Small Ubiquitin-like Modifier protein, SUMO regulates Jasmonic acid signalling by suppressing the activity of the Jasmonic acid receptor, CORONATINE INSENSITIVE 1, COI1.

Anjil Kumar Srivastava[1], Beatriz Orosa[1], Prashant Singh[2], Ian Cummins[1], Charlotte Walsh[1], Cunjin Zhang[1], Murray Grant[3], Michael R Roberts[2], Ganesh Srinivasan[4], Elaine Fitches[1*], Ari Sadanandom[1*]

Affiliations

[1]School of Biological and Biomedical Sciences, University of Durham, Durham, DH1 3LE, United Kingdom

[2]Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, United Kingdom

[3]Geoffrey Pope Building, Biosciences, College of Life and Environmental Sciences, University of Exeter, Stocker Road, Exeter EX4 4QD, UK

[4]Department of Biological Sciences, National University of Singapore, 14 Science Drive, Singapore 117543.

[*]Corresponding Authors
Elaine Fitches and Ari Sadanandom
Department of Biosciences,
University of Durham, Durham,
DH1 3LE, United Kingdom
Phone: +441913341263
Fax: +441913342000
Email: E.C.Fitches@durham.ac.uk; ari.sadanandom@durham.ac.uk

ABSTRACT

The sessile nature of plants dictates that they respond rapidly to sudden environmental cues often prior to changes in hormone levels that coordinate these responses. How this is achieved is not fully understood. The integrative role of the phytohormone Jasmonic Acid (JA) is reliant upon the plant's ability to control the levels of JASMONATE ZIM (JAZ) domain containing signalling repressor proteins. Here, we demonstrate that regardless of intrinsic JA levels, SUMO conjugated JAZ proteins inhibit the JA receptor COI1, from mediating non-SUMOylated JAZ degradation. SUMO deconjugating proteases, OTS1 and OTS2 regulate JAZ protein SUMOylation and stability. *ots1 ots2* double mutants accumulate both SUMOylated and non-SUMOylated JAZ repressor proteins but show no change in endogenous JA levels compared to wildtype plants. SUMO1 conjugated JAZ proteins bind to COI1 independently of the JA mimic coronatine. SUMO inhibits JAZ binding to COI1. We identify the SUMO interacting motif (SIM) in COI1 and demonstrate that this is vital to SUMO dependant COI1 inhibition. Necrotroph infection of Arabidopsis promotes SUMO protease degradation and this increases JAZ SUMOylation and abundance, which in turn inhibits JA signalling. This study reveals a mechanism for rapidly regulating JA responses allowing plants to adapt to environmental cues.

INTRODUCTION

The sessile nature of plants dictates that growth must be integrated with changes in the natural environment. Modulation of hormone signalling pathways plays a key role in this process. JA regulates a wide spectrum of plant growth, developmental and defence responses to pathogen attack. In this context JA is a major coordinator of both constitutive developmental processes and in defence responses activated upon pathogen invasion. Conjugation of JA to the amino acid L-isoleucine produces the bioactive signal (3R,7S)-jasmonoyl-L-isoleucine (JA-Ile) (Fonseca et al., 2009). JA-Ile is structurally and functionally imitated by the phytotoxin Coronatine produced by the bacterial pathogen, *Pseudomonas syringae* (Feys et al., 1994). An important step in the elucidation of the jasmonate-signalling pathway was made with the discovery of the JA receptor COI1 that encodes an F-box protein acting as part of a Skip-Cullin-F-box E3 ubiquitin ligase complex, targeting proteins for proteasomal degradation (Xie et al., 1998). The JASMONATE ZIM DOMAIN (JAZ) family of transcriptional repressors are the target substrates that associate with COI1 in a hormone-dependent manner (Chini et al., 2007; Thines et al., 2007; Katsir et al.,

2008; Fonseca et al., 2009). JAZ repressors directly interact with and govern the activity of transcription factors that include the basic helix-loop-helix (bHLH) proteins MYC2, MYC3, and MYC4 that act redundantly to regulate a plethora of JA-mediated responses (Fernandez-Calvo et al., 2011; Pauwels and Goossens, 2011). In the absence of a JA-Ile signal, JAZ proteins actively repress JA responsive transcription factors. In response to environmental cues that up-regulate JA signalling, the hormone binds to COI1 and stimulates specific binding to JAZ proteins. This leads to poly-ubiquitination and subsequent proteasomal degradation of JAZ proteins. JAZ degradation relieves repression of JA responsive transcriptional regulation leading to physiological changes. The integrative role of JA is heavily reliant on the plant's ability to control JAZ protein levels; to date this has been demonstrated to be controlled through modulating levels of JA-Ile. However, the static nature of plants dictates that they must respond rapidly to changing environments and often prior to changes in *de novo* JA levels. How this is achieved in plants is largely unknown.

Several other ubiquitin-like proteins have been described in plants, including SUMO that can act to stabilize proteins to which it is conjugated (Conti et al., 2008). Synthesized as an inactive precursor, SUMO proteins are processed to their mature form by SUMO proteases that cleave the C-terminal tail from the precursor. This exposes a di-Glycine motif where target attachment occurs in a series of enzymatic reactions very similar to ubiquitination, that includes activation, conjugation and ligation (Jentsch and Pyrowolakis, 2000; Kerscher et al., 2006; Capili and Lima, 2007). To regulate the effects of SUMO-conjugated proteins, SUMOylation can also be reversed by SUMO specific proteases, which release SUMO from their substrates (Hay, 2001). SUMO proteases are crucial as they function in both maturation and de-conjugation. These two activities share a common catalytic mechanism, although the substrates differ in so much as maturation involves hydrolysis of an amino-linked peptide bond and de-conjugation catalyzes the hydrolysis of lysine-glycine isopeptide bonds (Reverter and Lima, 2009). So far only a few bona fide SUMO proteases have been characterized in Arabidopsis and rice (Reeves et al., 2002; Conti et al., 2008; Conti et al., 2014; Srivastava et al., 2016; Srivastava et al., 2017). Previously, we identified two SUMO proteases, Overly Tolerant to Salt 1 (OTS1) and OTS2 that are localized in the nucleus and act redundantly to regulate salt stress responses in Arabidopsis (Conti et al., 2008). OTS1/OTS2 regulate the abundance

of SUMO conjugates in a salt stress dependant manner and overexpressing OTS1 alone reduces salt induced SUMO conjugate accumulation and can rescue the *ots1 ots2* double mutant sensitivity to high salinity (Conti et al., 2008).

Once covalently conjugated, SUMO affects protein-protein interactions, subcellular localization and stability of target proteins (Hay, 2001; Verger et al., 2003). Furthermore, SUMO may facilitate new protein-protein interactions through SUMO-interacting motifs (SIMs) and compete with other post-translational modifications such as ubiquitination and acetylation (Kerscher et al., 2006; Hickey et al., 2012). Previously, we demonstrated that the sequestration of the GA receptor GID1 by SUMO-conjugated DELLAs leads to an accumulation of non-SUMOylated DELLAs by blocking their ubiquitination thereby enabling beneficial growth restraint during stress (Conti et al., 2014). Here, we demonstrate a role for SUMOylation in stabilising JAZ proteins by inhibiting COI1 from mediating JAZ repressor degradation. The SUMO protease OTS1 regulates JAZ protein stability. *ots1 ots2* double mutants accumulate both SUMOylated and non-SUMOylated JAZ repressor proteins but show no change in endogenous JA levels as compared to wildtype plants. SUMO1 conjugated JAZ proteins bind to COI1 independently of the JA mimic coronatine. SUMO1 inhibits JAZ binding to COI1. *Botrytis cinerea* infection of Arabidopsis promotes OTS1 SUMO protease degradation and consequently increases JAZ SUMOylation and abundance, inhibiting JA signalling. Our data reveals a new SUMO dependent attenuation mechanism for JA signalling in plants.

RESULTS

OTS SUMO proteases are susceptible to the fungal pathogen *Botrytis cinerea* and the arthropod herbivore spider mite, *Tetranychus urticae*

Previously, we demonstrated that the *ots1 ots2* double mutant displayed enhanced resistance to virulent *P. syringae pv* tomato *(Pst)* and accumulated higher levels of Salicylic Acid (SA) compared to wildtype (WT) plants (Bailey et al., 2016). Furthermore, *ots1 ots2* mutants exhibited up-regulated expression of the SA biosynthesis gene *ICS1* and enhanced SA responsive *PR1* expression as compared to WT. SA stimulates OTS1/2 degradation and promotes accumulation of SUMO1/2 conjugates. These results indicate that OTS1 and -2 acts in a feedback loop in SA signalling and *de novo* OTS1/2 synthesis works antagonistically to SA-promoted

degradation, thereby adjusting the abundance of the OTS1/2 to moderate SA signalling.

The SA and JA signalling pathways often act antagonistically (Glazebrook, 2005; Kazan and Manners, 2008; Koornneef and Pieterse, 2008; Pieterse, 2012). Although there are exceptions, generally it can be stated that pathogens with a predominantly biotrophic lifestyle are more sensitive to SA induced defenses, whereas JA activates defense against necrotrophic pathogens and herbivorous insects (Glazebrook, 2005; Howe and Jander, 2008). Since, *ots1 ots2* mutants were more resistant to *Pst* due to increased SA levels, we wanted to ascertain if they were more susceptible to a necrotrophic fungal pathogen *B. cinerea,* that causes grey mold disease (Mengiste, 2012) and an arthropod herbivore (red spider mite; *Tetranychus urticae*) where JA is known to play a key role.

We compared the susceptibility of wild-type and *ots1 ots2* double mutant and OTS1 overexpressing (OTS1-OE) (Bailey et al., 2016) plants to *B. cinerea*. Initially, we scored the size of the necrotic lesions on detached leaves to assess the resistance of WT and *ots1 ots2* mutants to *B. cinerea* after inoculating plants with drops of fungal spore suspension onto the upper epidermis of rosette leaves. As shown in Figure 1A-B, disease lesions on detached leaves from the *ots1 ots2* plants were significantly larger than that of WT, confirming that these SUMO proteases are required for resistance against *B. cinerea*. The previously established OTS1-OE line was also included in this analysis and showed no significant difference when compared to wild type. The severity of symptoms seen in *ots1 ots2* double mutants was also reflected in the increased fungal biomass as indicated by quantitative-RT PCR (qRT-PCR) data which showed that *ots1 ots2* mutants had about 3-fold more fungal DNA as compared to WT or OTS1-OE lines (Figure 1C). Spider mite fecundity assays indicated that the female mites laid significantly more eggs on *ots1 ots2* mutant plants compared to the WT and OTS1-OE transgenic lines (Figure 1D) suggesting reduced resistance to insect herbivory in *ots1 ots2* mutants. The lack of any observable phenotypic differences in disease development in the OTS1-OE lines maybe be attributed to the fact that, the levels of OTS1 expression in these lines may not be significant enough to yield a tangible difference in defence phenotypes compared to WT plants but they were able to complement the salt stress sensitivity phenotype in *ots1 ots2* mutants (Conti et al., 2008; Bailey et al., 2016).

OTS SUMO proteases regulate JA responses

JA is well known to inhibit root growth and this growth inhibitory effect has been exploited in many genetic screens for plants with altered JA sensitivity. To determine the effect of JA on *ots1 ots2* root growth, we grew seeds of WT and mutant plants in the presence of 10 µM JA and monitored root growth. Exogenous JA treatment caused significant root growth retardation in WT plants; however, this effect was reduced in *ots1 ots2* plants (Figure 2A-B). *ots1 ots2* mutants were at least 30% more resistant to JA mediated root growth inhibition as compared to WT implying that *ots1 ots2* mutants are less sensitive to JA. This observation was substantiated by qRT-PCR data that demonstrated suppression of expression of downstream target genes of JA mediated defence in *ots1 ots2* mutant background (Figure 2C-F). Taken together our data demonstrates that *ots1 ots2* mutants have hampered JA sensitivity and/or signalling. Hence, the *ots1 ots2* mutant reveals a novel link between SUMOylation and JA signalling.

JAZ proteins are hyperSUMOylated and stabilised in ots1 ots2 mutants

The 13 members within the JAZ repressor protein family collectively act by interacting with and regulating various transcription factors to influence diverse JA responses. This provides an overall canonical mechanism for JA signalling repression. However, individual JAZ repressors affect specific aspects of JA signalling (Kazan and Manners, 2008). JAZ6 and 5 are known to be active in JA mediated defence while JAZ1 and 2 are more important for root growth (Grunewald et al., 2009; Ingle et al., 2015). Since the *ots1 ots2* mutants display reduced sensitivity to JA in both defence and root growth we wanted to ascertain the impact of the *ots1 ots2* mutations on JAZ1 and JAZ6 protein abundance as proof of concept of the increased repression of the canonical mechanism for JA signalling in the *ots1 ots2* mutants. Immunoblot experiments with anti-GFP antibodies revealed that 35S promoter driven GFP-tagged JAZ1 (35S::JAZ1:GFP) and JAZ6 (35S::JAZ6:GFP) proteins were more abundant in the *ots1 ots2* mutant plants compared to wildtype (Figure 3A and Supplemental Figure 1). There was no significant difference in the transcript levels of both transgenes in either genetic backgrounds (Supplemental Figure 2). OTS1 and OTS2 are SUMO proteases capable of cleaving SUMO from target proteins and therefore we wanted to ascertain whether SUMOylation of JAZ proteins could provide a mechanism for stabilising JAZ proteins in the *ots1 ots2*

196 background. We immunopurified the Arabidopsis JAZ6:GFP protein using GFP
197 antibody-coated beads. Immunoblotting of GFP immunoprecipitates with Arabidopsis
198 SUMO1–specific antibodies indicated that JAZ6:GFP was conjugated to SUMO1
199 (Figure 3A, upper panel). We also observed a similar pattern of SUMOylation for
200 JAZ1:GFP (Supplemental Figure 1). This evidence indicated that the stability of JAZs
201 as well as the SUMOylation of JAZ proteins are enhanced in the *ots1 ots2*
202 background. The increased abundance of JAZ6:GFP and JAZ1:GFP levels were not
203 due to changes in JA levels as hormone measurements indicated that there was no
204 significant difference in JA-Ile levels between *ots1 ots2* mutants and WT (Figure 3B).
205 This suggests a direct link between JAZ SUMOylation and its stability, a mechanism
206 consistent with increased repression of the JA responses observed in these mutant
207 plants. Intriguingly the lack of any significant change in JA levels in the *ots1 ots2*
208 mutant plants indicate that this repression mechanism operates independently of
209 intrinsic JA levels.
210 To determine the site of SUMO conjugation on JAZ6 we exploited the bacterial
211 SUMO conjugation system (Okada et al., 2009) to purify higher order SUMO1-JAZ6
212 conjugates and subjected them to mass spectrometry analysis (Supplemental Figure
213 3). Trypsin cleavage of SUMO conjugated peptides leaves a 4-specific amino acid
214 (QTGG) footprint when the mass spectrometry adapted SUMO1 (Miller et al., 2010)
215 is used to conjugate to target proteins. The peptide carrying this unique mass
216 footprint can be manually identified from fragmented ion mass spectra of a target
217 protein. Using this method, we successfully identified lysine 221 in JAZ6 as a
218 SUMO1 attachment site (Figure 3C). To test the hypothesis that SUMOylation on
219 JAZ6K221 was responsible for the increased stability of JAZ6 we produced
220 transgenic plants ectopically expressing via the 35S promoter, mutagenized versions
221 of JAZ6 lacking the relevant SUMO attachment site (lysine to arginine mutation at
222 position 221, K to R) (*35S::JAZ6*^{K221R}*:GFP*) in the WT and *ots1 ots2* backgrounds.
223 Anti-GFP immunoblot analysis revealed that JAZ6 levels in the *ots1 ots2* genetic
224 background reverted to those levels seen in WT background (Figure 3D), even
225 though there was no significant difference in transcript levels of JAZ6 in either
226 backgrounds (Supplemental Figure 4). We also observed a drastic reduction in the
227 SUMOylation of JAZ6^{K221R}:GFP (Figure 3D, upper panel). These observations,
228 together with the finding that JAZ6 repressor accumulates in *ots1 ots2* background
229 indicate that SUMOylation of JAZ repressors modulates JA signalling.

SUMOylation of JAZ6 modulates the stability of JAZ repressor after JA treatment

We investigated the interaction of mutated JAZ6 (JAZ6^{K221}R) with COI1 using co-immunoprecipitation with anti-GFP beads of JAZ6:GFP *and JAZ6^{K221R}:GFP* with myc tagged COI1 (myc-COI1) in the *Nicotiana benthamiana* transient assay system. Immunoblotting with anti-GFP and anti-myc antibodies allowed us to ascertain that the SUMO site mutated variant of JAZ6 actively interacted with myc-COI1 in a coronatine (JA mimic) dependant manner and thus is still functional (Figure 4A). JAZ6 is degraded in the presence of JA (Chini et al., 2007) therefore, we wanted to exploit this assay to determine the stability kinetics of JAZ6 and JAZ6^{K221R} in the presence of JA. In a JAZ degradation time-course experiment we treated JAZ6:GFP and JAZ6^{K221R}:GFP seedlings with JA for varying periods of time and, as indicated in Figure 4B, JAZ6^{K221R}:GFP was more rapidly degraded and was undetectable after 15 minutes as compared to JAZ6:GFP under the same conditions. JA treatment also promotes the accumulation of OTS1 protein (30 % compared to control mock treatment as quantified by imageJ against RubisCO), indicating that de-SUMOylation of JAZ6 protein is enhanced within 30 minutes of JA treatment (Figure 4C). The increase in HA:OTS1 protein levels could be due to the down regulation of a potential ubiquitin E3 ligase that targets OTS1 for ubiquitin dependent proteasomal degradation. We have previously shown that OTS1 is degraded by salt and ABA treatment in a proteasome dependent manner (Conti et al., 2008, Srivastava et al., 2017). Therefore, it is likely that JA treatment triggers the down regulation of a yet undiscovered E3 ligase.

Collectively, our data indicates that SUMOylation at K221 in JAZ6 is critical for its stability. The accumulation of OTS1 protein after JA treatment, further supports the role of this SUMO protease as a regulator of JAZ-SUMOylation

SUMOylation of JAZ6 is enhanced during Botrytis infection

Our data indicate a novel link between SUMOylation and JAZ protein stability through the *ots1 ots2* SUMO proteases. We therefore hypothesized that JAZ protein SUMOylation and deSUMOylation may be a naturally occurring mechanism by which pathogens attenuate JA signaling in plants. To test this hypothesis, we challenged *35S::JAZ6:GFP* transgenic plants with the virulent bacteria *Pst* and the fungal

pathogen *B. cinerea* to investigate the status of JAZ6 SUMOylation. As shown in Supplemental Figure 5, JAZ6:GFP degradation begins to occur within 2 hours post bacterial inoculation with a concomitant decrease in JAZ6:GFP SUMO conjugation. In contrast, *B. cinerea* infection leads to the accumulation of higher levels of JAZ6:GFP after 24 hours and a striking increase in SUMOylated JAZ6:GFP levels (Figure 5A). This coincided with the degradation of OTS1 protein during *B. cinerea* infection (Figure 5B). However, JA treatment reduces SUMOylation of JAZ6:GFP with the concomitant reduction of total JAZ6:GFP protein to a similar level to that observed following *Pst* inoculation (Figure 5C). Co-immunoprecipitation experiments using *Agrobacterium* mediated transient assays in *N. benthamiana* demonstrated that the OTS1 SUMO protease formed a protein complex with JAZ6:GFP (Figure 5D) indicating that OTS1 SUMO protease deSUMOylates JAZ repressors in the absence of pathogen infection. Taken together our data indicate that necrotrophic pathogen attack leads to the degradation of the OTS1 SUMO protease that otherwise targets JAZ proteins for deSUMOylation. This leads to the accumulation of SUMOylated JAZ proteins resulting in the attenuation of JA mediated defence pathway. Since the JA pathway is vital for defence against *B. cinerea* we postulate that targeted degradation of OTS1 resulting in JAZ6 protein accumulation is part of *B. cinerea's* virulence strategy.

