SHORT COMMUNICATION

Proteasomal Degradation of JAZ9 by Salt- and Drought-Induced Ring Finger 1 During Pathogen Infection

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E3 ubiquitin ligase salt- and drought-induced ring finger 1 (SDIR1) plays a novel role in modulating plant immunity against pathogens. The molecular interactors of SDIR1 during pathogen infection are not known. SDIR1-interacting jasmonate zinc-finger inflorescence meristem domain (JAZ) proteins were identified through a yeast two-hybrid (Y2H) screen. Full-length JAZ9 interacts with SDIR1 only in the presence of coronatine (a bacteria-secreted toxin) or jasmonic acid (JA) in a Y2H assay. The bimolecular fluorescence complementation and pull-down assays confirm the in planta interaction of these proteins. JAZ9 proteins, negative regulators of JA-mediated plant defense, were degraded during the pathogen infection by SDIR1 through a proteasomal pathway causing disease susceptibility against hemibiotrophic pathogens.

Keywords: avirulence factors, bacterial pathogenesis, ethylene, genomics, jasmonic acid, metabolomics, plant responses to pathogens, proteomics, salicylic acid

Pathogens suppress plant defense responses by promoting ubiquitination and degradation of target plant proteins during infection through the proteasome complex (Banfield 2015; Nomura et al. 2006; Singer et al. 2013; Üstün and Börnke 2014, 2015). During infection, pathogen effectors target the proteasome complex to

hijack the defense response (Üstün et al. 2016). Bacteria-secreted type 3 effectors (T3Es) XopJ from *Xanthomonas campestris* pv. *vesicatoria* and HopZ4 from *Pseudomonas syringae* pv. *lachrymans* inhibit regulatory particle triphosphatase 6, a subunit of the 19S recognition particle of proteasome to suppress plant defense (Üstün and Börnke 2014, 2015). T3Es HopM1, HopAO1, HopA1, and HopG1 from *P. syringae* were identified as putative inhibitors of proteasomal activity (Üstün et al. 2016). Proteasomal degradation of proteins involves ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3) (Ji et al. 2018). Really interesting new gene (RING)-finger proteins, involved in protein–protein interaction (Metzger et al. 2014) with E3 ligase catalytic activity, are activated by elicitor or avirulent factors and act as both positive and negative regulators of plant defense (Dreher and Callis 2007). The coronatine (COR)-insensitive protein 1 (COI1), a component of Skp1/Cullin/F-box (SCF^{COI1}) complex, mediates the ubiquitination and degradation of jasmonate zinc-finger inflorescence meristem (ZIM) domain (JAZ) proteins during jasmonate signaling (Delaure et al. 2008). JAZ proteins bind to different transcription factors and repress their activity in various developmental and pathogen infection conditions (Guo et al. 2018). The phyto-toxin COR secreted by a pathogen modulates plant defense by acting as a structural mimic of jasmonic acid isoleucine (JA-Ile) (He et al. 2004).

We previously showed that overexpression of salt- and drought-induced ring finger 1 (SDIR1), a RING-type E3 ubiquitin ligase, in *Arabidopsis*, makes it hypersusceptible to hemibiotrophic pathogen *P. syringae* pv. *tomato* DC3000 and resistant to necrotrophic pathogen *Erwinia carotovora* (Ramu et al. 2021). In contrast, the *Atsdir1* mutant plants exhibited resistance to *P. syringae* pv. *tomato* DC3000 and susceptibility to *E. carotovora* (Ramu et al. 2021). Overexpression of *AtSDIR1* in *Arabidopsis* showed drought stress tolerance by interacting and partially degrading SDIR1-interacting protein 1 (SDIRIP1), which acts upstream of abscisic acid (ABA) signaling (Zhang et al. 2007; Zhang et al. 2015). SDIR1 destabilizes F-box proteins EIN3-binding F-box protein 1 (EBF1) and EBF2 to promote accumulation of EIN3 involved in ethylene responses and temperature stress (Hao et al. 2021). Similar to *sdir1* mutants (Ramu et al. 2021), the *coil* ring domain ligase *rglg3* and *rglg4* mutants also showed pathogen resistance phenotype against hemibiotrophic bacterial pathogens (Xie et al. 1998; Zhang et al. 2012). The molecular interactors of SDIR1 during pathogen infection are not known. To establish the molecular mechanisms governed by SDIR1-mediated pathogen susceptibility, we identified several proteins that interact with

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SDIR1 using a yeast two-hybrid (Y2H) screen. We report that SDIR1 interacts with jasmonate ZIM-domain 9 (JAZ9) and degrades through a proteasome-mediated pathway during pathogen infection.

