Plant U-Box Proteins 25 and 26 are Involved in Regulation of Crosstalk Between Salicylate and Jasmonate Signaling Pathways

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PLANT U-BOX PROTEINS 25 AND 26 ARE INVOLVED IN REGULATION OF CROSSTALK BETWEEN SALICYLATE AND JASMONATE SIGNALING PATHWAYS

by

Dustin C. Rowland

A thesis submitted to the Graduate College in partial fulfillment of the requirements for the degree of Master of Science
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Thesis committee:

Jian Yao, Ph.D., Chair
Yan Lu, Ph.D.
Todd J. Barkman, Ph.D.
The jasmonic (JA) and salicylic acid (SA) signaling systems regulate diverse plant developmental processes and immune responses. Due to the opposite end-goals of various pathogen-response pathways, a certain level of antagonism between the JA and SA pathways is required. However, over-suppression of any single pathway would leave a plant susceptible to invasion by a specific class of pathogen. We investigate the regulation of crosstalk between the JA- and SA-mediated immune pathways and how the dilemma of specific susceptibility may be avoided. Manipulation of *Arabidopsis thaliana* genotypes was utilized to determine the effects of protein knockout on pathogen-specific susceptibility and resistance. The effects of hormone treatment on protein concentration and the effects of protein knockout on the plant transcriptome were investigated as well. Loss of the plant U-box protein 25/26 increased susceptibility of plants to necrotrophic pathogen *Pectobacterium carotovorum*, indicating that this protein is instrumental in regulation of immune pathways required for optimization of fitness.
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1 Introduction

Plant U-box proteins 25 and 26 make up a pair of proteins that are phylogenetically closely-related key regulatory elements in interactions between hormone subsystems. A number of other protein classes are relevant to these hormone signaling pathways, including jasmonate zim-domain (JAZ), coronatine-insensitive (COI), Myc, Myb, and non-expressor of PR genes (NPR).

1.1 The Jasmonate Signaling Pathway

The first immunity system relevant to the project is jasmonic acid signaling, which regulates plant responses to a specific type of plant pathogens. This is the necrotrophic class of pathogens, which destroy plant tissue and utilize the released nutrients. Necrotroph-responsive genes are repressed by a class of jasmonate-zim domain (JAZ) proteins that inhibit the MYC2 transcription factor. When the plant perceives a putative necrotroph molecular pattern, the level of JA-Ile increases. JA-Ile increases the affinity of E3 ubiquitin ligase COI1 for JAZ, resulting in polyubiquitylation and degradation of JAZ at the 26S proteasome. The released MYC2 transcription factor is then free to promote expression of necrotroph-responsive genes (Howe, 2010).

JAZ proteins represent a diverse class of plant proteins that repress transcriptional activators of the jasmonate-response genes. These proteins contain the eponymous ZIM domain, a conserved Jas domain, and another conserved domain at the N-terminus (NT) (Melotto et al., 2008). The Jas domain mediates interactions between JAZ, COI1, and MYC proteins while the ZIM domain mediates JAZ-JAZ interactions (Chini et al., 2009). These proteins are targeted by COI1 for degradation during jasmonate signaling, allowing MYC proteins to activate response genes.

COI1 is an F-box protein, a component of the SKP, CULLIN, F-box protein (SCF) complex that functions as an E3 ubiquitin ligase. The SCF complexes target a variety of proteins for degradation, including those in the jasmonate-zim domain (JAZ) class targeted by SCF$_{COI1}$ (Zhai et al., 2015). The affinity of COI1 for JAZ is heavily influenced by the availability of bioactive JA, jasmonyl isoleucine (JA-Ile), and requires the presence of co-activating molecule inositol pentakisphosphate (Sheard et al., 2010). Mimics of JA-Ile - such as the Pseudomonas syringae phytotoxin coronatine - may be secreted in order
to increase the affinity of COI1 for JAZ, increasing anti-nectrotroph signaling and allowing biotrophic pathogens to proliferate (Torres Zabala et al., 2016). Degradation of JAZ releases MYC proteins, allowing transcription of jasmonate-response genes to occur.

Activation of genes in the jasmonate signaling pathway is known to require the MYC class of transcription factors. These proteins are also involved in the ethylene signaling pathway (Boter et al., 2004). The proteins MYC2, MYC3, and MYC4 are all repressed by JAZ proteins and removal of this repression via any pathway results in activation of jasmonate-responsive genes (Fernández-Calvo et al., 2011). Unrepressed MYC proteins interact with MYB proteins, creating a cooperative transcriptional complex (Qi et al., 2015). MYB proteins also contain a DNA-binding domain and are transcriptional regulators of a diverse set of genes. The MYB-MYC interaction domain is highly conserved, and any alteration to its structure impairs creation of the cooperative transcriptional complex (Zimmermann et al., 2004).

