Life in the balance: Plant hormone signals orchestrate growth-defense dynamics under mechanical stimulation

by

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Table of Contents

Table of Contents i
Dedication iv
Acknowledgements v
Preface: Failure, and other beautiful things viii
List of abbreviations xi
Chapter 0.5: Touch stress, jasmonates, and the ecology of defense: a brief overview (1680 - present) 1
Touch stress and how we harness it for our purposes. 2
Introduction: raising questions 2
What is touch stress, really? 4
What happens to the plant after it's touched? 7
How does the plant regulate its interactions with plants and herbivores? 10
How ancient or conserved is jasmonate signaling? 15
References 22
Chapter 1: The jasmonate signaling pathway and its far-reaching impacts in plant physiology 25
Abstract 26
Part 1: Thigmomorphogenesis and how oxylipins mediate the relationship between growth and defense 27
Part 2: The oxylipin stress signaling pathway 28
Jasmonate biosynthesis 28
i. Initial chloroplast membrane lipid metabolism 29
ii. Fatty acid oxygenation 30
iii. Allene oxide metabolism 31
iv. Transporters 33
v. Peroxisomal processes 34
vi. JA fates and conjugation 35
Jasmonate perception 39
i. Transporters 39
ii. Transcription factors 40
iii. Mediator 41

iv. Co-receptor COI1 42
v. JAZ proteins 44
vi. Auxiliary repressors 46
vii. JA-mediated relief of repression 47
Phytohormonal crosstalk 49
i. JA and other hormones 49
ii. JA and GA 50
Other oxylipins 51
Part 3: Where do we go from here? 54
Knowledge gap 54
Roadmap 55
References 59
Chapter 2: Touch stimulation of *Arabidopsis* enhances defense but restricts growth via separate products of jasmonate biosynthesis 66
Abstract 67
Introduction 68
Results 71
Mechanically stimulated plants show reduced growth and increased JA responses 71
Touch-induced reduction in rosette growth is dependent on *LOX* and *AOS* but not *OPR3* 74
Touch-induced reduction in rosette growth is phenocopied by exogenous OPDA 78
OPDA alone is insufficient to increase touch-induced herbivory resistance, which is JA-Ile dependent 81
jar1 mutants are insensitive to touch stimulation 86
Discussion 87
Materials and methods 91
References 95
Supplementary information 99
Chapter 3: Crosstalk at the interaction of DELLA proteins and MYC co-repressors is at the center of growth reduction to touch stress 106
Abstract 107
Introduction 109
Results 117
Touch stress results in a marked reduction in vegetative growth and an increase in molecular markers of mechanical stress, and is OPDA-dependent 117

Touch-induced growth reduction is abolished in *coi1* mutants, which do not upregulate either oxylipin biosynthetic or oxylipin-dependent genes 119

MYC transcription factors are required for touch-induced growth stunting, and override the sensitive dwarf phenotype in higher-order *jaz* knockout plants 123

The JAZ auxiliary repressor protein TOPLESS is necessary for touch-induced growth stunting, but its associated co-repressor NINJA is not 125

DELLA proteins are required in addition to jasmonates for plants to orchestrate the growth response to touch 128

Discussion 131

Materials and methods 136

References 139

Supplementary information 143

Chapter 4: Perspectives, applications, and significance 148

Introduction 149

Divergence in functionality between OPDA and JA in response to touch stress 150

Elements of multiple signaling pathways converge to orchestrate growth reduction after touch stress 155

Future directions 159

References 161

This dissertation is dedicated to my mama, who didn’t raise no quitter.

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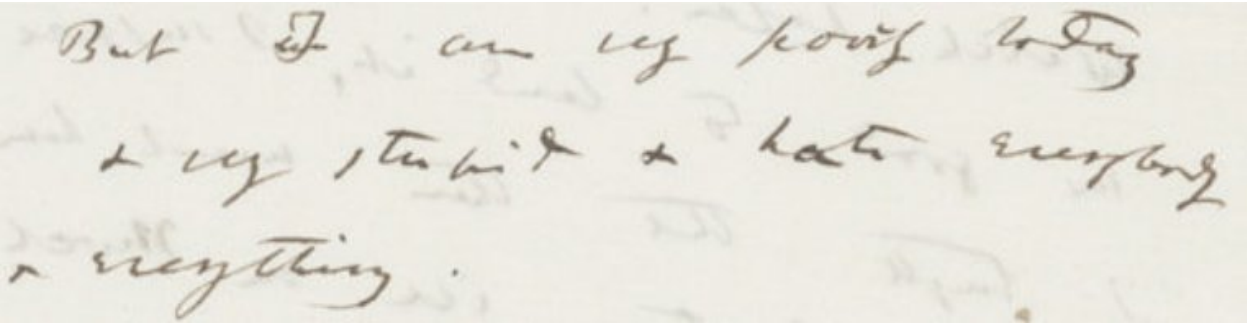
Preface: Failure, and other beautiful things

October 1, 1861. Charles Darwin, undeniably one of the most influential biologists of the 19th century and probably modern history, had a bad day and wrote a letter to his contemporary and friend Charles Lyell. He finished his correspondence with this paragraph:

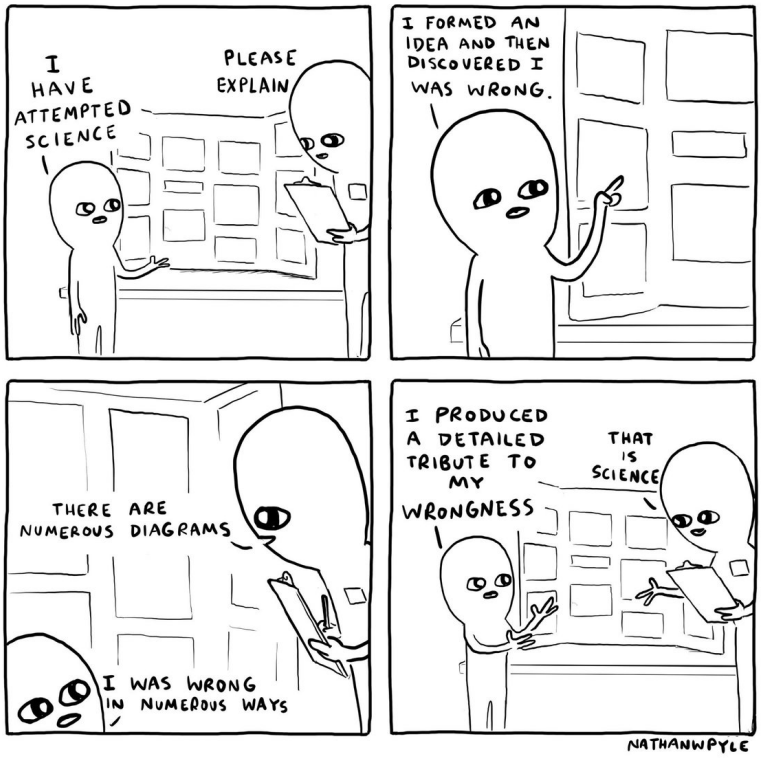


“But I am very poorly today & very stupid & I hate everybody & everything. One lives only to make blunders. I am going to write a little book for [John] Murray on orchids & today I hate them worse than everything so farewell & in a sweet frame of mind, I am.

Ever yours, C. Darwin.”

I cannot begin to fathom the number of times I’ve expressed nearly this exact sentiment to my friends, to my mother, even occasionally to my advisor (sorry). Others more sentimental than I may tell you that failure is never *really* failure, because every time you do something wrong is an opportunity to learn from the experience, grow as a person, and continually become a better version of yourself. I disagree. Failure is, more often than not, just that. Ideally, one learns from one’s failures and is less likely to fail in the future, of course. It’s just that in practice, pragmatically, this isn’t really the case every time we make a mistake. Sometimes mistakes are just that. Then you may lose an hour of your

time or months' worth of work for no intellectual gain. Qu'est-ce que c'est. Try again tomorrow.



The dissertation that follows this short monologue is a compilation of several of the successes from the past few years of my career, an anthology of the wins that happen in-between the blunders.

Science is a 300,000 year-long tradition of being wrong and failing over and over and over again until something - against the odds - goes right, and we learn a little more about the organisms, the ecosystems, the people, the world, or the universe around us. As natural scientists, we are simultaneously driven by, bound to, and empowered by the pursuit of knowledge about the world as we know it. That pursuit is rocky, the path that it requires us to take is leaky and full of pitfalls and hard lessons, and it's tempting but counterproductive to romanticize the process of science and pretend that it is something it's not. In his essay, "The importance of stupidity in scientific research," (which I have had printed out and taped up next to my desk for years) Martin Schwartz argues eloquently that stupidity is the entire point of science and the basis of all discovery. "Productive stupidity means being ignorant by choice." I agree with him here, though I may disagree with his take on whether you need to learn lessons from each and every time you fail at something. Knowledge comes from the cracks between our failures, and

can only be reached because of our willingness to accept feeling stupid. I have spent roughly the last decade and will hopefully spend the next several frequently feeling very, very stupid and sometimes even a little poorly. I like to think I'm carrying on an important tradition just like Darwin (because that makes me feel better about it). I used to think of myself as 'smart,' but I don't anymore; now I think of myself as 'persistent.' Depending on the day, that ranges from 'dedicated' to 'pigheaded.' Science demands that we get things wrong in our pursuit of getting things right. It requires that we get cozy with that feeling of ignorance as a baseline for pushing the boundaries of knowledge *just* a little further. Darwin had it right when he wrote, "One lives only to make blunders," but try reading that again in an optimistic light instead of a dreary one. Scientific progress is inseparably intertwined with failure, and that's what makes it remarkable.

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Darwin, C. Letter to Charles Lyell (1861). Compiled in "The Correspondence of Charles Darwin." Edited by Frederick Burkhardt et al. Cambridge University Press 1985–present.

Schwartz, M. A. (2008). The importance of stupidity in scientific research. Journal of Cell Science 121(11): 1771. https://doi.org/10.1242/jcs.033340

Artwork: "I have attempted science." From the *Strange Planet* comic series by Nathan Pyle. 2019.

List of abbreviations

13-HPOT - 13S-hydroperoxy octadecatrienoic acid
ABCD - Automated Botanical Contact Device
AOC - allene oxide cyclase
AOS - allene oxide synthase
DAMP - damage associated molecular pattern
dn-OPDA - dinor-OPDA
GA - gibberellic acid
HAMP - herbivore associated molecular pattern
HPL - hydroperoxide lyase
JA - jasmonic acid
JA-AA - jasmonic acid amino acid conjugate
JAR - Jasmonoyl-L-amino acid synthetase
MeJA - methyl jasmonate
MYC - Myelocytomatosis oncogene
OPDA - 12-oxo-phytodienoic acid
OPR - 12-oxo-phytodienoic acid reductase
PAMP - pathogen associated molecular pattern
PCR – polymerase chain reaction
PRR - pattern recognition receptor
qRT-PCR/qPCR - quantitative reverse transcription polymerase chain reaction
RLK - receptor-like kinase
SAR - systemic acquired resistance
SCF - Skp1/Cullin/F-box complex
TF - transcription factor
VFT – Venus fly trap

Chapter 0.5: Touch stress, jasmonates, and the ecology of defense: a brief overview (1680 - present)

Touch stress and how we harness it for our purposes.

My research, and therefore the contents of this dissertation, focused primarily on the plant physiological responses to touch stimuli and the ways in which hormonal signaling pathways control these responses. This prologue is intended to serve as a general introduction and broad contextual backdrop for this research. Here, I will discuss what is currently known of the plant touch response, as well as how touch, growth, and defense are connected by hormones, especially the jasmonate signaling system. I will also place these phenomena into a broader ecological and evolutionary context before honing in on the molecular mechanisms controlling the individual response to touch.

Introduction: raising questions

In the 17th century, a comprehensive agronomic textbook was written in the Tōkai region of Japan. That book was called *Hyakusho denki*, or roughly translated to English, *Farmers' Tales*. The farming advice contained within the *Hyakusho denki* has persisted to this day. Part of this agricultural handbook instructs the farmer to carefully tread on their barley and wheat seedlings in the winter, during a developmentally important early stage of their life cycle (reviewed in Iida 2014). Rather than harming the plant, this practice assures, treading on them actually should cause them to grow stronger and hardier. This practice is supposed to result in higher yields, provided it is performed at the correct developmental stage. It is called mugifumi, which translates to "stepping on wheat." Today, mugifumi (using feet, boards, or rollers) is still performed in the Tōkai region and other parts of Japan to improve performance of grain crops - mostly wheat, barley, and rice. Anecdotal evidence abounds for the efficacy of treading on wheat, but conventional

scientific evidence for or against it is sparse. In 1950, Yoshio Ohtani et al. showed experimentally that not only does treading result in a significant rise in shoot biomass (without an increase in shoot height) and number of roots, leaves, and stems; trodden seedlings also allocated proportionally more energy into grain (original results Ohtani et al. 1950, data translated and presented by Iida 2014). Clearly, higher biomass overall *and* higher proportional grain weight are a very desirable result and explains why this process has persisted as long as it has. In one of the only other studies available in English or accessible in major journals on the subject, seven decades later in 2022 (Mizumoto et al.), researchers provided a more comprehensive picture of the effects of treading on wheat. Mizumoto et al.'s treatments resulted in multiple temporal developmental delays, but ultimately higher yield. Initiation of the developmentally and agriculturally important spike, or inflorescence, phase was delayed by 5 days, and internode elongation lagged even further behind, at 12 days. This is a classic response to repeated stress, but could be useful for farmers – inflorescences are at higher risk to frost than are most other organs, so delaying flowering could allow farmers more temporal flexibility should the growing season's weather prove unpredictable. Interestingly, they show that their treatments prime cold hardiness, reducing the seedlings' susceptibility to frost damage. Hindhaugh et al. (2021) sought to determine if the age of mechanically stressed (brushed, not trodden) wheat seedlings had a significant influence on the results of mechanical stimulation on development. They found that seedlings were most susceptible to touch around two weeks old, and their mechanical stress application treatments started at this age resulted, similarly, in a shorter plant with more shoot biomass. Mugifumi is, traditionally, performed when seedlings are around two weeks old. These demonstrable

improvements in yield, biomass, and sturdiness provide more support for the ongoing implementation of the very old practice of mugifumi.

They also raise many more questions.

Fig. 1: Mugifumi, the ancient practice of treading on wheat seedlings to increase hardiness. This photograph (from Iida 2014) illustrates the longevity of the practice, as it is still performed in some parts of Japan today.

What is touch stress, really?

As every plant scientist knows all too well and doesn't need reminding, plants are sessile organisms. Their relationship with stresses is unique because they are incapable of escaping them, so they must adapt, outlast, or die. This requires a plant to be able to perceive its environment and changes to it and react appropriately. Both of those

processes require an ability to send signals from one cell to the next and from one organ to another, to coordinate a physiological response.

Mechanical stress in plants refers to the physical damage or disturbance that can occur when a plant is touched or subjected to physical forces. Those forces can come from weather conditions such as wind, rain, or hail; incidental or intentional animal damage (e.g. stepping or laying on plants, or eating them); and human activities, such as handling the plants or applying mechanical force during cultivation or weeding. In addition, plants can also experience touch stress as a result of their own growth and development, as they push against each other or against physical barriers in their environment or simply due to their own weight against gravity. The specific impacts of touch stress on a plant, much like most other disturbances, depend on the intensity and duration of the stress, as well as the plant's species and genetics. The general term for the morphological and physiological responses to touch stress is thigmomorphogenesis (Jaffe 1973). Thigmomorphogenesis, or the alteration of morphology in response to touch, is a phenomenon that has been observed for centuries (as exemplified by the practice of mugifumi) and gained its descriptive name within the last 50 years (Darwin and Darwin 1880, Jaffe 1973): created from the Greek root words for "touch"; "form", and "creation." It is the long-term physiological counterpart to thigmotropisms, and involves changing development in response to a touch stress. These changes often take the form of elongation stunting and radial expansion, which creates compact plants (Biddington 1986, Braam 2004). Thigmomorphogenesis occurs in herbaceous and woody plants alike (Jaffe 1973, Telewski and Jaffe 1986a, Telewski and Jaffe 1986b), although it may alter the structural integrity of affected plants differently depending on the species. What

is clear is that thigmomorphogenesis is adaptive and serves to protect the plant's tissues against the generalized mechanical stress it's experiencing (Biddington 1986, Telewski and Jaffe 1986a, Telewski and Jaffe 1986b, Braam 2004). Aside from the visible, vegetative morphological changes induced by touch stress, thigmomorphogenesis can also take the form of transcriptional changes and biochemical adjustments which lead to higher defense capabilities in stressed plants. Mechanical stress can cause structural trauma or damage to the plant, in the form of broken stems, wounds or torn leaves, but it can also be non-traumatic and non-wounding, a differentiation that some Japanese farmers have needed to draw for generations. The plant response to this non-traumatic touch stress, the thigmomorphogenetic response, is less well-studied than the wound response and is a primary focus of my work. Non-damaging touch stress is, in nature, often the result of rain, wind, or crowding, but it can also signal the presence of an herbivore – this is a signal, then, that has the potential to precede danger. Such a red flag needs to be perceived, interpreted, and responded to. Additionally, non-damaging touch stress offers more potential for long-term stress studies than does wounding. Touch has also been a larger research focus in recent years. Ghosh et al. (2021) reviewed a wide variety of literature on mechanostimulation, from wind to bending to sound, in several species, and conclude that it represents an encouraging potential for stronger, more resilient crops. We know from prior studies that touch stress produces both immediate and long-term physiological, transcriptional, and biochemical effects (Jaffe 1973, Braam 2004, Chehab et al. 2008, Chehab et al. 2012, Lange and Lange 2015).

What happens to the plant after it's touched?

Once a mechanical stimulus is perceived, a cascade of signaling needs to occur for the plant to respond. At the site of the stimulus, the first signals must be rapid. Changes in the levels of calcium are ubiquitous signals used by plant cells (Choi et al. 2016). Often, an increase in cytosolic Ca^{2+} is amongst the very first events in a signaling cascade, such as when a plant is challenged with wounding, herbivory, or contact with microbes. These signals are spatially and temporally varied based on the source and nature of the stress, and it is thought that downstream effects can be differentiated into pathways that elicit appropriate responses through decoding the so-called 'calcium signature' of the eliciting signal (McAinsh and Hetherington 1998). In recent years, new developments regarding proteins that recognize these calcium signatures have helped us understand the mechanisms by which plants propagate and appropriately respond to ion signals. The largest families of calcium sensors are the calmodulin (CaM) and calmodulin-like (CML) proteins, which can serve diverse roles in modulating plant stress responses (for review of these families and their roles, see Ranty et al. 2016). Braam and Davis (1990) demonstrated a marked increase in the expression of some CaM and CML coding genes, that are appropriately called *TOUCH (TCH)* genes, within ten to thirty minutes after a mechanical stimulation. The intensity of upregulation of some *TCH* genes appears to be dose-responsive, with higher expression increases associated with more mechanical stimulation (Braam and Davis 1990). Darwish et al. (2022) published on the calmodulin binding transcriptional activator CAMTA3 as another critical element of early touch signaling. Their *camta3* knockout mutants appear to essentially abolish the touch response (i.e., no effect of touch to reduce growth, delay flowering, or trigger touch-

responsive gene expression). CAMTAs bind CaMs early on in the response and promote touch-responsive expression of the *TCH* genes.

Wounded or otherwise mechanically stimulated cells in *Arabidopsis thaliana* (hereafter *Arabidopsis*) leaves release the amino acid glutamate into the apoplast immediately upon taking damage, which binds to glutamate receptor-like ion channels in order to send a burst of Ca^{2+} ions throughout the plant (Toyota et al. 2018). These calcium ions cause waves of depolarization events to send a signal cascade through tissue and into the vasculature of the plant, where it can rapidly propagate throughout the plant, alerting even distal tissues to danger (for a review of systemic signaling mechanisms, see Hilleary and Gilroy 2018). This represents the transition from localized, rapid signal transduction to slower, long-distance signaling that leads to transcriptional cascades and biochemical responses far from the site of damage. This is the process of systemic acquired resistance (SAR) in response to biotic stress, or systemic acquired acclimation (SAA) in response to abiotic stress, where the plant primes its defenses in anticipation of attack elsewhere or adjusts to withstand environmental stress. One of the key features of SAR is that it is, of course, systemic, meaning that it can spread throughout the entire plant, rather than being limited to the site of the initial stress. This allows the plant to mount a more comprehensive defense response and to protect itself from further attack. SAR promotes whole plant fitness against pathogen attack and herbivory. These physiological changes are known to be largely dependent on the action of multiple phytohormones, although the preeminent ones to which a massive amount of SAR/SAA is attributed are salicylic acid (SA) and jasmonic acid (JA). SA is primarily considered a key mediator in plant defense against biotrophic pathogens like *Pseudomonas syringae* and some herbivores, while JA

regulates defense against necrotrophic pathogens such as *Botrytis cinerea* and many other herbivores. These two hormonal signaling pathways are antagonistic towards one another (see Chapter 1), a relationship that has been exploited to the benefit of certain pathogens and insects that have evolved ways to trigger one of the pathways, which downregulates the actual defenses that the plant would need to implement against the attacker. The evolution of these molecular red herrings is a remarkable example of the evolutionary arms race between plants, pathogens, and herbivores. In the 1970s, Leigh Van Valen used the phrase “Red Queen hypothesis” to propose, in summary, that evolution is zero-sum, not a continuous addition and only occasional loss of fitness. Evolution and continual adaptation is therefore not only critical for reproductive advantage, but also for survival, since one’s enemies and competition are evolving too. Species constantly evolve, but in the context of other species around them, leading to an overall stasis in the fitness of a system (Van Valen, 1973). The name of this phenomenon comes from a novel published more than a century earlier: *Through the Looking-Glass* by Lewis Carroll. In one scene, the Red Queen, discussing the unusual mechanistic workings of Wonderland, tells Alice, “Now, here, you see, it takes all the running you can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!” (Carroll, 1872). In order to remain competitive, plants need to evolve ways to detect, respond to, and proactively protect against pathogens and herbivores, even as the same pathogens and herbivores evolve ways to circumvent or even manipulate those systems for their own gain.

Figure 2: The Red Queen hypothesis explains the continual coevolution of plants (left, as Alice) and their natural enemies, including lepidopteran herbivores (right, as the Red Queen). Illustration by author, based on artwork by John Tenniel from *Through the Looking-Glass*.

How does the plant regulate its interactions with plants and herbivores?

Like the animal immune system, the plant immune system needs to be able to tell when the plant is being invaded by pathogens, identify the attacker, and mount an appropriate response. Unlike animals, plants do not have specialized immune cells, and thus rely mainly on innate immunity, both constitutive and inducible. This is accomplished by a number of receptor-like kinases (RLKs) on the cell surface called pattern recognition receptors (PRRs) (reviewed in Macho and Zipfel 2014). In short, PRRs form an integral part of a transmembrane signal transduction system that is central to plant immunity, are capable of binding molecular patterns associated with certain pathogens (PAMPs) or

herbivores (HAMPs), and send specific instructions based on the patterns detected. PAMPs are conserved molecules that are found on the surface of many pathogens, including bacteria, fungi, and viruses. These molecules are essential for the survival and reproduction of the pathogen and are usually not found in plants or animals. Examples of PAMPs include bacterial flagellin, a protein found in the flagella of bacteria that helps them move; chitin, a polysaccharide found in the cell walls of fungi; and double-stranded RNA, which is a characteristic of many viruses. Pattern-based recognition of dangers isn't limited to microbes – plants have ways to detect herbivory, even to identify their attackers. These specific HAMPs may be found in insect saliva, eggs, or exoskeletal components. These are known to induce immune responses alone, but also amplify the intensity of the response to tissue damage.

Once a PRR binds a ligand, it begins a signal cascade that leads to the biosynthesis and mobilization of phytohormones and other systemic signals, and ultimately transcriptional changes resulting in the production of or changes to secondary metabolites, phytoalexins or other defensive molecules. The role of PRRs in anti-pathogen defenses has been studied in depth, and depending on the nature of the pathogen detected, the plant's response generally follows one of two signaling pathways: SA or JA. Herbivory primarily triggers the JA pathway; there are a wide variety of conserved molecules that serve as HAMPs, most of which lead to JA signaling. The receptor PRRs involved in HAMP signaling are increasingly a topic of interest to researchers (reviewed in Reymond 2021), but several of the ones that have so far been characterized have a demonstrated link to positive regulation of JA signaling (Hu et al. 2017, Gilardoni et al. 2011, reviewed in Erb and Reymond 2019).

Plants' use of HAMPs and PAMPs to recognize specific dangers and mount an appropriate defense provides an undeniable advantage: fewer metabolic resources are used because one primary defense is activated - the one that will be the most useful. However, some enterprising plant enemies have, as the Red Queen hypothesis would suggest, evolved ways to counteract these defenses and even hijack plant signaling to their advantage. Coronatine (COR) is a molecule produced by some bacteria including the biotrophic/hemibiotrophic plant pathogen *Pseudomonas syringae*. *P. syringae* is a prolific and opportunistic pathogen, with the capacity to infect woody and herbaceous plants alike. It is one of the most well-studied plant pathogens in existence. By employing effectors including coronatine, *P. syringae* has the ability to hijack plant defenses to divert signaling systems away from the salicylic acid defense pathway, which is useful against biotrophic pathogens, and to the jasmonic acid one, which is useless toward, or worse: makes the plant more susceptible to, biotrophic pathogens. COR is a structural mimic of JA-Ile, the primary bioactive form of JA, and uses this structural similarity, much like a Trojan horse, to initiate JA-responsive signaling through the canonical COI1-JAZ co-receptor complex (Katsir et al. 2008, Jiang et al. 2013). COR and JA-Ile compete for the same receptors and COR is three orders of magnitude more effective at inducing these responses. Upon perception of COR, COI1-JAZ interaction promotes the expression of, among others, genes that suppress salicylic acid signaling. Normally, this antagonistic crosstalk would be advantageous to the plant - one metabolically expensive demand at a time, please (See "Phytohormonal crosstalk" section for more information about hormonal crosstalk). But to protect against a biotrophic pathogen, salicylic acid is much more useful. The bacterium, then, benefits from targeting JAZ proteins and triggering JA-responsive

signaling (Zheng et al. 2012, Gimenez-Ibanez et al. 2014). The plants are then prepared to fend off an herbivore or a necrotrophic fungus, but left more vulnerable to bio/hemibiotrophic pathogens like *P. syringae*. Emerging evidence suggests, also, that *P. syringae* can affect ABA signaling, adding another dimension to the ways in which bacteria can enhance their own virulence through hormone manipulation (Mine et al. 2017).

Just as pathogens can hijack plant signaling systems and misdirect toward herbivore defense, so too can herbivores hijack signals and misdirect toward a contextually inappropriate pathogen defense, or transmit a false molecular “all-clear” signal (e.g. glucose oxidase suppressing nicotine production in tobacco [Musser et al. 2002]). Notably, plants often have a different reaction to wounding, even from herbivores without saliva, than they do to normal herbivore attack. This is a result of the evolution of red herring evasion mechanisms. Some of these mechanisms come from the associations between herbivores and symbiotic microbes found in their saliva, or from endogenous elicitors. One important mechanism by which herbivores can manipulate plant defenses comes from changing the crosstalk between hormone pathways, *especially* interactions involving or affecting JA signaling. One very interesting early finding in this realm was a marked SA-dependent induction of anti-biotrophic and antiiviral pathogen resistance in direct response to the oral secretions of *Pieris rapae* while at the same time not conferring any anti-fungal or anti-necrotroph protection (De Vos et al. 2006). Another suggests caterpillar saliva causes the downregulation of ‘induced resistance’ to herbivores via induction of SAR against pathogens, ultimately leading to a more favorable situation for herbivores (Weech et al. 2008). Induction of anti-fungal resistance would be indicative of

an increase in JA-dependent signaling, and generally comes with anti-herbivore effects. Interestingly, it appears that this is more common among generalist herbivores than among specialists (e.g. Diezel et al. 2009's beet armyworm vs. tobacco hornworm on tobacco plants). Unlike unicellular plant pathogens, herbivores have in their arsenal the ability to use symbiotic relationships with bacteria in their saliva to suppress anti-herbivore defenses in plants. Usually this comes in the form of the repression of JA signaling, often as an effect of eliciting SA signaling (Chung et al. 2013, Yamasaki et al. 2021). The ZIM domain, characteristic of all of the members of the large family of JAZ proteins (repressors of JA signaling, discussed in depth in Ch. 1), provides an efficient avenue for various interaction with effectors, including stabilization of JAZ against COI1 mediated degradation (Chen et al. 2019), should the herbivore use endogenous elicitors instead.

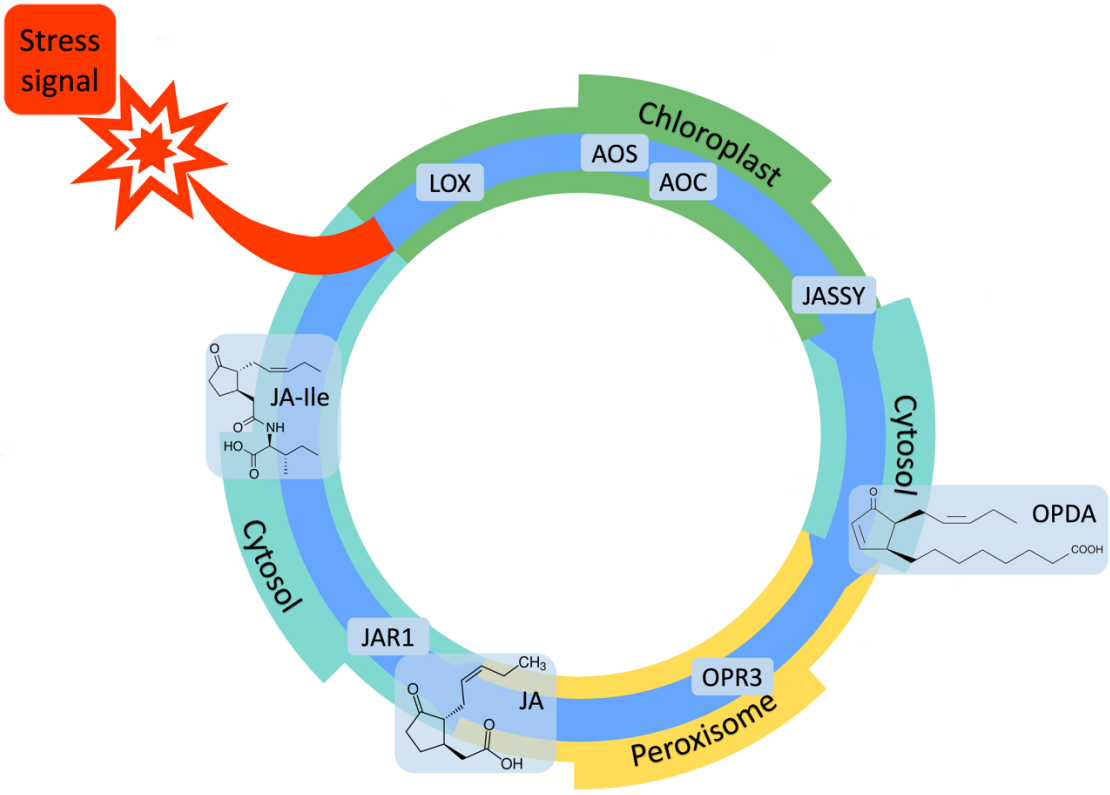

Fig. 3: The JA biosynthetic pathway. This diagram summarizes the major steps, enzymes, and intermediates of JA biosynthesis, as well as the subcellular localization of each step.

How ancient or conserved is jasmonate signaling?

While plants' exact origin point depends on one's definition of 'plant,' the rise of photosynthetic organisms can be seen as the beginning of the evolution of plants as we know them today. Early Earth conditions were anaerobic, and early life's metabolism likely relied on mineral nutrition for energy. Approximately 2.4 billion years ago, however, geochemical evidence suggests there was a Great Oxygenation Event (Bekker et al. 2004), where atmospheric oxygen rose sharply, marking the evolution of oxygenic photosynthesis. Early photosynthetic organisms were unicellular, gave rise to a myriad of extant lineages including cyanobacteria, and eventually through symbiosis became the chloroplasts that provide photosynthetic capabilities to today's green plants (a theory originally proposed by Mereschkowsky in 1905 and since largely adopted as the consensus on the evolution of plastids). Signaling is a ubiquitous necessity of any organism's survival, including unicellular organisms like cyanobacteria, for fundamental metabolic and reproductive activity. Multicellularity, however, imposes an additional requirement on more complex organisms that microbes can ignore: that of coordination of responses across tissues and organs. The evolution of multicellularity also allowed for new mechanisms of growth and reproduction that microbes are not able to achieve.

The emergence of primary producers from the seas onto dry land was arguably the single most influential event in evolutionary history. The new, harsh terrestrial environment presented a steep evolutionary challenge – multiple adaptive pressures and stresses that unicellular marine or aquatic organisms never faced. They encountered wind, drought, larger and more volatile temperature swings, and eventually enemies like pathogens and herbivores, all of which require coordination between cells, tissues, and eventually organs

to orchestrate physiological responses to inputs. One of the systemic signaling systems that help plants adapt to stressful conditions is phytohormones, and one of those phytohormones is JA. JA is nearly ubiquitous in extant terrestrial plants from ephemeral sidewalk dandelions to looming millennia-old conifers. Its absence affects the plant's ability to defend itself against herbivores and some pathogens; because JA plays a central role in the reproductive system of seed plants, its absence is also highly detrimental to reproduction (McConn and Browse 1996 and McConn et al. 1997). In reproduction, JA plays a role or roles in floral development, pollen viability, and anther dehiscence (reviewed in Huang et al. 2017). JA is also involved in trichome development (reviewed in Li et al. 2021), leaf senescence (reviewed in Hu et al. 2017), among a smattering of other physiological processes. JA is an interesting example of one hormonal signaling system mediating multiple distinct processes. It is unclear which function evolved first, but it is reasonable, given the evolutionary history of oxylipin signaling discussed in this section, that the stress-response signal JA was co-opted to coordinate reproduction and floral development. The exact origin of JA biosynthetic capabilities is a surprisingly contentious topic, but it is generally agreed that oxylipin signaling can be traced at least back to the last common ancestor of land plants, and JA signaling to that of seed plants. That does not mean that lower plants, the humble bryophytes for example, cannot have elements of the JA pathway. When examined in silico, there appear to be conserved binding sites present in the moss (*Physcomitrella patens*) and liverwort (*Marchantia polymorpha*) orthologs of *Arabidopsis* COI1, which is a crucial JA-Ile binding protein (Wang et al. 2015). However, biochemical analyses have failed to yield JA or JA-Ile from either of these species. Perhaps unsurprisingly, some lower plants that do not

produce JA *do* produce some of its metabolic precursors via the canonical JA synthesis pathway (discussed in depth in Chapter 1, but summarized in Fig. 3). Guan-Zhu Han (2017), using genomic and transcriptomic data, found analogs of lipoxygenase (*LOX*) genes and allene oxide synthase (*AOS*) in multiple species of photosynthetic organisms, including algae, and in all land plants examined. However, allene oxide cyclase (*AOC*) did not yield significant hits in the genomes of the several algae species included in the analysis, while it was present in all land plant species studied. The two lower land plant species (*P. patens* and *M. polymorpha*) compared by Wang et al. (2015) are shown to have functional AOC enzymes (the third major step in JA biosynthesis, see Section 2) and to accumulate the JA metabolic precursor 12-oxo-phytodienoic acid (OPDA). OPDA, in these plants, has a demonstrated role in reproductive development, and can even be induced upon wounding (Stumpe et al. 2010, Yamamoto et al. 2015), making it analogous to JA in multiple functions. Similarly, other bryophytic species have been shown to have AOC functionality and OPDA accumulation (for example the lycophyte spikemoss, *Selaginella martensii*, as shown by Ogorodnikova et al. 2015). Han (2017) found that some algae have *OPR3* orthologs, but none of them contained a crucial domain that is responsible for the specificity of OPR3 function in JA biosynthesis, a domain that was found in spikemoss and the vascular plants studied but *not* in the bryophytes *P. patens* or *M. polymorpha*. This indicates a potentially important evolutionary split between bryophytes and lycophytes in the potential ability to synthesize JA. Further, *JAR1* homologs were not found in most algae but were found in all land plants studied (Han 2017). This is an interesting result, given that only some of the land plants have a (for the purposes of JA biosynthesis) functional OPR3 gene/protein. However, as I will explain in

Chapter 1, Part 2, JAR1 may have multiple roles beyond the conjugation of JA to isoleucine, including a role that uses OPDA as a substrate. *S. martensii* and *M. polymorpha* lacked accumulation of JA or JA-Ile, despite having fully functional early JA biosynthetic enzymes and accumulating OPDA (Ogorodnikova et al. 2015, Yamamoto et al. 2015). Overall, these analyses point to an evolutionary branch point at approximately the evolution of flowering (or perhaps vasculature) where OPDA developed sometime around the last common ancestor between land plants and modern green algae, and another where JA began to become a very important signaling molecule in land plants. The JA perception pathway may only be partially present in algae, but appears to be mostly present in land plants. Han (2017) found analogs of *COI1,* the JA receptor protein responsible for nuclear perception and initiation of JA responses, in only a couple of algae species, and a hit in tBLASTn does not necessarily indicate the presence of a functional protein coded for by the gene searched. *JAZ* genes, which code for proteins that suppress JA-responsive signaling, were also not found in algae in this analysis; nor was the *JAZ* co-repressor *NINJA*. Homologs of genes coding for the transcription factor *MYC2,* which promotes JA-responsive genes, and the co-repressor *TOPLESS* (TPL) were found in all charophyte and embryophyte species examined, which is interesting but not surprising given MYC2's multifunctional nature and the ability of TPL proteins to interact with auxin signaling (Han 2017). All of these genes appear to have homologs in *P. patens* and *M. polymorpha*, however, despite the fact that these plants likely cannot synthesize JA. The MYC family in particular is extremely important in plant development and has accordingly been highly evolutionarily conserved; Peñuelas et al. (2019) demonstrated that multiple MYCs exist in *M. polymorpha*. Their phylogenetic analysis leads to the conclusion that

MYC function likely existed prior to the diversification of land plants as we know them, perhaps even in algae. The presence of proteins homologous to vascular plant JA perception proteins may be indicative of an ability for other molecules to, in some manner, affect the canonical JA perception machinery, or it may represent the evolutionary building blocks of modern seed plants' use of jasmonate signaling.

