GmDAD1, a Conserved Defender Against Cell Death 1 (DAD1) From Soybean, Positively Regulates Plant Resistance Against Phytophthora Pathogens

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Initially identified as a mammalian apoptosis suppressor, defender against apoptotic death 1 (DAD1) protein has conserved plant orthologs acting as negative regulators of cell death. The potential roles and action mechanisms of plant DADs in resistance against Phytophthora pathogens are still unknown. Here, we cloned GmDAD1 from soybean and performed functional dissection. GmDAD1 expression can be induced by Phytophthora sojae infection in both compatible and incompatible soybean varieties. By manipulating GmDAD1 expression in soybean hairy roots, we showed that GmDAD1 transcript accumulations are positively correlated with plant resistance levels against P. sojae. Heterologous expression of GmDAD1 in Nicotiana benthamiana enhanced its resistance to Phytophthora parasitica. NbDAD1 from N. benthamiana was shown to have similar role in conferring Phytophthora resistance. As an endoplasmic reticulum (ER)-localized protein, GmDAD1 was demonstrated to be involved in ER stress signaling and to affect the expression of multiple defense-related genes. Taken together, our findings reveal that GmDAD1 plays a critical role in defense against Phytophthora pathogens and might participate in the ER stress signaling pathway. The defense-associated characteristic of GmDAD1 makes it a valuable working target for breeding Phytophthora resistant soybean varieties.

Keywords: Glycine max, Phytophthora resistant, defender against apoptotic death 1 (DAD1), programmed cell death (PCD), ER stress

INTRODUCTION

As sessile organisms, plants are continually exposed to various biotic and abiotic stresses. Therefore, complex stress perception, signal transduction and adaptation strategies have evolved in plants to cope with adverse environmental conditions. In particular, the programmed cell death (PCD) pathway has been demonstrated to play key roles in plant responses to both abiotic and biotic stresses (Dickman et al., 2001; Lam et al., 2001; Williams et al., 2010). In plant defense against...
Our findings demonstrate that GmDAD1 overexpressing in Arabidopsis protoplast cells against ultraviolet-C-induced PCD (Danon et al., 2004) and DAD1 expression in Gladiolus decreases drastically during petal senescence (Yamada et al., 2004). Regarding the roles of DAD1 proteins in plant defense, Wang X. J. et al. (2011) reported that TaDAD2-silenced wheat leaves have attenuated resistance to Puccinia striiformis with down-regulated expression of several defense-related genes. However, how this protein modulates plant-pathogen interactions has not been well characterized overall.

In this study, a DAD1 orthologous gene was identified from soybean (Glycine max). Spatial and temporal expression of GmDAD1 upon P. sojae infection, as well as its protein subcellular localization, were investigated. The function of GmDAD1 in conferring Phytophthora resistance was dissected in soybean hairy roots with GmDAD1 specifically silenced by RNAi, and Nicotiana benthamiana transgenic lines overexpressing GmDAD1 or suppressing native NbDAD1. Our findings demonstrate that GmDAD1 plays a critical role in Phytophthora resistance probably via regulating ER stress signaling.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

Two soybean varieties were used in this research: Williams 82 carrying the gene Rps1k, which confers resistance to P. sojae race 2 (Bernard and Cremeens, 1988) and Williams which does not carry any known Rps resistance gene (Bernard and Lindahl, 1972). Seeds of Williams 82 and Williams were sown in small plastic pots containing disinfected soil and maintained in greenhouse at 25°C and 16h:8h light/dark photoperiod. N. benthamiana plants were grown under identical conditions as described above.

**Culture of Phytophthora Pathogens**

Phytophthora sojae isolates P6497 and P6497-RFP, which is a P. sojae strain constitutively expressing red fluorescence protein (RFP) (Xiong et al., 2014) were routinely cultured on 10% V8 juice agar plates at 25°C in the dark. Phytophthora parasitica was grown under the same conditions.

**P. sojae Inoculation and Soybean Samples Collection**

Root, stem and leaf samples of the soybean varieties Williams 82 and Williams were collected at seedling and pod-filling stages. Hypocotyl inoculation of P. sojae was performed on Williams 82 and Williams plants as described previously (Sun et al., 2014). Agar disks containing hyphae were cut from fresh cultures and inoculated onto hypocotyl incision. After inoculation, the seedlings were placed in growth chamber to keep moisture. Inoculated stems were collected at 0, 6, 12, 24, and 48 h post inoculation (hpi). All samples were frozen immediately in liquid nitrogen and stored at −70°C. Three biological replicates were performed for each time point.

**DNA and RNA Extraction and RT-qPCR**

Following supplier instructions, all DNA and RNA samples were extracted using the Hi-DNAssecure plant kit and the RNA simple Total RNA kit (Tiangen, China), respectively. For RNA samples, elimination of genomic DNA contamination and reverse transcription were performed using the HiScript II Q RT SuperMix reagent Kit (Vazyme, China). qPCR reactions were performed on an ABI PRISM 7500 real-time PCR system (Applied Biosystems, United States) using the ChamQ™ SYBR qPCR Master Mix reagent (Vazyme, China). Relative gene expression levels were calculated using the comparative 2−ΔΔCT method (Livak and Schmittgen, 2001). Statistical analysis was conducted using the Student’s t-test with Excel 2010 software and the data were considered statistically significant for P < 0.05. qPCR primers for GmDAD1 were designed from its conserved region. BTEF (GenBank ID EU079791) was selected for determining P. sojae biomass (Yan et al., 2014). GmCons4 (GenBank ID EU578186.1) was selected as...
endogenous reference in soybean (Libault et al., 2008). NbEF1a (GenBank ID AY206004) was used as N. benthamiana reference in the VIGS (virus-induced gene silencing) assay.

