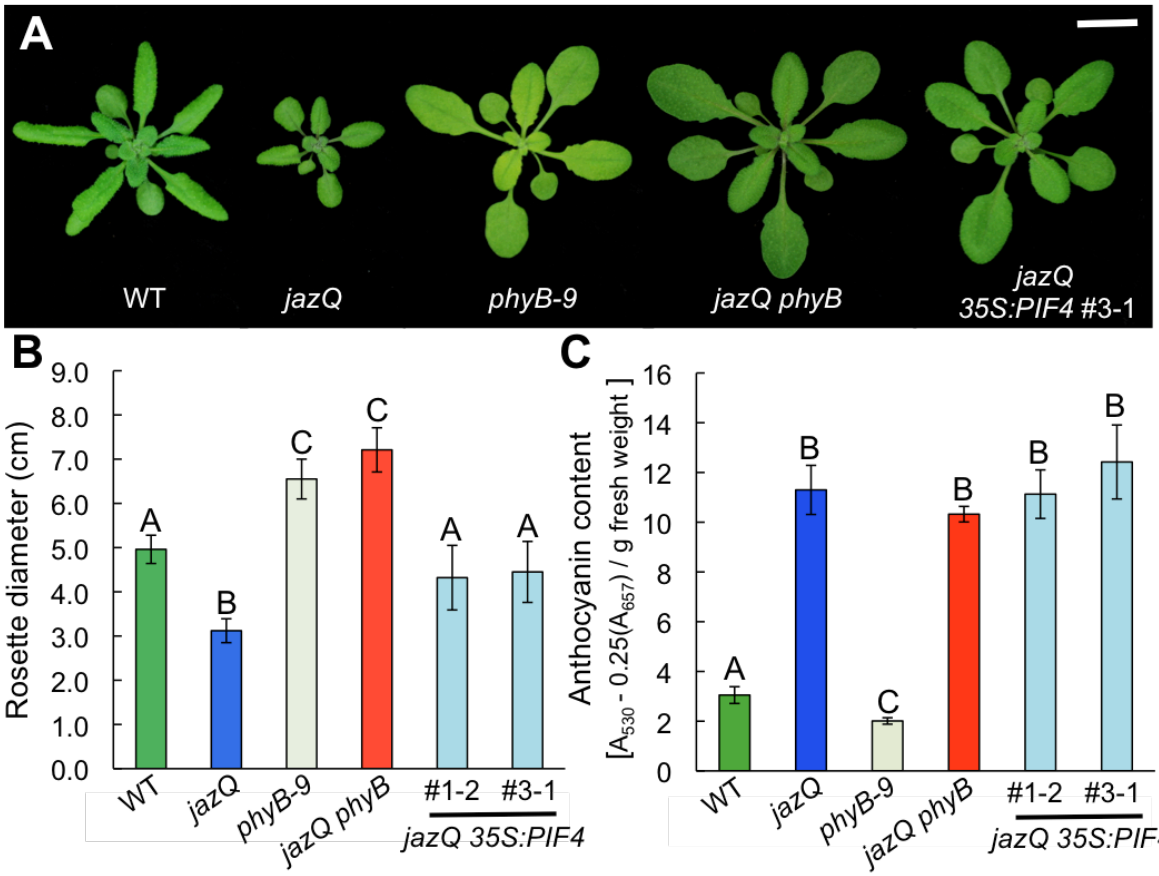


Figure 3.23. Overexpression of *PIF4* in *jazQ* leads to partial rescue of growth without affecting defense.

(A) Photograph of representative 21 days-old WT, *jazQ*, *phyB-9*, *jazQ phyB* and a representative line of *jazQ* overexpressing *PIF4* (#3-1). Scale bar = 1 cm.
(B) Rosette diameter of WT, *jazQ*, *phyB-9*, *jazQ phyB* and two independent *35S:PIF4 jazQ* lines (#1-2 and #3-1). Bars represent the mean ± SE (n>20). Letters indicate statistical differences according to Tukey HSD-test (*p*-value < 0.05).
(C) Anthocyanin content in petioles of WT, *jazQ*, *phyB-9*, *jazQ phyB* and the two *35S:PIF4 jazQ* lines (#1-2 and #3-1). Pigments were extracted from leaf petioles of 21days-old plants. Data represent the mean ± SE (n>10). Letters indicate statistical differences according to Tukey HSD-test (*p*-value < 0.05).

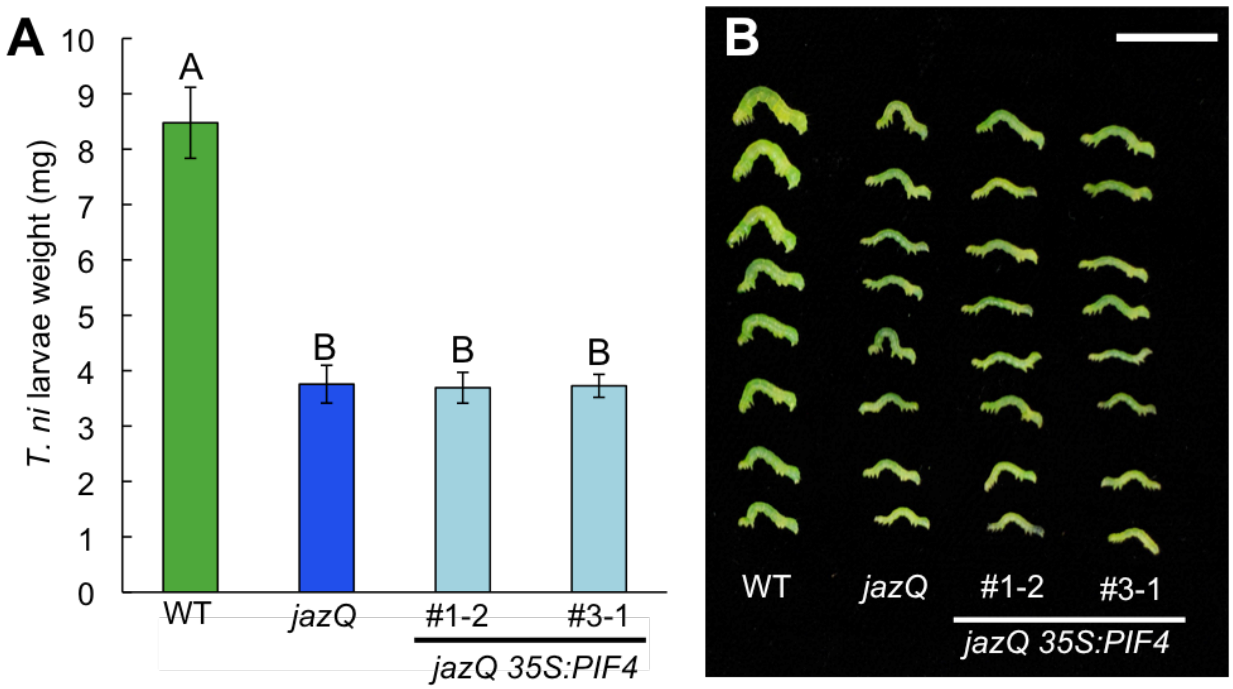


Figure 3.24. Overexpression of *PIF4* in *jazQ* does compromise resistance to insect herbivory.

(A) *Trichoplusia ni* larvae weight after feeding on WT, *jazQ* and two *35S:PIF4 jazQ* lines. *T. ni* neonate caterpillars were reared on six week-old for a period of 10 days. Data show the mean ± SE (n=12). Letters indicate statistical differences according to Tukey HSD-test (p-value < 0.05). **(B)** Photograph of representative *T. ni* larvae recovered from WT, *jazQ* and the two *jazQ 35S:PIF4* transgenic lines (#1-2 and #3-1) after 10 days of feeding. Scale bar = 1 cm.

some aspects of the slow-growth phenotype (e.g. petiole extension), but does not compromise the enhanced resistance of *jazQ* to insect feeding.

