

# The Ring-Type E3 Ubiquitin Ligase JUL1 Targets the VQ-Motif Protein JAV1 to Coordinate Jasmonate Signaling<sup>1</sup>[OPEN]

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Jasmonates regulate plant defense and development. In *Arabidopsis thaliana*, JASMONATE-ASSOCIATED VQ-MOTIF GENE1 (*JAV1/VQ22*) is a repressor of jasmonate-mediated defense responses and is degraded through the ubiquitin-26S proteasome system after herbivory. We found that *JAV1*-ASSOCIATED UBIQUITIN LIGASE1 (*JUL1*), a RING-type E3 ubiquitin ligase, interacted with *JAV1*. *JUL1* interacted with *JAV1* in the nucleus to ubiquitinate *JAV1*, leading to proteasomal degradation of *JAV1*. The transcript levels of *JUL1* and *JAV1* were coordinately and positively regulated by the CORONATINE INSENSITIVE1-dependent signaling pathway in the jasmonate signaling network, but in a manner that was not dependent on CORONATINE INSENSITIVE1-mediated signaling upon herbivory by *Spodoptera litura*. Gain or loss of function of *JUL1* modulated the expression levels of the defensin gene *PDF1.2* in leaves, conferring on the plants various defense properties against the generalist herbivore *S. litura*. Because neither the *JUL1* mutant nor overexpression lines showed any obvious developmental defects, we concluded that the *JAV1/JUL1* system functions as a specific coordinator of reprogramming of plant defense responses. Altogether, our findings offer insight into the mechanisms by which the *JAV1/JUL1* system acts specifically to coordinate plant defense responses without interfering with plant development or growth.

Jasmonates, a lipid-derived class of plant hormones, promote gene regulation for defense responses in plants. Jasmonates, including jasmonic acid (JA) and its active derivative jasmonyl-isoleucine (JA-Ile), play a vital role in defending plants against herbivorous insects and necrotrophic pathogens (McConn et al., 1997; Browse, 2009; Pieterse et al., 2012; Okada et al., 2015; Chini et al., 2016) and mediate many developmental processes in root growth, stamen development, and

senescence (Wasternack and Hause, 2013). Following JA signaling via the JA-Ile receptor CORONATINE INSENSITIVE1 (*COI1*), Jasmonate-ZIM domain proteins (*JAZs*) act as negative regulators of JA signaling (Chini et al., 2007; Thines et al., 2007). The JA-associated degradation of *JAZs* leads to transcriptional reprogramming of a vast array of genes controlled by transcription factors (TFs) such as *MYC2*, leading to the activation of defense responses and the modulation of numerous processes of plant growth and development, such as sexual maturation, trichome initiation, and root growth (Browse, 2009; Fernández-Calvo et al., 2011; Qi et al., 2011; Song et al., 2011, 2013; Wasternack and Hause, 2013; Goossens et al., 2017). Activation of JA signaling adversely affects plant growth through a tradeoff between JA-induced defense responses and gibberellin-promoted development of *Arabidopsis thaliana* plants, in which angiosperm plants prioritize JA-mediated defense over growth (Yang et al., 2012; Guo et al., 2018). It appears that JA signaling controls plant growth by suppressing the GA-mediated degradation of *DELTA*, a GA-responsive repressor of TFs (including phytochrome-interacting factors; Yang et al., 2012). Moreover, it appears that the plant growth-defense tradeoff is promoted via the relief of transcription repression rather than by the diversion of photoassimilates from growth to defense (Campos et al., 2016).

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Hu et al. (2013) identified JASMONATE-ASSOCIATED VQ-MOTIF GENE1 (JAV1, also known as VQ22) as a novel repressor of JA signaling in Arabidopsis. JAV1 is a member of a family of plant-specific proteins with the conserved short amino acid sequence motif FxxhVQxhTG, where x represents any amino acid and h represents a hydrophobic residue (Jing and Lin, 2015). This family plays various roles in plant defense responses, stress tolerance, and growth and development (Jing and Lin, 2015). JAV1 has been shown to specifically down-regulate jasmonate-mediated defense responses against a necrotrophic pathogen (*Botrytis cinerea*) and an herbivore (*Spodoptera exigua* larvae) without adversely impacting plant growth or development (Hu et al., 2013; Zhu and Zhu, 2013). Additionally, the JAV1-JAZ8-WRKY51 complex represses JA biosynthesis in healthy plants, but herbivory triggers the calmodulin-dependent phosphorylation of JAV1, which leads to JAV1 degradation and, consequently, to the release of WRKY51, thereby activating JA biosynthesis for plant defense (Yan et al., 2018).

Like JAZ degradation, JAV1 degradation requires JA signaling, which mediates JAV1 degradation through the ubiquitin-26S proteasome system (UPS) and, thereby, leads to the activation of defense genes, including *PDF1.2* (Hu et al., 2013). JAV1 is able to interact with not only WRKY51 but also WRKY28, a positive regulator of *PDF1.2* expression. However, the E3 ligase(s) specific to JAV1 degradation remain to be identified.

Ubiquitination is a posttranslational modification process that functions in all eukaryotes and is achieved by the consecutive action of three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). The Arabidopsis genome contains more than 1,400 genes encoding E3 ubiquitin ligases (Vierstra, 2009). These E3 ubiquitin ligases play critical roles in determining the substrate specificity of UPS for various target proteins, and the large number of E3 ligases implies their specific recognition of target substrates (Sadanandom et al., 2012). E3 ubiquitin ligases are classified into three families: the homology to E6-AP C-terminus E3 ligases, the really interesting new gene (RING) type of E3 ligases, and the U-box family members (Chen and Hellmann, 2013). RING-type E3 ligase family members can either ubiquitinate substrates independently or function as part of a multisubunit complex, which in plants includes Skp1-Cullin-F-box; Elongin B/C-Cul2/Cul5-SOCS-box protein; Broad-complex, Tramtrack, and Bric-à-brac; UV-damaged DNA-binding protein1; and anaphase promoting complex as independent complexes (Sadanandom et al., 2012).

There are more than 460 proteins in the RING-type protein family in Arabidopsis (Stone et al., 2005; Lee and Kim, 2011). These E3 ligases act in plant biotic stress tolerance (Marino et al., 2012; Duplan and Rivas, 2014) and play essential roles in the JA signaling pathway (Zhang et al., 2012; Nagels Durand et al., 2016b; Miricescu et al., 2018). However, the substrate targets of only a few E3 ligases have been identified. In this

study, we characterized the features of a RING-type E3 ligase that interacts with JAV1 in Arabidopsis. This E3 ligase, which we named JAV1-ASSOCIATED UBIQUITIN LIGASE1 (JUL1), was found to play a primary role in UPS for JAV1 degradation, leading to the positive regulation of defense responses.