Defense-related genes analyzed in this research include five pathogenesis-related (PR) genes: PR1a, PR2, PR3, PR4 and PR5 (Bertini et al., 2003; Chen et al., 2007; Mazarei et al., 2007; Maldonado et al., 2014); the JA-regulated defense gene

**plant defensin 1.2 (PDF1.2)** (Lorenzo and Solano, 2005); the ethylene (ET) signaling marker gene

**ethylene response factor 1** (ERF1) (Lorenzo et al., 2003); the reactive oxygen species (ROS) biosynthetic gene

**NADPH oxidase** (NADPHOX) and two ROS scavenging genes:

**catalase** (CAT) and **ascorbate peroxidase** (APX) (Perez and Brown, 2014). We employed the sequences of

**G. max** if the genes have been reported already, or obtained them by searching in the soybean EST and genome databases¹ using orthologous sequences from A. thaliana as queries. All primers were designed using the Primer Premier 5 software. Primer specificity was evaluated by sequence similarity comparison and melting curve results of RT-qPCR. The primers of ER related genes were designed used the same strategy. The analyzed ER-stress related genes were the

**binding immunoglobulin protein** (Bip), the **protein disulfide isomerase** (PDI), the **calnexin1** (CNX1), the **ER lumen-localized DnaJ protein3a** (ERdj3A), the **luminal binding domain/glucose-regulated protein 94** (GRP94), the **basic region/leucine zipper motif 17** (bZIP17) and the downstream gene

**vacuolar processing enzyme** (VPE) (Rojo et al., 2004; Gai et al., 2014; Tiziana and Roberto, 2014). All primers used in this study and detailed information were listed in

**Supplementary Table S1.**

### Subcellular Localization of the GmDAD1 Protein

For subcellular localization, the full-length coding sequence (CDS) of **GmDAD1** was amplified from cDNAs of the Williams variety using primer pair

**pBIN-G-DAD-F/R** (Supplementary Table S1). The 351-bp **GmDAD1** CDS was then translationally fused with GFP after cloning into pBIN-GFP (Zhang et al., 2014) using

**Kpn I** and **XbaI** sites. After sequencing validation, **GmDAD1-GFP** and **mCherry-HDEL constructs** were introduced into **Agrobacterium tumefaciens** strain GV3101. The two **Agrobacterium** liquid cultures were mixed and co-infiltrated into

**N. benthamiana** leaves using a blunt syringe. After maintained for 48 h in greenhouse, agroinfected leaves were detached and visualized with a laser scanning confocal microscope (Zeiss, GERMANY) at 488 and 591 nm for GFP and mCherry detection, respectively.

### Plasmid Construction for Soybean Cotyledon Transformation

The **pBIN-GFP-GmDAD1** construct was used to determine **GmDAD1** subcellular localization was also used to overexpress **GmDAD1** in soybean hairy roots, and the **pBIN-GFP empty vector** was used as control which allows expression of the **GFP** only. To make the **GmDAD1-RNAi construct**, partial **GmDAD1** gene was amplified (using primers p12-DAD-F and p12-DAD-R) and cloned into pDONR221 (Invitrogen, United States) and then entered in pHellsGate12:GFP via Gateway LR reaction. Modified from pHellsGate12 (Wesley et al., 2001), pHellsGate12:GFP harbors a 35S:GFP:nos expression cassette (Yan et al., 2014). After sequence validation, the **pBIN-GFP-GmDAD1, GmDAD1-RNAi**, the empty pBIN-GFP and pHellsGate12:GFP vectors were introduced into **Agrobacterium rhizogenes** strain K599 by electroporation.

### Plasmid Construction for N. benthamiana Transformation

To overexpress **GmDAD1** in **N. benthamiana**, the full length of **GmDAD1** CDS was obtained from cDNAs of the Williams variety using primer pair **pDONR-DAD-F/R** (Supplementary Table S1) and then cloned into the entry vector pDONR221 via Gateway BP reaction. After sequencing validation, the fragment was then entered in pEarleyGate202 via LR recombination reaction between the entry clone and the destination vector (Invitrogen, United States) (Earley et al., 2006). To make Tobacco Rattle Virus (TRV)-based VIGS construct targeting

**NbDAD1**, partial fragment of **NbDAD1** was amplified using primer pair TRV:NbDAD-F/R and cloned into pTRV2 (Liu et al., 2002) using **KpnI** and EcoRI sites. All constructs were validated by sequencing and transformed into A. tumefaciens strain EHA105 for N. benthamiana transformation and GV3101 for VIGS experiment.

### Soybean Cotyledon Transformation

Surface-sterilized soybean seeds were soaked in sterilized water overnight and then germinated on medium containing 0.5% sucrose and 1.2% agar in growth chamber with 16h:8h light/dark photoperiod. About 5 days after germination, unblemished cotyledons were harvested for A. rhizogenes-mediated transformation. Transformation was performed as described previously (Yan et al., 2014). After about 3 weeks of cultivation, transformed hairy roots became abundant at inoculated cotyledons. Positive transformants were selected by detecting GFP signal under fluorescence microscopy, cut off from cotyledons, and cultivated on White medium (Supplementary Table S2) for further verification and resistance level test.

### N. benthamiana Transformation and Virus-Induced Gene Silencing (VIGS)

**Nicotiana benthamiana** plants overexpressing **GmDAD1** were generated via A. tumefaciens mediated leaf disk transformation (Horsch et al., 1985). The T1 seeds harvested from self-pollinated T0 plants were surface-sterilized with 70% ethanol for 30 s, and 10% sodium hypochlorite solution for 5 min, then washed by sterilized water for five times. The sterilized seeds were germinated on MS medium with 100 mg/L glufosinate ammonium (Sigma, United States). T2 seeds were collected and sown in small plastic pots. After 2 weeks, the seedlings were sprayed with 100 mg/L glufosinate ammonium solution. Resistant were transplanted to new pots and confirmed by both genomic DNA and cDNA PCR using gene-specific

¹https://www.soybase.org/GlycineBlastPages/
Resistance Assay of *N. benthamiana* Against *Phytophthora parasitica*

Leaves from 5 to 6-week-old *N. benthamiana* plants were detached and inoculated with 20 µl *P. parasitica* zoospores (10⁴ ml⁻¹) per leaf. Inoculated leaves were then kept in a moist chamber and lesion diameters were measured at 36 and 60 hpi. Representative infected leaves were photographed at 60 hpi under a UV lamp and then stained with trypan blue to visualize the infected area. The experiment was repeated three times with similar results and at least 20 leaves were inoculated for each biological replicate. Two weeks after infiltration, leaves from TRV and NbDAD1-VIGS plants were inoculated with *P. parasitica* using the same strategy. Lesion diameters were measured at 36 and 48 hpi due to the semi-dwarf phenotype of NbDAD1-VIGS plants. At least 10 lesions per construct were measured with three biological repeats. Student’s *t*-test was used to analyze the significance of differences. Difference were considered as significant when *P* < 0.05.