1.2 The Salicylate Signaling Pathway

A second subsystem that governs plant immunity is the salicylic acid signaling system. Salicylic acid regulates genes that govern the plant’s response to biotrophic pathogens, such as viruses, that rely on living tissue. Plant perception of a putative biotrophic molecular pattern or effector causes an increase in intra- and extra-cellular salicylic acid. This alters the redox state of the cytosol via activation of various enzymatic intermediaries, causing disulfide bonds in NPR1 polymers to become reduced. This releases NPR1 monomers, which can enter the nucleus and activate transcription via binding to TGA-containing transcription factors, promoting the expression of biotroph-response genes (Zhou et al., 2015).

The key protein class in salicylate signaling is NPR. This class of proteins was originally discovered to mediate the beginning of plant systemic acquired resistance (SAR). Structurally, the conserved interaction domain is known to contain ankyrin repeats (Cao et al., 1997). NPR1 interacts with TGA transcription factors in order to activate SAR genes (Després et al., 2000). In order for this interaction to occur at significant levels, salicylic acid must be present (Fan & Dong, 2002). NPR1 binds cooperatively to salicylate and copper, causing oligomers of NPR1 to dissociate, allowing NPR1 to enter the nucleus, bind TGA2, and initiate gene activation (Y. Wu et al., 2012).
1.3 Jasmonate-salicylate signaling antagonism

The salicylic acid and jasmonic acid pathways can be antagonistic, although this antagonism is both concentration- and tissue-dependent. Pathogenic micro-organisms may intentionally release effectors to stimulate one of these pathways, causing suppression of the other pathway and increasing pathogen fitness. For example, the biotrophic pathogen *Pseudomonas syringae* releases coronatine, a molecular mimic of JA-Ile that causes rapid degradation of JAZ. This activates the JA pathway, resulting in suppression of the SA biotroph-response pathway and allowing bacteria to proliferate (Yang et al., 2015).

Strategies for dealing with biotrophic and necrotrophic pathogens are often diametrically opposed. Killing a cell infected with a (biotrophic) virus can prevent its replication via cellular machinery but would provide a necrotroph with an uncontested source of nutrients. Due to this antagonism, plants should theoretically be highly vulnerable to secondary infections by an alternate pathogen type. However, this isn’t observed categorically in nature - crop plants often deal with multiple challenge types at once, without a dramatic susceptibility increase. There must be a plant system that prevents this alteration, likely by degrading suppressor proteins in a time-dependent manner.

1.4 Plant U-box (PUB) proteins 25 and 26

PUB25 and PUB26 are part of a class of proteins named for their binding domain - the U-box. U-box containing proteins are known to facilitate ubiquitylation interactions. In ubiquitylation, a ubiquitin (Ub) residue is activated by an E1 ubiquitin-activating enzyme. This activated Ub is then transferred to an E2 ubiquitin-conjugating enzyme. Specificity between the E2 protein and protein targeted for ubiquitylation is granted by a class of proteins known as E3 ubiquitin ligases, a class which includes PUB, or plant U-box proteins (Ichimura et al., 2010). The E3 protein catalyzes the transfer of the activated Ub residue from the E2 protein to the substrate. This allows an organism to maintain a relatively small number of E1 and E2 enzymes, while using an array of E3 ligases to provide the necessary specificity. As is common with many types of covalent protein modification, ubiquitylation can alter protein fate in a variety of ways. Mono-ubiquitylation can facilitate protein-protein interactions, while poly-ubiquitylation has many possible outcomes dependent on which residues are involved. One of the most well-known pathways
controlled by poly-ubiquitylation is degradation at the 26S proteasome (Passmore, 2004).

PUB25/26 interact with the cytoplasmic kinase Botrytis-induced kinase 1 (BIK1), marking it for degradation. This decreases the ability of pathogen recognition receptors to transduce intracellular signals, decreasing immune response. PUB25/26 activity is increased by Calcium-dependent protein kinase 28 (CPK28) and decreased by G-protein-coupled receptors (GPCRs), allowing BIK1 to be stabilized or degraded as necessary (Wang et al., 2018). Increased PUB25/26 activity degrades BIK1 and represses pseudo-response regulator (PRR) response, which is similar to the known function of other PUB proteins, such as PUB22, 23, and 24 (Couto & Zipfel, 2016).

Previously, proteins with separate cytoplasmic and nuclear functions have been identified in *Arabidopsis thaliana* (G. Wu & Spalding, 2007). The goal of this project was identification of the nuclear function of PUB25 and PUB26, how this modified the stability of JAZ proteins, and the effect of PUB25/26 on gene expression.

2 Materials and methods

2.1 Plants and mutant lines

*Arabidopsis thaliana* and *Nicotiana tabacum* plants were both used in this study, with the great majority of experiments being performed on either 30-day-old *A. thaliana* grown in soil-containing pots or 10-day-old *A. thaliana* seedlings cultured in liquid-medium well plates. All *A. thaliana* lines were of Columbia-0 (Col-0) background. *Arabidopsis* germplasms most frequently used in this project were JAZ1, JAZ9, PUB25, and PUB26 overexpression lines, as well as the knockout mutants *pub25-1*, *pub26-1*, *pub25-1/pub25-1* (abbreviated as *pub25/26*), *coi1*, *coi1/pub25-1/pub26-1* (abbreviated as *coi-t*), *npr1*, and *npr1/pub25-1/pub26-1* (abbreviated as *npr-t*). JAZ1 and JAZ9 overexpression lines were also crossed with inducible PUB25 or PUB26 transgenic plants.
2.2 Growth conditions