The functional connections of oxylipin signaling, even in plants that only use OPDA, to JA are undeniable. One overarching theme emerges from looking at the jasmonate signaling system through time: oxylipin signaling molecules – jasmonates, broadly construed – are crucial in plants' adaptability and have persisted since nearly the origin of 'plants' as we know them.

Today, jasmonates persist as important stress and defense-related signaling molecules, but some plants have evolved to turn the tables on their enemies by repurposing signaling mechanisms already encoded in their genes. Plant carnivory is a phenomenon that has independently arisen several times in different lineages, and is an illustrious example of plants' ability to use different means to evolve functionally similar ends (Givnish 2015). It makes intuitive sense that the mechanisms that plants used to detect and respond to insect predators could give rise to a mechanism to detect and respond to insect prey. Plant chemical defenses can range from (fairly) innocuous bitter or noxious taste, like glucosinolates in the mustard family Brassicaceae (reviewed in Hopkins et al. 2008) which give cruciferous vegetables their signature taste, to potent poisons, like cardenolides (a subset of the medicinally useful but dangerous cardiac glycosides) in the dogbane family Apocynaceae (reviewed in Agrawal et al. 2012), to the structural disruptor of fungal cell walls (or arthropod exoskeletons) chitinases in, e.g., the grass family Poaceae (reviewed

in Grover 2012), and are regulated in large part by hormonal signaling including jasmonates. Known pathogen- and herbivore-related defense compounds have been found in the digestive fluids of multiple unrelated lineages of pit trap carnivores such as *Nepenthes* (Pavlovič and Mithöfer 2019), and many more appear to have been modified to better suit digestion over defense. Jasmonate is the hormone primarily associated with inducible defenses, often triggered after detection of herbivore or fungus-associated molecular signatures. Fascinatingly, in some carnivorous plants known to require induced digestion, like the Venus fly trap (VFT) and sundew, JA has a demonstrated role in triggering this process. Triggered VFT traps accumulate JA and JA-Ile in the same way as if they were wounded (Pavlovič et al. 2017) and exogenous JA application causes the chemical composition of VFT digestive fluid to change, increasing the relative proteolytic capability to improve prey breakdown (Libiaková et al. 2014). Similar responses are seen in sundew plants, where prey detection triggers jasmonate signaling and exogenous JA or JA-Ile can cause digestion initiation in the form of an 'outer stomach' (Krausko et al. 2016, Mithöfer et al. 2013, Nakamura et al. 2013). Pavlovič et al. 2017 suggest that this JA-dependent inducibility of digestive activity in response to mechanical triggers and/or insect detection is a mechanism for saving metabolic resources. Like inducible defenses, digestion uses resources that could otherwise be used for growth and development and may not be advantageous to have constitutively active. This is an even more convincing argument given that plant carnivory almost always evolves in environments that are nutrient-poor, placing additional limiting factors on resource availability (Givnish et al. 1984). Pavlovič and Mithöfer (2019) review comprehensively the connections between defense and carnivory in several different lineages of carnivorous plants, but one major

point that stands out repeatedly is the central role that JA plays in these responses. Undoubtedly, jasmonates are a multi-talented and extremely evolutionarily important class of signaling molecules deserving of close study.

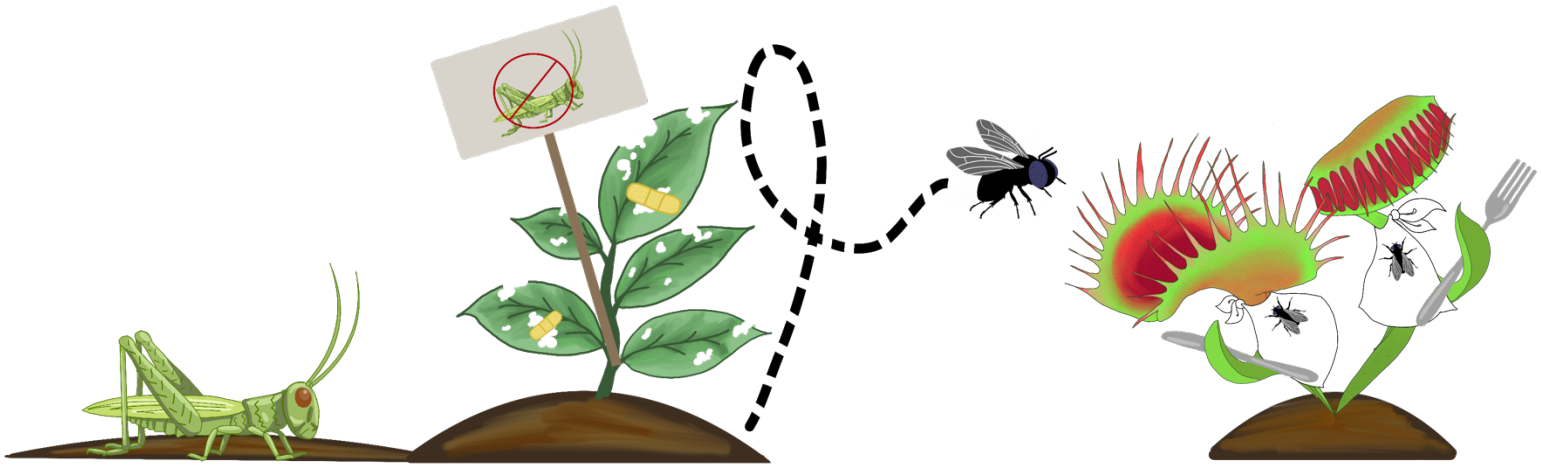

Figure 4: Jasmonates were an early development in the plant lineage, playing a role in stress response. This includes an upregulation of chemical defenses in response to herbivory. Later on, the jasmonate pathway was co-opted by carnivorous plants to better facilitate prey detection and digestion. Plants use jasmonates as defense, but also as offense against their enemies.

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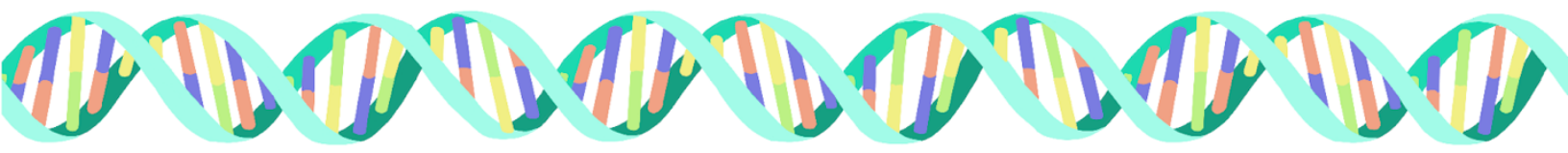
Chapter 1: The jasmonate signaling pathway and its far-reaching impacts in plant physiology



Abstract

The jasmonate phytohormones (JAs) are a family of fatty acid-derived molecules used by plants to coordinate several important physiological processes, including development, reproduction, and defense. The signaling pathway triggered by these molecules is as ancient as land plants themselves; the evolution of multicellularity and emergence onto the land combined with obligate immobility imposes a great deal of environmental stress on the newly terrestrial plants, with JAs representing a key element in the subsequent responses. JAs are most well-known as facilitators of defense against necrotrophic pathogens and herbivores, and their *de novo* synthesis and subsequent local and systemic defense induction is associated with a plant's perception of either of those possible enemies. There are decades of research into the jasmonate control of response to wounding and similar acute traumatic stress. However, despite anecdotal evidence of potential major induced responses to touch, there are still many open avenues of investigation as they relate to chronic, non-traumatic mechanical stress.

Jasmonates are far from the only phytohormonal signaling molecules that plants use to coordinate their functions. That said, they do play a major role as a crosstalk and regulation hub, interacting with nearly every known plant hormone in some way and affecting the ebb and flow of physiological processes. Of particular interest to plant researchers is the relationship between growth and defense, which is controlled primarily by JA and JA-related hormonal crosstalk. Touch stress represents an unexplored avenue for picking apart the complicated network of JA-related signals and then potential agricultural application to separate growth and defense to optimize both for sustainable agriculture in the future.

Part 1: Thigmomorphogenesis and how oxylipins mediate the relationship between growth and defense

Mechanical disturbance is among the myriad environmental stresses and stimuli plants must weather on a constant basis. Plants can't leave where they've put down roots, so their only option for survival when faced with environmental stress is to endure it. Mechanical stress can be indicative of a variety of potential situations or dangers; from the passing brush of a walking animal or another plant, to acute wounding that could signal that the plant is under attack from an herbivore. The literature abounds with information about the plant response to wounding; a relatively severe trauma that quickly induces a strong molecular and physiological response. However, plants can have dramatic responses to gentle mechanical stress too, especially when it is repetitive. Thigmomorphogenesis is the term used to describe the phenomenon where plants undergo changes in growth and morphology in response to non-wounding mechanical stimulation like touch stress (Jaffe, 1973). It can also refer to touch-induced changes in gene expression and biochemistry – for example, upregulation of anti-herbivory or anti-pathogen defensive compounds. Touch stress-induced physiological changes are linked to multiple signaling pathways, but the one that is among the most influential and well-studied in this context is jasmonate (JA). This signaling pathway is thought to be required for thigmomorphogenesis to occur, as these touch responses are abolished when JA biosynthesis is interrupted early in the pathway or when the plant is unable to perceive the JA it produces (Chehab et al. 2012). Mechanical stress leads to production and perception of JA in local *and* distal tissues, which then often induce defense-related gene expression, drawing a direct line from the stress to systemic defense induction (Chehab

et al. 2012). Touch stimulation, in general, inflicts a common set of physiological changes regardless of species: a reduction (in rate or in total size) of growth, and an induction of some sort of defense mechanism (Jaffe 1973; Jaffe 1983, Telewski and Jaffe 1986a, Telewski and Jaffe 1986b, Chehab et al. 2008, Ghosh et al. 2021). This leads to a logical hypothesis: growth and defense are tightly intertwined, are related to metabolic economy, and are controlled in a major way by the JA signaling pathway. While recent research has chipped away at the close relationship between growth and defense in an effort to uncouple them (Campos et al. 2016), the oxylipin (jasmonate) signaling pathway continues to be a major field of study because of its control over these responses.

Part 2: The oxylipin stress signaling pathway

Jasmonate biosynthesis

The biosynthesis of jasmonic acid is a complex process, and is summarized in Fig. 1. It takes place in two different organelles and the cytosol, requires the synthesis of multiple intermediates, and can ultimately produce one of several end products including JA, OPDA, their various amino acid conjugates, dinor-OPDA, and the volatile methyl ester of JA. The JA biosynthetic pathway is also referred to as the octadecanoid pathway, and the two terms may be used interchangeably in this chapter.

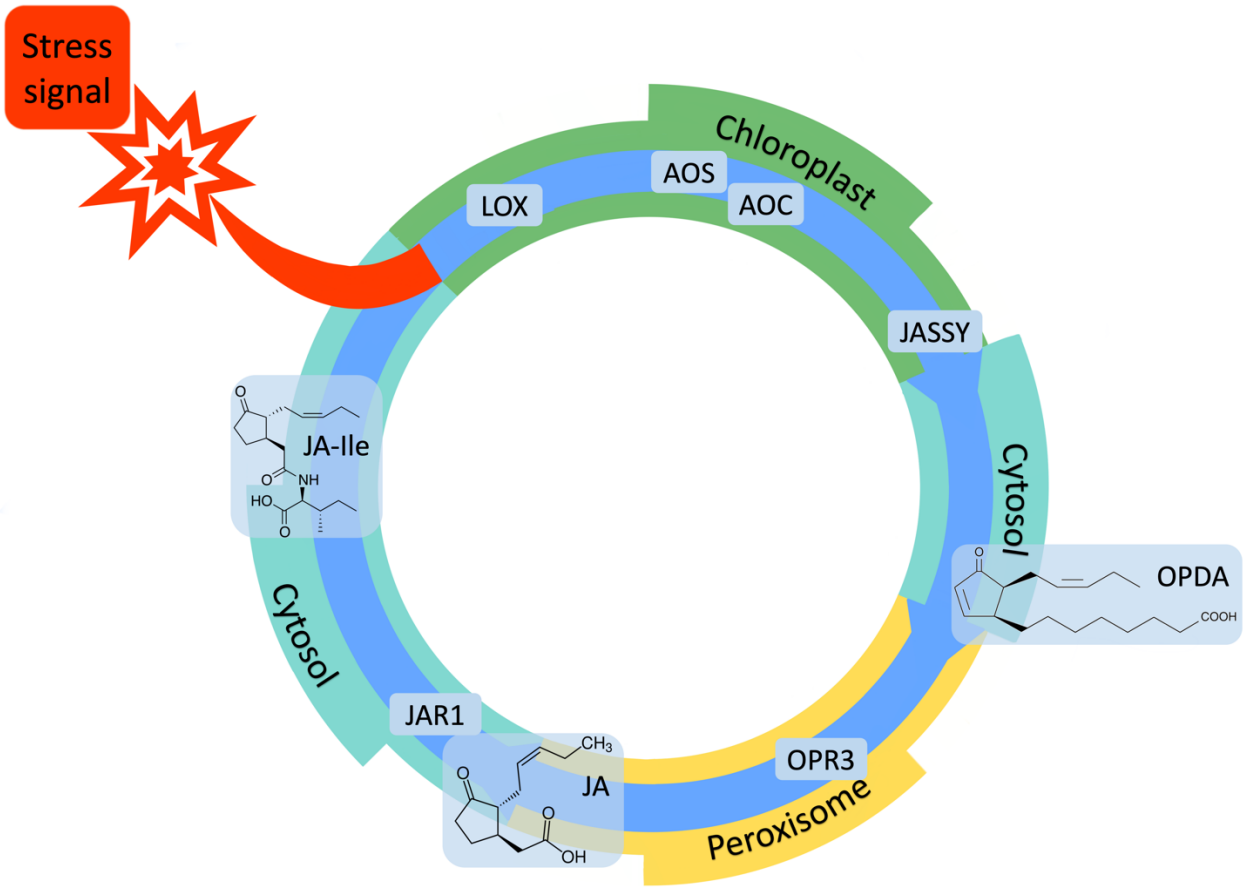


Figure 1: JA biosynthesis. This diagram illustrates the major steps, enzymes, and intermediates of JA biosynthesis, along with the subcellular localization of each major step.

i. Initial chloroplast membrane lipid metabolism

The initial steps of the JA biosynthetic pathway take place in the chloroplast. Injury signals reach the chloroplast, and first things first: in order for a hormone to be synthesized, the substrate needs to be available. Ishiguro et al. (2001) were among the first to identify a specific lipolytic enzyme (DAD1/PLA1) involved in JA biosynthesis, although it had been previously suggested, with only scant successful experimental evidence, that the fatty acid precursor was sourced from plastid membranes by a phospholipase. The mechanism of fatty acid release from membranes is still not completely well-characterized

due to the diversity and number of enzymes involved. However, there are multiple lipolytic enzymes that are activated by wounding, including forms of phospholipase A and phospholipase D (Wang et al. 2000, Ishiguro et al. 2001). The current state of knowledge of chloroplast lipid metabolism, including those used for JA biosynthesis, is reviewed in Cook et al. (2021). In short, there is a broad array of partially or fully functionally redundant lipase enzymes which are still under investigation and are responsible for releasing lipid precursors to oxylipin signaling molecules, a process which produces α-linolenic acid (α-LeA). α-LeA can then act as a substrate for lipoxygenase function.

ii. Fatty acid oxygenation

Linoleate:oxygen oxidoreductases, also called lipoxygenases (LOXs), are a major family of enzymes whose primary function is to catalyze polyunsaturated fatty acids' oxygenation into fatty acid hydroperoxides. These enzymes can be found in nearly every eukaryotic organism spanning kingdoms Plantae, Fungi, and Animalia (Brash 1999, Liavonchanka and Feussner 2006), and have relatively recently been documented in some bacteria (Hansen et al. 2013). The fact that these enzymes were either conserved or independently evolved in eukaryotic and prokaryotic lineages speaks to their crucial role in multiple physiologies – indeed, the variety of LOXs lends itself to a variety of functions, from signaling to developmental metabolism. Six LOX enzymes are present in the genome of *Arabidopsis*, which were originally described nearly a century ago and have been continuously studied since (Andre and Hou, 1937, Samuelsson et al. 1987, Bell and Mullet 1993, Bell et al. 1995, to name a few). Bannenberg et al. (2009) characterized the function of each of these enzymes, classifying them based on their primary substrate and the position that the target fatty acid is oxygenated. 9-LOX

enzymes LOX1 and LOX5 oxygenate the 9th carbon of linoleic or linolenic acid, while 13-LOX enzymes LOX2, LOX3, LOX4 and LOX6 oxygenate the 13th carbon of linolenic acid exclusively. Linoleic and linolenic acid are both 18-carbon polyunsaturated fatty acids, but linoleic acid ($C_{18}H_{32}O_2$) is an 18:2 fatty acid, meaning it contains two carbon-carbon double bonds breaking saturation and linolenic acid ($C_{18}H_{30}O_2$) is an 18:3 fatty acid with three double bonds. The primary enzymes involved in JA biosynthesis are the 13-LOXs, i.e. LOX2, 3, 4, and 6 – those that demonstrate a substrate specificity and preference for linolenic acid (reviewed in Feussner and Wasternack 2002). Upon release of α-linolenic acid (α-LeA), LOX2, 3, 4, and especially 6, are able to act on this substrate (Chauvin et al. 2013). Chauvin et al. discovered that LOX6 plays a unique part in JA biosynthesis – it is the only 13-LOX whose loss eliminates jasmonate production in tissues that are not in the immediate area of the wound. This cements LOX6 as central to the functional wound response since local JA signaling can only prepare local tissues for potential attack. Systemic signaling to distal tissues is an essential part of coordinating defenses and is one of the most critical aspects of jasmonate signaling. Regardless of which 13-LOX acts on α-LeA, the product is 13S-hydroperoxy octadecatrienoic acid (13-HPOT) (Wasternack and Feussner 2018, Liavonchanka and Feussner 2006).

iii. Allene oxide metabolism

The next step in the oxylipin synthesis pathway for LOX products is what determines the ultimate fate of the fatty acid intermediate. Two atypical cytochrome P450 enzymes compete for 13-HPOT and its metabolic relatives: allene oxide synthase (AOS), which commits the molecule to the octadecanoid pathway, or 13-hydroperoxide lyase (HPL), which commits the molecule to the green leaf volatile pathway. AOS and HPL are

remarkably similar in structure, as shown by Lee et al. (2008), who were able to alter the structure of *Arabidopsis* AOS by changing one amino acid to another of differing polarity to function like HPL and act as a green leaf volatile biosynthetic enzyme. The product of AOS acting on 13-HPOT is, as the name suggests, an unstable, reactive allene oxide molecule. Allene oxide intermediates in the lipid hydroperoxide metabolism are so unstable that the 13S-hydroperoxide of α-LeA, the product of AOS, has a half-life of only minutes at ambient temperature (Brash et al. 1988, Brash et al. 1990). Plant signaling can happen in a matter of minutes, but this instability likely prevents these intermediates from being used for anything but further metabolizing into other, more stable molecules. In some plants, including flax and *Arabidopsis*, AOS is encoded by a singular gene (Song et al. 1993, Laudert et al 1996), but some other species have multiple, including tomato, barley, and rice (Howe et al. 2000, Sivasankar et al. 2000, Maucher et al. 2001, Agrawal et al. 2004, Chehab et al. 2008). AOS transforms 13-HPOT by dehydration to 12,13-epoxy-octadecatrienoic acid and due to the instability of the latter compound the next step in JA biosynthesis begins basically immediately, catalyzed by allene oxide cyclase (AOC). AOC uses the product of AOS as a substrate, and as the name suggests, it cyclizes the allene oxide (Zimmerman and Feng 1978, Hamberg 1988, Hamberg and Fahlstadius 1990, Koetje 2003). The product of this reaction is 12-oxo-phytodienoic acid (OPDA), and it is the first molecule in the pathway to have any defined bioactivity beyond the biosynthetic pathway (see “Other oxylipins” section below for a discussion of OPDA’s capability as a signaling molecule).

iv. Transporters

OPDA production marks the last reaction in JA synthesis that occurs in the chloroplast. In order to proceed with the pathway, OPDA needs to be exported from the chloroplast, a process which until quite recently was mostly unknown. Like the nucleus, the chloroplast has two membranes that make up its envelope. That presents two barriers to molecular flux - although the inner membrane presents more of a challenge. The outer membrane is permeable to small molecules generally, but the inner membrane is specifically permeable and therefore requires transport molecules or proteins in order to facilitate crossing (Heldt and Sauer 1971). At the time of writing, no inner membrane transporter has been identified in *Arabidopsis* or any other herbaceous plant. However, in 2021, the first known inner envelope OPDA transport protein was characterized in *Populus trichocarpa*: OPDA Transporter 1 (OPDAT1) (Zhao et al. 2021). This transporter is essential to JA production in *P. trichocarpa* and localizes to the inner membrane. Its analog has yet to be discovered in *Arabidopsis* but presumably must exist for OPDA export. Guan et al. (2019) discovered that in *Arabidopsis*, the Bet v1-like protein JASSY is responsible in large part for transporting OPDA across the outer membrane. As a member of the Bet v1-like family, it is able to bind hydrophobic molecules. It binds OPDA and demonstrably acts as a transporter across the outer membrane of the envelope (Guan et al. 2019). Once exported from the chloroplast, OPDA either gets imported to the peroxisome for JA biosynthesis or it goes on to act as a standalone signaling molecule, sometimes in isoforms like OPDA-Isoleucine (OPDA-Ile). Little is known about the specifics of OPDA transport from the chloroplast to the peroxisome, but it is extremely likely that the peroxisomal ATP-binding cassette transporter protein COMATOSE (CTS)

is required to facilitate it (Theodoulou et al. 2005, Footitt et al. 2007). Mutants lacking CTS demonstrate extremely low, but interestingly non-zero, amounts of JA accumulation and an impairment of JA-dependent functions, although our knowledge of the transporter function of CTS in plants appears to be incomplete. This peroxisomal transporter brings OPDA in from the cytosol for it to undergo the next step of JA biosynthesis.

v. Peroxisomal processes

Upon import into the chloroplast, OPDA undergoes its next enzymatic reaction: a reduction to 3-2(2'(Z)-pentenyl) cyclopentane-1-octanoic acid (OPC-8) (Schaller et al. 2000). There exist in the *Arabidopsis* genome multiple genes encoding 'ODPA reductases' (OPRs), but only one of them is important to the JA biosynthesis pathway. OPDA REDUCTASE 3 (OPR3), also known as DELAYED DEHISCENCE 1, demonstrates localization to the peroxisome, unlike the other reductase isoforms which may be cytosolic (Stintzi and Browse 2000). OPR3 is also the only OPR to efficiently catalyze the reduction of OPDA at its 10,11 double bond for JA synthesis due to its substrate specificity (Strassner et al. 2002). OPR3 is the *Arabidopsis* enzyme but it has homologous enzymes in every plant that uses the octadecanoid pathway. In rice, the putative OPDA reductase that blocks JA synthesis, is OPEN GLUME 1 (OG1), and is required for the opening of inflorescences (glumes) which is a JA-responsive process (Li et al. 2018). OPC-8 goes on to prep for β-oxidation - Koo et al. (2006) discovered an enzyme they called OPC-8:0 CoA Ligase 1 (OPCL1) which, like other acyl-activating enzymes, converts its substrate to its CoA ester, which is then able to be oxidized (Koo et al. 2006, Kienow et al. 2008). Each of the successive rounds of β-oxidation is catalyzed by a different enzyme. The first round involves the action of one or more acyl-coenzyme

A oxidases (ACXs). Cruz Castillo et al. (2004) presented evidence that ACX1 is the main enzyme involved in this step of JA production, and Schilmiller et al. (2007) showed that while loss of ACX1 reduces JA production, loss of ACX1 and ACX5 abolished it. It is possible that there is a degree of functional redundancy between multiple enzymes in this family. Next, a multifunctional protein (MFP), likely AIM1, catalyzes the second round (Delker et al. 2007). The final round is facilitated by a 3-ketoacyl-CoA thiolase (KAT) - in *Arabidopsis*, this is likely KAT2 (Cruz Castillo et al. 2004, Afitlhile et al. 2005). The final product of these β-oxidation reactions is jasmonic acid (JA).

vi. JA fates and conjugation

After re-export into the cytosol, JA can undergo any of a wide variety of transformations. So far, at least hydroxylation, sulfation, glucosylation, methyl esterification, or conjugation to any of at least five different amino acids have been documented. Of these, methyl esterification and amino acid conjugation are of the most interest in long-distance signaling.

Methyl jasmonate (MeJA) was first identified as a component in the essential oils of jasmine (*Jasminum grandiflorum*). As a volatile ester, it is unsurprisingly a fragrant molecule, and contributes to the lovely scent of jasmine's inflorescences (Demole et al. 1962). MeJA is now recognized as an important interplant signaling molecule, due to its volatile nature. When wounded, plants release MeJA from their tissues as well as producing JA for intraplant communication and coordination of responses. It can then be perceived by nearby plants, which upon perception, even in the absence of further stressors, begin to respond as though they had experienced a stress like wounding that would increase JA signaling (Farmer and Ryan 1990, Farmer et al. 1992). MeJA could in

this way serve as a community survival mechanism. The enzyme responsible for the synthesis of MeJA is jasmonic acid carboxyl methyltransferase (JMT) (Seo et al. 2001). MeJA has a specific role in warning other plants of nearby danger, but plays much less of a role in systemic signaling within one plant.

Finally, JA can be conjugated to the nonpolar amino acids leucine (Leu), isoleucine (Ile), valine (Val), or phenylalanine (Phe) (Staswick and Tiryaki 2004), although of these JA-Ile is the most abundant and considered to be the most biologically active. JA-Ile is more effective in *Arabidopsis* in eliciting JA-responsive gene expression than are other isoforms, and many JA-related transcriptomic and physiological changes rely on JA-Ile. The roles of the other JA amino acid conjugates (JA-AAs) are not well-characterized or studied, but at least JA-Val and JA-Leu have very recently been demonstrated to have a similar effect to JA-Ile in rice (Fu et al. 2022). Fu et al. (2022) demonstrated that in rice, other JA-AAs in addition to JA-Ile are induced by herbivore feeding, which is interesting but perhaps not surprising given the general upregulation of

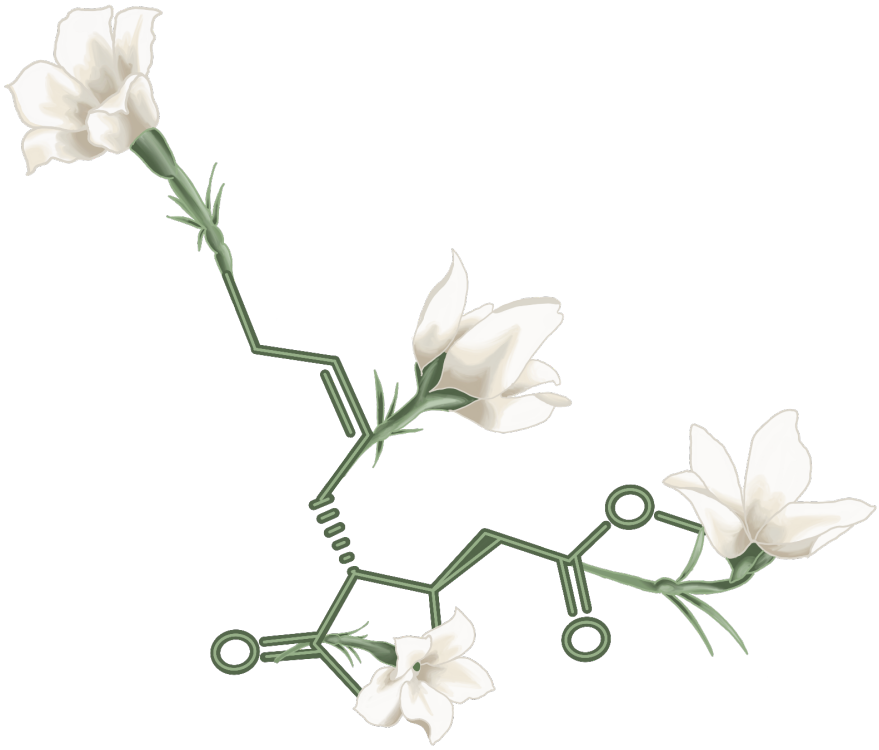


Figure 2: Methyl jasmonate, a volatile intraplant signaling molecule, was first isolated from jasmine plants as one component of the scent of jasmine flowers.

base JA biosynthesis. An increase in the substrate JA, if the endogenous basal level of JA is low enough that the conjugase enzymes are not completely occupied, would logically cause an increase in the conjugation product. They showed that JA-Val and JA-Leu also interact directly *in vitro* with the COI1 JA perception complex, which is what the plant uses to gauge JA-Ile level and is involved in the de-repression of JA-responsive signaling (see below). These two JA-AAs, similarly to JA-Ile, promoted the degradation of OsJAZ4 (a process mediated by COI1 and generally attributed to JA-Ile), and also inhibited seedling growth when seedlings were grown on media containing each JA-AA. In addition, both JA-AAs cause rice plants to increase transcription of JA-responsive genes, such as *OsJAZ8* and *OsMYC2*, and induce the production of defensive secondary metabolites (Fu et al. 2022). It appears that some other JA-AAs function much like JA-Ile does through the canonical signaling pathway and can at least partially induce similar responses. JAR1 is the enzyme primarily responsible for JA-Ile conjugation in *Arabidopsis* (Staswick and Tiryaki 2004) and has been proposed to be the conjugase that is responsible for multiple JA-AAs, and *in vitro* has demonstrable effects on the production of several of those compounds (Xiao et al. 2014). Knockout mutants deficient in OsJAR1 or OsJAR2 (in rice) and the analogous JAR4 or JAR6 (in *Nicotiana*), similarly, produced lower levels of JA-Leu, JA-Val, and/or JA-Ile (Wang et al. 2007, Wakuta et al. 2011, Svyatyna et al. 2014, Xiao et al. 2014). However, at this time, it appears that the effect of non-Ile conjugates of JA is much smaller than that of JA-Ile. They accumulate to a much lower level (73-90% less than JA-Ile levels in *Arabidopsis* according to Staswick and Tiryaki 2004) and have a lower binding affinity for COI1 in both tomato and rice and a lesser growth stunting effect (Katsir et al. 2008, Fu et al. 2022) than does JA-Ile. In

addition, there appear to be differences in the susceptibility of certain JAZ proteins to (non-Ile) JA-AAs over others – some JAZ are not affected, so there may be an evolved specificity of certain JAZ genes/proteins to be responsive only to JA-Ile (Katsir et al. 2008). JA-Ile is characterized as the primary bioactive JA because it, much more so than the other jasmonate products, leads to what is considered the canonical JA response, transcriptionally, chemically, and physiologically (Fonseca et al. 2009).

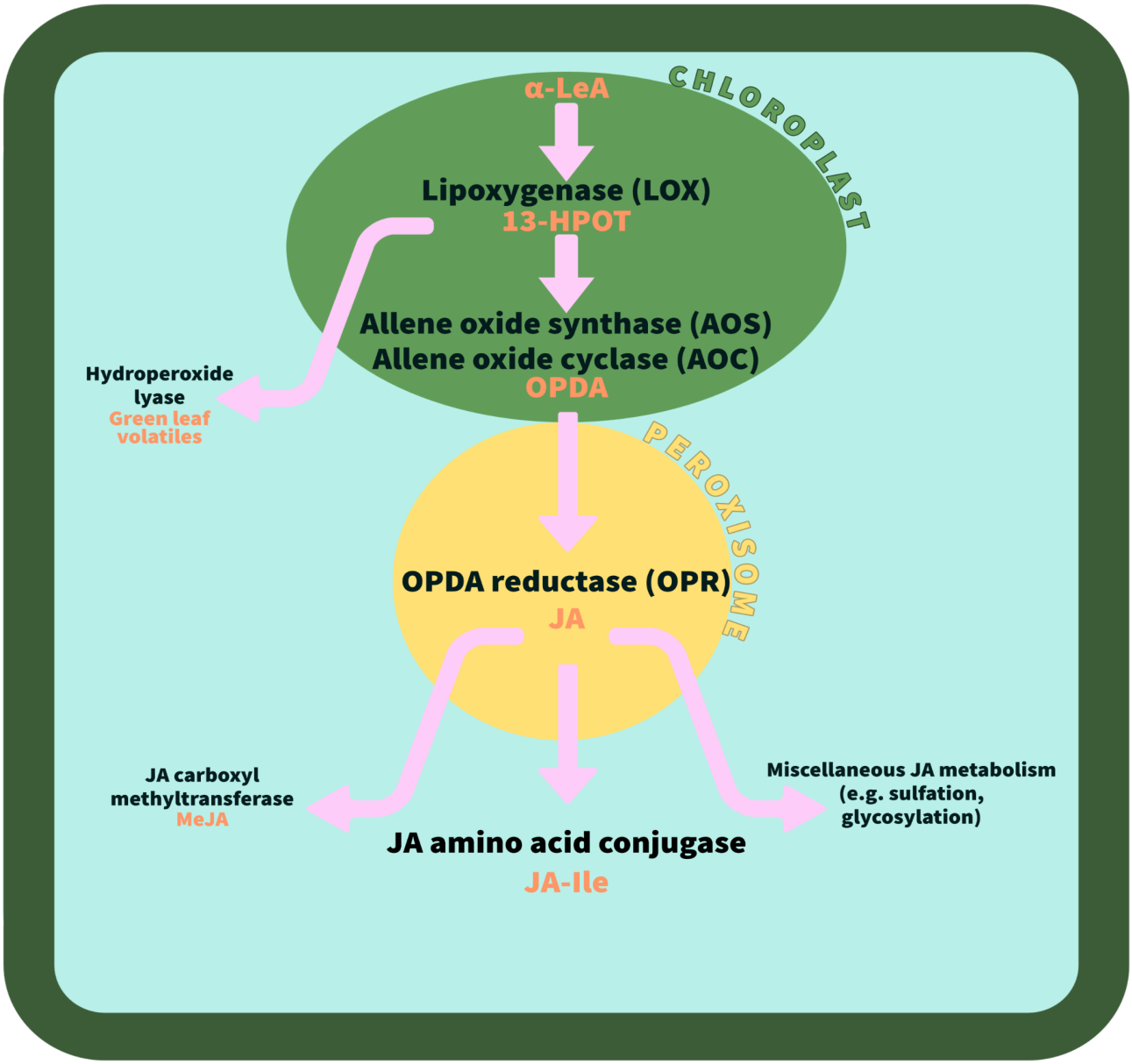


Figure 3: Schematic representation of the potential fates of α-LeA upon release. Points of metabolic divergence (committed steps) exist after LOX action for 13-HPOT, after chloroplastic export for OPDA, and after OPR3 action for JA. JA-Ile biosynthetic pathway is represented by the pink arrows flowing straight down.