Discussion

The ability to perceive, integrate and trigger proper responses to surroundings signals is an essential feature for any living organism to prosper in their environment. As sessile organisms, plants utilize their developmental plasticity to respond to adverse conditions, shaping their phenotype in response to the most variable external cues. The growth versus defense paradigm postulates that developmental plasticity is limited by resource availability, thus resource allocation to growth will come at the detriment of defense and vice-versa. Poor knowledge of the molecular players and mechanisms controlling resource allocation decisions has impeded further understanding on how plants adapt their metabolic fluxes to respond to their surroundings. However, recent empirical lines of evidence suggests that intricate regulatory circuits have evolved to finely tune the growth versus defense balance in response to dynamic environments (Chen et al. 2013; Denancé et al. 2013; de Wit et al. 2013; Huot et al. 2014, Leone et al. 2014; Moreno et al. 2009; Wild et al. 2012; Yang et al. 2012). We demonstrate the relevance of the JA-phytochrome B module as a master regulator of resource allocation decisions. Impairment of key signaling components belonging to this module altered a vast array of processes at the transcriptional, metabolic and morphological level, leading to a compromise in the balance between growth and defense. Surprisingly, through genetic manipulation we were able to uncouple the JA-phythochrome B module and activate growth and defense simultaneously. Our results suggest that the growth versus defense duality is not controlled by resource availability

per se, but rather by hard-wired transcriptional networks that evolved to precisely modulate the developmental plasticity in response to an ever-changing environment.

JAZ proteins inhibit defense and promote plant growth

Our starting point to better understand the growth versus defense paradigm was to obtain a plant that was clearly shifted in resource allocation decisions. For this purpose we focused on JAs, lipid-derived plant hormones that are largely characterized with respect to their involvement in defense processes (Campos et al. 2014; Dombrowski 2008; Goodspeed et al. 2012; Li et al. 2004; Howe and Jander 2008; Wasternack and Hause 2013). Studies on the JA signaling pathway have been partially hindered by the functional redundancy among the JAZ family of repressors. However, by genetically knocking out multiple *JAZ* genes, we were able to generate a mutant that is hypersensitive to exogenous JA treatment, upregulated in the expression of defense related genes and accumulation of secondary metabolites and finally, more resistant to insect herbivory. Even though other *jaz* single mutants have demonstrated some degree of JA hypersensitivity (Demianski et al. 2012; Grunewald et al. 2009; Sehr et al. 2010), *jazQ* is, to our knowledge, the first *jaz* mutant described where JA responses are constitutively active and defense processes are clearly upregulated without the need of exogenous hormone treatment. Our findings that growth parameters such as rosette growth and flowering time are hindered in *jazQ* also highlights the tradeoff caused by the constitutive activation of the JA-pathway and the increased allocation of resources in defensive traits. Eight functional *JAZ* genes are still present in the *jazQ* genome and further knockout of those can not only promote the intensification of these growth and defense parameters, but also lead to the discovery of distinct plant phenotypes

that may designate novel biological roles performed by the JAZ repressors (and the JA pathway itself).

The shift in the resource allocation decisions described for *jazQ* is in agreement with numerous described roles of JAs in plant development. For example, the higher anthocyanin and GS content in *jazQ* correlates with the described role of JA as a positively regulator of the biosynthesis of these secondary metabolites (Browse and Howe 2008; De Geyter et al. 2012; Qi et al. 2011; Shan et al. 2009; Schweizer et al. 2013). It is also known that the JAZ repressors can physically interact with and inhibit the action of TFs involved with anthocyanin and GS production (Fernández-Calvo et al. 2011; Qi et al. 2011; Schweizer et al. 2013). The expression of defense related genes such as *AOS*, *AOC*, *MYC* and *TGG2*, which are found to be upregulated in *jazQ*, is also under control of the JA pathway (Attaran et al. 2014; Chung et al. 2008). Recent evidences also indicate that the JAZ proteins can associate with different transcriptional modulators of growth-related pathways. One example is the DELLA, which act as repressors of numerous growth-related processes (Leone et al. 2014; Wild et al. 2012; Yang et al. 2012). DELLA interacts with PIFs, for example, to impede these TFs to promote the expression of growth-related genes. According to proposed models (Kazan and Manners 2012; Yang et al. 2012), JAZ removal by JA-mediated degradation (or by genetic manipulation as in *jazQ*) would disrupt the JAZ-DELLA interaction, releasing the DELLAs to further associate with PIFs (and other growth related TFs) and inhibit the activation of growth processes.

In conclusion, though a genetic manipulation that knocked out five *JAZ* genes from a plant's genome, we were able to overcome the functional redundancy among the JAZ family members and obtain a genotype that is shifted in the allocation of resources to growth and defense when compared to WT plants. The downregulation of growth processes caused by the

absence of multiple the *JAZ* genes indicate that they are essential promoters of plant growth, performing this function by inhibiting the allocation of resources to defense responses through repression of the JA signaling pathway.

Suppressors of *jazQ* identify new components involved with resource allocation decisions

In an attempt to isolate key components involved in resource allocation decisions, we EMS-mutagenized *jazQ* to isolated a total of 73 mutants that enhanced or suppressed its growth and defense parameters. Effort was given to further characterize mutants that suppressed the hindered growth observed in *jazQ* since these could provide novel mechanistic information on how the activation of JA pathway affect growth processes. Two of these lines carried mutations that impaired JA biosynthesis or signaling: *AOS* in *sjq10* and *COI1* in *sjq66*. These results demonstrate that the phenotypes observed in *jazQ* are mainly caused by a positive feedback mechanism that involves the upregulation of genes involved with JA biosynthesis and further degradation of the remaining JAZ repressors (Browse 2008; Campos et al. 2014; Kazan and Manners 2008). In agreement with this hypothesis, we did observe higher expression of genes involved with JA biosynthesis (such as *AOS*, *AOC* and *OPLC1*) and enrichment in the GO category "Jasmonic acid metabolic process" in *jazQ* (Table 3.1). Quantification of the endogenous levels of JAs in the high order mutant may confirm this hypothesis.

The identification of *phyB* as the causal mutation leading to upregulated growth and defense in *sjq11* was based on the long hypocotyl phenotype observed when this mutant was grown under constant white light (Figure 3.11). Interestingly, among the 51 *sjq* lines isolated, only *sjq11* showed this phenotype (data not shown), suggesting that impairment of other genes than *PHYB* also lead to suppression of the growth phenotype of *jazQ*. The identification of these

mutations can provide exciting evidence for the involvement of novel components and pathways in controlling the flux of resource allocation. Phenotypic characterization of the genetically reconstituted *sjq11*, *jazQ phyB*, confirmed that the large rosettes, early flowering phenotype and improved defense parameters observed in *sjq11* was caused by the impairment in *phyB* when in the *jazQ* genetic background. One interesting aspect of this finding is the observation that *phyB* mutant presents augmented growth (Figure 3.16; Mockler et al. 1999), but is severely impacted in defense processes (Figure 3.19; Cerrudo et al. 2012; Moreno et al. 2009). These phenotypes are contrasting as compared to *jazQ* (less defense, more growth), indicating that the activation of specific transcriptional circuits can culminate in strikingly different patterns of resource allocation. Surprisingly, results obtained with *jazQ phyB* suggest that both pathways could be activated in parallel to allow concomitant upregulation of growth and defense. This hypothesis is corroborated by the phenotype of transgenic *35S:PIF4 jazQ* plants, which are upregulated in defense responses, but show similar growth as WT (Figures 3.23 and 3.24). The observation that *35S:PIF4* plants do not display rosettes as large or flowering as early as *jazQ phyB* suggest that the expression of other PIF family members, such as *PIL1* and *REP1* (whose expression is found to be upregulated in *jazQ phyB*) may be fundamental to fully suppress the hindered growth in *jazQ* plants.

Uncoupling JA and phytochrome B transcriptional networks to activate growth and defense

The JA and phytochrome B transcriptional circuits evolved as important sensors of environmental signals and their action results in significant changes in plant development. Besides triggering antagonistic physiological responses (growth versus defense), these two

networks operate in a remarkably similar manner (Figure 3.25): Both pathways are regulated by environmental signals that are capable of inducing molecular changes in transcriptional regulators (JAZ and phyB), releasing a set of basic helix-loop-helix (bHLHs) TFs that bind similar DNA motifs (G-box) to induce developmental responses. In the case of phyB, alterations in R:FR light ratios lead to its dissociation from PIFs, allowing this family of TFs to bind DNA and trigger responses such as organ elongation and cell growth (Chen and Chori 2011, Lorrain et al. 2008). Environmental cues that activate the JA pathway (such as mechanical wounding) promote the degradation of the JAZ repressors, relieving TFs such as MYCs from repression to activate the expression of defense-related genes (Browse 2008; Campos et al. 2014). To correctly modulate developmental plasticity, these two transcriptional circuits evolved as a binary module, where activation of one is associated to deactivation (repression) of the other. For this purpose, plants utilize different nodes of crosstalk that precisely regulate the status of the module. One of the aforementioned nodes in this crosstalk are the DELLA proteins, which physically associate with the JAZ and PIFs to regulate growth and defense processes (Kazan and Manners 2010; Yang et al. 2012). A different scenario of regulation may occur at the DNA sequence level, where PIFs and MYCs would compete for binding to same gene promoters. Even though these two families of bHLH TFs regulate different sets of responses in the JA-phytochrome B module (growth for PIFs and defense for MYCs), they do bind to the same cis-regulatory elements (the G-boxes) and can act either as activators or repressors of transcription (Yadav et al. 2005; Zhang et al. 2013). Because the G-box element is enriched in promoters of growth-associated/light responsive genes and also defense-related genes (Martínez-Garcia et al. 2000; van der Burg et al. 2008), it is possible that when growth or defense processes are activated, the TFs associated with it (PIFs or MYCs) would promote the expression of the genes involved with that response but