Plants used at 30 days of growth were grown in either 2- or 1.5-inch square pots filled with soil. The 2-inch square pots used in infection assays were filled with soil past the top of the pot and then covered in Phiferglass mesh before seeding, preventing plant leaves from directly contacting the soil. After pots were filled with soil, seeds were sown onto the pots in a suspension of 0.1% agarose and moved into an unlit cold room (4 °C) for 3 days before being transferred to the growth chamber. After the seedlings were visually observed to have germinated and reach an appropriate size, all but one seedling was removed from each group. Flats were watered each Monday and Thursday at 10 am. Plants used at 10 days of growth were individually sown onto half-strength 2-morpholine-4-ethanesulfonic acid (MES)-buffered Murashige and Skoog medium with 1% sucrose (1/2 MMSS) agar plates, moved to the cold room for 3 days, then moved to the growth chamber. After 3 days in the growth chamber, four seedlings were transferred into each well of 24-well plates which contained 1 mL of liquid 1/2 MMSS. All plants were grown in a chamber at 26% relative humidity, 22 °C, and daytime irradiance of 130µmol photons m$^{-2}$s$^{-1}$ with a 12-hour light / 12-hour dark photoperiod.

2.3 Preparation of bacterial cultures for inoculation

Two bacterial pathogens were used in infection assays: *Pseudomonas syringae* pv. *tomato* (*Pst*) and *Pectobacterium carotovora* subsp. *carotovora* (*Pcc*). Bacterial pathogens were streaked out onto LM-agar plates from glycerol stocks and then transferred to liquid LM medium in sterile Erlenmeyer flasks the day before inoculation, incubating for 16 hours at 30 °C on a shaker platform. After incubation, total medium from the flasks was transferred to centrifuge tubes and spun down, with the bacterial pellet being re-suspended in 10mM MgCl$_2$ for *P. syringae* and 50mM NaCl for *P. carotovorum*. Spectrometry was used to determine the optical density at 600 nm (OD$_{600}$) of bacterial suspension, and the OD$_{600}$ was standardized to a level appropriate for the desired assay (Mittal & Davis, 1995).
2.4 *P. syringae* inoculation

Two strains of *P. syringae* were used in the infection assays: wild-type strain DC3000 and the coronatine-deficient mutant DC3118. Two methods of *P. syringae* inoculation were used: dipping and hand infiltration. In dipping inoculation, the OD of the *P. syringae* culture in 20 mM MgCl₂ was standardized to 0.1, roughly equivalent to 10⁸ CFU/mL. Large, mesh-covered pots, each containing 4 mature *A. thaliana* plants, were inverted and dipped into the bacterial solution for 15 seconds, then immediately placed into a covered, high-humidity flat. Hand infiltration proceeded in a similar manner, except that lower ODs were used, ranging from 0.0001 (10⁵ CFU/mL) to 0.01 (10⁷ CFU/mL) and bacterial suspension was directly inoculated into the underside of the leaves using a flat-tipped 5 mL syringe. In both cases, infection was allowed to proceed for 2-3 days before bacterial enumeration techniques were applied, dependent on the visible symptoms of disease in the plant (Yu et al., 1993).

2.5 *P. carotovorum* inoculation

Six strains of *P. carotovorum* were obtained from Dr. Amy Charkowski at Colorado State University and assessed for virulence. The most aggressive strain, WPP14, was identified and used in all subsequent assays. Various ODs of *P. carotovorum* in 50 mM NaCl were used, with the most common being 0.01, which was determined to be roughly 2 \( \times \) 10⁷ CFU/mL. During inoculation, 2-4 mesh-covered pots, each containing 4 mature plants, were placed into an autoclave bin with a small amount of water in the bottom. A dissecting needle was used to make a small hole in 4 leaves of each plant and 5 µL of bacterial suspension was inoculated directly onto the hole. After all plants were inoculated, clear plastic wrap was used to seal the bin, with 2 small holes created in the corners of the wrap to prevent ethylene buildup (Po-Wen et al., 2013). After 1-2 days, dependent on visible symptoms of infection, percent of surface area affected by pathogen was assessed. If no symptoms were visible on a leaf, it was given a score of 0. 1-25% of leaf surface area infected resulted in a score of 1, 25-50% resulted in a score of 2, 50-75% resulted in a score of 3, and 75% or more of leaf surface area infected resulted in a score of 4 (Rigault et al., 2017). After the leaves were scored, bacterial enumeration was utilized.
2.6 Bacterial enumeration

In order to assess bacterial growth, bacterial enumeration methods were applied. Leaves that had been marked prior to bacterial inoculation were collected. A hole-punch was used to remove a circular portion of the leaf 1 cm in diameter, with 2 discs each being placed into a 1.5-ml centrifuge tube in 100µL of water. A plastic drillbit was used to grind these discs until the solution appeared homogenous, with the drillbit being removed and 900µL of water being added to the tube before brief vortexing. The ground and mixed solution was serially diluted. These dilutions were plated in triplicate onto LM-agar plates, which were then placed into an incubator. Once bacterial colonies were visible under light microscopy, the colonies were counted and the CFU/cm² of leaf was calculated (Katagiri et al., 2002).