Jasmonate perception

i. Transporters

The transporters that regulate oxylipin transport into and out of the nucleus and therefore facilitate hormonal homeostasis and promote oxylipin responsive signaling were until recently almost entirely unknown. Li et al. (2017) discovered an ATP binding cassette (ABC) transporter - the same class of transporters that control peroxisomal OPDA transport and several other cellular molecular fluxes - which in *Arabidopsis* is called AtJAT1. Interestingly, this transporter serves a dual purpose, as it is localized to *both* the nuclear membrane and the plasma membrane. Its function appears to be critical to JA perception in the nucleus, and it has a specificity for JA-Ile as demonstrated by the use of *jar1* mutants and subcellular labeled JA tracking. As mentioned, AtJAT1 is not only located in the nuclear envelope, though - it facilitates the export of JA-Ile from the cell as well. As far as we are aware, AtJAT1 demonstrates no cellular import functionality, and although it has dual PM/NE localization it appears to only transport JA-Ile out of the cytosol in either direction (Li et al. 2017). Even more recently, the same group that discovered the function of AtJAT1 reported two other AtJAT transporters that contribute to intercellular JA transport: AtJAT3 and AtJAT4, providing evidence that ABC transporters are (one of/the) most important class(es) in JA transmission within and between cells (Li et al 2020). These transporters enable the inter-organ transmission of JA, likely via phloem, and drive long-distance signal transduction in response to perceived dangers.

ii. Transcription factors

JA arrival in the nucleus results in a molecular orchestra of regulatory proteins initiating a rapid, choreographed cascade of events, a machine with many moving parts. Some of the ways in which these moving parts (genetic and epigenetic mediators) interact with one another, and potentially some of the parts themselves, still represent an opportunity for further discovery. In the resting state (low endogenous JA-Ile concentration), so-called “JA-responsive” genes are expressed at a fairly low basal level - they are repressed through physical protein interaction with and functional repression of transcription factors (TFs) that promote expression of these “JA-responsive” genes (Chini et al. 2007, Yan et al. 2007) (Fig. 4). Of these, the MYC family of basic helix-loop-helix (bHLH) transcription factors is the most well-characterized grouping. MYC2, definitively characterized nearly twenty years ago (Lorenzo et al. 2004, Boter et al. 2004), is, in a sense, the representative of this group and has been thoroughly studied since its identification as a jasmonate-related TF. MYC3, MYC4, and MYC5 appear to have largely (but importantly, not completely) functionally redundant roles, highlighting the evolutionary necessity of complete MYC-mediated JA-related reproductive development (Fernández-Calvo et al. 2011, Wang et al. 2017). MYC3 and MYC4 particularly are capable of forming dimers with MYC2, which effectively facilitate the interactions that promote target gene expression (Fernández-Calvo et al. 2011). MYC2, MYC3, and MYC4 all exert effects over the transcription of JA-dependent signaling including pathogen resistance and root development (Major et al. 2017). Despite this, MYC2 remains the TF that is chiefly considered a major control over JA signaling (Dombrecht et al. 2007, Kazan and Manners 2013).

Structurally, MYC TFs are able to facilitate the expression of JA-responsive genes because they, like all proteins, contain distinct domains that serve different functions. MYC2, of course, contains a domain that binds it to target gene promoters, and at each terminus there is a domain that facilitates interactions with other proteins. The N-terminus is of particular relevance as it contains the domain that is involved in recruiting one of the Mediator subunits, MEDIATOR 25 (MED25) (Chen et al. 2012).

iii. Mediator

Mediator is a highly conserved protein complex that is essential for eukaryotic transcription, broadly construed. First identified in yeast (Kelleher III et al. 1990), this complex links transcription factors to RNA polymerase II. Mediator became somewhat well-characterized in yeast and in different animals/metazoans (reviewed in Kornberg 2005 and more recently in Allen and Taatjes 2015), but for quite some time the potential existence of a plant Mediator complex was only theorized. Bäckström et al. (2007) purified the plant Mediator from *Arabidopsis* and identified some of its subunits, linking many as homologous to other non-plant lineages and noting that some were less similar and potentially plant-specific (The plant Mediator complex is reviewed in Yang et al. 2016). Fortunately, one of the more definitively identified subunits, dubbed MED25, was demonstrated to be the same protein coded by the gene already known as *PHYTOCHROME AND FLOWERING TIME1 (PFT1)*, so named because of its initial discovery as an important modulator of light-dependent developmental signaling through phytochrome B (Cerdán and Chory 2003, Bäckström et al. 2007). Shortly thereafter, PFT1/MED25 was shown to play an important role in JA-related signal transduction and defense induction (Kidd et al. 2009). MED25 is not the only subunit implicated in defense

responses, but it is the one which seems to be particularly important to JA signaling (Çevik et al. 2012). As previously mentioned, when JA signaling is activated, MED25 links up with MYC2 and facilitates the attachment of RNA pol II to MYC2 target promoters, thus positively regulating JA-responsive gene transcription. Because these genes are, obviously, mostly only activated in the presence of JA-Ile, MED25 must be able to interact with MYC2 and recruit pol II rapidly when signals induce it but it also contributes to MYC2 repression and negative regulation of JA-responsive genes. Keeping defense signaling in check but able to be quickly mobilized requires a complex system of repressors, receptors, and recruiters.

iv. Co-receptor COI1

MED25 has multiple roles in the regulation of MYC function. One of them is association with COI1 via physical interactions near the sites of promoter regions of some genes that are directly affected by MYC2 (An et al. 2017).

coi1 knockout mutant *Arabidopsis* plants were first identified as being insensitive to the bacterial phytotoxin coronatine, hence CORONATINE INSENSITIVE 1 (COI1). In this same groundbreaking study, Feys et al. (1994) reported that not only was *coi1* insusceptible to the toxin, it was also male sterile, insensitive to methyl jasmonate application, and much more resilient against the biotrophic bacteria *P. syringae* than were wild type plants. The mechanism of COI1's involvement in jasmonate perception began to be uncovered only a few years later: COI1 is an F-box protein, associated with an SCF complex (Xie et al. 1998, Devoto et al. 2002, Xu et al. 2002) - it is the target protein-interacting component of a Skp1/Cullin/F-box complex which functions as an E3 ubiquitin ligase ubiquitin-tagging other proteins for 26S-proteasome degradation. The SCF^{COI1}

complex necessarily has to have a target or targets, or it would probably not have been evolutionarily advantageous enough to be conserved in the genome. Indeed, the complex targets a family of proteins called JASMONATE ZIM-DOMAIN (JAZ) proteins (more on JAZ below). Interestingly, endogenous COI1 levels are also maintained via the SCF^{COI1} complex. The complex's other proteins stabilize COI1 and free COI1 proteins are unstable and actually also degraded via 26S proteasome, though not directly targeted for degradation by SCF^{COI1}. Posttranslational regulation plays an important role here in keeping COI1 levels from becoming too high and ensures that COI1 proteins are stably maintained in SCF^{COI1} (Yan et al. 2013). MED25 links SCF^{COI1} to MYC2, although the SCF doesn't directly interact with MYC2 (An et al. 2017). Instead, it lies in wait for the right signal to activate.

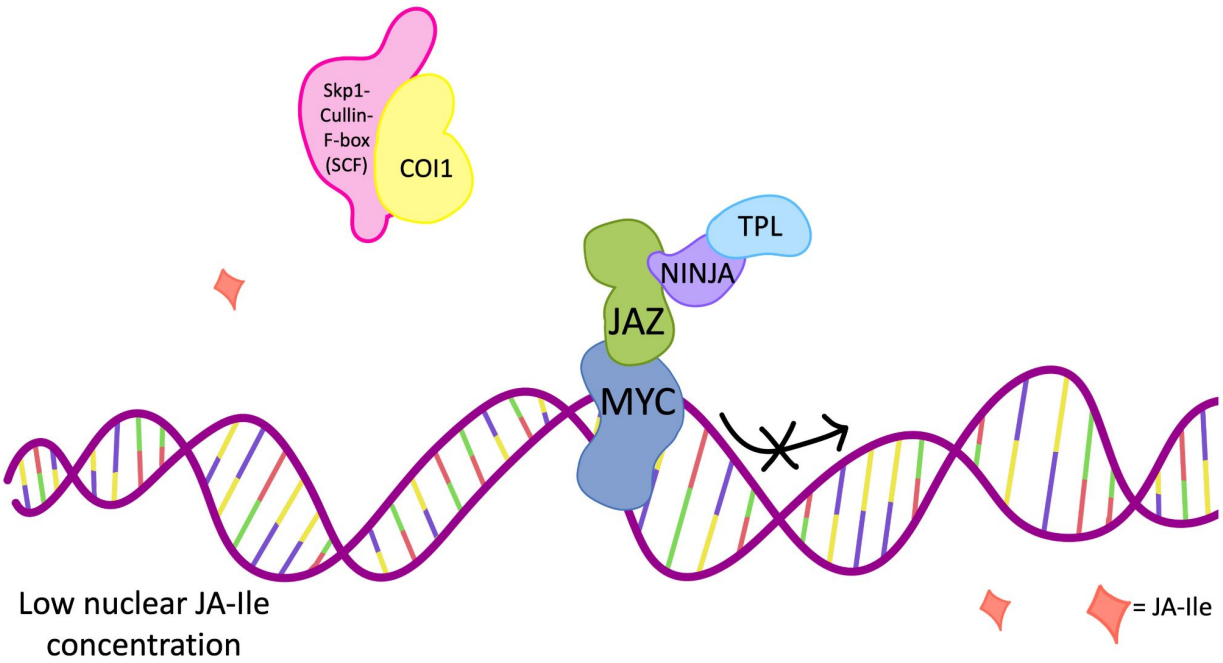


Figure 4: Resting-state repression of JA-responsive genes in a low endogenous JA-Ile environment. In this state, MYC transcription factors are bound by stable JAZ proteins, which in turn recruit NINJA and TPL as co-repressors, and MYCs are unable to recruit MED25 or by extension RNA polymerase to facilitate

transcription. The SCF-COI1 complex is not bound to JAZ proteins without the molecular glue that is JA-Ile.

v. JAZ proteins

The mechanism of repression that restricts MYC function and JA-influenced gene expression is complex and involves a family of 13 JAZ proteins and auxiliary co-repressors. While jasmonate signaling has been a topic of study for decades, the JAZ proteins were characterized less than twenty years ago. Some of the family were characterized in spurts, under a variety of names (e.g. Lorenzo et al. 2004) but among the first to publish about them as we now know and discuss them were three research groups that published similar discoveries simultaneously - with publication dates within 10 days of one another (Chini et al. 2007, Thines et al. 2007, Yan et al. 2007). In the resting state, as discussed earlier, JAZ proteins act as repressors on MYC TFs. These proteins directly interact with the target TFs, as well as other proteins, some of which act as MYC co-repressors alongside JAZ. These will be referred to broadly as JAZ auxiliary repressors when not discussing their individual functions. JAZ proteins require two specific domains in order to function properly as JA-responsive repressors of MYC: a TIFY-containing ZIM domain and a Jas domain (Shikata et al. 2004, Chini et al. 2007, Thines et al. 2007, Vanholme et al. 2007, Yan et al. 2007, Melotto et al. 2008). Mutations or alterations in these domains mostly render the proteins nonfunctional in this context. The Jas motif has an affinity for MYC TFs, and compete for MYC binding with MED25; in this way there is a molecular tug-of-war between initiation and inhibition. There are significant structural changes that occur when MYC binds to JAZ (detailed in Zhang et al. 2015). This region is also what facilitates JAZ-COI1 association upon JA-Ile increase (Melotto et al. 2008). The discovery that JAZ proteins regulate MYC TFs and are the

targets of the SCF^{COI1} complex upon JA perception connected several aspects of JA signaling that previously had seemed incongruous, and at once provided a much more complete understanding of how JA-Ile regulates transcriptional responses to stress. In repressing JA-responsive gene expression, JAZ proteins help plants maintain a sort of metabolic homeostasis and keep temporal and spatial developmental signals in check, leading to a more fit plant (Guo et al. 2018). There are 13 proteins in this family and although they are all different, most of them act at least somewhat functionally redundantly, highlighting the importance of keeping this balance at an appropriate level. Single knockout mutants in *JAZ* genes are usually not obviously phenotypically different than WT, but higher order mutants are progressively smaller and constitutively defended (as shown by the quintuple, decuple, and undecuple *jaz* mutants generated and reported by Guo et al. (2018), the latter two of which are *extremely* impaired in vegetative and reproductive development but very robustly defended). JAZ8 appears to be more unique among its family - it is protected against COI1-mediated degradation by the fact that it lacks the motif necessary for COI1 co-receptor complex formation upon JA-Ile perception (Shyu et al. 2012). It additionally, unlike most other JAZ proteins (and alongside four of them), contains an EAR motif that facilitates direct binding with TOPLESS (TPL) without the need for the interloping co-repressor NINJA (described below) (Shyu et al. 2012). However, these interesting structural differences seem to be anomalies among a small number of JAZ proteins rather than the general rule. The ability of JAZ proteins to form the co-receptor complex and be degraded is required to enable defense signaling, and is as important as the JAZ proteins' ability to repress defense signaling in the first place.

vi. Auxiliary repressors

JAZ proteins generally recruit auxiliary repressors with their ZIM domains. One of these, TPL, is a repressor that has roles in multiple important physiological processes; in fact, its name comes from the fact that its first published role is in regulating shoot apical meristem development (Long et al. 2002, Long et al. 2006). When *TPL* is knocked out, embryonic shoot tissues can be, depending on the temperature, forced to create root tissue instead. It is not difficult to imagine how this might be problematic for the plant, or where the gene's name came from. TPL has been shown to interact with auxin during embryonic development, as a phytohormone-dependent repressor of auxin-responsive genes (Szemenyi et al. 2008). A decade ago, an interactome tailored to TPL was published, highlighting the scope of TPL's influence (Causier et al. 2012). Its presence as a transcriptional repressor in so many processes, and the fact that its important domains are nearly ubiquitous all the way down the plant complexity hierarchy to algae (Martin-Arevalillo et al. 2017), indicates that at least its domains are probably ancient and conserved. Like the MYC TF family, TPL is not limited to only JA signaling, but it is extremely important to JA signaling.

TPL, as a general co-repressor protein, connects to other repressors via the other protein's terminal EAR motif, which is an active transcriptional repression motif that's not uncommon among repressor proteins (Kazan 2006, Szemenyei et al. 2008, Kagale and Rozwadowski 2011). As mentioned earlier, a small subset of JAZ proteins are able to directly recruit TPL with its own EAR motif, but this is not the norm for a majority of JAZ proteins (Shyu et al. 2012). One of the other proteins TPL interacts with in this manner is Novel INteractor of JAZ (NINJA), a co-repressor that interacts directly with TPL via

NINJA's EAR motif in a conserved A domain and with JAZ proteins via the JAZ's TIFY motif in the ZIM domain interacting with NINJA's conserved C domain (Pauwels et al. 2010). In this way, NINJA is capable of connecting TPL to JAZ, enhancing the repressor capabilities of JAZ through this recruitment (Fig. 4). This, additionally, provides an avenue for explaining why removal of the TIFY motif or ZIM domain of JAZ proteins renders them less- or non-functional in their repressor capabilities. NINJA interacts with most of the JAZ proteins directly, but doesn't associate with COI1 or MYC2 (Pauwels et al. 2010). NINJA is stable, and not degraded after JA-Ile perception and subsequent de-repression of MYC TFs, unlike JAZ proteins. Interestingly, in TPL's interaction with AUX/IAA proteins in a way very similar to its interaction with JAZ proteins, TPL does not require an additional coupling protein (such as NINJA), and instead binds directly to the AUX/IAA (Szemenyei et al. 2008). NINJA seems to play a unique role in jasmonate signaling that does not have an analog in otherwise very similar hormonal signaling pathways. Stress signaling is also not the only niche NINJA occupies. Although its name may imply otherwise, NINJA does more than just interact with JAZ; Baekelandt et al. (2018) demonstrated that it has a crucial role in regulating proper leaf development and the cell cycle. It is so far unclear which function evolved first: the developmental or the stress signaling role.

vii. JA-mediated relief of repression

Once JA-Ile concentrations in the nucleus rise above a threshold level, indicating some threat to the plant, JA-responsive transcriptional changes need to occur, which necessitates the de-repression of MYC TFs (Fig. 5). JA-Ile triggers the binding of COI1 to JAZ and the subsequent 26S-proteasome degradation of JAZ. Both SCF^{COI1} and JAZ are required for JA to act as a ligand for either of them - they form a co-receptor complex

that is required for JA perception (Yan et al. 2009, Sheard et al. 2010). COI1 contains a ligand-binding “pocket” region, which is capable of binding JA-Ile or coronatine (which is three orders of magnitude more active in facilitating this interaction, giving more information about how the phytotoxin is such a powerful weapon in bacterial pathogens’ arsenals) (Katsir et al. 2008). This pocket is partially covered by parts of the JAZ protein when the ligand binds, trapping JA-Ile inside and conferring a conformational change on the proteins in the receptor complex, subsequently releasing MYC TFs from the influence of the Jas domain of JAZ proteins and allowing association of MYC TFs with MED25, and therefore recruitment of Pol II and transcription of JA-responsive genes (Sheard et al. 2010, Zhang et al. 2015).

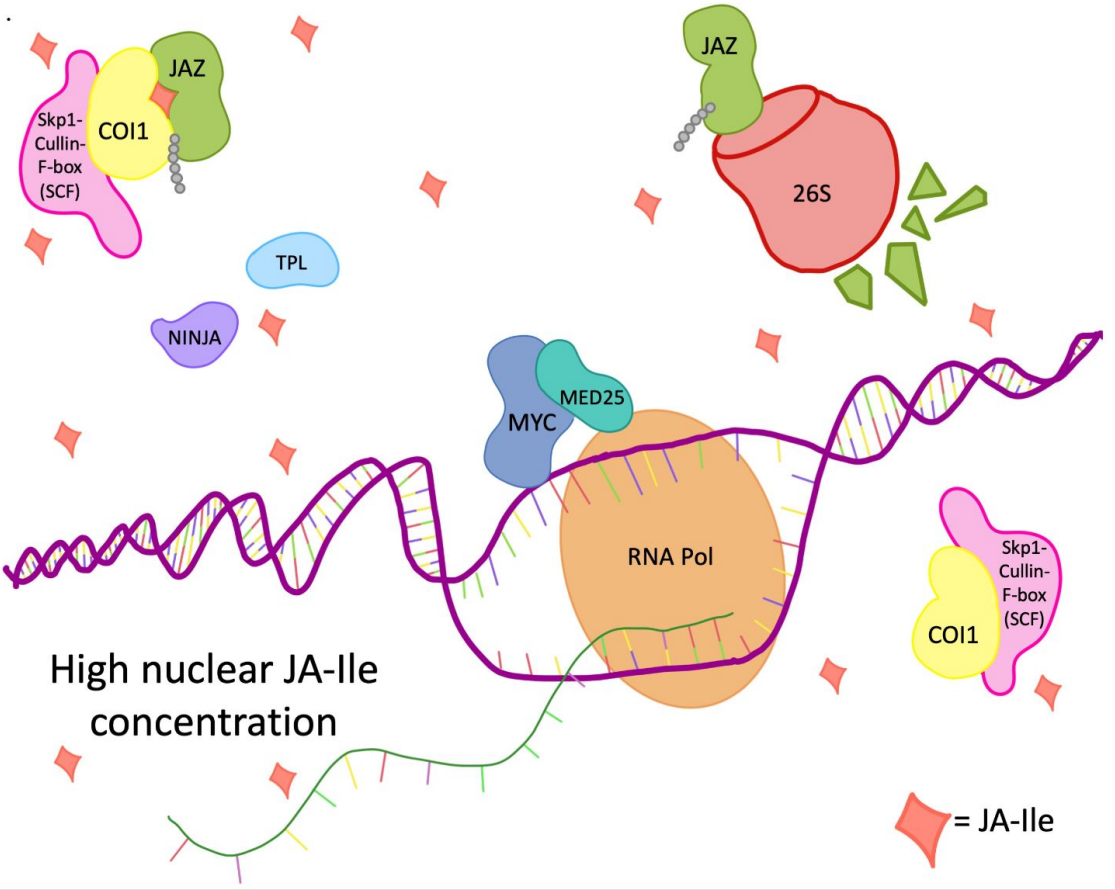


Figure 5: JA-Ile facilitates relief of repression of JA-responsive genes. An increase in JA-Ile concentration in the nucleus causes SCF-COI1 to form a co-receptor complex with JAZ proteins, breaking

the bond between JAZ and MYC transcription factors. MYC is then free to recruit MED25 and RNA polymerase, allowing transcription of JA-responsive genes. JAZ proteins are ubiquitin-tagged by SCF-COI1 and degraded via 26S proteasome.

Phytohormonal crosstalk

i. JA and other hormones

In the last couple of decades, it has become increasingly understood that phytohormonal signaling plays a massive role in plant development, defense, and reproduction that simply cannot be overstated. That being said, no signaling mechanism, in any organism, exists or operates in a vacuum. This is especially true in the context of phytohormones. A complex network of crosstalk between pathways is explicitly necessary in plant physiology - indeed, hormones and their crosstalk are needed for there to *be* a plant to have physiology at all. JA in particular appears to influence several other hormonal signaling pathways, and acts as a crucial molecule at the nexus of regulation of plant growth and defense. It has documented relevant interactions with abscisic acid (ABA), auxin (IAA), brassinosteroids (BRs), cytokinins (CKs), ethylene (ET), gibberellin (GA) and salicylic acid (SA). The ways that any two hormonal pathways interact with one another are far too complicated and context-dependent to reduce to simply “synergistic” or “antagonistic,” and usually could be classified as both.

This network of signals is numerous and complicated (and has already been reviewed recently [Yang et al. 2019, Liu & Timko 2021]), but of particular relevance to JA’s mediation of growth and defense is its interaction with GA, a hormone primarily known as a positive growth regulator. This relationship is mostly antagonistic - GA in general

induces expression of genes that have the eventual effect of suppressing JA signaling and vice versa.

ii. JA and GA

DELLA proteins are involved in modulating GA signaling in much the same way as JAZ do for JA signaling - by repressing GA-responsive genes and de-repressing/being degraded upon GA perception. DELLA proteins, in this way, are negative repressors of growth (Cao et al. 2006, Hou et al. 2008). GA and DELLAs also interfere in JA signaling - they are known to alter JA-responsive gene expression and contribute to pathogen-initiated physiological responses (Navarro et al. 2008, Hou et al. 2010). One important part of the crosstalk mechanism between the GA and JA pathways occurs at the interface between DELLA proteins and JAZ proteins. DELLAs have been known to interact with some bHLH transcription factors in other contexts, but they do not interact with MYC2 directly; instead, they compete with MYC2 for the Jas domain of JAZs (Hou et al. 2010). The ZIM domain was not required for this interaction to occur - only Jas and N-terminal domains. Recall that the Jas domain is the domain used not only for MYC2 interactions, but is *also* competed for by MED25 *and* is the domain bound by COI1. The DELLA domain in DELLA proteins facilitates this interaction. DELLAs are capable of initiating JA-responsive signaling in the absence of JA, through relief of repression of MYC2. Unlike the relief of repression via SCFCOI1 that occurs upon JA-Ile perception, JAZ proteins aren't degraded when they bind DELLAs. Therefore, repression can resume if the DELLAs are detached and the Jas domain freed. Fascinatingly, although DELLAs de-repress MYCs in a manner that involves competitive binding to JAZ, this interaction is COI1-dependent and phenotypes/transcriptional responses that occur in some DELLA

knockout mutants is abolished when *COI1* is also knocked out (Hou et al. 2010). *della* mutants have an attenuated sensitivity to JA, likely in a way that is associated with the DELLA/COI1 interaction. The DELLA domain that is required for binding to JAZ also facilitates JAZs' competitive inhibition of DELLAs' ability to bind *other*, growth-related transcription factors and therefore inhibit their ability to repress vegetative growth (Yang et al. 2012). There is a spiderweb of potential interactions that facilitate the complex crosstalk between JA and GA, but at the center of it all is the interaction between DELLA proteins, JAZ proteins, and the MYC repression or facilitation machinery.

Other oxylipins

OPDA, the oxylipin precursor to JA, does not have one simple role. Like JA, it can meet a variety of fates after synthesis, and only one of those fates is further synthesis into JA. OPDA has multiple roles as a standalone signaling molecule, from development to stress signaling. It does not interact with the SCF^{COI1} complex, so its target genes are often said to be regulated in a COI1- and JA-independent manner (Taki et al. 2005). OPDA's unique signaling properties can be attributed structurally to two distinct differences between it and JA, each of which has a distinct effect: the double bond in OPDA's cyclopentenone ring and the un-reduced, longer carbon chain (Taki et al. 2005).

Some of OPDA's signaling capabilities are associated with abiotic stresses, marking a potentially important distinction between its roles and those of JA. This difference makes sense in an evolutionary context; OPDA signaling, if it evolved before JA signaling, would have been useful for facilitating plant adaptation to the environmental stresses that early land plants would have needed to deal with before they may have needed to defend against herbivores or pathogens. For example, Savchenko and Dehesh (2014) found a

unique function for OPDA in coordinating, alongside ABA, stomatal closure in response to drought stress. They established that this role for OPDA is probably unlinked from JA. OPDA's crosstalk with ABA also has implications for root development and seed development and germination (Dave et al. 2011, Barros-Galvão et al. 2019), and there likely exists a JA-independent role for OPDA in tomato embryonic development as well (Goetz et al. 2012). The developmental signaling roles of OPDA are reviewed in Maynard et al. (2018) and Liu and Park (2021).

Some of the roles that have been shown or suggested for OPDA are non-defensive, and actually involve mechanostimulation and the associated signal transduction that goes along with it (partially reviewed in Dave and Graham 2012). For example, it has been implicated in in the tendril coiling response of the cucurbit vine bryony (*Bryonia dioica*) and grapevine (*Vitis vinifera*), up to and including potentially as the primary communicatory molecule (Weiler et al. 1993, Weiler et al. 1997, Stelmach et al. 1999, Blechert et al. 1999, Malabarba et al. 2019).

Although there are multiple roles for OPDA in plant development and responses to abiotic stress, that doesn't mean that it isn't involved in the response and acclimation to biotic threats. In the absence of JA, plants may be capable of building some resistance against herbivores and pathogens via OPDA signaling, via both COI1-dependent and -independent mechanisms (Stintzi et al. 2001, Stotz et al. 2011). Further, OPDA has been implicated in regulation of induced resistances and mediation of the mutualistic interaction of maize with the microbe *Trichoderma virens* (Wang et al. 2020).

As previously mentioned, OPDA and later JA are known to require the initial step of the desaturation of an 18:3 fatty acid. However, in some cases, a plastid-derived 16:3 fatty

acid can give rise to an isomer of OPDA called dinor-cis-OPDA (dnOPDA). dnOPDA is found across plant species and could represent a potential bypass for jasmonate synthesis even in the absence of 18:3 fatty acid substrates - a hexadecanoid pathway rather than an octadecanoid one (Weber et al. 1997). Fascinatingly, the recent discovery of dnOPDA biosynthesis from 18:3 fatty acids - directly from OPDA itself - further illustrates the flexibility of the jasmonate pathway and the importance of these signaling molecules - such that multiple back channels for oxylipin biosynthesis have evolved (Chini et al. 2018). dnOPDA, regardless of a hexadecanoic or octadecanoic origin, is also a substrate for an OPR3-independent JA biosynthesis workaround, where it instead is converted to 4,5-didehydrojasmonate (4,5-ddh-JA) which can either then go on to transmit signals by itself or can be further reduced to JA by OPR2 in *Arabidopsis*.

JA has a more potent and bioactive isoform in JA-Ile, and likewise, so does OPDA. OPDA-Ile is an amino acid conjugate of OPDA and is also biologically active (Arnold et al. 2016, Floková et al. 2016). OPDA on its own can be used as a substrate for OPR3 to continue JA biosynthesis, but OPDA-Ile is more stable and resistant against being enzymatically altered. In particular, this suggests that OPDA-Ile is the, or one of the, isoforms used for JA-independent oxylipin signaling. JAR1 has been proposed to be the enzyme that facilitates OPDA-Ile conjugation, largely because *jar1 Arabidopsis* mutants have decreased OPDA-Ile accumulation. Importantly, this is a *reduced* but not *abolished* accumulation, which indicates the possibility of multiple enzymes acting partially redundantly. Thus, this role for JAR1 has not been conclusively demonstrated (Floková et al. 2016).

Part 3: Where do we go from here?

Knowledge gap

Although some of the mechanisms by which JA, directly or indirectly through crosstalk with other hormones, modulates the growth-defense relationship have been extensively studied, the system is by no means exhaustively understood. Current jasmonate research tides appear to be shifting to focus on potential roles of OPDA and OPDA-Ile, as well as on the crosstalk between multiple hormonal signaling pathways in mediating defense responses and vegetative growth, as well as how these molecular mechanisms could affect plant-pathogen interactions. These research directions have clear applications in agricultural settings. Current mechanical stimulation research is slowly beginning to incorporate non-traumatic touch stress, but largely still focuses on wounding. This is understandable as it's directly related to herbivory and acute threats to the plant, but it does leave open potential investigations for soft mechanical stimulation and touch, which is also ecologically relevant as discussed above. The ancient practice of applying this kind of treatment to crops to increase hardiness, change phenology, and alter biomass allocation away from vegetative growth is a unique example of direct application of the physiology of touch stress (reviewed in Ghosh et al. 2021). Touch stress remains an understudied phenomenon. Inherently, the roles of different jasmonate molecules, their signaling pathways, and the hormonal crosstalk that underlies the plant response to touch also have yet to be understood in depth. The groundwork laid by our current, relatively more robust understanding of the molecular and physiological responses to traumatic mechanical stress and wounding provides a bit of blueprint for studying these responses

in the context of touch – e.g. we know that JA plays a major role in those responses, and that similar results have been published demonstrating the importance of JA in the touch response (Chehab et al. 2012). In particular, the roles of individual oxylipins and the molecular mechanisms by which they mediate growth and defense in response to touch are not yet well-characterized. Therefore, they remain open questions and gaps in our knowledge of plant stress response.

Roadmap

In **Chapter 1**, we traversed the complex world of mechanical stress signaling: how plants perceive and respond to touch stimuli. I discussed the importance of understanding touch stress and the phenomena that occur between the rapid initial signal sent immediately after perception and before long-term changes in morphology and biochemistry. While this is an extraordinarily complicated network of several types of signaling systems, the developmental changes induced by repeated stress are largely dependent on the action of phytohormones – particularly jasmonates/oxylipins/octadecanoid molecules. I discussed what is known and some of what is left to discover about octadecanoid biosynthesis, transport, perception, catabolism, and crosstalk with other phytohormones in coordinating the relationship between growth and defense in response to mechanical stress.

In **Chapter 2**, I demonstrate that repeated touch stress imposes on *Arabidopsis* plants a clear physiological response: growth restriction and anti-herbivore defense priming, which are oxylipin-dependent. I will show that prior models of growth and defense responses to mechanical stress that center JA-Ile as the crucial molecule in orchestrating them are only partially correct in the case of chronic touch stress. In fact, I demonstrate that JA is not

actually required for plants to translate repeated touch stimulation into a growth restriction response. Rather, one of the upstream metabolic precursors – OPDA – is primarily responsible. In particular, an OPDA-AA (likely OPDA-Ile) conjugated by JAR1, the established JA-AA conjugase, underlies these responses. I will show that OPDA is insufficient to induce touch-responsive defense priming (determined by herbivory) in *Arabidopsis* and that, specifically, JA-Ile is required. This is in agreement with established models that center JA-Ile in defense induction. All in all, I will demonstrate that there exists a disparity in how growth and defense are regulated in response to touch stress, and that more attention should be allocated to examining all pieces of a pathway to search for critical steps that a different resolution study could miss.

In **Chapter 3**, I present a case that the growth response to touch stress is dependent on crosstalk between the JA and GA pathways at the point of nuclear oxylipin perception. I will demonstrate that the plant's ability to constrict growth in response to touch relies on several, but not all, parts of the canonical JA perception machinery as well as on the function of the DELLA proteins. This ability is reliant on COI1 and MYC TFs, although perhaps not in the way I expected. *coi1* and *myc* knockout mutants show an insensitivity to touch stress, so this could indicate that both of these factors are necessary for OPDA perception and the transcriptional changes that result from it. However, it could be attributable to oxylipin biosynthetic genes' reliance on COI1 and MYC function to promote their induction in response to touch. Also among those JA perception elements that are necessary for the previously established OPDA-mediated response is TOPLESS, but interestingly not NINJA, TPL's associated co-repressor. I discuss some possible models of OPDA perception and growth following prolonged touch stress.

In **Chapter 4**, I will bring this story full circle to summarize my findings presented in Chapters 2 and 3 and discuss the implications of those findings for basic plant science and some aspirations for agricultural applications of my work. I will present pathways forward from my work, directions which would pave the way for further research into the complex signaling network that governs plant-pest interactions and the regulation of plant growth under touch stress. The IPCC estimates that within the next 80 years, global mean surface temperatures will rise between 4 and 6 degrees Fahrenheit. These temperature changes are going to affect crop yield and food production, and by extension compromise global food security, in several ways - one being direct physiological stress on plants, compromising biomass production. Another, often overlooked, one is through changes in pest insect physiology and populations. Insects are already a major agricultural problem, but in the future, models predict an additional loss of grain yield to insects of up to 40% *per degree Fahrenheit*. Those numbers, combined with the projected increase of 4 to 6 degrees, highlight the urgent need for us to understand how plants manage growth and defense so that we can optimize and safeguard global food supply against diminishing yields and insecurity.