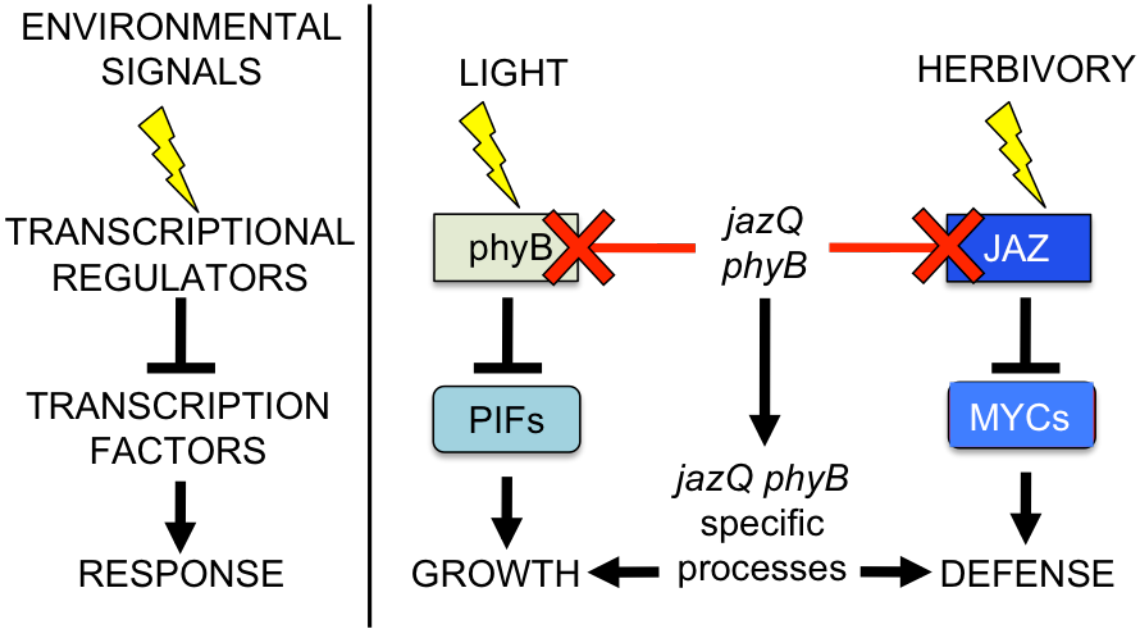


Figure 3.25. Removal of transcriptional regulators rewires a regulatory network to allow concomitant activation of growth and defense.

Phytochrome B (phyB) and JAZ proteins work as transcriptional regulators that inhibit the action of transcription factors associated with growth (e.g. PIFs) and defense (e.g. MYCs). Environmental signals such as red light and herbivory trigger alterations in the molecular status of phyB and JAZ, respectively, releasing the transcription factor association that promote growth or defense. Genetic removal of phyB and five JAZ proteins in *jazQ phyB* rewire these regulatory networks, promoting the concomitant activation of growth and defense and also the activation of processes that are now active only in the *jazQ phyB* background.

concomitantly repress the expression of other genes associated with the opposing process. Indeed, it has been shown, for example, that MYC2 can bind to and negatively regulate the expression of light-responsive genes (Yadav et al. 2005).

Surprisingly, our results with *jazQ phyB* indicate that the JA-phytochrome B module can be genetically uncoupled. The concomitant removal of multiple transcriptional regulators (*phyB* and five *JAZ* genes) allowed the activation of these two transcriptional circuits in parallel, resulting in activation of both growth and defense in *jazQ phyB* (Figure 3.25). Epistatic relationships between *jazQ* and *phyB* are generally consistent with a model in which the MYC and PIF transcriptional programs in *jazQ phyB* are uncoupled and operate independently of one another. In general, *jazQ* was epistatic to *phyB* with respect to defense phenotypes (insect resistance, secondary metabolism, and JA hypersensitivity) whereas *phyB* was epistatic to *jazQ* with respect to growth (rosette diameter, petiole and hypocotyl length, and flowering time) (Table 3.3). An interesting exception was rosette biomass; although both *jazQ* and *phyB* parental lines have low biomass relative Col-0, the biomass of *jazQ phyB* is similar to that of Col-0. This finding suggests that the combined transcriptional output of the MYC and PIF transcriptional programs act synergistically to drive biomass accumulation through metabolic pathways that are unique to *jazQ phyB*. In future studies, it will be interesting to analyze the specific components of biomass in this mutant. Our findings suggest that growth-defense antagonism may not be controlled by limitations on metabolic resources but rather by hard-wired transcriptional regulatory programs that exert control over resource partitioning in response to external signals. At this point it still uncertain if the parallel activation of these two pathways may result in a fitness penalty when these plants are grown in more dynamic conditions such as natural environments. However, it is clear that these two regulatory networks are interesting targets for

Table 3.3. Epistatic relationships between *jazQ* and *phyB*.

	TRAIT	RELATIVE TO WILD TYPE		
		jazQ	*phyB-9*	*jazQ phyB*
Growth	Petiole length	↓	↑	↑
	Hypocotyl length[1]	n.a	↑	↑
	Rosette diameter	↓	↑	↑
	Rosette biomass	↓	↓	n.a
	Flowering time	Late	Early	Early
Defense	Glucosinolate accumulation[2]	↑	↓	↑
	Anthocyanin accumulation	↑	↓	↑
	Sensitivity to exogenous JA	↑	n.a	↑
	Expression of defense-related genes	↑	↓ / n.a	↑
	T. ni resistance	↑	↓	↑

n.a., not affected relative to WT

[1]Indicates hypocotyl length of seedlings grown in red light.

[2]Indicates bulk accumulation of indole and aliphatic glucosinolates. The levels of specific glucosinolates did not always fit this pattern.

bioengineering research, in efforts to produce crop cultivars with increased productivity and reduced necessity of pesticide application.

Methods

Plant material and growth conditions

The Columbia ecotype (Col-0) of *Arabidopsis thaliana* was used as a wild-type (WT) parent for all experiments. Soil-grown plants were maintained at 20 +/- 1^{o}C under 16 h light/8 h dark photoperiod and 120 μE m^{-2} s^{-1} light intensity, unless otherwise noted. For the initial 10 days after seed sowing, trays containing potted plants were covered with a transparent plastic dome to increase humidity. For experiments involving growth of seedlings on agar plates, seeds were surface sterilized for 15 min in a solution containing 50% (v/v) bleach and 0.1% (v/v) Triton X-100, washed 10 times with sterile water and then stratified in dark at 4^{o}C for 2 days. Seeds were then sown on 0.7% (w/v) agar media containing half-strength Murashige and Skoog (MS; Caisson Labs) salts supplemented with 0.8% (w/v) sucrose. Transfer DNA (T-DNA) insertion mutants used for construction of *jazQ* were obtained from the *Arabidopsis* Biological Research Center (ABRC; The Ohio State University) and are named as follows: *jaz1-SM* (*jaz1*, JIC-SM.22668), *jaz3-GK* (*jaz3*, GK-097F09), *jaz4-1* (*jaz4*, SALK_141628), *jaz9-GK* (*jaz9*, GK-265H05) and *jaz10-1* (*jaz10*, SAIL_92_D08). The position of T-DNA insertion within each gene is shown diagrammatically in Figure 3.1A. *jaz3-GK* and *jaz9-GK* lines were backcrossed to Col-0 to remove unlinked T-DNA insertions. *jaz10-1* was backcrossed to Col-0 to remove a *qrt1-2* mutation present in SAIL lines (McElver et al. 2001). *jaz4-1* and *jaz10-1* mutants were previously described (Jiang et al. 2014; Demianski et al. 2013; Sehr et al. 2010). The *jazQ phyB*

sextuple mutant was obtained from a genetic cross between *jazQ* and the *phyB* reference allele *phyB-9* (Reed et al. 1993).