Root Infection and Observation

After verification by detection of GFP fluorescence and qPCR, transgenic hairy roots of similar length (approximately 3 cm) were excised and dipped into the zoospore suspension (10⁴ zoospores per ml) of *P. sojae* race P6497-RFR for 5 min as described previously (Xiong et al., 2014). Inoculated roots were placed in Petri dishes containing 0.6% agar in the dark at room temperature. At 12, 24, and 36 hpi, the infection progression was monitored under an OLYMPUS MVX10 (OLYMPUS, Japan) fluorescence microscope via RFP fluorescence detection at 535 nm. The *P. sojae*-specific gene *PsTEF* was used for qPCR quantification of the relative biomass of *P. sojae*. For each sample, about 10 infected hairy roots were collected and pooled for DNA/RNA extraction which helps to reduce bias and increase statistical accuracy (Graham, 1991; Subramanian et al., 2005; Graham et al., 2007).

Western Blotting Assay

About 10 transgenic roots with GFP fluorescence were collected and ground in liquid nitrogen. Total proteins were extracted with the extraction buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2 mM DTT, 1% triton, 2% polyvinylpolyprrolidone and Roche complete protein inhibitor tablets). The samples were boiled for 10 min in 6× sodium dodecyl sulfate (SDS) loading buffer. SDS-PAGE and immunoblotting were performed in a mini-gel apparatus and submarine gel transfer systems (Bio-Rad, United States), respectively. Proteins were then transferred onto polyvinylidene fluoride (PVDF) membranes and then membranes were blocked with 5% non-fat dry milk in 0.01 M PBST for 1 h and then incubated with anti-GFP (1:1,000) (Sigma, United States) for 2 h at room temperature. After washing by TBST three times, the membrane was incubated with IRDye®800CW Goat anti-rabbit IgG (LI-COR, United States) secondary antibody at room temperature for 1 h. Protein bands were detecting using the Odyssey® CLx quantitative fluorescence imaging system (LI-COR, United States).

Sequence Analysis and Alignment

The conserved and transmembrane domains of GmDAD1 were analyzed with InterProScan and TMPRED respectively (Hofmann and Stoeck, 1993; Jones et al., 2014). Multiple sequence alignment was performed using the BioEdit software (Hall, 1999).

RESULTS

**ER-Located GmDAD1 Shares Conserved Regions With Other Plant DAD1 Orthologs**

*GmDAD1* (Gma.7542.2.S1_at) was identified from an Affymetrix Genechip microarray data analysis on soybean and *P. sojae* interaction (Zhou et al., 2009). *GmDAD1* was up-regulated in soybean varieties with different degrees of resistance to *P. sojae* (Zhou et al., 2009). Sequence analysis of *GmDAD1* (cloned from the Williams variety) revealed that its open reading frame (ORF) encodes a protein of 117 amino acid residues. GmDAD1 shares 91, 54, and 36% identities with DAD1 orthologs in *Arabidopsis thaliana*, *Homo sapiens*, and *Saccharomyces cerevisiae*, respectively. Similar to other plant DAD1 orthologs, GmDAD1 contains three transmembrane regions (residues 27–52, 61–81, and 95–115) and a subunit of OST (residues 13–116) (Figure 1A). To investigate the subcellular localization of GmDAD1, a *GmDAD1*-GFP fusion construct driven by the CaMV 35S promoter was expressed in *N. benthamiana* leaves. GmDAD1-GFP co-localized in the cytoplasm with mCherry-HDEL, an endoplasmic reticulum (ER) marker, demonstrating the ER localization of GmDAD1 (Figure 1B).

**GmDAD1 Expression Is Induced Upon *P. sojae* Infection**

*GmDAD1* transcript can be detected ubiquitously in roots, stems and leaves during plant development in cv Williams, with root being the organ exhibiting highest expression (Figure 2A). Interestingly, leaves showed much higher *GmDAD1* transcript accumulation at pod filling stage than seedling stage (Figure 2A). Similar *GmDAD1* expression pattern was detected in Williams 82 variety in the seedling stage (Supplementary Figure S1). On the contrary, the expression of *GmDAD1* is higher in roots at the pod filling stage in Williams 82 than in Williams.

After inoculation with P6497, a *P. sojae* isolate of race 2, the compatible variety Williams showed...
**FIGURE 1** | Molecular characterization and subcellular localization of GmDAD1 protein. (A) Sequence alignment of GmDAD1 and other defender against cell death (DAD) proteins. The darkblue (100%), pink (75%), and cyan (50%) boxes represent levels of amino acid identity or similarity. TM, transmembrane domain; OT, oligosaccharyltransferase domain. At, *Arabidopsis thaliana*; Hv, *Hordeum vulgare*; Zm, *Zea mays*; Os, *Oryza sativa*; Ta, *Triticum aestivum*. The asterisk indicates the stop codon. (B) Subcellular localization of GmDAD1 was performed via transient expression system in *Nicotiana benthamiana*. Green and red fluorescence represent the signal of GFP fusion protein and ER marker mCherry-HDEL, respectively. The reticulate fluorescence pattern of GmDAD1-GFP and its co-localization with mCherry-HDEL indicate accumulation in the ER.

**FIGURE 2** | Gene expression analysis of GmDAD1. (A) GmDAD1 mRNA levels in various tissues of soybean cultivar Williams. Leaves, roots, and stems were harvested from plants at the seedling and pod filling stage. (B) Expression profiles of GmDAD1 in Williams (compatible interaction) at 0, 6, 12, 24, 48 h post inoculation (hpi) with *P. sojae* (P6497). The relative expression level was normalized to soybean GmCons4 (GenBank: BU578186.1). Means and standard deviations were calculated from three independent biological replicates. Data were analyzed by using Student’s t-tests (∗∗P < 0.01).

