

A DUF3455 domain-containing protein promotes *Ustilaginoidea virens* infection and functions as a plant immune elicitor

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Abstract

Rice false smut (RFS), caused by *Ustilaginoidea virens*, poses a serious threat to global rice production by forming false smut balls and reducing grain yield and quality. Although numerous putative effectors have been predicted in the *U. virens* genome, only a limited number have been functionally characterized. Here, we identify and characterize *U. virens* DUF domain-containing protein 1 (UvDUF1), a secreted, ascomycete-conserved protein harboring a DUF3455 domain. UvDUF1 is strongly induced during the early stages of infection, and its deletion significantly reduces the virulence of *U. virens*. Pathogenicity is restored by complementation with the wild-type UvDUF1 gene but not with a signal peptide-deficient variant, demonstrating that secretion is indispensable for its virulence function. Recombinant UvDUF1 elicits early immune responses, including reactive oxygen species bursts and activation of defense-related genes, and enhances rice resistance to *Magnaporthe oryzae*. Moreover, UvDUF1 induces immune responses in the nonhost *Nicotiana benthamiana* and increases resistance to *Phytophthora* species. Collectively, results show that UvDUF1 contributes to pathogen virulence and plant immunity induction, providing new avenues for the development of environmentally friendly strategies for crop disease management.

KEYWORDS

defense responses, effector, pathogenicity, rice false smut, *Ustilaginoidea virens*

1 | INTRODUCTION

Rice false smut (RFS), caused by the fungal pathogen *Ustilaginoidea virens* (teleomorph: *Villosiclava virens*), has emerged as one of the most destructive diseases in major rice-growing regions. It not only causes substantial yield losses by reducing grain weight but also poses serious risks to human and animal health through the production of mycotoxins, such as

ustiloxins and ustilaginoidins, which contaminate harvested grains.^{1,2} As a biotrophic fungus, *U. virens* invades spikelets through the gap between the lemma and palea, colonizes stamen filaments, and hijacks host nutrients, ultimately forming characteristic false smut balls at late stages of infection.³ However, effective control of RFS remains a major challenge due to the limited availability of resistant rice germplasm and the lack of efficient management strategies.⁴ Therefore, a

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deeper understanding of the molecular mechanisms by which *U. virens* invades rice and modulates plant immunity is essential for developing novel and sustainable disease control strategies.

Like many plant pathogenic fungi that deploy extensive repertoires of secreted effectors to manipulate host immunity and facilitate infection, the *U. virens* genome encodes more than 600 putative effector proteins.¹ However, only a small fraction of these candidates has been experimentally characterized. Recent studies have identified several *U. virens* effectors that function as suppressors of host defense. For example, the secreted laccase UgsL enters rice cells, where it interacts with OsSRF3 and promotes its degradation via the ubiquitin–26S proteasome pathway, thereby inhibiting OsBAK1–OsSRF3-mediated PAMP-triggered immunity (PTI).⁵ Similarly, the glycoside hydrolase UvGH18.1 binds and hydrolyzes chitin, disrupting the chitin-induced dimerization of the receptor OsCEBiP and its co-receptor OsCERK1, thereby attenuating host immune signaling in the lemma and palea to facilitate fungal invasion.⁶ In contrast, relatively few *U. virens* effectors have been shown to activate plant immunity. SGP1, STOM, and UvGHF1 function as elicitors or PAMPs that trigger defense responses in *Nicotiana benthamiana*. Among them, only UvGHF1 can induce disease resistance in rice spikelets—the primary infection site of *U. virens*.^{7–9} Given the functional diversity of fungal effectors, elucidating the roles of additional *U. virens* effectors—whether they act as virulence factors that suppress host immunity or as elicitors that activate defense responses—is essential for advancing our understanding of RFS pathogenicity and for developing innovative and sustainable disease management strategies.