PCR and qPCR analysis

PCR-based genotyping of *jazQ* and lower-order mutants relied on primer sets flanking T-DNA insertion sites, together with a primer recognizing the border of the inserted T-DNA (Table 3.4). Reverse transcription-PCR (RT-PCR) was used to confirm the presence or absence of *JAZ* transcripts in WT and *jazQ* plants (Figure 3.1B). For this purpose, RNA was extracted from 8-day-old seedlings grown on MS plates containing 20 μM of MeJA. Frozen tissue was homogenized with a mortar and pestle and RNA was extracted using an RNeasy kit (Qiagen) followed by on-column DNase (Qiagen) treatment. cDNA was reverse transcribed from 1μg total RNA with a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, ABI). RT-PCR was performed using primers designed to amplify the five *JAZ* genes and the internal control ACTIN1 (AT2G37620) (Table 3.4). RT-PCR reactions were performed with the following conditions: 94^{o}C for 5 min, followed by 30 cycles of 45 sec at 94^{o}C for denaturation, 30 sec at 52^{o}C for annealing and 1.5 min at 72^{o}C for elongation. Final elongation step was performed at 72^{o}C for 10min and completed reactions were maintained at 12^{o}C. Forty elongation cycles were used to detect *JAZ4* transcripts, which accumulate at low levels in WT plants (Chung et al. 2008).

qPCR-based measurement of mRNAs was performed with RNA extracted from WT and *jazQ* 8-d-old seedlings grown on MS medium not supplemented with JA. RNA extraction and cDNA synthesis was performed as described above. Transcript quantification was evaluated on a

Table 3.4. List of PCR primers used in this chapter.

TARGET	NAME	PRIMER SEQUENCE	NOTES
		Genotyping	
JAZ1	JAZ1_GenFw	5'-ACCGAGACACATTCCCGATT-3'	
	JAZ1_GenRv	5'-CATCAGGCTTGCATGCCATT-3'	
	dSpm32_alt	5'-ACGAATAAGAGCGTCCATTTTAGAG-3'	
JAZ3	JAZ3_GenFw	5'-ACGGTTCCTCTATGCCTCAAGTC-3'	
	JAZ3_GenRv	5'-GTGGAGTGGTCTAAAGCAACCTTC-3'	
	pAC161-LB1	5'-ATAACGCTGCGGACATCTACATT-3'	
JAZ4	JAZ4_GenFw	5'-TCAGGAAGACAGAGTGTTCCC-3'	
	JAZ4_GenRv	5'-TGCGTTTCTCTAAGAACCGAG-3'	
	pROK2-LB3	5'-TTGGGTGATGGTTCACGTAG-3'	
JAZ9	JAZ9_GenFw	5'-TACCGCATAATCATGGTCGTC-3'	
	JAZ9_GenRv	5'-TCATGCTCATTGCATTAGTCG-3'	
	35S-rseq1	5'-CTTTGAAGACGTGGTTGGAACG-3'	
JAZ10	JAZ10_GenFw	5'-ATTTCTCGATCGCCGTCGTAGT-3'	
	JAZ10_GenRv	5'-GCCAAAGAGCTTTGGTCTTAGAGTG-3'	
	pCSA110-LB4	5'-GTCTAAGCGTCAATTTGTTTACACC-3'	
		Quantification of transcript levels by qRT-PCR	
JAZ1	JAZ1_RTFw	5'-ATGTCGAGTTCTATGGAATGTTCTG-3'	
	JAZ1_RTRv	5'-TCATATTTCAGCTGCTAAACCGAGCC-3'	
JAZ3	JAZ3_RTFw	5'-ATGGAGAGAGATTTTCTCGGG-3'	
	JAZ3_RTRv	5-'TTAGGTTGCAGAGCTGAGAGAAG-3'	
JAZ4	JAZ4_RTFw	5'-ATGGAGAGAGATTTTCTCGG-3'	
	JAZ4_RTRv	5'-CAGATGATGAGCTGGAGGAC-3'	40 cycles of elongation in PCR cycle were used to detect a JAZ4 transcript.
JAZ9	JAZ9_RTFw	5'-ATGGAAAGAGATTTTCTGGGTTTG-3'	
	JAZ9_RTRv	5'-TTATGTAGGAGAAGT AGAAGAGTAATTCA-3'	
JAZ10	JAZ10_RTFw	5'-ATGTCGAAAGCTACCATAGAAC-3'	
	JAZ10_RTRv	5'-GATAGTAAGGAGAT GTTGATACTAATCTCT-3'	
ACT1	ACT1_RTFw	5'-ATGGCTGATGGTGAAGACATTCAA-3'	
	ACT1_RTRv	5'-TCAGAAGCACTTCCTGTGAACAAT-3'	
		qPCR	
AOS	AOS_Fw	5'-GGAGAACTCACGATGGGAGCGATT-3'	As in Attaran et al., 2014.
	AOS_Rv	5'-GCGTCGTGGCTTTCGATAACCAGA-3'	As in Attaran et al., 2014.
CAB3	CAB3_Fw	5'-CGGAAAGTGAGCCAAGTTTTATCAG-3'	As in Attaran et al., 2014.
	CAB3_Rv	5'-AGTCTCAAACCATCACATACAACCT-3'	As in Attaran et al., 2014.

Table 3.4 (cont'd).

		Quantification of transcript levels by qRT-PCR	
LHCB2.4	LHCB2.4_Fw	5'-GGCCACTTCAGCAATCCAAC-3'	
	LHCB2.4_Rv	5'-GACGGTACGACGCATGATGA-3'	
MYC2	MYC2_Fw	5'-AGAAACTCCAAAT CAAGAACCAGCTC-3'	
	MYC2_Rv	5'-CCGGTTTAATCGA AGAACACGAAGAC-3'	
PIF4	PIF4_Fw	5'-GCCGATGGAGATGTTGAGAT-3'	
	PIF4_Rv	5'-CCAACCTAGTGGTCCAAACG-3'	
THAS	THAS_Fw	5'-ATGTACGGGGTCAGCGATTG-3'	
	THAS_Rv	5'-ATGAACCATCCACCGTTTGC-3'	
TGG2	TGG2_Fw	5'-CAGCACAGAAGCTCGCCTTT-3'	
	TGG2_Rv	5'-GACCAGGGGGTTGACCATTT-3'	
		Identificaton of causal mutations in *sjq*s	
AOS	AOS_F1	5'-CAAAATATGGATACGGGACA-3'	As in Niu et al. 2011
	AOS_F3	5'-AAAACTAGTATGGCT TCTATTTCAACCCCT-3'	
	AOS_F4	5'-CTTCCTCCTCAAGTCATCTCG-3'	
	AOS_R3	5'-AAAACTAGTCTAAAAG CTAGCTTTCCTTAACG-3'	
	AOS_R4	5'-CGTAGAAAGCTCGAGCCAAG-3'	
COI1	COI1-f3	5'-ATGGAGGATCCTGATATCAAG-3'	
	COI1-f5	5'-GTAGCTGAGATCTGACCACTGCAA-3'	
	COI1-fseq1	5'-AGCATCGTTACACACTGCAGGA-3'	
	COI1-rseq1	5'-TTGCATTCATATCCCTTATCTCC-3'	
	COI1-r2	5'-ATTGCTCGCTCACTGAAGCAAC-3'	
	COI1-r5	5'-GCTCTCAGAAGTCAACACCATGGA-3'	
PHYB	PHYBgenFw	5' GAAGAAACCAAACTTGTATAGTACG-3'	
	PHYBgenRv	5' AATTTCAACTTTTTGGACGG-3'	

7500 Fast Real-Time PCR system (Applied Biosciences) using the protocol described by Attaran et al. (2014). Primers utilized for experiments are listed in Table 3.4.

Root growth assays

The effect of exogenous JA on seedling root growth inhibition was determined as previously described (Shyu et al. 2012). Seedlings were grown on square Petri plates (Fisher) containing MS medium supplemented with the indicated concentration of methyl-JA (MeJA; Sigma-Aldrich). Plates were incubated vertically in a growth chamber maintained at 21^{o}C under continuous light for 8d. The primary root length was measured with the use of ImageJ software (http://imagej.nih.gov/ij/). WT and mutant lines were grown on the same plate to control for plate-to-plate variation.