**GmDAD1 Enhances Resistance to *P. sojae* in Soybean Hairy Roots**

RT-qPCR analysis of ten mixed hairy roots displaying GFP fluorescence indicated that expression of GmDAD1 in GmDAD1-GFP overexpression (OE) plants was nearly 14-fold higher than in the control (GFP) (Figure 3A). Western blotting also showed the accumulation of the GmDAD1-GFP fusion protein (Figure 3B). When OE and GFP hairy roots were inoculated with *P. sojae* P6497-RFP (Xiong et al., 2014), the biomass of *P. sojae* was significantly and consistently less in OE hairy roots than in GFP samples at 12, 24, and 36 hpi (Figure 3C). In the GFP control, the invasion hyphae emerged at 12 hpi, rapidly extended at 24 hpi, and almost filled the entire tissue at 36 hpi (Figure 3D). In contrast, hyphal growth was limited and the invasion hyphae were much sparser in GmDAD1-GFP overexpression roots (Figure 3D), which is consistent with the lower accumulation of *P. sojae* biomass (Figure 3C).

**Silencing of GmDAD1 Reduces Resistance to *P. sojae* in Soybean Hairy Roots**

RNAi-directed silencing of GmDAD1 in soybean hairy roots (Figure 4A) was performed as described previously
Yan et al. 2014). Both GmDAD1-RNAi (RNAi) and EV control (EV) roots were inoculated with P. sojae P6497-RFP. Compared with control, GmDAD1-RNAi roots showed gradually increased P. sojae biomass accumulation over time (Figure 4B). Furthermore, a greater hyphal growth and higher oospore germination can be observed in GmDAD1-RNAi roots (Figure 4C). Our results indicated that GmDAD1 is important for soybean resistance against P. sojae.

GmDAD1 Affects the Expression of Multiple Defense-Related Genes

To further determine whether the expression of defense-related genes was affected by GmDAD1 silencing, we assessed the expression of several genes in hairy roots inoculated with P. sojae, including the marker genes of SA, and JA/ET signaling pathways, ROS generation and scavenging. The expression of PR1a, PR2, PR3, PR5 and ERF1 were decreased in GmDAD1-RNAi roots after P. sojae inoculation. It is to note that the expression of PR1a was also dramatically suppressed without inoculation (Figure 5). In contrast, the expression of PDF1.2, PR4, and two ROS scavenging genes, CAT and APX, were induced in the GmDAD1 silencing roots infected with P. sojae (Figure 5). No significant change of NADPHOX expression was observed when GmDAD1 was silenced (Figure 5).

**GmDAD1 Is Involved in P. sojae-Activated ER Stress Signaling**

Since DAD1 catalyzes the first step of protein N-linked glycosylation, disruption of GmDAD1 is expected to trigger unfolded protein response (UPR), which facilitates proper protein folding in ER via inducing the expression of a series of relevant genes (Li et al., 2011). After P. sojae inoculation, the transcript accumulations of six UPR marker genes were examined in soybean hairy roots, including Bip, PDI, CNX1, ERdj3A, GRP94, and bZIP17. All these genes are induced at the onset of ER stress and mark the activation of adaptive UPR. Expression changes of VPE were also monitored since its protein product possesses caspase-1-like activity and acts downstream of UPR and is part of the ER-PCD pathway. Compared to EV control, GmDAD1-RNAi roots showed significantly higher transcript accumulations of all seven UPR/ER stress marker genes at both 24 and 36 hpi (Figure 6). VPE was upregulated at 12 hpi and its expression decreased at 24 and 36 hpi in EV hairy roots. On the contrary, different trend was observed in GmDAD1 silencing hairy roots. The expression increased continuously through the selected time course, and was significantly higher at 24 and 36 hpi (Figure 6).
FIGURE 4 | Silencing of GmDAD1 reduces resistance to *P. sojae* in soybean hairy roots. (A) Relative expression of GmDAD1 was determined by RT-qPCR in inoculated hairy roots in which GmDAD1 was silenced via RNAi (GmDAD1-RNAi) or empty vector (EV) at 0, 12, 24, and 36 hpi. (B) Relative biomass of *P. sojae* was determined in inoculated hairy roots GmDAD1-RNAi or EV at 12, 24, and 36 hpi. Values represent the means of three replicates ± SD. Data were analyzed by using Student’s *t*-tests (*P* < 0.05, **P** < 0.01 compared with the control). (C) Microscopic analysis of *P. sojae* colonization in soybean hairy roots. The control EV and GmDAD1-RNAi hairy roots were inoculated with zoospore suspension (10⁴ zoospore/ml) of the *P. sojae* P6497-RFP. Photos were taken at 12, 24, 36 hpi. The white arrows indicate germinating oospores.

FIGURE 5 | GmDAD1 affects the expression of multiple defense-related genes. RT-qPCR analysis of the expression patterns of defense-related genes in the EV and GmDAD1-RNAi transgenic hairy roots after inoculation with *P. sojae*. Values represent the means of three replicates ± SD. Data were analyzed by using Student’s *t*-tests (*P* < 0.05, **P** < 0.01 compared with the control).
**GmDAD1 Enhances Resistance to* P. parasitica *in* N. benthamiana**

To test whether GmDAD1 confers resistance against other Phytophthora pathogens, transgenic *N. benthamiana* plants overexpressing GmDAD1 were generated and verified (Supplementary Figure S2). Compared to wild-type (WT) and empty vector controls (EV) both GmDAD1 overexpression lines tested (4-1 and 8-4) showed reduced disease symptoms (Figures 7A,B) and significantly smaller lesion diameters on leaves (Figure 7C) when infected with *P. parasitica* zoospores. The results suggest that GmDAD1 overexpression enhances *N. benthamiana* resistance against *P. parasitica*.

**Silencing of NbDAD1 in* N. benthamiana *Reduces Resistance to* P. parasitica**

Since plant DADs are highly conserved, the native NbDAD1 in *N. benthamiana* was silenced via TRV-based VIGS system for functional analysis. Compared to TRV-infected controls, plants infiltrated with TRV-NbDAD1 displayed a semi-dwarf phenotype with increased branching (Figures 8A,B), which implies a possible role of NbDAD1 in modulating growth and development. Three verified NbDAD1 knock-down lines and TRV-infected controls were challenged with *P. parasitica* zoospores on detached leaves (Figure 8C). Silencing of NbDAD1 led to significantly larger lesion diameters at both 36 and 48 hpi (Figures 8D–F), which indicates that NbDAD1 is similar as GmDAD1 in the function of conferring resistance against *P. parasitica*.