2.7 Seedling treatment and protein analysis

10-day-old seedlings in 24-well plates were subjected to a number of treatments including various concentrations of salicylic acid, jasmonic acid, cycloheximide, plant protease inhibitor, beta-estradiol, and abscisic acid. Treatments were administered in a sterile hood and the well plates were placed onto a shaker for 1-24 hours with samples collected periodically. Seedlings from each well were collected as one sample and flash-frozen in 1.5-mL grinding tubes using liquid nitrogen. Protein extraction buffer used for the experiments contained 50 mM Tris-HCL pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM DTT, 1% protease inhibitor, and 50µM MG-132. Samples were retrieved directly from the liquid nitrogen and 80µL of protein extraction mix was immediately added before grinding of the samples using plastic drillbits. After grinding, the samples were briefly vortexed and placed on ice. After grinding of all samples, a centrifuge at 4 °C was used to spin down the samples for 30 minutes at 13,000 rpm. 42µL of the supernatant was transferred to a new 1.5-mL centrifuge tube, and 1µL was used in an RC/DC protein light spectrometry assay with 2 technical replicates in order to determine protein concentration. Protein concentration was standardized across all samples using water and 4x SDS sampling buffer. 10µL of these standardized samples were used for SDS-PAGE followed by Western Blot analysis (Wawer et al., 2010).
2.8 RNAseq analysis

Samples for RNAseq were extracted using the protocol found in Huot et al. (2017). Four samples from wild-type Col-0 plants and 4 samples from the pub25/26 mutant were sequenced at Michigan State University’s RTSF facility using Illumina sequencing. The resulting FASTQ files were cleaned of contaminants and aligned to representative A. thaliana transcripts from Araport generated by the R module BioPer1. Expression analysis was performed using the edgeR and limma packages in R (Ritchie et al 2015; Robinson et al 2010). Genes with fewer than 10 counts per million in at least 3 of the samples were removed from analysis. Expression data were normalized using the TMM method and corrected for heteroscedasticity using the voom function (Law et al 2014). Moderated t-statistics were calculated, and only genes with log-fold change greater than 1.1 and adjusted p-values less than 0.05 were considered differentially expressed. The results were written to a table showing RPKM for each of the 8 total samples. These results were subjected to a PANTHER over-representation test using the Gene Ontology Database for A. thaliana (Mi et al 2013). Fold enrichment of genes over standard values was calculated in order to determine which plant responses were most altered.

2.9 Alternaria brassicicola and Botrytis cinerea inoculation and evaluation of infection

A. alternata and B. cinerea were cultured on 1/2 potato dextrose medium. B. cinerea was subjected to a cycle of ultraviolet light as described in Tan & Epton (1973) in order to induce sporulation. Total spores from fungal plates were extracted using 20% Tween 20 and filtered with cheese cloth. Samples were spun down for 10 minutes at 6000 rpm and re-suspended in 0.1% agarose. Light microscopy was used to calculate the spore concentration and additional agarose was added to standardize the sample to $10^4$ spores/mL. Five microliters of standardized spore solution was added to 4 leaves per plant of small-pot flats, with each pot containing one plant. Flats were covered and placed in a high-humidity condition for 2-3 days, with the spread of fungal hyphae closely monitored. When any leaves were noted to be close to 100% infected, all inoculated leaves on every plant were detached and scanned. ImageJ pixel blockout was used to calculate the spread of fungus on each detached leaf leaf (Ferrari et al., 2003).
2.10 qPCR

Entire-seedling samples were taken from soil-containing pots 2 weeks after they had been placed in the growth chamber. Seedlings were flash-frozen with liquid nitrogen. High velocity bead beating with the PowerLyzer 24 Homogenizer followed by usage of Qiagen RNeasy Mini Kit was used for RNA extraction. cDNA was synthesized according to the procedure in Huot et al. (2017) and 1.5 ng of cDNA was used for qPCR.

3 Results

3.1 JAZ1 is not stable

To assay JAZ stability, HA-tagged JAZ1 (HA-JAZ1) overexpressing seedlings were treated with both a mock and cycloheximide condition (Figure 1). In the mock condition, JAZ1 levels remained constant. With the cycloheximide treatment, new protein synthesis was inhibited and a decrease in JAZ1 levels was noted. By the one-hour mark, most JAZ1 was gone. A stable level of JAZ1 in the mock condition contrasted with a rapidly-decreasing level of JAZ1 in cycloheximide-treated samples indicates that JAZ1 is undergoing basal turnover. COI1 is the first candidate for the mechanism of basal turnover, as it has already been categorized as an E3 ubiquitin ligase with affinity for JAZ1.

