**Phytophthora sojae** effector Avr1d functions as an E2 competitor and inhibits ubiquitination activity of GmPUB13 to facilitate infection

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Oomycete pathogens such as *Phytophthora* secrete a repertoire of effectors into host cells to manipulate host immunity and benefit infection. In this study, we found that an RxLR effector, Avr1d, promoted *Phytophthora sojae* infection in soybean hairy roots. Using a yeast two-hybrid screen, we identified the soybean E3 ubiquitin ligase GmPUB13 as a host target for Avr1d. By communoprecipitation (Co-IP), gel filtration, and isotothermal titration calorimetry (ITC) assays, we confirmed that Avr1d interacts with GmPUB13 both in vivo and in vitro. Furthermore, we found that Avr1d inhibits the E3 ligase activity of GmPUB13. The crystal structure Avr1d complexed with GmPUB13 was solved and revealed that Avr1d occupies the binding site for E2 ubiquitin conjugating enzyme on GmPUB13. In line with this, Avr1d competed with E2 ubiquitin conjugating enzymes for GmPUB13 binding in vitro, thereby decreasing the E3 ligase activity of GmPUB13. Meanwhile, we found that inactivation of the ubiquitin ligase activity of GmPUB13 stabilized GmPUB13 by blocking GmPUB13 degradation. Silencing of GmPUB13 in soybean hairy roots decreased *P. sojae* infection, suggesting that GmPUB13 acts as a susceptibility factor. Altogether, this study highlights a virulence mechanism of *Phytophthora* effectors, by which Avr1d competes with E2 for GmPUB13 binding to repress the GmPUB13 E3 ligase activity and thereby stabilizing the susceptibility factor GmPUB13 to facilitate *Phytophthora* infection. This study unravels the structural basis for modulation of host targets by *Phytophthora* effectors and will be instrumental for boosting plant resistance breeding.

**Significance**

Ubiquitination acts as a crucial regulator in plant immunity. Accordingly, microbial pathogens secrete effectors to hijack the host ubiquitination system. However, the molecular mechanisms by which effectors modulate the host ubiquitination system are not yet clear. Here, we found that the *Phytophthora sojae* effector Avr1d physically binds to the U-box-type E3 ligase GmPUB13, which proved to be a susceptibility factor. The crystal structure of Avr1d complexed with GmPUB13 revealed that Avr1d occupies the binding site in GmPUB13 for E2 ubiquitin conjugating enzyme and competes with E2 for binding to GmPUB13. Avr1d stabilized GmPUB13 by suppressing the self-ubiquitination activity of GmPUB13 and thereby promoting *Phytophthora* infection. This study reveals a structural basis for modulation of host targets by *Phytophthora* effectors.

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roots. During *P. sojae* infection, Avr1d targets two highly homologous U-box ARMs-repeats-type E3 ligases in soybean, Glyma12g06860 and Glyma11g14910, which are phylogenetically close to AtPUB13 and were named as GmPUB13 and GmPUB13-like (GmPUB13L), respectively. Silencing GmPUB13 and GmPUB13L in soybean hairy roots increased the resistance to *P. sojae*. Avr1d physically binds to GmPUB13 and blocks the ubiquitin ligase activity of GmPUB13 by competing with E2 ubiquitin conjugating enzyme for the U-box domain. This study reveals the physical basis for modulation of host targets by a *Phytophthora* effector and provides mechanistic insights into the promotion of host susceptibility by *Phytophthora* effectors.

**Results**

**Avr1d Promotes *P. sojae* Infection.** To determine whether Avr1d acts as a virulence effector, we overexpressed Avr1d (without signal peptide) fused with a N-terminal enhanced green fluorescence protein (eGFP) in soybean hairy roots and performed infection assays using mRFP-labeled *P. sojae*. *P. sojae* infection was evaluated by quantifying the number of produced oospores and *P. sojae* biomass at 2 d postinoculation. Both the produced oospores and biomass of *P. sojae* were much greater in the hairy roots overexpressing eGFP-Avr1d than in the eGFP control (Fig. 1 A). These results suggested that Avr1d could promote *P. sojae* infection.

To confirm secretion of Avr1d by *P. sojae*, Avr1d with native signal peptide fused with monomeric red fluorescence protein (mRFP) at the C terminus was overexpressed in the wild-type *P. sojae* strain P6497. The mRFP-labeled *P. sojae* was used as a control (10). Protein expression was confirmed by Western blot with anti-mRFP (*SI Appendix*, Fig. S1). Confocal microscopy visualization of infected hypocotyl epidermal cells showed that the red fluorescence of Avr1d-mRFP concentrated specifically at the haustoria, while the control mRFP localized in the cytosol, without significant concentration in the haustoria (Fig. 1 B). This result suggested that the haustoria were primary sites of Avr1d secretion during infection.