Quantification of secondary metabolites

Anthocyanins were quantified as described by Spitzer-Rimon et al. (2010), with minor modifications. Petioles were excised from 4-week-old plants and extracted in 1 ml methanol containing 1% (v/v) HCl. Samples were incubated overnight at 4°C with constant agitation. Anthocyanin pigments in the resulting extract were measured spectrophotometrically and calculated as A_{530} - $0.25(A_{657}).g^{-1}$ fresh weight. Glucosinolates were quantified as described by Barth and Jander (2006) with minor modifications. Eight-day-old seedlings grown on MS plates in the absence of MeJA were collected into two-mL tubes (approximately 50 seedlings per tube) and immediately frozen in liquid nitrogen. WT and mutant lines were grown on the same plate to control for plate-to-plate variation. Frozen tissue was lyophilized, ground to a fine powder and extracted with 1 mL of 80% MeOH containing an internal standard (25 nmol sinigrin Sigma-

Aldrich). Samples were briefly vortexed, incubated at 75°C for 15 min, and then centrifuged at room temperature at 10,000 x g for 10 min. Resulting supernatants were applied to Sephadex A-25 columns (Amersham). Desulfoglucosinolates were eluted with a solution containing 30 μL of sulfatase (3.0mg mL^{-1}; Sigma) and 70 μL water (HPLC-graded). Following an overnight incubation in the dark at room termperature, 200 μL 80% MeOH and 200 μL water were added to each sample. Samples were then lyophilized for 2 h and dissolved in 100 μL water. Desulfoglucosinoaltes were detected by HPLC and quantified as described (Barth and Jander 2006). For each independent experiment, ten biological replicates per genotype were used for quantification of anthocyanin and glucosinolates levels.

Insect feeding assays

Insect feeding assays were performed with soil-grown plants maintained in a growth chamber at 19°C and a photoperiod of 8 hrs light (120 $\mu M.m^{-2}.s^{-1}$)/16 hrs dark. Neonate *Trichoplusia ni* larvae (Benzon Research) were transferred to the center of fully expanded rosette leaves of six-week-old plants, as previously described (Herde et al. 2013). Four larvae were reared on each of 12 plants per genotype. Plants were then covered with a transparent dome and returned to the chamber for ~10 days, after which larval weights was measured.

Rosette phenotypes and flowering time

Rosette and flowering phenotype characterization was performed with three to four week-old soil-grown plants using at least 10 plants per measurement, unless indicated otherwise. Petiole length of the excised 3rd true leaf was measured with a caliper. At this time stage in development, the total number of true leaves on each rosette was counted to assess the developmental status of

each genotype. Bolting time was measured in a separate set of plants by scoring the number of true leaves on the main stem and the number of days from sowing until bolting (i.e., flower buds visible in the center of the rosette). The same set of plants was subsequently used to measure the length of time to opening of the first flower. Rosette diameter and projected leaf area were determined by photographing rosettes with a Nikon D80 camera. The resulting images were used to calculate Feret diameter using ImageJ (http://imagej.nih.gov/ij/) and total area leaf using GIMP software (http://www.gimp.org). Leaf dry weight was determined by weighing the excised rosette (without roots) after freeze drying for two days in a lyophylizer.

jazQ mutagenesis experiment and identification of causal mutations in *sjq* plants

Approximately 50,000 *jazQ* seeds were mutagenized by immersion in a solution of 0.1% or 0.2% (v/v) ethyl methanesulfonate (EMS, Sigma-Aldrich) for 16 hr at room temperature, with constant agitation. Seeds (M_1 generation) were thoroughly washed with H_2O, stratified in dark at 4^oC for two days and then immediately sown on soil. M_2 seed was obtained from 16 pools of self-pollinated M_1 plants (approximately 1,000 M_1 plants/pool). Soil-grown M_2 plants (~2000 plants/pool) were visually screened for individuals in which one or more *jazQ* phenotypes, including compact rosette size, delayed flowering time and anthocyanin accumulation were altered. Putative *sjq* (*suppressors of the jazQ*) and *ejq* (*enhancers of the jazQ*) mutants were rescreened in the M_3 generation to confirm heritability of phenotypes.

Identification of causal mutations in *sjq10* and *sjq66* was based on JA-phenotypes observed for plants grown in soil and MS plates containing 25 μM MeJA. PCR primers for amplification *AOS* and *COI1* genes in *sjq10* and *sjq66*, respectively, are described in Table 3.4. Insight into the causal mutation in *sjq11* was obtained from hypocotyl elongation assays

performed with monochromatic light as described by Fankhouser and Casal (2004), with minor modifications. WT, *jazQ* and *sjq11* (M_3 generation) seeds were plated on MS medium lacking sucrose and stratified at 4°C in dark for two days. To improve synchronous seed germination, a 3 hr pulse of white light was administered. Plates were then returned to darkness for one day at 21°C and then transferred to monochromatic LED chambers outfitted to emit blue (470 ± 20 nm – 5 μE), red (670 ± 20 nm – 25 μE) or far-red (740 ± 20 nm – 5 μE) light. As controls, the light-sensing mutants *phyA-75*, *phyB-9* and *cry1-400* were included (gift from Dr. Rob Larkin, Michigan State University, see Reed et al. 1993; Ruckle et al. 2007) and a set of plates containing each genotype was maintained in darkness. WT and mutant lines were grown on the same plate to control for plate-to-plate variation. Following three days of grown under the specified monochromatic light condition, seedling hypocotyls were scanned for length measurement using the ImageJ software.

Allelism tests were performed with F_1 plants obtained from the cross between *sjq11* and *phyB-9*. Seedlings were grown on MS medium plates and incubated horizontally in a growth chamber maintained at 21°C under continuous light for 3 d.

Global gene expression profiling (RNA-seq)

Global gene expression profiling in 8-day-old whole seedlings was assessed by mRNA sequencing (RNA-seq) performed on the Ilumina HiSeq 2000 platform. Seedlings were grown on solid MS medium supplemented with sucrose but not JA, and harvested for RNA extraction (~200 seedlings per RNA extraction). WT and mutant seedlings were grown on the same plate to minimize plate-to-plate variation. Two independent RNA-seq experiments were performed. The first experiment compared expression profiles of WT and *jazQ* only, whereas the second

experiment compared transcript abundances in WT, *jazQ*, *phyB-9* and *jazQphyB*. In both experiments, three independent RNA samples (biological replicates) were sequenced per genotype. Total RNA was isolated as described above and RNA integrity was assessed on a 2100 Bioanalyzer (Agilent Technologies). All samples used for sequencing had an integrity score of at least 7.0.

Single-end (50 bp) sequencing was performed at the Michigan State University Research Technologies Service Facility (https://rtsf.natsci.msu.edu). Barcoded sequencing libraries were constructed using the Illumina RNAseq kit according to the manufacturer's instructions. Samples were multiplexed in six libraries per lane. The average number of sequencing reads were 22.1 ± 1.6 and 18.42 ± 4.3 million reads per sample in the first and second experiment, respectively. Raw sequencing reads were assessed with Illumina quality control tools filters and FASTX toolkit (http://hannonlab.cshl.edu /fastx_toolkit/). Reads were mapped to gene models in the TAIR10 with the program RSEM (version 1.2.11) set for default parameters (Li and Dewey 2011). Data was expressed as transcripts per million reads (TPM; Wagner et al. 2012). DESeq, (version 1.18.0; Anders and Huber 2010) was used to assess differential gene expression by comparing TPM values in WT to that in the mutant line (Figure 3.26). Gene onthology (GO) analysis of enriched functional categories was performed using BinGO (version 2.44, Maere et al. 2005). The default Benjamini & Hochberg multiple testing correction was used to calculate over- and underrepresented GO categories among differentially expressed genes, using a *P* value of <0.05. The same RNA utilized for high throughput sequencing was used in experiments to validate sequencing data by qPCR (Figure 3.27), as described in Attaran et al. (2014). Primers utilized for experiments are listed in Table 3.4.