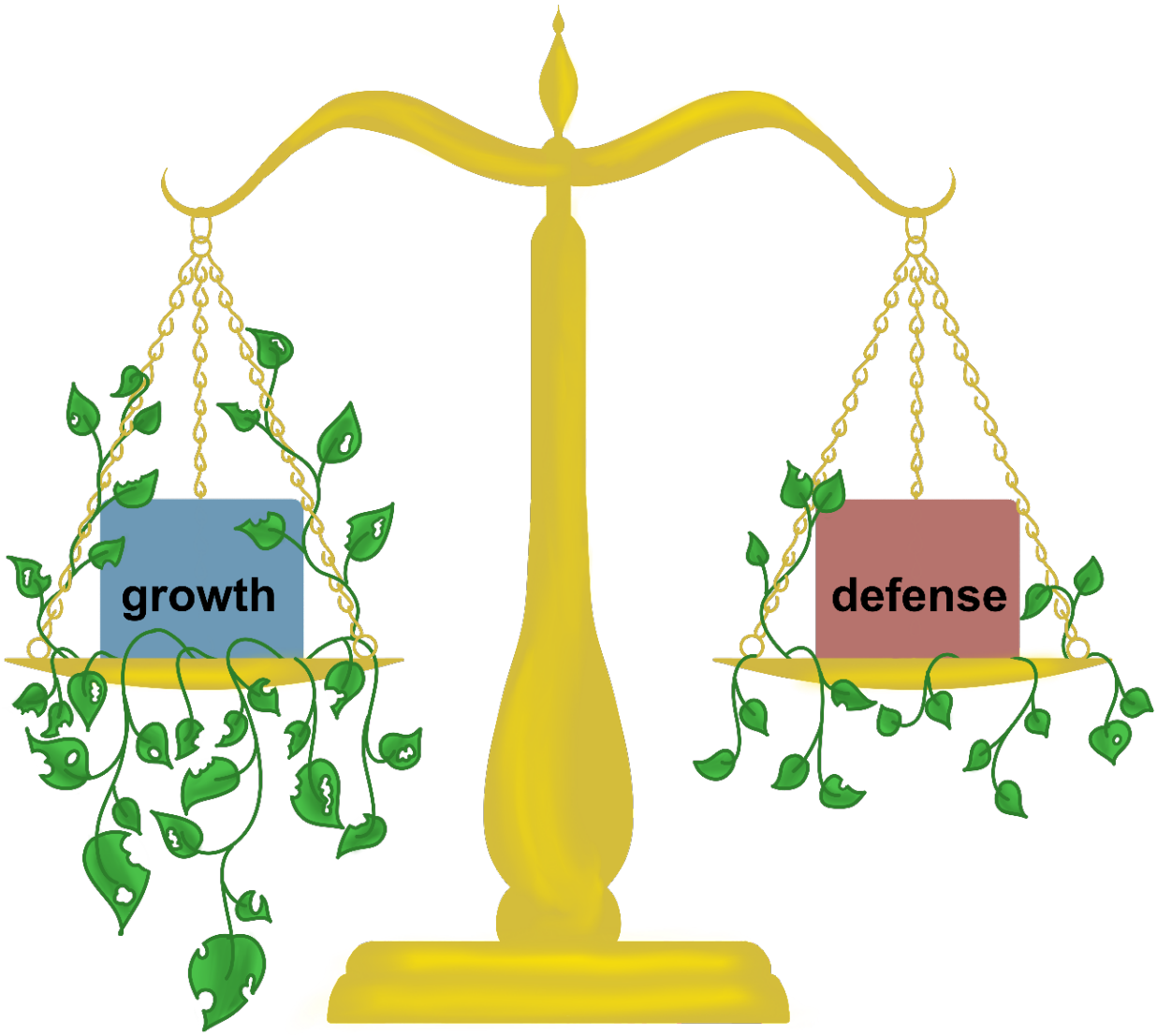


Figure 6: Traditional understanding of the relationship between growth and defense has come in the form of a tradeoff or a balance. Left: High growth, at the cost of low defenses against herbivory. Right: High defenses against herbivory, at the cost of constricted growth. Illustration by author.

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Chapter 2: Touch stimulation of *Arabidopsis* enhances defense but restricts growth via separate products of jasmonate biosynthesis

Abstract

Touch stress induces a suite of morphological and physiological changes in plants such as restricting vegetative growth, delaying flowering, and increasing pest and pathogen resistance. These phenotypic responses have been linked to a wide array of signaling systems ranging from Ca^{2+} and reactive oxygen species to hormonal regulation via e.g., jasmonic acid (JA). Here, we demonstrate that mechanical induction of defense response is dependent on the production of JA, whereas the mechanical effect on growth is independent of JA itself but dependent on 12-Oxo-Phytodienoic Acid (OPDA), a metabolic intermediate in JA biosynthesis. Thus, plants impaired in the initial steps of JA production that lead to the formation of OPDA are unable to either restrict growth or induce defenses in response to touch stress. However, plants impaired in the transformation of OPDA to JA exhibit touch-induced reduction in vegetative growth similar to that of wild-type, yet fail to trigger herbivore defenses. Treatment with exogenous OPDA mimics touch stimulation on vegetative growth, even in plants where mutation in their *OPR3* reductase means this OPDA cannot be transformed into JA. Such divergence in regulation by OPDA and JA – separate components of a common biosynthetic pathway – likely provides a mechanism whereby the plant can separately modulate defense responses and growth, while also maintaining coordination between each process.

Introduction

A host of sensory and regulatory networks allow plants to monitor changes in their environment and dynamically adapt characteristics ranging from growth and development to physiology and biochemistry. One of the most pervasive of these signals is mechanical stimulation, be it from wind, rain, the touch of passing animals, contact with neighboring vegetation, or simply the mechanical loads imposed from the plant's own weight. Collectively, the growth effects of mechanostimulation constitute thigmomorphogenesis, with touch stress generally reducing vegetative growth and delaying floral development (reviewed in Jaffe 1973; Kouhen et al., 2023). However, the range of mechanical responses is diverse, with, e.g., touch also causing an increase in resistance against pathogen and herbivore attack and, at a molecular level, changes in the expression of many hundreds of genes (e.g., Chehab et al. 2012, Benikhlef et al. 2013; Darwish et al 2022). Despite the ubiquitous nature of such mechanical stimuli and the far-reaching effects on plant growth and physiology, the regulatory networks that control how plants respond to touch are only just beginning to be unraveled.

A variety of factors have been identified as being involved in plant touch perception. These putative signaling elements include mechanosensitive ion channels such as members of the MSL, MCA and Piezo families (reviewed in Basu and Haswell 2017); a role for cytosolic Ca^{2+} signaling (e.g., Matsumura et al. 2022); the action of receptor-like kinases such as FERONIA (Shih et al. 2014; Darwish et al. 2022); the MAP kinase signaling cascade (Wang et al. 2018); and a range of transcription factors such as members of the WRKY, MYC and CAMTA families (reviewed in Li et al. 2020; Darwish et al. 2022). Hormonal regulation also appears important in this response system with, e.g.,

mutants in *GIBBERELLIN 2-BETA-DIOXYGENASE 7* (a gibberellin degrading enzyme) and *ALLENE OXIDE SYNTHASE* (*AOS*, required for jasmonic acid production) showing reduced effects of touch on growth and flowering time, implying a role for gibberellin (GA) and jasmonic acid (JA) in the touch response (Chehab et al., 2012; Lange and Lange, 2015). However, the hormonal pathways mediating the plant's mechanical response networks are recognized as being multifaceted. For example, MYC transcription factors are part of a well-defined network linking JA to altered gene expression (Boter et al. 2004; Lorenzo et al. 2004; reviewed in Hau et al. 2021) but elements of touch-responsive transcription are maintained in the *myc* mutant backgrounds (Van Moerkercke et al., 2019; Darwish et al., 2022). Similarly, classic markers of the transcriptional response to touch such as *TOUCH2*/*CALMODULIN-LIKE 24* (*TCH2/CML24*) and *TOUCH4/XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 22 (TCH4/XTH22)* are still upregulated in JA biosynthesis mutants (Chehab et al., 2012; Darwish et al., 2022) leading to a model where there are both JA-dependent and -independent events. Similarly, depending on developmental stage, ethylene also appears important in mediating shoot growth responses to mechanical impedance but, again, is involved in the induction of only a subset of molecular responses to touch (Johnson et al. 1998; Wu et al 2020).

In addition to their likely role in touch signaling jasmonates are involved in a host of other plant responses, including the regulation of growth and development, and of pathogen and herbivore defense (Creelman et al. 1992; Farmer and Ryan 1992; Thomma et al. 1998; Wasternack and Hause 2013; Ruan et al. 2019). Indeed, jasmonates have generally been considered as one of the major switches between prioritizing defense or

growth in a phenomenon named the growth-defense tradeoff. In this scenario, JA directs increased resources to defense, limiting those available for growth (Huot 2014; He et al. 2022). Such a tradeoff would provide a compelling explanation for the phenotype of reduced vegetative growth alongside induction of JA production that often accompanies touch stimulation of the plant. However, analyses of JA signaling related to wound responses has shown that in certain mutant backgrounds, growth retardation and defense induction can be uncoupled (e.g., Campos et al., 2016; reviewed in He et al., 2022). This observation implies that these phenomena are not always obligately linked via competition for shared resources.

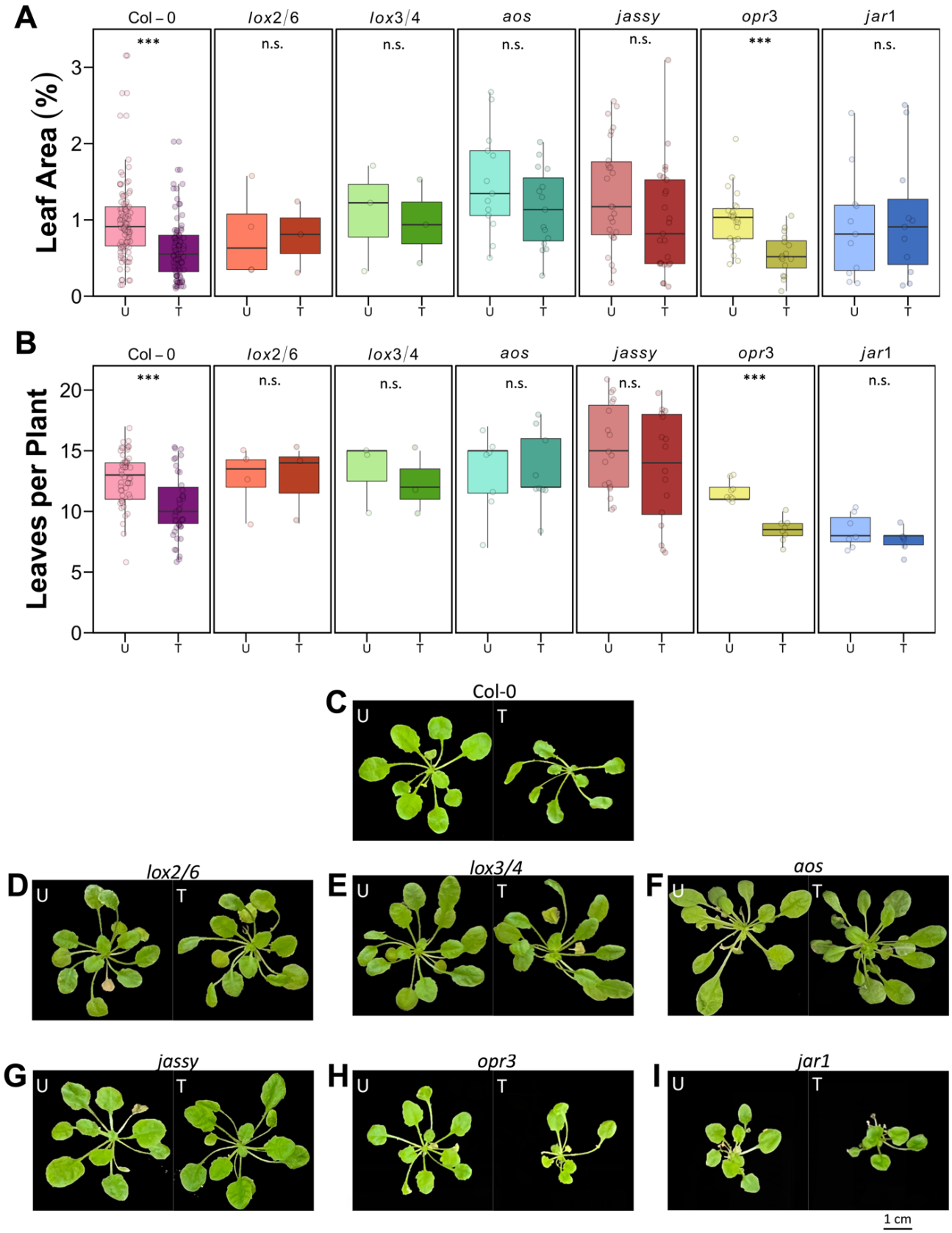
Production of JA involves coordination between biochemical pathways of oxylipin synthesis located in the chloroplast, peroxisome and cytoplasm. The JA produced is then processed further by e.g., methylation to the volatile meJA, or conjugated to the amino acid isoleucine. The resulting JA-Ile can then trigger a nuclear perception machinery triggered by the JA-Ile receptor CORONITINE INSENSITIVE 1 (COI1; reviewed in Ghosh et al.,2016; Ruan et al. 2019;). These well-defined steps in the biochemistry of JA biosynthesis and its subsequent signal transduction provide a strong framework within which to dissect precisely how JA might mediate responses. We report that in *Arabidopsis*, although the induction of herbivore defenses by touch stimulation relies upon the production of JA, touch regulates growth through production of OPDA, a precursor of JA biosynthesis. The use of OPDA and JA as biochemically related but separate signals may provide a mechanism for the plant to coordinate the effects of touch on growth and defense, while retaining the ability to uncouple these processes when necessary.

Results

Mechanically stimulated plants show reduced growth and increased JA responses

To better define the effects of mechanical stimulation, we took advantage of the Automated Botanical Contact Device (ABCD, Fitzgerald et al., 2022). The ABCD is a custom-built robot that repeatedly draws plastic sheets across the rosettes of *Arabidopsis* plants to provide highly reproducible, repetitive touch stimulation to multiple plants (Supplemental Fig. S1). The system allowed for an experimental setup with alternating rows of stimulated plants and paired untreated controls (Supplemental Fig. S1). Although touch stimulation has been shown to trigger both reduced vegetative growth and delayed flowering (e.g., Chehab et al. 2012), we focused our experiment design to a duration that would reveal effects on rosette growth. This was because we wished to ask questions about the tradeoff between growth and anti-herbivore defense and insect attack is principally manifested as damage to the vegetative tissues of the leaves. In addition, the other commonly monitored morphological effect of touch, a delay in flowering time, has been proposed to be a downstream effect of the inhibition of vegetative growth. In this model, delay in flowering arises from the slower accumulation of rosette leaves in the mechanically-stimulated plants to the threshold that triggers floral induction (Chehab et al. 2012). Therefore, we programmed the ABCD to run every 15 minutes for 7 days, a period defined from initial testing as leading to a reduction in rosette diameter of 40-50% in Col-0 wild type plants (WT; Fitzgerald et al, 2022). Under these growth conditions both touched and untouched plants remained vegetative throughout the experiment.

Fig. 1a-c shows that touch-stimulated WT *Arabidopsis* exhibited the expected ~40% reduction in rosette area alongside an ~20% decrease in leaf number. These growth responses were accompanied by an elevation in molecular markers of oxylipin-biosynthesis, OPDA response, and JA response (*LIPOXYGENASE 6*, *LOX6;*, *GLUTAREDOXIN480, GRX480*; and *JASMONATE-ZIM DOMAIN 7*, *JAZ7* respectively; Supplemental Fig. S2). In addition, this treatment caused upregulation of the classic touch-response marker genes *CML24* and *XTH22* (Supplemental Fig. S2), consistent with the triggering of well-documented touch responsive molecular response pathways (e.g., Braam and Davis 1990; Braam 2005; Darwish et al., 2022). Expression of each of these genes followed a common pattern: a spike in transcript level in the first hour of treatment followed by a lower, but significant, sustained upregulation throughout the 7 days of the experiment. Having established our touch stimulation system was effectively triggering expected touch-responses, we next asked how JA might be contributing to these events.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Leaf Area (%)
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0
5
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Col-0
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lox2/6
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Fig. 1: Phenotypes of touched *Arabidopsis* plants illustrate the importance of oxylipin biosynthesis.
A: The leaf area of control (U) and touched (T) plants after one week of touch treatment. In order to control for variable environmental conditions, all measurements were normalized within each experiment to the corresponding wild-type and presented as a normalized value. Wild-type plants show a dwarfing phenotype ($p<0.001$) in response to touch stress, a phenotype shared only by *opr3* mutants ($p<0.001$). *lox2/lox6*, *lox3/lox4, aos, jassy,* and *jar1* show no significant changes in rosette area in response to touch. B: The number of live leaves on each plant after one week of touch treatment. The results mirror that of total rosette area: wild-type plants and *opr3* mutants show a reduction in total leaf number in response to touch stress, while *lox2/lox6*, *lox3/lox4, aos, jassy,* and *jar1* show no significant changes in leaf number in response to touch. Significance between treatments was determined using a two-sample Student's *t*-test. *=$p<0.05$; **=$p<0.01$; ***=$p<0.001$
C-I: Representative photographs of control (U) and touched (T) plants of wild-type and six JA biosynthetic mutants.

Touch-induced reduction in rosette growth is dependent on *LOX* and *AOS* but not *OPR3*

Previous research (e.g., Chehab 2012) used mutants in AOS, one of the initial steps in JA production to show that a functional JA biosynthetic pathway is required for touch responses in *Arabidopsis*. In order to more fully screen for points in oxylipin biosynthesis critical to the touch response, we tested mutant plants deficient in various stages of JA production. For reference, the JA biosynthetic pathway is summarized in Chapter 1, Fig. 1. 13-Lipoxygenase (LOX) enzymes are involved in the early stages of the formation of jasmonate precursors in the chloroplast. The *Arabidopsis* genome encodes 4 *LOX* genes capable of generating these intermediates: *LOX 2*, *3*, *4*, and *6* (Chauvin et al., 2012), with *LOX3,* and to a lesser extent *LOX4,* being thought to play the major roles in JA-dependent growth restriction in response to repetitive wounding (Yang et al., 2020). We therefore tested the effects of touch on single and double knockouts in all 4 of these LOX genes. Mutants in *lox2*, *lox3* and *lox6*, as well as *lox2/lox6* and *lox3/lox4* double mutants failed to show inhibition in rosette growth or reduction in leaf number in response to touch stimulation suggesting they had lost touch responsiveness (Fig. 1A-E and Supplemental

Fig. S3). In contrast, *lox4* knockouts showed a wild type-like reduction in rosette size (though not leaf number) in response to touch stress (Supplemental Fig. S3). In addition, we found that when *lox2/6* double mutants were subjected to touch stress they showed no touch-responsive upregulation of *JAZ7* (Fig. 2), In *lox3/4* mutants, although this *JAZ7* upregulation response was attenuated, it was still statistically significant. The single *lox* mutants showed that *lox3* and *lox4* both upregulated *JAZ7* in response to touch, whereas *lox2* and *lox6* both failed to. These observations confirm that neither LOX3 nor LOX4 is explicitly required for the complete JA response to touch stress (Supplemental Fig. S3). Further, although touch stimulation requires LOX action to trigger changes in downstream signaling events that lead to a reduction in vegetative growth, unlike wounding, *LOX3* and *LOX4* may not represent major isoforms responsible for these responses.

The next step in JA biosynthesis is the enzyme AOS that is required to process the product of LOX action (13-S-hydroperoxyoctadeca-9, 11, 15-trienoic acid, 13-HPOT) into *cis*-(+)-12-oxo-phytodienoic acid (OPDA). *aos* knockout mutants are known to lack all products of the JA metabolic pathway downstream of HPOT (Park et al. 2002, Chehab et al. 2011). When subjected to touch stress treatment, *aos* plants continued to grow to the same size, and produce the same number of rosette leaves as their untouched counterparts (Fig. 1A, B, F) and failed to show touch-induced upregulation of *GRX480*, *ZAT10*, or *JAZ7* expression (Fig. 2). Identical phenotypic responses from a second *AOS* knockout allele are shown in Supplemental Fig. S4.

The biochemical steps described above all occur in the chloroplast, which then exports OPDA to the cytosol using a transport system dependent on the plastid outer membrane protein JASSY (Guan et al., 2019). We found that *jassy* mutants also do not show reduced

rosette size or change in leaf number in response to touch stress (Fig. 1A-B, G), consistent with the reports that *jassy* mutants accumulate very low levels of JA (Guan et al. 2019). Taken together, these results suggest that the growth response to touch stress requires the synthesis and export of a chloroplast-synthesized oxylipin (most likely OPDA).

Upon exiting the chloroplast, OPDA is imported into the peroxisome where it is reduced to JA by the enzyme OPDA REDUCTASE 3 (OPR3). Thus, while knockouts in *OPR3* accumulate OPDA, they are unable to produce JA (Stintzi and Browse 2000). When subjected to touch stress treatments, *opr3* mutants demonstrated a significant dwarfing effect on the rosette as well as a reduction in leaf number that resembled the touch response of wild-type plants (Fig. 1A, B, H); identical responses in a second allele are shown in Supplemental Fig. S4). o*pr3* mutant plants upregulated *ZAT10* and, to a lesser extent, *GRX480* in response to touch stress. These genes are known to be responsive to OPDA, which *opr3* is capable of producing. However, these plants did not show touch-induction of *JAZ7,* consistent with an inability to synthesize JA.

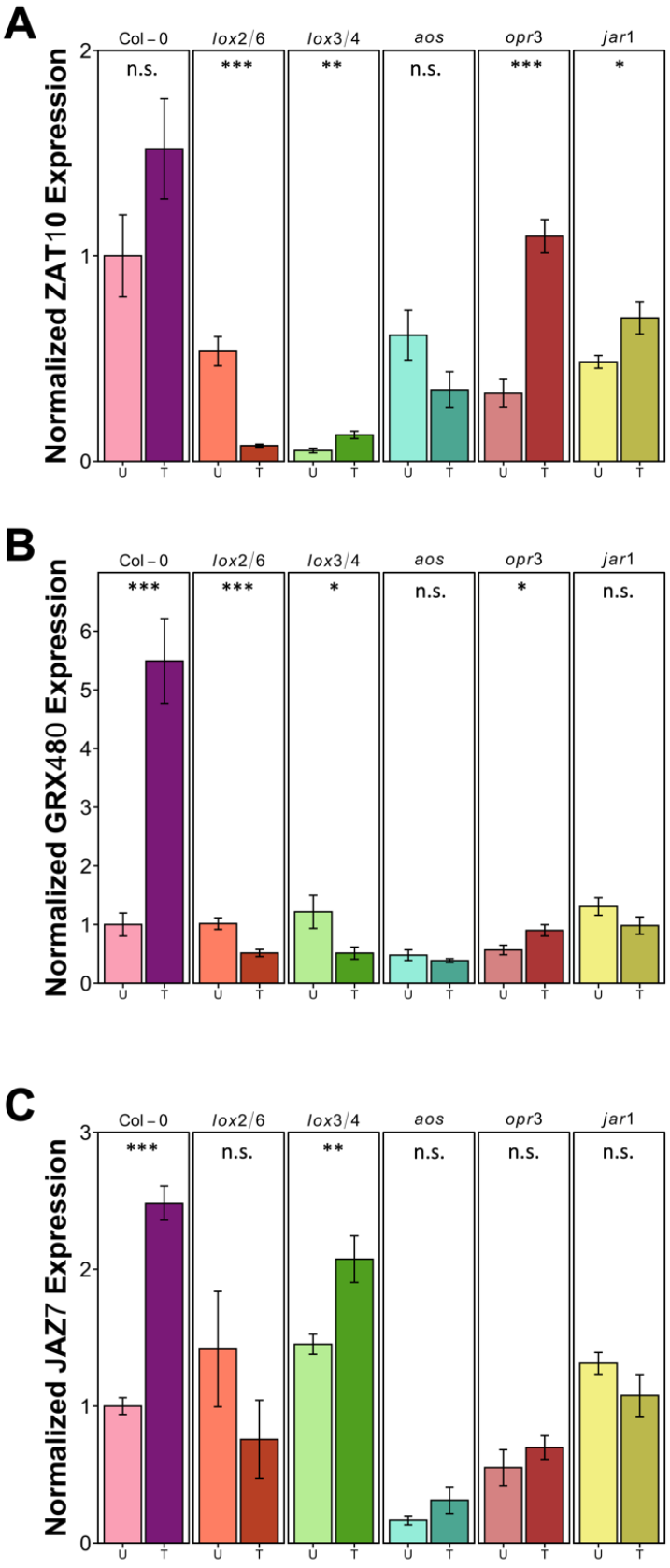
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Fig. 2: qRT-PCR analyses of three oxylipin-related genes in oxylipin biosynthetic mutants. A: ZAT10, known OPDA-responsive gene marker. *lox3/lox4, opr3,* and *jar1* upregulate this gene, while *lox2/lox6* downregulates it and both WT and *aos* show no significant change in response to touch. B: GRX480, known OPDA-responsive gene marker. WT and *opr3* upregulate its expression in response to touch, with *opr3*'s upregulation being significantly smaller than WT. *lox2/lox6* and *lox3/lox4* both downregulate the gene in response to touch, while *aos* and *jar1* show no response. C: JAZ7, known JA-responsive gene marker. WT and *lox3/lox4* upregulate its expression in response to touch, while *lox2/lox6, aos,* and *jar1* show no significant change. Significance between treatments was determined using a two-sample Student's *t*-test. *=p<0.05; **=p<0.01; ***=p<0.001

Touch-induced reduction in rosette growth is phenocopied by exogenous OPDA

Taken together, the results described so far suggested to us that production of OPDA, but not further processing to JA is required to elicit touch-related effects on vegetative growth. Indeed, assaying the OPDA-response marker genes *GRX480* and *ZAT10* (Liu and Park, 2021) showed they were induced in touch-stimulated wild type and *opr3* mutants but not in the *lox2, lox6, lox2*/*lox6*, and *aos* knockout backgrounds, i.e., not in mutants that also failed to show reduction in vegetative growth in response to touch (Fig. 2, Supplemental Fig. S3).

To directly test the idea that OPDA production was responsible for the vegetative growth reduction upon touch stimulation, we asked whether exogenous OPDA could trigger touch-like effects on plant growth. We therefore sprayed plants daily for 7 days with either water (control) or 100 µM MeJA or 250 µM OPDA and monitored the effects on both growth and patterns of gene expression with and without touch stimulation. These treatment levels were chosen based on the concentrations at which responses appear to saturate in published data on the treatment of plants with exogenous MeJA or OPDA (e.g., Zalewski et al. 2019; Knieper et al. 2021). Wild-type *Arabidopsis* plants sprayed with OPDA or MeJA showed significantly reduced rosette growth when compared to the

water control (Fig. 3). In the same experiment, when touched using the ABCD, the water sprayed controls showed reduced growth. Touch treatment also further dwarfed the MeJA-treated plants by an additional 56% but did not further affect growth of OPDA-treated plants.

As expected from our previous results, *aos* mutants showed no growth response to touch treatment in the water sprayed controls. However, OPDA-treated *aos* plants exhibited a significant reduction in growth similar to the effect on WT. Further, and as predicted if OPDA were indeed mediating the touch response, touch did not further alter this dwarfing effect of OPDA treatment. MeJA treated *aos* mutants grew to the same size as water controls (i.e., did not phenocopy a touch response). Additionally, these plants showed no reduction in growth to touch stimulation. In contrast, touch stimulated a significant dwarfing in the water controls of the *opr3* mutants (Fig. 3). When sprayed with OPDA, *opr3* mutants showed a significant growth reduction in rosette size of 66% that was not responsive further to touch treatment. MeJA, similarly, dwarfed *opr3* plants by 68% and these MeJA treated plants did not further respond to touch.

These results are consistent with a model whereby OPDA triggers a reduction in vegetative growth that is not dependent on the conversion of OPDA to JA by OPR3. MeJA is known to lead to feedback induction of the JA biosynthetic pathway such as upregulation of *LOX* expression (Ruan 2019), potentially explaining why MeJA was capable of dwarfing the plant's rosette growth but only when a functional AOS was present, i.e., AOS action is required to convert the newly synthesized products of any MeJA-activated LOX activity into OPDA.

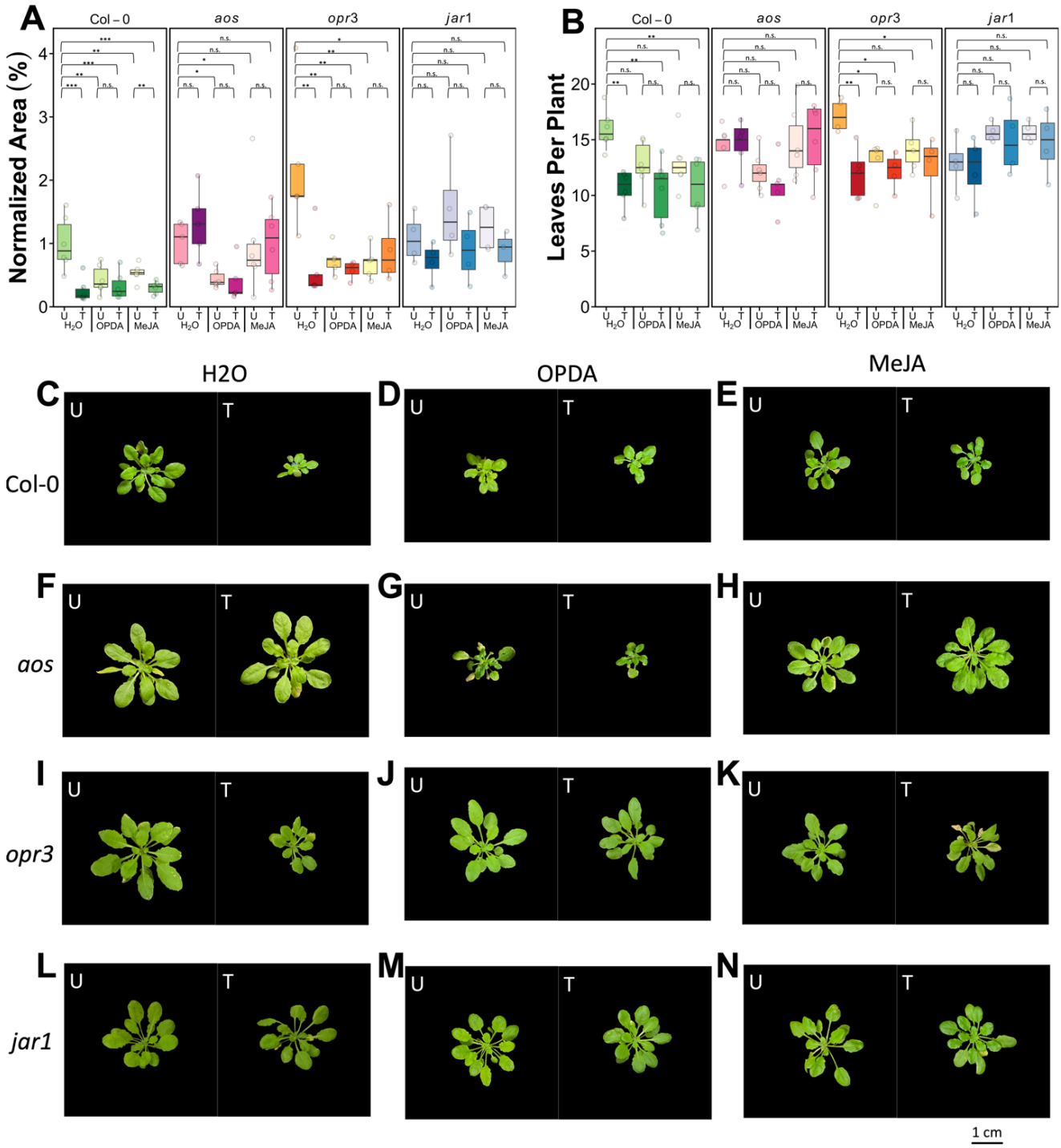


Fig. 3: Exogenous application assay results highlight the importance of OPDA in growth regulation. A: Rosette area of plants of four genotypes (WT, *aos, opr3, jar1*), with three chemical treatment groups each (H2O, OPDA, MeJA), split into control (U) and touched (T). B: Live leaf number of the same plants. Significance was determined using a one-way ANOVA test with Dunnett's post hoc test to compare multiple sample groups to a singular control group. *=p<0.05; **=p<0.01; ***=p<0.001. C-N: Representative photos of individuals of each combination of genotype and treatment. Shown is one control (U) and one touch-stressed (T) plant.

OPDA alone is insufficient to increase touch-induced herbivory resistance, which is JA-Ile dependent

Wild-type *Arabidopsis* subjected to touch treatment have been reported to display an upregulation of defense (Chehab et al. 2012; Benikhlef et al. 2013). Similarly, using the ABCD we found that repeated touch stimulation resulted in wild-type *Arabidopsis* seedlings resistant to herbivory by *Trichoplusia ni* (cabbage looper) as shown by a reduction in the amount of the rosette consumed (Fig. 4). This touch-induced defense priming was negated in both *aos* and *opr3* plants (Fig. 4, second alleles in Supplemental Fig. S4). WT plants treated with OPDA or with MeJA varied greatly in the extent to which they experienced herbivory compared to their water controls, although a trend towards a decrease in herbivory could be observed (Fig. 5). We attribute this variation to the extremely small size of the plants after 1 week of OPDA or MeJA treatment, leading to extremes of herbivory where many plants were either ignored, or completely consumed by the *T. ni* larvae.

A significant reduction in herbivory did occur in MeJA-treated *aos* mutants, and this response was not touch sensitive, i.e., no further defense induction occurred upon treatment with the ABCD (Fig. 5). This observation is in agreement with JA rather than OPDA being specifically required for touch-induction of anti-herbivore defenses. However, after treatment with OPDA or MeJA, *opr3* mutants demonstrate a trend to upregulation of defense resulting in a reduction in herbivory compared to their water-treated controls (Fig. 5). Unlike water controls, neither the OPDA nor MeJA treated plants were further responsive to touch stress for their defense response. The effect of MeJA is consistent with the predicted responsiveness of this mutant to exogenous JA. However,

opr3 mutants are incapable of converting OPDA to JA and so should not be able to elicit JA-dependent defense responses. This result therefore suggests that OPDA may have some role in orchestrating plant defenses after touch stress in the absence of JA. Indeed, OPDA-triggered defense in the absence of JA has been reported previously against both nematode attack (Gleason et al., 2016) and insect herbivory (Bosch et al., 2014).