**DISCUSSION**

Being one of the most important crops worldwide, soybean can be infected by several major diseases, including the *Phytophthora* stem and root rot caused by *P. sojae* (Tyler, 2007). Continual efforts have been made to characterize novel defense genes against *Phytophthora* pathogens (Sugimoto et al., 2012). Here we identified GmDAD1, an ER-membrane protein from soybean, and dissected its function in plant–*Phytophthora* interactions.
so far. The Arabidopsis to bacterial pathogens (Haweker et al., 2010). Several reports on elicitation capacity resulting in higher susceptibility of the plant bleaches its ligand binding and results in oxidative burst triggered by host defense. DAD1 (Wang X. J. et al., 2011).

2008), temperature (Lee et al., 2003), and pathogen infection (Yan et al., 2011; Gilmore, 1997; Sanjay et al., 1998). In both animals and plants, the expression of DADI orthologs responds to a wide range of STI-signaling motifs. The expression of N. benthamiana NbEF1a gene was used as a reference for normalization. Data are the means ± SD calculated from three replicates. **P < 0.01. (D, E) Leaf phenotypes of TRV control and NbDAD1-VIGS after P. parasitica zoospores inoculation. Pictures were taken under UV illumination at 48 hpi. The experiments were repeated three times with similar results and representative images are shown. (B) Relative expression of NbDAD1 in N. benthamiana plants inoculated with TRV and three NbDAD1 knock-down plants. The samples were collected 1 week after inoculation. N. benthamiana NbEF1a gene was used as a reference for normalization. Data are the means ± SD calculated from three replicates. **P < 0.01.

FIGURE 8 | Silencing of NbDAD1 in N. benthamiana reduces resistance to P. parasitica. (A, B) Side- and top-view of the TRV-infected control and NbDAD1 silencing plants. (C) Relative expression of NbDAD1 in N. benthamiana plants inoculated with TRV and three NbDAD1 knock-down plants. The samples were collected 1 week after inoculation. N. benthamiana NbEF1a gene was used as a reference for normalization. Data are the means ± SD calculated from three replicates. **P < 0.01. (D, E) Leaf phenotypes of TRV control and NbDAD1-VIGS after P. parasitica zoospores inoculation. Pictures were taken under UV illumination at 48 hpi. The experiments were repeated three times with similar results and representative images are shown. (F) Average lesion size of NbDAD1 knock-down leaves and TRV control after P. parasitica zoospores inoculation. Averages were calculated from at least 10 lesions per construct. Data are the means ± SD. Data were analyzed by using Student’s **P < 0.01.

Being evolutionary conserved across plant and animal species, DAD1 is a subunit of the OST complex, which catalyzes the first step of protein N-linked glycosylation in ER (Kelleher and Gilmore, 1992; Sanjay et al., 1998). In both animals and plants, the expression of DADI orthologs responds to a wide range of adverse environmental stimuli, including injury (Zhu et al., 2008), temperature (Lee et al., 2003), and pathogen infection (Wang X. J. et al., 2011). DADI inhibits undesired cell death triggered by host defense.

N-glycosylation has been reported to play a critical role in plant–pathogen interactions. For example, site-mutation on the N-glycosylation motif of A. thaliana receptor kinase EFR bleaches its ligand binding and results in oxidative burst elicitation capacity resulting in higher susceptibility of the plant to bacterial pathogens (Haweker et al., 2010). Several reports on the role of DAD proteins in plant defense have been published so far. The Arabidopsis dadi mutant shows reduced secretion of PR proteins and resistance against pathogens (Wang et al., 2005).

In wheat, knock-down of TaDADI2 suppresses the expression of PR1, PR2, and PR5 in response to the infection of Puccinia striiformis f. sp. tritici (Wang X. J. et al., 2011). We hence propose that GmDADI1 may also play a role in soybean disease resistance.

In soybean, GmDADI1 expression can be induced by P. sojae infection in both compatible and incompatible varieties, which indicates that GmDADI1 serves as a non-specific defense gene to some extent. However, GmDADI1 has consistently higher expression after P. sojae inoculation in the incompatible variety Williams 82, and its expression does not drop dramatically afterward at 48 hpi, as it happens in the compatible variety Williams. Therefore, GmDADI1 may be subjected to distinct transcriptional regulations in P. sojae compatible and incompatible soybean varieties.

Since GmDADI1 has highest transcript accumulation in roots, we adopted the soybean hairy root infection system for P. sojae resistance test. GmDADI1 gain- and loss-of-function mutants exhibit opposite P. sojae resistance phenotypes, which indicates that GmDADI1 contributes to the resistance of soybean against P. sojae. Similarly, knock-down of NbDADI1, the native DADI ortholog in N. benthamiana, reduces plant resistance to another Phytophthora pathogen, P. parasitica. Heterologous expression of GmDADI1 in N. benthamiana enhances resistance to P. parasitica. Our results reveal that DADI1 is a potential valuable defense gene against Phytophthora pathogens and this disease resistance function is conserved across plant species.

Phytohormone signaling, which is mediated by SA during biotrophic and hemibiotrophic plant–pathogen interactions and JA and ET for necrotrophic plant pathogens, plays important roles in plant resistance (Glazebrook, 2005). Previously studies demonstrated that the resistance to P. sojae is mediated by the SA and ET signaling pathways (Moy et al., 2004; Sugano et al., 2014). Therefore, we assessed the expression of several key defense related genes by RT-qPCR. When GmDADI1 silencing hairy roots were inoculated with P. sojae, the transcription of PR1a, PR2, PR3, PR5, and ERF1 were significantly reduced. Since the PR genes are generally regarded as early markers of defense response, the suppressed expression of these genes may be responsible for the compromised resistance at the begin of the infection process (from 0 to 24 hpi). Moreover, the two JA-dependent signal marker genes PDF1.2 and PR4 were up-regulated after P. sojae infection in the silenced hairy roots (later than 24 hpi). We inferred that this JA resistance signaling activation might be lately induced, and the up-regulation might be caused by the antagonistic effect of JA and SA pathways.