## RESULTS

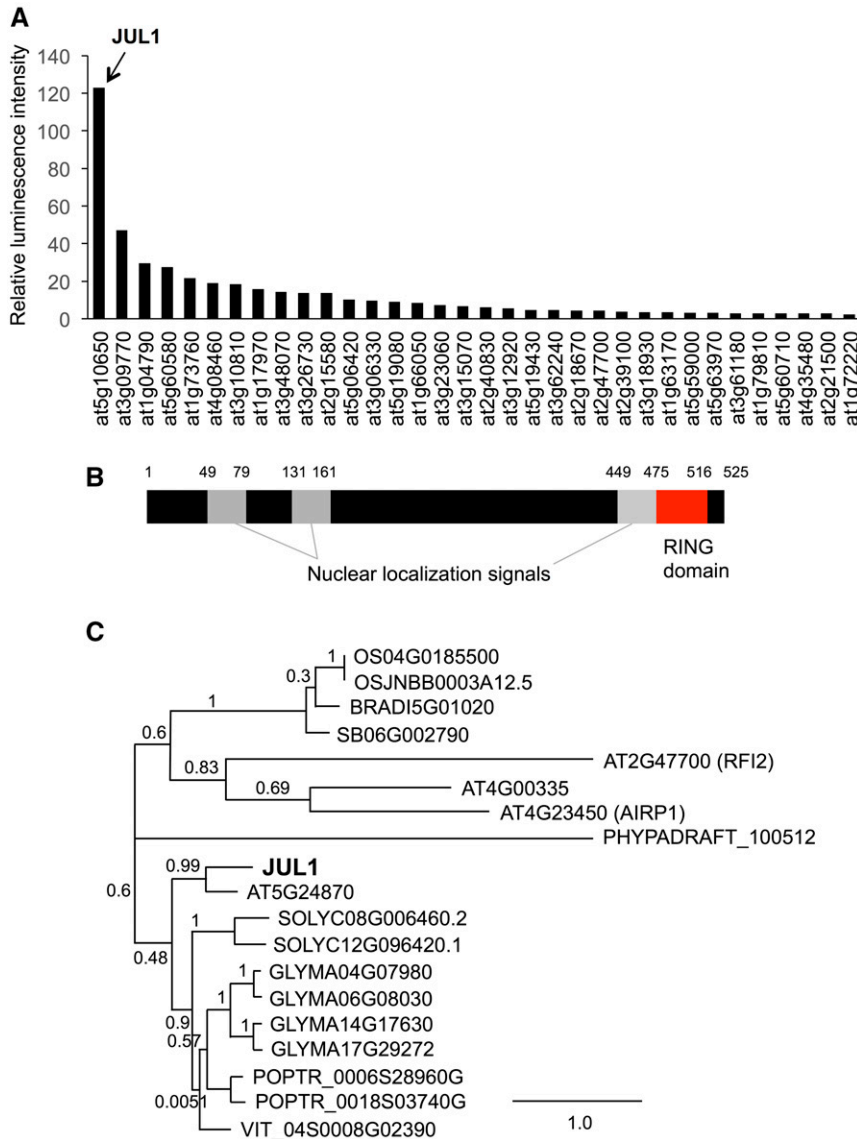
### Screening of JAV1-Interacting Ubiquitin Ligases

To construct a protein library of putative Arabidopsis RING-type E3 ligases, we prepared 210 cDNA clones from the RIKEN Arabidopsis full-length library (Ramadan et al., 2015), as shown in Supplemental Table S1. Transcription templates with an N-terminal FLAG-tag sequence were used to synthesize proteins using a cell-free protein synthesis system. To identify characteristic(s) of E3 ligase(s) that interact with JAV1 from the library proteins, we used the AlphaScreen-based detection system. Among the 210 synthesized proteins, JAV1 interacted most strongly with an E3 ligase (at5g10650), hereafter designated JUL1 (Fig. 1A). JUL1 has three predicted nuclear localization signals and a RING domain at the C terminus (Fig. 1B). Among the crustacean homologs exhibiting  $E < 10^{-5}$ , none of the proteins whose functions have been predicted or characterized have sequence similarities to JUL1 (Fig. 1C). For instance, the well-characterized E3 ligases RFI2 (Chen and Ni, 2006) and AIPR1 (Ryu et al., 2010) did not have similarity to JUL1. Instead, since JUL1 showed high similarity to a predicted E3 ligase (at5g24870; 58% identity) that was not present in our RIKEN Arabidopsis full-length library, we performed the AlphaScreen assay again for interaction between JAV1 and the JUL1 homolog. However, the JAV1 and JUL1 homologs did not show interaction (Supplemental Fig. S1). Likewise, the JAV1 homolog at4g15120 did not interact with JUL1.

### JUL1 and JAV1 Interact in the Nucleus

JAV1 is a nuclear protein (Hu et al., 2013). Therefore, we assessed the subcellular localization of JUL1 using a JUL1-GFP fusion protein expressed in Arabidopsis mesophyll protoplast cells (Fig. 2A). In contrast to the ubiquitous localization of the native GFP protein, which served as a control, the fusion protein showed nuclear localization.

Moreover, in order to assess the interaction in vivo between JUL1 and JAV1 proteins, a bimolecular fluorescence complementation (BiFC) assay was conducted. JUL1 fused to the N-terminal portion of yellow fluorescent protein (YFP; JUL1-nYFP) and JAV1 fused to the C-terminal portion of YFP (JAV1-cYFP) were transiently expressed in Arabidopsis mesophyll protoplast cells (Fig. 2B). The fluorescent signals of these recombinant YFP proteins were detected in the nuclei of



**Figure 1.** Screening and structure of JUL1. A, Luminescence-based protein-protein interaction detection (AlphaScreen) was used for high-throughput screening of 210 FLAG-conjugated putative Arabidopsis E3 ligases for interaction with biotinylated-JAV1 (Bio-JAV1). The data for the top 34 E3 ligases that interacted strongly with Bio-JAV1 are shown (for the full set of data, see Supplemental Table S2). Luminescence intensities were normalized by those of *Escherichia coli* dihydrofolate reductase. Data represent means of three replicates. B, JUL1 protein is represented schematically with the nuclear localization signals and RING domain and Lys residues. C, Phylogenetic tree inferred from deduced amino acid sequences of JUL1 and other proteins from several plant taxa: Arabidopsis; BRAD, *Brachypodium distachyon*; GLYMA, *Glycine max*; OS, *Oryza sativa*; PHYPADRAFT, *Physcomitrella patens*; POPTR, *Populus trichocarpa*; SB, *Sorghum bicolor*; SOLYC, *Solanum lycopersicum*; VIT, *Vitis vinifera*.

Arabidopsis protoplast cells only when MG132, a proteasome inhibitor, was applied to the cells (Fig. 2B). However, fluorescence was not detected when JAV1-cYFP was coexpressed with JUL1-ring domain mutant (JUL1<sup>RM</sup>) fused to the N-terminal portion of YFP. These results indicated that the persistent access of JUL1 to JAV1 caused rapid UPS-dependent JAV1 degradation.

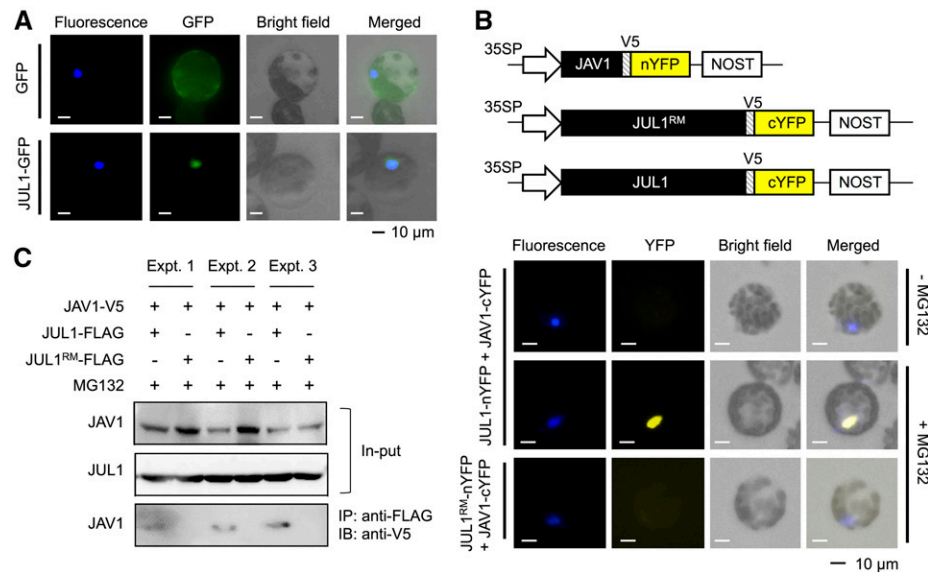
Moreover, immunoprecipitation assays confirmed that V5-tagged JAV1 interacted with FLAG-tagged JUL1 but not JUL1<sup>RM</sup> in the presence of MG132 (Fig. 2C).

### Ubiquitin Ligase Activity of JUL1

To assess the autoubiquitination activity of JUL1 in vitro, biotinylated JUL1 protein (Bio-JUL1) was incubated with HA-tagged ubiquitin (HA-Ub) in either the presence or absence of UBCH5b (Fig. 3A). UBCH5b

is an E2 ubiquitin ligase that is involved in the poly-ubiquitination of a suite of E3 ligases, including K48 (David et al., 2011). Immunoprecipitation with anti-biotin and subsequent anti-HA antibody detection revealed that Bio-JUL1 underwent autoubiquitination in the presence of UBCH5b. Moreover, when biotinylated JAV1 protein (Bio-JAV1) was incubated with FLAG-tagged ubiquitin (FLAG-Ub), UBCH5b, and JUL1, the Bio-JAV1 protein was ubiquitinated, as was the positive control, biotinylated P53 protein (Bio-P53), when it was incubated with FLAG-Ub, UBCH5b (E2), and MDM2, a human p53-specific E3 ligase (Fig. 3B). In contrast, when biotinylated dihydrofolate reductase was used as a substrate, it was not ubiquitinated by either MDM2 or JUL1.