![Figure 1: JAZ1 is degraded rapidly during cycloheximide treatment.](image)

10-day-old seedlings were treated with 0.2% DMSO (mock) or 200 µg/ml cycloheximide (CHX) for 6 hours; 35 µg of total proteins were used for SDS/PAGE electrophoresis followed by Western blot analysis.
3.2 Removal of COI1 does not block all degradation of JAZ proteins

In order to determine the contribution of COI1 to the observed basal turnover of JAZ proteins, similar experiment was performed with the addition of a coi1 mutant (Figure 2). In the coi1-30 line, addition of cycloheximide still results in a significant decrease in JAZ1 after 6 hours. It is unsurprising that removal of COI1 increases stability of JAZ1, but the continued degradation of JAZ1 indicates that another mechanism is still involved in JAZ1 turnover.

Figure 2: JAZ1 is rapidly degraded in coi1-30. 10-day-old seedlings were treated with 0.2% DMSO (M, mock), 10µM methyl jasmonate (J), and 200µg/ml cycloheximide (C) for 6 hours; 35µg of total proteins were used for SDS/PAGE electrophoresis followed by Western blot analysis

3.3 Basal turnover is mediated by 26S proteasome

A third seedling treatment experiment was performed in order to determine the mechanism of COI1-independent JAZ1 degradation (Figure 3). Two treatments were added in combination with cycloheximide to determine their effect on JAZ1 degradation. Treating the seedlings with MG132, a 26S proteasome inhibitor, blocked a significant portion of JAZ1 degradation. However, treating the seedlings with plant protease inhibitor (PI) failed to cause noticeable inhibition of JAZ1 degradation. This indicates that the agent of JAZ protein basal turnover is targeting the proteins to the 26S proteasome, and is not a protease itself.
3.4 PUB25/26 interact with multiple JAZ proteins in yeast

To determine what protein could be causing turnover of JAZ, besides COI1, yeast-2-hybrid screening of JAZ-interacting proteins was performed (Figure 4). PUB25 and PUB26 both displayed strong binding to a variety of JAZ proteins.

3.5 PUB25/26 interact with JAZ in plants

Transient co-expression of CFP-tagged PUB25/26 in the leaves of *N. tabacum* along with HA-tagged JAZ9 was performed. The leaves were sampled and co-immunoprecipitation was performed (Figure 5).
Pulldown with alpha-GFP antibody yielded JAZ protein, and pulldown with alpha-HA antibody yielded PUB25/26 protein. This indicates that PUB25/26 interact with JAZ9 in plant.

![Figure 5: Both PUB25 and PUB26 co-immunoprecipitate with JAZ9. PUB25, PUB26, and JAZ9 were transiently co-expressed in the leaves of a tobacco plant. After induction of the proteins, co-IP with antibodies targeting known protein tags was performed, followed by SDS/PAGE electrophoresis and Western blot analysis.](image)

3.6 PUB25/26 and JAZ both localize to the nucleus

Localization of PUB25/26 and JAZ9 was assayed via fluorescent tagging and microscopy (Figure 6). Both PUB25 and PUB26 localized to the nucleus, and so did JAZ9. JAZ must be degraded in the nucleus in order for its effects to be alleviated. PUB25/26 localization to the nucleus - along with yeast-2-hybrid and co-IP interaction data - makes it an excellent candidate for JAZ degradation.

3.7 PUB26 poly-ubiquitylates JAZ9

In order to assay ubiquitylation activity between PUB26 and JAZ9, the necessary components of the ubiquitylation system were combined in vitro. Modification of JAZ will alter its molecular weight, changing the position of the band on the gel. Mono-ubiquitylation will result in two distinct bands - modified and unmodified - while poly-ubiquitylation will result in a smeared band. Figure 7 displays the results...
Figure 6: PUB25, PUB26, and JAZ9 each localize to the nucleus. Localization of fluorescent-tagged PUB25/26 and JAZ9, expressed in mutant A. thaliana and viewed via confocal microscopy.

of a ubiquitylation assay; the result from each lane is described below.

Lane 1) Combination of E2, PUB26, and JAZ9 did not result in any ubiquitylation, as indicated by no signal on the UBQ blot and no smearing of the JAZ9 band.

Lane 2) Combination of E1, PUB26, and JAZ9 did not result in ubiquitylation

Lane 3) Combination of E1, E2, and JAZ9 did not result in ubiquitylation

Lane 4) Combination of E2, PUB26, and MBP did not result in ubiquitylation

Lane 5) Combination of E1, E2, PUB26 and JAZ9 resulted in a smear of the JAZ9 band, indicating modification is occurring. The UBQ blot indicates that the modification is poly-ubiquitylation

The results shown in Figure 7 indicate that the PUB protein is able to ubiquitylate JAZ, and acts as part of the E1-E2-E3 ubiquitylation system. If PUB25/26 is responsible for basal turnover of JAZ, pub25/26 double mutants should display decreased basal turnover. This should stabilize JAZ, decreasing JA-sensitive gene expression. SA-JA pathway antagonism should then raise resistance to biotrophic pathogens.
3.8 Induction of PUB26 decreases JAZ9

JAZ9 overexpression mutants were combined with PUB26-inducible mutants to produce seedlings that could be used to assess the effect of artificially increasing PUB26 on JAZ9 levels. 10-day-old seedlings were treated with a mock condition and an inducing agent (β-estradiol) and harvested after 6 hours of exposure. Total protein was collected via previously-outlined methods and used for SDS/PAGE and Western Blot analysis. Induction of PUB26 resulted in a marked decrease in JAZ9, as shown in Figure 8. Two different mutant lines were used, 1T2 and 2T. Figure 8 shows the results of induction in both lines. The inverse correlation between PUB26 and JAZ9 levels indicates that PUB26 facilitates JAZ9 degradation.