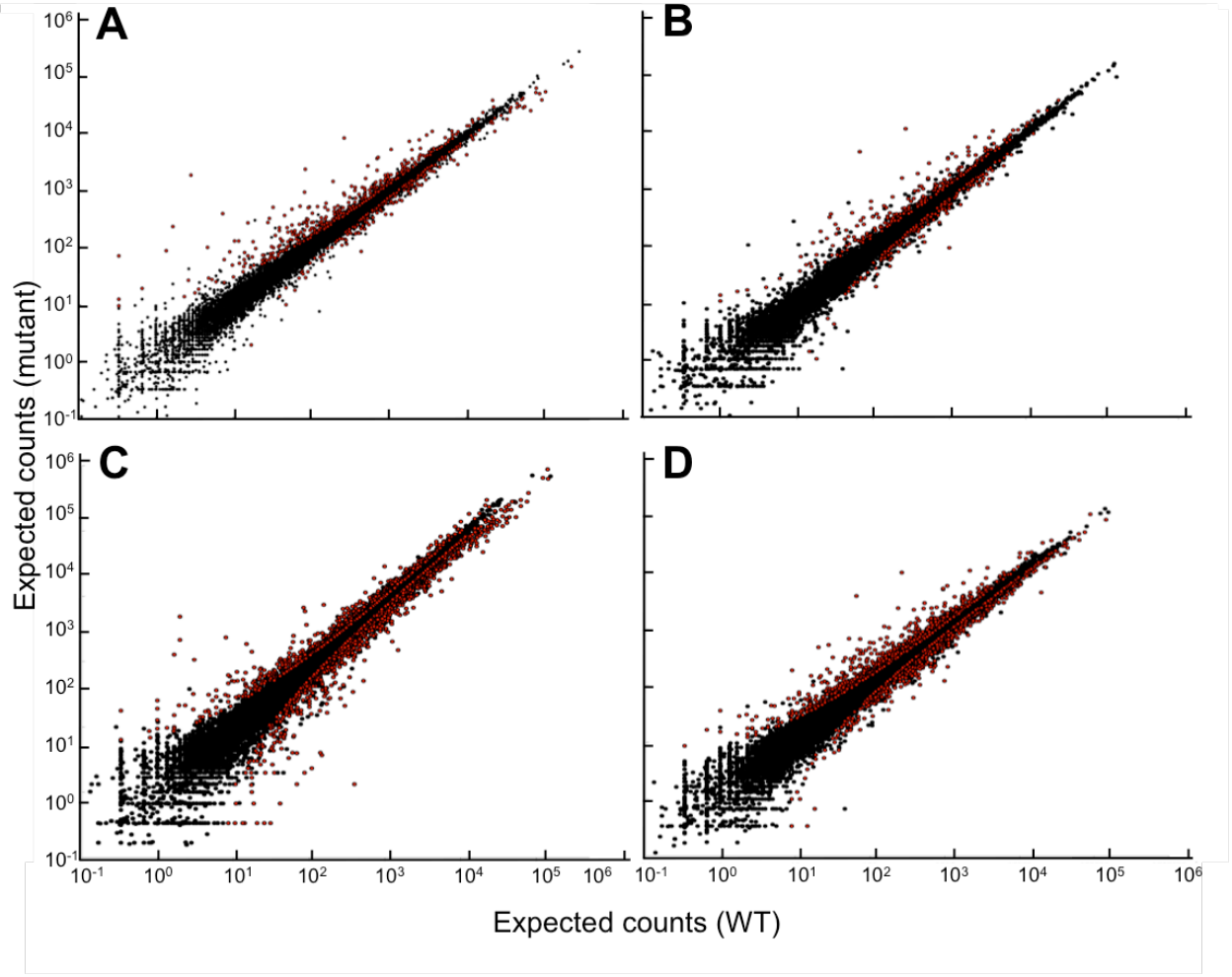


Figure 3.26. Comparison of gene expression profiles between wild type (WT) and various mutants analyzed in this study.

Scatter plots of expected counts reads obtained by full transcriptome sequencing (RNA-seq) for WT versus the mutants studied in this work: **(A)** WT versus *jazQ*, experiment 1, **(B)** WT versus *jazQ*, experiment 2 (see methods), **(C)** WT versus *phyB-9* and **(D)** WT versus *jazQ phyB*. Red dots indicate genes called as differentially expressed in the mutants according to DESeq statistical package (p-value<0.05).

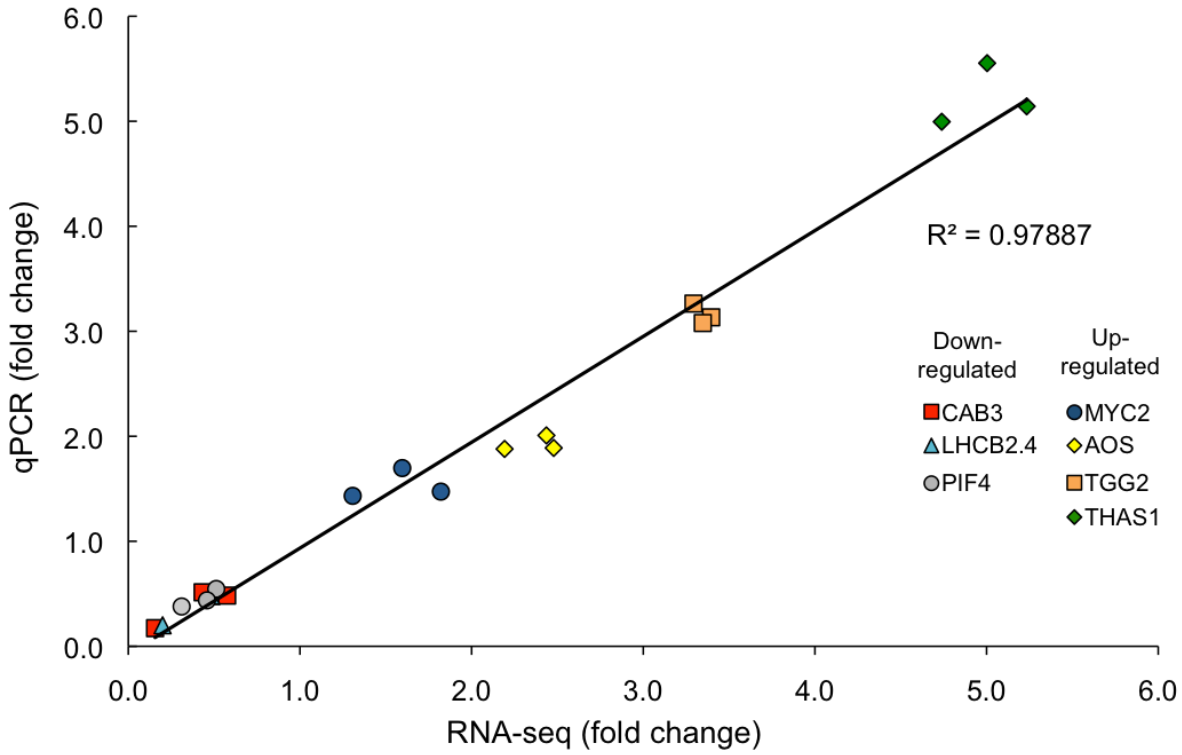


Figure 3.27. Validation of RNA-seq data by qPCR.

Samples submitted for RNA-seq analysis were evaluated for the expression of specific genes by qPCR. Values represent the fold change in expression of *jazQ* over WT measured by qPCR (y-axis) or RNA-seq (x-axis).

Overexpression of *PIF4* in the *jazQ* background

The *35S::PIF4*-TAP overexpression construct was kindly provided by Dr. Michael Thomashow and is previously described (Lee and Thomashow 2012). Transformation of WT and *jazQ* plants with *Agrobacterium tumefaciens* (strain C58C1) was performed using the flower dip method (Clough and Bent 1998). Multiple independent transformed lines (T1 generation) were selected on MS plates containing gentamycin and transferred to soil for subsequent analysis. Homozygous lines were selected by testing the T3 progeny for gentamycin resistance.

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CHAPTER FOUR

Summary and future perspectives

Summary of dissertation

From its first isolation in the early 1960's (Demole et al. 1962) to the recent crystallization of its co-receptor complex (Sheard et al. 2010), scientific interest in jasmonates (JAs) has made a transition from being a simple constituent of the jasmine flower scent to a ubiquitously occurring hormones that plays essential roles in plant development and immune function. The identification of the JASMONATE ZIM-domain (JAZ) family of proteins marked a major step towards understanding JA signaling (Chini et al. 2007; Thines et al. 2007, Yan et al. 2007), but, at the time this dissertation research started, little was understood about the molecular mechanisms of JAZ function. Functional redundancy among the JAZ family members hindered research to elucidate the biological relevance of these repressors in plant growth and defense. In this dissertation, I first describe how alternative splicing of a specific *Arabidopsis thaliana JAZ* gene, *JAZ10*, produces stabilized splice variants that function to attenuate JA responses upon induction. This function is dependent on an intron-retention event that truncates the C-terminal end of the degron, thus impairing interaction of the splice variant isoform with COI1. Because this mechanism of protein stabilization is distinct from that of other JAZ repressors (e.g. *JAZ8*, Shyu et al. 2012), these findings suggest a degree of functional specificity among the JAZ family members.

This dissertation research also employed the higher order *jazQ* mutant to address the function of JAZ genes. I showed how constitutive activation of the JA signaling in this mutant leads to a shift in the allocation of resources between growth and defense processes. Screening of an EMS-mutagenized population of *jazQ* plants lead to the identification of a *phyB* mutantion that suppresses the slow-growth phenotype of *jazQ* without significantly altering the enhanced defense phenotype. Characterization of *jazQ phyB* plants indicated that a genetic uncoupling of

the JA-phyB binary module triggers the concomitant activation of growth and defense processes. Taken together, my findings suggest that the JAZ proteins are essential modulators of plant growth and defense.