SUMO inhibits JA- receptor COI1 binding to JAZ6 protein

We have established that JAZ6:GFP protein is SUMOylated during *B. cinerea* infection and this leads to enhanced JAZ6:GFP stability. We also demonstrated that OTS SUMO proteases play a direct role in JAZ6:GFP protein stability by deSUMOylating JAZ6:GFP. We next investigated whether the SUMOylated JAZ6:GFP protein could interfere with the function of the JA receptor, COI1 F-box protein. Inspection of the Arabidopsis COI1 protein sequence revealed a conserved putative SUMO interaction motif (SIM) at its C-terminus (position 550-558 in Arabidopsis COI1; Figure 6A-B) which is also conserved in *Brassica napus* COI1. SIM motifs on proteins are specific consensus sequences that bind to SUMO and therefore mediate distinct protein-protein interactions (Minty et al., 2000). Depending on the ability to interact with the SIM containing proteins, SUMOylated proteins may influence cell functions (Hecker et al., 2006). These facts led us to hypothesize that SUMOylation of JAZ6 protein and the SIM in COI1 might have a significant role on

COI1-JAZ interaction that results in modulating JA signalling pathway. Furthermore, a structural model of COI1 (Sheard et al., 2010) developed using PyMOL Graphics software based on the resolved structures of COI1 and JAZ suggested that free SUMO and SUMOylated JAZ proteins can occupy the same interaction face as non-SUMOylated JAZ for COI1 binding. This suggests that there may be competition between SUMOylated and non-SUMOylated JAZ proteins for COI1 binding through the SIM motif (Figure 6A). To investigate the potential role of a SIM in COI1-JAZ6 interaction, we first used Glutathione S-transferase (GST) pull down assays to examine possible interactions between COI1 and SUMO1 proteins. In the first *in-vitro* binding experiment, COI1 was expressed in *Escherichia coli (E. coli)* as a GST fusion (GST:COI1) and immobilized on glutathione beads. SUMO1 (His:SUMO1) was expressed in *E. coli* and purified using nickel beads. Possible interaction between GST:COI1 and His:SUMO1 was examined by incubating the His-tagged SUMO1 protein with beads immobilized with GST:COI1 or GST-only. After extensive washing of unbound molecules, the bound SUMO1 was detected by immunoblotting using a monoclonal anti-His antibody. As shown in Figure 6C, His:SUMO1 was retained on the GST:COI1 beads but not on the GST control beads indicating that COI1 indeed possesses a *bona fide* SIM motif. To further validate the significance of COI1-SIM for SUMO1 binding, we mutated the core SIM amino acid residue, Valine at position 553 of COI1 to Alanine through site directed mutagenesis (GST:COI1^{V553A}) to potentially eliminate SUMO binding to COI1. GST:COI1^{V553A}, showed markedly decreased interaction with SUMO1 in comparison with its corresponding WT COI1 demonstrating the critical nature of the SIM motif in COI1 for SUMO1 binding (Figure 6D).

SUMOylated JAZ6 inhibits non-SUMOylated JAZ and COI1 interaction

Coronatine (COR) is a major high affinity analogue of JA-Ile (Katsir et al., 2008; Fonseca et al., 2009; Sheard et al., 2010) produced by pathogens to overcome SA-induced resistance (Brooks et al., 2004; 2005). Coronatine can mimic JA-Ile to relieve transcriptional repression of JA-responsive genes by promoting the interaction of the COI1 F-box protein with the JAZ transcriptional repressors. To test whether SUMO1 affected the interaction between COI1 and JAZ6 we performed *in vitro* GST pull-down assays between GST:COI1 and His:JAZ6 with increasing amounts of recombinant His:SUMO1. This experiment demonstrated that COR

dependent interaction of GST-COI1 with His:JAZ6 can be inhibited by His:SUMO1 (Figure 7A). However, the inhibitory effect of His:SUMO1 was significantly less efficient when GST-tagged COI1^{V553A} SIM mutant was used instead of the WT COI1 with His:JAZ6 (Figure 7B). We also examined the possible *in vivo* interaction of SUMOylated JAZ6 and COI1 *in planta* via co-immunoprecipitation assays. SUMOylated JAZ6:GFP interacts with myc-COI1 *in planta* independently of coronatine (Figure 7C) suggesting that this mechanism operates regardless of endogenous JA levels.

The enhanced interaction between GST tagged SIM mutant of COI1 (COI1^{V553A}) and His:JAZ6, even in the presence of His:SUMO1, raises the possibility that JAZ proteins may be degraded more rapidly in the presence of COI1^{V553A} SIM mutant. We therefore tested HA:COI1 and HA:COI1^{V553A} mediated degradation of JAZ6:GFP in *N. benthamiana* transient assays. Results showed that plants expressing HA:COI1^{V553A} degrade JAZ6:GFP more rapidly than plants expressing wildtype HA:COI1 in the presence of coronatine (Figure 7D and Supplemental Figure 6). This data demonstrates that HA:COI1^{V553} is not only active as a JA receptor but, since it is not under the repression of SUMO1, HA:COI1^{V553A} is more potent in mediating JAZ6:GFP degradation. These observations provide a mechanism for SUMOylated JAZ to disrupt the interaction of COI1 with non SUMOylated JAZ allowing the accumulation of the repressor. Since SUMOylated JAZ6:GFP interacted with myc-COI1 even in the absence of the JA mimic coronatine it is highly likely that this COI1 inhibition by SUMO1 is JA independent.

COI1 SIM mutant suppresses JA insensitivity of SUMO protease (*ots1 ots2*) mutants

COI1 forms a functional E3 ubiquitin ligase SCF^{COI1} and acts as an essential component of JA perception machinery by stimulating the degradation of JAZ proteins (Chini et al. 2007; Pauwels et al. 2011). Results from Figure 7 indicate that SUMO inhibits COI binding to JAZ proteins via the SIM motif therefore we postulated that distrupting the SIM motif in COI1 should lead to increased JA signalling by promoting faster degradation of JAZ repressors as seen in the *N. benthamiana* transient assays (Figure 7D). To test the significance of the SIM motif of COI1 in JA signalling, we generated Arabidopsis transgenics overexpressing (under the 35S promoter) wild type COI1 (35S::COI1:GFP) or the SIM variant COI1^{V553A}

(35S::COI1^{V553A}:GFP) in the *ots1 ots2* double mutant background where there are higher levels of both SUMOylated JAZ and non-SUMOylated JAZs. We anticipated that by overexpressing the SIM disrupted COI1^{V553A}:GFP we should overcome JA insensitivity mediated by increased JAZ levels in the *ots1 ots2* double mutant. As controls we also expressed wildtype COI1 (35S::COI1:GFP) in the wildtype Col-0 background. Both in *B. cinerea* infection assays (Figure 8A-C) and in root growth inhibition assays (Figure 9A-C) we observed that the transgenics expressing COI1^{V553A}:GFP (in *ots1 ots2* background) were more sensitive to JA than the corresponding WT COI1:GFP expressing plants (Supplemental Figure 7). The comparable protein levels of the respective transgenes (Figure 9C) demonstrated that SUMOylated JAZ proteins suppress JA signalling by inhibiting COI1 from targeting non-SUMOylated JAZ proteins for ubiquitin dependent degradation.

DISCUSSION

Given the importance of jasmonates as endogenous developmental regulators in plants, and as primary responders against pathogen attack, improving our understanding of their mechanisms of recognition and signalling has far-reaching importance for plant biology. This study reveals a new control feature of the JA pathway with demonstrable implications for developmental processes and adaptive responses in plants.

The SUMO-SIM interaction is emerging as a key theme in molecular signalling in a wide range of organisms (Geiss-Friedlander and Melchior, 2007). This study describes how the SUMO–SIM 'molecular glue' paradigm operates within plants to block ubiquitination of target proteins (sequestering COI1 needed for ubiquitinating JAZ repressors). Through this study, we unravel a mechanism for attenuating JA signalling through SUMOylation of the JAZ repressor proteins. Three clear lines of evidence support this conclusion. Firstly, hormone analysis indicates that there is no significant change in JA levels in the *ots1 ots2* double mutants although these mutants accumulate JAZ1 and 6 repressor proteins as well as their SUMOylated forms. Secondly, in Figure 7D we provide data on JAZ6 degradation kinetics demonstrating that, at the same level of JA, the COI1 SIM mutant (that is no longer under SUMO mediated repression) is more efficient in causing the degradation of JAZ6:GFP as compared to WT. Thirdly, Figure 7C shows that only SUMOylated JAZ6:GFP, but not JAZ, is able to interact with myc-COI1 in the absence of the JA mimic (coronatine). This study identifies a mechanism that can operate

independently of JA to suppress COI1 activity. This provides direct evidence that intrinsic hormonal levels *in planta* do not affect SUMOylated JAZ from inhibiting COI1 and degrading non-SUMOylated JAZ and therefore attenuating JA signalling. This mechanism may allow plants to develop a rapid adaptive response prior to changes in JA levels. Attenuation of JA signalling has been reported to occur by an increase in JAZ repressor gene expression (Chico et al., 2008) and by the degradation of Je-Ile (Aubert et al., 2015; Smirnova et al., 2017). Here, we provide a new post-translational mechanism for repressing JA signalling that operates within hours of *Botrytis* infection that does not require changes in JA levels. This process affords a new layer of regulation in hormone signalling, allowing plants to rapidly apply “brakes” on JA responses without the need for changes in hormone levels.

The JA mimic coronatine is an important component of the armoury of phytopathogenic *Pseudomonas* used to infect and cause disease in Arabidopsis. Mutants deficient in JA signalling were found to be more resistant to virulent *Pst* as in *ots1 ots2* double mutants that also have elevated SA levels (Bailey et al., 2016; Zabala et al., 2016). Coronatine mediated activation of JA signalling contributes to disease development and Arabidopsis challenged with coronatine deficient *Pst* also have enhanced levels of SA (Geng et al., 2012, Laurie-Berry et al., 2006). A JA signalling repression mechanism that operates independently of coronatine will allow plants to counter *Pst* infection and evidence that *de novo* JA levels accumulated very late in *Pst* infected Arabidopsis argues for the existence of such a mechanism (Zabala et al., 2016). Here, we postulate that plants have exploited the SUMO system to attenuate JA signalling to enhance defence against biotrophic and hemi-biotrophic phytopathogens.

In our model (Figure 10) we suggest that virulent *Pst* infection promotes deSUMOylation of JAZ repressors which promotes COI1-JAZ interaction to activate JA signalling. Activation of JA signalling pathways results in the suppression of SA signalling. In contrary, during necrotrophic infection such as *B. cinerea,* OTS SUMO protease is degraded and this upregulates SUMOylation of JAZ6:GFP protein possibly by the change in equilibrium in favour of SUMO E2s which have been known to SUMOylate targets directly and/or SIZ1 SUMO E3 which have been implicated in pathogen responses in Arabidopsis (Lee et al., 2007). The consequent

re-SUMOyation of JAZ proteins would inhibit JAZ6-COI1 interaction preventing JAZ6 degradation thus repressing JA signalling.

Interestingly, the SUMO site in JAZ6 is located in the C-terminal JAS motif that has been shown to interact with, not only COI1, but also a range of transcription factors **(**Melotto et al., 2008; Staswick, 2008; Yan et al., 2009) whose activity is repressed by JAZ proteins. The impact of SUMOylation on JAZ repressor interaction with cognate transcription factors is not known and requires further investigation.

DELLA growth regulators restrain plant growth, whereas gibberellic acid (GA) promotes growth by targeting DELLAs for destruction. Different studies have demonstrated that DELLA restraint is a crucial mechanism for plants to modulate growth according to environmental cues (Achard et al., 2008; de Lucas et al., 2008; Achard and Genschik, 2009). We previously demonstrated that a proportion of DELLAs are conjugated to the SUMO protein and the extent of conjugation increases during stress, similar to JAZ1 and JAZ6. We identified a SUMO interacting motif (SIM) in the GA receptor GID1 and demonstrated that SUMO-conjugated DELLA binds to this motif in a GA-independent manner (Conti et al., 2008). The consequent sequestration of GID1 by SUMO-conjugated DELLAs leads to an accumulation of non-SUMOylated DELLAs resulting in beneficial growth restraint during stress. For example, DELLAs sequester light responsive and phytochrome interacting transcription factors such as PIF3 and PIF4 and inhibit hypocotyl elongation in the light (de Lucas et al., 2008; Feng et al., 2008). In this context, the JAZ proteins appear to play an analogous role in inhibiting transcription factor activity. Primary root growth of *ots1 ots2* mutants are less hindered by exogenous JA treatment and this is likely to be due to the suppression of the inhibitory effect of MYC2 on root development due to the accumulation of JAZ proteins in the *ots1 ots2* mutants.

In all of these cases, the common central thread is the relative abundance of DELLAs and JAZ repressors, which is modulated by changes in GA and JA levels, respectively. We have demonstrated that dwarfism can be reversed independently of GA levels by modifying the SUMOylation status of DELLAs and that this mechanism is particularly important for plant growth under stress (Conti et al., 2014). Recent evidence indicates that DELLA and JAZ proteins directly interact to mediate cross talk between GA and JA. The discovery that both DELLA and JAZ proteins are SUMOylated leads to the possibility that SUMO may provide a new facet to this

cross talk. Thus, this study provides an important insight into the integrative role of hormones in controlling plant growth and defence.

METHODS

Plant Material and growth conditions

All Arabidopsis lines used in this study are in Col-0 (WT) background. Seeds were plated on 0.5 x Murashige and Skoog (MS) medium, and 0.8 % agar (w/v) on vertical plates, stratified for 2 days at 4 $^{\circ}$C then transferred to growth chambers at 21 $^{\circ}$C under long day (16 h light/8 h dark) cycle. The *ots1 ots2* double mutants were used as described previously (Bailey et al., 2016).

Agrobacterium mediated transformation of Arabidopsis for the generation of transgenic plants

The constructs were transformed into *Agrobacterium tumefaciens* GV1301 and transferred into Arabidopsis using the floral dip method. *Agrobacterium* cells containing the appropriate construct were collected by centrifugation and re-suspended using 5 % (w/v) sucrose solution until the OD_{600} of the cell suspension was 0.8. Silwet L-77, as a strong surfactant, was added to the sucrose solution to obtain a final concentration of 0.05 % (v/v). Developing Arabidopsis inflorescences were dipped into *Agrobacterium* cell suspensions for about 15 seconds and transformed plants were subsequently grown in darkness horizontally for 16–24 h. The seeds of treated plants were harvested after *Agrobacterium*-mediated transformation and seedlings of transformants were obtained by glufosinate screening. Transgenic lines expressing *JAZ6-GFP* and *COI1-GFP* and their mutant variants in the ots1 ots2 double mutant and wildtype Col-0 were generated by floral dips of the respective constructs in Agrobacterium as described above. Two independent lines containing single insertions in T3 generation homozygous transgenic plants with comparable level of transcripts (supplemental figures 2 and 4) were used for further experiments.

Analysis of disease and herbivore resistance

Pseudomonas syringae pv. tomato DC3000 (*Pst*) was grown on King's B medium plates with appropriate antibiotics and incubated for 2 days at 28 $^{\circ}$C. *Pst* infection was performed as previously described (Bailey et al., 2016). Briefly, bacterial cells were collected by centrifugation (2500 g) and re-suspended in 10 mM $MgCl_2$. Pressure infiltration of *Pst* (cfu 1x 10^6; $OD_{600\ nm}$ = 0.002) was carried out using a

needleless syringe. Whole leaves were harvested at the indicated time point after bacterial challenge, frozen immediately in liquid nitrogen and used for western blotting. Three independent experiments were performed for the protein accumulation analysis. Each replicate consisted of rosette leaves of at least three plants grown in individual pots. Collection of *B. cinerea* spores and plant inoculation was performed as described previously (Bailey et al., 2016). In short, *B. cinerea* was sub-cultured on sterile petri dishes with potato dextrose agar medium 2 weeks prior to use of the spores. Subcultures were incubated in the dark at 25 $^{\circ}$C. Spores were harvested in water, inoculums were filtered to remove hyphae and then re-suspended in potato dextrose broth to a concentration of 10^5 spores/mL. Leaf 7 from each of the plants was detached and placed on a bed of 0.8 % (w/v) agar in three plates. Half of the leaves were inoculated with 5 µl droplets of *B. cinerea* inoculum and the other half were mock inoculated with 5 µl of sterile potato dextrose broth. Each plate contains 24 infected and 24 uninfected randomly arranged leaves. Trays were covered with lids and kept under the same conditions as for plant growth, except that the relative humidity was raised to 90%. Lesion perimeters were determined from photographs taken 48 and 72 h post inoculation using image analysis software ImageJ (http://rsb.info.nih.gov/ij/). Mean lesion perimeters of 20 leaves from 20 plants of different genotypes were compared. Cultures of red spider mite (*Tetranychus urticae*) were maintained on French bean plants. Adult female mites were collected from stock plants and released onto leaves (5 mites per plant) of Arabidopsis plants grown in controlled environments under standard conditions. After 5 days, eggs were counted using a binocular microscope.

Site Directed Mutagenesis

Wildtype sequences of JAZ6 and COI1 were amplified by PCR from Arabidopsis and cloned into pENTR/D-TOPO (Invitrogen). Mutated versions of JAZ6 and COI1 were generated by site-directed mutagenesis using the pENTR/D-TOPO clones as template. Oligonucleotide primers used to introduce the mutations are listed in Table S1. The introduction of mutations was confirmed by sequencing, performed both before and after introduction of the mutated JAZ6 and COI1 coding sequences into pEarlyGate103/201/203 destination vectors using LR Clonase (Invitrogen). The pEarlyGate 103/201/203 vector drives expression with the cauliflower mosaic virus 35S promoter with GFP, HA and myc tags. The GFP tagged constructs were

introduced into Columbia-0 (WT) and *ots1 ots2* double mutant background plants via *Agrobacterium*-mediated transformation.

Generation of JAZ expression constructs and transgenic lines

To generate the 35S::JAZ6 construct, the JAZ6 cDNA was cloned into the pENTR/D-TOPO (Invitrogen) vector and recombining the plasmid pENTR/D-TOPO with the binary vector pEarlygate-103 vectors to generate overexpression constructs. The JAZ6^{K221R} allele was generated according to the quick-change Site-Directed Mutagenesis Kit with mutagenic oligos (JAZ6^{K221R} FP/RP). The resulting plasmid was recombined with the pEarlygate 103 vector to obtain the 35S::JAZ6^{K221R}:GFP fusion. The COI1 ORF was amplified by PCR from whole cDNAs from seedlings with COI1 specific oligos and cloned into pENTR/D-TOPO to yield entry clone. The 35S::COI1:GFP construct was generated by recombining the plasmid entry vector with the binary vector pEarlygate 101 vector. The COI1^{V553A} allele was generated according to the quick-change Site-Directed Mutagenesis Kit with mutagenic oligos (COI1^{V553A}FP/RP). For GST pull down assays, fusion constructs GST:COI1, His:JAZ6 and His:SUMO1: were generated by recombining entry vector plasmids with destination vectors pDEST15 (GST tag) and pDEST17 (His tag) vectors. Transgenic plants were generated and analysed as described above.

Total RNA extraction and quantitative RT-PCR

Twelve-day-old wildtype (WT), *ots1 ots2* plant leaves were frozen in liquid nitrogen and ground to a fine powder in pestle and mortar. RNA was extracted using the Spectrum^{TM} Plant Total RNA kit (Sigma-Aldrich) following the manufacturer's recommendations. RNA was quantified by measuring absorbance at wavelengths of 260 and 280 nm using a NanoDrop^{TM} 1000 Spectrophotometer (Thermo Scientific). RNA was DNase treated with Promega DNase I and cDNA synthesis conducted using Invitrogen SuperScript-II Reverse Transcriptase following manufacturer's guidelines. Seedlings were exposed to mock (MS) or 50 µM MeJA before being harvested and RNA extracted. One microgram of total RNA was used for cDNA synthesis and qRT-PCR analysis was performed.

The qRT-PCR assay was conducted as described previously (Conti et al. 2008), using SYBR green master mix (Applied Biosystem) and used for qPCR with a Rotor-Gene-Q (Qiagen). Amplification was followed by a melt curve analysis. The $2^{-\Delta\Delta Ct}$ method was used for relative quantification (Livak and Schmittgen, 2001). To detect

transcript levels, oligos for specific genes were used (Supplemental table 1). Oligonucleotides amplifying Actin were used for normalization.

Quantification of JA-Ile from Arabidopsis tissues

JA-Ile was quantified essentially as previously described (Forcat et al., 2008) with slight modification. Twelve-day-old seedlings grown on 0.5x MS plates were harvested into liquid nitrogen. Samples were ground using a mortar and pestle, and 10 mg powdered tissue aliquots were weighed into microcentrifuge tubes and extracted with 400 µl of 10% (v/v) methanol containing 1 % (v/v) acetic acid to which internal standards (10 ng of JA) had been added. Following removal of the supernatant, the pellet was re-extracted (400 µl of 10 % methanol; 1 % acetic acid). Following a 30 minute incubation on ice, the extract was centrifuged and the supernatants pooled. Samples were then analysed by mass spectrometry using a Sciex Q TRAP 6500 hybrid triple-quadrupole analyser linked to Shimadzu Nexera UHPLC system. Samples were separated on a Phenomenex Luna Omega Polar column (1.6 µm 100 x 2.1 mm) using mobile phases of 0.1% (v/v) formic acid (A) and 0.1 % formic acid in methanol (B) at a flow rate of 200 $\mu L.min^{-1}$, starting at 5 % B, held for 2 minutes, with a linear gradient to 95 % B at 9 minutes, held for 2.9 minutes, with a total run time of 12.2 minutes. The column was equilibrated at 5% B for 5 minutes between runs.

Bioinformatical analysis of protein structures

The structural model of the COI1 SIM site interaction was developed using PyMOL software. The co-ordinates for each structure were downloaded from the PDB (files 3OGK and 1A5R) and the binding sites of SUMO1 and COI1 were mapped onto the protein.