RESULTS

SDIR1 interacts with JAZ9 in the presence of COR or jasmonic acid.

The *Arabidopsis* *AtSDIR1* overexpression plants are hypersusceptible to hemibiotrophic bacterial pathogens and resistant to necrotrophic bacterial pathogens (Ramu et al. 2021). To understand the molecular mechanisms of SDIR1-mediated disease susceptibility or resistance, a Y2H screening was performed using an *Arabidopsis* mixed elicitor-induced cDNA library (Lee et al. 2017) to identify SDIR1-interacting proteins. SDIR1 possesses N-terminal transmembrane (TM) domains (78 amino acids [aa]), a C-terminal RING domain, a metal-binding site, and a palmitoylation (PAL) site (Fig. 1). When full-length SDIR1 was used for the Y2H screen, we did not find any SDIR1-interacting plant proteins. We speculated that deletion of the RING domain would prevent the degradation of interacting proteins and, also, deleting the TM domain would help in identifying the interactors in the yeast system. The Y2H screening was performed with $\Delta^{\text{TM}}\text{SDIR1}\Delta\text{RING}$ (79 to 210 aa) and identified several SDIR1-interacting proteins, including JAZ proteins (7, 18, 38, and 53 other clones) (Supplementary Table S1). Because JAZ proteins have been shown to play a role in defense against bacterial pathogens (Zhang et al. 2017), we further characterized them. Interestingly, the full-length JAZ9 protein did not interact with $\Delta^{\text{TM}}\text{SDIR1}$, $\Delta^{\text{TM}}\text{SDIR1}\Delta\text{Metal}$, $\Delta^{\text{TM}}\text{SDIR1}\Delta\text{PAL}$, and $\Delta^{\text{TM}}\text{SDIR1}\Delta\text{RING}$ proteins in minus leucine/tryptophan/histidine triple drop-out media (TDO) even though a partial clone of JAZ9 interacted with $\Delta^{\text{TM}}\text{SDIR1}\Delta\text{RING}$ in the Y2H screening (Fig. 1B). We speculate that a structural difference in full-length and partial JAZ9 proteins could be contributing to this discrepancy. We anticipated that there may be a cofactor required for SDIR1 interaction with JAZ9. Melotto et al. (2008) previously reported that JAZ9 interacts with COI1 in Y2H when growth medium is externally supplied with cofactors COR or JA-Ile. External supplementation of COR, jasmonic acid (JA), or methyl jasmonate (MeJA) facilitated interaction of full-length JAZ9 and SDIR1 proteins and the 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) staining confirmed these interactions (Fig. 1B). However, the interaction with MeJA was weak (Fig. 1B) and the interaction in the absence of cofactor was also found when proteasomal inhibitor MG132 was added to the yeast growth media (Fig. 1B). These results suggest that SDIR1–JAZ9 interaction could be occurring only in specific conditions when the pathogen secretes COR or when phytohormone JA accumulates in the plants.

The interaction of SDIR1 with JAZ9 was further confirmed by pull-down assay. The *AtSDIR1*-glutathione-S-transferase (GST) protein (expressed and purified from *Escherichia coli*) was incubated with total protein from stable *HA-JAZ9*-expressing *Arabidopsis* plants (Lee et al. 2018). Hemagglutinin (HA) resin was used to pull down HA-JAZ9 and its interacting proteins. The SDIR1-GST pull-down proteins were detected using GST and HA tag antisera and the interaction of SDIR1 with JAZ9 was evident (Fig. 1C; Supplementary Fig. S1). These results demonstrate that SDIR1 interacts with JAZ9 both in vitro and in vivo.