Reactive oxygen species are important messenger molecules in disease resistance regulation. The expression of ROS-generating gene NADPHox showed no difference between EV and GmDADI1-RNAi hairy roots, however, the ROS-scavenging genes CAT and APX were statistically significant up-regulated after P. sojae infection in the silencing roots, this means that the ROS signaling was not completely affected by GmDADI1 silencing.

As a core subunit of OST complex, DADI plays an important role in protein N-glycosylation (Peristera and Stephen, 2012), the defeat of protein N-glycosylation cause accumulation of misfolded proteins in ER and subsequently ER stress (Li et al., 2011; Cai et al., 2014). In soybean hairy roots infected by P. sojae,
we found that GmDAD1 acts as a repressor for multiple UPR marker genes. In detail, all tested genes became up-regulated at later stages of the infection when GmDAD1 is silenced, indicating severe ER stress. We believe that this situation is caused by a less efficient or delayed defense signaling transduction. However, whether the suppression of defense-related genes was directly caused by the ER stress due to GmDAD1 silencing need to be further investigated.

Under extreme condition such as pathogen infection, a prolonged ER stress is known to eventually activate the ER-PCD pathway. Phytophthora pathogens are hemibiotrophic. They initially establish a biotrophic relationship with their hosts, and switch to necrotrophic phase later than 15 hpi (Enkerli et al., 1997). In EV hairy roots, a sharp increase of VPE, a cystein proteinase mediating PCD via the maturation and activation of vacuolar proteins, was observed at 12 hpi most likely to limit and overcome the biotrophic phase of P. sojae infection. In GmDAD1-RNAi roots, VPE expression was relatively suppressed at the same infection stage, suggesting the failure of PCD induction. However, elevated expression of same infection stage, suggesting the failure of PCD induction. VPE overcame the biotrophic phase of vacuolar proteins, was observed at 12 hpi most likely to limit and overcome the biotrophic phase of P. sojae infection. In GmDAD1-RNAi roots, VPE expression was relatively suppressed at the same infection stage, suggesting the failure of PCD induction. However, elevated expression of VPE was detected at 24 and 36 hpi indicating a later activation of ER-PCD pathway. This late apoptosis overlaps with the necrotrophic phase of P. sojae, which may be one of the reasons of the increased P. sojae accumulation in GmDAD1 silencing hairy roots.

Disruption of DADI causes growth defect or even embryonic lethality in animal systems (Brewster et al., 2000; Zhang et al., 2016). In this study, we have observed significantly reduced transformation rate when silencing GmDAD1 in soybean hairy roots (Supplementary Figure S3). Moreover, knock-down of NbDADI by VIGS caused a semi-dwarf phenotype in N. benthamiana. These results suggest that DADI may play a similar role of regulating growth in plants most likely by acting on the N-glycosylation pathway of key proteins involved in plant development.

**REFERENCES**

Babaeizad, V., Imani, J., Kogel, K. H., Eichmann, R., and Huckelhoven, R. (2009). Over-expression of the cell death regulator BAX inhibitor-1 in barley confers reduced or enhanced susceptibility to distinct fungal pathogens. Theor. Appl. Genet. 118, 455–463. doi: 10.1007/s00122-008-0912-2

Bernard, R. L., and Creemens, C. R. (1988). Registration of ‘Williams 82’ soybean. Crop Sci. 28, 1027–1028. doi: 10.2135/cropsci1988.0011183X002800060049x

Bernard, R. L., and Lindahl, D. A. (1972). Registration of williams soybean1 (reg. no. 94). Crop Sci. 12:716. doi: 10.2135/cropsci1972.0011183X001200050067x

Berti, L., Leonardi, L., Caporale, C., Tucci, M., Cascone, N., Di Berardino, I., et al. (2003). Pathogen-responsive wheat PR4 genes are induced by activators of systemic acquired resistance and woundind. Plant Sci. 164, 1067–1078. doi: 10.1016/j.plantsci.2003.03.0112-2

Brewster, J. L., Martin, S. L., Toms, J., Goss, D., Wang, K., Zachrone, K., et al. (2000). Deletion of Dadi in mice induces an apoptosis-associated embryonic death. Genesis 26, 271–278. doi: 10.1002/(SICI)1526-9452(200004)26:4<271::AID-GENE90>3.0.CO;2-E

Cai, Y. M., Jia, Y., and Patrick, G. (2014). Endoplasmic reticulum stress-induced PCD and caspase-like activities involved. Front. Plant Sci. 5:41. doi: 10.3389/fpls.2014.00041

Chen, Y. P., Xing, L. P., Wu, G. J., Wang, H. Z., Wang, X. E., Cao, A. Z., et al. (2007). Plastidial glutathione reductase from Hauyaldia villosa is an enhancer of powdery mildew resistance in wheat (Triticum aestivum). Plant Cell Physiol. 48, 1702–1712. doi: 10.1093/pcp/pcm142

**CONCLUSION**

We observed that GmDAD1, a conserved component of the OST complex, via participating in the ER-PCD and UPR pathways and affecting the expression of multiple defense-related genes, confers resistance to Phytophthora pathogens. Moreover, GmDAD1 regulates plant growth and development likely by the effect on the N-glycosylation pathway. Taken together, GmDAD1 can be considered as a promising target for the molecular breeding of Phytophthora-resistant soybean varieties.