Recombinant protein and GST Pull down assay

Recombinant protein expression and production in *E. coli* were as previously described (Srivastava et al., 2015) with slight modifications. COI1 (GST:COI1), SUMO1 (His:SUMO1) and JAZ6 (His:JAZ6) were expressed in BL21 (DE3) cells. GST-COI1 protein was over-expressed and purified from *E. coli* using Glutathione sepharose 4B beads (GE, USA). His:SUMO1 and His:JAZ6 protein was overexpressed and purified from *E. coli* (BL21) cells using Ni-NTA Agarose beads (Qiagen). For *in vitro* binding experiments, GST and GST-COI1 (2.0 µg) protein was bound to a GST column by incubating with *in-vitro* pull-down buffer for 2h at 4 $^{\circ}C$. Excess unbound protein was washed off and His:SUMO1 proteins were added in

equimolar ratio and incubated in 500 µl *in vitro* pull-down (IVPD) buffer (50 mM Tris-Cl (pH 7.5), 100 mM NaCl, 0.2 % [v/v] glycerol, 1 mM EDTA, 0.1% NP 40 [v/v], 1mM PMSF and 1x protease inhibitor cocktail (Sigma) at 4 $^{\circ}$C. The GST beads were collected by brief centrifugation and (input was collected separately) washed three times with 1 ml of IVPD buffer. Pellets were re-suspended in 1x SDS loading buffer, boiled for 5 minutes and analysed by SDS-PAGE for protein binding. Both input (2%) and pull-down samples were probed with anti-GST and anti-His antibodies.

Reconstitution of SUMOylation in *E. coli*

In order to perform SUMOylation reactions in *E. coli,* we transformed the His:JAZ6 plasmid in two different strains containing SUMO conjugation machinery with SUMO1 modified to expose the C-terminal Gly-Gly (GG) sequence and as a negative control, SUMO1 with the C-terminal Gly-Gly mutated to Ala-Ala (AA) (Okada et al., 2009). For SUMOylation reactions, proteins were purified from freshly transformed *E. coli* using 1ml His-Trap nickel affinity columns (GE Healthcare) and probed with anti-MBP and anti-AtSUMO1 antibodies to investigate the SUMOylation of JAZ6 *in vitro*.

Mass Spectrometry Analysis

The reaction was performed in a single cell system and the protein purified using His-Trap columns and samples were loaded with 4× SDS loading buffer. Five individual reactions were combined and separated by 10% acrylamide SDS-PAGE gel. Gels were stained for total proteins with coomassie brilliant blue and subsequently de-stained with 10 % acetic acid; 40 % methanol; 50 % water and washed with double distilled water (ddH_2O). Protein bands were sliced for MS analysis.

***In vivo* protein degradation assays**

Protein degradation assays were performed as described previously with slight modifications (Bueso et al., 2014). For *in vivo* protein degradation experiments, *A. tumefaciens* cultures containing constructs that express JAZ6:GFP, HA:COI1/ HA:COI1^{V553A} or HA:SUMO1 and the silencing suppressor p19 were co-infiltrated at different ratios in tobacco leaves. Three days after infiltration, samples were collected, ground in liquid nitrogen and immediately placed on ice in lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% NP-40, 10 mM $MgCl_2$, protease inhibitor tablets) for protein extraction. Homogenates were cleared by centrifugation at 13 000

rpm at 4 °C for 15 minutes, and supernatants were used for protein immunoblot analysis.

Protein extraction and Western blot analysis

Frozen plant tissue was ground to a fine powder with a chilled pestle and mortar. Protein extraction buffer (50 mM Tris/HCl, pH 8.5, 4% SDS (w/v), 2% β-mercaptoethanol (v/v), 10 mM EDTA) and protease inhibitor tablet was added at a ratio of 1:1 w/vol. The mixture was centrifuged at 12 000 g at 4 °C for 10 min. The protein concentration was determined using a Direct Detect TM Infra-red Spectrometer (EMD Millipore) and samples were equalized with the addition of extraction buffer. Protein loading dye (4x) was added and the samples were separated on SDS-PAGE gels. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes and blocked with 5 % (w/v) semi-skimmed milk powder at room temperature and probed with the respective antibodies. Secondary horseradish peroxidase (HRP)-conjugated antibodies were applied before developing the blots with X-ray film using an automated developer.

Statistical Analysis

All statistical analysis was performed using GraphPad Prism 6 software. One-way or Two-way ANOVAs were performed at a significance level of $P<0.05$ or $P<0.01$ or $P<0.001$. All root phenotype experiments had at least an N of 25-30 seedlings in each biological replication. Data are representing an average of three individual biological replicates.

Supplemental Data

Supplemental Figure 1 JAZ1 protein accumulation and SUMOylation.

Supplemental Figure 2 Relative transcript levels of JAZ1/JAZ6 in different transgenic plants.

Supplemental Figure 3 Reconstituted *in-vitro* SUMOylation assay of JAZ6:MBP fusion protein.

Supplemental Figure 4 Relative transcript levels of JAZ6 in different transgenic plants.

Supplemental Figure 5 *Pst* DC 3000 infection negatively regulates JAZ6:GFP SUMOylation and accumulation.

Supplemental Figure 6 COI1^{V553A}:GFP SIM mutant plants show significantly increased resistance to *B. cinerea* infection and spider mite infestation in the *ots1 ots2* genetic background.

Supplemental Table 1 List of DNA oligonucletides used in this study.

AUTHOR CONTRIBUTIONS AND ACKNOWLEDGEMENTS

AKS and AS designed the research and analysed the data. AKS performed most of the experiments assisted by BO, CW, PS, GS and CZ. AKS, MG, MRR, EF and AS wrote the paper. All Authors read and commented on the manuscript. AS and AKS were supported by the BBSRC and ERC for research funding.

References

Achard, P., and Genschik, P. (2009). Releasing the brakes of plant growth: how GAs shutdown DELLA proteins. J Exp Bot **60,** 1085-1092.

Achard, P., Gong, F., Cheminant, S., Alioua, M., Hedden, P., and Genschik, P. (2008). The cold-inducible CBF1 factor-dependent signaling pathway modulates the accumulation of the growth-repressing DELLA proteins via its effect on gibberellin metabolism. Plant Cell **20,** 2117-2129.

Aubert, Y., Widemann, E., Miesch, L., Pinot, F., and Heitz, T. (2015). CYP94-mediated jasmonoyl-isoleucine hormone oxidation shapes jasmonate profiles and attenuates defence responses to Botrytis cinerea infection. J Exp Bot **66,** 3879-3892.

Bailey, M., Srivastava, A., Conti, L., Nelis, S., Zhang, C., Florance, H., Love, A., Milner, J., Napier, R., Grant, M., and Sadanandom, A. (2016). Stability of small ubiquitin-like modifier (SUMO) proteases OVERLY TOLERANT TO SALT1 and -2 modulates salicylic acid signalling and SUMO1/2 conjugation in Arabidopsis thaliana. J Exp Bot **67,** 353-363.

Brooks, D.M., Bender, C.L., and Kunkel, B.N. (2005). The Pseudomonas syringae phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in Arabidopsis thaliana. Mol Plant Pathol **6,** 629-639.

Brooks, D.M., Hernández-Guzmán, G., Kloek, A.P., Alarcón-Chaidez, F., Sreedharan, A., Rangaswamy, V., Peñaloza-Vázquez, A., Bender, C.L. and Kunkel, B.N. (2004). Identification and characterization of a well-defined series of coronatine biosynthetic mutants of *Pseudomonas syringae* pv. tomato DC3000. Mol. Plant Microbe Interact. **17,** 162–174.

Bueso, E., Rodriguez, L., Lorenzo-Orts, L., Gonzalez-Guzman, M., Sayas, E., Munoz-Bertomeu, J., Ibanez, C., Serrano, R., and Rodriguez, P.L. (2014). The single-subunit RING-type E3 ubiquitin ligase RSL1 targets PYL4 and PYR1 ABA receptors in plasma membrane to modulate abscisic acid signaling. Plant J **80,** 1057-1071.

Capili, A.D., and Lima, C.D. (2007). Structure and analysis of a complex between SUMO and Ubc9 illustrates features of a conserved E2-Ubl interaction. J Mol Biol **369,** 608-618.

Chico, J.M., Chini, A., Fonseca, S., and Solano, R. (2008). JAZ repressors set the rhythm in jasmonate signaling. Curr Opin Plant Biol **11,** 486-494.

Chini, A., Fonseca, S., Fernandez, G., Adie, B., Chico, J.M., Lorenzo, O., Garcia-Casado, G., Lopez-Vidriero, I., Lozano, F.M., Ponce, M.R., Micol, J.L., and Solano, R. (2007). The JAZ family of repressors is the missing link in jasmonate signalling. Nature **448,** 666-671.

Conti, L., Price, G., O'Donnell, E., Schwessinger, B., Dominy, P., and Sadanandom, A. (2008). Small ubiquitin-like modifier proteases OVERLY TOLERANT TO SALT1 and -2 regulate salt stress responses in Arabidopsis. Plant Cell **20,** 2894-2908.

Conti, L., Nelis, S., Zhang, C., Woodcock, A., Swarup, R., Galbiati, M., Tonelli, C., Napier, R., Hedden, P., Bennett, M., and Sadanandom, A. (2014). Small Ubiquitin-like Modifier protein SUMO enables plants to control growth independently of the phytohormone gibberellin. Dev Cell **28,** 102-110.

de Lucas, M., Daviere, J.M., Rodriguez-Falcon, M., Pontin, M., Iglesias-Pedraz, J.M., Lorrain, S., Fankhauser, C., Blazquez, M.A., Titarenko, E., and Prat, S. (2008). A molecular framework for light and gibberellin control of cell elongation. Nature **451,** 480-484.

Feng, S., Martinez, C., Gusmaroli, G., Wang, Y., Zhou, J., Wang, F., Chen, L., Yu, L., Iglesias-Pedraz, J.M., Kircher, S., Schafer, E., Fu, X., Fan, L.M., and Deng, X.W. (2008). Coordinated regulation of Arabidopsis thaliana development by light and gibberellins. Nature **451,** 475-479.

Fernandez-Calvo, P., Chini, A., Fernandez-Barbero, G., Chico, J.M., Gimenez-Ibanez, S., Geerinck, J., Eeckhout, D., Schweizer, F., Godoy, M., Franco-Zorrilla, J.M., Pauwels, L., Witters, E., Puga, M.I., Paz-Ares, J., Goossens, A., Reymond, P., De Jaeger, G., and Solano, R. (2011). The Arabidopsis bHLH transcription factors MYC3 and MYC4 are targets of JAZ repressors and act additively with MYC2 in the activation of jasmonate responses. Plant Cell **23,** 701-715.

Feys, B., Benedetti, C.E., Penfold, C.N., and Turner, J.G. (1994). Arabidopsis Mutants Selected for Resistance to the Phytotoxin Coronatine Are Male Sterile, Insensitive to Methyl Jasmonate, and Resistant to a Bacterial Pathogen. Plant Cell **6,** 751-759.

Fonseca, S., Chico, J.M., and Solano, R. (2009). The jasmonate pathway: the ligand, the receptor and the core signalling module. Curr Opin Plant Biol **12,** 539-547.

Forcat, S., Bennett, M.H., Mansfield, J.W., and Grant, M.R. (2008). A rapid and robust method for simultaneously measuring changes in the phytohormones ABA, JA and SA in plants following biotic and abiotic stress. Plant Methods **4,** 16.

Geiss-Friedlander, R., and Melchior, F. (2007). Concepts in sumoylation: a decade on. Nat Rev Mol Cell Biol **8,** 947-956.

Geng, X., Cheng, J., Gangadharan, A., and Mackey, D. (2012). The coronatine toxin of Pseudomonas syringae is a multifunctional suppressor of Arabidopsis defense. Plant Cell **24,** 4763-4774.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu Rev Phytopathol **43,** 205-227.

Grunewald, W., Vanholme, B., Pauwels, L., Plovie, E., Inze, D., Gheysen, G., and Goossens, A. (2009). Expression of the Arabidopsis jasmonate signalling repressor JAZ1/TIFY10A is stimulated by auxin. EMBO Rep **10,** 923-928.

Hay, R.T. (2001). Protein modification by SUMO. Trends Biochem Sci **26,** 332-333.

Hecker, C.M., Rabiller, M., Haglund, K., Bayer, P., and Dikic, I. (2006). Specification of SUMO1- and SUMO2-interacting motifs. J Biol Chem **281,** 16117-16127.

Hickey, C.M., Wilson, N.R., and Hochstrasser, M. (2012). Function and regulation of SUMO proteases. Nat Rev Mol Cell Biol **13,** 755-766.

Howe, G.A., and Jander, G. (2008). Plant immunity to insect herbivores. Annu Rev Plant Biol **59,** 41-66.

Ingle, R.A., Stoker, C., Stone, W., Adams, N., Smith, R., Grant, M., Carre, I., Roden, L.C., and Denby, K.J. (2015). Jasmonate signalling drives time-of-day differences in susceptibility of Arabidopsis to the fungal pathogen Botrytis cinerea. Plant J **84,** 937-948.

Jentsch, S., and Pyrowolakis, G. (2000). Ubiquitin and its kin: how close are the family ties? Trends Cell Biol **10,** 335-342.

Katsir, L., Chung, H.S., Koo, A.J., and Howe, G.A. (2008). Jasmonate signaling: a conserved mechanism of hormone sensing. Curr Opin Plant Biol **11,** 428-435.

Kazan, K., and Manners, J.M. (2008). Jasmonate signaling: toward an integrated view. Plant Physiol **146,** 1459-1468.

Kerscher, O., Felberbaum, R., and Hochstrasser, M. (2006). Modification of proteins by ubiquitin and ubiquitin-like proteins. Annu Rev Cell Dev Biol **22,** 159-180.

Koornneef, A., and Pieterse, C.M. (2008). Cross talk in defense signaling. Plant Physiol **146,** 839-844.

Laurie-Berry, N., Joardar, V., Street, I.H., and Kunkel, B.N. (2006). The Arabidopsis thaliana JASMONATE INSENSITIVE 1 gene is required for suppression of salicylic

acid-dependent defenses during infection by Pseudomonas syringae. Mol Plant Microbe In **19,** 789-800.

Lee, J., Nam, J., Park, H.C., Na, G., Miura, K., Jin, J.B., Yoo, C.Y., Baek, D., Kim, D.H., Jeong, J.C., Kim, D., Lee, S.Y., Salt, D.E., Mengiste, T., Gong, Q., Ma, S., Bohnert, H.J., Kwak, S.S., Bressan, R.A., Hasegawa, P.M., and Yun, D.J. (2007). Salicylic acid-mediated innate immunity in Arabidopsis is regulated by SIZ1 SUMO E3 ligase. Plant J **49,** 79-90.

Livak, K.J. and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods. **25,** 402–408.

Melotto, M., Mecey, C., Niu, Y., Chung, H.S., Katsir, L., Yao, J., Zeng, W., Thines, B., Staswick, P., Browse, J., Howe, G.A. and He S.Y. (2008). A critical role of two positively charged amino acids in the JAS motif of Arabidopsis JAZ proteins in mediating coronatine- and jasmonyl isoleucine-dependant interactions with the COI1 F-box protein. Plant J **55,** 979-988.

Mengiste, T. (2012). Plant immunity to necrotrophs. Annu Rev Phytopathol **50,** 267-294.

Miller, M.J., Barrett-Wilt, G.A., Hua, Z., and Vierstra, R.D. (2010). Proteomic analyses identify a diverse array of nuclear processes affected by small ubiquitin-like modifier conjugation in Arabidopsis. Proc Natl Acad Sci U S A **107,** 16512-16517.

Minty, A., Dumont, X., Kaghad, M., and Caput, D. (2000). Covalent modification of p73alpha by SUMO-1. Two-hybrid screening with p73 identifies novel SUMO-1-interacting proteins and a SUMO-1 interaction motif. J Biol Chem **275,** 36316-36323.

Okada, S., Nagabuchi, M., Takamura, Y., Nakagawa, T., Shinmyozu, K., Nakayama, J., and Tanaka, K. (2009). Reconstitution of Arabidopsis thaliana SUMO pathways in E. coli: functional evaluation of SUMO machinery proteins and mapping of SUMOylation sites by mass spectrometry. Plant Cell Physiol **50,** 1049-1061.

Pauwels, L., and Goossens, A. (2011). The JAZ proteins: a crucial interface in the jasmonate signaling cascade. Plant Cell **23,** 3089-3100.

Pieterse, C.M. (2012). Prime time for transgenerational defense. Plant Physiol **158,** 545.

Reeves, P.H., Murtas, G., Dash, S., and Coupland, G. (2002). early in short days 4, a mutation in Arabidopsis that causes early flowering and reduces the mRNA abundance of the floral repressor FLC. Development **129,** 5349-5361.

Reverter, D., and Lima, C.D. (2009). Preparation of SUMO proteases and kinetic analysis using endogenous substrates. Methods Mol Biol **497,** 225-239.

Sheard, L.B., Tan, X., Mao, H., Withers, J., Ben-Nissan, G., Hinds, T.R., Kobayashi, Y., Hsu, F.F., Sharon, M., Browse, J., He, S.Y., Rizo, J., Howe, G.A., and Zheng, N. (2010). Jasmonate perception by inositol-phosphate-potentiated COI1-JAZ co-receptor. Nature **468,** 400-405.

Smirnova, E., Marquis, V., Poirier, L., Aubert, Y., Zumsteg, J., Menard, R., Miesch, L., and Heitz, T. (2017). Jasmonic Acid Oxidase 2 Hydroxylates Jasmonic Acid and Represses Basal Defense and Resistance Responses against Botrytis cinerea Infection. Mol Plant **10,** 1159-1173.

Srivastava, A.K., Zhang, C., Caine, R.S., Gray, J., and Sadanandom, A. (2017). Rice SUMO protease Overly Tolerant to Salt 1 targets the transcription factor, OsbZIP23 to promote drought tolerance in rice. Plant J **92,** 1031-1043.

Srivastava, A.K., Zhang, C., Caine, R.S., Gray, J., and Sadanandom, A. (2017). Rice SUMO protease Overly Tolerant to Salt 1 targets the transcription factor, OsbZIP23 to promote drought tolerance in rice. Plant J **92,** 1031-1043.

Srivastava, A.K., Zhang, C., Yates, G., Bailey, M., Brown, A., and Sadanandom, A. (2016). SUMO Is a Critical Regulator of Salt Stress Responses in Rice. Plant Physiol **170,** 2378-2391.

Staswick P.E. (2008). JAZing up jasmonate signaling. Trends Plant Sci **13,** 66-71.

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., Nomura, K., He, S.Y., Howe, G.A., and Browse, J. (2007). JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. Nature **448,** 661-665.

Verger, A., Perdomo, J., and Crossley, M. (2003). Modification with SUMO. A role in transcriptional regulation. EMBO Rep **4,** 137-142.

Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M., and Turner, J.G. (1998). COI1: an Arabidopsis gene required for jasmonate-regulated defense and fertility. Science **280,** 1091-1094.

Yan, Y., Stolz, S., Chetelat, A., Reymond, P., Pagni, M., Debugnon, L. and Farmer E.E. (2007). A downstream mediator in the growth repression limb of the jasmonate pathway. Plant Cell **19,** 2470-2483.

Zabala, M.D., Zhai, B., Jayaraman, S., Eleftheriadou, G., Winsbury, R., Yang, R., Truman, W., Tang, S.J., Smirnoff, N., and Grant, M. (2016). Novel JAZ co-operativity and unexpected JA dynamics underpin Arabidopsis defence responses to Pseudomonas syringae infection. New Phytol **209,** 1120-1134.

Figure legends

Figure 1. OTS SUMO proteases regulate JA mediated defence responses

(**A**) White light images of representative WT, *ots1 ots2* and OTS1-OE leaves showing cell death lesions at 72 hours post inoculation with *B. cinerea*. Scale bars represent 1 cm.

(**B**) Analysis of leaf lesion diameter of WT, *ots1 ots2* and OTS1-OE at 72 hours post-inoculation (hpi) with *B.cinerea*. Histograms represent the mean lesion diameter ± SD of at least 50 lesion sites from 10 plants for each genotype. Error bars represent standard deviation (SD) from five biological replicates per experiment based on three independent experiments. Asterisks denote statistical significance of the differences between WT and *ots1 ots2* calculated using student t-test (***P ≤ 0.001).

(**C**) Quantification of fungal growth by quantitative real-time PCR of *B. cinerea. Cutinase gene*-specific primers using genomic DNA at 72 hpi. Histograms represent the mean ± SD from three biological replicates. Asterisks denote statistical significance of the differences between WT and *ots1 ots2* calculated using students t-test (***P ≤ 0.001).

(**D**) *Tetranychus urticae* egg counts on Arabidopsis plants 5 days post infestation with adult female mites. The average number of eggs produced per female on each genotype shown, along with standard errors. Asterisks denote statistical significance of the differences between WT and *ots1 ots2* calculated using student t-test (*** P ≤ 0.001).