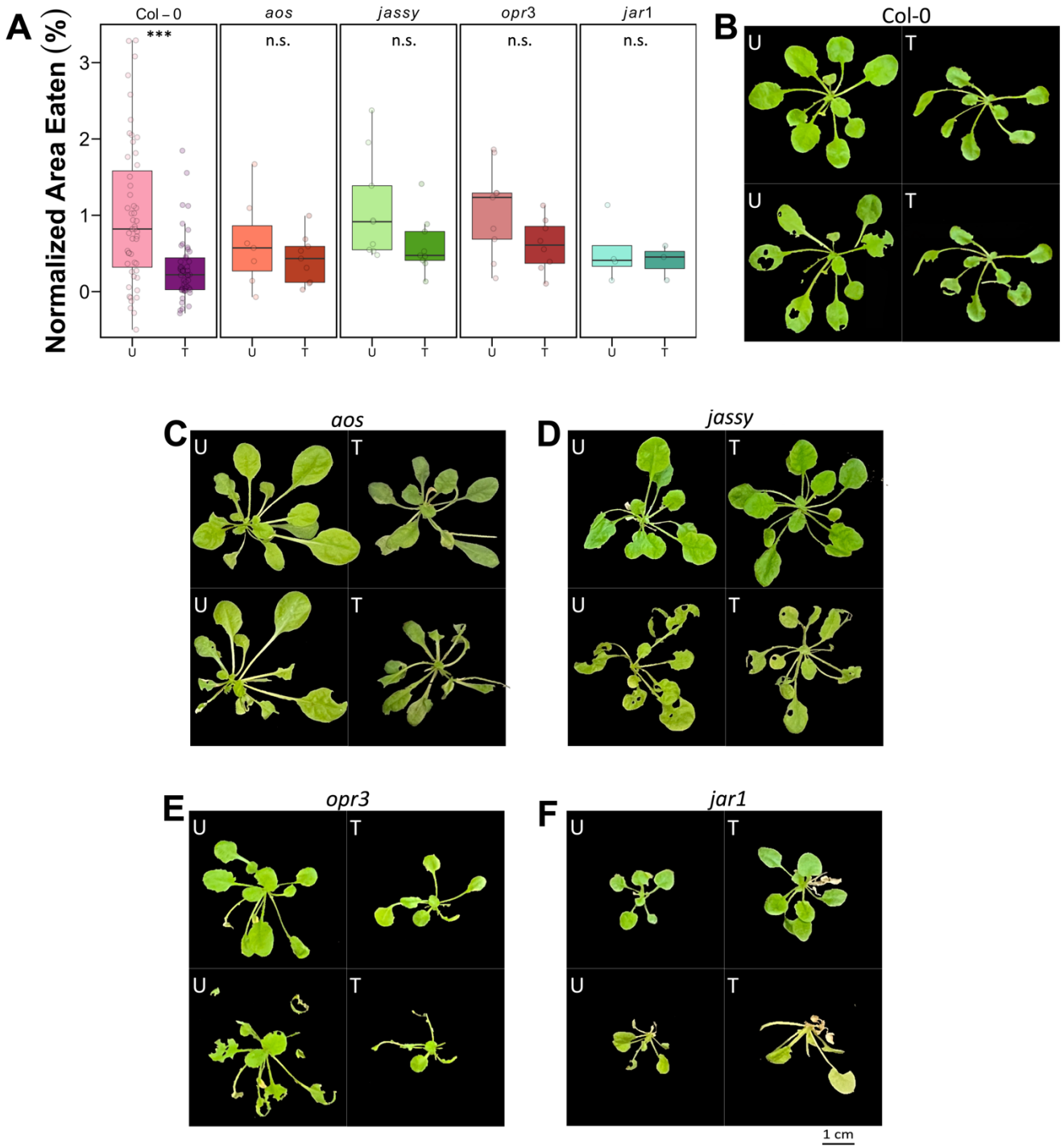
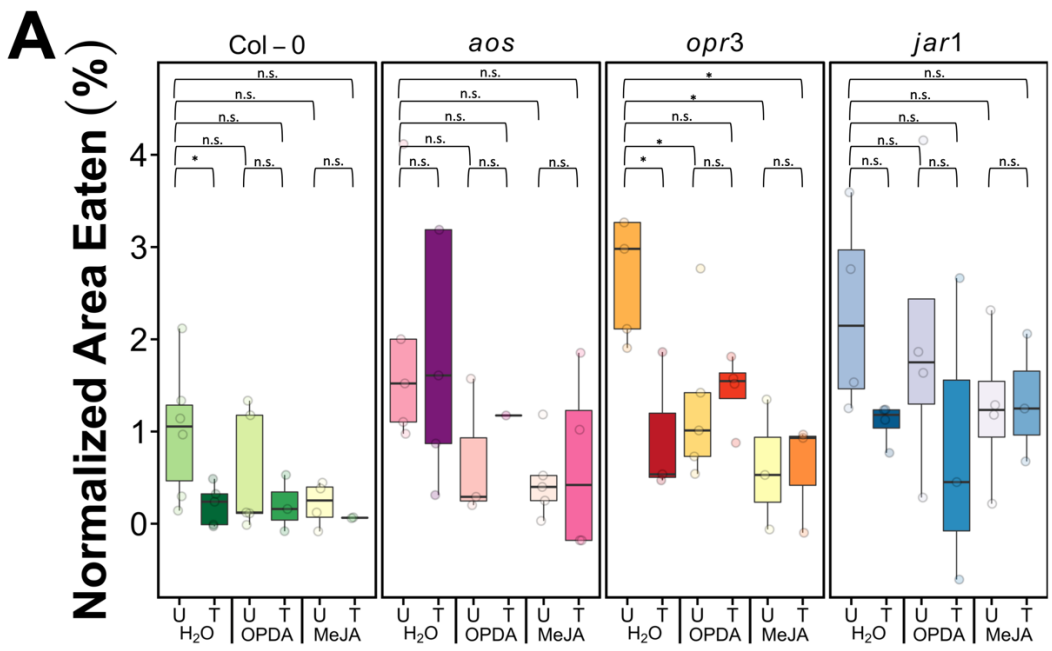


Fig. 4: Herbivory defense priming occurs in WT in response to touch, but not in mutants deficient in JA-Ile biosynthesis. A: Leaf area eaten by *T. ni* larvae, normalized to the amount eaten in each experiment's corresponding WT control to control for exact age of larvae and environmental factors. B-F: Representative images of control (U) and touched (T) individuals of the same genotypes before (top row) and after (bottom row) herbivory assay. Significance between treatments was determined using a two-sample Student's *t*-test. *=p<0.05; **=p<0.01; ***=p<0.001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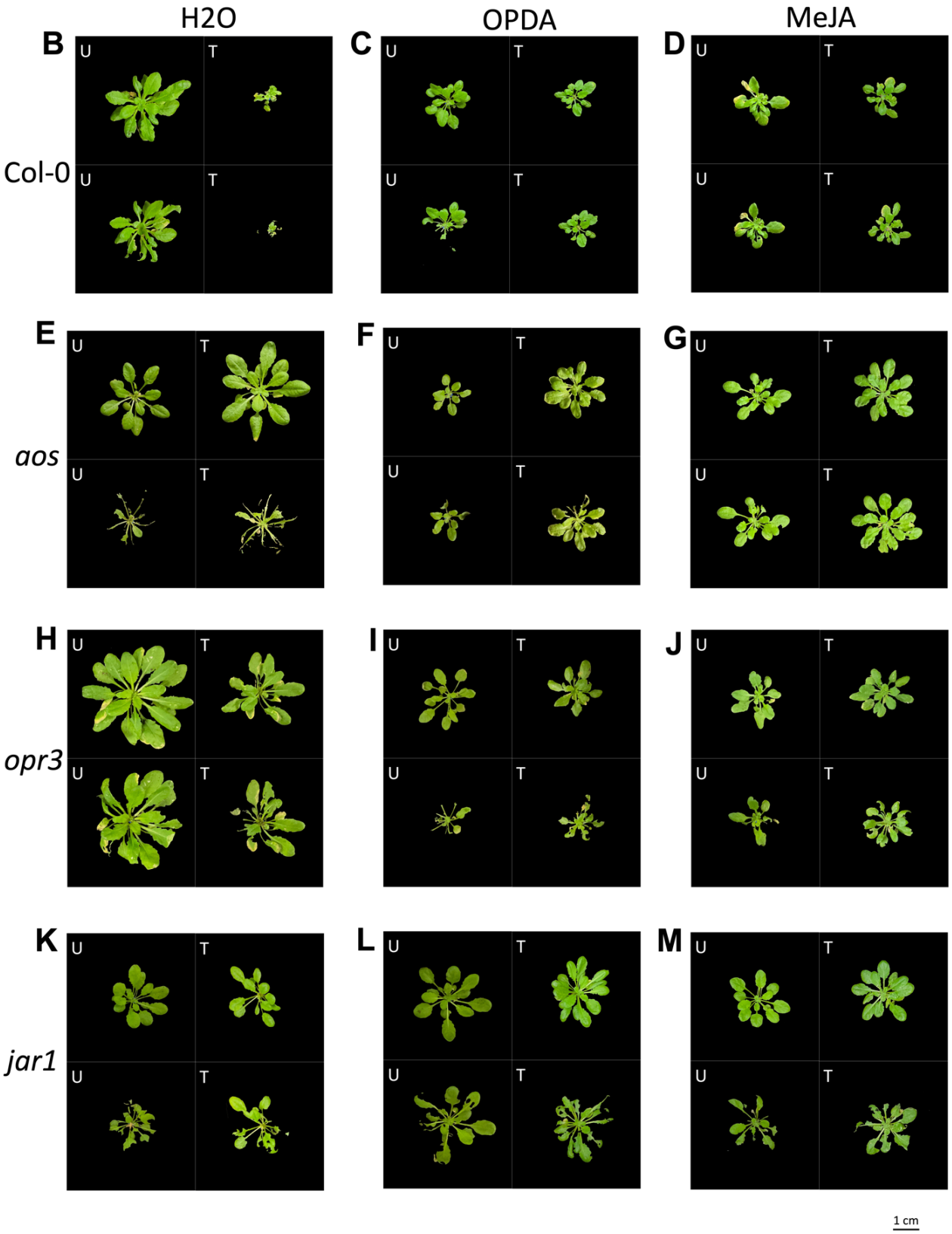
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Fig. 5: Exogenous application of MeJA can induce herbivory defenses. A: Leaf area eaten by *T. ni* larvae of plants of four genotypes (WT, *aos, opr3, jar1*), with three chemical treatment groups each (H2O, OPDA, MeJA), split into control (U) and touched (T). B-M: Representative images of control (U) and touched (T) individuals of each combination of genotype and treatment before (top row) and after (bottom row) herbivory by *T. ni* larvae. *=p<0.05; **=p<0.01; ***=p<0.001

jar1 mutants are insensitive to touch stimulation

JAR1, the synthetase responsible for creating JA-Ile has been reported to be required for the vegetative dwarfing response to touch stress (Chehab et al, 2012). We also found that touched *jar1* knockout mutants do not differ significantly in size from their untouched counterparts. In our exogenous application experiments, *jar1* plants sprayed with water were the same size as WT and also non-responsive to touch. Further, when treated with OPDA or with MeJA, there was no significant reduction in rosette size for any of these treatments. These results all indicate JAR1 is required for the touch effects on vegetative growth.

We found that *jar1* mutants also lacked a defense priming response to touch stress (Fig. 4). Thus, there was no difference in the amount of tissue consumed by herbivores between touched and untouched *jar1* seedlings. In addition, defense in *jar1* plants was not significantly affected by spraying with OPDA or MeJA in our exogenous application assay (Fig. 5). Taken together, these results also suggest that JAR1 is required for the effects of touch on the induction of defense to herbivores.

jar1 mutants demonstrated a marked increase in expression of the OPDA-responsive gene *ZAT10*, but not another OPDA-responsive gene *GRX480* or the JA response marker *JAZ7* (Fig. 2). It is possible that *ZAT10* and *GRX480* are differently responsive to separate isoforms of OPDA and that *GRX480* responds to a JAR1-dependent isoform.

Discussion

In this study, we show that touch treatment promotes oxylipin phytohormone signaling, and that touch stress responses leading to altered growth and to defense are differentially regulated. Previous research has linked touch response to JA action (e.g., Chehab et al., 2012) with touch regulating a suite of JA biosynthetic genes (e.g. Darwish et al., 2022) and triggering the accumulation of JA (Chehab et al. 2012), suggesting a potential growth-defense tradeoff where touch shifts the plant towards defense-related effects at the expense of growth. However, we have now been able to extend these findings to show that it is the oxylipin precursor for JA, OPDA, that appears primarily responsible for the coordination of growth reduction. Thus, *lox2, lox3, lox6, lox2/6, lox3/4, aos,* and *jassy* knockout mutants lost the dwarfing phenotype observed in the wild-type plants in response to touch stress. These genes all encode enzymes that are part of the chloroplast localized steps of oxylipin metabolism to the point of export of OPDA from the chloroplast. In contrast *opr3* plants showed a wild-type-like response, dwarfing in response to touch stress. Therefore, the final steps of JA biosynthesis that are dependent on peroxisomal processing of OPDA do not appear to be required for the signal transduction pathways which control growth reduction in response to mechanical stress to function. Further, exogenous application of MeJA to *aos* knockout mutants did not cause a significant dwarfing response, again indicating that production of OPDA rather than the direct action of JA is responsible for modulating the vegetative growth response.

Our results do indicate that conversion of OPDA to JA is required for orchestrating the major fraction of touch-induced defense priming, although OPDA itself appears capable of triggering some level of herbivore defense in the absence of JA production. However,

it is possible to divorce touch-induced reduction in vegetative growth from the induction of defense by interrupting JA production after OPDA production but before conversion to JA with the *aos* mutant. Thus, in this case, there is no competition between growth and defense and so it appears that independent regulatory networks rather than tradeoffs due to competition for resources likely explain touch effects on growth vs defense.

Of the four 13-lipoxygenases in *Arabidopsis*, LOX3 and 4 have been shown to be the major enzymes responsible for wound-induced JA production (Young et al., 2020). However, for touch-induced oxylipin production, LOX2, 3 and 6 all appear quantitatively important for the final vegetative response, with mutants in *LOX4* behaving like wild type. Thus, the touch-triggered vegetative growth response appears separable from the wound response system at the earliest steps in oxylipin production. These results are consistent with previous literature documenting a degree of functional redundancy among some of the *LOX* genes (Bell et al. 1995, Chauvin et al. 2016 and Grebner et al. 2013). Thus, mutants lacking *LOX6*, including the *lox2/6* double mutants used in our experiments, are expected to show the most severe phenotypes as they accumulate dramatically less OPDA, JA, or JA-Ile at a basal level and in response to wounding. In contrast, *lox3/4* double mutants are less deficient in accumulating JA and JA-Ile (Bell et al. 1995, Chauvin et al. 2016 and Grebner et al. 2013) and appear more important in the growth response to repetitive wounding (Young et al., 2020) than to touch stimulation .

JA signaling positively regulates itself in a feedback loop, with JA-Ile perception activating the transcription of the *13-LOXs, AOS,* and *OPR3*, leading to additional OPDA and JA accumulation (Wasternack 2006). In combination with the idea that OPDA orchestrates

the touch response this feedback induction may explain why treatment with exogenous JA induces a dwarfing response in WT and in *opr3* mutants but not *aos* (Fig 3).

Oxylipin production is an ancient biochemical pathway in plants and genes encoding elements of the JA biosynthetic pathway can be found in the genomes of all major land plant lineages, in some chlorophyte and charophyte alga and even scattered through various prokaryote lineages (Han, 2017). OPDA has been suggested to alter patterns of growth in both *Physcometrium patens* and *Marchantia polymorpha* (Stumpe *et al.*, 2010; Ponce De Leon *et al.*, 2012; Yamamoto *et al.*, 2015), raising the possibility of a potentially ancient role for OPDA in controlling growth and development. Whether touch-stimulation dependent modulation of growth mediated by OPDA is a similarly ancient regulatory mechanism is an important question for future research.

JAR1 family members can also be found in all plants (Han 2017) and this enzyme is classically thought to be the amino acid conjugase that attaches Ile to JA to make the bioactive signaling molecule JA-Ile. (Han 2017). However, JAR1 has also been proposed to act on OPDA, producing OPDA-Ile (Arnold et al. 2016; Floková et al. 2016; Guranowski et al. 2007). Our *jar1* results (the loss of response to touch stress, and to exogenous OPDA in *jar1* mutant plants; Fig. 1, Fig. 3) indicate that if OPDA is acting as a signaling molecule for growth reduction in response to touch stress, it may take the form of an amino acid conjugate formed by JAR1 action, such as OPDA-Ile. Previous studies (e.g., Staswick and Tiryaki 2004) show that, as expected for a JA-Ile conjugase, *jar1* knockout mutants accumulate similar amounts of JA as wild-type plants but JA-Ile production is significantly diminished. However, *jar1* mutants have also been demonstrated to similarly

accumulate OPDA, but to have significantly lower OPDA-Ile levels (Floková et al. 2016), consistent with JAR1 activity towards OPDA.

It is also important to note that in the absence of JA, OPDA is capable of inducing systemic resistance against insect herbivory, although it is less effective that JA under these circumstances (Stintzi et al., 2001; Zhang and Turner, 2008). This defense-related activity of OPDA potentially explains some of the touch-induced defense induction we observed in the *opr3* mutants (Fig. 4).

Fig. 6 summarizes the point at which OPDA may exit the JA biosynthetic pathway to modulate the vegetative responses to touch stress. Non-damaging mechanical stress is an agroecologically relevant phenomenon, particularly in the context of balancing or engineering the relationship between biomass and natural defense (Ghosh et al., 2021). For example, gently walking on emerging wheat seedlings to improve their stress resilience in the ancient Japanese practice of mugifumi (Iida, 2014) is likely capitalizing these mechanical response systems of the plant. Thus, the finding that OPDA represents a likely important regulator of growth in these mechanical signaling systems provides a key molecular point where we may be able to engineer plants to protect themselves against pests and diseases without sacrificing biomass and yield.

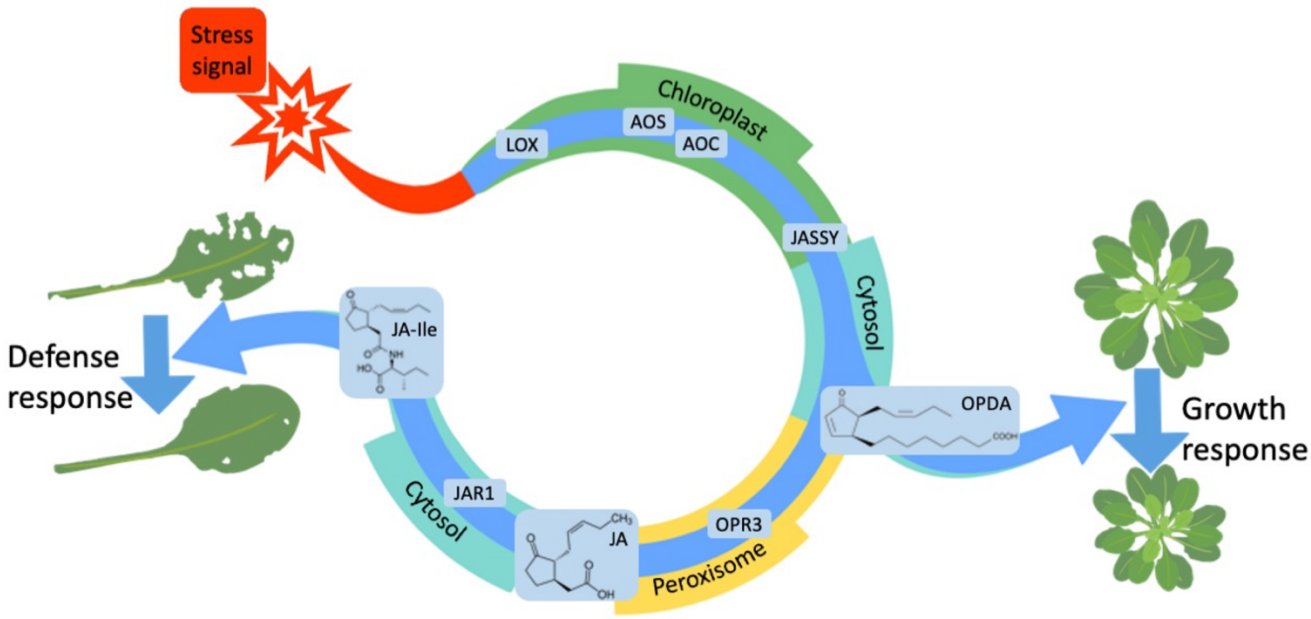

Fig. 6: A model illustrating the conclusions from this study. Once synthesized, OPDA can go on to mediate the growth response to touch independently, or go on to become JA which can in turn mediate the defense upregulation response.

Materials and methods

Arabidopsis lines

Several mutant lines were obtained from the *Arabidopsis* Biological Research Center (Ohio State University, USA) (Supplemental Table S1). *aos-TJ* mutants were kindly provided by Dr. Greg Howe (MSU-DOE Plant Research Laboratory, Michigan State University, USA*); lox6a, lox2/6a, lox 3/4, lox2/3/4/6a* mutants by Dr. Ted Farmer (Department of Plant Biology, University of Lausanne, Switzerland); and *jassy* mutants by Dr. Serena Schwenkert (Plant Sciences, Graduate School of Life Science, Ludwig Maximilian University of Munich, Germany). Mutant lines were genotyped by PCR (Promega, Madison, WI, USA) according to O'Malley et al. (2015). The border and gene-specific primers used are presented in Supplemental Table S2.

Growth conditions and touch stimulation

Arabidopsis thaliana plants were grown on 1:1 (v/v) SunGro propagation mix:MiracleGro potting mix in #1801 pot inserts (3x6 cells per insert that fits a standard 1020 plant seedling flat) and maintained under 12 h light/12 h dark (~100 µmol/m2/s, ~22˚ C) for 5 weeks. The Automated Botanical Contact Device (ABCD; Fitzgerald et al. 2022) was used to apply standardized mechanical stress by drawing a plastic sheet of 0.08 mm thickness across plants at 15-min intervals for 7 d (Supplemental Fig. S1). Half of the plants received mechanical stimulation, while half remained untouched as controls (Supplemental Fig. S1). On day 7 of treatment, plants were removed, photographed, and used for one of the response assays described below. Rosette area and leaf number were extracted from the images using ImageJ (Schindelin et al. 2012).

Exogenous hormone and touch stress assays

To test the effects of oxylipin hormones while touch stress is applied, 6-week-old Arabidopsis seedlings grown under 12-hour photoperiod and transplanted into individual pots as above were distributed into groups for regular application of one of the following: deionized water, 50 µM JA or 250 µM 12-oxo-phytodienoic acid (OPDA). OPDA stock was carried in 100% ethanol, leading to an ethanol concentration of approximately 1%, so we adjusted the water and MeJA solutions to 1% ethanol to control for any possible effects not attributable to OPDA. Plants were placed into the ABCD for a touch assay as described above. Exogenous hormone was applied by spray mist to entire 18-pot flats at once, separate from the other flats to avoid cross-contamination, once per day for seven days and afterward photographed and used for response assays. The configuration,

carefully constructed to avoid cross-contamination by plastic sheet, is below. Once per day beginning with day 0, all plants were removed from the ABCD, separated into their respective treatment groups, treated with water, MeJA, or OPDA with a gentle spray, and replaced in the ABCD. No exogenous application took place on day 7, to avoid unrelated effects of potentially unpalatable chemical spray on herbivory.

Gene expression analysis

RNA extraction and qPCR was performed and standardized against UBQ10 as described previously (Hilleary et al., 2020) using the primers described in Supplemental Table S2.

Insect rearing conditions

Trichoplusia ni larvae (Frontier Scientific, Newark, DE or from a lab colony) were raised on soy-wheat germ general Lepidoptera diet with vitamins and agar (Frontier Scientific) to the second instar stage. For herbivory assays, the larvae were starved for 3 h before being placed onto *Arabidopsis* plants covered with a transparent plastic cup (Supplemental Fig. S6). Larvae were removed after 2 h. Anti-herbivory defense response was evaluated by measuring leaf area consumed after 2 h from images of the plants using ImageJ.

Statistical analysis

Homogeneity of variance was confirmed using Bartlett's test. Significance between touched and untouched sample groups was determined by independent two-sample Student's *t*-test with an α of 0.05 to compare treatment within genotypes. One-way

ANOVA followed by post-hoc Dunnett's test was used for analysis when multiple treatments were used with a single control but individual pairwise comparisons are not needed (e.g. exogenous hormone application).

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Supplementary information

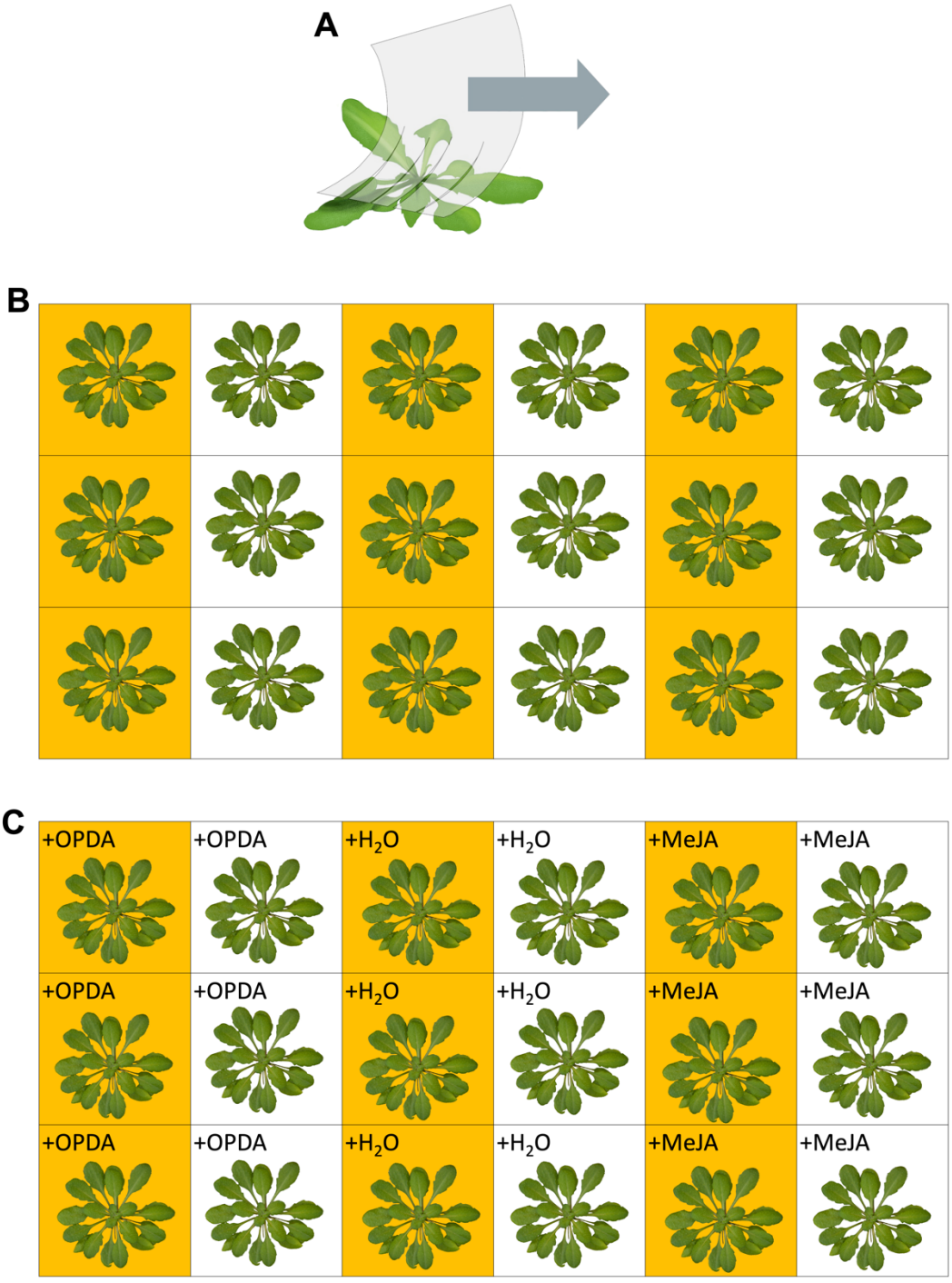


Fig. S1: ABCD experimental setup. A: Illustration of touch stress treatment: plastic sheet being dragged across a rosette. B: Layout of plants in the ABCD flats in regular touch experiments. Pots in yellow represent touch-stressed plants, while pots in white represent controls. C: Layout of plants in the ABCD flats in exogenous treatment experiments. After each chemical treatment, plants were replaced in the flat with the same touch layout as regular experiments (B).

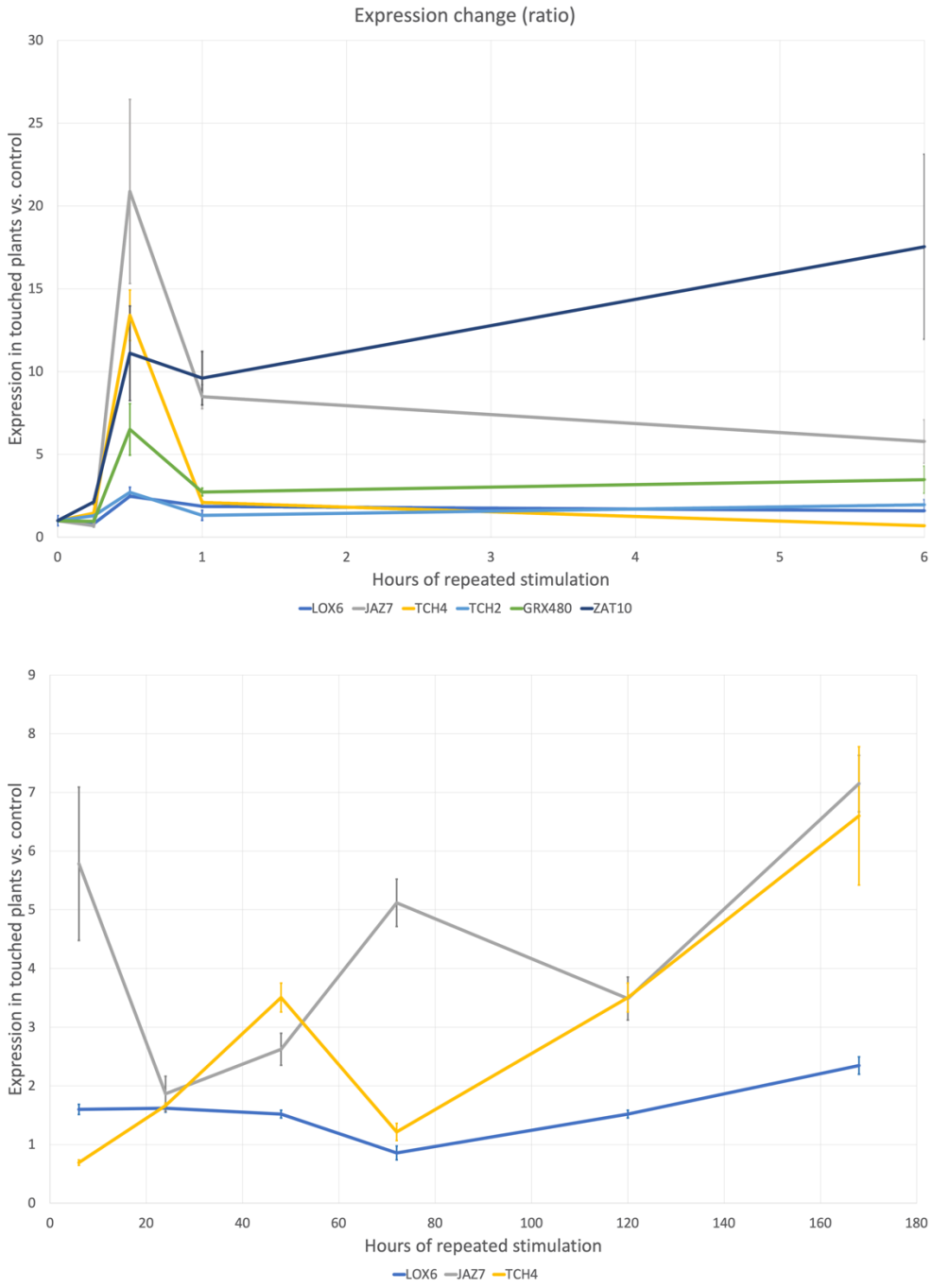


Fig. S2: Expression changes of touch- and oxylipin-related genes following touch treatment. Following initial touch treatment, *TCH2, TCH4, LOX6, GRX480, ZAT10,* and *JAZ7* are upregulated, indicating touch perception, initiation of oxylipin biosynthesis, and perception of both OPDA and JA. Expression of *LOX6*, *JAZ7*, and *TCH4* remains modestly elevated throughout the week of touch treatment, indicating a continuous touch perception and upregulation of oxylipin biosynthesis and signaling.

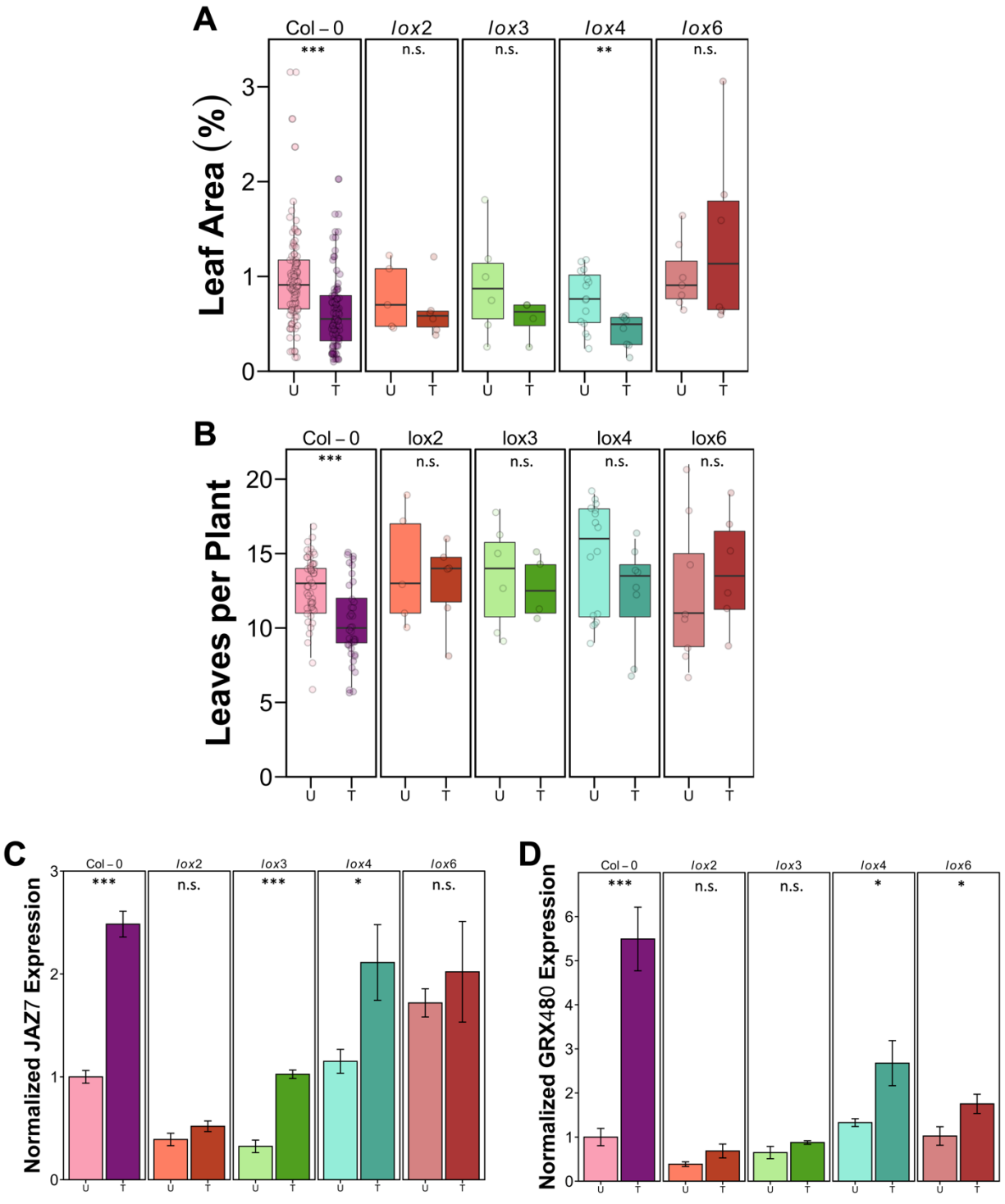


Fig. S3: Responses of individual *lox* knockout mutants to touch stress. A: Leaf area of WT plants and four *lox* individual mutants. WT plants show a dwarf phenotype in response to touch ($p<0.001$), as does *lox4* ($p<0.01$). *lox2, lox3,* and *lox6* show no response. B: Live leaves per plant. WT plants have a reduction in leaf number ($p<0.001$) and each *lox* individual mutant does not. C: Expression levels of *JAZ7*, a known JA-responsive gene. WT ($p<0.001$), *lox3* ($p<0.001$), and *lox4* ($p<0.05$) all upregulate *JAZ7* in response to touch treatment, while *lox2* and *lox6* do not. D: Expression levels of GRX480, a known OPDA-responsive

gene. WT ($p<0.001$), *lox4* ($p<0.05$), and *lox6* ($p<0.05$) upregulate GRX480 in response to touch, while *lox2* and *lox3* do not. Significance between treatments was determined using a two-sample Student's *t*-test. *=p<0.05; **=p<0.01; ***=p<0.001

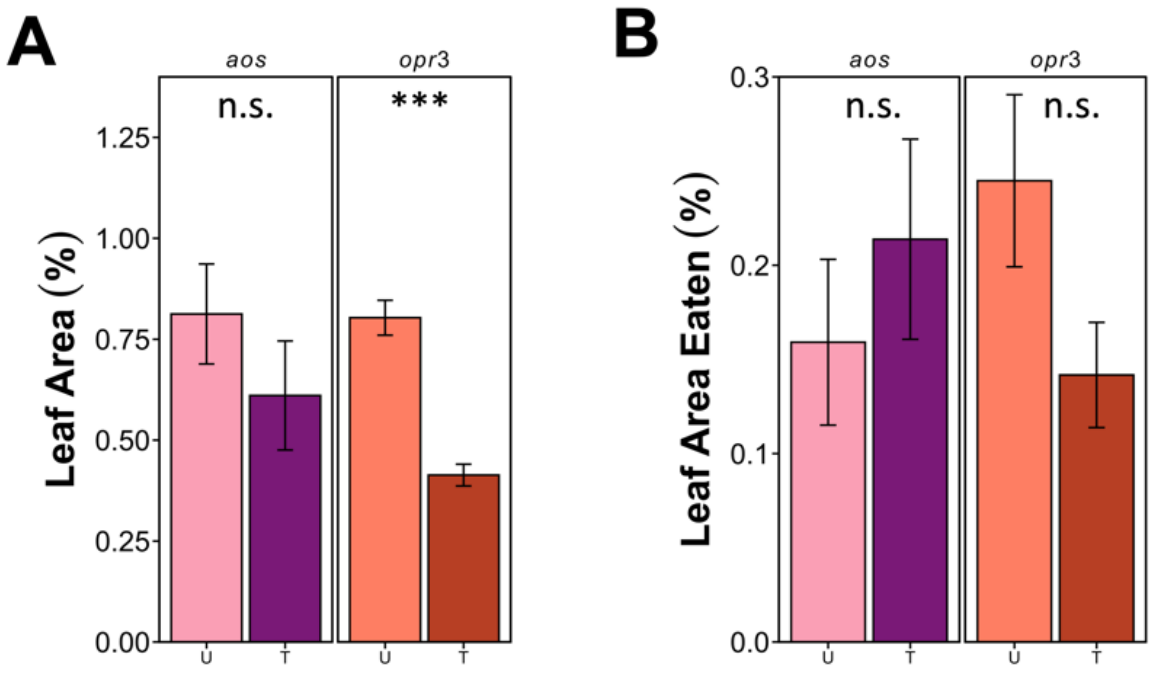


Fig. S4: Leaf area and herbivory data for second confirmatory alleles of *aos* and *opr3*. A: Leaf area data reflects other alleles, with *aos* not responding to touch treatment and *opr3* having a dwarfed phenotype. B: Leaf number data reflects other alleles, with neither *aos* nor *opr3* reducing leaf number in response to touch. Significance between treatments was determined using a two-sample Student's *t*-test. *=p<0.05; **=p<0.01; ***=p<0.001

Fig. S6: *T. ni* herbivory assay setup. Two larvae were placed on each rosette, covered with a plastic cup, and allowed to eat for 2 hours before removal.