JAV1 contains two Lys residues, Lys-52 and Lys-179. The level of ubiquitination of JAV1 by JUL1 decreased when a JAV1 mutant, in which either Lys-52 or Lys-179 was replaced by Thr, was used as the substrate in the



**Figure 2.** Subcellular localization and interaction of JAV1 and JUL1. A, The vector containing the *Cauliflower mosaic virus* 35S promoter (35SP)::GFP or JUL1 fused to GFP (JUL1-GFP) was used to transform Arabidopsis leaf protoplast cells. B, BiFC analysis of JUL1-JAV1 interactions. The vector constructs of 35SP::JUL1 or the JUL1-ring domain mutant (JUL1<sup>RM</sup>) fused to the N-terminal fragment of YFP (JUL1-nYFP or JUL1<sup>RM</sup>-nYFP) and JAV1 fused to the C-terminal fragment of YFP (JAV1-cYFP), as shown at the top, were cotransformed into Arabidopsis leaf protoplast cells. The protoplasts were treated with a potent proteasome inhibitor, MG132 (+MG132), or not treated with the inhibitor (-MG132). V5, V5 epitope tag. C, The vector constructs of 35SP $\Omega$ ::V5-tagged JAV1 (JAV1-V5) and FLAG-tagged JUL1 (JUL1-FLAG) or JUL1<sup>RM</sup> (JUL1<sup>RM</sup>-FLAG) were cotransformed into Arabidopsis leaf protoplast cells, and the cells then were treated with MG132 during the incubation. The extracted JAV1 and JUL1 or JUL1<sup>RM</sup> proteins (input) were immunoprecipitated with anti-FLAG antibody, subjected to SDS-PAGE, and detected using anti-V5. Three independent experiments (Expt. 1, 2, and 3) were performed. IB, immunoblotting; IP, immunoprecipitation.

ubiquitination assay (Fig. 3C). The ubiquitination signal was more markedly decreased when a JAV1 mutant in which both of the Lys residues were replaced with Thr was used as the substrate.

Next, *in vivo* degradation of JAV1 by the JUL1-UPS was evaluated. FLAG-tagged JAV1 protein (JAV1-FLAG) was expressed with V5-tagged JUL1 or JUL1<sup>RM</sup> (JUL1-V5 or JUL1<sup>RM</sup>-V5) in wild-type leaf protoplast cells (Fig. 4A). Irrespective of the presence or absence of MG132, the levels of the transiently expressed JAV1 proteins were well maintained when JUL1<sup>RM</sup>-V5 was coexpressed. In contrast, JAV1 protein levels decreased when JUL1-V5 was coexpressed in the absence of MG132. In the presence of MG132, JAV1 protein levels tended to recover slightly in comparison with those in the absence of MG132. Such JAV1 degradation in the absence of MG132 also was observed in wild-type and *coi1-1* protoplast cells (Supplemental Fig. S2), indicating that COI1-dependent JA signaling is not required for JUL1 ubiquitination activity toward JAV1.

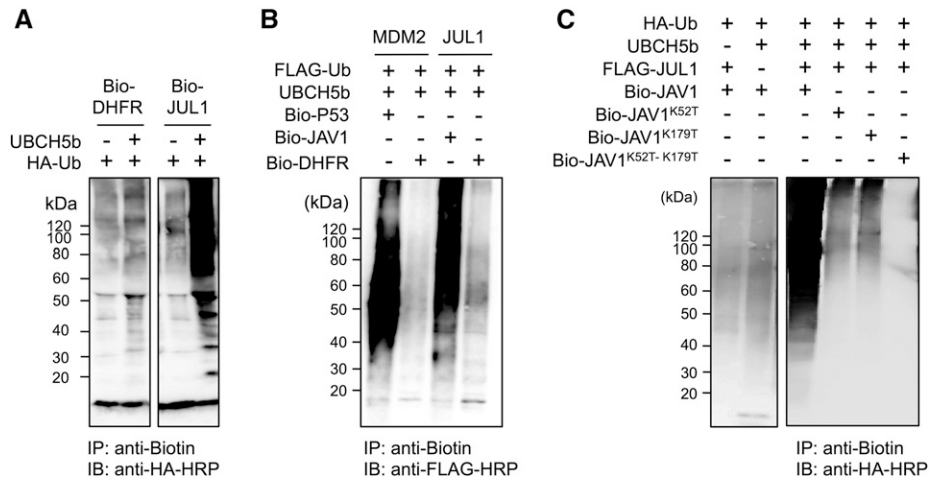
In additional assays, JAV1-FLAG was expressed in leaf protoplast cells prepared from the Arabidopsis wild type and the *jul1* mutant line (see below; Fig. 4B). Irrespective of the presence or absence of MG132, the JAV1 protein levels were elevated in the *jul1* cells compared with the wild-type cells (Fig. 4B). Moreover, JAV1-FLAG was expressed in leaf protoplast cells prepared from the Arabidopsis wild type and a transgenic

line overexpressing JUL1 (JUL1-OX1; see below; Fig. 4C). In comparison with the level in the wild type, the JAV1 protein level was decreased in the JUL1-OX1 cells incubated in the absence of MG132 but not in those incubated in the presence of MG132. These findings confirmed that JUL1 contributes to JAV1 degradation through the UPS in leaf cells.

Finally, we evaluated the level of the JAV1 protein in Arabidopsis T87 cells constitutively overexpressing JAV1, with or without JUL1, using an anti-JAV1 antibody, and found a decreased level of JAV1 protein in the cells expressing both JAV1 and JUL1 in comparison with the cells expressing JAV1 without JUL1 (Fig. 5A). In contrast, the JAV1 protein level was elevated in the leaves of the *jul1* mutant, in comparison with that in wild-type leaves (Fig. 5B).

#### Regulation of Transcript Levels of JUL1 and JAV1 via the COI1 System

Transcript levels of *JUL1*, *JAV1*, and *PLANT DEFENSIN1.2* (*PDF1.2*, a JA-associated defense gene) were elevated in wild-type leaves treated with methyl jasmonate (MeJA) or damaged by larvae of the generalist herbivore *Spodoptera litura* (Fig. 6). In contrast, the *coi1-1* mutant showed defective elevation of these transcript levels in leaves after MeJA treatment. Upon



**Figure 3.** In vitro ubiquitination of JUL1 on JAV1 as substrate. A, Autoubiquitination activity of JUL1. Biotinylated JUL1 proteins (Bio-JUL1) were incubated with HA-Ub in the presence or absence of UBCH5b (E2). Biotinylated dihydrofolate reductase (Bio-DHFR) served as a negative control. B, Ubiquitination activity of JUL1 on JAV1 substrate. JUL1 or MDM2 (a human p53-specific E3 ligase), which served as a positive control, was incubated in the presence or absence of FLAG-Ub, UBCH5b (E2), and biotinylated substrate proteins (Bio-p53 or Bio-JAV1). Bio-DHFR served as a substrate-negative control. For these assays, the incubated reaction mixtures were immunoprecipitated using streptavidin Alexa Fluor 647 (anti-Biotin), subjected to SDS-PAGE, and probed with anti-HA-HRP (HA-Ub) or anti-FLAG-HRP (FLAG-Ub) antibody. C, Wild type of the biotinylated JAV1 protein (Bio-JAV1) and its mutants, in which either the Lys-52 or Lys-179 residue or both Lys residues in the VQ motif domain were replaced with Thr (Bio-JAV1<sup>K52T</sup>, Bio-JAV1<sup>K179T</sup>, and Bio-JAV1<sup>K52T-K179T</sup>), were incubated with UBCH5b (E2) or FLAG-tagged JUL1 (FLAG-JUL1). Assays in the absence of either UBCH5b or Bio-JAV1 served as controls. The incubation mixtures were immunoprecipitated using streptavidin Alexa Fluor 647 (anti-Biotin), subjected to SDS-PAGE, and probed using anti-HA-HRP (HA-Ub) antibody. IB, immunoblotting; IP, immunoprecipitation.

herbivory, however, the transcript levels of *JUL1* and *JAV1* in *coi1-1* leaves showed no significant defect of elevation in comparison with the levels in wild-type leaves, whereas the *PDF1.2* transcript level was significantly lower in *coi1-1* leaves in comparison with that in wild-type leaves.