To further validate in planta interaction of SDIR1 with JAZ9, we performed bimolecular fluorescence complementation (BiFC) assay in *Nicotiana benthamiana*. Full-length *AtSDIR1* fused to the N-terminal half of the enhanced yellow fluorescent protein

(*eYFP*) and *AtJAZ9* fused to the C-terminal half of *eYFP* and was coexpressed in *N. benthamiana*. The reconstitution of yellow fluorescence suggests interaction of SDIR1–JAZ9 in endoplasmic reticulum (ER) or plasma membrane (PM) (Fig. 2A; Supplementary Fig. S2A). SDIR1 and JAZ9 are supposed to be localized to the ER membrane and nucleus, respectively (Withers et al. 2012; Zhang et al. 2007; Zhang et al. 2015). However, we did not see interaction in the nucleus. We anticipated interaction in the nucleus can happen if the TM domain is deleted in SDIR1. Surprisingly, upon deletion of the TM domain of SDIR1, interaction of nEYFP- $\Delta^{\text{TM}}\text{SDIR1}$ and *AtJAZ9*-cEYFP was detected in the ER membrane in the background of ER-specific marker and in cytosol (Fig. 2A; Supplementary Fig. S2B). Pearson coefficient data clearly suggest the colocalization of interaction on PM or ER (Fig. 2B). As a negative control, we used the *AtGCN4* protein (Kaundal et al. 2017) that showed no interaction with SDIR1 (Supplementary Fig. S2C).

SDIR1 degrades JAZ9 via the proteasome-mediated pathway during pathogen infection.

The importance of the RING domain for E3 ligase activity of SDIR1 was shown previously (Zhang et al. 2007; Zhang et al. 2015). We speculated that JAZ9 is a target of SDIR1-mediated polyubiquitination and subsequent degradation. To confirm the polyubiquitination of JAZ9 before degradation by SDIR1, the full-length SDIR1 fused with GST was expressed in *E. coli* and purified. A truncated SDIR1 protein in which a C-terminus RING domain was deleted and fused with GST ($\Delta^{\text{TM}}\text{SDIR1}\Delta\text{RING}$ -GST) was purified, to determine the role of this domain in polyubiquitination. C-terminal His-tagged JAZ9 was expressed and purified from *E. coli*. The ubiquitination activity was determined in the presence of ubiquitin-conjugating enzymes, wheat (*Triticum aestivum*) E1 and a human E2 (UBCh5b), purified GST-SDIR1 or GST- $\Delta^{\text{TM}}\text{SDIR1}\Delta\text{RING}$, and His-JAZ9-tagged proteins (Fig. 3A). Interestingly, the self-polyubiquitination of SDIR1 was detected by nickel-horseradish peroxidase (for detecting HA-ubiquitin) (Fig. 3A). In the absence of a RING domain, self-ubiquitination of SDIR1 was completely abolished due to loss of E3 ligase activity. These results are similar to previously reported assays on other ring finger proteins, which showed that the RING motif is essential for E3 ligase activity (Xi et al. 2002). The in vitro ubiquitination assay shows an SDIR1-dependent ladder of ubiquitinated forms when JAZ9 is present (Fig. 3Aii). When probed with His antibodies, the JAZ9 also appeared to be ubiquitinated, as shown by the presence of high molecular weight JAZ9 protein (Fig. 3Aiii; Supplementary Fig. S3).

To demonstrate the degradation of JAZ9 by SDIR1, GST-SDIR1 fusion protein was purified from *E. coli*. *Arabidopsis* transgenic plants expressing HA-JAZ9 were infected with host pathogen *P. syringae* pv. *tomato* DC3000 and, after 24 h postinoculation (hpi), total plant protein was extracted. Purified GST-SDIR1 and plant proteins were mixed and incubated at room temperature for 0, 1, 2, 3, and 4 h with or without MG132. The JAZ9 protein degradation started from 1 h and was completely degraded after 3 h of incubation (Fig. 3Bi). Proteasomal inhibitor MG132 was able to inhibit the HA-JAZ9 degradation by SDIR1, suggesting that JAZ9 is degraded via the 26S proteasome system during pathogen stress (Fig. 3Bi). In the absence of SDIR1, there was no degradation of JAZ9 (Fig. 3Bii). No degradation of JAZ9 was observed in SDIR1-treated samples in the absence of the pathogen (Fig. 3Biii; Supplementary Fig. S3). Therefore, both SDIR1 and pathogen infection are needed for degradation of JAZ9. These results suggest that SDIR1 degrades JAZ9 through a proteasome-mediated pathway.