Figure 2. OTS SUMO proteases regulate JA mediated growth responses

(**A**) Images of Arabidopsis seedlings of different genotypes on MS agar plates with and without JA indicating that loss of function SUMO protease mutant *ots1 ots2* shows decreased sensitivity to exogenous JA. WT and *ots1 ots2* mutants were grown on MS medium without or with 10 µM JA for 8 days. Scale bar represents 1 cm.

(**B**) Quantification of root growth under exogenous JA treatment against that without JA treatment (designated as 100 %). Values are mean ± SD of at least 20 plants of each genotype. Error bars represent SD from three biological replicates. Asterisks denote statistical significance of the differences between WT and *ots1 ots2* calculated using student t-test (*P ≤ 0.05).

(C-F) Relative transcript levels of JA responsive genes *PDF1.2, ERF1, ZAT10* and *LOX2* were measured in WT and *ots1 ots2* mutant with and without JA treatment.

Twelve-day old seedlings were treated with 50 µM JA for 6 hours and seedlings without JA treatment were used as a mock control. Values are means ± SD of three biological replicates. At least 50 seedlings were combined into one replicate.

Figure 3. Arabidopsis JAZ6 protein is SUMOylated

(**A**) Immunoprecipitations (IP: αGFP) from total proteins derived from 4 week old plant leaves of wildtype (WT) or 35S::JAZ6:*GFP* (WT background) or *35S::JAZ6:GFP (ots1 ots2* background). Immunoprecipitated proteins were immunoblotted (IB) and probed with anti-GFP (αGFP) or anti-AtSUMO1/2 antibodies. S1-JAZ6:GFP indicates SUMOylated JAZ6:GFP proteins. Molecular weights are indicated on the left in kiloDaltons (kD). Ponceau staining indicating Rubisco levels was employed to determine protein loading for the immunoprecipitation. WT (non-transgenic) plants served as a negative control.

(B) Estimation of JA-Ile concentrations through mass spectrometry analysis from twelve-day old seedlings of WT and *ots1 ots2* mutant. Data presented are mean from three biological replicates. Error bars indicate standard deviation of the means and no significant difference was observed between the genotypes after Student-t-test analysis.

(C) Relevant section of mass spectra obtained from JAZ6-SUMO1 conjugated peptide fragmentation experiments. The peak representing JAZ6 peptide sequence carrying a SUMO1 signature peptide fragment QTGG on residue K221 is indicated on the amino acid sequence.

(**D**) Immunoblots indicating reduced SUMOylation and protein abundance of 35S::JAZ6^{K221R}:GFP in WT or 35S::JAZ6^{K221R}:GFP in the *ots1 ots2* backgrounds compare to the 35S::JAZ6:GFP in WT and *ots1 ots2* background. Proteins were immunoblotted (IB) and probed with anti-GFP (αGFP) or anti-AtSUMO1/2 (αSUMO1) antibodies. S1-JAZ6:GFP indicates SUMOylated JAZ6:GFP proteins. Molecular weights are indicated on the left in kiloDaltons (kD). Ponceau staining indicating Rubisco levels was employed to determine protein loading for the immunoprecipitation (IP:αGFP). WT (non-transgenic) plants served as a negative control.

Figure 4. JAZ6 sumo site mutation affects the stability of JAZ6 protein but does not affect its interaction with COI1

(A) Co-immunoprecipitation of myc:COI1 with GFP only, JAZ6:GFP and JAZ6^{K221R}:GFP was performed *in planta* using *N. benthamiana* transient assays to

investigate the interaction of JAZ6:GFP and JAZ6^{K221R}:GFP with myc:COI1 protein. Immunoprecipitates (IP: αGFP) were analyzed by SDS-PAGE and immunoblots were probed with αGFP to detect JAZ6:GFP and JAZ6^{K221R}:GFP and GFP alone and with αmyc to detect myc:COI1 proteins. Ponceau staining indicating Rubisco levels was employed to determine protein loading for the immunoprecipitation (IP:αGFP).

(B) JA mediated degradation of JAZ6:GFP and JAZ6^{K221R}:GFP proteins. Immunoblot probed with anti-GFP antibodies showing protein levels of 35S::JAZ6:GFP and 35S::JAZ6^{K221R}:GFP in respective seedlings treated with and without (mock treatment) JA (100 µM). Seedling samples were collected at the indicated time points. Ponceau staining indicating Rubisco levels was employed to determine protein loading for the immunoprecipitation (IP:αGFP).

(C) Immunoblots probed with αHA (IB: αHA) indicating the accumulation of HA:OTS1 protein in 12 day old seedlings expressing 35S promoter driven *HA-OTS1* transgene. Seedlings were treated with and without (mock) JA. Protein samples from seedlings were collected at the indicated time points. Ponceau red stained Rubisco protein was used to indicate total protein levels.

Figure 5. SUMOylation of JAZ6 is enhanced during *Botrytis* infection

(A) Immunoblots indicating significantly increased SUMOylation and protein abundance of GFP tagged JAZ6 from 4-week-old (35S:JAZ6:GFP transgenics in WT background) plants infected with *B. cinerea*. Samples were collected at different time points post infection and mock treated samples were used for immunoprecipitation with anti-GFP antibodies (IP: αGFP). Immunoblots (IB) were probed with GFP (IB:αGFP) or AtSUMO1/2 antibodies (IB:αSUMO1). Ponceau staining indicating Rubisco levels was employed to determine protein loading for the immunoprecipitation (IP:αGFP).

(B) Immunoblots probed with anti-HA antibodies showing HA-OTS1 levels in *35S::OTS1-HA* transgenic Arabidopsis lines infected with *B. cinerea*. Four-week old *35S::OTS1-HA* transgenic Arabidopsis leaves were pressure infiltrated with *B. cinerea* and mock treated with Magnesium chloride solution. Protein extracts were harvested from leaf samples collected at different time points after infection. Ponceau red stained Rubisco protein was used to indicate total protein levels.

(C) Immunoblots indicating greatly reduced SUMOylation and protein abundance of GFP tagged JAZ6 from 15 day-old seedlings (35S:JAZ6:GFP transgenics in WT background) treated with 100 µM JA for 30 minutes. Protein samples were collected

for immunoprecipitation with anti-GFP antibodies (IP: αGFP) at 0 and 30 minutes after treatment. Immunoblots (IB) were probed with GFP (αGFP) or AtSUMO1/2 antibodies (αSUMO1). Ponceau staining indicating Rubisco levels was employed to determine protein loading for the immunoprecipitation (IP:αGFP).

(D) Co-immunoprecipitation of HA-OTS1 with JAZ6:GFP *in planta*. *Agrobacterium* cultures containing 35S::HA-OTS1 were mixed with *Agrobacterium* cultures containing either 35S::GFP or 35S::JAZ6:GFP and transiently expressed in *N. benthamiana*. Total protein was extracted for immunoprecipitation with anti-GFP beads. Immunoprecipitates were analyzed by immunoblotting using anti-HA and anti-GFP antibodies to detect for the presence of OTS1-HA or JAZ6:GFP, respectively. Ponceau red stained Rubisco protein was used to indicate total protein levels used in the immunoprecipitation in the time points

Figure 6. SUMO inhibits JA- receptor COI1 binding to JAZ proteins

(**A**) Side view of the COI1 JA receptor (beige) allows the identification of the location of the flexible loop forming the COI1 SIM motif (blue) residing at the interface between COI1 and the JAZ degron binding site (green). The binding of SUMO1 (pink) via its β-sheet (red), at this position can mask the COI1 domain that binds JAZ proteins. The binding of COI1 to SUMOylated JAZ through its SIM is therefore predicted to be able to disrupt binding of the non-SUMOylated JAZ to COI1.

(**B**) Cross species alignment of COI1 SIM from Arabidopsis and Brassica. *At*COI1; *Arabidopsis thaliana* COI1; *Br*COI1; *Brassica rapa* COI1 Residues are colored according to properties: red, hydrophobic; blue, acidic; magenta, basic; green, hydrophilic.

(**C**) GST pull down assays between recombinant His:SUMO1 with recombinant GST:COI1 or GST only indicate that GST:COI1 binds to SUMO1.

(**D**) GST pull down assays between recombinant His:SUMO1 with recombinant GST:COI1, SIM site mutated GST:COI1^{V553A}; or GST only. The data indicates that Valine at position 553 is critical for SUMO1 binding.

Figure 7. SUMOylated JAZ6 negatively regulates COI1-JAZ6 interaction

(A) GST pull down assays indicate that interaction between His:JAZ6 and GST:COI1 is weakened by the addition of increasing amounts of His:SUMO1 protein. His:JAZ6 protein mixed with different amounts of His:SUMO1 and pulled down with either GST:COI1 or GST alone in the presence or absence of coronatine (10 μM). The

eluates were then probed with anti-His tag (αHis) or anti-GST (αGST) antibodies to detect His:JAZ6 or GST tagged proteins, respectively.

(B) GST pull down assays performed as above but with GST:COI1 replaced by the COI1 SIM mutant GST:COI1^{V553} indicate that mutation of Valine to Alanine rescues the interaction between GST:COI1^{V553A} and His:JAZ6 even in the presence of His:SUMO1 protein. The eluates were probed with anti-His tag (αHis) or anti-GST (αGST) antibodies to detect His-JAZ6 or GST tagged proteins, respectively.

(C) Co-immunoprecipitation of JAZ6:GFP with myc:COI1 *in planta* indicates that SUMOylated JAZ6:GFP binds to myc:COI1 even in the absence of JA mimic coronatine. *Agrobacterium* culture containing 35S::JAZ6:GFP was mixed with *Agrobacterium* cultures containing both 35S::myc:COI1 and 35S::HA:SUMO1 and transiently expressed in *N. benthamiana.* Total protein was extracted for immunoprecipitation with anti-myc antibodies (IP; αmyc) to pull down myc-COI1 and the immunoprecipitates were probed with anti-SUMO, anti-myc (IB: αmyc) and anti-GFP(IB: αGFP) antibodies to detect for the presence of SUMOylated and Non-SUMOylated JAZ6:GFP and myc:COI1. Ponceau staining indicating Rubisco levels was employed to determine protein loading for the immunoprecipitation (IP:αGFP).

(D) *In vivo* degradation of JAZ6 was observed in co-infiltration experiments with increasing amounts of HA:COI1 or HA:COI1^{V553A} in presence of 50 µM coronatine. The ratio of the relative concentration of agrobacteria used in the different co-infiltrations is indicated by numbers (top). Cell extracts were analysed by immunoblot analysis with anti-GFP and anti-HA antibodies. Immunoblot analysis indicated that JAZ6:GFP was more unstable in plants transiently expressing HA:COI1^{V553A} when compared to plants expressing HA:COI1. Ponceau red stained Rubisco protein was used as a loading control.

Figure 8. COI1 SIM mutant suppresses *Botrytis* susceptibility

(A) White light images of representative leaves from 4 week old transgenic plants expressing 35S::COI1:GFP in the wildtype (WT), *ots1 ots2* and 35S::COI1^{V553A} :GFP in the *ots1 ots2* background at 72 hours post infection after inoculation with mock (left panel) or *B. cinerea* spores (right panel).

(**B**) Quantification of lesion sizes on rosette leaves at 72 hours post infection with *B. cinerea* spores. Values represent the means $\pm$ SD of three biological replicates of 4 week old transgenic plants. The letters indicate averages that are statistically significantly different from each other (*P < 0.05, **P< 0.01).

(C) Quantification of fungal growth by real-time PCR on *Botrytis* genomic DNA with *B. cinerea cutinase gene* specific primers at 72 hours post infection. Histograms represent the means +_SD of three biological replicates of 4 week old transgenic plants. The letters indicate averages that are statistically significantly different ($P < 0.05$) from each other. n = 15 to 20, four week old plants in each replicate.

Figure 9. COI1 SIM mutant restores JA sensitivity in *ots1 ots2* mutant background

(**A**) Image of representative 10-day-old seedlings grown in MS and MS + JA (10 μM) and the effect of JA on root length of different transgenic plants. Scale bar represents 1 cm.

(**B**) Mean root length of 10-day-old seedlings in the presence of 10 μM JA relative to the controls. Values represent the means ±SD of three biological replicates. The letters indicate significant differences between WT and the transgenic lines of COI1 (WT), COI1 (*ots1 ots2*) and $COI1^{V553A}$ (*ots1 ots2)* in presence of JA. n = 35 to 40 seedlings each replicate.

(**C**) Immunoblots probed with αGFP indicating COI1:GFP and $COI1^{V553A}$:GFP protein levels in WT and *ots1 ots2* background.

Figure 10. A model for JA signaling repression by SUMOylated JAZ proteins.

During biotroph infection, such as by *Pst*, bacterial coronatine promotes JA signalling by activating the 26S proteasome mediated degradation of JAZ repressors by the JA receptor CORONATINE INSENSITIVE1 (COI1). Part of this process involves JA mediated accumulation of the SUMO de-conjugating protease, OTS1 that rapidly deSUMOylates JAZ repressors and facilitates COI1 access to JAZ for degradation. JAZ repressor turnover activates JA-responsive gene expression through the transcriptional regulators such as MYC2/MYC3/MYC4.

On the other hand infection by necrotrophs such as the fungal pathogen, *Botrytis cinerea* stimulates, degradation of the SUMO deconjugating protease OTS1. This leads to the accumulation of SUMOylated JAZ proteins (this does not preclude increased SUMO conjugating via hitherto unknown mechanisms) that inhibit COI1 mediated degradation of non-SUMOylated JAZ repressors, consequently, suppressing JA signalling.

Supplemental Figure 1. JAZ1 protein accumulation and SUMOylation (Supports Figure 3).

Immunoblots probed with Immunoprecipitations (IP: αGFP) of total proteins derived from 4 week old plant leaves of wildtype (WT) or *35S::JAZ1:GFP* (WT background) or *35S::JAZ1:GFP (ots1 ots2* background). Proteins were immunoblotted (IB) and probed with anti-GFP (αGFP) or anti-AtSUMO1/2 antibodies (αSUMO1). S1-JAZ1:GFP indicates SUMOylated JAZ1:GFP proteins. Molecular weights are indicated on the left in kiloDaltons (kD). Ponceau staining indicating Rubisco levels was employed to determine protein loading for the immunoprecipitation. WT (non-transgenic) plants served as a negative control. Right panel indicates the full blot probed with αGFP (lower) and αSUMO1 (Upper).

Supplemental Figure 2. Relative transcript levels of *JAZ1 and 6* in the different transgenic plants (Supports Figure 3).

Quantitative real time PCR was used to analyse *JAZ1* mRNA from twelve day old seedlings of transgenic plants expressing 35S::JAZ1:GFP in WT and *ots1 ots2* genetic background and **(b)** *JAZ6* gene expression in 35S::JAZ6:GFP in WT and *ots1 ots2* genetic backgrounds. Actin was used as the internal control. Histograms represent the mean $\pm$ SD from three independent biological replicates.

Supplemental Figure 3. Reconstituted *in-vitro* SUMOylation assay of JAZ6:MBP fusion protein (Supports Figure 3).

Immunoblots probed with either anti-MBP and anti-AtSUMO1/2 antibodies show definitive SUMOylation of JAZ6:MBP. SUMO1-GG is the wildtype SUMO1 while SUMO1-AA is a mutant form of SUMO1 that cannot be conjugated to target proteins. Immunoblots were probed with anti-MBP (IB: αMBP) and anti-AtSUMO1 (IB: αSUMO1) antibodies to detect JAZ6:MBP or SUMO1-JAZ6:MBP

Supplemental Figure 4. Relative transcript levels of JAZ6 in different transgenic plants. (Supports Figure 3)

Quantitative real time PCR was used to analyze $JAZ6^{K221R}$:GFP mRNA from twelve-day old seedlings of transgenic plants expressing 35S::$JAZ6^{K221R}$:GFP in WT and *ots1 ots2* genetic backgrounds. Actin was used as the internal control. Data are mean $\pm$ SD of three biological replicates.

Supplemental Figure 5. *Pst* DC3000 infection negatively regulates JAZ6:GFP SUMOylation and accumulation (Supports Figure 5).

Immunoblots indicating SUMOylation and protein accumulation of JAZ6:GFP from four-week-old transgenic 35S::JAZ6:GFP plants pressure infiltrated with virulent *Pst*. Samples were collected for immunoprecipitation with anti-GFP antibodies (IP: αGFP) at the indicated time points. Immunoblots (IB) were probed with GFP (αGFP) or AtSUMO1/2 antibodies (αSUMO1). Ponceau staining indicating Rubisco levels was employed to determine protein loading for the immunoprecipitation (IP:αGFP).

Supplemental Figure 6. JAZ6:GFP is more unstable in plants transiently expressing the SIM mutant COI1^{V553A} (Support Figure 7).
The DNA constructs HA:COI1, HA:COI1^{V553A} and GFP:JAZ6 were transiently co-expressed in *N. benthamiana* leaves in the presence of coronatine. Total protein extracted at different time points after coronatine treatment were analysed by immunoblot analysis with anti-GFP and anti-HA antibodies. Immunoblot analysis indicated that JAZ6:GFP was more unstable in plants transiently expressing HA:COI1^{V553A} when compared to plants expressing HA:COI1. Ponceau red stained Rubisco protein was used as a loading control.

Supplemental Figure 7. COI1^{V553A}:GFP SIM mutant plants show significantly increased resistance to *B. cinerea* infection and spider mite infestation in the *ots1 ots2* genetic background (Supports Figure 9).

(a) White light Images of representative leaves from 4 week old transgenic plants expressing 35S::COI1:GFP and 35S::COI1^{V553A}:GFP in wildtype background at 72 hours post-infection with *B. cinerea* spores.

(b) Quantification of lesion sizes on rosette leaves at 72 hours post-infection with *B. cinerea* spores.

(c) Egg counts from adult female mite infestation for 5 days *on Arabidopsis plants.* Data shows mean ± SD of eggs laid on seven independent plants from each genotype. Asterisks denote statistical significance of the differences between COI1 (WT), COI1 (*ots1 ots2*) and COI1^{V553A} (*ots1 ots2)* using Two-way-ANOVA (*P < 0.05, ***P< 0.001).

Supplementary Table 1. List of DNA oligonucleotides used in the study.

Table of DNA primers employed for generating DNA constructs and real-time PCR.

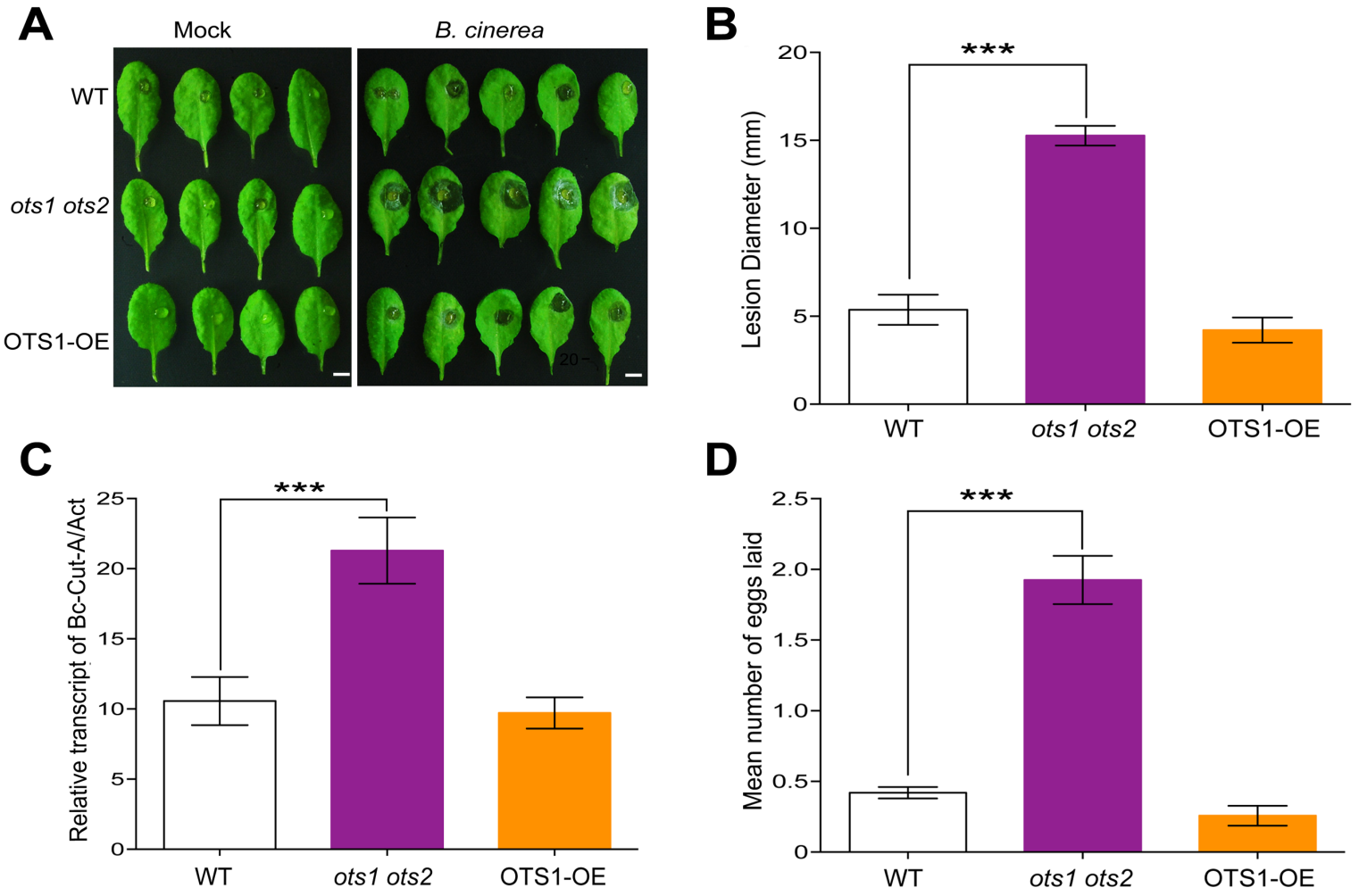


Figure 1. OTS SUMO proteases regulate JA mediated defence responses

(A) White light images of representative WT, *ots1 ots2* and OTS1-OE leaves showing cell death lesions at 72 hours post inoculation with *B. cinerea*. Scale bars represent 1 cm.