Intriguingly, it also should be noted that the transcript level of *JAV1* was higher in unstressed *coi1-1* leaves than in wild-type leaves.

#### Phenotypic Characterization of JUL1 Mutants and JUL1-Overexpressing Lines

To assess the function of JUL1 in plants, we generated two JUL1-overexpressing lines (JUL1-OX1 and JUL1-OX2), exhibiting 96- and 101-fold increased levels of *JUL1* expression, respectively, compared with the level in wild-type leaves (Fig. 7D). These transgenic lines, as well as the *jul1* and *jav1* knockdown mutants (Supplemental Fig. S3), showed no obvious differences in plant growth, development, or morphology, including root growth, vegetative stage development, or seed number, with or without MeJA treatment, in comparison with wild-type plants (Fig. 7, A and B; Supplemental Figs. S4 and S5).

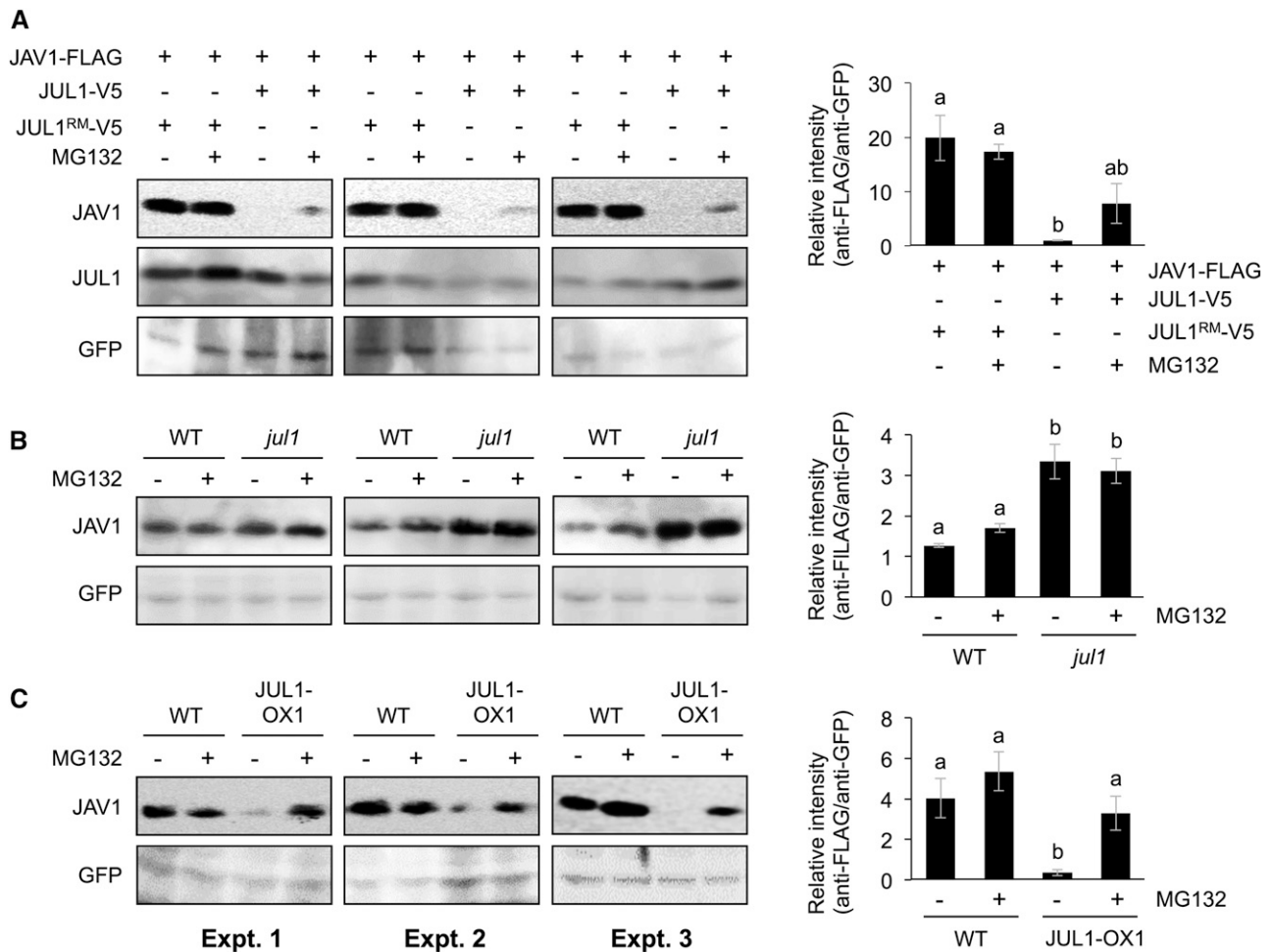
The defense ability of these lines was changed compared with that of wild-type plants. JA-inducible *PDF1.2* was highly expressed in the JUL1-OX line and *jav1* mutant leaves (Fig. 7C). Another JA-inducible

gene, *VEGETATIVE STORAGE PROTEIN2 (VSP2)*, was highly expressed in *jav1* mutant leaves but only tended to be slightly more highly expressed in the JUL1-OX line leaves compared with wild-type leaves (Fig. 7C). Conversely, the salicylate (JA-antagonist)-inducible *PATHOGENESIS-RELATED1* (Spoel et al., 2003; Beckers and Spoel, 2006) was highly expressed only in *jul1* mutant leaves (Fig. 7C).

Next, to confirm the plant defense properties of those lines against larvae of the generalist herbivore *S. litura*, we assessed the growth of larvae on the potted plants. The larvae on JUL1-OX and *jav1* plants exhibited lower weight gain for up to 3 d after the larvae were released, compared with that exhibited by the larvae on wild-type plants (Fig. 7D). In turn, the larvae on *jul1* mutant plants exhibited higher weight gain after 2 and 3 d, compared with the weight gains exhibited by the larvae on wild-type plants. The feeding behavior observed at 1 d showed a similar trend to the leaf damage area when JUL1-OX and *jav1* leaves were challenged with *S. litura* larvae for 2 h (Supplemental Fig. S6). Moreover, similar trends were observed for the leaf lesion sizes after infection with the necrotic pathogen *B. cinerea* for 3 d (Fig. 7E).

#### DISCUSSION

Arabidopsis JAV1 has been reported to be a negative regulator of JA-dependent defense responses that acts

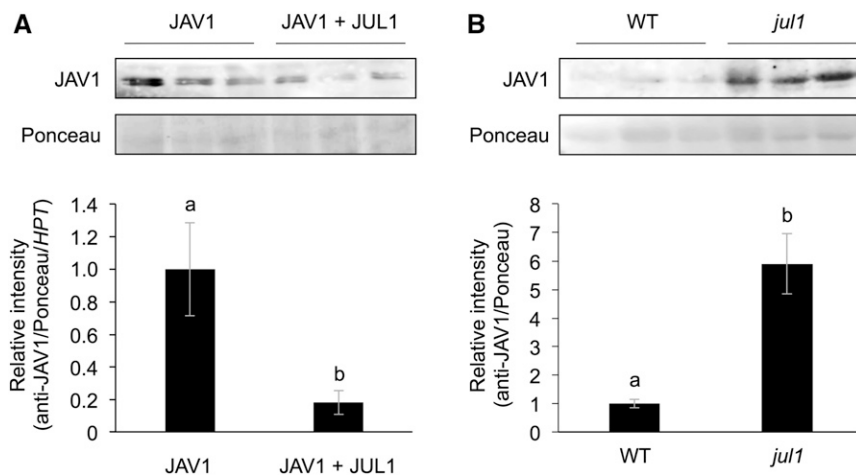


**Figure 4.** In vivo protein levels of JAV1 regulated by the JUL1-26S proteasome system. A, The vector constructs of 35SP $\Omega$ ::FLAG-tagged JAV1 (JAV1-FLAG) and V5-tagged JUL1 or JUL1<sup>RM</sup> (JUL1-V5 or JUL1<sup>RM</sup>-V5) were cotransformed with 35SP $\Omega$ ::GFP into Arabidopsis leaf protoplast cells, and the cells then were treated with MG132 during the incubation. B and C, 35SP $\Omega$ ::JAV1-FLAG and 35SP $\Omega$ ::GFP were cotransformed into protoplast cells prepared from wild-type plants (WT) and either the *jul1* mutant (B) or transgenic plants overexpressing *JUL1* (JUL1-OX1; C), with (+) or without (–) MG132 treatment. Total proteins extracted from the cells were subjected to SDS-PAGE and immunoblotted with FLAG-HRP and GFP antibodies. Mean and SE values from three independent experiments (Expt. 1, 2, and 3) are presented in the graphs at right. Means indicated by different letters are significantly different, based on one-way ANOVA with posthoc Tukey's honestly significant difference ( $P < 0.05$ ).