3.9 pub25/26 is less susceptible to *P. syringae*

To assay the role of PUB25/26 in resistance, pub25/26 double mutant plants were used. A bacterial infection assay comparing pub25/26 and wild-type yielded the results depicted in Figure 9. The hemibiotrophic pathogen *P. syringae* was utilized in both a dipping and direct-infiltration assay. DC3000 dipping inoculation was used to compare susceptibility between wild-type and mutant plants; the results are in Figure 9a. The reduced susceptibility of pub25/26 mutant to *P. syringae* wild-type (DC3000) in-
Figure 9: pub25/26 mutant plant contains less DC3000 or DC3118 P. syringae than wild-type at 3 dpi. 30-day-old A. thaliana were dipped into OD 0.1 (10^8 density) solutions of DC3000 or DC3118 P. syringae. Three days post-inoculation, 1 cm disks of pre-marked leaves were sampled and CFU measured via serial dilution and plating on selective media. Experiment was replicated three times.

Inoculated by dipping indicated that altered JAZ signaling may be present. Because wild-type P. syringae produces coronatine, this may confound assessment of resistance by altering COI-1 activity and stomatal opening (Brooks et al, 2005). Both of these possible confounding factors were circumvented by using the coronatine-deficient P. syringae strain DC3118, and by utilizing the previously-described hand-infiltration method of inoculation. Using this method, pub25/26 mutant plants still displayed decreased susceptibility to the biotrophic pathogen challenge, when compared to wild type (Figure 9b). This lines up with the concept of PUB25/26 as responsible for a portion of JAZ degradation. In order to determine how this increase in resistance to biotrophs was being mediated at a genetic level, transcriptome analysis was considered.
Figure 10: Immune response-related RNA frequencies are heavily modified in pub25/26 mutant plants. RNAseq frequency analysis of altered gene ontologies in a comparison between Col-0 and pub25/26

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Change</th>
<th>Keyword</th>
<th>Go Term</th>
</tr>
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<td>jasmonate-zim-domain protein 1</td>
<td>2.4</td>
<td>defense response to bacterium</td>
<td>42742</td>
</tr>
<tr>
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<td>2.2</td>
<td>defense response</td>
<td>6952</td>
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<td>AT1G18570 MYB51</td>
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<td>defense response to bacterium</td>
<td>42742</td>
</tr>
<tr>
<td>AT1G02450 NIM1</td>
<td>NIM1-interacting 1</td>
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<td>defense response to bacterium</td>
<td>42742</td>
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<tr>
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</tr>
<tr>
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<td>6950</td>
</tr>
<tr>
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<td>9620</td>
</tr>
<tr>
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<td>RING/U-box superfamily protein</td>
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</table>

Table 1: Example of differentially-regulated genes in comparative transcriptome analysis between Col-0 and pub25/26. These genes all displayed statistically significant alteration between wild-type and mutant lines, and are related to jasmonic or salicylic acid response.

3.10 Comparative RNAseq of pub25/26 and Col-0

Eight samples, four from Col-0 and four from pub25/26 were sent to the Michigan State University Research Technology Support Facility. Sequencing data were obtained in FASTA format. Using the R modules limma, edgeR, and Rsubread, the data were aligned to specific mRNA sequences and subread count was determined in reads per kilobase per million mapped reads (RPKM) format. In order to increase the signal-to-noise ratio, a noise threshold was established and all genes with average RPKM below this threshold for error in both strains were removed. From this list of genes, alteration magnitude was calculated by comparing average RPKM between strains on a per-gene basis. This list was then sorted and each gene was weighted by post-sort location. Using this list, a frequency analysis of transcript alter-
3.11 Salicylic acid affects both JAZ and PUB protein concentrations

The effects of salicylic acid on JAZ and PUB proteins were initially assayed via seedling treatment (Figure 13). Mock treatment resulted in no significant alteration in JAZ9 levels. Salicylic acid treatment at 100 micromolar concentration showed a significant increase in JAZ9 at the 6-hour mark, followed by a return to normal levels.
3.12 pub25/26 loses protection from SA priming prior to B. cinerea challenge

As salicylic acid affected levels of JAZ protein in seedlings, an assay to determine the effect of salicylic acid on pathogen resistance was utilized. pub25/26 plants were included for comparison to wild type, to determine what portion of resistance was mediated by PUB25/26. The four groups of plants were: wild-type (SA-treated), wild-type (mock-treated), pub25/26 (SA-treated), pub25/26 (mock-treated). Twenty-four hours after spray treatment with 100µM salicylic acid, the necrotrophic fungal pathogen Botrytis cinerea was inoculated onto the leaves of A. thaliana plants and lesion size was measured three days post-inoculation. An average value for lesion size was obtained across each of the four plant groups. The average area for each SA-treated group was divided by the area for each mock-treated group, to determine how salicylic acid treatment altered susceptibility. In wild-type plants, SA treatment decreased susceptibility by roughly 20%, while in pub25/26 SA treatment did not affect susceptibility (Figure 14). If PUB25/26 play a key role in degradation of JAZ, this result indicates that their removal does prevent expression of JA-responsive genes.
## 3.13 PUB25 and PUB26 are not stable; stability is altered by salicylic acid