Alternative splicing of *JAZ* transcripts expands the spectrum of repressors that participate in the JA-signaling pathway. It was previously shown that *JAZ10* is subjected to alternative splicing, producing three protein isoforms (JAZ10.1, JAZ10.3 and JAZ10.4) that differentially interact with CORONATINE INSENSITIVE1 (COI1) in the presence of JA-Ile (Chung and Howe 2009; Chung et al. 2010). However, the biological function of these variants was still unknown. In Chapter Two of this dissertation, I describe the functional characterization of the *JAZ10* splice variants and show that, upon induction by mechanical wounding, the JA-labile isoform JAZ10.1 is quickly removed from cells. In contrast, the stable isoforms JAZ10.3 and JAZ10.4 accumulate and are retained for longer periods of time in JA-elicited cells. In a series of complementation assays utilizing the *jaz10-1* null mutant, which is insensitive to exogenous JA (Demianski et al. 2012), I demonstrated that JAZ10.3 and JAZ10.4 but not JAZ10.1 function to attenuate various JA responses, including inhibition of root elongation and expression of JA-responsive genes. The observation that JAZ8 cannot complement the ability of stable JAZ10 splice variants to attenuate JA responses point to a unique role of JAZ10 in the regulation of JA signaling. Furthermore, these results suggest that alternative splicing can lead to production of a range of JAZ variants that, based on differences in stability, perform different functions.

I then focused my studies on understanding the mechanism by which the most abundant of the JAZ10 protein isoforms in JA-elicited cells, namely JAZ10.3, acts as a potent repressor. JAZ10.3 lacks the seven C-terminal amino acids of the Jas motif, an alteration that impairs its interaction with COI1 in the presence of JA-Ile. I found that addition of a single amino acid to

the C-terminus of JAZ10.3 (to generate JAZ10.3+L^{Jas21}) is sufficient to fully restore ligand-dependent interaction with COI1. Interestingly, this affected region of the Jas motif corresponds to the C-terminal end of an alpha-helical region that was proposed to dock the JAZ substrate to COI1 in a manner that facilitates JA-Ile dependent formation of the COI1-JAZ co-receptor complex (Sheard et al. 2010). My results therefore suggest that the intron-retention event that gives raise to JAZ10.3 impedes COI1-JAZ interaction by disrupting the integrity of this alpha-helix. Furthermore, the observation that the intron-retention event is conserved among the majority of *JAZ* genes in Arabidopsis and likely *JAZ* genes in other plants as well indicates an evolutionary importance for these type of events.

In Chapter Three of the dissertation, I describe a strategy to overcome the functional redundancy in the JAZ family by genetic removal of multiple *JAZ* genes. Construction of the *jazQ* mutation resulted, for the first time, in constitutive activation of JA responses and upregulation of defense processes. As described by the growth versus defense paradigm (Herms and Mattson 1992; Huot et al. 2014), *jazQ* is also hindered in the growth of above- and below-ground organs. Transcriptional, metabolic and morphological characterization of *jazQ* illustrates how the constitutive activation of the JA pathway shifts the growth-defense equilibrium towards defense processes and highlights the significance of this hormone and the JAZ proteins as regulators of resource allocation.

The phenotypes of *jazQ* provide a powerful tool to identify regulatory genes that control growth-defense tradeoffs. I isolated an EMS-mutagenized supressor line (*sjq11*) that retains defense-related phenotypes (e.g. anthocyanin accumulation) of *jazQ* but exhibits a robust growth phenotype comparable to WT plants. I identified the genetic basis of the *sjq11* phenotype, which was tracked to a non-sense mutation that in *PHYB*. Genetic reconstitution of *sjq11* through

construction of the *jazQ phyB* sextuple mutant confirmed that this combination of mutations results in uncoupling of growth-defense antagonism. These results were surprising because *phyB* mutants are known to exhibit increased extension-type growth (e.g. petiole elongation) and impaired defense against insects and pathogens (Cerrudo et al. 2012; Moreno et al. 2009). Moreover, current models indicate that the JA-phyB crosstalk is a binary module that perceives external signals and modulate resource allocation in response to changing environmental conditions (Ballaré 2014; Kazan and Manners 2012; Leone et al, 2014; Moreno et al. 2009). In this context, activation of one branch of this module is typically associated with repression of the counterpart branch. However, I found that the combination of the *jazQ* and *phyB* mutations simultaneous increases both growth and defense. This uncoupling of growth-defense antagonism results from a transcriptional reprogramming that allows not only the simultaneous expression of defense- and growth-related genes, but also the activation of new transcriptional circuits that are not active in either *jazQ* and *phyB* parental genotypes. My findings suggest that growth-defense antagonism may not be dictated by constraints on metabolic resources but rather by hard-wired regulatory programs that exert control over resource partitioning in dynamic environments.

To summarize, research in this dissertation demonstrates how plants utilize the JAZ proteins to regulate growth and defense processes upon environmental fluctuations (Figure 4.1). In the absence of stress (resting state), JAZ proteins interact with and inhibit the action of MYC2 transcription factors (TFs) associated with defense responses. JAZ also interact with negative regulators of growth (DELLAs), thereby allowing PIF TFs to regulate the expression of growth-associated genes (Yang et al. 2012). In this resting state, resources are spent primarily on growth processes. However, under conditions of environmental stress such as mechanical wounding or insect herbivory, accumulation of bioactive JA (JA-Ile) promotes the formation of the COI1-JA-

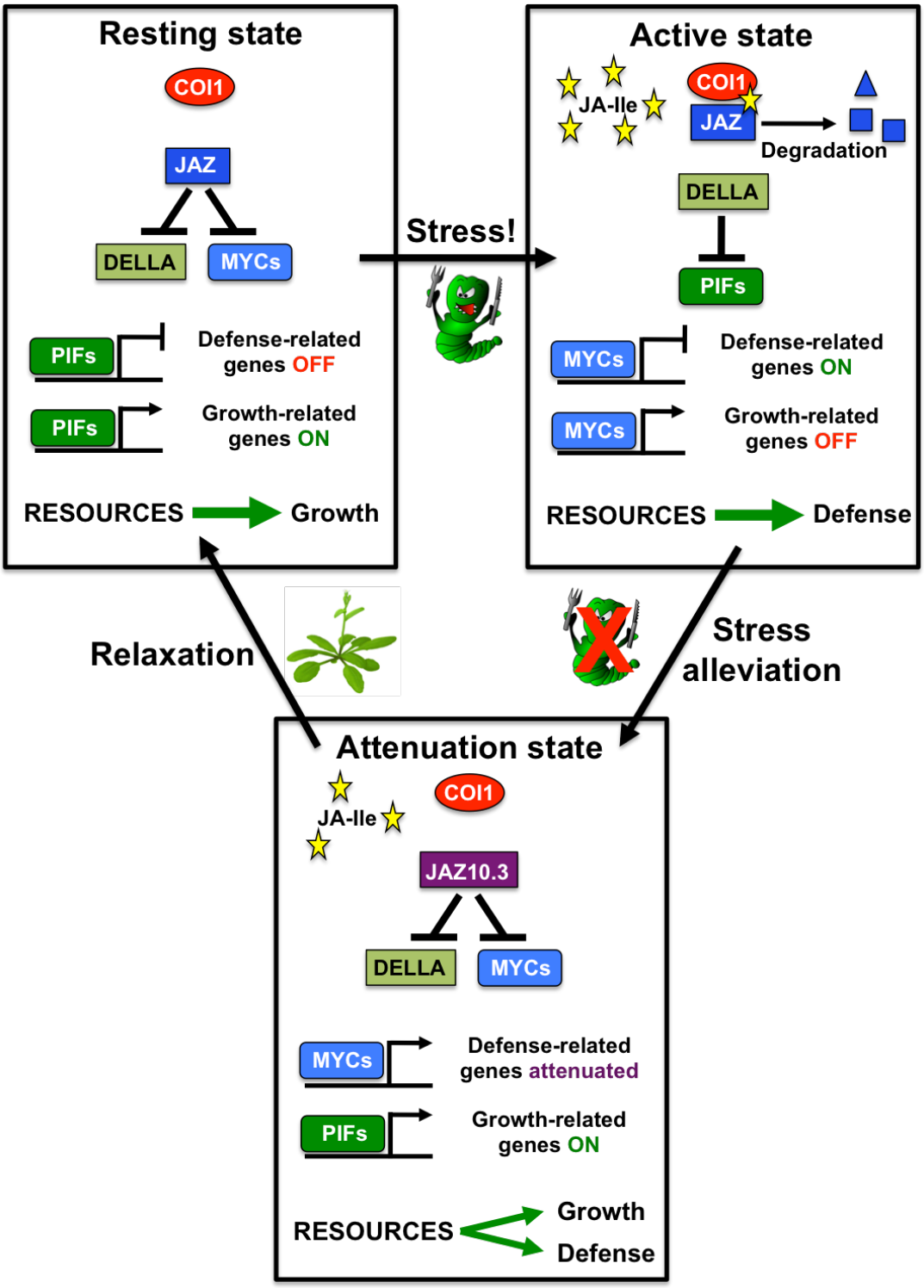

Figure 4.1. JAZ proteins are modulators of plant growth and defense.