Mutant	Allele/Line	Accession
lox2	CS3748	AT3G45140
lox3	SALK_147830.50.15.X	AT1G17420
lox4	SALK_017873.48.10.X	AT1G72520
lox6	SALK_001035.39.35.X	AT1G67560
lox2/lox6	*lox2-1/lox6A*	AT3G45140/AT1G67560
lox3/lox4	*lox3B/lox4A*	AT1G17420/AT1G72520
aos	*aos-TJ*	AT5G42650
	dde2-2	
jassy	*jassy*	AT1G70480
opr3	SALK_053805	AT2G06050
	SALK_201355	
jar1	*jar1-11*	AT2G46370

Table S1: Mutant *Arabidopsis* lines used in experiments.

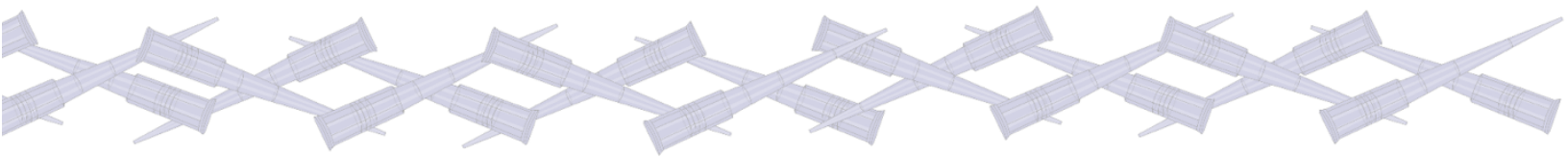
Gene	Direction	Sequence 5' to 3'	Reference
UBQ10	F	CACACTCCACTTGGTCTTGCGT	
	R	TGGTCTTTCCGGTGAGAGTCTTCA	
TCH2	F	AGAAGATGATGAGTAATGGTGGTG	Braam and Davis 1990
	R	CGCCGTCACTAAAATTAATCTGC	Braam and Davis 1990
TCH4	F	ATCTACAATCTCTAGAAAATGGCGATC	Braam and Davis 1990
	R	GCTGAAACAGAGGAGGTGATGATAAGA	Braam and Davis 1990
JAZ7	F	GATCCTCCAACAATCCCAAA	Mengarelli et al. 2021
	R	TGGTAAGGGGAAGTTGCTTG	Mengarelli et al. 2021
LOX2	F	ATTACGGTAGAAGACTACGCACAAC	Chauvin 2016
	R	GTAATTTAAGCTCTACCCCCTTGAG	Chauvin 2016
LOX6	F	CGGGGTACCGGTTGTTGAAATTTCTGATGCT	Chauvin 2012
	R	TTCCCCCCCGGGTTTTGTTTGGAGTTTGGCA	Chauvin 2012
AOC3	F	ACTCTCAACAATCTCTCTCGTAAT	
	R	GAGGAGAAATCTGGACCGTTA	
GRX480	F	TGATTGTGATTGGACGGAGA	Arnold et al. 2016
	R	TAAACCGCCGGTAACTTCAC	Arnold et al. 2016
ZAT10	F	AGGCTCTTACATCACCAAGATTAG	Arnold et al. 2016
	R	TACACTTGTAGCTCAACTTCTCCA	Arnold et al. 2016

Table S2: qRT-PCR primers used in experiments.

Gene	Direction	Sequence 5' to 3'
AOS	L	AACATATGCTCAAGGGATGGAGCTAAAAG
	R	CGAACATGTAGAGCAGCAACTGATTATACA
OPR3	L	TCCTCCGTATTGGTCAGTACG
	R	AATAAAAATGATTGAATACCATTGG
JAR1	L	CTCGGAAATTCAAATGGATCC
	R	TAGAATCGGCTGCAAAGAGAC
WiscDsLox_P745_LB	L	AACGTCCGCAATGTGTTATTAAGTTGTC
SALK_LBb1.3_LB	L	ATTTTGCCGATTTCGGAAC

Table S3: Genotyping primers used in experiments.

Chapter 3: Crosstalk at the interaction of DELLA proteins and MYC co-repressors is at the center of growth reduction to touch stress



Abstract

Touch stimulation, especially repetitively, of plants results in a diverse array of physiological effects that occur in close connection with one another, from growth restriction to delayed flowering to induction of anti-herbivore and anti-pathogen defenses. Hormonal signal transduction pathways (e.g. gibberellin (GA) and jasmonic acid (JA)) have been demonstrated to play a major part in orchestrating these responses. GA and JA in particular are commonly thought of as major coordinators of growth and defense, respectively. These pathways are deeply interwoven with one another, acting synergistically or antagonistically towards one another at varying points of crosstalk. External stress perception, like touch, throws the baseline balance of GA and JA signaling into disharmony and requires the pathways to together modulate the changing metabolic priorities of the plant. We have previously demonstrated that the growth reduction and defense initiation that occur as a result of touch stress are regulated by different products of the oxylipin (jasmonate) biosynthetic pathway, with OPDA (or OPDA-Ile) primarily mediating the growth response. Here, we present evidence that the mechanism of action of this control requires some, but not all, of the canonical JA-Ile perception molecular machinery. We show that the MYC transcription factors, along with the JA-Ile receptor COI1, are necessary for the growth response to occur and provide possible explanations for this phenomenon. Further, we show that the promiscuous repressor TOPLESS (TPL) is necessary for this response, but its more specific, JA-related co-repressor NINJA is not. Finally, we show that the growth response to touch stress is not entirely dependent on the JA signaling pathway – GA mediates elements of this response by altering the function and abundance of DELLA proteins. DELLA proteins are known to interface with the JA perception machinery to de-repress transcription of JA-related genes, and JA affects the balance of GA signaling by interfering with the basal level of DELLA proteins, so this result offers more perspective into the balance struck by the two signaling pathways. Together, these

results provide possible mechanisms through which the JA and GA pathways work with or against one another to alter the balance of growth and defense under mechanical stress.

Introduction

Because they are incapable of escaping environmental stresses and are tied to where they have put roots down, plants' relationship with those stresses is different from our own. Fleeing is not an option, so they must instead weather any stress that comes their way. Using molecular and environmental cues, the plant diagnoses the nature of the threat and mounts a physiologically appropriate response to it. Mechanical stress is known to induce both a growth and a defense response - this response to touch is called thigmomorphogenesis (Jaffe 1973, Chehab et al. 2012). Growth and defense are both orchestrated in plants through the action of hormones, which create a system where several different pathways exist and act standalone and in conjunction with each other. Thigmomorphogenesis in particular has been shown to involve multiple signaling pathways: jasmonic acid (JA), which is induced (Chehab et al. 2012) and gibberellin (GA), which is suppressed (Lange and Lange 2015). When these pathways are interrupted (e.g. blocking biosynthesis of either hormone), plants show impaired thigmomorphogenesis, and so it is important to understand the nature of those signaling pathways' involvement in touch response, which is the objective of this chapter.

One of the primary phytohormones involved in orchestrating the physiological response to external threats is JA. Upon the perception of (among other stimuli) mechanical stress including wounding and touch, chloroplastic fatty acids are recruited into the octadecanoid biosynthetic pathway, eventually resulting in 12-cis-oxo-phytodienoic acid (OPDA), JA, or the various isoforms thereof including methyl esters and amino acid conjugates (reviewed in Erb and Raymond 2019; Koo and Howe 2009). These molecules serve unique roles and orchestrate signal pathways which result in unique physiological responses. We have

previously demonstrated that the canonical oxylipin (JA) biosynthetic pathway is required in order for the plant to respond to touch stress; the JA precursor OPDA exerts control in the growth response and JA-Ile exerts it in the defense response. Having established that oxylipin biosynthesis is crucial to the touch response, the next important step to understand is how those molecules are perceived within the plant and lead to thigmomorphogenetic physiological changes. In order to do this we must investigate the nuclear perception components that turn a signal into a response.

Once the primary bioactive form of JA, JA-Ile, is produced and transported into the nucleus, its presence sets into motion a cascade of signal transduction events that eventually lead to defense and growth responses throughout the plant (reviewed in Wasternack and Hause 2013). The first of those events is the perception of JA-Ile by a receptor complex and transcriptional changes in JA-responsive genes.

Central to the entire system is the actual transcription factor being repressed or promoted: in jasmonate signaling, these are principally the MYC transcription factors. MYC TFs have a wide variety of essential functions in plant growth and development, from stress signaling to root development to reproduction to senescence (reviewed in Chen et al. 2019). MYC TFs play a central and essential role in jasmonate signaling: In the default state (i.e., low endogenous JA-Ile concentration in the nucleus), “JA-responsive” genes are expressed at a relatively low basal level. The MYC TFs are functionally suppressed by a variety of repressors and thus cannot promote the expression of these genes (Chini et al. 2007, Yan et al. 2007). Fig. 1 illustrates the basal state of repression under low endogenous JA-Ile levels. This TF family is well-characterized. Although there is a high degree of functional redundancy among these TFs, there are individual roles that appear

unique to different MYCs (Fernández-Calvo et al. 2011, Wang et al. 2017) and they appear to function in a quantitatively additive role (Zhang et al. 2020). MYC2, 3, and 4 are involved in mediating JA-responsive gene expression, but MYC2 is studied in greater depth than other MYCs and is considered an important controller of JA signaling (Dombrecht et al. 2007, Kazan and Manners 2013, Major et al. 2017). Left to their own devices and unrepressed, MYC TFs recruit, via the Mediator subunit MED25, RNA polymerase II to promote transcription of JA-responsive genes (Chen et al. 2012, Çevik et al. 2012). Controlling this interaction is the central purpose of the involved repressors centered around JAZ proteins.

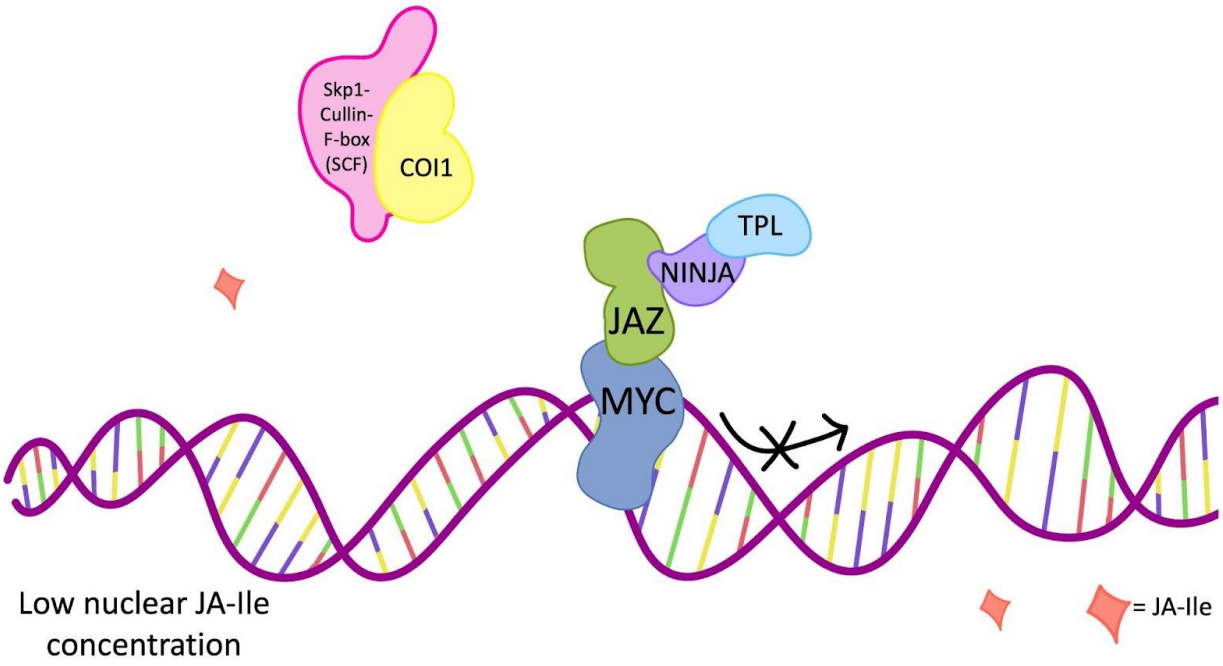


Fig. 1: A simple representation of the basal state of MYC TF repression, and therefore JA-responsive gene suppression, under low JA-Ile conditions. JAZ proteins are the primary repressors of MYC TFs. JAZ recruits NINJA, which in turn recruits TOPLESS, and all three act as co-repressors. Skp1-Cullin-F-box complex, with the specialized targeting protein COI1, are stable and do not interact with the repressors.

The JAZ proteins are a family of transcriptional repressors that are degraded upon JA perception, leading to the activation of JA-responsive genes (Chini et al., 2007). JAZ

proteins interact with a range of transcription factors, including MYC2 (Kazan and Manners, 2013). As the name alludes, nearly all JAZ proteins contain a conserved TIFY motif-containing ZIM domain near the central portion of the protein, which plays multiple roles in regulating jasmonate signaling. The TIFY motif and ZIM domain are required for several interactions between JAZ and other proteins, as well as for JAZ dimerization. JAZ proteins contain a conserved N-terminal domain (NT) as well as a C-terminal Jas domain that is required for interaction with the protein CORONATINE INSENSITIVE 1 (COI1) that acts as the JA-Ile receptor (Thines et al., 2007). When JA-Ile binds to COI1 as a ligand, the resulting complex recruits JAZ proteins, leading to their ubiquitination and subsequent degradation by the 26S proteasome (Chini et al., 2007; Fonseca et al., 2009). This degradation releases transcription factors from JAZ-mediated repression, allowing them to activate JA-responsive genes (Wasternack and Song, 2017).

COI1 is a subunit of a Skp1-Cullin-F-box (SCF) E3 ubiquitin ligase complex, which are involved in the degradation of a range of proteins in plants (Devoto et al., 2002, Xu et al. 2002). COI1 specifically contains an F-box domain that is responsible for its interaction with the other components of the SCF complex, as well as a Leu-rich repeat (LRR) domain that is required for its interaction with JAZ proteins (Sheard et al. 2010). The LRR domain of COI1 binds to the Jas motif of JAZ proteins, leading to the recruitment and ubiquitination of JAZ proteins (Chini et al., 2007; Fonseca et al., 2009). Fig. 2 illustrates this relief-of-repression mechanism that promotes JA-responsive gene expression after stress perception.

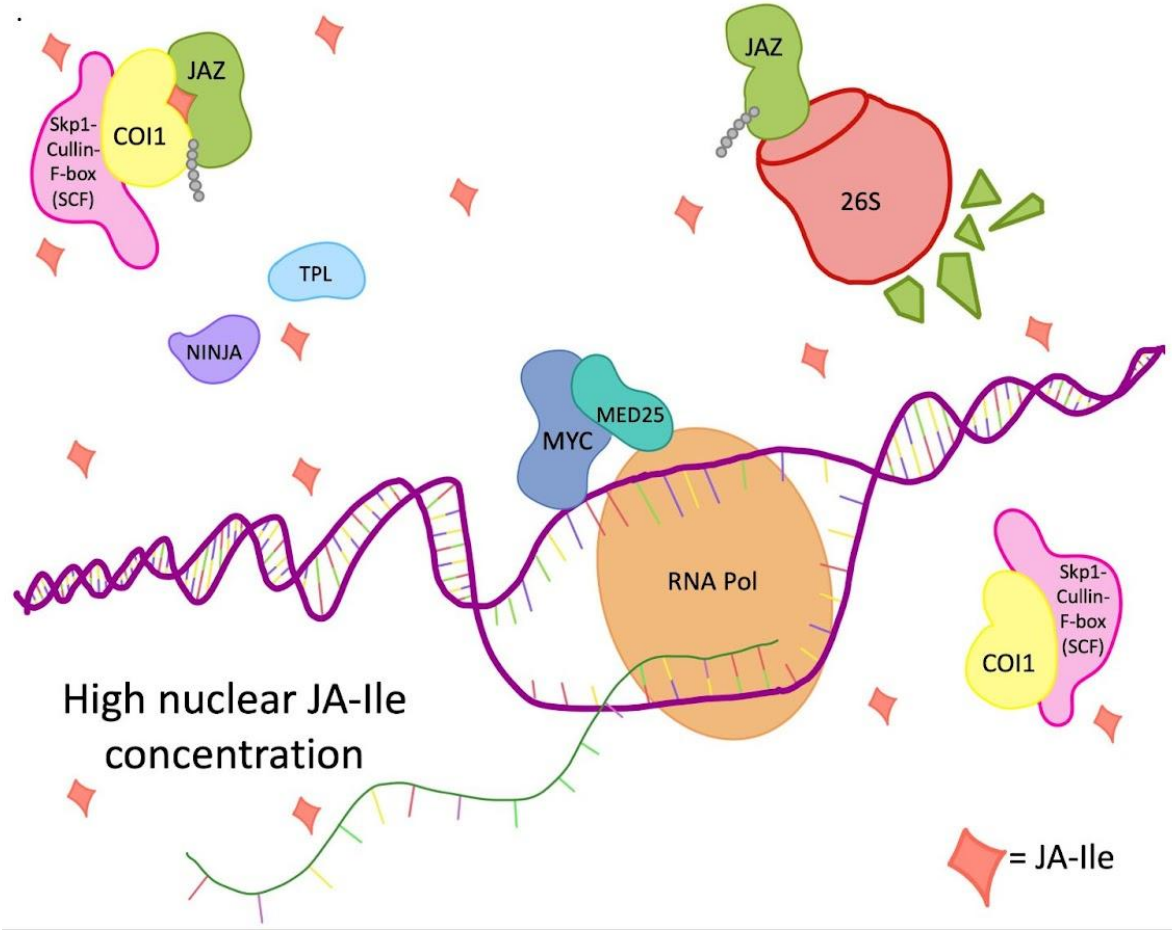


Fig. 2: A simple representation of the relief of repression on MYC TFs upon heightened JA-Ile presence. SCF^{COI1} forms a co-receptor complex for JA-Ile, which acts as a molecular glue to bind JAZ to COI1. JAZ is no longer able to repress MYC, which recruits MED25 and RNA polymerase to begin transcription of JA-promoted genes. JAZ proteins, now bound to COI1, are ubiquitin-tagged by the SCF^{COI1} complex and taken for subsequent degradation via 26S proteasome.

NINJA (Novel INteractor of JAz) and TPL (TOPLESS) are transcriptional co-repressors that play a crucial role in regulating the activity of the MYC family of transcription factors in plants. MYC proteins are key regulators of multiple developmental processes and responses to environmental stimuli, including the response to jasmonic acid (JA) signaling, which is involved in defense against herbivores and pathogens (Kazan and Manners, 2013). NINJA is a JAZ-interacting protein that was first identified in *Arabidopsis* (Pauwels et al., 2010). NINJA interacts specifically with the conserved TIFY motif in the

ZIM domain of JAZ proteins (Chini et al., 2007; Thines et al., 2007). NINJA interacts endogenously with JAZ proteins in a manner that is independent of COI1 function, and which, unlike the JAZ-COI1 interaction, does not require the presence of JA-Ile. This interaction leads to the recruitment of the TPL co-repressor to the JAZ-MYC transcriptional complex, though NINJA does not interact directly with MYC2 (Pauwels et al., 2010). Aside from its role in JA signaling, NINJA also plays a role in leaf shape development through cell cycle regulation (Baekelandt et al. 2018).

TPL is a member of the Groucho/Tup1 family of transcriptional co-repressors and functions to repress transcription of target genes by interfering with the assembly of transcriptional activation complexes (Long et al., 2002 and 2006). TPL interacts with NINJA, as it does with several other proteins, through a conserved ethylene response factor-associated amphiphilic repression (EAR) domain present in the A domain of NINJA, allowing it to repress JAZ proteins by way of NINJA without necessitating a direct interaction with JAZ (Pauwels et al. 2010).

Despite NINJA being apparently necessary for JAZ recruitment of TPL in most circumstances, there are some NINJA-independent mechanisms through which TPL can contribute to JAZ repression. Some JAZ proteins - JAZ8 and JAZ13 - lack the conserved TIFY motif that otherwise defines the ZIM domain in other JAZ proteins, or have an altered version. JAZ8 and JAZ13, which do not bind NINJA via TIFY motif/ZIM domain, instead contain an EAR motif which binds TPL directly (Shyu et al. 2012, Thireault et al. 2015). JAZ8 and JAZ13 are also stabilized against JA-Ile mediated destruction, compared to other JAZ proteins.

TPL is a multi-talented protein with multiple important roles in various hormone systems (Causier et al. 2012). It plays a similar role in auxin signaling as it does in JA signaling – as a transcriptional repressor released upon auxin perception. There is no analogous protein to NINJA in this system – instead, TPL interacts directly with Aux/IAA repressor proteins (which function similarly to JAZ proteins) via the Aux/IAA's EAR motif (Szemenyei et al. 2008). Given that some JAZ proteins contain EAR motifs that facilitate TPL recruitment and others do not, there are interesting questions that arise about NINJA's potential importance in other signaling pathways. Although jasmonate signaling is crucial to several essential physiological functions, jasmonates also influence several other hormonal signaling pathways, and through these interactions can act as a point of crosstalk in the relationship between growth and defense.

As previously mentioned, gibberellins are another signaling cascade implicated in the touch response. GAs are hormones that are closely related to growth and development. GA promotes growth by causing the degradation of DELLA proteins, endogenous negative growth regulators. DELLA proteins affect GA signaling in a similar manner to how JAZ do for JA signaling – by repressing GA-responsive genes and being degraded upon GA perception by the receptor GID1 and releasing the repression (Cao et al. 2006, Hou et al. 2008, Sun 2010). The suppression or promotion of growth in response to abiotic stress is of particular interest in studying touch response. In particular, Lange and Lange (2015) presented a model of thigmomorphogenesis that relies on the presence *and* conditional absence of GA. They demonstrated that *ga2ox7*, a knockout mutant impaired in GA biosynthesis, are non-responsive to touch, as are *della global* mutants lacking the DELLA protein family and therefore GA-responsive gene suppression capability. Further,

they showed that the stunted growth caused by touch stress can be rescued by exogenous GA application. Given that both GA and JA pathways have been shown to influence touch response in plants, the possibility of crosstalk and pathway interaction should be considered.

GA and DELLAs also affect JA signaling and JA-responsive gene expression (Navarro et al. 2008, Hou et al. 2010). In particular, the interaction between DELLA and JAZ proteins represents a major point of crosstalk between the two pathways. Although they fill similar roles in their respective signaling systems, these two protein families have direct, documented interactions with one another. The DELLA domain of DELLA proteins competitively bind to the Jas domain of JAZ proteins – the same domain used to bind JAZ to MYC TFs (Hou et al. 2010). This binding necessarily also prevents DELLA binding of other growth-related TFs, leading to an inability to repress vegetative growth (Yang et al. 2012). DELLA proteins, by providing an alternative way for relief of repression, can initiate JA-responsive signaling in the absence of JA. The presence of GA causes the degradation of DELLA proteins, leading to the re-repression of JA-responsive genes. Phytohormonal crosstalk, especially at the point of interaction between DELLA, JAZ, and MYC, is an undeniably important aspect of the relationship between plant stress, growth, and defense, and understanding the mechanisms behind and context of these molecular interactions will help us better understand this broader physiological relationship.

Our results from Chapter 2 demonstrated that oxylipin signaling is crucial to the plant response to mechanical stress. We found that defense induction in response to touch stress is reliant on JA-Ile, and from this we have concluded that defense is reliant on the already-characterized JA pathway in touch in a similar way as wounding. We found a

potential deviation between touch and wounding, however, concerning the governance of growth changes associated with touch stress. OPDA (and possibly OPDA-Ile) is the primary signaling molecule involved in this response. OPDA's standalone signaling capabilities do not necessarily come as a surprise – it has documented COI1-independent (and some COI1-dependent) roles in stress response and development. However, the precise mechanisms that control the growth response to touch remain poorly understood. A potential set of novel candidates for elements in the touch response have emerged from both Chapter 2 and the literature previously discussed. Parts of the canonical JA and GA pathways are promising possibilities, and in this chapter I report that parts of both pathways are required in order for the plant to orchestrate the complex growth response to touch stress.

Results

Touch stress results in a marked reduction in vegetative growth and an increase in molecular markers of mechanical stress, and is OPDA-dependent

Mechanical stimulation is known to be associated with growth reduction, but the effects of touch stress specifically have not been fully elucidated. In order to better describe the signaling systems involved in orchestrating this growth restriction in response to mechanical stress, we used the Automated Botanical Contact Device (ABCD, Fitzgerald et al. 2022), a computerized high-throughput system for applying repeated and replicable mechanical stress to plants. This system is used to define the effects of touch stress on

plants. The ABCD draws a thin plastic sheet across the entire aerial portion of the plant at pre-determined intervals. We used stimulus repetition every 15 minutes, for a total period of 7 days of treatment (see Ch. 2 for detailed methods). Repeated gentle touch stimulation every 15 minutes for 7 days results in a growth-stunting phenotype in WT *Arabidopsis* which was again observed in WT *Nicotiana benthamiana* (Fig. 3 and Supplemental Fig. S1, respectively). These results, which are very close to those from Chapter 2, are being reproduced for context for the following results. In Fitzgerald et al. 2022, this specific ABCD treatment resulted in a 50% reduction of *Arabidopsis* rosette diameter; similarly, we observed a 40% reduction in total rosette leaf area and 20% reduction in leaf number. In Chapter 2 I demonstrated that this gentle chronic touch stress, in addition to inflicting an observable quantitative change in growth in *Arabidopsis* seedlings, initiates a variety of molecular markers of mechanical stress. These markers include an upregulation of touch-responsive gene expression, OPDA/JA biosynthesis, and both OPDA- and JA-responsive gene expression within the first 30 minutes, and the repetitive nature of the ABCD treatment maintains a consistent and elevated response in all of these markers throughout the trial (Fitzgerald et al. 2022).

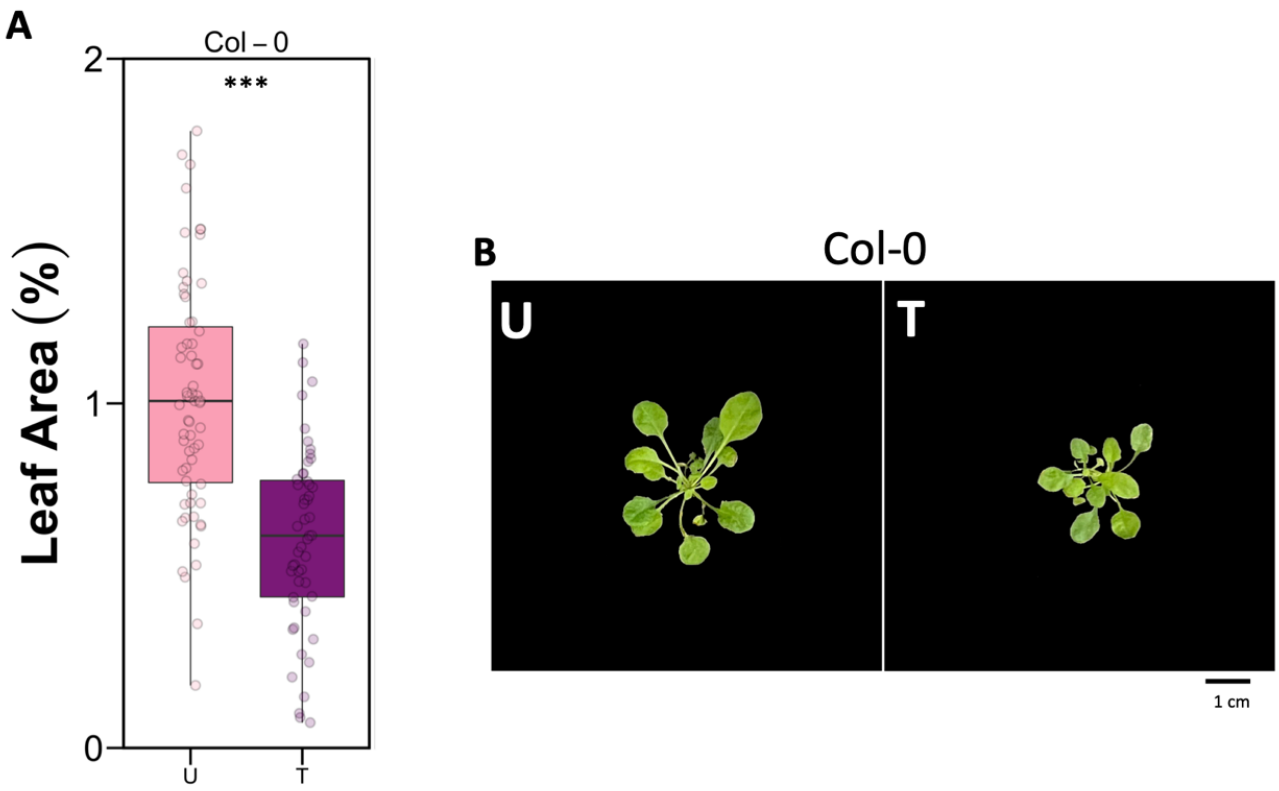


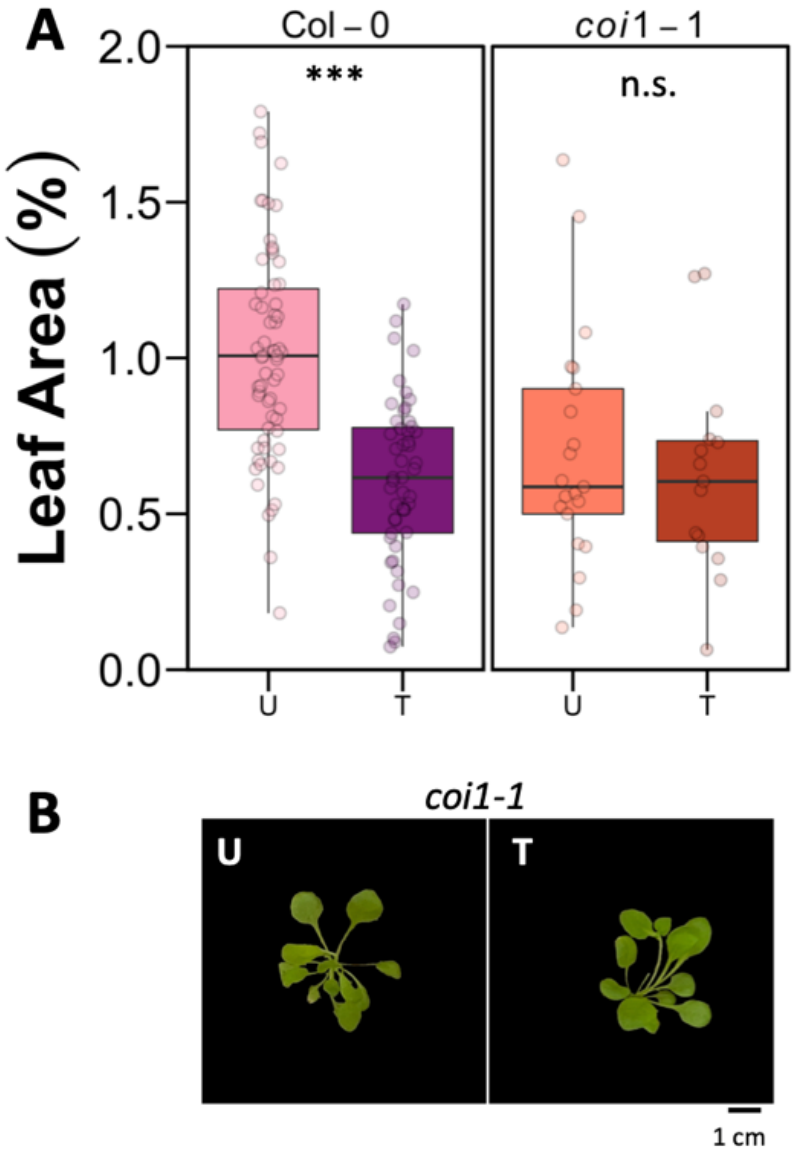
Fig. 3: Wild-type (Col-0) *Arabidopsis* plants have a growth-stunted phenotype after one week of touch treatment. A: Graphic representation of rosette areas of untouched (U) and touched (T) plants. All leaf area measurements have been normalized to their respective WT control averages in order to control for variation between experiments (environmental conditions, exact age, etc). WT control therefore has an average size value of 1. Significant dwarfing can be seen in the touched plants ($p<0.001$). B: Representative photographs of Col-0 plants after one week of no treatment (U) vs. touch stress (T). Scale bar=1 cm. The touched plant is visually smaller than its corresponding control. Significance between treatments was determined using a two-sample Student's *t*-test. *=$p<0.05$; **=$p<0.01$; ***=$p<0.001$

Touch-induced growth reduction is abolished in *coi1* mutants, which do not upregulate either oxylipin biosynthetic or oxylipin-dependent genes

We tested the role of the JA receptor COI1 in the growth stunting response to touch stress using two common knockout mutant alleles: *coi1-1* and *coi1-30*. Both alleles had a smaller unstimulated plant size phenotype than WT plants. Consistent with previous literature that centers COI1 as a critical protein for coordinating multiple abiotic stress responses, including the restriction of vegetative growth in response to wounding (Zhang and Turner

2008), neither *coi1* knockout mutant allele demonstrated a further dwarfing response to touch stress (Fig. 4 shows results from *coi1-1*, identical phenotypic results from *coi1-30* in Supplemental Fig. S2).