Stability of PUB25 was assayed via time-course cycloheximide treatment (Figure 14). PUB25 was found to degrade almost completely within 14 hours, despite displaying a fairly constant concentration across all times in the mock-treated condition. Treatment of a PUB25-FLAG overexpression mutant with DMSO and salicylic acid caused no alteration in PUB25 levels until 24 hours later, where a large increase was observed (Figure 15). PUB26 overexpression mutants displayed a similar pattern of degradation (Figure 16), indicating that both PUB25 and PUB26 undergo a constant basal level of turnover within the plant. However, PUB26 response to plant hormone treatment differed from PUB25 response. PUB26 displayed a decrease in degradation immediately in response to salicylic acid treatment, while PUB25 showed no response until a sudden spike in concentration at 24 hours. In addition, treatment of PUB26 overexpression mutants with jasmonic acid appeared to increase stability of PUB26 across a 24 hour period, preventing circadian alterations in protein concentration. These results indicate that both PUB25 and PUB26 are able to vary in concentration rapidly, and this variation is altered by the presence of plant hormones associated with pathogen-reponse.

**Figure 14:** PUB25 is rapidly degraded during cycloheximide treatment 10-day-old seedlings were treated with 250μM CHX or DMSO. 40μg of total protein was used for SDS/PAGE followed by Western Blot analysis.

**Figure 15:** PUB25 is increased by 24 hours of salicylic acid treatment 10-day-old seedlings were treated with 250μM CHX or DMSO. 40μg of total protein was used for SDS/PAGE followed by Western Blot analysis.

**Figure 16:** PUB26 is rapidly degraded during cycloheximide treatment and increased by salicylic acid 10-day-old seedlings were treated with 250μM CHX or DMSO. 40μg of total protein was used for SDS/PAGE followed by Western Blot analysis.
3.14 **pub26-1** is more susceptible to *P. carotovorum* compared to wild type as assayed by lesion counting

30-day-old plants were inoculated with $10^6$ CFU/ml *P. carotovorum* in 100mM NaCl. 4 leaves per plant were inoculated with 4 plants being used for the mutant and the wild-type condition, resulting in 16 lesions developing. The previously-described lesion assessment scale was used to determine what portion of the leaf was affected by the pathogen and the results are shown in Figure 18. The wild-type primarily displayed lesions that affected less than 25% of the surface area of the leaf (counted as 1). More than 60% of lesions present on the *pub26-1* single mutant scored above 1 on assessment scale, showing that deficiency of the PUB26 protein affects the visible spread of necrotrophic pathogens.

![Figure 17: pub26-1 displays larger lesions 24 hpi than wild-type. Wild-type and mutant plants were both inoculated with *P. carotovorum*. Lesion size was calculated the next day and a lesion-counting scheme applied to determine relative susceptibility.](image)

![Figure 18: Knockout of PUB25/26 increases visible *P. carotovorum* lesion area. Puncture inoculation was used on 4 *A. thaliana* lines; Col-0, compared to single- and double- knockout lines of PUB25/26. All knockout lines visibly lesioned more rapidly.](image)

3.15 **pub25, pub26,** and **pub25/26** all display increased leaf lesion size when inoculated with *P. carotovorum*

Wild-type (Columbia-0) *A. thaliana*, as well as *pub25-1, pub26-1,* and *pub25-1/pub26-1* were inoculated with *P. carotovorum* at an OD of 0.01, using the procedure described previously. 24 hours-post-inoculation, leaves were collected from each of the represented plant groups; the results are shown in Figure 19. Both
single mutants displayed much larger necrotic lesions than the wild type, and the double mutant pub25/26 displayed the largest lesion, as a percentage of leaf surface area. No inoculated leaves in the wild-type A. thaliana scored more than a 1 on the evaluation scale for infection, as all lesions had spread to less than 25% of the total leaf surface area.

Figure 19: pub25/26 displays greater disease index than wild-type visibly and by bacterial enumeration. 30-day-old wild-type Columbia-0 and pub25/26 were infected with P. carotovorum via direct puncture inoculation at $2 \times 10^7$ CFU/ml. All measures were taken at 24hpi. A) Visible symptoms of nectroph infection. B) Lesion assessment. C) Average disease index from assessment of visible damage. D) Results of bacterial enumeration.