Many fungal proteins contain conserved domains that determine their molecular or biological functions.¹⁰ Approximately 25% of Pfam families are annotated as domains of unknown function (DUFs).¹¹ Although their functions remain largely uncharacterized, DUF-containing proteins can play essential biological roles. For example, the DUF630 and DUF632 domains are critical for proper regulation of leaf shape in rice.¹² Similarly, the DUF2193 domain functions as an Fe-binding module in methanogens and may participate in iron acquisition and/or trafficking.¹³ Elucidating the functions of DUF-containing proteins is therefore crucial for advancing our understanding of DUF-mediated biological mechanisms in *U. virens*.

In this study, we systematically characterized the *U. virens* DUF domain-containing protein 1 (UvDUF1), a putative immune elicitor of *U. virens*,⁷ by analyzing its expression dynamics, secretion activity, contribution to fungal pathogenicity, and capacity to activate plant immunity and disease resistance. This study not only provides new insights into the pathogenic mechanisms of *U. virens* but also establishes a theoretical

foundation for developing effector-based strategies for sustainable disease control.

2 | MATERIALS AND METHODS

2.1 | Microbial strains and culture conditions

The *U. virens* strain HWD-2 and its derived mutants were cultured on potato sucrose agar (PSA) plates at 28°C. Mycelial plugs (5 mm in diameter) were excised from the edge of actively growing colonies and incubated for 15 days to assess mycelial growth. *Phytophthora parasitica* strain P26 and *Phytophthora capsici* strain LT1534 were maintained on a 10% (v/v) vegetable juice (V8) medium at 25°C in darkness. The wild-type *Magnaporthe oryzae* strain Guy11 was grown on a complete medium at 28°C. For sporulation, *M. oryzae* culture plugs were transferred to a straw decoction and corn medium (100 g straw, 40 g corn powder, and 15 g agar per liter of distilled water) and incubated at 28°C in the dark, followed by 3 days of continuous fluorescent light exposure to induce conidiation.¹⁴ *Agrobacterium tumefaciens* strain GV3101 was cultured in a Luria-Bertani (LB) medium at 30°C by shaking at 200 rpm.

2.2 | Pathogenicity and plant infection assays

Rice cultivar “Wanxian-98” was used for *U. virens* virulence assays and was grown at the experimental station in Nanjing, Jiangsu, China. Inoculation with *U. virens* was performed as previously described.¹⁵ At the booting stage (5–7 days before heading), panicles were inoculated by injecting 1–2 mL of mycelial with conidial suspension (10^6 conidia/mL) using a sterile syringe. Inoculated plants were subsequently maintained in a humidified environment, and the number of false smut balls per panicle was recorded at 25 days postinoculation (dpi).¹⁶

Rice seedlings (*Oryza sativa* cv. CO39) were used for pathogenicity assays with *M. oryzae*. Two-week-old seedlings were spray-inoculated with 5 mL of conidial suspension (10^5 conidia/mL). After inoculation, plants were kept in darkness at high humidity for 24 h to facilitate infection and then transferred to a transparent growth chamber under a 12 h light/12 h dark photoperiod at 25°C. Disease symptoms were photographed at 7 dpi.¹⁷ Fungal biomass in infected leaves was quantified by real-time quantitative PCR (qPCR) to evaluate lesion development.

N. benthamiana plants were grown in a growth chamber at 25°C with 60% relative humidity under a 14 h light/10 h dark photoperiod. Fully expanded leaves

of 4–5-week-old plants were inoculated with 0.5 cm-diameter mycelial plugs from 3-day-old *Phytophthora* spp. cultures and incubated at 25°C. Lesion size was measured at 2 dpi.

2.3 | Yeast secretion assay

The signal peptide (SP) secretion assay was performed as previously described.¹⁸ The predicted SP sequence of UvDUF1 was cloned into the pSUC2 vector to generate pSUC2-UvDUF1-SP, which was then introduced into the yeast strain YTK12. Transformants were selected on a CMD-W medium and subsequently incubated on a YPRAA medium containing raffinose as the sole carbon source. The empty pSUC2 vector and pSUC2-Avr1b-SP were used as negative and positive controls, respectively. Invertase secretion activity was assessed by monitoring the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to a red 1,3,5-triphenylformazan (TPF) precipitate.