(B) Analysis of leaf lesion diameter of WT, *ots1 ots2* and OTS1-OE at 72 hours post-inoculation (hpi) with *B. cinerea*. Histograms represent the mean lesion diameter ± SD of at least 50 lesion sites from 10 plants for each genotype. Error bars represent standard deviation (SD) from five biological replicates per experiment based on three independent experiments. Asterisks denote statistical significance of the differences between WT and *ots1 ots2* calculated using student t-test (***P < 0.001).

(C) Quantification of fungal growth by quantitative real-time PCR of *B.cinerea.* Cutinase gene-specific primers using genomic DNA at 72 hpi. Histograms represent the mean ± SD from three biological replicates. Asterisks denote statistical significance of the differences between WT and *ots1 ots2* calculated using students t-test (***P < 0.001).

(D) Egg counts from adult female mite infestation for 5 days on Arabidopsis plants. The average number of eggs produced per female on each genotype shown, along with standard errors. Asterisks denote statistical significance of the differences between WT and *ots1 ots2* calculated using student t-test (*** P < 0.001).

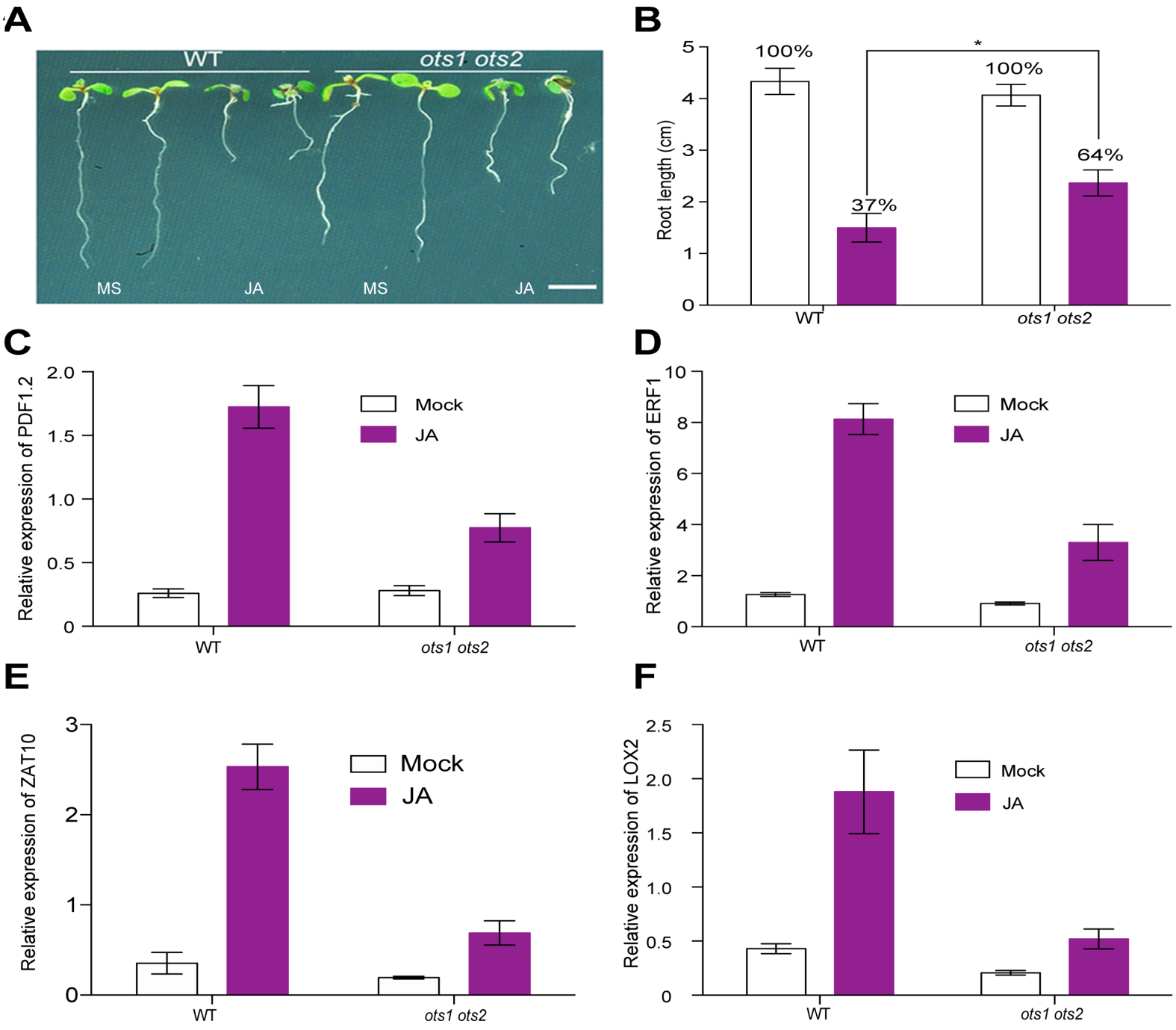


Figure 2. OTS SUMO proteases regulate JA mediated growth responses

(A) Images of Arabidopsis seedlings of different genotypes on MS agar plates with and without JA indicating that loss of function SUMO protease mutant *ots1 ots2* shows decreased sensitivity to exogenous JA. WT and ots1 ots2 mutants were grown on MS medium without or with 10μM JA for 8 days. Scale bar represents 1 cm.

(B) Quantification of root growth under exogenous JA treatment against that without JA treatment (designated as 100%). Values are mean ± SD of at least 20 plants of each genotype. Error bars represent SD from three biological replicates. Asterisks denote statistical significance of the differences between WT and *ots1 ots2* calculated using student t-test (*P < 0.05).

(C-F) Relative transcript levels of JA responsive genes PDF1.2, ERF1, ZAT10 and LOX2 were measured in WT and *ots1 ots2* mutant with and without JA treatment. Twelve-day old seedlings were treated with 50μM JA for 6 hours and seedlings without JA treatment were used as a mock control. Values are means ± SD of three biological replicates. At least 50 seedlings were combined into one replicate.

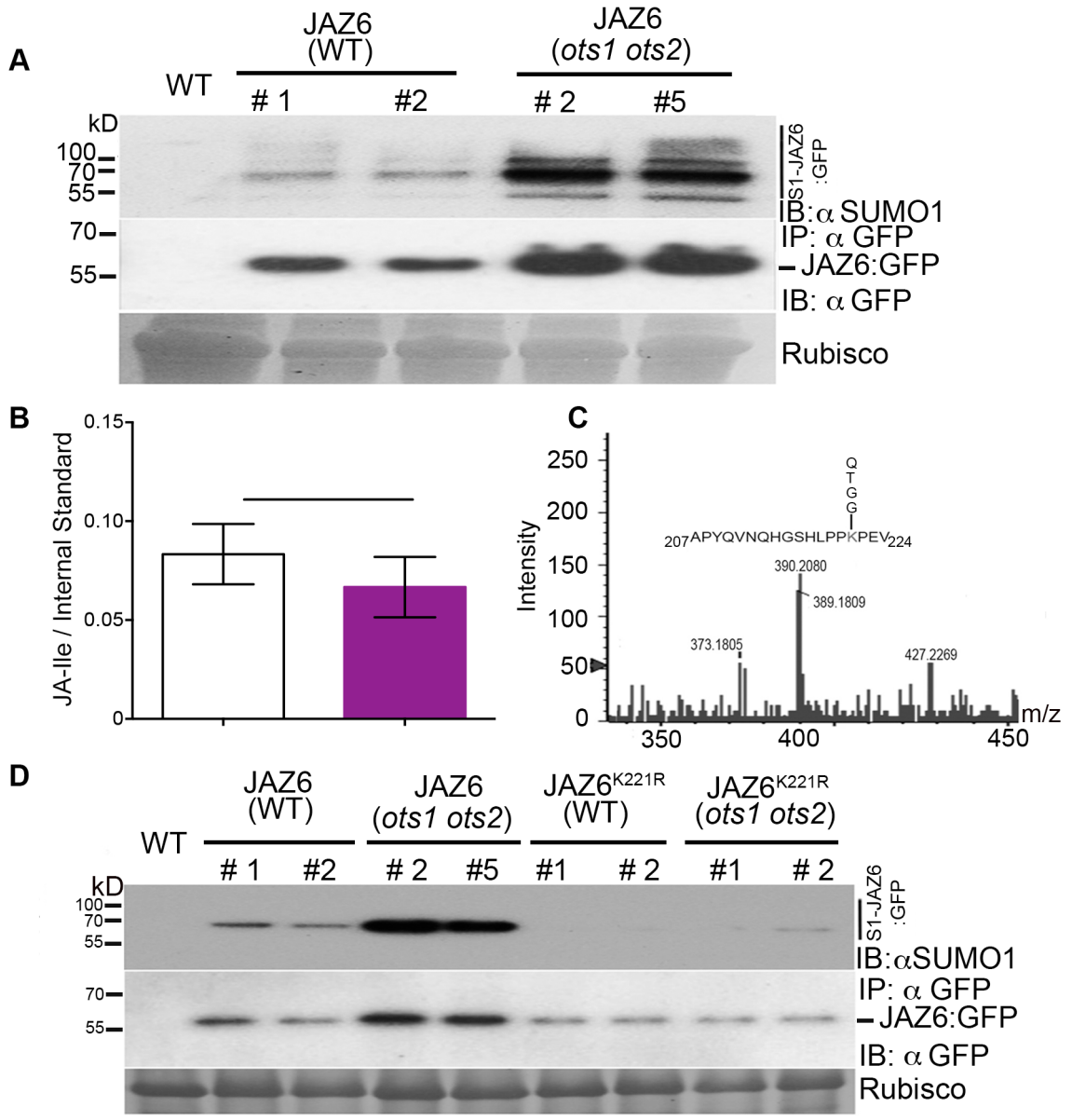


Figure 3. Arabidopsis JAZ6 protein is SUMOylated

(A) Immunoprecipitations (IP: αGFP) from total proteins derived from 4 week old plant leaves of wildtype (WT) or 35S::JAZ6:GFP (WT background) or 35S::JAZ6:GFP (*ots1 ots2* background). Immunoprecipitated proteins were immunoblotted (IB) and probed with anti-GFP (αGFP) or anti-AtSUMO1/2 antibodies. S1-JAZ6:GFP indicates SUMOylated JAZ6:GFP proteins. Molecular weights are indicated on the left in kiloDaltons (kD). Ponceau staining indicating Rubisco levels was employed to determine protein loading for the immunoprecipitation. WT (non-transgenic) plants served as a negative control.

(B) Estimation of JA-Ile concentrations through mass spectrometry analysis from twelve-day old seedlings of WT and *ots1 ots2* mutant. Data presented are mean from three biological replicates. Error bars indicate standard deviation of the means and no significant difference was observed between the genotypes after Student-t-test analysis.

(C) Relevant section of mass spectra obtained from JAZ6-SUMO1 conjugated peptide fragmentation experiments. The peak representing JAZ6 peptide sequence carrying a SUMO1 signature peptide fragment QTGG on residue K221 is indicated on the amino acid sequence.

(D) Immunoblots indicating reduced SUMOylation and protein abundance of 35S::-JAZ6^{K221R}:GFP in WT or 35S::JAZ6^{K221R}:GFP in the *ots1 ots2* backgrounds compare to the 35S::JAZ6:GFP in WT and *ots1 ots2* background. Proteins were immunoblotted (IB) and probed with anti-GFP (αGFP) or anti-AtSUMO1/2 (αSUMO1) antibodies. S1-JAZ6:GFP indicates SUMOylated JAZ6:GFP proteins. Molecular weights are indicated on the left in kiloDaltons (kD). Ponceau staining indicating Rubisco levels was employed to determine protein loading for the immunoprecipitation (IP:αGFP). WT (non-transgenic) plants served as a negative control.

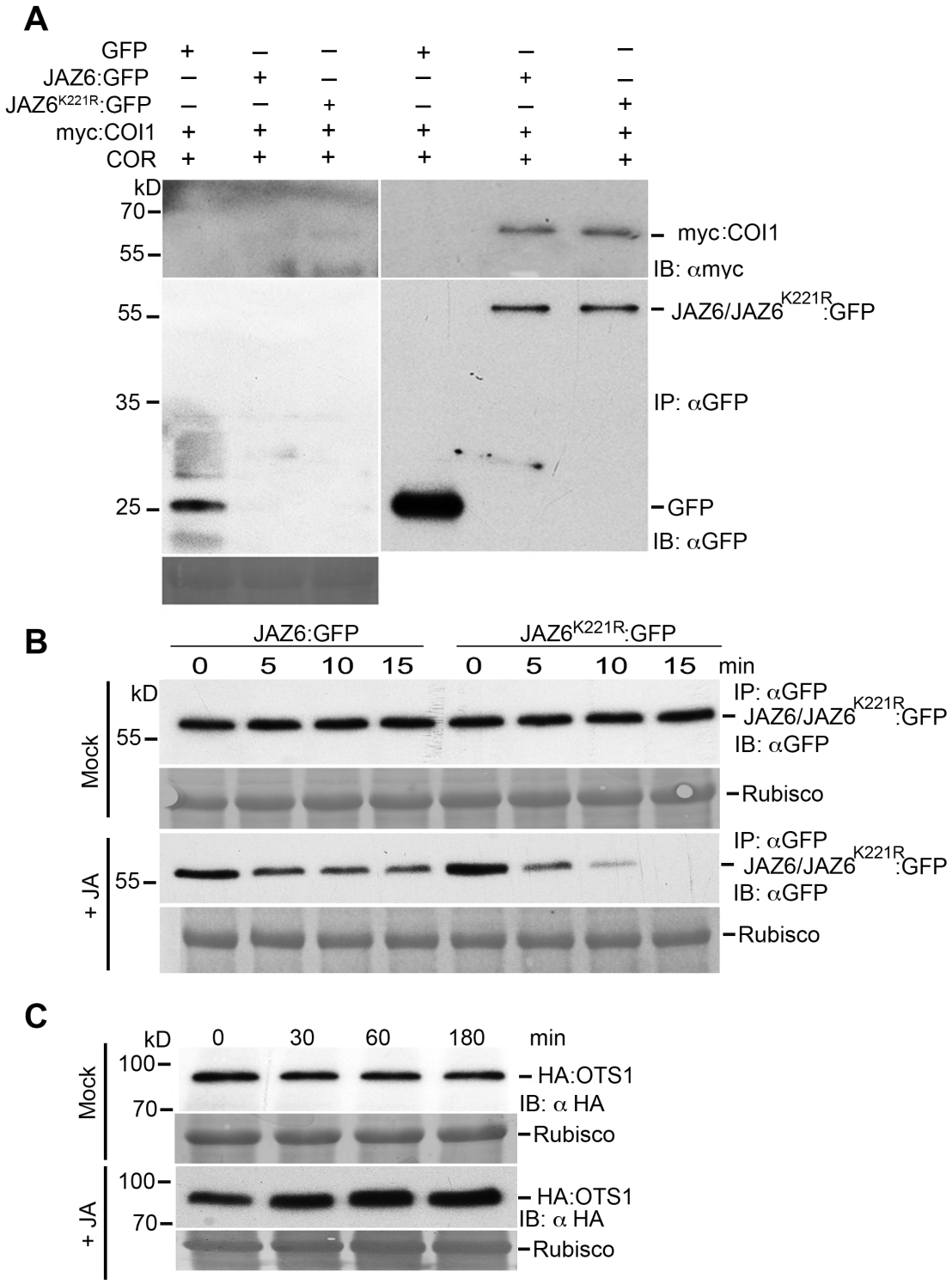


Figure 4. JAZ6 sumo site mutation affects the stability of JAZ6 protein but does not affect its interaction with COI1

(A) Co-immunoprecipitation of myc:COI1 with GFP only, JAZ6:GFP and JAZ6^{K221R}:GFP was performed in planta using N. benthamiana transient assays to investigate the interaction of JAZ6:GFP and JAZ6^{K221R}:GFP with myc:COI1 protein. Immunoprecipitates (IP: αGFP) were analyzed by SDS-PAGE and immunoblots were probed with αGFP to detect JAZ6:GFP and JAZ6^{K221R}:GFP and GFP alone and with αmyc to detect myc:COI1 proteins. Ponceau staining indicating Rubisco levels was employed to determine protein loading for the immunoprecipitation (IP:αGFP).

(B) JA mediated degradation of JAZ6:GFP and JAZ6^{K221R}:GFP proteins. Immunoblot probed with anti-GFP antibodies showing protein levels of 35S::JAZ6:GFP and 35S::JAZ6^{K221R}:GFP in respective seedlings treated with and without (mock treatment) JA (100μM). Seedling samples were collected at the indicated time points. Ponceau staining indicating Rubisco levels was employed to determine protein loading for the immunoprecipitation (IP:αGFP).

(C) Immunoblots probed with αHA (IB: αHA) indicating the accumulation of HA:OTS1 protein in 12 day old seedlings expressing 35S promoter driven HA-OTS1 transgene. Seedlings were treated with and without (mock) JA. Protein samples from seedlings were collected at the indicated time points. Ponceau red stained Rubisco protein was used to indicate total protein levels.

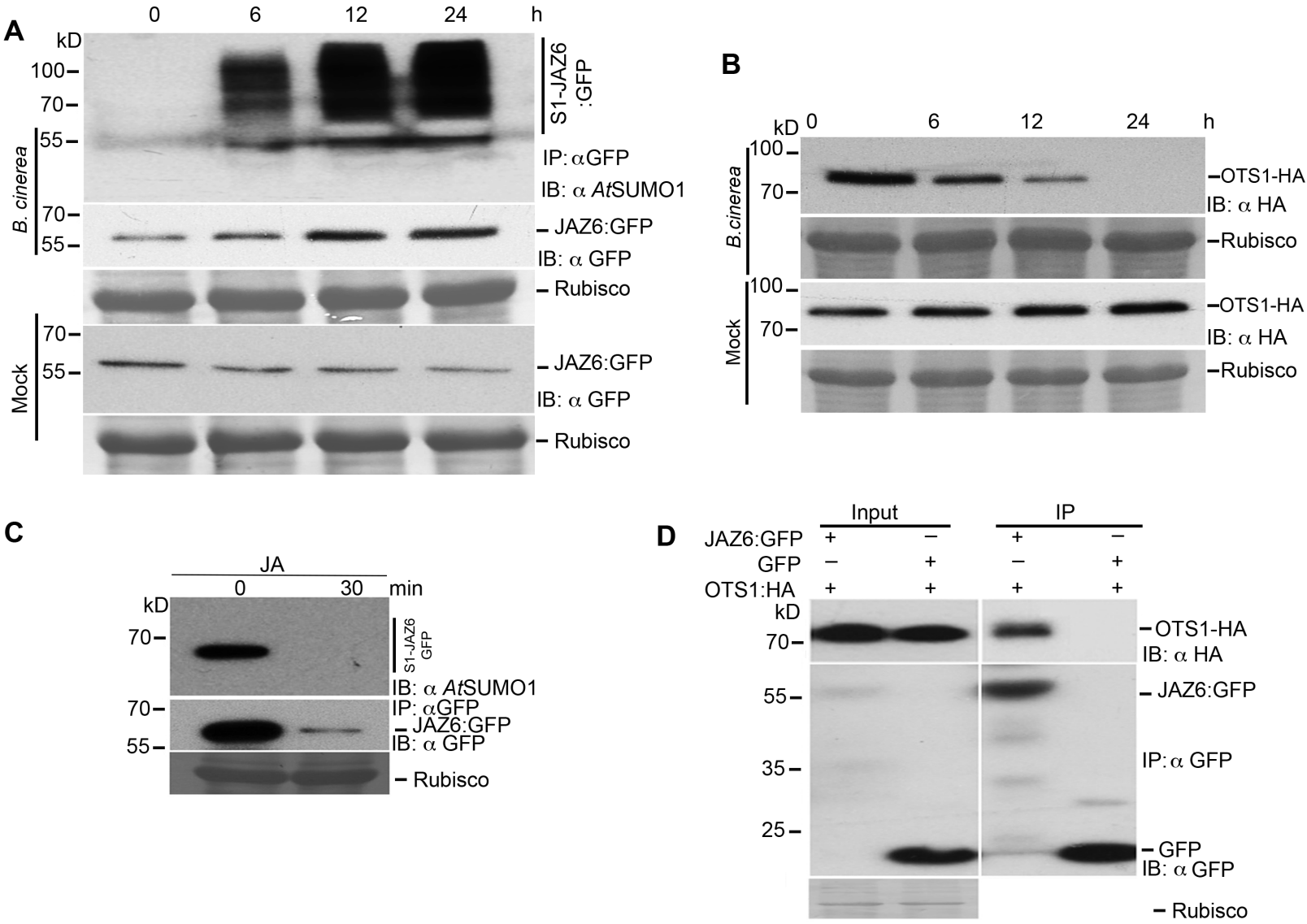


Figure 5. SUMOylation of JAZ6 is enhanced during Botrytis infection

(A) Immunoblots indicating significantly increased SUMOylation and protein abundance of GFP tagged JAZ6 from 4-week-old (35S:-JAZ6:GFP transgenics in WT background) plants infected with *B. cinerea.* Samples were collected at different time points post infection and mock treated samples were used for immunoprecipitation with anti-GFP antibodies (IP: αGFP). Immunoblots (IB) were probed with GFP (IB:αGFP) or *At*SUMO1/2 antibodies (IB:αSUMO1). Ponceau staining indicating Rubisco levels was employed to determine protein loading for the immunoprecipitation (IP:αGFP).