Fig. 1. Salt- and drought-induced ring finger 1 (SDIR1) interacts with jasmonate zinc-finger inflorescence meristem domain 9 (JAZ9). **A**, Schematic representation of different SDIR1 gene constructs used for yeast two-hybrid and bimolecular fluorescence complementation assays. Numbers 1 to 78 indicate N-terminal transmembrane (TM) domain (red), 210 to 251 is really interesting new gene (RING) domain (blue), 251 is 259 metal-binding region (pink), and 259 to 273 is palmitoylation (PAL) domain (yellow); aa = amino acid. **B**, AtSDIR1 interacts with JAZ9 protein in a yeast two-hybrid assay. The *AtSDIR1* TM-domain-deleted ($\Delta^{\text{TM}}\text{SDIR1}$), TM and RING domain ($\Delta^{\text{TM}}\text{SDIR1}\Delta\text{RING}$), metal-binding region ($\Delta^{\text{TM}}\text{SDIR1}\Delta\text{Metal}$), and PAL domain ($\Delta^{\text{TM}}\text{SDIR1}\Delta\text{PAL}$) deleted constructs were expressed as bait protein in *pDEST32*; and *AtJAZ9* was expressed as a prey protein in *pDEST22* vector and cotransformed to MaV203 yeast cells. Cells were grown on double drop-out (DDO) or triple (TDO) drop-out media supplemented with different concentrations (1.5 and 60 μM) of coronatine (COR), methyl jasmonate (MeJA), or jasmonic acid (JA). The interaction stability of two proteins was confirmed by 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) staining. MG132 (10 μM), a proteasome degradation inhibitor, was added to the quadruple drop-out media to inhibit any degradation of target proteins. *Krev1 RalGDS* and *Krev1 RalGDS-m1* were used as positive and negative controls, respectively, and grown on DDO or TDO media. Negative control did not grow on TDO medium. **C**, Biochemical interaction of AtSDIR1 with AtJAZ9 in glutathione-S-transferase (GST) pull-down assay. *GST-AtSDIR1* was expressed in *pDEST17* in *Escherichia coli* and the *HA-AtJAZ9* was expressed in *Arabidopsis*. Proteins were purified and confirmed by Western blotting with hemagglutinin (HA) or GST antisera. An equal amount of GST-AtSDIR1 was used in pull-down assays, with GST resin as the input control. HA antibody was used to detect input and bound forms of JAZ9. Pull-down assays were performed at 4°C, and protein gel blot analysis was performed using anti-HA antibody. Experiments were conducted with a minimum of three replicates and repeated three times with similar results.

DISCUSSION

The *SDIR1* overexpression in *Arabidopsis* led to hypersusceptibility to hemibiotrophic pathogens and resistance to necrotrophic pathogens (Ramu et al. 2021). *SDIRIP1*, *EBF1*, and *EBF2* were identified previously as targets of *SDIR1* that acts upstream of ABA- and ethylene-signaling pathways (Hao et al. 2021; Zhang et al. 2007; Zhang et al. 2015). During pathogen infection, we found that *SDIR1* interacts with many defense-responsive JAZ proteins. The interaction of *SDIR1* with *JAZ9* was observed in the presence of COR or JA. MeJA, JA-phenylalanine, 12-oxo-phytodienoic acid, and JA-Ile are known to provide active signals during pathogenesis (Melotto et al. 2008; Xin and He 2013). Similar to this scenario, the interaction of *COI1* with *JAZ1*, *JAZ3*, and *JAZ9* was observed only in the presence of COR and JA-Ile (Melotto et al. 2008; Thines et al. 2007). The interaction of *JAZ9* with *SDIR1* occurs at the PM and cytosolic fraction (Fig. 2). *JAZ9* localization to the nucleus is compromised in the *myc2* mutant; *JAZ9* interaction with *MYC2* represses the JA-dependent gene expression (Withers et al. 2012). Previously, in *JAZ9* protein RKJas, two *COI1*-interacting residues were identified, and mutation in this region resulted in resistance to SCF^{COI1}-mediated degradation (Browse 2009; Song et al. 2011; Withers et al. 2012).