We performed qRT-PCR on *coi1-1* mutants and found that they do not show touch induction of *JAZ7* or *GRX480* (known JA- and OPDA- responsive genes, respectively) (Fig. 4). JAZ7 expression is known to be COI1-dependent, so its lack of expression is expected. Previous literature has shown that oxylipin biosynthetic genes *LOX2* and *AOS* are also JA-responsive, are COI1-dependent, and do not show an increase in transcript level upon wounding in *coi1* mutants (Reymond et al. 2000). We therefore asked whether this phenomenon was similar under touch stress as it is after wounding and found that touch-stressed *coi1* mutants also do not upregulate two early steps in JA biosynthesis *LOX2* and *AOC3* (the enzyme acting immediately after AOS in the paired AOS/AOC allene oxide reactions) (Fig. 4). It is therefore perhaps unsurprising that, although it is not directly promoted by COI1, *GRX480* transcripts would not increase following touch stress as induction of the pathway that produces OPDA could not be triggered by touch in the *coi1* background.


A
Col – 0
coi1 – 1
***
n.s.
Leaf Area (%)
2.0
1.5
1.0
0.5
0.0
U
T
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coi1-1
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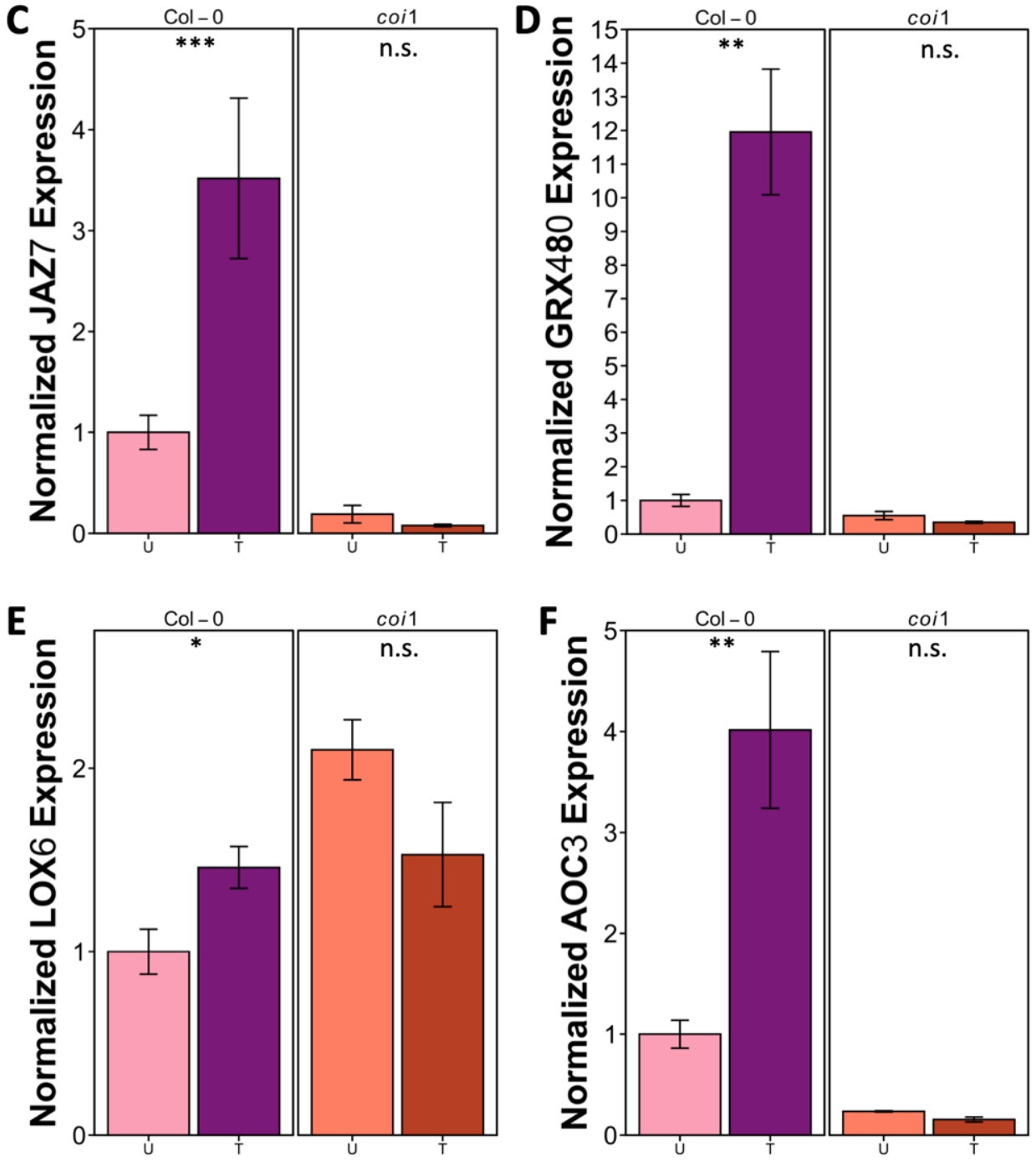

Fig. 4: Wild-type (Col-0) and coi1-1 knockout mutants display a different touch response phenotype and respond differently at the transcriptional level. A: Graphical representation of WT (left) and *coi1-1* (right) growth response to touch stress. WT displays a dwarfing response to touch ($p<0.001$), while *coi1-1* shows no difference between the two treatments. B: Representative photos of *coi1-1* plants after ABCD treatment. There is no visible difference in area between the two plants. Scale bar = 1 cm. C: Known JA-responsive marker gene JAZ7 expression in touched WT plants (T) after one week of repetitive stress is elevated more than threefold compared to untouched controls (U) ($p<0.001$), while in coi1 plants it remains significantly lower than WT as a baseline and does not show a response to touch. D: Known OPDA-responsive marker gene GRX480 in touched WT plants after one week of touch stress is elevated more

than elevenfold compared to untouched controls ($p<0.01$), while it remains lower and unchanged in *coi1-1.* E: Early JA biosynthetic gene *LOX6* expression in touched WT plants (T) after one week of repetitive stress is elevated compared to untouched controls (U) ($p<0.05$), while in *coi1* plants it does not show a response to touch. F: Early JA biosynthetic gene *AOC3* expression in touched WT plants after one week of touch stress is elevated fourfold compared to untouched controls ($p<0.01$), while it remains lower and unchanged in *coi1-1*. Significance between treatments was determined using a two-sample Student's *t*-test. *=$p<0.05$; **=$p<0.01$; ***=$p<0.001$

MYC transcription factors are required for touch-induced growth stunting, and override the sensitive dwarf phenotype in higher-order *jaz* knockout plants

We tested *Arabidopsis* mutants lacking three (*mycT*, i.e. *myc2,-3,-4*) or four (*mycQ*, i.e. *myc2,-3,-4,-5*) MYC transcription factors. Given that MYC is extremely important to both the repression and activation of JA signaling, we anticipated a loss of response to touch stress and possibly a larger plant compared to WT. There was no difference in size between WT controls and *mycT* or *mycQ*, but *mycT* and *mycQ* abolished the typical wild-type-like vegetative growth response to the ABCD and did not dwarf in response to touch. Fig. 5 shows this lack of response in *mycT*. Identical phenotypic results from *mycQ* are found in Supplemental Fig. S2.

The JAZ proteins make up a central and important component of JA signaling, and it is a large family with a high degree of functional redundancy. Single knockout mutants lacking individual *JAZ* genes have subtle phenotypic differences from WT, if at all (Chini et al. 2016, Thireault et al. 2015). Progressively higher-order *jaz* knockout mutants lose the benefits of this redundancy, and as more *JAZ* are knocked out, phenotypes become more evident and begin to mimic the effects of exogenous JA (Guo et al. 2018). Higher-order mutants up to an undecuple mutant, *jazU* (lacking *JAZ1* through *10* and *JAZ13*) exist

(Guo et al. 2018), but *jazU* plants have a very severe constitutive JA response and were not suitable for a mechanical stress experiment in the ABCD due to their extremely small size. The ABCD applies a non-structurally traumatic touch stress but does require roots sufficient to anchor the plant when it withstands some force. We tested *jazD* (a decuple mutant lacking *JAZ1-7, -9, -10, and -13*) for a touch response, but these plants are also constitutively severely stunted. Touched *jazD* plants had low survival because their size left them unable to avoid being torn from the soil. Those that survived were not smaller than their untouched counterparts, so we can very tentatively infer that they may be insensitive to touch. However, *jazD* might be unresponsive to additional touch because losing 10 of the 13 JAZ repressors saturates the stress response. Beyond that threshold, additional stimuli may be perceived, but no more phenotypic changes can be observed (Supplemental Fig. S3). Untouched *jazQ* (a quintuple mutant lacking *JAZ1, -3, -4, -9, and -10*)(Campos et al. 2016), on the other hand, grows relatively similarly to WT plants. Fig. 5 shows that when subjected to touch treatment *jazQ* mutants dwarfed by approximately 18%.

To investigate any potential interactions between or competing phenotypes involving JAZ proteins and MYC transcription factors, we tested *jazQ mycT* octuple mutants, impaired in expression of all of the same genes as *jazQ* and *mycT* described above. The effect of the loss of MYC transcription factors (insensitivity to touch) overrides the effect of the loss of JAZ proteins (constitutive stress response), as *jazQ mycT*, much like *mycT*, do not dwarf either constitutively or in response to touch (Fig. 5). *jazQ mycT* plants are roughly 30% larger in the control (untouched) treatment than corresponding WT plants (and 52%

larger than control *jazQ*), while the touch-stressed mutants are intermediate in size as they are not distinctly larger or smaller than control WT or control *jazQ mycT*.

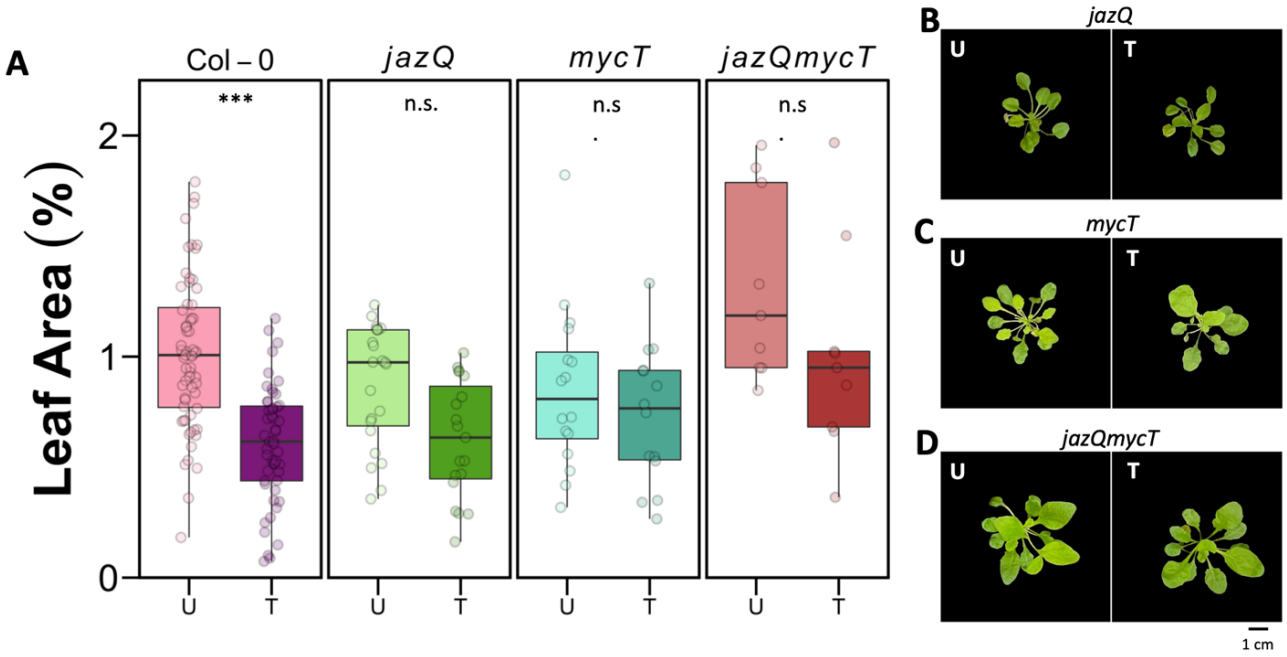

Fig. 5: Growth responses of *jazQ, mycT,* and *jazQmycT* mutants in response to stress show that losing MYC TFs abolishes touch response and overrides any sensitivity imposed by loss of JAZ. A: Graphical representation of (from left) WT, *jazQ, mycT,* and *jazQmycT* growth response to touch stress. WT displays a dwarfing response to touch ($p<0.001$), *jazQ* displays a slight dwarfing phenotype ($0.05<p<0.1$) *mycT* and *jazQmycT* show no difference between the two treatments. B-D: Representative photos of *jazQ, mycT,* and *jazQmycT* plants. There is a slight difference between the control (U) and touched (T) *jazQ* plants, and no visible difference between the control and touched of either *mycT* or *jazQmycT.* Significance between treatments was determined using a two-sample Student's *t*-test. *=$p<0.05$; **=$p<0.01$; ***=$p<0.001$

The JAZ auxiliary repressor protein TOPLESS is necessary for touch-induced growth stunting, but its associated co-repressor NINJA is not

Having determined that the MYC transcription factors *and* COI1 are required for *Arabidopsis* to coordinate a marked vegetative growth reduction phenotype in response to touch, we next asked if the other repressors on MYC TFs play a critical role in this response. JAZ proteins are associated with two main co-repressors: Novel INteractor of

JAz (NINJA) and TOPLESS (TPL). NINJA interfaces directly with JAZ proteins, and serves as a link between JAZ and TPL, as in most cases (barring JAZ8 and JAZ13) JAZ are unable to directly bind TPL due to a lack of EAR motif. In our tests, TPL is a critical component of the plants' ability to constrict growth in response to touch stress. Our *tpl* knockout mutant phenotype was overall smaller than the WT phenotype (by 32%), but does not significantly dwarf further in response to touch (Fig. 6). This puts TPL in the same category as MYC and COI1 as a putative requirement for this response. Unlike TPL, NINJA, despite being similarly constitutively dwarfed (at 31% smaller than WT controls), retains the capacity to further restrict growth under touch stress. Touched *ninja* mutants are 56% smaller than their untouched counterparts (Fig. 6).

Although *tpl* and *ninja* mutants are roughly the same size as each other and as touched WT plants under the control condition, it is unlikely that *tpl* is simply incapable of further dwarfing in response to stress given that touched *ninja* plants demonstrated a further dwarfing phenotype.

These results represent a divergence between the roles played by TOPLESS and NINJA; given TOPLESS's demonstrated role in multiple signaling systems beyond JA, it is possible that this capacity for multiple roles is a connecting point between JA and other hormonal signaling pathways. NINJA also has other documented roles in plant physiology beyond repressing JA-responsive genes; however, those roles likely are not a deciding factor in the growth-related touch response. Because TPL may act as a crux in multiple signaling cascades, because GA has been implicated in the touch response (Lange and Lange 2015), and because it is known that JAZ proteins are a direct crosstalk point

between JA and GA signaling, we decided to investigate whether the GA/DELLA growth signaling system is involved in regulating the growth response to touch stress.

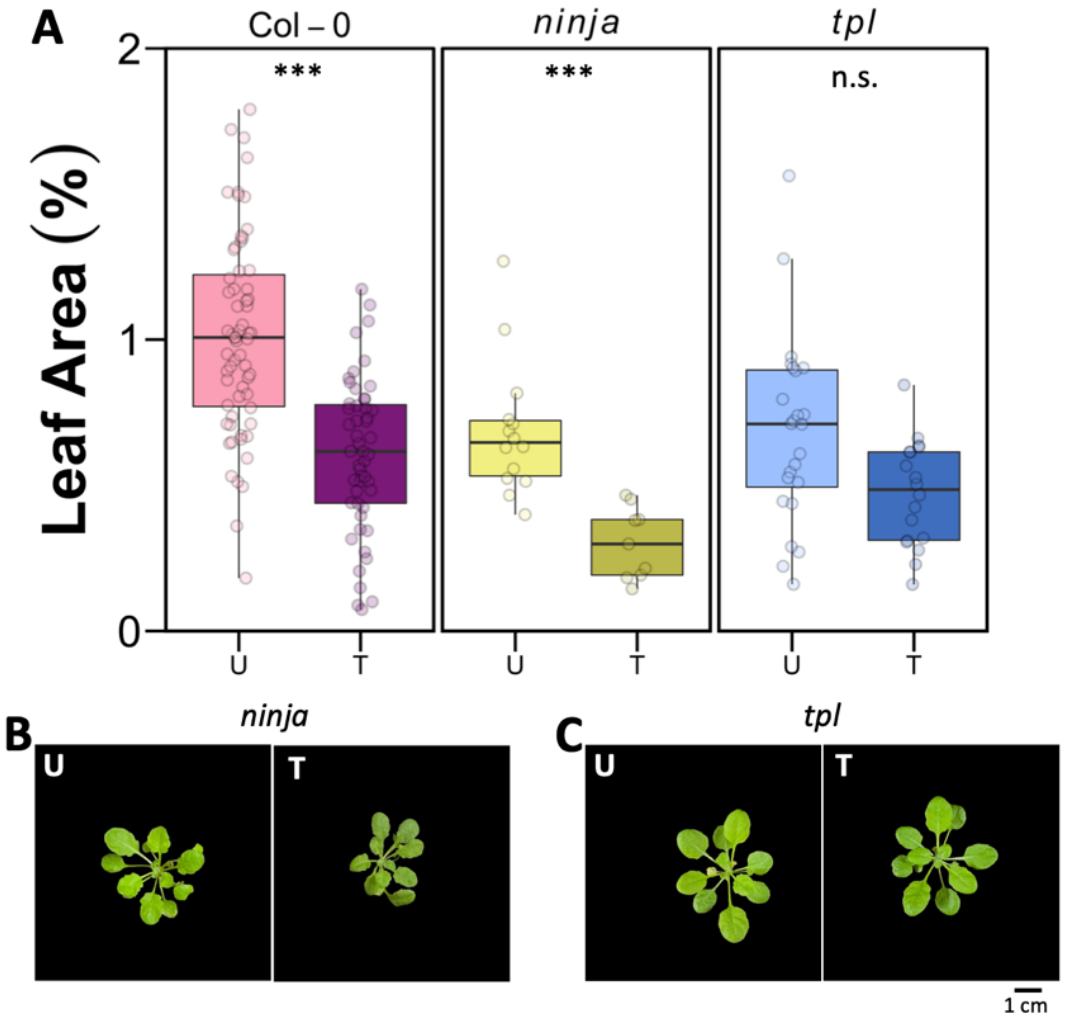

Fig. 6: Growth responses of *ninja* and *tpl* mutants in response to stress reflect a difference in role between the two co-repressors. A: Graphical representation of (from left) WT, *ninja,* and *tpl* growth response to touch stress. WT displays a dwarfing response to touch ($p<0.001$), *ninja* displays a dwarfing response to touch ($p<0.001$) *tpl* shows no difference between the two treatments. B-C: Representative photos of *ninja* and *tpl* plants. *ninja* plants demonstrate a dwarfing response in touched (T) plants compared to controls (U), and there is no visible difference between *tpl* control and touch stressed plants. Significance between treatments was determined using a two-sample Student's *t*-test. *=$p<0.05$; **=$p<0.01$; ***=$p<0.001$

DELLA proteins are required in addition to jasmonates for plants to orchestrate the growth response to touch

To determine the effects of alteration of GA signaling on growth in response to touch stress, we touch-stressed knockout mutants lacking each of the five DELLA proteins individually, all five DELLA proteins, and the three gibberellin receptors found in *Arabidopsis*: GID1a, GID1b and GID1c.

Fig. 7 shows that *della* mutants, lacking all five DELLA proteins, are constitutively smaller than WT plants, but do not dwarf further in response to touch. This result indicates that the DELLA proteins may play an important role in regulating growth reduction in response to mechanical stress, but offers little further insight into any individual roles played by the proteins. To investigate the individual contributions to the touch response, we tested single knockout mutants in *rga, rgl1, rgl2,* and *rgl3* (Tyler et al. 2004).

We found that each DELLA protein tested is required for plants to display a rosette growth phenotype that is responsive to touch stress. *rga* single mutants are smaller than WT plants in general, while in our experiments *rgl1*, *rgl2*, and *rgl3* plants are similar in size to WT. Each DELLA knockout mutant tested is insensitive to touch stress and does not display a further dwarfing phenotype (Fig. 7).

Analysis of a knockout mutant of the last DELLA, GAI, is underway. However, we also used a *gai-1* gain-of-function mutant to investigate if the reverse - an overabundance of a DELLA protein - causes a touch response phenotype more similar to the DELLA knockouts or to WT. *gai1-1* has a mutation in the DELLA domain of GAI, allowing it to retain functionality as a repressor but resist GA-induced degradation (Willige et al. 2007). *gai-1* mutants have a severely dwarfed, dark green phenotype consistent with an

impairment in GA-promoted processes (Koorneef et al. 1985). Additionally, they were insensitive to our touch stress treatment. Plants were, on average, nearly 80% smaller than WT under control conditions (Fig. 8). Added touch stress did not affect these plants and we did not observe any additional dwarfing.

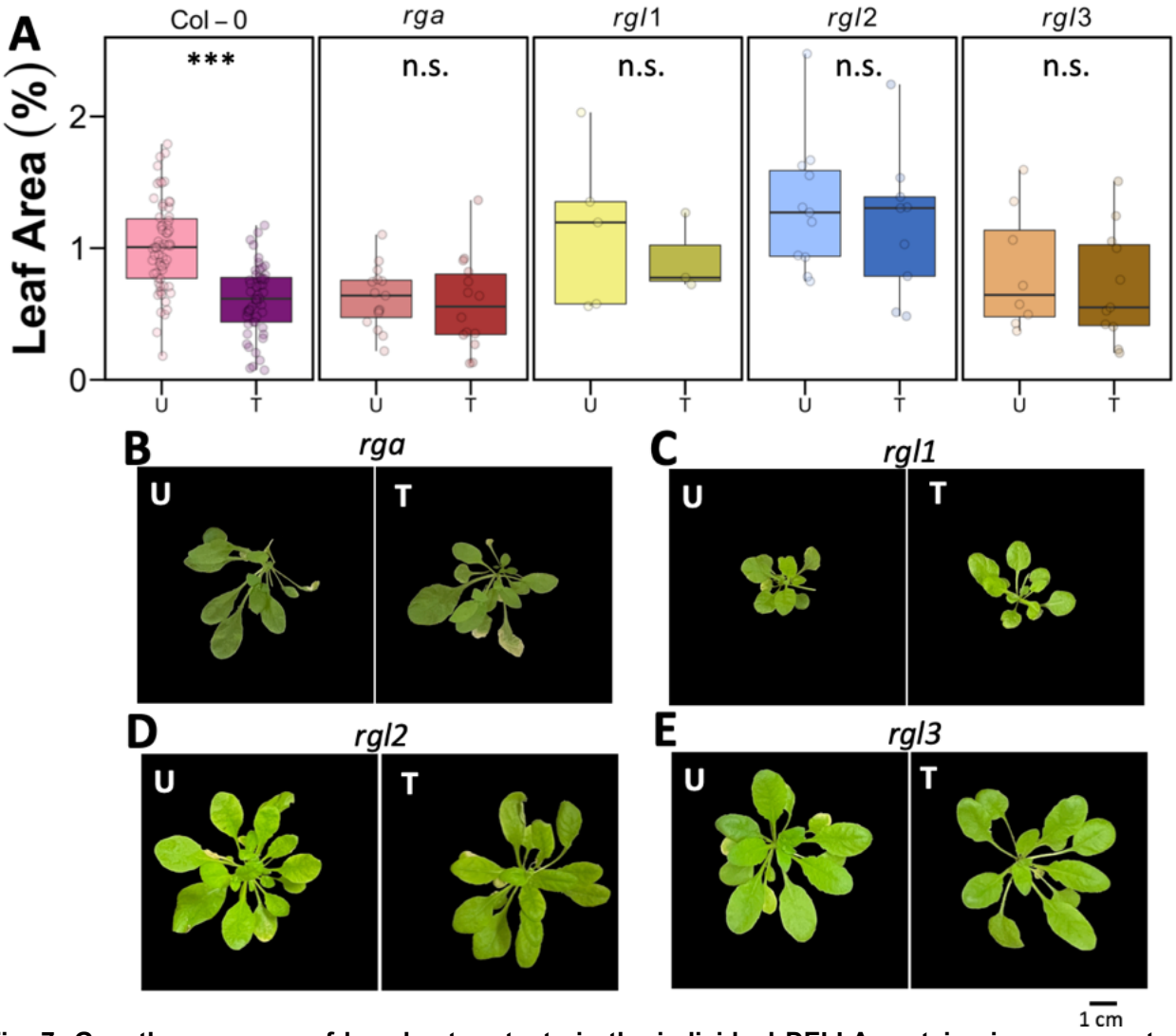

Fig. 7: Growth responses of knockout mutants in the individual DELLA proteins in response to stress show that each shows loss of dwarfing phenotype. A: Graphical representation of WT, *rga, rgl1, rgl2,,* and *rgl3* growth response to touch stress. WT displays a dwarfing response to touch ($p<0.001$), *rga, rgl1, rgl2,* and *rgl3* show no difference between the two treatments. B-E: Representative photos of *rga, rgl1, rgl2,,* and *rgl3* plants. There is no visible difference between the control (U) and touched (T) plants in any genotype. Significance between treatments was determined using a two-sample Student's *t*-test. *=$p<0.05$; **=$p<0.01$; ***=$p<0.001$

Because these results suggest that DELLA proteins play a central role in the growth response to touch stress, we next set out to determine if the DELLAs' involvement relate to their integration with GA signaling. In order to do this, we investigated whether the DELLA-associated GID1s are involved in the response.

GID1 was first characterized in rice as a GA receptor protein that enhances the SCF^{SLY1} complex's ability to target DELLA proteins for degradation and promote GA-responsive signaling (Ueguchi-Tanaka et al. 2005; Griffiths et al. 2006). In *Arabidopsis*, there are three homologs of GID1: *GID1a*, *GID1b*, and *GID1c*. Individual single knockout mutants in these *GID1* genes have subtle growth phenotypes, but the triple *gid1a/b/c* knockout mutant is constitutively dwarfed. We tested *gid1abc* mutants and found that they demonstrate touch stress-induced growth restriction. Our *gid1abc* mutants were significantly smaller (46%) than WT under control conditions. Unlike the constitutively dwarfed but touch-unresponsive *rga* mutants described above, in our experiments, *gid1abc* mutants were capable of further touch stress-induced growth constriction being approximately half the size (47%) of their untouched counterparts (Fig. 8). Thus, we conclude that none of the *GID1* homologs are critical for touch-induced growth restriction to occur, nor are they together critical or apparently functionally redundant.

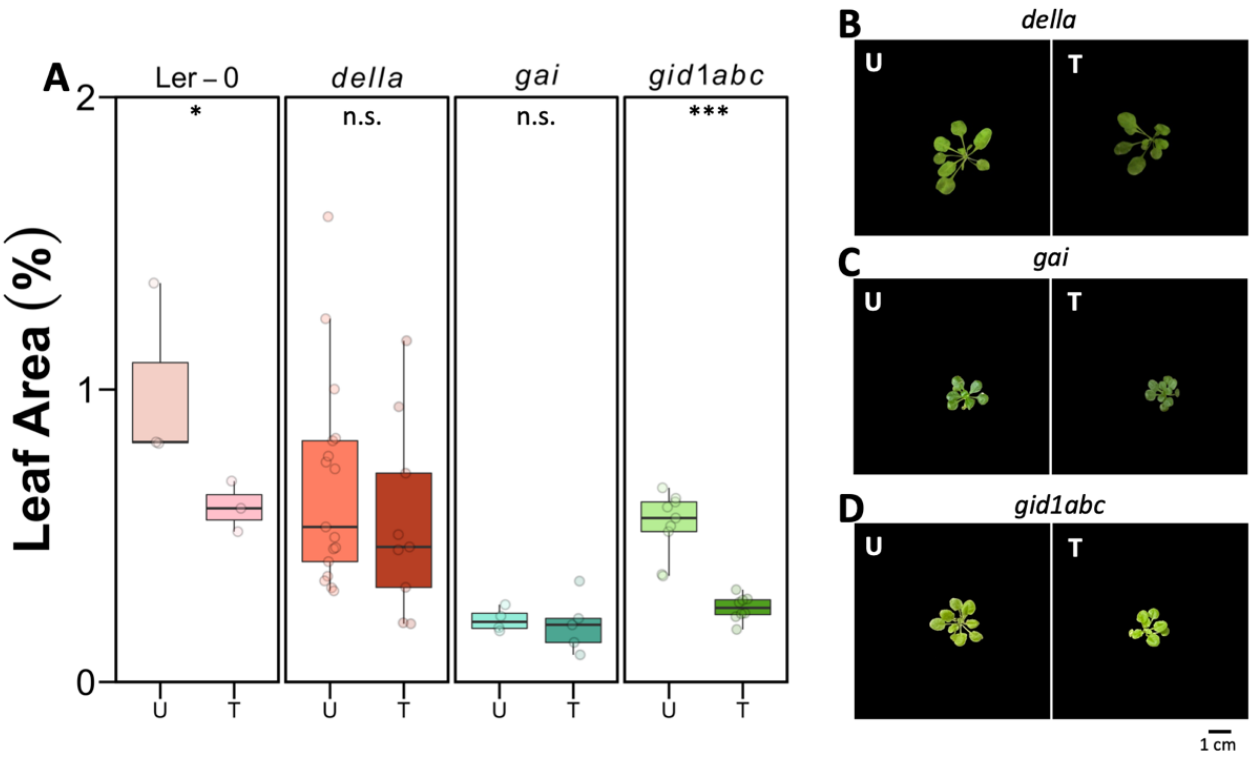

Fig. 8: Growth responses of *della global* knockout mutants, *gai-1* gain-of-function mutants, and *gid1abc* triple knockout mutants highlights the importance of DELLA in the touch response. A: Graphical representation of WT, *della, gai-1,* and *gid1abc* growth response to touch stress. WT displays a dwarfing response to touch ($p<0.01$), *della* and *gai* do not show any response, and *gid1abc* does demonstrate a further response to touch ($p<0.001$). B-D: Representative photos of *della, gai-1,* and *gid1abc* plants. There is no visible difference between the control (U) and touched (T) plants in the *della global* or *gai-1* genotype, and *gid1abc* plants are sensitive to touch. Significance between treatments was determined using a two-sample Student's *t*-test. *=$p<0.05$; **=$p<0.01$; ***=$p<0.001$

Discussion

Here, we have presented evidence that plant growth restriction in response to touch stress is regulated in part by aspects of both the octadecanoid (broadly jasmonate) signaling pathway and the gibberellin signaling pathway, although in both cases, the canonical signaling pathways drawn from the literature do not completely explain how these response networks are acting in response to touch.

Our results indicate that the MYC family of bHLH transcription factors (MYC2,-3,-4, and -5) are indispensable in the growth response to touch stress. This is consistent with previous literature highlighting their importance in the wound response and defense (e.g. Van Moerkercke et al. 2019, Darwish et al. 2022). It is not outside of possibility that OPDA-dependent transcriptional changes would rely on these TFs: in the bryophyte *Marchantia polymorpha*, it does just that (Peñuelas et al. 2019).

MYC transcription factors and TOPLESS appear to be critical parts of the plant's ability to turn OPDA signals from touch stress into a physiological response, but NINJA is likely not required and JAZ proteins appear highly functionally redundant for this response. TPL's deep involvement in multiple signaling systems indicate that signal crosstalk may be an important aspect of the plant's ability to coordinate systemic physiological responses to stress, which is further supported by the apparently minor role played by NINJA. NINJA does not play a role in other signaling systems TPL is involved in, including auxin (Szemenyei et al. 2008) or gibberellin signaling (Fukazawa et al. 2014), in which TPL plays central roles as a repressor. In any case, because of this divergence between NINJA and TPL, the coordination of the touch response isn't fully reliant on the canonical JA perception/repression machinery operating as we understand it to function in JA-Ile signaling.

OPDA's previous signaling roles have been described as COI1-independent, because COI1 does not use OPDA as a ligand, but we found that *coi1* knockout mutants lack the growth restriction after touch stress displayed by WT. Our results, at face value, may suggest that the growth restriction in response to touch is dependent on the action of

COI1 at the point of nuclear OPDA perception, or that COI1 exerts a different important effect on growth responses to touch stress.

A potential explanation for our results is that COI1 is able to bind OPDA-Ile as a ligand in the same manner as JA-Ile. This has yet to be documented in *Arabidopsis*, but has been in *Marchantia polymorpha* (Monte et al. 2018). Thus, MpCOI1 is capable of accepting dinor-OPDA (dn-OPDA) as a ligand. MpCOI1 and AtCOI1 have similar functions, and the ligand specificity is dependent on a single residue, which differs between MpCOI1 and AtCOI1 (Monte et al. 2018). dn-OPDA signaling is also repressed by a JAZ protein in *M. polymorpha* that functions very similarly to its counterparts in *Arabidopsis* (Monte et al. 2019). Further, MYC TFs are present and required for the OPDA response in *M. polymorpha* (Peñuelas et al. 2019). These changes in ligand and target specificity presumably represent a point in plant evolution where JA rose in prominence relative to OPDA as a key oxylipin signaling molecule.

It remains unknown whether COI1 plays a role in the nuclear perception and action of OPDA signaling in *Arabidopsis*.

It is also possible that the result we observed is due to *coi1* mutants' inability to upregulate oxylipin biosynthesis in general, not due to a particular requirement for COI1 to include binding ability to OPDA or OPDA-Ile. Previous literature has provided evidence that JA biosynthetic genes are COI1-dependent and not induced upon wounding in *coi1* mutants (Devoto et al. 2005, Chung et al. 2008). Similarly, we found that they are not induced upon touch in *coi1* mutants. Our model, therefore, does not include COI1 as an essential part of OPDA perception and touch signal translation into the physiological response observed in WT plants. Under this model, the canonical JA perception molecular

machinery, in which COI1 is deeply involved, likely plays some role in orchestrating the vegetative response to touch stress in a manner at least partially analogous to wounding stress. COI1's role in this response would be to upregulate OPDA or JA biosynthesis, rather than a direct role in the nuclear perception of either molecule. Alternatively, Ribot et al. (2008) presented an interesting OPDA-mediated, JA-independent *and* COI1-dependent pathway for transcriptional regulation (a wound-induced upregulation of a *PHO1* gene) that provides a further alternative explanation for the lack of touch response in *coi1* mutants.

This same phenomenon could also explain our results showing that *myc* mutants lack a growth response to touch: Dombrect et al. (2007) showed that JA biosynthesis, and specifically *AOC4*, is promoted by MYC2. Van Moerkercke et al. (2019) presented a JA-mediated positive feedback loop dependent on MYC2, 3 and 4 for the continued expression of JA biosynthetic genes. It is entirely possible that either MYC TFs are more responsive to OPDA/OPDA-Ile than previously known or that *myc* knockout mutants cannot promote oxylipin (including OPDA) biosynthesis in response to touch stress, and therefore lose the OPDA-mediated growth restriction phenotype without a strict requirement that MYCs actually ever interact in any meaningful way with OPDA/OPDA-Ile.

DELLA proteins have been proposed to have an evolutionarily ancient and conserved promiscuity, lending itself to the ability to mediate multiple transcriptional regulatory networks (Briones-Moreno et al. 2023). These functions have specialized with further speciation from an ancestral state, but DELLA proteins appear to have maintained an ability to mediate multiple processes through interactions with various transcription

factors. These functions are evidence of DELLAs' flexibility and central role in hormonal signaling and crosstalk. Our results suggest that DELLA proteins are, additionally, responsible for mediating the relationship between touch stress and vegetative growth. RGA, RGL1, RGL2, and RGL3 each had a demonstrable role, as single knockout mutants in each abolished the dwarfing response to growth. In addition, a *gai* gain-of-function mutant with degradation-resistant GAI proteins did not demonstrate any response to touch stress. The DELLA proteins have partially functionally redundant roles, but each also performs some distinct function (Sun and Gubler 2004). We propose a model in which stress-induced growth restriction occurs because of interactions between the JA and GA pathways facilitated by DELLA proteins. Our results further suggest that this phenomenon is more specific than simply GA signaling; because none of the 3 *Arabidopsis* GID1 homologs appear to be involved, the crosstalk in the touch response may involve DELLA proteins acting in a possibly different role than they are known to have in GA signaling.