(B) Immunoblots probed with anti-HA antibodies showing HA-OTS1 levels in 35S::OTS1-HA transgenic Arabidopsis lines infected with *B. cinerea*. Four-week old 35S::OTS1-HA transgenic Arabidopsis leaves were pressure infiltrated with B. cinerea and mock treated with Magnesium chloride solution. Protein extracts were harvested from leaf samples collected at different time points after infection. Ponceau red stained Rubisco protein was used to indicate total protein levels.

(C) Immunoblots indicating greatly reduced SUMOylation and protein abundance of GFP tagged JAZ6 from 15 day-old seedlings (35S:-JAZ6:GFP transgenics in WT background) treated with 100μM JA for 30 minutes. Protein samples were collected for immunoprecipitation with anti-GFP antibodies (IP: αGFP) at 0 and 30 minutes after treatment. Immunoblots (IB) were probed with GFP (αGFP) or *At*SUMO1/2 antibodies (αSUMO1). Ponceau staining indicating Rubisco levels was employed to determine protein loading for the immunoprecipitation (IP:αGFP).

(D) Co-immunoprecipitation of HA-OTS1 with JAZ6:GFP in planta. Agrobacterium cultures containing 35S::HA-OTS1 was mixed with *Agrobacterium* cultures containing either 35S::GFP or 35S::JAZ6:GFP and transiently expressed in *N. benthamiana*. Total protein was extracted for immunoprecipitation with anti-GFP beads. Immunoprecipitates were analyzed by immunoblotting using anti-HA and anti-GFP antibody to detect for the presence of OTS1-HA or JAZ6:GFP respectively. Ponceau red stained Rubisco protein was used to indicate total protein levels used in the immunoprecipitation in the time points

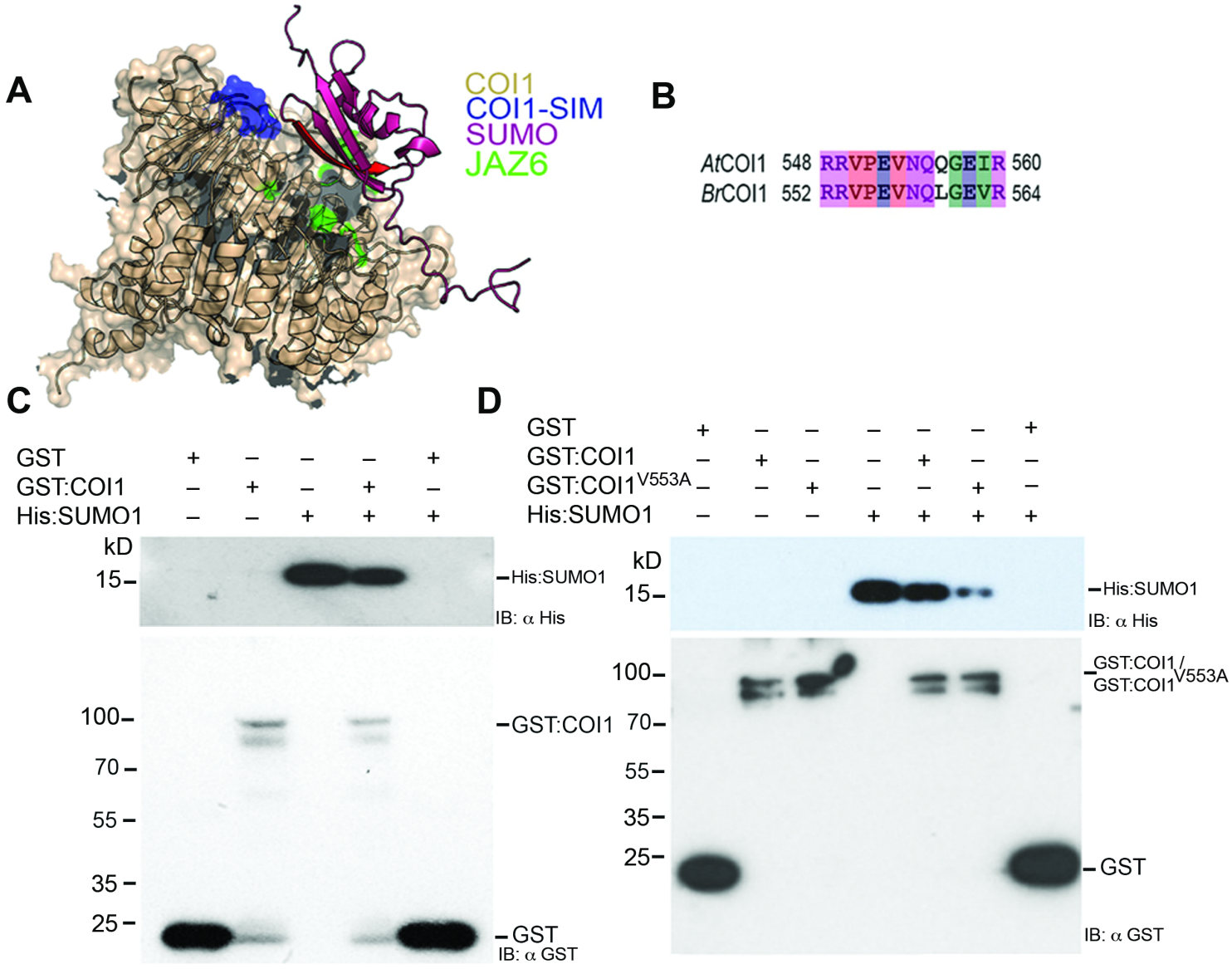


Figure 6. SUMO inhibits JA- receptor COI1 binding to JAZ proteins

(A) Side view of the COI1 JA receptor (beige) allows the identification of the location of the flexible loop forming the COI1 SIM motif (blue) residing at the interface between COI1 and the JAZ degron binding site (green). The binding of SUMO1 (pink) via its β-sheet (red), at this position can mask the COI1 domain that binds JAZ proteins. The binding of COI1 to SUMOylated JAZ through its SIM is therefore predicted to be able to disrupt binding of the non-SUMOylated JAZ to COI1.

(B) Cross species alignment of COI1 SIM from Arabidopsis and Brassica. *At*COI1; *Arabidopsis thaliana* COI1; *Br*COI1; *Brassica rapa* COI1 Residues are colored according to properties: red, hydrophobic; blue, acidic; magenta, basic; green, hydrophilic.

(C) GST pull down assays between recombinant His:SUMO1 with recombinant GST:COI1 or GST only indicate that GST:COI1 binds to SUMO1.

(D) GST pull down assays between recombinant His:SUMO1 with recombinant GST:COI1, SIM site mutated GST:COI1V553A; or GST only. The data indicates that Valine at position 553 is critical for SUMO1 binding.

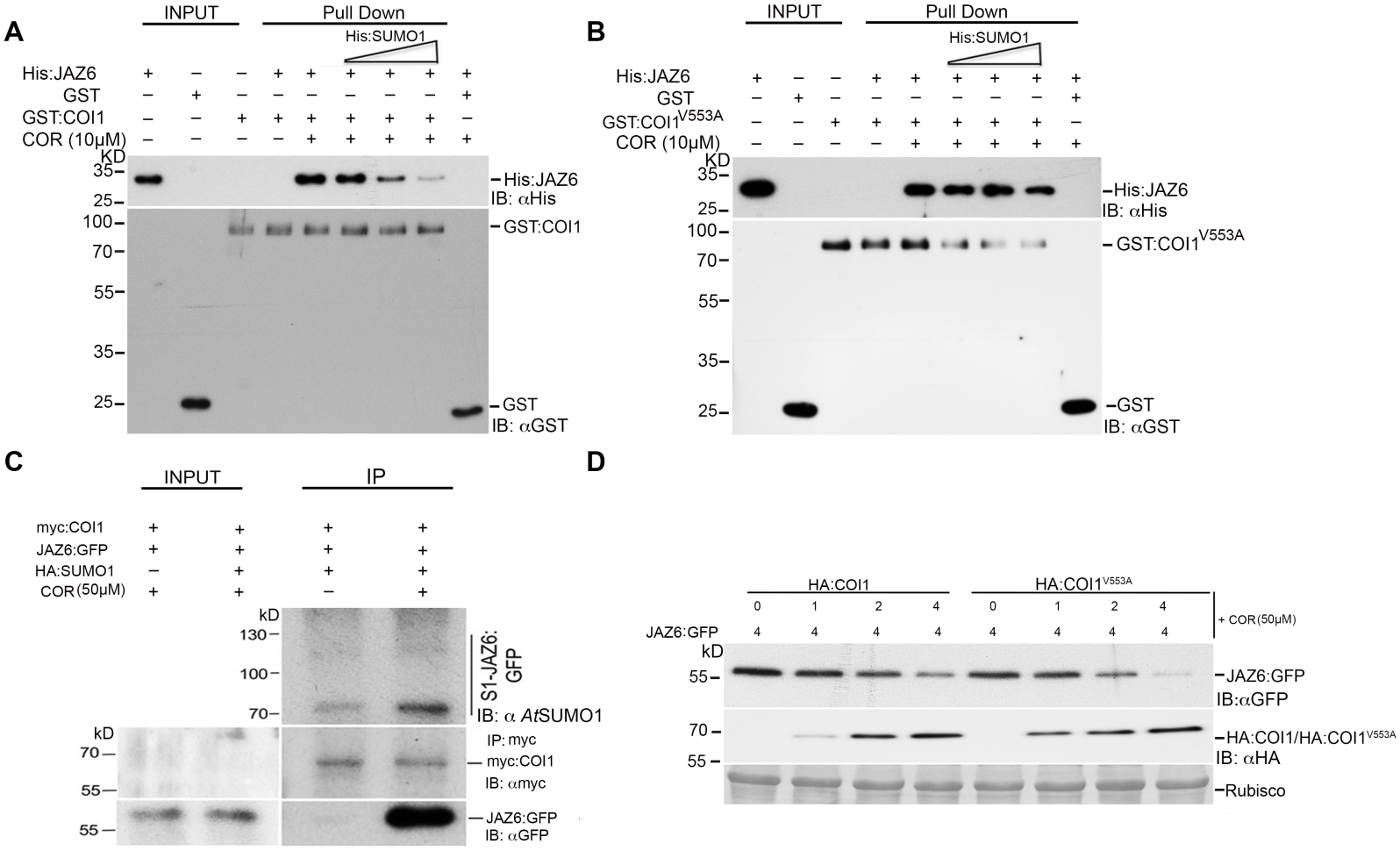


Figure 7. SUMOylated JAZ6 negatively regulates COI1-JAZ6 interaction

(A) GST pull down assays indicate that interaction between His:JAZ6 and GST:COI1 is weakened by the addition of increasing amounts of His:SUMO1 protein. His:JAZ6 protein mixed with different amounts of His:SUMO1 and pulled down with either GST:COI1 or GST alone in the presence or absence of coronatine (10μM). The eluates were then probed with anti-His tag (αHis) antibody or anti-GST (αGST) antibodies to detect His:JAZ6 or GST tagged proteins respectively.

(B) GST pull down assays performed as above but with GST:COI1 replaced by the COI1 SIM mutant GST:COI1^{V553A} indicate that mutation of Valine to Alanine rescues the interaction between GST:COI1^{V553A} and His:JAZ6 even in presence of His:SUMO1 protein. The eluates were probed with anti-His tag (αHis) antibody or anti-GST (αGST) antibodies to detect His-JAZ6 or GST tagged proteins respectively.

(C) Co-immunoprecipitation of JAZ6:GFP with myc:COI1 in planta indicates that SUMOylated JAZ6:GFP binds to myc:COI1 even in the absence of JA mimic coronatine. Agrobacterium culture containing 35S::JAZ6:GFP was mixed with *Agrobacterium* cultures containing both 35S::myc:COI1 and 35S::HA:SUMO1 and transiently expressed in *N. benthamiana*. Total protein was extracted for immunoprecipitation with anti-myc antibodies (IP; αmyc) to pull down myc-COI1 and the immunoprecipitates were probed with anti-SUMO, anti-myc (IB: αmyc) and anti-GFP(IB: αGFP) antibodies to detect for the presence of SUMOylated and Non-SUMOylated JAZ6:GFP and myc:-COI1. Ponceau staining indicating Rubisco levels was employed to determine protein loading for the immunoprecipitation (IP:αGFP).

(D) In vivo degradation of JAZ6 was observed in co-infiltration experiments with increasing amounts of HA:COI1 or HA:COI1^{V553A} in presence of 50μM coronatine. The ratio of the relative concentration of agrobacteria used in the different co-infiltrations is indicated by numbers (top). Cell extracts were analysed by immunoblot analysis with anti-GFP and anti-HA antibodies. Immunoblot analysis indicated that JAZ6:GFP was more unstable in plants transiently expressing HA:COI1^{V553A} when compared to plants expressing HA:COI1. Ponceau red stained Rubisco protein was used as a loading control.

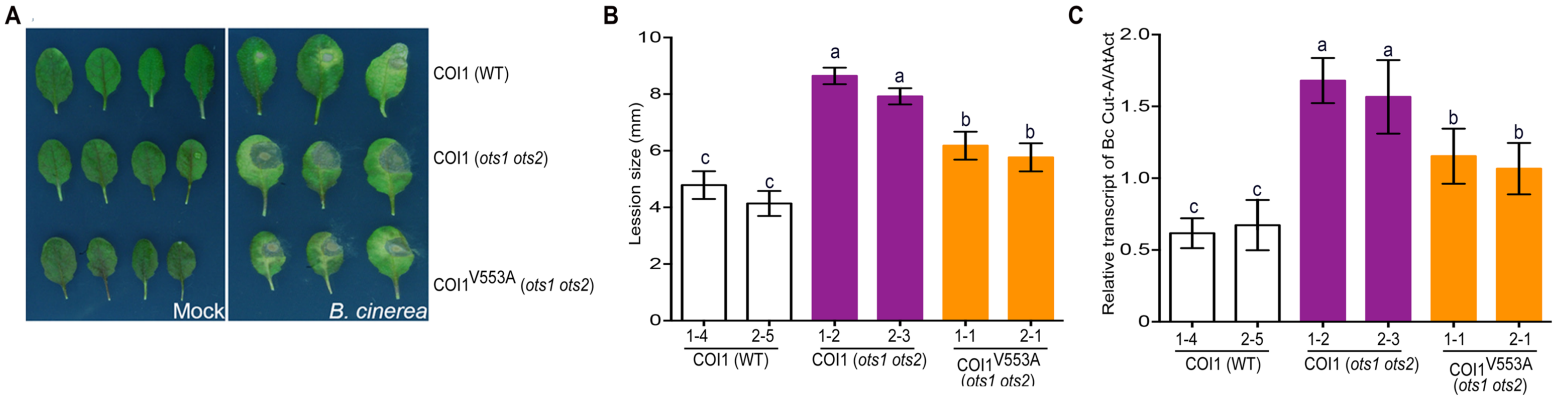


Figure 8. COI1 SIM mutant suppresses *Botrytis* susceptibility

(A) White light images of representative leaves from 4 week old transgenic plants expressing 35S::COI1:GFP in the wildtype (WT), *ots1 ots2* and 35S::-COI1^{V553A} :GFP in the *ots1 ots2* background at 72 hours post infection after inoculation with mock (left panel) or *B. cinerea* spores (right panel).

(B) Quantification of lesion sizes on rosette leaves at 72 hours post infection with *B. cinerea* spores. Values represent the means + SD of three biological replicates of 4 weeks old transgenic plants. The letters indicate averages that are statistically significantly different from each other (*P < 0.05, **P< 0.01).

(C) Quantification of fungal growth by real-time PCR on *Botrytis* genomic DNA with B. cinerea cutinase specific primers at 72 hours post infection. Histograms represent the means $\pm$ SD of three biological replicates of 4 weeks old transgenic plants. The letters indicate averages that are statistically significantly different (P < 0.05) from each other. n = 15 to 20, four weeks old plant in each replicate.

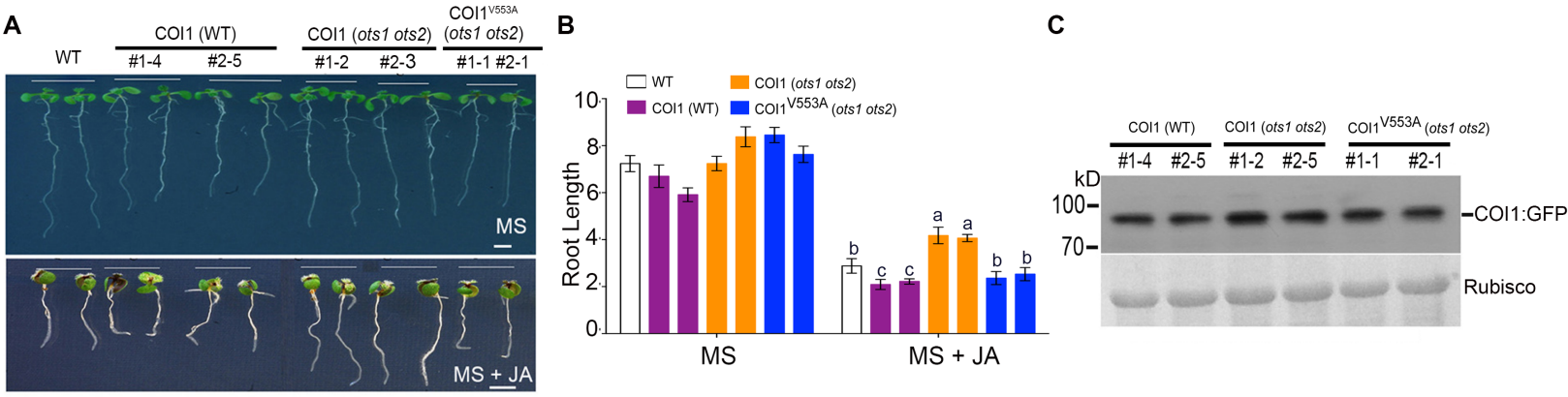


Figure 9. COI1 SIM mutant restores JA sensitivity in ots1 ots2 mutant background

(A) Image of representative 10-day-old seedlings grown in MS and MS + JA *(10 μM) and the effect of JA on root length of different transgenic plants. Scale bar represents* 1 cm.

(B) Mean root length of 10-day-old seedlings in the presence of 10 μM JA relative to the controls. Values represent the means ±SD of three biological replicates. The letters indicate significant difference between WT and the transgenic lines of COI1 (WT), COI1 (*ots1 ots2*) and COI1^{V553A} (*ots1 ots2*) in presence of JA. n = 35 to 40 seedlings each replicate.

(C) Immunoblots probed with αGFP indicating COI1:GFP and COI1^{V553A}:GFP protein levels in WT and *ots1 ots2* background.