The polyubiquitination of *SDIR1* is enhanced in the presence of *JAZ9* in the ubiquitination assay. The *JAZ9* protein was completely degraded by *SDIR1* after 3 h when coexpressed together in *N. benthamiana*, and proteasome inhibitor MG132 prevents the degradation. The hypersusceptibility of *AtSDIR1* overexpression lines to hemibiotrophic pathogens and compromised nonhost resistance of *jaz9* mutant (Ramu et al. 2021) could be attributed to the role of *SDIR1* in JA or pathogen-mediated response to trigger the proteasome complex. JAZ proteins are known to be negative regulators of gene expression in JA-mediated responses (Withers et al. 2012; Zhang et al. 2015). The degradation of *JAZ9* mediated by *SDIR1* could be activating the *MYC2* transcription factor and other proteins, leading to

susceptibility against hemibiotrophic pathogen *P. syringae* pv. *tomato* DC3000. *SDIR1* may also be involved in pathogen-associated molecular pattern-mediated plant defense, which depends on JA signaling components of the SCF complex such as Cullin 1, JAZs, *COI1*, and F-box proteins for modulating downstream transcription factors such as *MYC2* (Browse 2009; Burgess et al. 2012). *JAZ9* is known to act as a repressor for the MYB transcription factors under cold- and pathogen-stress conditions (Lv et al. 2017; Qi et al. 2011). The transcriptomic data from *Arabidopsis SDIR1* overexpression plants showed higher transcript levels of *MYB30* and *MYB28* genes (Ramu et al. 2021). The higher levels of these genes could be due to removal of the repression activity of *JAZ9* by *SDIR1* through proteasome-mediated degradation. It is evident that JA, ABA, and ethylene signaling events are modulated by *SDIR1* through JAZ, *SDIRIP1*, and *EBF* target proteins, respectively (Hao et al. 2021; Ramu et al. 2021; Zhang et al. 2015). Therefore, *SDIR1* could be acting as a common regulator of phytohormone signaling in plants exposed to multiple environmental stresses.

MATERIALS AND METHODS

Y2H assays.

Y2H assays were performed using ProQuest Two-Hybrid System (Thermo Fisher Scientific). *AtSDIR1* was fused to the GAL4 DNA-binding domain in *pDEST32* as the bait construct. *pEXP-AD502* was used to develop a cDNA library from mixed-elicitor-treated *Arabidopsis* for screening. *AtJAZ9* was fused to the GAL4 activation domain in *pDEST22* as prey protein using a Gateway cloning method. The TM ($\Delta^{\text{TM}}\text{SDIR1}$), RING ($\Delta^{\text{TM}}\text{SDIR1}\Delta\text{RING}$), metal-binding ($\Delta^{\text{TM}}\text{SDIR1}\Delta\text{Metal}$), and PAL ($\Delta^{\text{TM}}\text{SDIR1}\Delta\text{PAL}$) domain-deleted constructs were expressed in *pDEST32* vector. Bait and prey constructs were cotransformed into yeast *MaV203* yeast-competent cells (Thermo Fisher Scientific). Positive clones were identified on TDO or minus leucine/tryptophan/histidine/uracil quadruple drop-out medium