to coordinate the primary COI1-JAZ signaling pathway (Hu et al., 2013; Zhu and Zhu, 2013). JAV1 can form a complex with JAZ8 and TFs, including WRKY51, in order to suppress JA biosynthesis genes under normal conditions. However, in response to herbivory, all of these molecules are dissociated from the complex, JAV1 is then degraded via the UPS, and the free WRKY51 is able to access JA biosynthesis-related genes (Yan et al., 2018). In this study, JUL1 was found to have the characteristics of a RING-type E3 ubiquitin ligase that catalyzes ubiquitination of JAV1 as a substrate, thereby leading to the degradation of JAV1 via the UPS (Figs. 4 and 5). According to data from Hu et al. (2013) and our preliminary AlphaScreen-based assays for interactions between JAV1 and 566 TF proteins (Supplemental Table S2), it appears that the JAV1/JUL1 system is able to control a suite of WRKY family (e.g. WRKY28,

WRKY48, and WRKY51) members involved in promoting the transcription of defense-related genes, including *PDF1.2* (Zhu and Zhu, 2013). Such a regulatory system involving JAV1 is in accord with the basic nature of the VQ family, whose members interact specifically with the WRKY domain of groups I and IIc WRKY TFs (Cheng et al., 2012). Although Arabidopsis SIB1 (VQ23)/SIB2 (VQ16) and VQ10 have been shown to contribute to the activation of WRKY33 and WRKY8 in plant defense responses (Lai et al., 2011; Chen et al., 2018), respectively, an array of other Arabidopsis VQ proteins, including not only JAV1 but also VQ21/MKS1, VQ12, and VQ29, act as negative regulators of plant defense responses (Andreasson et al., 2005; Petersen et al., 2010; Hu et al., 2013; Wang et al., 2015).

WRKYs function as positive (WRKY28) or negative (WRKY48 and WRKY51) regulators of defense genes



**Figure 5.** In vivo protein levels of JAV1 in Arabidopsis cells overexpressing JAV1 and Arabidopsis leaves. JAV1 protein levels in Arabidopsis T87 cells overexpressing JAV1 with or without JUL1 (A) and leaves of Arabidopsis wild-type (WT) and *Jul1* mutant plants (B) are shown. Total proteins extracted from the cells and mature leaves were subjected to SDS-PAGE and immunoblotted with an anti-JAV1 antibody. For A, data were normalized by transcript levels of the hygromycin phosphotransferase (*HPT*) gene. Mean and se values from three independent experiments are presented at bottom. Means indicated by different letters are significantly different, based on Student's *t* test ( $P < 0.05$ ).

(Xing et al., 2008; Gao et al., 2011; Wu et al., 2011). Based on these facts, and given our finding here that both a loss of JAV1 function and a gain of JUL1 function result in enhanced expression of *PDF1.2* in leaves (Fig. 7D), we conclude that the orchestration of WRKY-based transcriptional machinery, according to the level of JAV1-based defense, is ultimately related to the positive regulation of the defense response. However, this should be flexible to enable adjustment to the circumstances of the plants, for example, whether they are being stressed, in which case the transcriptional modes can be drastically reprogrammed in plant cells.

It also appeared that *JUL1* and *JAV1* are constitutively expressed during several stages of Arabidopsis development, based on the microarray database GENEVESTIGATOR (Zimmermann et al., 2004; Supplemental Fig. S7). Remarkably, in the leaf senescence stage, transcripts of *JUL1* and *JAV1* showed high and low levels, respectively, indicating that *JUL1* might play a role in senescence. Moreover, both *JUL1* and *JAV1* transcript levels were found to be attenuated in *coi1-1* leaves as compared with wild-type leaves in response to MeJA (Fig. 6), indicating that those genes are positively regulated by a COI1-dependent signaling cascade. Intriguingly, when plants were challenged with herbivores, however, neither *JUL1* nor *JAV1* transcripts appeared to be induced in a COI1-dependent manner. Taking all these results together, we suggest that COI1-independent factor(s) may strongly contribute to the regulation of *JUL1* and *JAV1* transcript levels in response to herbivory. Interestingly, the level of *JAV1* transcripts was found to be negatively regulated by the COI1-dependent signaling cascade (Fig. 6). Our observation of the negative impact of COI1 on *JAV1* expression is in accord with data in the microarray database GENEVESTIGATOR (Zimmermann et al., 2004; <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE21762>) showing that *JAV1* transcript levels are higher in *coi1-2* leaves than in wild-type leaves under normal growth conditions (Supplemental Fig. S8). However, what this regulation implies for the JAV1/JUL1 module remains to be clarified.

RING-type E3 ligases play vital roles in the regulation of physiological and morphological processes in plants in response to various internal and external stimuli (Duplan and Rivas, 2014; Shabek and Zheng, 2014; Ding et al., 2015; Lazaro et al., 2015; Zhang et al., 2015). Nonetheless, to the best of our knowledge, RGLG3/4 and KEG are the only JA-associated RING-type E3 ligases reported thus far (Zhang et al., 2012; Pauwels et al., 2015). However, it was reported recently that RGLG3 and RGLG4 can ubiquitinate GRXS17, and thereby control the redox state of proteins, and that the RGLG system is not strongly relevant to the JA response module, in contrast to the findings in the previous report (Nagels Durand et al., 2016a). The findings of our study clearly showed that *JUL1* is a novel RING-type E3 involved in JA signaling. Notably, some RING-type E3 ligases have been shown to have a diverse range of substrate targets. One of the most striking examples is CONSTITUTIVE PHOTOMORPHOGENIC1, which is involved in a wide range of plant growth and developmental processes (Kim et al., 2017). It thus remains possible that *JUL1* has multiple substrate targets involved in an array of functions in plant defense, stress tolerance, and other physiological processes, and this issue should be assessed in the future.