In absence of stress (Resting state), JAZ proteins act as growth promoters, hindering the action of defense-related transcription factors (TFs) (MYCs) and negative regulators of growth (DELLAs). TFs associated with growth processes (PIFs) are free to bind to and activate trans-

Figure 4.1 (cont'd). cription of growth-related genes, but also to repress the expression of defense-related genes. In this resting condition, plants prioritize the allocation of resources to growth. However, stressful situations such as insect herbivory trigger a burst of JA-Ile, which allows the formation of COI1-JAZ complexes that promote JAZ degradation. In this condition (Active state), MYCs are freed from repression and activate defense-related genes. Removal of JAZ also promotes DELLA interaction with PIFs as a further mechanism to repress growth. In this active state, plants allocate their resources mainly to defense. Stress alleviation leads to accumulation of JA-stable JAZ proteins (e.g. JAZ10.3), which interact with MYCs TFs and DELLAs even in the presence of high JA-Ile levels. In this condition (Attenuation state), defense-related processes are attenuated and growth is resumed. Finally, upon stress removal (relaxation), JA-stable JAZ proteins are removed by an unknown mechanism, allowing the system to be JA-sensitized again if necessary.

JAZ complex that targets the JAZ proteins from degradation. JAZ removal allows the MYC family of TFs to bind to JA-related genes and activate defense responses, and also inhibit growth-related gene expression (Yadav et al. 2005). Removal of JAZ also promotes DELLA-PIF interaction as another proposed mechanism of JA-mediated growth suppression. In this scenario, resources are invested mainly in defense processes. As part of a negative feedback loop to attenuate JA responses, *de novo* synthesis of stable JAZ such as JAZ10.3 serves to inhibit the action of defense-related TFs even in the presence of JA-Ile. This attenuation of JA responses presumably balances growth-defense tradeoffs and also permits return to resting state when stress conditions subside.

Future perspectives

In the past few years, numerous discoveries have advanced our understanding of JA biology and how this hormone evolved as a mechanism to maximize plant fitness in an ever-changing environment. The JAZ proteins were discovered less than 10 years ago as the missing link in the JA signaling pathway (Chini et al. 2007; Thines et al. 2007, Yan et al. 2007). (+)-7-*iso*-JA-Ile was identified as the bioactive form of the hormone (Fonseca et al. 2009; Sheard et al. 2010; Staswick and Tiryaki 2004) and biochemical pathways for catabolism of this bioactive molecule are now understood (Heitz et al. 2012; Koo et al. 2011; Koo and Howe 2012; Koo et al. 2014). The crystal structure of the COI1-JAZ JA-Ile co-receptor complex was resolved, unraveling the mechanism of JA-Ile perception (Sheard et al., 2010). A rapidly expanding list of TFs and co-repressors involved with JA-signaling is providing additional insight into how JA responses are coordinated (Fernández-Calvo et al. 2010; Nakata et al. 2013; Pauwels et al. 2010; Qi et al. 2011; Song et al. 2011; Song et al. 2013; Yang et al. 2012). Several nodes for crosstalk between the JA

and other signaling pathways have been identified (Campos et al. 2009; Grunewald et al. 2009; Lorenzo et al. 2002; Moreno et al. 2009; Song et al. 2014; Thaler et al. 2012; Yang et al. 2012) and there is now a firm mechanistic understanding of JA-triggered plant immunity (Howe and Jander 2009; Campos et al. 2014). While these discoveries are helping us to understand the broad biological relevance of JA, they also raise new and exciting questions.

Recent studies have identified several JAZ proteins that interact weakly or not at all with COI1 (Chung et al. 2010; Moreno et al. 2014; Shyu et al. 2012; Thireault et al. 2015). The mechanisms involved in turnover of these stable JAZ proteins, which include JAZ10.3, JAZ10.4, JAZ8 and JAZ13, remain to be determined. Such mechanisms presumably exist in order for cells to recover from JA-induced stress and to become "re-sensitized" to the hormone (Figure 4.1). One hypothesis is that a ligand other than JA-Ile can promote COI1 interaction with these proteins. Hydroxylated and carboxylated forms of JA-Ile that are synthesized in response to wounding have little or no capacity to promote the formation of COI1-JAZ complexes (Heitz et al. 2012; Koo et al. 2011). However, it remains to be tested whether these JA-Ile derivatives can promote COI1 interaction with the stable JAZ repressors referred above. This function can also be performed by different endogenously found JA-amino acid conjugates (other than JA-Ile), which have been shown to promote COI1-JAZ interaction in vitro (Katsir et al. 2008). These alternatives suggest that the JA-stable JAZ form a different binding pocked that allows the utilization of a different compound other than JA-Ile as the molecular glue to promote the formation of COI1-JAZ complexes.

Identification of novel *JAZ* genes and splice variants may greatly expand our understanding of the biology of JA. Recent homology studies with JAZ8 lead to the description a new *JAZ* gene in Arabidopsis, called *JAZ13*. *JAZ13* possesses unique features not described for

other JAZ family members (Thireault et al. 2015). One example is a C-terminal Ser-rich tail that is a likely site for protein phosphorylation, suggesting a new mechanism to regulate JAZ function. The increasing availability of genome-scale datasets is facilitating the identification of new *JAZ* genes and JAZ splice variants in diverse species (Chung et al. 2010; Hong et al., 2014; Ye et al. 2009). Molecular characterization of these genes promises to improve our comprehension of the versatility of JA as a regulator of plant growth and development. An elegant recent example was demonstrated in rubber tree (*Hevea brasiliensis*) where characterization of the JAZ gene family provided new insight into the role of JA signaling in wound-induced latex production (Hong et al. 2014).

In Chapter Three of this dissertation, I described the JA-phyB transcriptional network as a binary module that is rewired upon recognition of specific external signals to modulate the tradeoffs between growth and defense. Similar regulatory modules involving other signaling pathways possibly exist to control the allocation of finite resources in the face of changing environmental conditions. In this sense, the molecular characterization of crosstalk nodes between JAs and other hormone response will allow us to better understand the regulatory circuits involved in plant growth and defense. Analysis of crosstalk between JA and growth-related hormones such as auxin, gibberellin and brassinosteroids are already leading to the identification of signaling components that are essential for proper tuning of plant development (Campos et al. 2009; Grunewald et al. 2009; Yang et al. 2012). It is tempting to speculate that some of the *jazQ* suppressor mutants identified during the course of this dissertation research (Chapter Three) are indeed affected in these networks. Further characterization and identification of the causal suppressor mutations is needed to test this hypothesis.

Experimental designs that more accurately reflect the dynamic condition in natural environments will ultimately be needed to discern the complexity of regulatory circuits involved in resource allocation. Phenotypic characterization of *jazQ phyB* and related mutants in a broad range of environmental conditions (e.g. fluctuating temperature and light), including field studies will likely provide valuable insight into this question. Empirical evidences obtained from these types of experiments may indicate, for example, how plants prioritize specific developmental programs in the face of multiple stresses (Moreno et al. 2009). It is known that natural populations of herbivores dictate polymorphisms in defense traits (Züst et al. 2012) and that competition with naturally occurring, fast growing plants can trigger a shift in the growth-defense equilibrium (Agrawal et al. 2012). This ecological perspective provides a plausible strategy to bridge basic and applied science, which may lead to the development of crops that are optimized in growth and defense processes.

The development of new technological tools and current pace of discovery mark a new era in understanding the biological function of JA in resource allocation. Interdisciplinary studies that combine large-scale -omics analysis, synthetic biology, mathematical modeling and systems biology will aid in the quest to untangle the intricate web of regulatory circuits in which JA participates to maximize plant fitness in response to myriad of environmental conditions. In conclusion, there is no better time to be studying JA biology.

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