2.4 | Gene knockout and complementation in *U. virens*

To generate *UvDUF1* deletion mutants, approximately 1 kb upstream and downstream flanking sequences of the target gene were amplified and ligated into the pKD1 vector, resulting in the deletion construct pKD1-UvDUF1. This construct was introduced into *A. tumefaciens* strain AGL-1 and subsequently transformed into conidia of the *U. virens* strain HWD-2 via *A. tumefaciens*-mediated transformation (ATMT). Hygromycin-resistant transformants were selected and verified by PCR using three primer pairs listed in Table S1.

For complementation assays, the native promoter region and the full-length *UvDUF1* coding sequence, as well as a version lacking the SP sequence, were cloned into the pKO1-NEO vector to generate pKO1-UvDUF1 and pKO1-UvDUF1^{ΔSP}, respectively. These constructs were introduced into the $\Delta UvDUF1$ mutants via ATMT. Positive transformants were selected on PSA plates supplemented with 800 $\mu\text{g/mL}$ Geneticin (G-418, Macklin) and further confirmed by reverse transcription quantitative PCR (RT-qPCR).¹⁶

2.5 | Prokaryotic expression and purification of recombinant UvDUF1 protein

For prokaryotic expression, the coding sequence of *UvDUF1* was cloned into the pET32a vector and transformed into *Escherichia coli* BL21 (DE3) cells. Transformed cells were cultured overnight at 37°C in an

LB medium supplemented with the appropriate antibiotics. The overnight culture was then diluted 1:50 into a fresh LB medium and grown at 37°C until the optical density at 600 nm (OD_{600}) reached 0.6. Protein expression was induced by the addition of 0.1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG), followed by incubation at 20°C for 12 h. Cells were harvested by centrifugation and lysed using a high-pressure homogenizer. The lysate was centrifuged at $12,000\times g$ for 30 min at 4°C to remove cell debris. His-tagged recombinant proteins were purified from the supernatant using Ni-NTA affinity resin (Novagen). Protein concentration was determined using a BCA Protein Assay Kit (Solarbio). Purified proteins were separated by 10% SDS-PAGE and visualized by Coomassie brilliant blue staining.

2.6 | RT-qPCR analysis

Total RNA was extracted using the RNeasy Pure Plant Plus Kit (TIANGEN). Approximately 800 ng of total RNA was used for first-strand cDNA synthesis with the HiScript III RT SuperMix for RT-qPCR (+gDNA wiper) kit (Vazyme) according to the manufacturer's instructions. RT-qPCR was performed in a 10- μL reaction volume containing 10 ng of cDNA, 0.2 μL of each gene-specific primer (10 μM), 5 μL of ChamQ Universal SYBR qPCR Master Mix (Vazyme), and 3.6 μL of sterile deionized water. Amplification was carried out on an Applied Biosystems 7500 Real-Time PCR System. All primers used for RT-qPCR analysis were listed in Table S1. Relative gene expression levels were calculated using the $2^{-\Delta\Delta\text{CT}}$ method.¹⁹

2.7 | ROS burst assay

Reactive oxygen species (ROS) burst assays were performed as previously described with minor modifications.^{20,21} Leaf discs (0.5 cm in diameter) were excised from *N. benthamiana* leaves and preincubated overnight in a 96-well plate (one disc per well) containing 200 μL of sterile distilled water. After removing the water, 200 μL of reaction mixture containing 35.4 $\mu\text{g/mL}$ luminol, 10 $\mu\text{g/mL}$ horseradish peroxidase, and purified protein, was added to each well. Chemiluminescence was recorded immediately at 1-min intervals for 80 min using a FLUOstar Omega microplate reader (BMG LABTECH). For ROS detection in rice, 3-mm segments were collected from the tender stems of 10-day-old seedlings and individually placed into each well of the 96-well plates and treated with the same reaction solution described above. Luminescence was measured under identical conditions, and three independent biological replicates were analyzed.