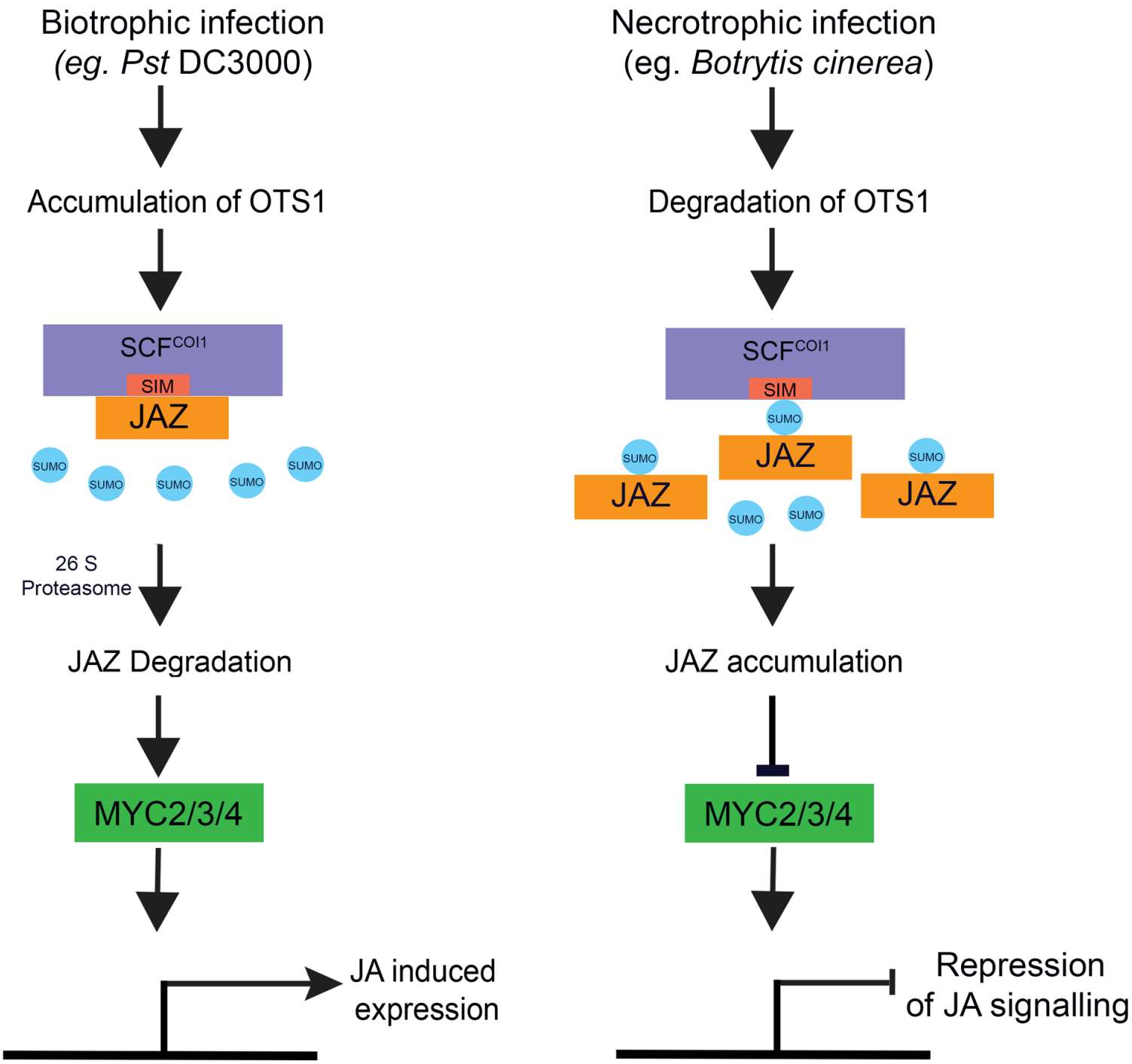
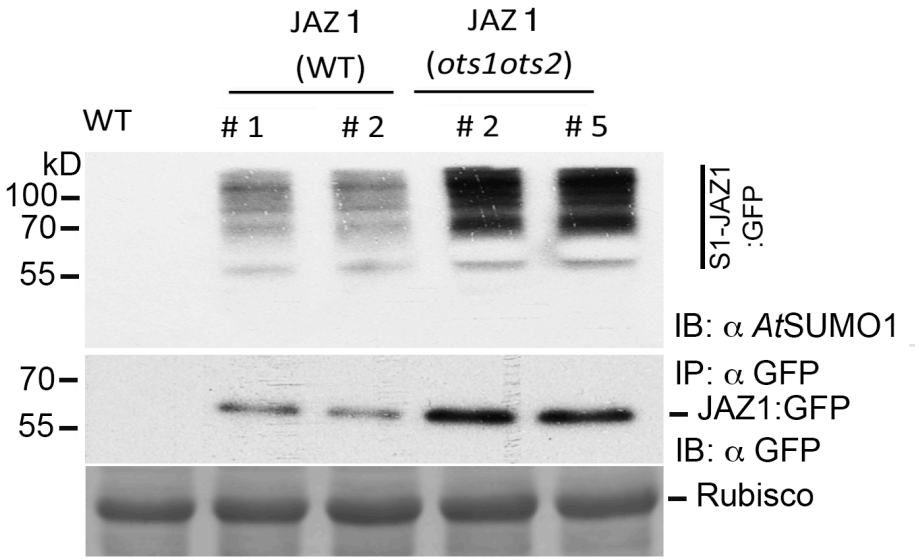
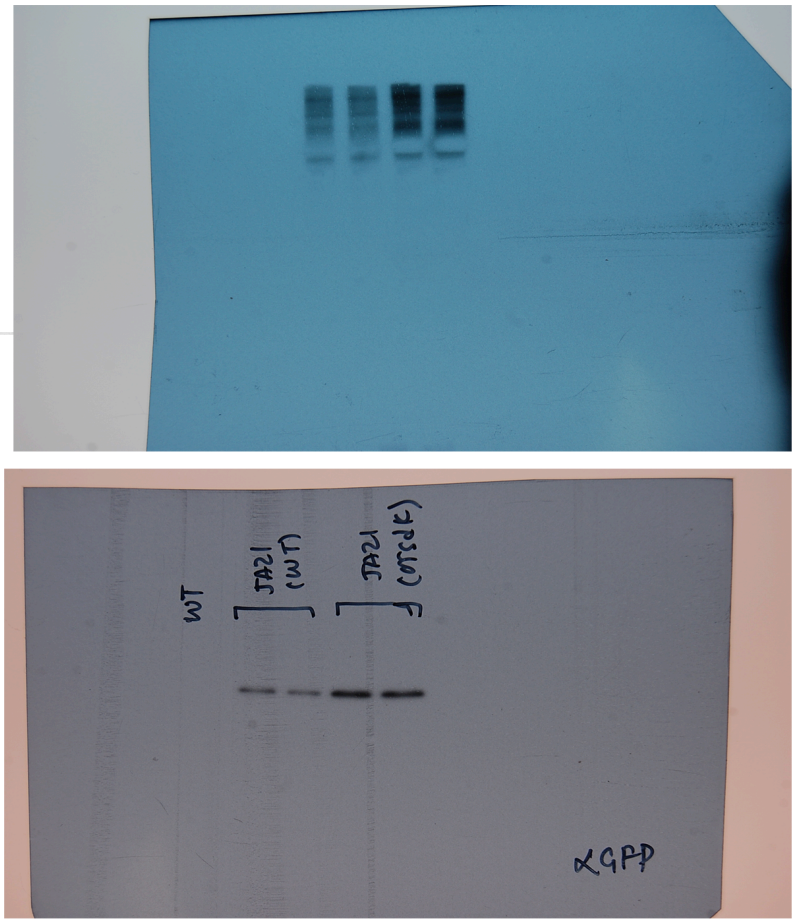


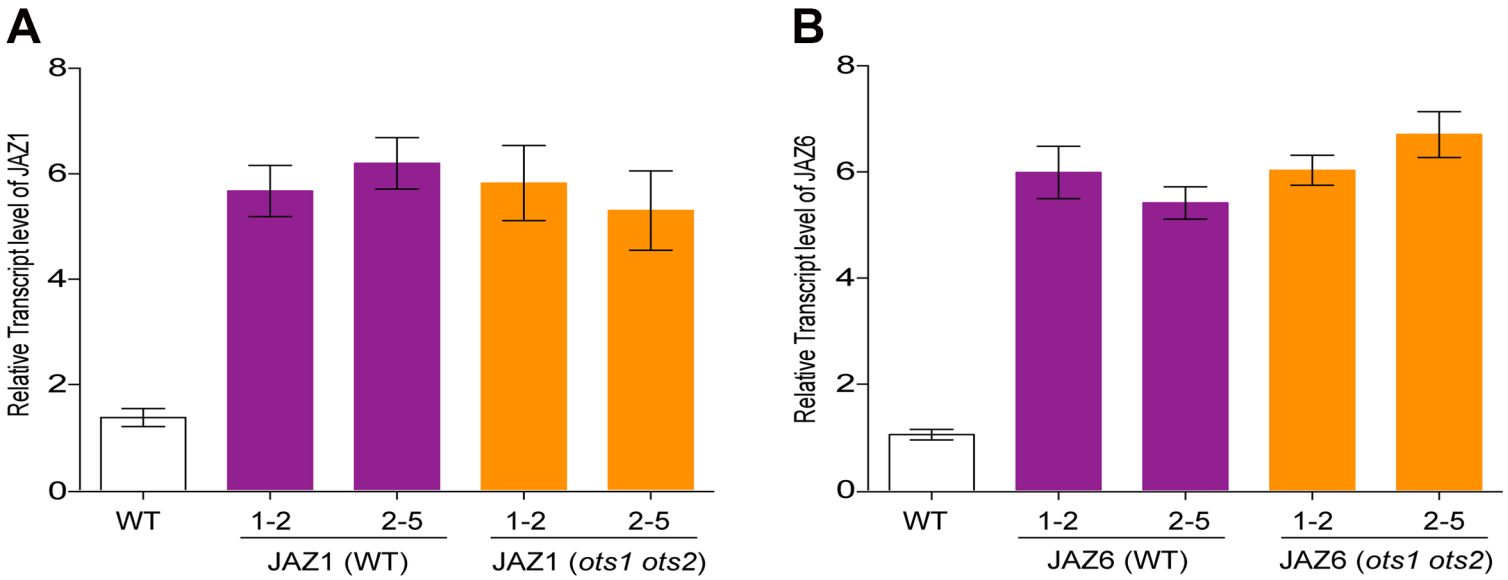
Figure 10. A model for JA signaling repression by SUMOylated JAZ proteins.
During hemibiotroph infection, such as by *Pst*, bacterial coronatine promotes JA signaling by activating the 26S proteasome mediated degradation of JAZ repressors by the JA receptor CORONATINE INSENSITIVE1 (COI1). Part of this process involves JA mediated accumulation of the SUMO deconjugating protease, OTS1 that rapidly deSUMOylates JAZ repressors and facilitates COI1 access to JAZ for degradation. JAZ repressor turnover activates JA-responsive gene expression through the transcriptional regulators such as MYC2/MYC3/-MYC4. On the other hand infection by necrotrophs such as the fungal pathogen, Botrytis cinerea stimulates, degradation of the SUMO deconjugating protease OTS1. This leads to the accumulation of SUMOylated JAZ proteins (this does not preclude increased SUMO conjugating via hitherto unknown mechanisms) that inhibit COI1 mediated degradation of non-SUMOylated JAZ repressors. Consequently, suppressing JA signaling.



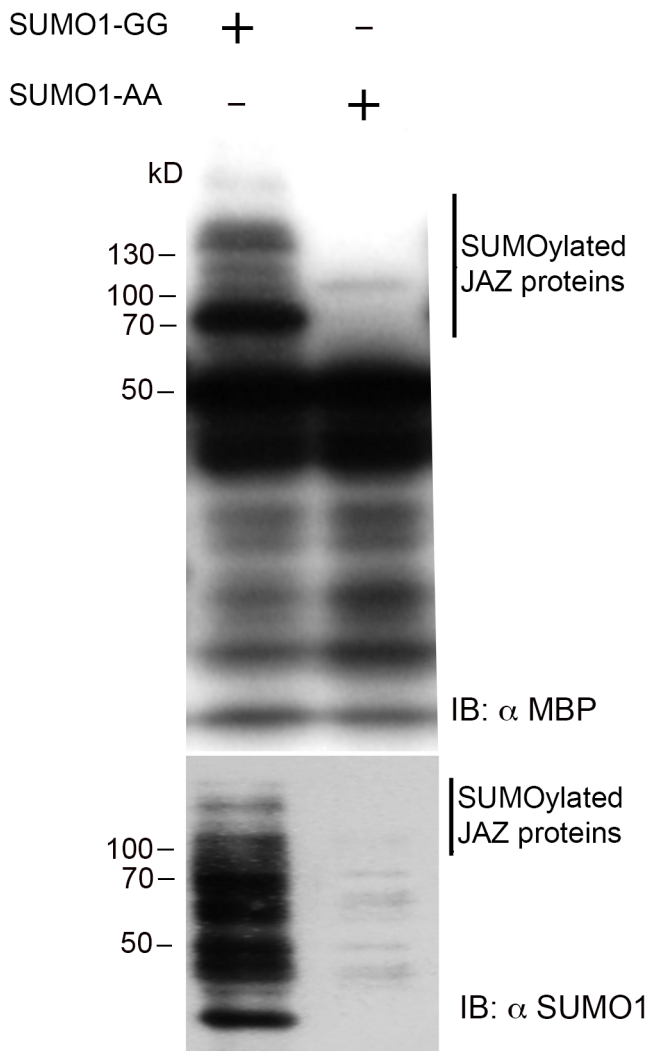


Supplemental Figure 1. JAZ1 protein accumulation and SUMOylation (Supports Figure 3).

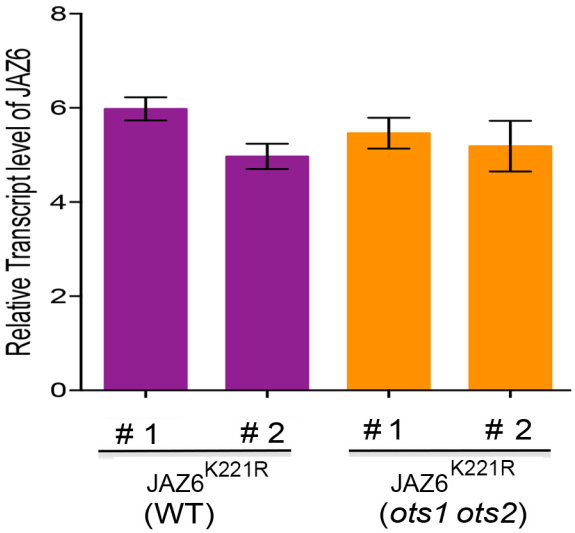
Immunoblots probed with Immunoprecipitations (IP: αGFP) of total proteins derived from 4 week old plant leaves of wildtype (WT) or 35S::JAZ1:GFP (WT background) or 35S::JAZ1:GFP (*ots1 ots2* background). Proteins were immunoblotted (IB) and probed with anti-GFP (αGFP) or anti-AtSUMO1/2 antibodies (αSUMO1). S1-JAZ1:GFP indicates SUMOylated JAZ1:GFP proteins. Molecular weights are indicated on the left in kiloDaltons (kD). Ponceau staining indicating Rubisco levels was employed to determine protein loading for the immunoprecipitation. WT (non-transgenic) plants served as a negative control. Right panel indicates the full blot probed with αGFP (lower) and α SUMO1 (Upper).



Supplemental Figure 2. Relative transcript levels of JAZ1 and 6 in the different transgenic plants (Supports Figure 3). **(A)** Quantitative real time PCR was used to analyse JAZ1 mRNA from twelve day old seedlings of transgenic plants expressing 35S::JAZ1:GFP in WT and *ots1 ots2* genetic background and **(B)** JAZ6 gene expression in 35S::JAZ6:GFP in WT and *ots1 ots2* genetic backgrounds. Actin was used as the internal control. Histograms represent the mean ± SD from three independent biological replicates.

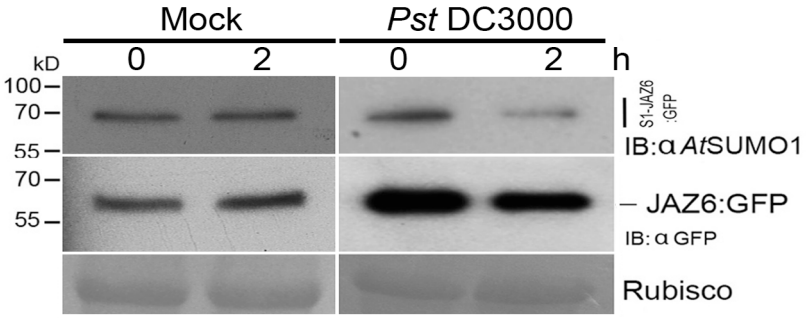


Supplemental Figure 3. Reconstituted in-vitro SUMOylation assay of JAZ6:MBP fusion protein (Supports Figure 3). Immunoblots probed with either anti-MBP and anti-AtSUMO1/2 antibodies show definitive SUMOylation of JAZ6:MBP. SUMO1-GG is the wildtype SUMO1 while SUMO1-AA is a mutant form of SUMO1 that cannot be conjugated to target proteins. Immunoblots were probed with anti-MBP (IB: αMBP) and anti-AtSUMO1 (IB: αSUMO1) antibodies to detect JAZ6:MBP or SUMO1-JAZ6:MBP



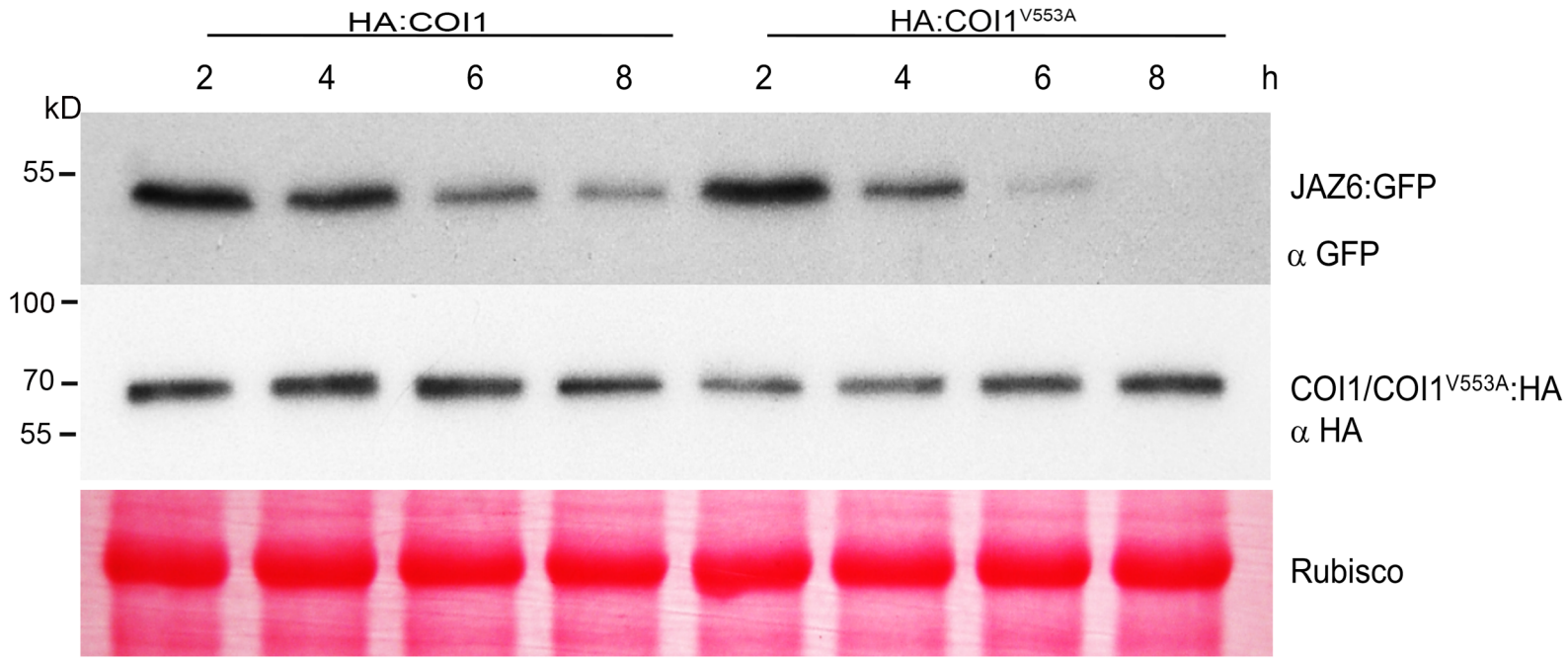
Supplemental Figure 4 (Supports Figure 3). Relative transcript levels of JAZ6 in different transgenic plants.

Quantitative real time PCR was used to analyze JAZ6^{K221R}:GFP mRNA from twelve-day old seedlings of transgenic plants expressing 35S::-JAZ6^{K221R}:GFP in WT and *ots1 ots2* genetic backgrounds. Actin was used as the internal control. Data are mean ± SD of three biological replicates.