Fig. 2. Bimolecular fluorescence complementation (BiFC) assay confirming the interaction of *AtSDIR1* with *AtJAZ9* in planta. **A**, Interaction of salt- and drought-induced ring finger 1 (*SDIR1*) with jasmonate zinc-finger inflorescence meristem domain 9 (*JAZ9*). The *pSITE-nEYFP-SDIR1* (full-length; upper panel) or *pSITE-nEYFP- $\Delta^{\text{TM}}\text{SDIR1}$* (lower panel) construct was coexpressed transiently with *pSITE-JAZ9-cEYFP* and plasma membrane (PM) red fluorescent protein (RFP) marker genes in *Nicotiana benthamiana* using *Agrobacterium*-mediated transient transformation. Images were taken after 3 days using a Leica-SP5 confocal laser-scanning microscope. DIC = differential interference contrast image. Scale bars indicate 50 μm . Experiments were repeated three times with similar results. **B**, Colocalization of *SDIR1*-*JAZ9* interaction showing high Pearson coefficient of yellow fluorescent protein (YFP) over PM-RFP. More than 10 images from a minimum of three biological replicates were used for analysis. Confocal images were processed in the Leica-SP5 image analysis tool.

(QDO) synthetic-defined medium containing 20 mM 3-amino-1,2,4-triazole. The liquid media contained X-gal to detect interaction by development of blue color. During growth in TDO and QDO media, 1.5 and 60 μ M COR, JA, and MeJA (Sigma-Aldrich, St. Louis) were included in the drop-out media. MG132 (10 μ M), a proteasome degradation inhibitor, was added to the QDO.

BiFC assay.

AtSDIR1 and $\Delta^{TM}SDIR1$ were fused to N-terminal *eYFP* in *pSITE-nEYFP* vector and *AtGCN4* was used as negative control. The C-terminal part of *eYFP* in *pSITE-cEYFP* was fused to *AtJAZ9* in *pSITE-nEYFP* (Kudla and Bock 2016). *Agrobacterium* strain GV3101 harboring these vectors along with PM or ER (*RFP-PM/RFP-ER*) markers were infiltrated to 3-week-old *N. benthamiana* leaves in 1:1 ratio at an optical density of 0.6 in morpholineethanesulfonic acid (MES) buffer. Sixteen hours prior to imaging, 10 μ M MG132 was infiltrated into the leaf to avoid protein degradation. Three days after infiltration, RFP (red fluorescent protein) or YFP fluorescence was detected at 561 or 514 nm, respectively, under $\times 63$ oil immersion using a confocal laser-scanning microscope (Leica-TCS SP5 AOBs and Leica-SP8). To analyze the colocalization, more than 10 images from a minimum of three biological replications were

processed for Pearson's correlation coefficient analysis in Leica SP5 software (Dunn et al. 2011).

Pull-down assay.

For the pull-down assay, *AtSDIR1* was expressed in *pDEST15* with a GST tag, and N-terminal *HA-JAZ9* was expressed in *Arabidopsis* stable lines. Leaf tissue from *Arabidopsis* Col-0 plants and HA-tagged *JAZ9*-overexpressing transgenic plants were homogenized in protein extraction buffer (50 mM Tris-HCl [pH 7.5], 75 mM NaCl, 0.2% Triton X-100, 5 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, 100 μ M MG132, 10 mM NaF, 2 mM Na_2VO_4 , and 1% protease inhibitor cocktail). *AtSDIR1* was purified with GST resin (Thermo Fisher Scientific) and Pierce 660 nm of protein assay reagent (Thermo Fisher Scientific) was used for quantifying the protein content. The recombinant *SDIR1* protein of 2.5 μ g was added to 5 μ g of total crude protein from *HA-JAZ9*-overexpressing *Arabidopsis* plants and the mix was incubated overnight at 4°C. The mixture was later incubated for 2 h at 4°C with Anti-HA Agarose conjugated resin (Thermo Fisher Scientific). Precipitated samples were resolved by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis by washing with Tris-buffered saline with 0.1% Tween 20, and dissolving in 2 $\times$ SDS protein loading buffer. Immunoblot assays were performed with anti-GST or anti-HA (Abcam, Cambridge, MA,