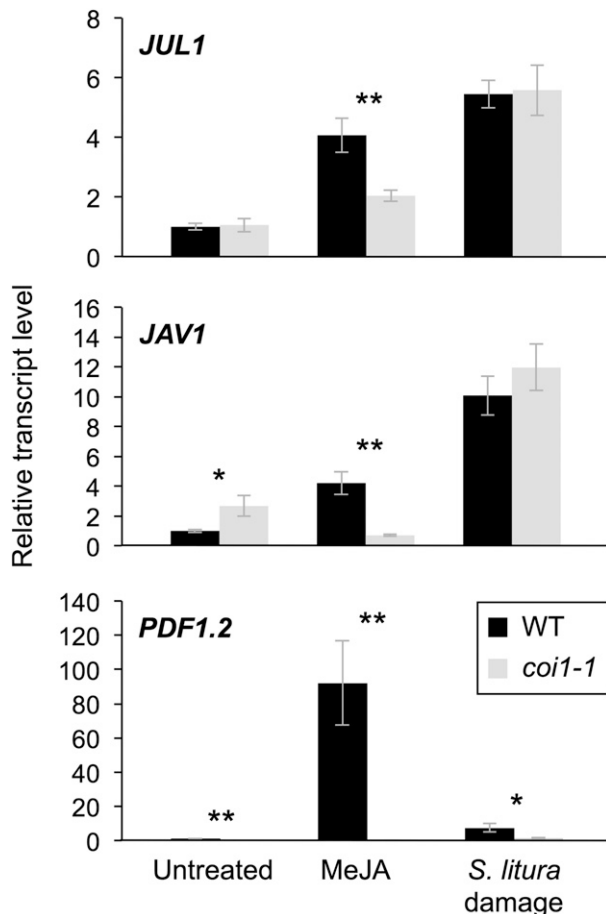
## MATERIALS AND METHODS

### Plant Materials

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia-0, its mutants (SALK\_146039C [*jav1*] and SALK\_101465C [*Jul1*]), and transgenic plants overexpressing *JUL1* (see below) were grown in plastic pots for 4 to 5 weeks in a growth chamber at  $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$  with a photoperiod of 14 h ( $80 \mu\text{E m}^{-2} \text{s}^{-1}$ ). The *coi1-1* seeds were germinated on one-half-strength Murashige and Skoog (1/2 MS) medium supplemented with 0.5% (w/v) Suc, 0.8% (w/v) agar, and  $50 \mu\text{M}$  MeJA (Wako Pure Chemical Industrials) to screen the individuals showing normal root growth for 2 weeks (Xie et al., 1998). Homozygous SALK lines were confirmed according to the method described at <http://signal.salk.edu/tdnaprimers.2.html>.

### Primers

Primers used for all the polymerase chain reactions (PCR) in this study are listed in Supplemental Table S3.



**Figure 6.** Regulation of transcript levels of *JUL1*, *JAV1*, and *PDF1.2*. Transcript levels are shown for *JUL1*, *JAV1*, and *PDF1.2* in leaves of Arabidopsis wild-type (WT) and *coi1-1* mutant plants in response to MeJA treatment for 4 h and damage with *S. litura* larvae for 24 h. Transcript levels of genes were measured by reverse transcription quantitative PCR and normalized by those of *ACT8*. Data represent means and SE ( $n = 5-7$ ). Asterisks indicate significant differences between wild-type and *coi1-1* mutant plants, based on Student's *t* test (\*\*,  $P < 0.01$  and \*,  $0.01 \leq P < 0.05$ ).

### Phylogeny Analysis

The phylogenetic tree was generated using the one-click mode of the Phylogeny.fr pipeline (<http://www.phylogeny.fr/>; Dereeper et al., 2008).

### Generation of Transgenic Arabidopsis Plants

The full-length coding region of *JUL1* (at5g10650) was inserted into binary plasmid pMDC32 (2 $\times$  cauliflower mosaic virus 35S promoter [35SP]::the Gateway cassette [GW] region::*nopaline synthase* terminator [NOST]) using the Gateway cloning system (Thermo Fisher Scientific). The resulting vector, pMDC32-*JUL1*, was transformed into *Agrobacterium tumefaciens* strain EHA105 by electroporation. Columbia-0 wild-type Arabidopsis plants that had been grown for 6 to 7 weeks were transformed via the floral dip transformation method (Clough and Bent, 1998). Transgenic T1 seeds from each transformant were tested for germination on 1/2 MS medium supplemented with 30 mg L<sup>-1</sup> hygromycin. T2 seeds were harvested from each individual. T2 plants that showed a segregation ratio of about 3:1 were tested for hygromycin resistance again. T3 homozygous plant lines were used for further analyses.

### Chemical and Herbivore Treatment

For chemical treatment, potted Arabidopsis plants were sprayed evenly with 1 mL of aqueous solution (0.5% [v/v] ethanol) of MeJA (0.2 mM) and incubated in a growth chamber at 22°C  $\pm$  1°C (14-h photoperiod at a light intensity of 80  $\mu$ E m<sup>-2</sup> s<sup>-1</sup>) for 4 h.

*Spodoptera litura* were reared on an artificial diet (Insecta LF; Nihon Nosan Nogyo) in the laboratory at 24°C  $\pm$  1°C with a photoperiod of 16 h. For herbivore treatment, four third-instar larvae were released on potted Arabidopsis plants in a growth chamber at 22°C  $\pm$  1°C (14-h photoperiod at a light intensity of 80  $\mu$ E m<sup>-2</sup> s<sup>-1</sup>) for 24 h.

### Cell-Free Protein Synthesis

Cell-free protein synthesis was carried out according to the method described previously (Ramadan et al., 2015; Takahashi et al., 2016). Primers used are listed in Supplemental Table S3.

### AlphaScreen System

We performed the AlphaScreen-based protein-protein interaction assay to screen the interaction of 210 Arabidopsis RING-type E3 ligases (Ramadan et al., 2015) and 566 Arabidopsis TFs (Nozawa et al., 2009) with JAV1. Assays for interaction between *JUL1* and the JAV1 homolog (at4g15120) or between the *JUL1* homolog (at5g24870) and JAV1 also were carried out. Assays were performed in 10  $\mu$ L of 100 mM Tris-HCl (pH 8), 0.1% (v/v) Tween 20, 1 mg mL<sup>-1</sup> BSA (AlphaScreen buffer), and 1  $\mu$ L of biotinylated JAV1 (or JAV1 homolog) and FLAG-adapted E3 ligase (either *JUL1* or a *JUL1* homolog) or TFs per well at 25°C for 1 h on a 384-well OptiPlate (Perkin-Elmer). In accord with the AlphaScreen IgG (Protein A) detection kit (Perkin-Elmer) instruction manual, 10  $\mu$ L of detection mixture containing AlphaScreen buffer, 5  $\mu$ g mL<sup>-1</sup> anti-FLAG M2 antibody (Sigma-Aldrich), 0.1  $\mu$ L of streptavidin-coated donor beads, and 0.1  $\mu$ L of Protein A-coated acceptor beads was added to each well of a 384-well OptiPlate, followed by an additional incubation at 25°C for 1 h. Luminescence was analyzed using the AlphaScreen detection program (Perkin-Elmer). All data represent averages of three independent experiments, and the background was controlled using a DHFR from *Escherichia coli*.

### Mutagenesis

JAV1 point mutations (Lys-52 and Lys-179) were obtained by inverse PCR using In-Fusion HD Cloning Plus (Takara Bio), primers (JAV1-K1-fw/JAV1-K1-rv and JAV2-K1-fw/JAV1-K2-rv [Supplemental Table S3]), and JAV1 cDNA as template.

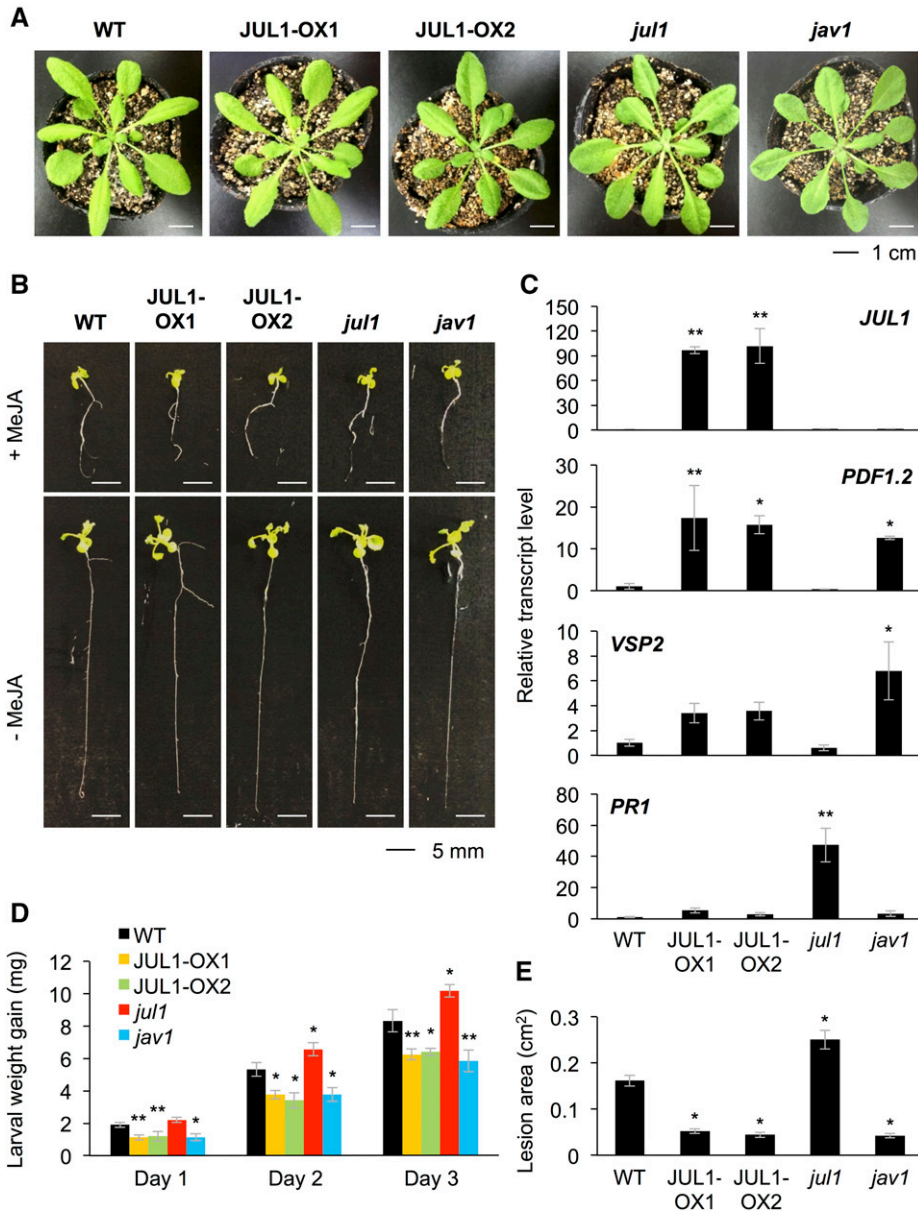
Similarly, *JUL1* protein mutations were obtained at Cys-493, His-495, and His-498 of the RING domain using primers (*JUL1*-RING-M-F and *JUL1*-RING-M-R [Supplemental Table S3]) and *JUL1* cDNA as template, to obtain *JUL1*<sup>RM</sup> based on Stone et al. (2005).