**Avr1d Physically Interacts with GmPUB13 In Vitro and In Vivo.** To uncover the molecular mechanism underlying the function of Avr1d in promoting *P. sojae* infection, we performed yeast two-hybrid assays to identify potential targets of Avr1d. Through screening, we identified two highly homologous genes, Glyma12g06860 and Glyma11g14910 that encode U-box ARM-repeats-type E3 ligases. Phylogenetic analysis indicated that these two genes were homologous to AtPUB13 of *A. thaliana* (*SI Appendix*, Fig. S2A). Glyma12g06860 and Glyma11g14910 shared 97.0% amino acid sequence identity (*SI Appendix*, Figs. S2B and S3A) and we named them GmPUB13 and GmPUB13L, respectively. Further, yeast two-hybrid assays supported that Avr1d interacted with both GmPUB13 and GmPUB13L (Fig. 1 C). In support of the interaction between Avr1d and GmPUB13, we coexpressed Avr1d and GmPUB13 in *Nicotiana benthamiana* and found eGFP-Avr1d and GmPUB13-HA-mRFP colocalized to the cytoplasm under the confocal fluorescence microscopy (Fig. 1 D). Furthermore, GmPUB13-HA-mRFP communoprecipitated from *N. benthamiana* cell extracts with eGFP-Avr1d, but not with eGFP (Fig. 1 E). To determine whether Avr1d physically interacted with GmPUB13 in vitro, we performed pull-down assays and found His-GmPUB13 could be pulled down by GST-Avr1d, but not by GST (Fig. 1 F). The physical interaction between Avr1d and GmPUB13 was further confirmed by gel filtration chromatography (*SI Appendix*, Fig. S3B).

Together, these data demonstrated that Avr1d physically interacted with GmPUB13 both in vivo and in vitro.

**Crystall Structure of the Complex of Avr1d with the GmPUB13 U-Box Domain.** Avr1d belongs to the class of RxLR effectors that carry a signal peptide followed by the RxLR motif and an effector domain (*SI Appendix*, Fig. S4A) (9). To characterize the molecular mechanism by which Avr1d contributes to the virulence of *P. sojae*, we ascertained the structure of the effector domain of Avr1d complexed with the U-box domain of GmPUB13 (Fig. 2 A and *SI Appendix*, Fig. S4C). The structure of the complex was determined at 2.5-Å resolution using single wavelength anomalous diffraction. The model was refined to final *R*work and *R*free values of 23.5% and 24.9%, respectively (*SI Appendix*, Table S1). The asymmetric unit of the crystals contained one Avr1d-GmPUB13 U-box complex (PDB: 7C96).

Avr1d displayed the structural characteristic of a WY motif, adopting a three alpha helix bundle, in which the highly conserved Trp96 and Try118 formed a hydrophobic core. It shared structural similarity with Avr3a11 (ZRR5) (11), another WY motif-containing RxLR effector protein, yielding a root-mean-square deviation (rmsd) of 1.95 Å, despite low sequence similarity (*SI Appendix*, Fig. S4B).

The U-box domain of GmPUB13 consisted of a central α-helix (α1), a C-terminal helix (α2), a small antiparallel β-sheet (β1 and β2) and two prominent loops (loop1 and loop2) (Fig. 2 A), resembling the structure of the U-box of the eukaryotic ubiquitin ligase, the C-terminus of Hsp70 interacting protein (CHIP) (PDB: 2OXQ) (12); it could be superimposed well with the CHIP U-box with a rmsd of 1.66 Å over 72 matching Cα atoms (Fig. 2 B). The hydrophilic groove formed by loop1, loop2, and central α-helix was essential for the interaction with Avr1d. Five residues of GmPUB13, P264, I265, L267 (from loop1), W290 (from helix α1), and P299 (from loop2) formed hydrophobic interactions with five residues of Avr1d, I87, F90, F93 (from helix α2), A117, and I120 (from helix α3). In addition to the hydrophobic interactions, hydrogen bonds and a salt bridge formed between GmPUB13 D259 and Avr1d R123 also stabilized the complex (Fig. 3 A). Intriguingly, the residues in CHIP corresponding to the Avr1d-binding residues of GmPUB13 in CHIP are F218, I216, H241, and P250, and these four residues are necessary for the interaction of CHIP with the E2 subunit, UbcH5a (*SI Appendix*, Fig. S4C) (12). Therefore, by similarity to the UbcH5a-CHIP complex, the Avr1d-binding site of GmPUB13 was predicted also to be its E2 binding site, and suggesting that Avr1d may compete with E2 for GmPUB13 binding. Using yeast two-hybrid screening over 35 soybean E2s (GmE2s) from 14 different groups, we found seven GmE2s could interact with GmPUB13 (*SI Appendix*, Fig. S5A). As expected, the GmPUB13-Avr1d-binding affinity was stronger than those of GmPUB13-GmE2s in the yeast two-hybrid assay (*SI Appendix*, Fig. S5B). Two of the seven GmE2s and Avr1d were further assayed for the binding affinity with GmPUB13 using the isothermal titration calorimetry (ITC) assay. As shown in Fig. 2 C and *SI Appendix*, Fig. S5C, the binding affinity of GmPUB13-Avr1d (Kd 89 nM) was much stronger than those of GmPUB13-GmE2s (Kd 39.1 μM and 46.6 μM). Altogether, these results suggested that Avr1d tightly occupied the E2-binding site of GmPUB13 to compete with E2 for GmPUB13 binding.

**The Phenylalanine 90 of Avr1d Is Required for Interaction with GmPUB13 and Virulence Function.** The crystal structure revealed that hydrophilic interactions played major roles in Avr1d-GmPUB13 complex formation, in which the phenyl ring of Avr1d F90 inserted into the hydrophobic groove formed by P264, I265, W290, and P299 of GmPUB13 (Fig. 3 A). Substitution of these residues in GmPUB13, GmPUB13-V90A in the yeast two-hybrid assay (*SI Appendix*, Fig. S5D). On the other side, the mutation F90A in Avr1d likewise disrupted GmPUB13 binding as measured by ITC, gel filtration, and yeast two-hybrid assays, indicating that F90 was essential for the interaction with GmPUB13 (*SI Appendix*, Fig. S6 A and B). To evaluate the effect of this mutation on the potential virulence contribution of Avr1d, we overexpressed Avr1dF90A and wild-type Avr1d in soybean hairy roots, then inoculated the roots with mRFP-labeled *P. sojae*. We observed that *P. sojae* oospore production in hairy roots expressing Avr1dF90A was reduced, compared with wild-type Avr1d (*SI Appendix*, Fig. S7 A and B). These findings suggested that Avr1dF90A was less pathogenic than wild-type Avr1d.
not significantly different from in hairy roots expressing eGFP (Fig. 3C). Avr1d<sup>DAK</sup> protein expression in the transformed hairy roots was confirmed by Western blotting with anti-GFP antibodies (SI Appendix, Fig. S7). Thus, Avr1d<sup>DAK</sup> failed to promote <i>P. sojae</i> infection. Together, these results demonstrated that F90 of Avr1d was required for the interaction with GmPUB13 and for its potential virulence contribution.