Fig. 3. Salt- and drought-induced ring finger 1 (SDIR1) degrades jasmonate zinc-finger inflorescence meristem domain 9 (JAZ9) through proteasome-mediated pathway. **A**, E3 ubiquitin ligase activity of full-length *AtSDIR1* (SDIR1 FL) and transmembrane (TM) and ring domain-deleted *AtSDIR1* ($\Delta^{TM}SDIR1\Delta RING$) fused to glutathione-S-transferase (GST) were used in the presence of ATP, 6 $\times$ hemagglutinin (HA) tag ubiquitin, E1 (from wheat), and E2 (from human-UBCH5b). His-tagged JAZ9 was used to assess ubiquitin transfer to the substrate by SDIR1. $\Delta^{TM}SDIR1\Delta RING$ did not show any E3 ligase activity, even in the presence of JAZ9. (i) Polyubiquitination of SDIR1-blot exposed for only 5 s; (ii) the same blot exposed for only 15 s to increase the signal intensity; (iii) detection of JAZ9 using His antibody; and (iv) detection of SDIR1 using GST antisera. Numbers on the left indicate the molecular mass of marker proteins in kilodaltons. Samples were resolved on 4 to 20% criterion protein gel. **B**, Protein gel blot analysis showing (i) SDIR1 promoting JAZ9 degradation in the presence of pathogen infection and levels of JAZ9 without SDIR1 at different time points after pathogen infection. (ii) Ratio of JAZ9 over Actin was estimated using image J band intensities and JAZ9 protein levels without pathogen at different time points, band. (iii) Intensities of JAZ9 over Actin showed no significant difference (control). *Escherichia coli*-expressed GST-SDIR1 fusion protein was incubated with HA-JAZ9-tagged protein isolated from *Arabidopsis* lines expressing *HA-JAZ9* that was treated with or without host pathogen *Pseudomonas syringae* pv. *tomato* DC3000 for 24 h postinoculation (hpi). Band intensities of JAZ9 over Actin showed no significant difference. Both proteins were mixed and incubated at room temperature. Samples were taken at different time points during incubation to load on protein gels. MG132 (10 μ M) was used in one of the samples from the start of the assay (0 hpi) to 3 hpi to inhibit proteasome degradation.

U.S.A.). The enhanced chemiluminescence system (GE Healthcare Bio-Sciences, Pittsburgh, PA, U.S.A.) was used for detection.

In vitro ubiquitination assay.

Crude extract containing recombinant wheat (*T. aestivum*) E1 (Sigma-Aldrich), E2 UBCH5b (Sigma-Aldrich), purified SDIR1 fused with GST tag, Δ^{TM} SDIR1 Δ RING-GST, JAZ9-His tags, and HA-ubiquitin (Sigma-Aldrich) were used. Reactions were performed using the protocol described previously (Xi et al. 2002). The reaction was stopped by adding protein-loading buffer and boiling for 10 min. Later, proteins were transferred to the nitrocellulose membrane and the anti-HA antibody (Abcam) was used for detection.

Protein degradation and gel blot analyses.

AtSDIR1-GST was expressed in BL21 *E. coli* and *HA-JAZ9* was expressed in transgenic *Arabidopsis* lines. SDIR1-GST was purified from *E. coli*. After 24 hpi in *HA-JAZ9*-expressing *Arabidopsis* with or without the host pathogen *P. syringae* pv. *tomato* DC3000, the leaf samples were ground in extraction buffer (50 mM Tris-MES [pH 7.5], 80 mM NaCl, 10 mM MgCl₂, 10% glycerol, 0.2% NP40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail) (Sigma-Aldrich) for total protein and quantified using Pierce 660 nm protein assay reagent (Thermo Fisher Scientific). Equal concentrations were taken for the assay. Both SDIR1-GST- and HA-JAZ9-tagged protein samples were incubated for 0, 0.5, 1, 2, 3, and 4 h at room temperature. MG132 (1.6 μ M) (Sigma-Aldrich) was added to one of the samples (incubated for 3 h) to inhibit the proteasome-mediated protein degradation. Later, proteins were blotted on nitrocellulose membrane, and GST antisera (Abcam) or HA antisera (Sigma-Aldrich) were used to detect the SDIR1 and JAZ9 protein levels. The GST and HA antisera were diluted to 1:10,000, followed by a secondary goat antirabbit antibody conjugated to horseradish peroxidase. The protein gel blots were visualized using ECL solution (GE Healthcare Bio-Sciences).

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