### Subcellular Localization and BiFC Assays in Protoplast Cells

The full-length coding regions of *JUL1* and *JAV1* (except for the stop codons) were cloned into Gateway destination plasmid pGWB451 (35SP::GW::GFP; Nakagawa et al., 2007), resulting in the construction of the vector pGWB451-*JUL1*-GFP. This vector, plus pGWB452 (35SP::GFP::GW), which served as a control, were transiently transformed into Arabidopsis mesophyll protoplasts as described previously (Yoo et al., 2007; Wu et al., 2009). The transformed protoplasts were incubated for 18 to 20 h in the dark at 25°C, and GFP fluorescence was observed in the protoplasts with a BZ-X700 fluorescence microscope (Keyence).

The full-length coding regions of *JUL1*, *JUL1*<sup>RM</sup> (see below), and *JAV1* were inserted (separately) into the Gateway destination vector 35SP $\Omega$ -GW-V5-nYFP, along with the N-terminal fragment of YFP, and 35SP $\Omega$ -GW-V5-cYFP with the C-terminal fragment of YFP, respectively (Nemoto et al., 2017). Ten micrograms of each vector was cotransformed into Arabidopsis protoplast cells. The transformed protoplasts were incubated for 14 h in the dark at 25°C, and then 50  $\mu$ M MG132 (Wako) in 0.1% (v/v) dimethyl sulfoxide (DMSO) or 0.1% (v/v) DMSO solution (control) was applied. Following an additional 6-h incubation in the dark at 25°C, the protoplast cells were incubated in WI solution (5 mM MES [pH 5.7], 0.4 M mannitol, and 20 mM KCl) with 1  $\mu$ g mL<sup>-1</sup>





**Figure 7.** The JAV1/JUL1 system is responsible for defense responses but not plant growth or development properties. A and B, Phenotypes of rosette plants (A) and seedlings with (+) or without (-) MeJA treatment (B) of the wild type (WT), JUL1-overexpressing lines (JUL1-OX1 and JUL1-OX2), and *jul1* and *jav1* mutants. C, Transcript levels of *JUL1*, *PDF1.2*, *VSP2*, and *PR1* in leaves of the wild type, JUL-OX lines, and mutants ( $n = 5-6$ ). D, *S. litura* larvae were put onto potted plants. The net body weight that larvae gained during 3 d after they had been placed on the plants was determined ( $n = 11-13$ ). E, Likewise, the fungus *B. cinerea* was inoculated onto leaves of potted plants, and areas of induced lesions were determined after 3 d ( $n = 10$ ). For C to E, data represent means and se. Data marked with asterisks are significantly different from those of the wild type, based on one-way ANOVA with Holm's sequential Bonferroni posthoc test (\*\*,  $P < 0.01$  and \*,  $0.01 \leq P < 0.05$ ).

4',6-diamino-phenylindole (Sigma-Aldrich) for 5 min before observation. The resultant YFP fluorescence was observed in the protoplasts using a fluorescence microscope (BZ-X700).

### In Vitro Ubiquitination Assay

The ubiquitination assays for JUL1 were carried out as described previously (Ramadan et al., 2015). The assays were performed in a 10- $\mu$ L reaction mixture containing 1  $\mu$ L of recombinant MDM2 (AF527840; mouse double minute 2 homolog) or JUL1 protein, 1  $\mu$ L of recombinant substrate (P53 [AB082923; p53 tumor suppressor targeted by MDM2 E3 ubiquitin ligase], JAV1 or JAV1 mutant [see above]), 1.2  $\mu$ M UbcH5b (Enzo Life Sciences), 20 mM Tris-HCl, pH 7.5, 0.2 mM DTT, 5 mM MgCl<sub>2</sub>, 10  $\mu$ M zinc acetate, 3 mM ATP, 1 mg mL<sup>-1</sup> BSA, and 400 nM FLAG-tagged or HA-tagged human ubiquitin (FLAG-Ub or HA-Ub; Boston Biochem) at 37°C for 3 h. The reactions were terminated by the addition of 5  $\mu$ L of 3 $\times$  SDS sample buffer and boiling for 5 min, and the products then were analyzed by 10% SDS-polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting (see below) using anti-FLAG-HRP (Sigma-Aldrich) or anti-HA-HRP (clone 3F10) antibody (Roche Applied Science).

### In Vivo Protein Degradation Assay

Polyethylene glycol-mediated DNA transfection of Arabidopsis mesophyll protoplasts from wild-type, *jul1*, and JUL1-OX1 backgrounds was performed as described previously (Yoo et al., 2007; Wu et al., 2009) with slight modifications. The full-length coding regions of *JAV1* (except for the stop codons) were cloned into Gateway destination plasmid p35S $\Omega$ ::GW::3 $\times$ FLAG::NOST (Nemoto et al., 2017), resulting in the construction of the vector p35S $\Omega$ ::JAV1 tagged with 3 $\times$ FLAG at the C terminus (JAV1-FLAG)::NOST. Likewise, the full-length coding regions of *JUL1* and *JUL1<sup>RM</sup>* (except for the stop codons) were cloned into Gateway destination plasmid p35S $\Omega$ ::GW::V5::NOST (Nemoto et al., 2017), resulting in the construction of the vector p35S $\Omega$ ::*JUL1* tagged with V5 at the C terminus (JUL1-V5)::NOST and p35S $\Omega$ ::*JUL1<sup>RM</sup>* tagged with V5 at the C terminus (JUL1<sup>RM</sup>-V5)::NOST, respectively. Thirty micrograms of JAV1-FLAG vector, along with 6  $\mu$ g of pGWB452 (35SP::GFP::GW) vector (Nemoto et al., 2017), were transformed into  $2 \times 10^5$  wild-type, *jul1*, or JUL1-OX1 cells. Otherwise, 30  $\mu$ g of JAV1-FLAG vector and 20  $\mu$ g of JUL1-V5 vector, along with 6  $\mu$ g of pGWB452 (35SP::GFP::GW) vector, were transformed into  $2 \times 10^5$  wild-type cells. Following incubation overnight in the dark at 22°C  $\pm$  1°C, 6 h before harvest, protoplasts were treated with 50  $\mu$ M MG132 in 0.1% (v/v) DMSO or

with 0.1% (v/v) DMSO (control). Total proteins were extracted from the protoplasts with 3× sample buffer. JAV1-FLAG protein was analyzed by immunoblotting using anti-FLAG HRP (see below).