Avr1d Inactivates the Ubiquitin Ligase Activity of GmPUB13 In Vitro and In Vivo. According to our structure and biochemical assays, Avr1d could compete with E2 for GmPUB13 binding. To determine whether Avr1d affects the ubiquitin ligase activity of GmPUB13, we performed a ubiquitination assay using GmPUB13 in yeast. Avr1d interacted with GmPUB13 and GmPUB13L in yeast, as indicated by yeast two-hybrid assays. EV, empty vector; SD<sup>-</sup>2 medium: SD<sup>−</sup>/−Leu/<sup>−</sup>Trp medium; SD<sup>-</sup>4+/X-α-gal: SD<sup>−</sup>/−Ade/<sup>−</sup>His/<sup>−</sup>Leu/<sup>−</sup>Trp + X-α-gal medium. (D) eGFP-Avr1d and GmPUB13-HA-mRFP coexpressed in <i>N. benthamiana</i> leaves (infiltrated with 50 μM MG132 12 h before observation) by agroinfiltration. eGFP-Avr1d and GmPUB13-HA-mRFP were coexpressed in <i>N. benthamiana</i> leaves by agroinfiltration. The leaves were observed under confocal microscopy 2 d postinfiltration. The smaller pictures on the Upper Left are a bigger view of the nucleus region in the main picture. (Scale bar, 50 μm.) (F) Confirmation of the interaction between Avr1d and GmPUB13 by GST pull-down. Protein extracts of GST-Avr1d and His-GmPUB13 were mixed and incubated with glutathione Sepharose 4B beads, respectively, and followed by washing with PBS buffer five times. Proteins in the input samples and pull-down samples were detected by Western blot with anti-His and anti-GST antibodies. Red dot indicated the protein size of His-GmPUB13. Molecular mass markers are shown (in kilodaltons).
no smeared bands were detected in the reaction containing E1, E2, GST, or in the reactions containing GmPUB13 but lacking E1 or E2 (Fig. 4A), showing GmPUB13 was an active ubiquitin ligase. It has been reported that cysteine 262 and tryptophan 289 of AtPUB13 in the U-box domain are required for its ligase activity (5). GmPUB13 with mutations on these sites, such as GmPUB13C263A or GmPUB13W290A lost ubiquitin ligase activity since no smeared bands of polyubiquitin were detected in these reactions (Fig. 4A), supporting the importance of the U-box domain for enzyme activity. To test whether Avr1d could modulate the enzyme activity of GmPUB13, Avr1d was added into the reaction mixture. The smeared bands were much weaker compared with the control (Fig. 4B). Thus, Avr1d indeed inhibited ubiquitin ligase activity of GmPUB13. In line with this, Avr1d decreased the ubiquitin ligase activity of GmPUB13 in a dose-dependent manner since the smeared polyubiquitin bands became progressively weaker as the concentration of Avr1d was increased (Fig. 4B and SI Appendix, Fig. S8A). However, even high amounts of Avr1dP90A protein could not block the ubiquitin ligase activity of GmPUB13 since Avr1dP90A failed to bind GmPUB13 (Fig. 4C and SI Appendix, Fig. S8B). To further evaluate the effect of Avr1d on the ligase activity of GmPUB13, we performed a ubiquitination assay in N. benthamiana. In line with our in vitro assay, smeared bands of polyubiquitin in leaves coexpressing Avr1d and GmPUB13 were weaker than that in leaves expressing GmPUB13 and eGFP or Avr1dP90A with similar amount of GmPUB13 protein loaded, respectively (Fig. 4D and SI Appendix, Fig. S8D). Altogether, Avr1d inhibited the ubiquitination activity of GmPUB13 in vitro and in vivo. GmPUB13 and

Fig. 2. Avr1d interacts with GmPUB13 by occupying the E2-binding site of GmPUB13. (A) Overall structure of Avr1d-GmPUB13 U-box. Avr1d (orange) and GmPUB13 U-box (cyan) are shown in cartoon form. (B) Superposition of crystal structures of Avr1d-GmPUB13 U-box and UbcH5a-CHIP U-box (PDB: 2OXQ). Avr1d (orange), GmPUB13 U-box (cyan), UbcH5a (gray), and CHIP U-box (blue white) are shown in cartoon form. (C) Binding affinity of Avr1d or GmE2 with GmPUB13. ITC-binding curve between Avr1d and GmPUB13(252 to 630) (Left). ITC-binding curve between GmE2 and GmPUB13(252 to 630) (Right). The ITC experiments were repeated twice independently with similar results. GmE2: Glyma06g13020.

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Lin et al. https://doi.org/10.1073/pnas.2018312118
GmPUB13 shared 97% sequence identity. Especially within the U-box domain, the region that interacts with Avr1d, the sequences are exactly the same (SI Appendix, Fig. S3A). In addition, we tried to test the interaction between Avr1d with GmPUB13L using gel filtration and in vitro ubiquitination assays. In these assays, Avr1d also interacted with GmPUB13L and inhibited its ubiquitin ligase activity (SI Appendix, Figs. S3C and S8C). Together, these data demonstrate that GmPUB13 and GmPUB13L probably function redundantly in soybean.