**AUTHOR CONTRIBUTIONS**

DD and QY designed the project. QY, JS, and XnC performed the experiments and analyzed the data. XnC, HX, and DD guided the experimental work. DD, QY, HP, and MJ wrote the manuscript. All authors read and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/articles/10.3389/fpls.2019.00107/full#supplementary-material

Danon, A., Rotari, V. I., Gordon, A., Mailhac, N., and Gallois, P. (2004). Ultraviolet-C overexposure induces programmed cell death in Arabidopsis, which is mediated by caspase-like activities and which can be suppressed by caspase inhibitors, p35 and defender against apoptotic death. J. Biol. Chem. 279, 779–787. doi: 10.1074/jbc.M304468200

Dickman, M. B., Park, Y. K., Oltersdorf, T., Li, W., Clemente, T., and French, R. (2001). Abrogation of disease development in plants expressing animal antipaptotic genes. Proc. Natl. Acad. Sci. U.S.A. 98, 6957–6962. doi: 10.1073/pnas.091108998

Doukanina, E. V., Chen, S., Van Der Zalm, E., Godzik, A., Reed, J., and Dickman, M. B. (2006). Identification and functional characterization of the BAG protein family in Arabidopsis thaliana. J. Biol. Chem. 281, 18793–18801. doi: 10.1074/jbc.M511794200

Earley, K. W., Haag, J. R., Pontes, O., Oppe, K., Juehne, T., Song, K., et al. (2006). Gateway-compatible vectors for plant functional genomics and proteomics. Plant J. 45, 616–629. doi: 10.1111/j.1365-313X.2005.02617.x

Eichmann, R., Bischof, M., Weis, C., Shaw, J., Lacomme, C., Schweizer, P., et al. (2010). BAX INHIBITOR-1 is required for full susceptibility of barley to powdery mildew. Mol. Plant Microbe Interact. 23, 1217–1227. doi: 10.1094/MPmi-23-9-1217

Enkerli, K., Mims, C. W., and Hahn, M. G. (1997). Ultrastructure of compatible and incompatible interactions of soybean roots infected with the plant pathogenic oomycete Phytophthora sojae. Can. J. Bot. 75, 1493–1508. doi: 10.1139/b 97-864
Fu, D. Q., Zhu, B. Z., Zhu, H. L., Jiang, W. B., and Luo, Y. B. (2002). Virus-induced gene silencing in tomato. Plant J. 43, 299–308. doi: 10.1046/j.1365-313X.2002.02441.x

Gallois, P., Makishima, T., Hecht, V., Després, B., Leduc, M., Nishimoto, T., et al. (1997). An Arabidopsis thaliana cDNA complementing a hamster apoptosis suppressor mutant. Plant J. 11, 1325–1331. doi: 10.1046/j.1365-313X.1997.11061325x

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu. Rev. Phytopathol. 43, 205–227. doi: 10.1146/annurev.phyto.43.040204.135923

Graham, T. L. (1991). A rapid, high resolution high performance liquid chromatography profiling procedure for plant and microbial aromatic secondary metabolites. Plant Physiol. 95, 584–593. doi: 10.1104/pp.95.2.584

Graham, T. L., Graham, M. Y., Subramanian, S., and Yu, O. (2007). RNAi silencing of genes for elicitation or biosynthesis of 5-deoxysphingolipids suppresses race-specific resistance and hypersensitive cell death in Phytophthora sojae infected tissues. Plant Physiol. 144, 728–740. doi: 10.1104/pp.107.097865

Hall, T. A. (1999). Bioedit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/nt. Nucleic Acids Symp. Ser. 41, 95–98. doi: 10.1021/bk-1999-0734.ch008

Haweker, H., Rips, S., Koiwa, H., Salomon, S., Saijo, Y., Chinchilla, D., et al. (2010). Pattern recognition receptors require N-glycosylation to mediate plant immunity. J. Biol. Chem. 285, 4629–4636. doi: 10.1074/jbc.M109.063073

Hofmann, K., and Stoel, W. (1993). Timbase-A database of membrane spanning protein segments. Biol. Chem. Hoppe Seyler 374:166. doi: 10.1056/NEJM19990104220121

Horsch, R. B., Rogers, S. G., and Fraley, R. T. (1985). Transgenic Plants. Maldonado, A., Youssef, R., McDonald, M., Brewer, E., Beard, H., Matthews, B. J. P., et al. (2014). Overexpression of four Arabidopsis thaliana NHL genes in soybean (Glycine max) roots and their effect on resistance to the soybean cyst nematode (Heterodera glycines). Physiol. Mol. Plant P 86, 1–10. doi: 10.1111/pmpp.2014.02.001

Matsunura, H., Nirasawa, S., Kiba, A., Usaki, S., Saitoh, H., Ito, M., et al. (2003). Overexpression of Bax inhibitor suppresses the fungal elicitor-induced cell death in rice (Oryza sativa L.) cells. Plant J. 33, 425–434. doi: 10.1046/j.1365-313x.2003.01639.x

Mazarei, M., Elling, A. A., Maier, T. R., Puthoff, D. P., and Baum, T. J. (2007). GmFERPI is a transcription factor activating defense genes in soybean and Arabidopsis. Mol. Plant Microbe Interact. 20, 107–119. doi: 10.1094/MPMI-20-2-0107

Moy, P., Qutob, D., Chapman, B. P., Atkinson, I., and Gijzen, M. (2004). Patterns of gene expression upon infection of soybean plants by Phytophthora sojae. Mol. Plant Microbe Interact. 17, 1051–1062. doi: 10.1094/MPMI.2004.17.10.1051

Nakashima, T., Sekiguchi, T., Kuraoka, A., Fukushima, K., Shibata, Y., Komiyama, S., et al. (1993). Molecular-cloning of a human cDNA-encoding a novel protein, Dad1, whose defect causes apoptotic cell-death in hamster cell lines. J. Biochem. 113, 299–308. doi: 10.1242/jcs.113.037234

Perizonov, A. F., and Brown, P. J. (2014). The role of ROS signaling in cross-tolerance: from model to crop. Front. Plant Sci. 5:754. doi: 10.3389/fpls.2014.00754

Peristera, R., and Stephen, H. (2012). The oligosaccharyltransferase subunits OST48, DAD1 and KCP2 function as ubiquitous and selective modulators of mammalian N-glycosylation. J. Cell Sci. 125, 3474–3484. doi: 10.1242/jcs.103952

Plotnikova, J., et al. (2004). Patterns of gene expression upon infection of soybean plants by Phytophthora sojae. Mol. Plant Microbe Interact. 17, 1051–1062. doi: 10.1094/MPMI.2004.17.10.1051

Suganuma, S., Sugiura, M., Usaki, S., Itoh, M., Gijzen, M., et al. (2014). Induction of resistance to Phytophthora sojae in soybean (Glycine Max) by salicylic acid and ethylene. Plant Pathol. 62, 1048–1056. doi: 10.1111/ppa.12011