3.16 pub25/26 is more susceptible than wild-type to high-OD P. carotovorum

If PUB25 and PUB26 do play an instrumental role in degrading JAZ proteins, removal of both in the pub25/26 double mutant should increase JAZ. This should lead to a corresponding drop in plant im-
munity to necrotrophic pathogens at a correct concentration. Both wild-type and pub25/26 plants were inoculated with *P. carotovorum* at OD 0.01, equivalent to $2 \times 10^7$ CFU/ml. A small puncture wound was created on 4 leaves per plant, and *P. carotovorum* was inoculated and evaluated using procedures previously outlined. 4 separate measures showed that pub25/26 displays a higher baseline level of susceptibility to *P. carotovorum* than wild-type. Figure 20A shows that pub25/26 displays visibly larger necrotic lesions than wild-type at 24hpi. Figure 20B shows that, while all leaves inoculated on wild-type *A. thaliana* showed establishment of bacterial presence, all leaves displayed visibly lesioned surface area of less than 25%. In the same graph, pub25/26 shows a significant number of entire-leaf lesions, as well as more than half of all established colonies causing more than 25% visibly lesioned surface area. Figure 20C shows that the average disease index of the mutant plant is more than twice that of the wild-type. Figure 20D verifies the results of the visible-assessment assays by showing that the difference in bacterial number between wild-type and mutant is roughly equivalent to 1.5-log.

### 4 Discussion

#### 4.1 JAZ proteins are targeted to the proteasome by a non-COI1-dependent pathway

Although the current understanding of jasmonate signaling involves a complex of COI1, JAZ, and inositol pentakisphosphate (Sheard et al., 2010), removal of COI1 only reduces degradation of JAZ proteins. Blocking protease function in coi1 mutant plant does not prevent additional degradation, but does prevent proteasome degradation. JAZ proteins must be being degraded at the proteasome, and are being targeted for degradation by a protein other than COI1.

#### 4.2 PUB25 and PUB26 are likely candidates for targeting JAZ proteins to the proteasome

E3 ubiquitin ligases are a likely candidate class of enzymes that can target JAZ proteins to the proteasome. Due to the ability of E3 ligases to act as a target-specific adaptor in coordination with E1 and
E2 proteins (Ardley & Robinson, 2005), any putative E3 ligase that binds JAZ proteins with high affinity can cause degradation. Screening this class of proteins via yeast-2-hybrid resulted in the identification of PUB25 and PUB26 as E3 ligases that can bind a number of different JAZ proteins with a high affinity \textit{in vitro}. This was confirmed by co-immunoprecipitation using antibodies against JAZ9 and then antibodies against PUB25 and PUB26. \textit{In vivo} localization data shows that JAZ9, PUB25, and PUB26 all localize to the nucleus, meaning that interaction in a full plant system is plausible. The ability of PUB26 to polyubiquitylate JAZ9 in conjunction with E1, E2, and free ubiquitin was also confirmed. Both PUB25 and PUB26 are excellent candidates for instigators of JAZ degradation by protein class, binding activity, localization, and function.

![Figure 20: Proposed model for the function of PUB25/26 in regulating the cross-talk of JA-SA signaling.](image)

4.3 PUB25 and PUB26 are instrumental in both biotroph- and necrotroph-response pathways

The activity of JAZ proteins in defense pathways against both necrotrophic and biotrophic pathways has been well-studied. JA-response pathways are activated by removal of JAZ, increasing efficacy of plant defense against necrotrophic pathogens (Pieterse et al., 2014). The antagonistic SA-response pathway for defense against biotrophic pathogens increases JAZ levels, upregulating anti-biotroph plant de-
fenses (Kazan & Manners, 2012). The pub25/26 double mutant shows both increased susceptibility to necrotrophic pathogens and decreased susceptibility to biotrophic pathogens. This phenotype is similar to the coi1 mutant plant (Feys et al., 1994), indicating that a similar alteration of JAZ response occurs in both. In addition, removal of PUB25 and PUB26 alters the transcriptome of *A. thaliana* in a manner similar to removal of COI1 (Devoto et al., 2005). Therefore, PUB25 and PUB26 are both instrumental in regulation of both biotroph- and necrotroph-response pathways at the molecular-genetic level (Figure 21).

### 4.4 PUB25 and PUB26 are part of the salicylic acid signaling pathway

The downstream function of PUB25 and PUB26 is JAZ degradation via proteasomal targeting, but the upstream activators of these proteins are not known. However, RNAseq data indicates that many genes associated with salicylic acid response (Glazebrook, 2001) are significantly altered in pub25/26. The finding that salicylic acid at 100µM concentration can affect both JAZ and PUB protein levels indicates that these proteins are downstream of a biotroph-response pathway. Response to salicylic acid in alteration of defense response was found to be absent in pub25/26. Given the antagonistic nature of the salicylate and jasmonate signaling pathways and the loss of salicylate response in knockout plants, it is likely that PUB25 and PUB26 are a part of the protein interface between different defense pathways.

### 4.5 Future experiments

Although this study has characterized the effects of PUB25 and PUB26 on pathogen response, transcriptome, and protein concentrations, many more questions remain to be answered. If PUB25 and PUB26 are downstream of salicylic acid signaling, the mechanism of signal transduction remains unknown. Effects on water retention, dessication recovery, and germination indicate that pub25/26 mutant plants also display altered abscisate signaling (Giraudat, 1995). How these proteins affect the interaction between the jasmonate, salicylate, and abscisate pathways, and the molecular mechanism of this activity, may be determined in future studies.
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