GmPUB13 Self-Ubiquitination Regulates Its Abundance. To explore whether GmPUB13 could regulate its protein abundance by self-ubiquitination, we expressed the inactive mutants GmPUB13C263A and GmPUB13W290A in N. benthamiana leaves. The protein abundance and fluorescence of GmPUB13C263A-HA-mRFP, GmPUB13W290A-HA-mRFP, and control HA-RFP were much stronger than GmPUB13-HA-mRFP (Fig. 4E and SI Appendix, Figs. S9A and B). The protein abundance of GmPUB13-HA-mRFP was significantly increased when the infiltrated leaves were treated with 50 μM MG132, a 26S proteasome complex inhibitor (Fig. 4F). Together, these data suggest that GmPUB13 may regulate its protein abundance by self-ubiquitination and 26S proteasome-mediated degradation.

GmPUB13 Promotes Plant Susceptibility. To determine the role of GmPUB13 in soybean resistance, we silenced GmPUB13 and GmPUB13L in soybean hairy roots. The qRT-PCR assay revealed that GmPUB13/GmPUB13L gene expression level decreased around 60% in the silenced hairy roots with little effect on a homologous gene Glyma10g35220 when compared to the empty vector control (SI Appendix, Fig. S10A and B). The GmPUB13/GmPUB13L silenced hairy roots showed less oospore production and P. sojae biomass compared to the control hairy roots when inoculated with eGFP-labeled P. sojae (Fig. 5A). In addition, the relative expression levels of several resistance-related marker genes, such as GmPR1a, GmPR2, and GmPR3, were slightly increased in GmPUB13/GmPUB13L silenced hairy roots (SI Appendix, Fig. S10C), suggesting that GmPUB13 and GmPUB13L are susceptibility factors for P. sojae (13). To further verify the conclusion, HA-GmPUB13 and derived mutants were overexpressed in soybean hairy roots. The protein abundance of GmPUB13 mutants was greater than wild-type GmPUB13 as detected by Western blot (Fig. 5B). The hairy roots were inoculated with mRFP-labeled P. sojae and Avr1d knockout P. sojae mutants (SI Appendix, Fig. S12A–D). The results showed that the oospore production was slightly increased in the hairy roots expressing GmPUB13 than EV control when inoculated with mRFP-labeled P. sojae. There was also a slight, albeit not significant, increase of P. sojae biomass in the hairy

**Fig. 3.** The 90th F of Avr1d is the key residue for the interaction with GmPUB13 and its virulence function. (A) Interaction residues between Avr1d and GmPUB13. The I87, F90, F93, A117, and I120 of Avr1d (orange) contribute to the hydrophobic interaction surface with P264, I265, L267, W290, and P299 of GmPUB13 U-box (cyan). R123 of Avr1d (orange) forms hydrogen bonds and salt bridge with D259 of GmPUB13 U-box (cyan). (B) ITC-binding curve between Avr1dW90A and GmPUB13(252 to 630). The ITC experiments were repeated twice independently with similar results. (C) Overexpression of Avr1dW90A in soybean hairy roots fails to promote P. sojae infection. The fluorescence hairy roots expressing eGFP, eGFP-Avr1d, and eGFP-Avr1dW90A were inoculated with mRFP-labeled P. sojae hyphae on the tips (Left). (Scale bar, 500 μm.) The oospore production was observed under fluorescence microscopy at 2 d postinoculation (Right). Asterisks indicate t test P < 0.05. These experiments were repeated three times with similar results.
Fig. 4. Avr1d inhibits GmPUB13’s ubiquitin ligase activity to promote its accumulation. (A) Ubiquitination assays of GmPUB13, GmPUB13C263A, and GmPUB13W290A in vitro. GmPUB13 and mutants were fused with GST-tag and incubated with or without wheat E1, ATUBCB as E2 in the buffer with ubiquitin. Reaction mixtures were detected by Western blot with anti-ubiquitin and anti-GST. Red dots indicate the protein size of GST-GmPUB13. Molecular mass markers are shown (in kilodaltons). (B) Avr1d inhibits the ubiquitin ligase activity of GmPUB13. Avr1d and His-MBP proteins were added into the reaction mixtures with E1, E2, and GST-GmPUB13 as E3. Reaction mixtures were detected by Western blot with anti-ubiquitin and anti-GST. Red dots indicate the protein size of GST-GmPUB13. (C) Avr1dF90A fails to inhibit the ubiquitin ligase activity of GmPUB13. Avr1d or Avr1dF90A protein were respectively added into the reaction mixture with E1, the soybean predicted ubiquitin conjugating enzyme-related protein Glyma06g13020 as E2, flag-ubiquitin, and GST-GmPUB13(252 to 630) as E3. Reaction mixtures were detected by Western blot with anti-GST and anti-flag. Molecular mass markers are shown (in kilodaltons). (D) Avr1d inhibited the ubiquitination activity of GmPUB13 in N. benthamiana. eGFP, eGFP-Avr1d, and eGFP-Avr1dF90A were coexpressed with GmPUB13-HA-mRFP, Myc-GmE2 (Glyma06g13020), and p19 in N. benthamiana leaves, respectively. Leaves were injected with 50 μM MG132 12 h before harvest. The input samples were detected by anti-HA, anti-Myc, and anti-GFP antibody and the output samples were detected by anti-RFP and anti-ubiquitin antibody. The smear bands indicate polyubiquitin chains. Ponceau S: Ponceau staining indicates the rubisco protein. Molecular mass markers are shown (in kilodaltons). (E) Protein accumulation of GmPUB13 and its mutants. GmPUB13-HA-mRFP, GmPUB13C263A-HA-mRFP, and GmPUB13W290A-HA-mRFP were expressed in N. benthamiana leaves by agroinfiltration. The total protein was extracted at 1.5, 2, 3, and 4 d postinfiltration followed by Western blot detection with anti-HA antibody. Ponceau S: Ponceau staining indicates the rubisco protein. Molecular mass markers are shown (in kilodaltons). (F) MG132 treatment increases GmPUB13-HA-mRFP protein abundance. The leaves expressing GmPUB13-HA-mRFP were infiltrated with 50 μM MG132 0, 4, 8, and 12 h before harvest and 0.5% DMSO as control. The total protein was detected by Western blot with anti-HA antibody. Ponceau S: Ponceau staining indicates the rubisco protein. Molecular mass markers are shown (in kilodaltons). (G) Avr1d, but not Avr1dF90A, can stabilize GmPUB13. GmPUB13-HA-mRFP was coexpressed with eGFP, eGFP-Avr1d, or eGFP-Avr1dF90A in soybean hairy roots. The total proteins of the hairy roots were detected by Western blot with anti-HA and anti-GFP. Ponceau S: Ponceau staining. Molecular mass markers are shown (in kilodaltons).
roots expressing HA-GmPUB13 than EV control, when inoculated with Avr1d knockout mutants (Fig. 5 C–E). In addition, we found that the oospore production and P. sojae biomass was significantly increased in the hairy roots expressing GmPUB13C263A or GmPUB13W290A than expressing GmPUB13 or EV control when infected with mRFP-labeled P. sojae or Avr1d knockout mutants (Fig. 5 C–E). We also overexpressed GmPUB13-HA-mRFP and mutants in N. benthamiana leaves and then performed infection assays using Phytophthora capsici. Compared with the HA-mRFP control, N. benthamiana leaves expressing GmPUB13 showed no difference in lesion area (SI Appendix, Fig. S11A). However, the leaves expressing GmPUB13C263A or GmPUB13W290A showed much bigger lesions than those expressing GmPUB13 (SI Appendix, Fig. S11 B and C) or HA-mRFP control (SI Appendix, Fig. S11 D and E). These results suggested that the stable inactivated mutants of GmPUB13 could promote P. sojae and P. capsici infection when overexpressed in soybean hairy roots or N. benthamiana and that the inactive mutants did not need Avr1d to promote infection.