Subramanian, S., Graham, M. Y., Yu, O., and Graham, T. L. (2005). RNA interference of soybean isoflavone synthase genes leads to silencing in tissues distal to the transformation site and to enhanced susceptibility to Phytophthora sojae. Plant Physiol. 137, 1345–1353. doi: 10.1104/pp.104.075257

Suganuma, S., Sugiura, T., Takatsuji, H., and Jiang, C. J. (2014). Induction of resistance to Phytophthora sojae in soybean (Glycine Max) by salicylic acid and ethylene. Plant Pathol. 62, 1048–1056. doi: 10.1111/ppa.12011

Sugiura, T., Kato, M., Yoshida, S., Matsumoto, I., Kobayashi, T., Kaga, A., et al. (2012). Pathogenic diversity of Phytophthora sojae and breeding strategies to develop phytophthora-resistant soybeans. Breed. Sci. 61, 511–522. doi: 10.1270/jsbsb.61.111
Sun, J., Li, L., Zhao, J., Huang, J., Yan, Q., Xing, H., et al. (2014). Genetic analysis and fine mapping of RpsJs, a novel resistance gene to Phytophthora sojae in soybean [Glycine max (L.) Merr]. *Theor. Appl. Genet.* 127, 913–919. doi: 10.1007/s00122-014-2266-2

Tanaka, Y., Makishima, T., Sasabe, M., Ichinose, Y., Shiraishi, T., Nishimoto, T., et al. (1997). dad-1, a putative programmed cell death suppressor gene in rice. *Plant Cell Physiol.* 38, 379–383. doi: 10.1093/oxfordjournals.pcp.a029179

Tiziana, A., and Roberto, S. (2014). Protein quality control in the early secretory pathway. *Embo J.* 27, 315–327. doi: 10.1038/sj.emboj.7601974

Tyler, B. M. (2007). *Phytophthora sojae*: root rot pathogen of soybean and model oomycete. *Mol. Plant Pathol.* 8, 1–8. doi: 10.1111/j.1364-3703.2006.00373.x

Wang, D., Weaver, N. D., Kesarwani, M., and Dong, X. (2005). Induction of protein secretory pathway is required for systemic acquired resistance. *Science* 308, 1036–1040. doi: 10.1126/science.1108791

Wang, X. J., Tang, C. L., Zhang, H. C., Xu, J. R., Liu, B., Lv, J., et al. (2011). TaDAD2, a negative regulator of programmed cell death, is important for the interaction between wheat and the stripe rust fungus. *Mol. Plant Microbe Interact.* 24, 79–90. doi: 10.1094/MPMI-06-10-0131

Watanabe, N., and Lam, E. (2009). Bax Inhibitor-1, a conserved cell death suppressor, is a key molecular switch downstream from a variety of biotic and abiotic stress signals in plants. *Int. J. Mol. Sci.* 10, 3149–3167. doi: 10.3390/ijms10073149

Wesley, S. V., Helliwell, C. A., Smith, N. A., Wang, M., Rouse, D. T., Liu, Q., et al. (2011). TaDAD2, a negative regulator of programmed cell death, is important for the interaction between wheat and the stripe rust fungus. *Mol. Plant Microbe Interact.* 24, 79–90. doi: 10.1094/MPMI-06-10-0131

Williams, B., Kabbage, M., Britt, R., and Dickman, M. B. (2010). AtBAG7, an *Arabidopsis* Bcl-2-associated atahogene, resides in the endoplasmic reticulum and is involved in the unfolded protein response. *Proc. Natl. Acad. Sci. U.S.A.* 107, 6088–6093. doi: 10.1073/pnas.0912670107

Xiong, Q., Ye, W., Choi, D., Wong, J., Qiao, Y., Tao, K., et al. (2014). Phytophthora suppressor of RNA silencing 2 is a conserved RxLR effector that promotes infection in soybean and *Arabidopsis thaliana*. *Mol. Plant Microbe Interact.* 27, 1379–1389. doi: 10.1094/MPMI-06-14-0190-R

Yamada, T., Takatsu, Y., Kasumi, M., Marubashi, W., and Ichimura, K. (2004). A homolog of the defender against apoptotic death gene (DAD1) in senescing gladiolus petals is down-regulated prior to the onset of programmed cell death. *J. Plant Physiol.* 161, 1281–1283. doi: 10.1016/j.jplph.2004.06.005

Yan, A., Wu, E., and Lennarz, W. J. (2005). Studies of yeast oligosaccharyl transferase subunits using the split-ubiquitin system: topological features and in vivo interactions. *Proc. Natl. Acad. Sci. U.S.A.* 102, 7121–7126. doi: 10.1073/pnas.0502669102

Yan, Q., Cui, X., Su, L., Xu, N., Guo, N., Xing, H., et al. (2014). GmSGT1 is differently required for soybean Rps genes-mediated and basal resistance to *Phytophthora sojae*. *Plant Cell Rep.* 33, 1275–1288. doi: 10.1007/s00299-014-1615-6

Zhang, M., Rajput, N. A., Shen, D., Sun, P., Zeng, W., Liu, T., et al. (2014). *Phytophthora sojae* cytoplasmic effector mediates disease resistance and abiotic stress tolerance in *Nicotiana benthamiana*. *Sci. Rep.* 5:10837. doi: 10.1038/srep10837

Zhang, Y., Chang, C., and Lai, Z. C. (2016). The defender against apoptotic cell death 1 gene is required for tissue growth and efficient N-glycosylation in *Drosophila melanogaster*. *Dev. Biol.* 420, 186. doi: 10.1016/j.ydbio.2016.09.021

Zhou, L., Mideros, S. X., Bao, L., Hanlon, R., Arredondo, F. D., Tripathy, S., et al. (2009). Infection and genotype remodel the entire soybean transcriptome. *BMC Genomics* 10:49. doi: 10.1186/1471-2164-10-49

Zhu, L., Song, L., Zhang, H., Zhao, J., Li, C., and Xu, W. (2008). Molecular cloning and responsive expression to injury stimulus of a defender against cell death 1 (DAD1) gene from bay scallops *Argopecten irradians*. *Mol. Biol. Rep.* 35, 125–132. doi: 10.1007/s11033-007-9061-y

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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