DELLA proteins are known to be able to interact with JAZ proteins directly and competitively against MYC TFs. TPL is an important co-repressor of MYC TFs alongside JAZ proteins, even directly physically interacting with JAZ sometimes. TPL is also known to associate with the DELLA-binding TF GAI-ASSOCIATED FACTOR1 (GAF1) (Fukazawa et al. 2014). When endogenous DELLA levels are low (i.e. when GA levels are high), TPL represses GAF1 and by extension shuts down transcription of GAF1's target genes. As GA levels decrease, DELLAs accumulate, and GAF1-TPL becomes GAF1-DELLA and is activated (Fukazawa et al. 2014). Our *tpl* mutants abolished the touch response similarly to how *della* mutants did, including being slightly smaller than

WT. This may be due to an inability to regulate GA signaling by removing important regulators on GA-related TFs. Even though DELLA and TPL play dueling roles directly or indirectly in GA (competing for GAF1) and JA (competing for JAZ) signaling, disrupting the homeostasis between the proteins throws the balance of growth signaling into disarray. However, growth regulation in response to external stresses is undoubtedly very complex, and likely involves a spiderweb of interactions; in this study, we have shown that some of the connections in that network likely rely on the DELLA-JAZ interface, TPL repression, and the function of MYC TFs but these are clearly just a small part of a complex network that translates touch sensing into the control of growth and development.

Materials and methods

Mutant lines

Arabidopsis thaliana mutant lines used were T-DNA insertion mutants. *ninja, tpl, rga, rgl1, rgl2, rgl3, gai-1* were procured from the Ohio State University *Arabidopsis* Biological Research Center (Lines available in Supplementary Table S1). *coi1-1, coi1-30, jazQ, jazD, mycT, mycQ, jazQmycT* mutants were kindly provided by the Howe lab at MSU-DOE Plant Research Laboratory, Michigan State University, USA. *della global* and *gid1abc* mutants were kindly provided by Dr. Richard Barker. PCR-based genotyping was performed as in Chapter 2. Primers are available in Supplementary Table S2.

Arabidopsis growth conditions

Soil-grown *Arabidopsis* seeds were grown as in Chapter 2.

Media-grown *Arabidopsis* seeds were grown on 1% (w/v) agar plates containing ½ strength Murashige and Skoog medium (Murashige and Skoog 1962) and 2% (w/v) sucrose with modifications for screening; *coi1* seeds were germinated on plates additionally containing 25 μM MeJA and homozygous *coi1* mutant plants were identified using root length and cotyledon size and color.

Touch stress

Touch stress was applied with the Automated Botanical Contact Device (ABCD) (Fitzgerald *et al.* 2020). Treatments were carried out as in Chapter 2.

Image analysis

To determine total rosette area, photographs were taken and analyzed in Fiji (ImageJ) as in Chapter 2. All rosette measurement normalizations were performed within each individual experiment.

Gene expression analysis

RNA isolation and gene expression analysis by qPCR were performed as in Chapter 2. qPCR primers available in Supplemental Table S2

Normalization and statistical analysis

All environmental variables and conditions were consistent within each assay. Possible variance due to seasonal fluctuation in the non-controlled growth environment was a

concern, and necessitated that values be normalized within each experiment. Leaf area measurements are provided as a value relative to the average untouched WT leaf area in each experiment.

Homogeneity of variance was confirmed using Bartlett's test. Significance between touched and untouched sample groups was determined by independent two-sample Student's t-test with an α of 0.05 to compare treatment within genotypes.

We excluded those plants from analysis which suffered stem breakage, leaf damage, or other significant structural trauma as a result of the touch treatment, as this may introduce damage responses and compromise results.

Some data points in the WT dataset are also presented in Chapter 2. This is because there were several experiments containing multiple mutants relating to the material in Chapter 2 as well as Chapter 3 that use the same wild-type control.

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Supplementary information

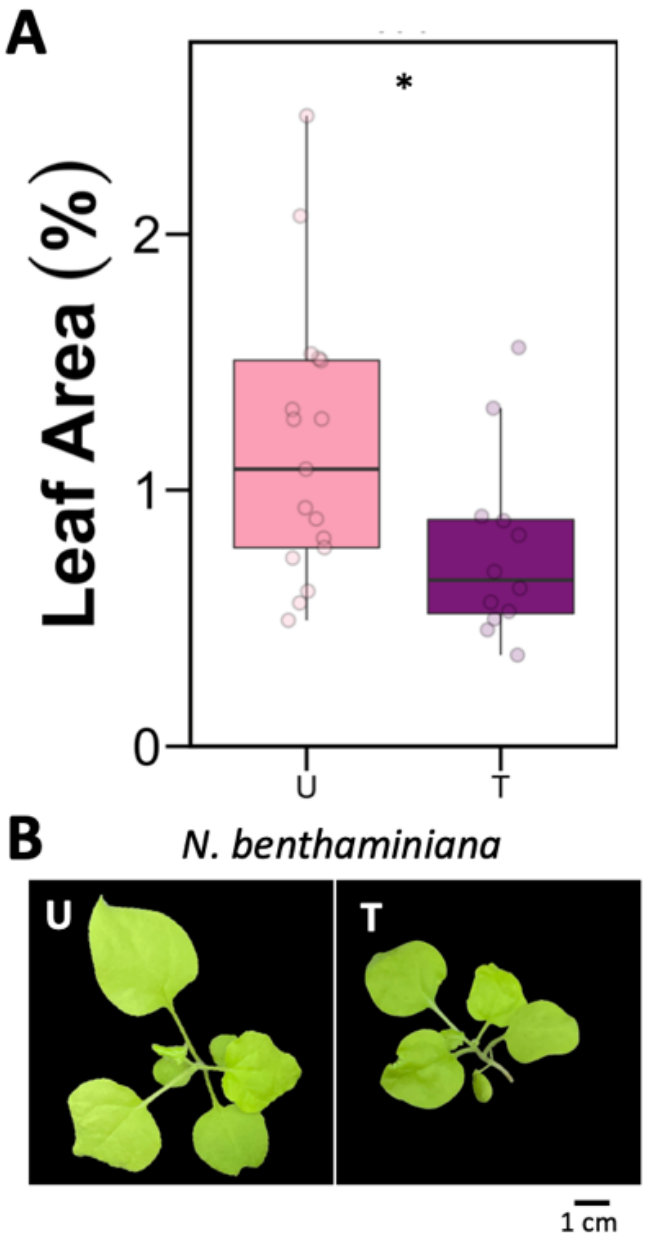


Fig. S1: Wild-type *Nicotiana benthaminiana* plants have a growth-stunted phenotype after one week of touch treatment. A: Graphic representation of rosette areas of untouched (U) and touched (T) plants. All leaf area measurements have been normalized to their respective WT control averages in order to control for variation between experiments (environmental conditions, exact age, etc). WT control therefore

has an average size value of 1. Significant dwarfing can be seen in the touched plants ($p<0.05$). B: Representative photos of control (U) and touched (T) *N. benthaminiana* plants. There is visible dwarfing in the touched plants. *=$p<0.05$; **=$p<0.01$; ***=$p<0.001$

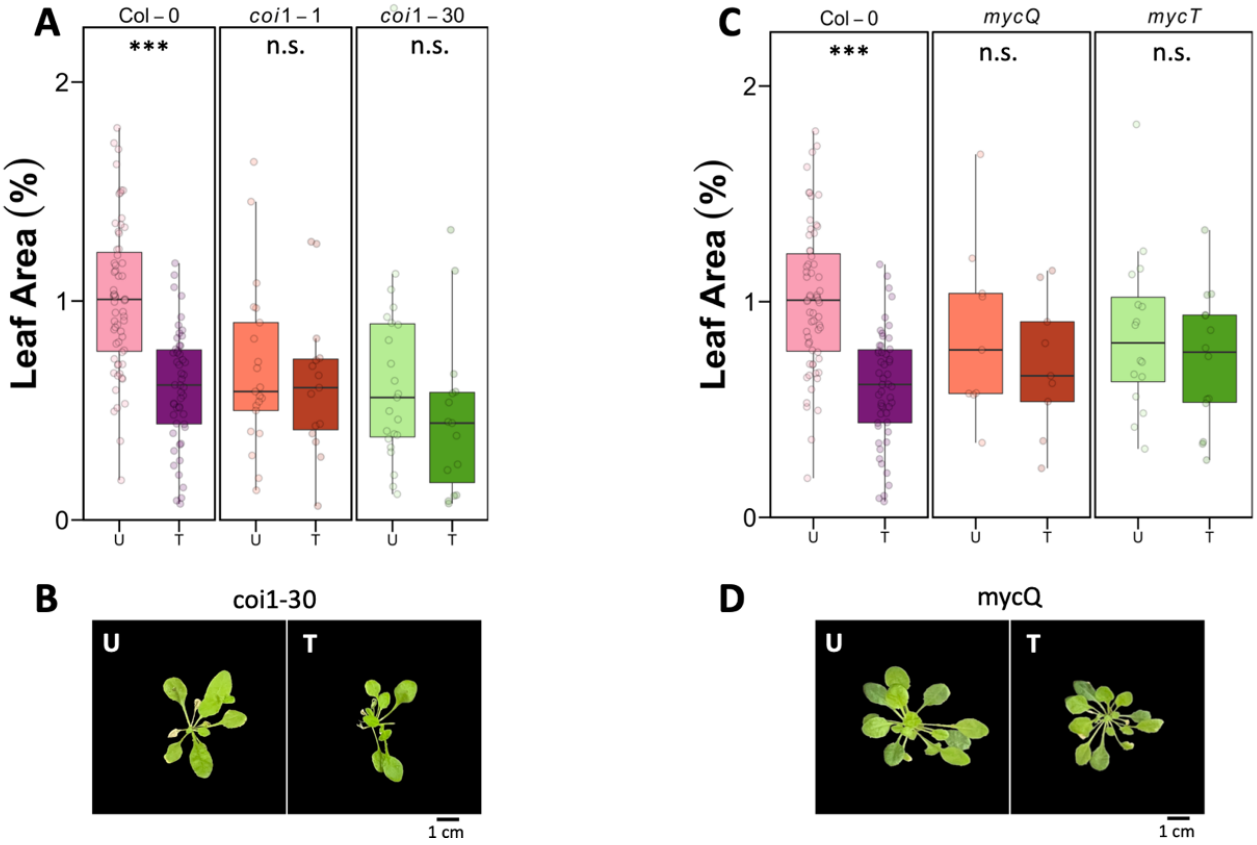


Fig. S2: A secondary allele of *coi1*, *coi1-30*, shows an identical response to touch as *coi1-1*, while *mycQ* is similarly phenotypically identical to *mycT*, supporting *coi1-1* and *mycT* results. A: Wild-type (Col-0) plants dwarf in response to touch compared to conrols ($p<0.001$).There is no difference in the leaf area of control and touched plants of either *coi1-1* or *coi1-30*. B: Representative images of control (U) and touched (T) *coi1-30* plants show that there is no visible difference in size. C: Wild-type (Col-0) plants dwarf in response to touch compared to controls ($p<0.001$).There is no difference in the leaf area of control and touched plants of either *mycT* or *mycQ.* D: Representative images of control (U) and touched (T) *mycQ* plants show that there is no visible difference in size. *=$p<0.05$; **=$p<0.01$; ***=$p<0.001$

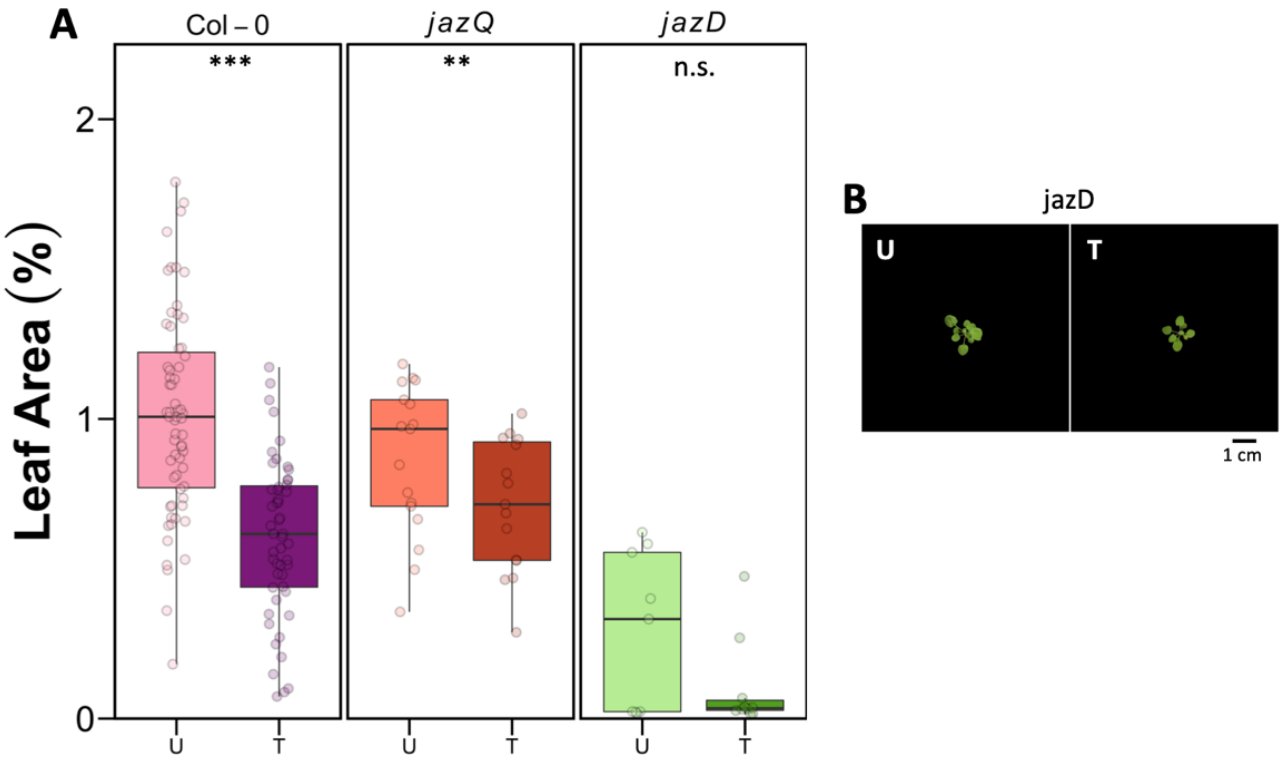


Fig. S3: The decuple *jaz* mutant, *jazD*, is severely constitutively dwarfed, and does not dwarf further in response to touch. A: Wild-type (Col-0) plants dwarf in response to touch compared to controls ($p<0.001$), as do *jazQ* plants ($p<0.01$).*There* is no difference in the leaf area of control and touched plants of jazD plants. *jazD* are also smaller overall than WT. B: Representative images of control (U) and touched (T) *jazD* plants show that there is no visible difference in size. *=$p<0.05$; **=$p<0.01$; ***=$p<0.001$

Table S1: Mutant lines used in this study, alleles, and corresponding gene accessions.

Mutant line	Allele(s)	Accession(s)
ninja	WISCDSLOX386G12	AT4G28910
tpl	SALK_034518C	AT1G15750
rga	*rga-28*	AT2G01570
rgl1	*rgl1-2*	AT1G66350
rgl2	*rgl2-13*	AT3G03450
rgl3	*rgl3-3*	AT5G17490
gai	*gai-1*	AT1G14920
coi1	*coi1-30* (SALK_035548)	AT2G39940
coi1	*coi1-1*	AT2G39940
della	*gai-t6; rga-t2; rgl1-1; rgl2-1; rgl3-1*	AT1G14920; AT1G66350; AT2G01570; AT3G03450; AT5G17490
jazQ	*jaz1/3/4/9/10*	AT1G19180 ; AT3G17860; AT1G48500; AT1G70700; AT5G13220
jazD	*jaz1–7/9/10/13*	AT1G19180; AT1G74950; AT3G17860; AT1G48500; AT1G17380; AT1G72450; AT2G34600; AT1G70700; AT5G13220; AT3G22275
mycT	*myc2/3/4*	AT1G32640; AT5G46760; AT4G17880
mycQ	*myc2/3/4/5*	AT1G32640; AT5G46760; AT4G17880; AT5G46830
jazQmycT	*jaz1/3/4/9/10 myc2/3/4*	AT1G19180 ; AT3G17860; AT1G48500; AT1G70700; AT5G13220; AT1G32640; AT5G46760; AT4G17880
gid1abc	*gid1a/b/c*	AT3G05120; AT3G63010; AT5G27320

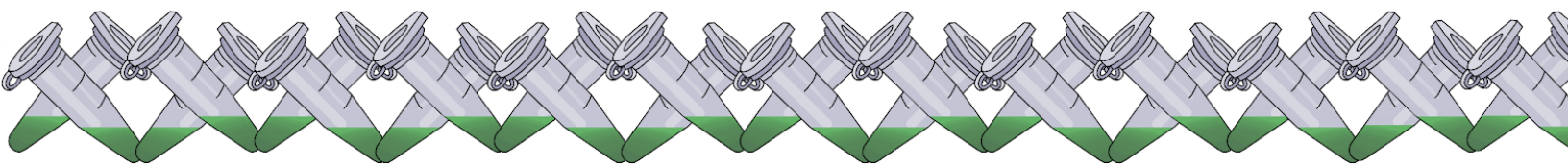
Table S2: Genotyping primers used in this study.

Gene ID	Direction	Sequence 5' to 3'
WiscDsLox_P745_LB	L	AACGTCCGCAATGTGTTATTAAGTTGTC
SALK_LBb1.3_LB	L	ATTTTGCCGATTTCGGAAC
NINJA	L	AATGATAACGTCGCCTGAGAG
	R	TCAGATTAAAACCCTGACCCC
TPL	L	GCTATGCTGTTTATTGCAGGC
	R	GTTCTGAATTGCAGGAGTTGC

Table S3: qRT-PCR primers used in this study and corresponding references.

Gene ID	Direction	Sequence 5' to 3'	Reference
UBQ10	F	CACACTCCACTTGGTCTTGCGT	
	R	TGGTCTTTCCGGTGAGAGTCTTCA	
TCH2	F	AGAAGATGATGAGTAATGGTGGTG	Braam and Davis 1990
	R	CGCCGTCACTAAAATTAATCTGC	Braam and Davis 1990
TCH4	F	ATCTACAATCTCTAGAAAATGGCGATC	Braam and Davis 1990
	R	GCTGAAACAGAGGAGGTGATGATAAGA	Braam and Davis 1990
JAZ7	F	GATCCTCCAACAATCCCAAA	Mengarelli et al 2021
	R	TGGTAAGGGGAAGTTGCTTG	Mengarelli et al 2021
GRX480	F	TGATTGTGATTGGACGGAGA	Arnold et al. 2016
	R	TAAACCGCCGGTAACTTCAC	Arnold et al. 2016
ZAT10	F	AGGCTCTTACATCACCAAGATTAG	Arnold et al. 2016
	R	TACACTTGTAGCTCAACTTCTCCA	Arnold et al. 2016

Chapter 4: Perspectives, applications, and significance



Introduction

All of the scientific pursuits I undertook and present in this dissertation are intended to dig a little deeper into one massive, overarching, important question in plant biology: How do plants perceive and interact with the world around them? Specifically, I investigated how plants sense mechanical stress (touch), and how they, in response, perform what has been presumably the most ecologically (and ultimately evolutionarily) appropriate physiological processes in response to that stimulus. I used a novel technology, the Automated Botanical Contact Device (Fitzgerald et al. 2022), to apply a regular and reproducible gentle touch stimulus to *Arabidopsis thaliana* plants and measured their responses in a variety of ways. First, I monitored their vegetative growth. This is a simple measure to quantify, but turned out to be abundantly informative. From the area of these plants, I was able to describe the reliably consistent growth reduction seen in touched wild-type plants and compare it to the presence and magnitude of the same response in a range of mutant plants. The exact number of plants I put into the ABCD isn't exactly clear because essentially every experiment had some tragic loss of plant life or plants that suffered structural trauma and those individuals were not included in my final data, but it is somewhere in the range of 2,000. This method combined with reverse genetics screening helped us understand the genetic basis of the plant's innate growth restriction response. Second, I measured the degree of induction of chemical defenses in the plants. In order to do that, I decided to use a proxy: herbivory. Measuring the glucosinolate (GS) content in *Arabidopsis* plants would have been another approach, and one that should be done in the future in order for us to have a more robust and accurate understanding of this species' chemical responses to touch stress. However, I

used herbivores instead because of the agroecological relevance of direct plant-herbivore relationships. *Arabidopsis* produces several different GSs, but altogether they contribute to the real interaction between it and its enemies, which is what we described when we quantified the extent of herbivory across many genotypes and treatments. Finally, I tested for transcriptional changes indicative of the molecular response. Transcription, of course, changes rapidly after essentially any stimulus, and is often a necessary precursor to the physiological or chemical changes observed in response. I performed qPCR on plant samples to measure the degree to which various touch response markers and genes known to be specifically responsive to JA and its precursor, OPDA, were altered. Those responses were then compared to the physiological responses we found from the other major measures to corroborate or challenge the models and assumptions we formed using reverse genetics.

Divergence in functionality between OPDA and JA in response to touch stress

In Chapter 2, I dissected the jasmonic acid signaling pathway and screened mutants deficient in (nearly) each individual step of JA biosynthesis, from the initial fatty acid oxygenation event in the chloroplast, to amino acid conjugation after JA is formed. Wild-type plants, when subjected to touch stress for an extended period of time, severely stunt their growth. This is what we've referred to as the vegetative, or growth, response. Previous studies (many reviewed in Koo and Howe 2009) have generally attributed the growth response to mechanical stress to JA. Touch stress, though, is an understudied phenomenon, and most literature about a growth response to mechanical stress involves

wounding. This leaves open questions about how touch may evoke a different response, or a similar response via different means, than wounding. I found that the early, chloroplast-localized steps of JA biosynthesis are all required for the plant to demonstrate a growth stunting response to touch. In particular, LOX2, LOX3, LOX6, AOS, and JASSY must be intact and functional. From this, we can deduce that the JA precursor 12-oxo-phytodienoic acid (OPDA) needs to be synthesized *and* exported from the chloroplast. However, what we found next challenges the current understanding of mechanical stress response. OPR3, the peroxisomal enzyme required for converting OPDA into its next metabolic derivative OPC-8 (only one step away from JA), was *not* required in order for the plant to dwarf in response to touch. This is a significant finding that suggests that either our prior understanding of the growth response to stress was incomplete *or* that the touch response is fundamentally regulated in a different way than wounding. Previous studies claiming JA's governance over the response have largely used *aos* as their JA-deficient mutants (e.g. Chehab et al. 2012), but in my experiments I took a finer-resolution approach to the JA biosynthetic pathway. This approach uncovered an as-yet undescribed mechanism where plants use OPDA to constrict their growth. We found that *opr3* knockout mutants, capable of producing OPDA but not JA, show a wild-type response to touch stress, unlike mutants which do not produce or export OPDA (e.g. *aos, lox6, jassy*). In order to verify this conclusion, we performed exogenous application experiments, where we treated wild-type, *aos*, *opr3*, and *jar1* plants with MeJA and with OPDA. If OPDA, but not JA, were primarily responsible for constricting growth in response to external stress, we would expect to see WT and *opr3* dwarf in response to either treatment, but *aos* to only dwarf when exposed to OPDA. This is the result we observed;

when treated with MeJA, *aos* mutants did not dwarf and were still insensitive to touch stress but did dwarf in response to OPDA treatment, while WT and *opr3* dwarfed in response to both. Further, OPDA application removed WT plants' sensitivity to touch stress, while MeJA + touch treated plants demonstrated a further dwarf phenotype. We verified that these genotypes were capable of the expected OPDA and JA responsive gene expression changes using qPCR, and found that WT upregulated OPDA and JA-responsive genes in response to touch, *aos* did not upregulate either, and *opr3* upregulated only OPDA-responsive genes and not JA-responsive ones.

After prolonged touch stress, WT plants demonstrated a pronounced reduction in herbivory from *Trichoplusia ni* larvae. This, along with the finding that these plants also reduce their growth, lends evidence to the idea that growth and defense are in a zero-sum metabolic competition, stemming from a resource allocation crisis. Our findings further support the canonical explanation that defense induction in response to stress is a JA-Ile-dependent process (e.g. Devoto and Turner 2003, Howe 2004). Using several mutants deficient in JA-Ile, we showed that this response seen in WT is abolished when any step in the biosynthetic pathway is interrupted, and rescued when mutants are treated with MeJA, with the notable exception of *jar1* mutants. Therefore, we concluded that JA-Ile is required for the plant to orchestrate this defensive response to touch. This finding is similar to what has been previously demonstrated in response to wounding (Chehab et al. 2012, Wang et al. 2019).

Another novel finding from Chapter 2 is the importance of JAR1 in what we have demonstrated is likely an OPDA-dependent vegetative response. JAR1's involvement in the JA-dependent defense induction was not surprising, but given that JA does not appear

to be required for plants to dwarf after touch stress, we did not expect JAR1 to be involved in the growth response. If the pathway deviates after OPDA export from the chloroplast, it seems counterintuitive that a step later on in the JA pathway should be required. However, OPDA has a documented bioactive isoform conjugate with isoleucine. Previous published results have shown that *jar1* mutants are not only deficient in JA-Ile accumulation, but that they also accumulate significantly lower levels of OPDA-Ile than do WT after wounding (Floková et al. 2016). The conjugase responsible for OPDA-Ile production has not been definitively identified, but it has been proposed to be JAR1 (Guranowski et al. 2007, Floková et al. 2016). Taken alongside the results from the literature, our results support the hypothesis that JAR1 is at least partially responsible for OPDA-Ile conjugation *and* brings an additional interesting conclusion: OPDA-Ile, specifically, is the bioactive signal used to constrict growth in response to touch stress.

My research has demonstrated that there exists a split in the octadecanoid pathway, where OPDA-Ile and JA-Ile control growth and defense responses to touch, respectively. Further, there is a possibility that the way the response to prolonged touch is regulated is fundamentally different than the wound response. This could be due to a severity-dependent response from the outset/infliction of stress, but may also be a result of the long-term stress that our touch treatment inflicts and reflect an acute vs. chronic rather than a severe vs. gentle stress difference.

Because we have identified a point at which defense and growth can be effectively uncoupled, future research could pursue this possibility. Understanding the nature of the disparity between touch and wounding will be very informative for any potential future agricultural applications. At what points in the pathway do the responsive signal cascades

differ? When (at what length of time/at what severity) does this difference take effect? Future research could investigate external factors that challenge the plant – a spectrum of differing perturbation intensity or frequency. If there is a difference in how touch and wounding are regulated, on a basic level, logically there needs to be an inflection point at which the plant switches responses. Less intuitive but no less plausible is the possibility that, just like stress severity can be a spectrum, the response mechanisms may bleed together at medium intensity stimulation. One approach to that question is to survey gene expression changes across the transcriptome in response to a variety of mechanical stresses from infrequent, gentle touch through frequent rough touch, bending, or even light wounding. A bioinformatics-informed model based on RNA-seq assays can be revealing on its own, or provide an informative and useful base from which to investigate candidate pathways. I would also like to see further experiments using an ABCD-like system with non-model crop plant species, probably after we have a deeper understanding of the divergence that is emergent from my dissertation work. Since jasmonate signaling is ancient, but the anatomy and physiology of various plant families, including how they respond to JA, is widely different, it would be very interesting to investigate the effects of altering the octadecanoid pathway in different species. The grass family, Poaceae, from which we get grains, has different needs from the nightshade family Solanaceae, from which we get a lot of (culinary) vegetables like potatoes and eggplant. These families' defense responses are different as well - for example, I would be very interested to see how touch may alter toxic defense mechanisms, like the alkaloid content of solanaceous plants or the toxic glycoside benzoxazinoids of grasses. Nicotine production is inducible as well as constitutive in tobacco, a solanaceous commodity crop

(Baldwin 1989, Baldwin et al. 1994, Baldwin 1996) and benzoxazinoids are inducible in various grasses, including maize (Robert et al. 2012), so the goal of enhancing endogenous defense might actually be at odds with the additional goal of improving food quality. Perhaps this would also be a concern with Brassicaceae, *Arabidopsis*'s family, and their bitter GS content. GS have, after all, been the target of a recent breeding campaign meant to reduce the GS content, and therefore the bitterness, of Brussels sprouts. I would also like to know the agroecological repercussions of such efforts, such as the success of herbivorous insect populations, and how this ultimately affects yield and production (e.g. Hondelmann et al. 2020). The ancient Japanese practice of mugifumi used touch stress to alter biomass allocation in wheat and prime hardiness, though there is extremely little scientific evidence in the available literature to support or investigate it. If there is a way to harness touch application, alone or in conjunction with genetic engineering or chemical manipulation, to alter plant-pest interactions and optimize crop yield, that is an avenue that should be pursued.

Elements of multiple signaling pathways converge to orchestrate growth reduction after touch stress

Having established that the defense response matches the canonical understanding of JA-Ile control, in Chapter 3 I focused on the downstream signal pathway involved in touch-induced vegetative/developmental changes. In a similar fashion to how I systematically investigated the importance of steps in the JA biosynthetic pathway, I chose to use knockout mutants in various components of JA perception to learn through deduction the roles played by those components. In doing so, I found that the response

appears at first to be COI1-dependent. However, it's been demonstrated that COI1 probably does *not* interact with OPDA (Thines et al. 2007, Katsir et al. 2008). Naturally, this was intriguing, and opened the possibility that OPDA-Ile could act as a secondary, less-than-ideal ligand for COI1. The binding potential (or lack thereof) between COI1 and OPDA-Ile has not been well-documented, and notably, COI1 also preferentially binds with JA-Ile instead of JA or MeJA, neither of which are capable of promoting the formation of the COI1-JAZ complex (Thines et al. 2007). This remains an open possibility, and I would be very interested in further experiments using *in silico* approaches to determine if there is a possibility, physically, for COI1 to accommodate OPDA-Ile in the JA-Ile binding site. I would also be interested in seeing *in vitro* experiments similar to those that have already successfully demonstrated the JA-Ile promotion of COI1-JAZ interaction (Thines et al. 2007), with OPDA-Ile. Protein interactions as determined by, e.g., pull-down assays, under various conditions could prove informative whether or not they show OPDA-Ile promotes COI1-JAZ interactions. From the literature, we know that COI1 and MYC TFs both are involved in a positive feedback loop in which JA triggers its own continued biosynthesis, and that in knockout mutants of either component, JA biosynthesis is not upregulated after stimulation (Reymond et al. 2000, Devoto et al. 2005, Chung et al. 2008, Dombrect et al. 2007, Van Moerkercke et al. 2019). This presents another interesting hypothesis about why our *coi1* and *myc* knockout mutants failed to dwarf in response to touch stress despite a lack of known interactions with OPDA/OPDA-Ile. Indeed, we also found that *coi1* mutants failed to upregulate *LOX6* or *AOC3* at the end of the touch stimulation period, where WT did. Thus, these mutants are impaired in oxylipin biosynthesis. Another potential explanation for the *coi1* insensitivity results that we saw

is an OPDA-responsive, COI1-dependent transcriptional regulation pathway. Previous literature contains evidence for such a pathway induced after wounding: Ribot et al. (2008) showed that a stress-related, wounding-induced gene in the *PHO1* family is induced upon wounding in WT and in *opr3*, but not in *aos* and *coi1* mutants, a result that mimics the responses that I saw in response to touch stress. Of these potential models, the one that I find the most compelling is that *coi1* and *myc* mutants are impaired in oxylipin biosynthesis to begin with. This possibility was backed up by my own data, which I collected in order to corroborate the Devoto et al. 2005 & Van Moerkercke 2019 findings. However, further studies could change that conclusion.

A result from Chapter 3 that surprised me was the difference between my *tpl* and *ninja* mutants' response to touch. They are both co-repressors on JAZ proteins, and if one were going to be more important than the other I would have predicted NINJA would be the most important, given that it directly interfaces with JAZ and TPL (generally) does not. This result perhaps makes more sense when we consider TPL in its full context: as a prolific repressor of several TFs across multiple signaling pathways, including JA and GA. The presence of high endogenous JA leads to TPL de-repression of MYC TFs and induction of JA-responsive gene transcription, leading to growth constriction and defense induction (Wasternack and Song 2017). In contrast, high endogenous GA leads to induced TPL repression of GAF1, leading to promotion of growth (Fukazawa et al. 2014). TPL plays a central role in regulating both JA and GA responsive signaling pathways, and its absence likely causes disarray and imbalance between the (sometimes) competing interest of growth and defense promotion. TPL's role in different signaling systems is well-documented (Causier 2012), but how it reacts with other elements of those pathways

under stress is still an open question. Future research pursuing that question could track TPL after (e.g.) touch stress. Does it interact with DELLA proteins at any point during the relief of repression in response to JA or enforcement of repression in response to GA? *In vitro* protein interaction assays, e.g. yeast two-hybrid or pull-down assays could be sufficient to demonstrate if there is a strong enough physical interaction. Alternatively, *in planta* experiments might use a fluorescence resonance energy transfer (FRET) bimolecular fluorescence complementation (BiFC) to track DELLA interactions with TPL and/or with the TFs TPL regulates.

Given NINJA's apparent lack of role in the growth response to touch, I have questions about the evolutionary importance of its role in JA signaling. Not all JAZ proteins require NINJA to recruit TPL; some JAZ contain their own EAR motifs, while others seem to have lost them or to have not evolved them. TPL doesn't require a NINJA analogue for its roles in other systems like auxin signaling, and JA signaling seems to depart from similar systems in needing that co-repressor. NINJA's role in leaf development shows another context in which NINJA could have evolved before being possibly co-opted by JA signaling. To my knowledge, nobody has studied the evolution of NINJA to determine how ancient or recent it may be. It would be interesting to look into the evolution of the protein itself, but also into the evolution of its role in JA signaling. Current evidence leads me to suspect that the incorporation of NINJA into JA signaling may have occurred alongside the disappearance of the EAR motif in some JAZ proteins.

Future directions

From a larger, whole-plant physiological perspective, uncoupling growth from defense could allow greater insight into the economics of plant development. Even if there may not be an immediate metabolic appropriation crisis in normal conditions (in nature or in cultivation, depending on the species), we have seen that development generally follows the growth-defense “tradeoff” pattern. I would very much like to know how tissue value and age affect plants’ resource allocation. There exist a handful of explanations for this pattern, but two of the most popular are the optimal defense hypothesis, which states that plants will allot resources to be used for defense, e.g. secondary metabolism, specifically to those tissues which are the most “valuable”; and the growth-differentiation balance hypothesis, which pits carbon and nitrogen against one another by stating that the slowest-growing photosynthetic tissue will have the highest defenses thanks to a different available C:N ratio (Givnish 1986, Stamp 2003). There is a very wide range of experiments that could link touch to these explanations, including measuring photosynthesis changes, growth rates, and biochemical makeup in tissues of varying age and function in plants that have been touch-stressed. Any and all of these approaches has the potential to tell us much about the effect of touch stress on the fundamental regulation of growth and defense homeostasis on a very different level than I have chosen to focus on, but that can bridge the observable physiology to the molecular mechanisms that underlie it.

My research has brought up, and answered, interesting questions about the mechanisms underlying plant responses to touch stress and the relationship between growth and defense. The pursuit of uncoupling plant growth, development, and defense has been a

major research focus in plant biology since the Green Revolution. A huge amount of progress has been made in understanding the relationships between these phenomena on a basic level, and it has been exciting to add to that growing body of research. Ultimately, one day we will be able to grow crops that grow quickly, grow large, and grow unhindered by attack by pathogens or herbivores.

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