Avr1d Can Stabilize GmPUB13 In Plant. Since GmPUB13 could undergo self-ubiquitination to regulate its protein abundance and Avr1d inhibited the ubiquitination activity of GmPUB13, we tested whether Avr1d could stabilize GmPUB13 in planta. When we coexpressed Avr1d and GmPUB13 in soybean hairy roots, we observed that the red fluorescence of GmPUB13-HA-mRFP was stronger when coexpressed with Avr1d than with eGFP or Avr1dF90A (SI Appendix, Fig. S13A). This also held true when GmPUB13-HA-mRFP was expressed with Avr1d, eGFP, or Avr1dF90A in N. benthamiana (SI Appendix, Fig. S13B). We also detected stronger bands of GmPUB13-HA-mRFP in the total proteins of hairy roots or N. benthamiana leaves coexpressing GmPUB13-HA-mRFP with eGFP-Avr1d than with eGFP by anti-HA (Fig. 4G and SI Appendix, Fig. S13C). Unlike Avr1d, coexpressing Avr1dF90A failed to stabilize GmPUB13 in soybean hairy roots and N. benthamiana leaves (Fig. 4G and SI Appendix, Fig. S13C). This is consistent with the previous results in Fig. 4B–D suggesting that Avr1d could stabilize GmPUB13.

Discussion

Plant pathogens secrete large arsenals of effectors to modulate host cell processes and enable a successful infection. Studies on the host targets of such effectors provide molecular clues for...
understanding the outcome of plant–pathogen interactions. Thus far, multiple host targets have been identified for effectors secreted by oomycete pathogens. However, structural evidence needed to unravel the mechanisms underlying interactions of effectors and host targets is still missing. In this study, we determined that *P. sojae* avirulence effector Avr1d physically binds to the soybean U-box-type E3 ligase GmPUB13 and we dissected the structural basis of this interaction.

A typical feature of most E3 ligases is the ability to catalyze their own ubiquitination. The biological role of self-ubiquitination was proposed to be to target the ligase for degradation, which could function as a means of negative feedback of E3 ligases (14, 15). In fact, many E3 ligases, even those that catalyze their own ubiquitination, are targeted by an exogenous ligase, which makes the regulation of E3 ligases rather complex. In addition, self-ubiquitination of E3 ligases could be involved in nonproteolytic functions (16) and could enhance substrate ubiquitin ligase activity (17). In this study, we found that GmPUB13 was a substrate of its own E3 ubiquitin ligase activity and this self-ubiquitination induced degradation of GmPUB13 by the proteasome.

Ubiquitination contributes crucially to the intricate and precise molecular mechanisms that govern plant immune responses and therefore the ubiquitination system is a hub targeted by multiple pathogen virulence effectors. For example, several effectors possess E3 ubiquitin ligase activity, such as AvrPtoB from *Pseudomonas syringae* (18, 19) and XopK from *Xanthomonas oryzae pv. oryzae* (20) that degrade host targets to attenuate plant immunity.