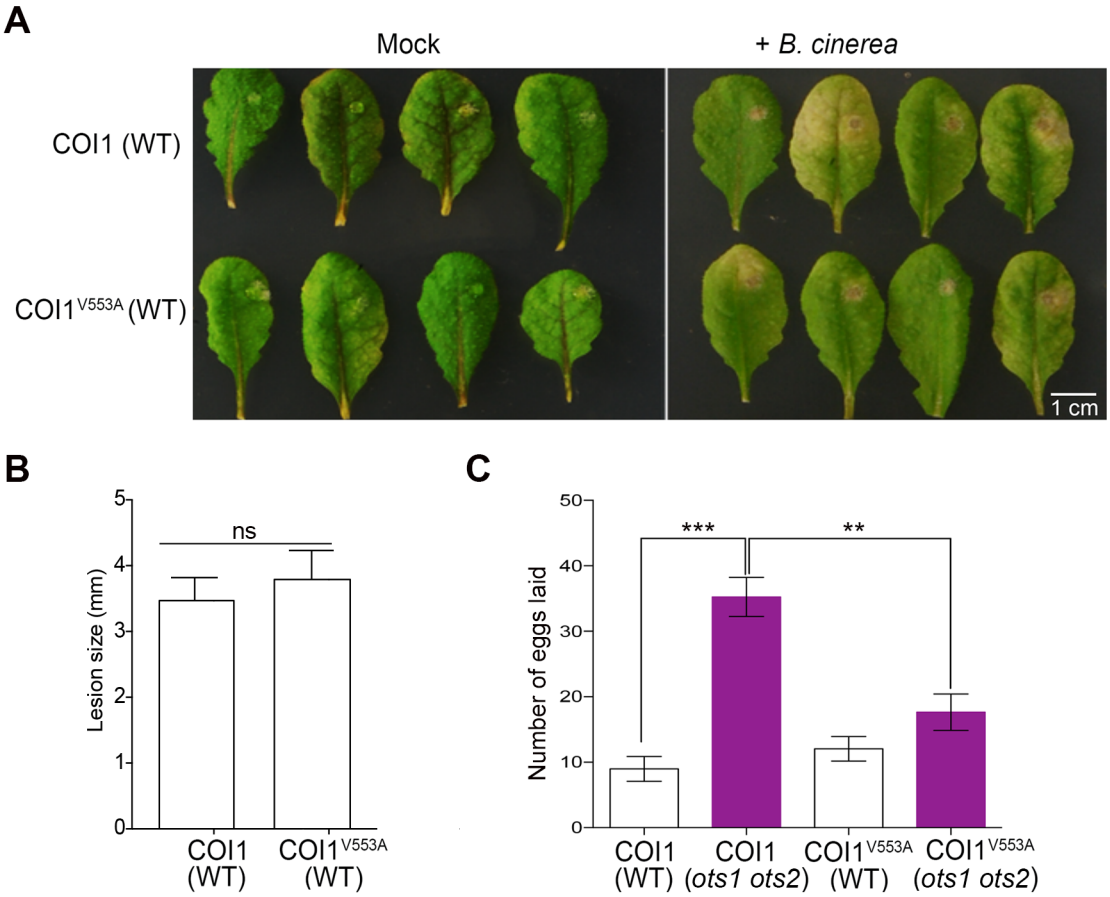


Supplemental Figure 5. Pst DC3000 infection negatively regulates JAZ6:GFP SUMOylation and accumulation (Supports Figure 5).

Immunoblots indicating SUMOylation and protein accumulation of JAZ6:GFP from four-week-old transgenic 35S::JAZ6:GFP plants pressure infiltrated with virulent *Pst*. Samples were collected for immunoprecipitation with anti-GFP antibodies (IP: α GFP) at the indicated time points. Immunoblots (IB) were probed with GFP (αGFP) or *At*SUMO1/2 antibodies (αSUMO1). Ponceau staining indicating Rubisco levels was employed to determine protein loading for the immunoprecipitation (IP:αGFP).



Supplemental Figure 6. JAZ6:GFP is more unstable in plants transiently expressing the SIM mutant COI1^{V553A} (Support Figure 7).
The DNA constructs HA:COI1, HA:COI1^{V553A} and GFP:JAZ6 were transiently co-expressed in *N. benthamiana* leaves in the presence of coronatine. Total protein extracted at different time points after coronatine treatment were analysed by immunoblot analysis with anti-GFP and anti-HA antibodies. Immunoblot analysis indicated that JAZ6:GFP was more unstable in plants transiently expressing HA:COI1^{V553A} when compared to plants expressing HA:COI1. Ponceau red stained Rubisco protein was used as a loading control.



Supplemental Figure 7 (Supports Figure 9). COI1V553A:GFP SIM mutant plants show significantly increased resistance to *B. cinerea* infection and spider mite infestation in the *ots1 ots2* genetic background.

(a) White light Images of representative leaves from 4 week old transgenic plants expressing 35S::COI1:GFP and 35S::COI1^{V553A}:GFP in wildtype background at 72 hours post-infection with *B. cinerea* spores.

(b) Quantification of lesion sizes on rosette leaves at 72 hours post-infection with *B. cinerea* spores.

(c) Egg counts from adult female mite infestation for 5 days on Arabidopsis plants. Data shows mean ± SD of eggs laid on seven independent plants from each genotype. Asterisks denote statistical significance of the differences between COI1 (WT), COI1 (*ots1 ots2*) and COI1^{V553A} (*ots1 ots2*) using Two-way-ANOVA (*P < 0.05, ***P< 0.001).

Table S1. List of DNA oligonucleotides used in the study.

Table of DNA primers employed for generating DNA constructs and real-time PCR.

Name	Forward	Reverse
JAZ6 FL	CACCATGTCAACGGGACAAGCGCC	AAGCTTGAGTTCAAGGTTTTTGG
JAZ1 FL	CACCATGTCGAGTTCTATGGAATG	TATTTCAGCTGCTAAACCG
COI1 FL	CACCATGGAGGATCCTGATATCAAG	TATTGGCTCCTTCAGGAC
JAZ6K221R	TCTTCCTCCCAGGCCAGAGATGG	CCATCTCTGGCCTGGGGAGGAAGA
COI1V553A	CCCGGAAGCGAATCAAC	GTTGATTCGCTTCCGGG
JAZ6 RT	GAACTCGCCGGAAAATTCGA	TGCTACTTTTGCCGGTTCAC
JAZ1 RT	AGCTTCACTTCACCGGTTCT	TGAAGACGCTTTGGCTGGA
COI1 RT	GAGATGGAGCATCCGGCTCA	TGGCTCCTTCAGGACTCTAACA
PDF1.2 RT	CACCCTTATCTTCGCTGCTCTT	TACACTTGTGTGCTGGGAAGAC
ERF1 RT	TTCCCCTTCAACGAGAACGA	GTTTGTTGCGTGGACTGCT
LOX2 RT	AATGAGCCTGTTATCAATGC	CATACTTAACAACACCAGCTCC
ZAT 10 RT	ACA TCC CTC CGA TCC CTG AA	ACC GGA AAG TCA AAC CGA GG
Actin RT	CTGGAATGGTGAAGGCTGGT	GTGCCTAGGACGACCAA
BOT_CUT_A RT	GATGTGACGGTCATCTTTGCCC	AGATTTGAGAGCGGCGAGG

Parsed Citations

Achard, P., and Genschik, P. (2009). Releasing the brakes of plant growth: how GAs shutdown DELLA proteins. J Exp Bot 60, 1085-1092.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Achard, P., Gong, F., Cheminant, S., Alioua, M., Hedden, P., and Genschik, P. (2008). The cold-inducible CBF1 factor-dependent signaling pathway modulates the accumulation of the growth-repressing DELLA proteins via its effect on gibberellin metabolism. Plant Cell 20, 2117-2129.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Aubert, Y., Widemann, E., Miesch, L., Pinot, F., and Heitz, T. (2015). CYP94-mediated jasmonoyl-isoleucine hormone oxidation shapes jasmonate profiles and attenuates defence responses to Botrytis cinerea infection. J Exp Bot 66, 3879-3892.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Bailey, M., Srivastava, A., Conti, L., Nelis, S., Zhang, C., Florance, H., Love, A., Milner, J., Napier, R., Grant, M., and Sadanandom, A. (2016). Stability of small ubiquitin-like modifier (SUMO) proteases OVERLY TOLERANT TO SALT1 and -2 modulates salicylic acid signalling and SUMO1/2 conjugation in Arabidopsis thaliana. J Exp Bot 67, 353-363.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Brooks, D.M., Bender, C.L., and Kunkel, B.N. (2005). The Pseudomonas syringae phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in Arabidopsis thaliana. Mol Plant Pathol 6, 629-639.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Brooks, D.M., Hernández-Guzmán, G., Kloek, A.P., Alarcón-Chaidez, F., Sreedharan, A., Rangaswamy, V., Peñaloza-Vázquez, A., Bender, C.L. and Kunkel, B.N. (2004). Identification and characterization of a well-defined series of coronatine biosynthetic mutants of Pseudomonas syringae pv. tomato DC3000. Mol. Plant Microbe Interact. 17, 162–174.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Bueso, E., Rodriguez, L., Lorenzo-Orts, L., Gonzalez-Guzman, M., Sayas, E., Munoz-Bertomeu, J., Ibanez, C., Serrano, R., and Rodriguez, P.L. (2014). The single-subunit RING-type E3 ubiquitin ligase RSL1 targets PYL4 and PYR1 ABA receptors in plasma membrane to modulate abscisic acid signaling. Plant J 80, 1057-1071.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Capili, A.D., and Lima, C.D. (2007). Structure and analysis of a complex between SUMO and Ubc9 illustrates features of a conserved E2-Ubl interaction. J Mol Biol 369, 608-618.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Chico, J.M., Chini, A., Fonseca, S., and Solano, R. (2008). JAZ repressors set the rhythm in jasmonate signaling. Curr Opin Plant Biol 11, 486-494.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Chini, A., Fonseca, S., Fernandez, G., Adie, B., Chico, J.M., Lorenzo, O., Garcia-Casado, G., Lopez-Vidriero, I., Lozano, F.M., Ponce, M.R., Micol, J.L., and Solano, R. (2007). The JAZ family of repressors is the missing link in jasmonate signalling. Nature 448, 666-671.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Conti, L., Price, G., O'Donnell, E., Schwessinger, B., Dominy, P., and Sadanandom, A. (2008). Small ubiquitin-like modifier proteases OVERLY TOLERANT TO SALT1 and -2 regulate salt stress responses in Arabidopsis. Plant Cell 20, 2894-2908.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Conti, L., Nelis, S., Zhang, C., Woodcock, A., Swarup, R., Galbiati, M., Tonelli, C., Napier, R., Hedden, P., Bennett, M., and Sadanandom, A. (2014). Small Ubiquitin-like Modifier protein SUMO enables plants to control growth independently of the phytohormone gibberellin. Dev Cell 28, 102-110.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

de Lucas, M., Daviere, J.M., Rodriguez-Falcon, M., Pontin, M., Iglesias-Pedraz, J.M., Lorrain, S., Fankhauser, C., Blazquez, M.A., Titarenko, E., and Prat, S. (2008). A molecular framework for light and gibberellin control of cell elongation. Nature 451, 480-484.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Feng, S., Martinez, C., Gusmaroli, G., Wang, Y., Zhou, J., Wang, F., Chen, L., Yu, L., Iglesias-Pedraz, J.M., Kircher, S., Schafer, E., Fu, X., Fan, L.M., and Deng, X.W. (2008). Coordinated regulation of Arabidopsis thaliana development by light and gibberellins. Nature 451,

475-479.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Fernandez-Calvo, P., Chini, A., Fernandez-Barbero, G., Chico, J.M., Gimenez-Ibanez, S., Geerinck, J., Eeckhout, D., Schweizer, F., Godoy, M., Franco-Zorrilla, J.M., Pauwels, L., Witters, E., Puga, M.I., Paz-Ares, J., Goossens, A., Reymond, P., De Jaeger, G., and Solano, R. (2011). The Arabidopsis bHLH transcription factors MYC3 and MYC4 are targets of JAZ repressors and act additively with MYC2 in the activation of jasmonate responses. Plant Cell 23, 701-715.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Feys, B., Benedetti, C.E., Penfold, C.N., and Turner, J.G. (1994). Arabidopsis Mutants Selected for Resistance to the Phytotoxin Coronatine Are Male Sterile, Insensitive to Methyl Jasmonate, and Resistant to a Bacterial Pathogen. Plant Cell 6, 751-759.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Fonseca, S., Chico, J.M., and Solano, R. (2009). The jasmonate pathway: the ligand, the receptor and the core signalling module. Curr Opin Plant Biol 12, 539-547.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Forcat, S., Bennett, M.H., Mansfield, J.W., and Grant, M.R. (2008). A rapid and robust method for simultaneously measuring changes in the phytohormones ABA, JA and SA in plants following biotic and abiotic stress. Plant Methods 4, 16.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Geiss-Friedlander, R., and Melchior, F. (2007). Concepts in sumoylation: a decade on. Nat Rev Mol Cell Biol 8, 947-956.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Geng, X., Cheng, J., Gangadharan, A., and Mackey, D. (2012). The coronatine toxin of Pseudomonas syringae is a multifunctional suppressor of Arabidopsis defense. Plant Cell 24, 4763-4774.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu Rev Phytopathol 43, 205-227.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Grunewald, W., Vanholme, B., Pauwels, L., Plovie, E., Inze, D., Gheysen, G., and Goossens, A. (2009). Expression of the Arabidopsis jasmonate signalling repressor JAZ1/TIFY10A is stimulated by auxin. EMBO Rep 10, 923-928.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Hay, R.T. (2001). Protein modification by SUMO. Trends Biochem Sci 26, 332-333.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Hecker, C.M., Rabiller, M., Haglund, K., Bayer, P., and Dikic, I. (2006). Specification of SUMO1- and SUMO2-interacting motifs. J Biol Chem 281, 16117-16127.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Hickey, C.M., Wilson, N.R., and Hochstrasser, M. (2012). Function and regulation of SUMO proteases. Nat Rev Mol Cell Biol 13, 755-766.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Howe, G.A., and Jander, G. (2008). Plant immunity to insect herbivores. Annu Rev Plant Biol 59, 41-66.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Ingle, R.A., Stoker, C., Stone, W., Adams, N., Smith, R., Grant, M., Carre, I., Roden, L.C., and Denby, K.J. (2015). Jasmonate signalling drives time-of-day differences in susceptibility of Arabidopsis to the fungal pathogen Botrytis cinerea. Plant J 84, 937-948.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Jentsch, S., and Pyrowolakis, G. (2000). Ubiquitin and its kin: how close are the family ties? Trends Cell Biol 10, 335-342.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Katsir, L., Chung, H.S., Koo, A.J., and Howe, G.A. (2008). Jasmonate signaling: a conserved mechanism of hormone sensing. Curr Opin Plant Biol 11, 428-435.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Kazan, K., and Manners, J.M. (2008). Jasmonate signaling: toward an integrated view. Plant Physiol 146, 1459-1468.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Kerscher, O., Felberbaum, R., and Hochstrasser, M. (2006). Modification of proteins by ubiquitin and ubiquitin-like proteins. Annu Rev Cell Dev Biol 22, 159-180.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Koornneef, A., and Pieterse, C.M. (2008). Cross talk in defense signaling. Plant Physiol 146, 839-844.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Laurie-Berry, N., Joardar, V., Street, I.H., and Kunkel, B.N. (2006). The Arabidopsis thaliana JASMONATE INSENSITIVE 1 gene is required for suppression of salicylic acid-dependent defenses during infection by Pseudomonas syringae. Mol Plant Microbe In 19, 789-800.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Lee, J., Nam, J., Park, H.C., Na, G., Miura, K., Jin, J.B., Yoo, C.Y., Baek, D., Kim, D.H., Jeong, J.C., Kim, D., Lee, S.Y., Salt, D.E., Mengiste, T., Gong, Q., Ma, S., Bohnert, H.J., Kwak, S.S., Bressan, R.A., Hasegawa, P.M., and Yun, D.J. (2007). Salicylic acid-mediated innate immunity in Arabidopsis is regulated by SIZ1 SUMO E3 ligase. Plant J 49, 79-90.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Livak, K.J. and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCt method. Methods. 25, 402–408.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Melotto, M., Mecey, C., Niu, Y., Chung, H.S., Katsir, L., Yao, J., Zeng, W., Thines, B., Staswick, P., Browse, J., Howe, G.A. and He S.Y. (2008). A critical role of two positively charged amino acids in the JAS motif of Arabidopsis JAZ proteins in mediating coronatine- and jasmonyl isoleucine-dependant interactions with the COI1 F-box protein. Plant J 55, 979-988.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Mengiste, T. (2012). Plant immunity to necrotrophs. Annu Rev Phytopathol 50, 267-294.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Miller, M.J., Barrett-Wilt, G.A., Hua, Z., and Vierstra, R.D. (2010). Proteomic analyses identify a diverse array of nuclear processes affected by small ubiquitin-like modifier conjugation in Arabidopsis. Proc Natl Acad Sci U S A 107, 16512-16517.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Minty, A., Dumont, X., Kaghad, M., and Caput, D. (2000). Covalent modification of p73alpha by SUMO-1. Two-hybrid screening with p73 identifies novel SUMO-1-interacting proteins and a SUMO-1 interaction motif. J Biol Chem 275, 36316-36323.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Okada, S., Nagabuchi, M., Takamura, Y., Nakagawa, T., Shinmyozu, K., Nakayama, J., and Tanaka, K. (2009). Reconstitution of Arabidopsis thaliana SUMO pathways in E. coli: functional evaluation of SUMO machinery proteins and mapping of SUMOylation sites by mass spectrometry. Plant Cell Physiol 50, 1049-1061.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Pauwels, L., and Goossens, A. (2011). The JAZ proteins: a crucial interface in the jasmonate signaling cascade. Plant Cell 23, 3089-3100.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Pieterse, C.M. (2012). Prime time for transgenerational defense. Plant Physiol 158, 545.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Reeves, P.H., Murtas, G., Dash, S., and Coupland, G. (2002). early in short days 4, a mutation in Arabidopsis that causes early flowering and reduces the mRNA abundance of the floral repressor FLC. Development 129, 5349-5361.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Reverter, D., and Lima, C.D. (2009). Preparation of SUMO proteases and kinetic analysis using endogenous substrates. Methods Mol Biol 497, 225-239.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Sheard, L.B., Tan, X., Mao, H., Withers, J., Ben-Nissan, G., Hinds, T.R., Kobayashi, Y., Hsu, F.F., Sharon, M., Browse, J., He, S.Y., Rizo, J., Howe, G.A., and Zheng, N. (2010). Jasmonate perception by inositol-phosphate-potentiated COI1-JAZ co-receptor. Nature 468, 400-405.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Smirnova, E., Marquis, V., Poirier, L., Aubert, Y., Zumsteg, J., Menard, R., Miesch, L., and Heitz, T. (2017). Jasmonic Acid Oxidase 2 Hydroxylates Jasmonic Acid and Represses Basal Defense and Resistance Responses against Botrytis cinerea Infection. Mol Plant 10, 1159-1173.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Srivastava, A.K., Zhang, C., Caine, R.S., Gray, J., and Sadanandom, A. (2017). Rice SUMO protease Overly Tolerant to Salt 1 targets the transcription factor, OsbZIP23 to promote drought tolerance in rice. Plant J 92, 1031-1043.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Srivastava, A.K., Zhang, C., Caine, R.S., Gray, J., and Sadanandom, A. (2017). Rice SUMO protease Overly Tolerant to Salt 1 targets the transcription factor, OsbZIP23 to promote drought tolerance in rice. Plant J 92, 1031-1043.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Srivastava, A.K., Zhang, C., Yates, G., Bailey, M., Brown, A., and Sadanandom, A. (2016). SUMO Is a Critical Regulator of Salt Stress Responses in Rice. Plant Physiol 170, 2378-2391.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Staswick P.E. (2008). JAZing up jasmonate signaling. Trends Plant Sci 13, 66-71.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., Nomura, K., He, S.Y., Howe, G.A., and Browse, J. (2007). JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. Nature 448, 661-665.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Verger, A., Perdomo, J., and Crossley, M. (2003). Modification with SUMO. A role in transcriptional regulation. EMBO Rep 4, 137-142.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M., and Turner, J.G. (1998). COI1: an Arabidopsis gene required for jasmonate-regulated defense and fertility. Science 280, 1091-1094.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Yan, Y., Stolz, S., Chetelat, A., Reymond, P., Pagni, M., Debugnon, L. and Farmer E.E. (2007). A downstream mediator in the growth repression limb of the jasmonate pathway. Plant Cell 19, 2470-2483.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Zabala, M.D., Zhai, B., Jayaraman, S., Eleftheriadou, G., Winsbury, R., Yang, R., Truman, W., Tang, S.J., Smirnoff, N., and Grant, M. (2016). Novel JAZ co-operativity and unexpected JA dynamics underpin Arabidopsis defence responses to Pseudomonas syringae infection. New Phytol 209, 1120-1134.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title