For coimmunoprecipitation assays, 30 μg of JAV1-V5 vector (p35S<sub>Ω</sub>::JAV1 tagged with V5 at the C terminus) and 20 μg of JUL1-FLAG vector (p35S<sub>Ω</sub>::JUL1 tagged with 3×FLAG at the C terminus) were transformed into 1 × 10<sup>6</sup> wild-type cells; the protoplasts were incubated overnight and treated with 50 μM MG132 for 6 h before being homogenized with immunoprecipitation lysis/wash buffer (Thermo Fisher Scientific) supplemented with 50 μM MG132 and 1% (v/v) Protease Inhibitor Cocktail (Sigma-Aldrich). Immunoprecipitation of JUL1-FLAG protein from the protoplast lysate was performed using a Pierce Crosslink Magnetic IP/Co-IP Kit (Thermo Fisher Scientific) and anti-FLAG (DYKDDDDK) monoclonal primary antibody (1E6; Wako) following the manufacturer's protocol. After the beads were washed twice with immunoprecipitation lysis/wash buffer, the immunoprecipitated proteins were eluted with 50 μL of elution buffer. JAV1-V5 protein was analyzed by immunoblotting using anti-V5 HRP (see below). RbL was visualized as a loading control using Ponceau S staining solution.

### Generation and Analysis of Transgenic Arabidopsis T87 Cultured Cells Expressing JAV1-GFP and JUL1

The full-length coding region of *JAV1* (at3g22160) was inserted into binary plasmid pGWB5 (35S<sub>P</sub>::GW:NOST) to construct pGWB5-*JAV1*. Additionally, the expression cassette of *JUL1* in pMDC32-*JUL1* was subcloned into pGWB5-*JAV1* to construct pGWB5-*JAV1*-*JUL1*. pGWB5-*JAV1* and pGWB5-*JAV1*-*JUL1* were transformed into *A. tumefaciens* strain GV3103 by electroporation. Arabidopsis T87 cells were transformed and cultured in Jouanneau and Péaud-Lenoël medium supplemented with 10 mg L<sup>-1</sup> hygromycin according to the method described previously (Ogawa et al., 2008). Total proteins were extracted from the transgenic cells with extraction buffer (50 mM HEPES [pH 7.4], 5 mM EDTA, 5 mM EGTA, 50 mM β-glycerophosphate, 10 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM NaF, 2 mM DTT, and 1% (v/v) Triton-X100). JAV1-GFP protein was analyzed by immunoblotting using anti-JAV1 antibody (see below). Data were normalized by transcript levels of the hygromycin phosphotransferase gene.

### Immunoblot Analysis

Ten- to 15-μL samples of the boiled proteins in SDS sample buffer were subjected to 10% SDS-PAGE, and the proteins were transferred to a polyvinylidene difluoride membrane (ATTO) using the Trans-BlotTurbo Blotting System (Bio-Rad). Immunoblotting was carried out with anti-FLAG-HRP-conjugated antibody (Wako Pure Chemical Industries), anti-V5-HRP-conjugated antibody (Wako Pure Chemical Industries), or anti-GFP chicken IgY primary antibody (GeneTex) and then with anti-chicken IgY-HRP-conjugated secondary antibody (Promega). Detection was performed using Immobilon Western Chemiluminescent HRP Substrate (ECL; Millipore) according to the manufacturer's procedure. The signals were visualized using ImageQuant LAS 4000 mini (GE Healthcare).

Immunoblotting for JAV1 was carried out with anti-JAV1 antibody obtained by immunizing rabbits with a peptide antigen (with peptide sequence CWQYNFQPHAPLQPPQR; Cosmo Bio). Unlabeled anti-rabbit IgG (#3678; Cell Signaling Technology) and HRP-linked anti-mouse IgG (#7076) antibodies were used as secondary and tertiary antibodies, respectively. The membranes were soaked with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific), and the signals were detected with an ImageQuant LAS-4000 imaging system (GE Healthcare).

### RNA Extraction, cDNA Synthesis, and Reverse Transcription Quantitative PCR

Approximately 100 mg of leaf tissues or 1 mL of cell culture was homogenized in liquid nitrogen, and total RNA was isolated and purified using Sepasol-RNA I Super G (Nacalai Tesque) following the manufacturer's protocol. First-strand cDNA synthesis and quantitative PCR were performed according to the method described previously (Uemura et al., 2018). Relative transcript abundances were determined after normalization of raw signals with the transcript abundance of the housekeeping gene *ACT8* (at1g49240). We did not use samples and data when sufficient amounts of RNA were not isolated from leaves or when abnormal quantification cycle values for the actin gene were obtained.

Finally, replicate analyses were conducted with five or six independent samples, except for the data in Figure 5B (three independent samples).

### Root Length Measurement

Plant seedlings (14 d old) were grown on 1/2 MS medium with or without 50 μM MeJA. Root lengths were determined using ImageJ software (version 1.50i; Schneider et al. (2012)).

### Herbivore Assay

We performed assays to assess the growth of *S. litura* larvae at 22°C ± 1°C (14-h photoperiod at 80 μE m<sup>-2</sup> s<sup>-1</sup>). Third-instar larvae were initially weighed (1.6–2.1 mg), and each larva was released onto a potted plant for 3 d. The net body weight that *S. litura* larvae gained each of the following 3 d was determined. When a larva died or was lost during the assay, we excluded that sample, and final replicate analyses were conducted with 12 to 15 independent samples.

For leaf-consumption assays, five third-instar larvae, starved overnight, were released onto the shoots of potted plants for 2 h. The leaves then were scanned, and the total leaf area and the consumed leaf area were determined using ImageJ. Replicate analyses were conducted with eight to 10 independent samples.

### Pathogen Infection Assay

*Botrytis cinerea* (strain IuRy-1) was grown on potato Suc agar under black light at 25°C for 1 week to promote the formation of conidia. For inoculation, 10 μL of conidial spore suspension (2 × 10<sup>5</sup> conidia mL<sup>-1</sup>, in 2.5% Glc) was spotted onto the left and right sides of the upper surface of a leaf of the potted plants. The inoculated plants were covered with a plastic sheet to enable the maintenance of humidity. The plants were placed in a growth chamber at 24°C ± 1°C for 3 d. The leaf lesion area was analyzed using ImageJ.

### Statistical Analyses

We performed Student's *t* tests for pairwise analysis and one-way ANOVA with Holm's sequential Bonferroni posthoc test or Tukey's honestly significant difference test using the program ([http://astatsa.com/OneWay\\_Anova\\_-with\\_TukeyHSD/](http://astatsa.com/OneWay_Anova_-with_TukeyHSD/)) for comparing multiple samples.

### Accession Numbers

The sequences and detailed information for the genes in this study can be found in the Arabidopsis Biological Resource Center database under the accession numbers listed in Supplemental Table S3.

### Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** Interactions between JUL1 and JAV1 homolog (at4g15120) and between JAV1 and JUL1 homolog (at5g24870).

**Supplemental Figure S2.** COI1-independent protein levels of JAV1 regulated by the JUL1-26S proteasome system.

**Supplemental Figure S3.** Molecular analysis of T-DNA insertion mutants and gene expression profiling in the mutants.

**Supplemental Figure S4.** Root length of the seedlings of the wild type, JUL1-overexpressing lines, and *jul1* and *jav1* mutants with or without MeJA treatment.

**Supplemental Figure S5.** Root length of the seedlings of wild type, JUL1-overexpressing lines, and *jul1* and *jav1* mutants following with or without MeJA treatment.

**Supplemental Figure S6.** Leaf-consumption of *S. litura* larvae.

**Supplemental Figure S7.** Transition of transcript levels of *JUL1* and *JAV1* during the Arabidopsis life cycle, based on the GENEVESTIGATOR database.

**Supplemental Figure S8.** Transcript levels of *JAV1* in Arabidopsis wild-type and *coi1-2* mutant leaves 4 h after application of MeJA, based on the GENEVESTIGATOR database.

**Supplemental Table S1.** AlphaScreen-based binding intensity to *JAV1*.

**Supplemental Table S2.** AlphaScreen-based binding strength of TFs to *JAV1*.

**Supplemental Table S3.** Primers.

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