In addition, effectors may hijack the host ubiquitination complex to reprogram the host targets. AvrPiz-t from *Magnaporthe oryzae* interacts with the RING-type E3 ubiquitin ligase APIP6, a positive regulator of rice immunity, to promote the degradation of APIP6 (21). The *Phytophthora infestans* RxLR effector PI02860 interacts with a potato susceptibility factor SNRL1, a putative substrate adaptor of a CULLIN3-associated ubiquitin E3 ligase complex, to degrade a guanine nucleotide exchange factor called SWAP70, which is essential for potato immunity (22, 23). In addition, *P. infestans* effector AVR3a interacted with a U-box-type ubiquitin E3 ligase CMPG1 and manipulated plant immunity by stabilizing potato CMPG1 (24). Nevertheless, how AVR3a mediate the stability of CMPG1 is thus far unclear.

In this study, we found that the *P. sojae* avirulence effector Avr1d physically binds to the soybean U-box-type E3 ligase GmPUB13 and could stabilize GmPUB13 by suppressing its ubiquitin ligase activity. We deciphered the molecular mechanism underlying inhibition of GmPUB13 by Avr1d by determining the molecular structure of the Avr1d-GmPUB13 complex, combined with biochemical assays. Based on the crystal structure, we successfully identified the interface required for interaction between GmPUB13 and Avr1d and showed that the residues at the interface were required for Avr1d to bind to GmPUB13 and inhibit its ligase activity. Since GmPUB13 functions as a susceptibility factor, stabilization of GmPUB13 by Avr1d-mediated inhibition of its ligase activity is the key to the contribution of Avr1d to *P. sojae* virulence.

**Materials and Methods**

*Phytophthora, Bacteria, and Plant Growth Condition.* *P. sojae* and *P. capsici* were grown in 10% (V/V) V8 medium with 1.5% agar or without agar in the dark at 25 °C. *Escherichia coli* JM109 for vector construction were grown on Luria–Bertani (LB) medium with agar or shaking 220 rpm in the dark without agar. 37 °C. *Agrabacterium tumefaciens* and *Agrobacterium rhizogenes* were grown on LB medium with 1.5% agar or with shaking 220 rpm in the dark without agar containing 50 μg/mL rifampicin or streptomycin, respectively, at 30 °C. *N. benthamiana* were grown on vermiculite from seeds in a climate chamber under long-day conditions (14 h light and 10 h dark) at 24 °C. Glycine max etiolated seedlings were grown on vermiculite medium from seeds in the dark, at 25 °C for 3 to 4 d in a climate chamber.

*P. sojae* Transformation, Zoospore Producing, and Infection Assay. Avr1d-mRFP driven by the Hm34 promoter (pGFMRFΔ9) was transformed into *P. sojae* strain Avr1d-mRFP using a PEG (polyethylene glycol-mediated protoplast transformation system (25). Transformants were selected on 10% (V/V) V8 medium containing 50 μg/mL genetin (G418) and further confirmed by red fluorescence selection under fluorescence microscopy and Western blot detection of Avr1d-mRFP protein by anti-mRFP antibody. Avr1d was knocked out using the CRISPR-Cas9 system mediated gene editing following the protocols described by Fang et al. (26). The transformants were screened by PCR using the genome DNA of transformants with primers listed in SI Appendix, Table S2. Mutants were confirmed by sequencing. *P. sojae* zoospores were produced by washing the 3- to 5-d-old *P. sojae* hyphae grown in 10% V8 liquid medium with sterilized tap water three times and then incubated in the dark (25 °C) to stimulate sporulation and zoospore releasing. The droplets containing about 100 zoospores were then inoculated to the hypocotyl of soybean etiolated seedlings. The inoculated samples were kept in wet and dark conditions at 25 °C. For a 5- to 10-h infection, the epidermal cells of soybean hypocotyl were observed under confocal microscopy.

**Hairy Roots Transformation and Infection Assay.** Soybean hairy root transformation was performed by using *A. rhizogenes* K599-mediated T-DNA transformation (27). Cotyledons of 6-d-old soybeans grown under long-day conditions (14 h light and 10 h dark) at 25 °C on vermiculite were used as explant for transformation. The lower epidermis of the cotyledons was wounded by cutting a 3- to 5-mm diameter wound. The wounding sites were inoculated with K599 strains carrying cognate plasmids for overexpression or silencing. After incubation for 3 to 4 wk on Murashige and Skoog (MS) medium at 25 °C, hairy roots were collected from the wall of the wounded sites. The transformed hairy roots were chosen under fluorescent microscopy. To express Avr1d and the related mutants in the soybean hairy root, each was fused with N terminus eGFP and cloned into the T-DNA region of pBGFPI2 plasmid with the CaMV 35S promoter. To coexpress eGFP, eGFP-Avr1d, or eGFP-Avr1dΔNOS with GmPUB13-HA-mRFP in soybean hairy roots, eGFP and GmPUB13-HA-mRFP, eGFP-Avr1d and GmPUB13-HA-mRFP, and eGFP-Avr1dΔNOS and GmPUB13-HA-mRFP were cloned into two open reading frames separately in the T-DNA region of pFGCS941 plasmid driven by mannopine synthase promoter. For these three plasmids, both of DNA fragments were digested with EcoRI and NotI, respectively. The green fluorescence hairy roots selected under fluorescence microscopy were regarded as transformed hairy roots. The protein expression of the fluorescence hairy roots was detected by Western blot with anti-GFP (C M C T A G ) or anti-HA.

For silencing of GmPUB13 and GmPUB13L in soybean hairy roots, a 100-bp cDNA sequence that specifically matches to GmPUB13 and GmPUB13L (blasted by the VIGS tool in https://solgenomics.net/) was constructed with forward and reverse sequence linked by chalcone synthase intron from *Petunia hybrida* into pFGCS941mCherry plasmid with CaMV 35S promoter. The T-DNA region contains another open reading frame for mCherry expression driven by a mannopine synthase promoter. The red fluorescence hairy roots under fluorescence microscopy were regarded as silenced hairy roots. The total RNA was extracted using the Total RNA Kit (Cat. No. R6834, OMEGA). The silencing efficiency and the relative expression levels of resistance marker genes were detected by real-time quantitative PCR (qPCR) relative to the gene expression of GmCYP2 (8).

To test the susceptibility of transformed soybean hairy roots, more than 30 transformed hairy roots were collected and infected with eGFP or mRFP-labeled *P. sojae*. The hairy roots were incubated in humid and dark conditions for 2 d at 25 °C (28). The oospore production was assayed under fluorescence microscopy. The relative biomass of *P. sojae* grown in transformed hairy roots was detected by quantitative PCR and indicated by the ratio of *P. sojae* actin gene and soybean GmCYP2 gene.

**Yeast Two-Hybrid Assay.** The PEG-mediated transformation protocol described in the Clontech Yeast Protocol Handbook was used to transform plasmids into yeast AH109 and Matchmaker GAL4 two-hybrid system to screen for targets or check the protein-to-protein interaction. To screen host targets of Avr1d, Avr1d without signal peptide was constructed into vector pGBK7 as the bait. cDNA libraries derived from soybean hypocotyl and root RNA were constructed to pGADT7 derived from soybean hypocotyl and root RNA were constructed to pGADT7 plasmid fused with GAL4 activation domain (AD) by the Clontech company. A total of 6 × 106 clones were screened. The yeast clones were selected on SD-/His−/−Leu−/−Trp medium for medium stringency interaction or on SD−/−His−/−His−/−Leu−/−Trp−/−Gal medium for high stringency interaction. The AD plasmids of the yeast were extracted by the Qiagen Plasmid Mini Kit (Cat. No. 12931). The AD plasmids were confirmed by restriction enzyme digestion. The yeast clones were selected with yeast AH109 and Matchmaker GAL4 two-hybrid system to screen for targets or check the protein-to-protein interaction. To screen host targets of Avr1d, Avr1d without signal peptide was constructed into vector pGBK7 as the bait. cDNA libraries derived from soybean hypocotyl and root RNA were constructed to pGADT7 plasmid fused with GAL4 activation domain (AD) by the Clontech company. A total of 6 × 106 clones were screened. The yeast clones were selected on SD-/His−/−Leu−/−Trp medium for medium stringency interaction or on SD−/−His−/−His−/−Leu−/−Trp−/−Gal medium for high stringency interaction. The AD plasmids of the yeast were extracted by the Qiagen Plasmid Mini Kit (Cat. No. 12931). The AD plasmids were confirmed by restriction enzyme digestion.
confirm the interaction of Avr1d or E2 conjugating enzyme-related genes and full-length GmPUB13, Avr1d and E2 conjugating enzyme-related genes were constructed into pBGK77 plasmid, while full-length GmPUB13 and GmPUB13L were constructed into pGADT7 plasmid from soybean cDNA. The plasmids were cotransformed into yeasts and then tested on SD-Leu–Tryp medium and SD−Ade−His−Leu–TrpXα-Gal medium.

**GST Pull Down and Co-IP Assay.** For GST pull down, Avr1d was cloned into pGEX-4T-2 with a C terminus GST tag, while GmPUB13 was cloned into pET28a fused with a C-terminal His-tag. Both Avr1d and GmPUB13 were expressed in E. coli BL21, induced by 0.2 μM IPTG (β-D-galactopyranoside). The bact­erial lystate containing GST-Avr1d was incubated with 10 μl glutathione Sepharose 4B beads (Cat. No. 45-000-139, GE Healthcare) for 3 h and purified by washing with 1 × PBS (phosphate buffered saline, Cat. No. ST448, Beyotime Biotechnology) five times. Then the beads were incubated with bacteria lystate containing His-GmPUB13 for another 3 h and purified by washing with PBS five times. The proteins eluted from the beads were determined by Western blot with anti-GFP (CMC tag) and anti-His. For Co-IP, Avr1d without signal peptide was cloned into pBluescript plasmid fused with eGFP at its C terminal and GmPUB13 was cloned into pGFC914HAmRFP fused with 3× HA-mRFP at its C terminal. Avr1d and GmPUB13 were coexpressed in N. benthamiana leaves as A. tumefaciens-mediated transformation. The total protein of N. benthamiana leaves was extracted in IP buffer (50 mM Tris·HCl pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 1% protease inhibitor cocktail [Cat. No. P9599, Sigma-Aldrich]) that contained 10% Fetal bovine serum (FBS) (phenylmethylsulfonyl fluoride) and then incubated for 4 h with 10 μl agarose beads conjugated anti-HA mouse monoclonal antibody (Cat. No. AT0079, CMC tag). After washing with 1× PBS buffer five times, the agarose beads with proteins were boiled in 1× SDS (sodium dodecyl sulfate)-polyacylamide gel electrophoresis) loading buffer for 10 min. Then the protein samples were determined by Western blot with anti-HA (Abmart) and anti-GFP (CMC tag).

Relative Quantification of P. sojae Biomass. The genome DNA of infected soybean hairy roots was extracted using the New Plant Genome Extraction Kit (Cat. No. DP305, Tiangen Biotech) and used for qPCR with ChamQ SYBR qPCR Master Mix (Cat. No. P505, Vazyme) from cDNA of P. sojae (Cat. No. Q311, Vazyme). The P. sojae biomass was calculated by using the P. sojae PsActin gene relative to the soybean GmCY2P2 gene. Primer sequences are listed in [SI Appendix, Table S2].

**Transient Expression in N. benthamiana.** Avr1d, GmPUB13, and related mutant sequences were amplified by Phanta Max Super-Fidelity DNA Polymerase (Cat. No. P505, Vazyme) from cDNA of P. sojae or G. max, respectively. The fragments were then cloned into vector pBluescript and pGFC914HAmRFP using ClonExpress II One Step Cloning Kit (Cat. No. C0112, Vazyme). A. tumefaciens strains with different plasmids were infiltrated into N. benthamiana leaves. The leaves were harvested 1.5, 2, 3, and 4 days after infiltration. Total proteins were extracted with 1× TBS 500 buffer (50 mM Tris, 200 mM NaCl, 20 mM imidazole) and lysed with a homogenizer. The lysate was then washed with 1× TBS buffer (50 mM Tris pH 7.5, 200 mM NaCl) four times and boiled in 100 °C for 5 min in SDS-PAGE loading buffer. The input samples were determined by Western blot with anti-HA (Abmart), anti-GFP (CMC tag) and the output samples were determined by Western blot with anti-RFP (Cat. No. 6G6, ChromoTek) and anti-ubiquitin (Cat. No. ab7254, Abcam).

**Protein Expression and Purification.** The GmPUB13 U-box and Avr1d were both cloned into the modified pet32a vector (Novagen) after adding a cleavage site for tobacco etch virus (TEV) protease to the 5′ end of the GmPUB13 and GmPUB13 gene through PCR amplification. For His-GmPUB13 U-box and Avr1d protein coexpression, the constructs were transformed into E. coli BL21 (DE3), and cells were grown at 37 °C to OD600 = 0.6 to 0.8 and induced with 0.1 mM IPTG for 8 to 10 h at 16 °C. The cells were harvested by centrifugation for 15 min at 4,000 × g at 4 °C. Cells were resuspended in Ni-lys buffer (50 mM Tris pH [8.0], 200 mM NaCl, 20 mM imidazole) and lysed with a homogenizer. The lysate was then centrifuged twice using 13,000 × g for 30 min. The supernatant was loaded on Ni-affinity column (GE Healthcare). His-GmPUB13 U-box and Avr1d were recovered by gradient elution with Ni-elution buffer (50 mM Tris pH [8.0], 200 mM NaCl, 200 mM imidazole). Fractions containing His-GmPUB13 U-box and Avr1d were verified by SDS-PAGE and pooled for tag cleavage to cleave the His tag overnight at 4 °C with 1:20 tobacco etch virus (TEV) protease. The tag-removed His-GmPUB13 U-box and Avr1d were loaded on a Superdex 200 gel-filtration column (GE Healthcare) and then stored at −20 °C. The fractions (50 mM NaCl, 1 mM TCEP [tris(2-carboxyethyl)phosphine]) were collected using the AKTA Explorer FPLC system (GE Healthcare). The protein concentration was determined to 7.5 mg/mL.

**Crystallization, Data Collection, and Structure Determination.** Crystallization was conducted using the sitting-drop vapor diffusion method at 4 °C. Avr1d-GmPUB13 U-box yielded crystals with good diffraction quality in 12% wt/vol polyethylene glycol 20,000, 0.1 M BICINE pH 8.5 and 3% wt/vol dextrose sulfate sodium salt. Before data collection, all crystals were soaked in the reservoir solution supplemented with 25% glycerol and flash cooled in liquid nitrogen. The diffraction data were collected at beamlines BL17U1 and BL19U1 at the Shanghai Synchrotron Radiation Facility and processed using the XDS program. A summary of the statistical methods used for data collection and analysis is provided in [SI Appendix, Table S1]. Phases were obtained experimentally with data from selenomethionine-substituted Avr1d-GmPUB13 U-box. The PHENIX software suite was used for initial model building. The final model was built by performing iterative manual model building with Coot and maximum likelihood refinement in PHENIX. Images and structural alignments were generated by using PyMOL.

**Size Exclusion Chromatography (SEC) Assay.** Purified GmPUB13(252-630) (roughly 40 μM) and Avr1d (roughly 40 μM) proteins were incubated at 4 °C for 1 h in the buffer containing 20 mM Tris (pH 8.0), 300 mM NaCl, 1 mM TCEP. GmPUB13(252-630), Avr1d, GmPUB13(252-630), and Avr1d, three samples were then injected onto a Superdex 200 gel-filtration column (GE) for analysis in line at a flow rate of 0.4 mL/min. The fractions (0.5 mL/fraction) were analyzed by SDS-PAGE electrophoresis and visualized by Coomassie brilliant blue staining. Mutant samples such as Avr1d were analyzed using the same method as described above.

**Lin et al.** Phytophthora sojae effector Avr1d functions as an E2 competitor and inhibits ubiquitination activity of GmPUB13 to facilitate infection
Isothermal Titration Calorimetry. ITC-binding curves were measured by using a Microcal ITC-200 instrument (Malvern). Purified proteins were transferred to buffer containing 20 mM Hepes pH 7.5, 100 mM NaCl, and 2 mM β-mercaptoethanol by 5 mL desalting column (GE). Titrations were performed at 20 °C. Titration of GmPUB13(252-630) in the cell was performed by sequential addition of Avr1d, GmE2, and Avr1dF90A separately. Data were analyzed using Origin 7.0.

Data Availability. Protein structure data have been deposited in the RSCB Protein Data Bank (7C96).

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