2.8 | Bioinformatics analysis

Homologous protein sequences of UvDUF1 from diverse fungal species were retrieved from the NCBI database. SP prediction was performed using the SignalP 5.0 server (<http://www.cbs.dtu.dk/services/SignalP/>). Functional domains were identified using the Pfam database (<https://pfam.xfam.org/>). Phylogenetic analysis was conducted using the maximum likelihood method based on protein sequences. The phylogenetic tree was drawn to scale, with branch lengths representing the number of substitutions per site. Evolutionary analyses were conducted using MEGA 10.0. GraphPad Prism software was used to present graphs and perform statistical analysis.

3 | RESULTS

3.1 | UvDUF1 is a secreted protein that is broadly distributed and evolutionarily conserved in ascomycetes

UvDUF1, previously identified in the culture filtrate of *U. vires* as a cell death-inducing protein in *N. benthamiana*,⁷ encodes a 250-amino-acid protein containing an N-terminal SP and a C-terminal DUF3455 domain but lacking predicted transmembrane helices (Figure 1A), suggesting that it functions as a secreted protein.

To experimentally validate SP-mediated secretion, we performed a yeast invertase secretion assay. In this system, secretion of invertase enables the *Saccharomyces cerevisiae* strain YTK12 to grow on the raffinose-containing medium (YPRAA) and to reduce TTC to the insoluble red compound TPF. The SP of UvDUF1, or Avr1b (used as a positive control), was fused in-frame to the invertase gene in the pSUC2 vector and introduced into the invertase-deficient yeast strain YTK12. Yeast transformants expressing the SP-invertase fusion proteins grew on a YPRAA medium and efficiently converted TTC to TPF, whereas negative controls (YTK12 alone or carrying the pSUC2 empty vector) failed to grow on YPRAA and remained colorless after TTC treatment (Figure 1B). These results demonstrate that the N-terminal SP of UvDUF1 is functional and sufficient to mediate protein secretion.

To assess the evolutionary conservation of UvDUF1, homologous sequences were identified across diverse taxa. UvDUF1 homologs were detected exclusively in ascomycete fungi and were absent from prokaryotes, oomycetes, and plants (Figure 1C, Table S2). Multiple sequence alignment of UvDUF1 and its homologs from 16 representative ascomycete species revealed strong sequence conservation (Figure S1). Consistent with phylogenetic analysis, these results indicate that UvDUF1 is broadly distributed and evolutionarily conserved within ascomycetes, suggesting that this conserved protein plays an important role during *U. vires* infection.

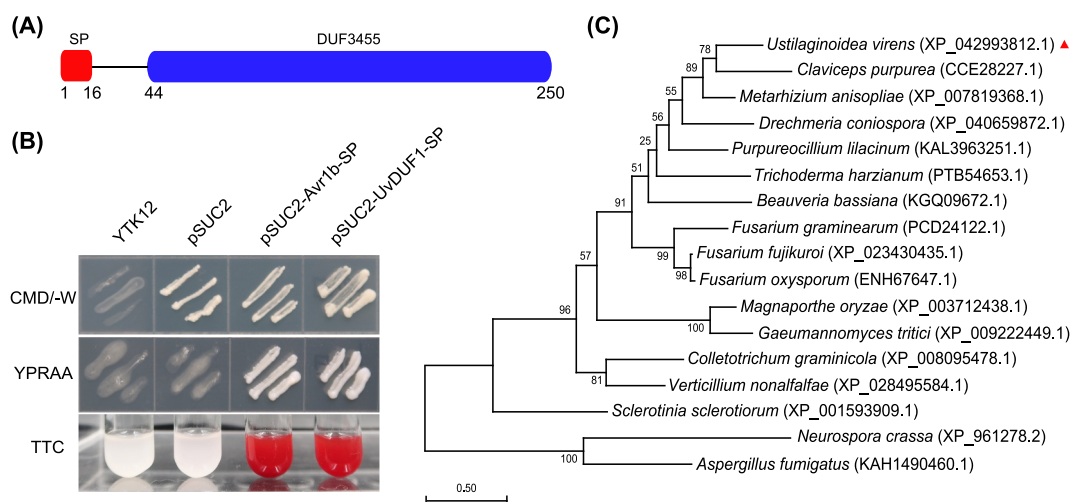


FIGURE 1 UvDUF1 is a secreted and evolutionarily conserved fungal protein. (A) Schematic representation of the UvDUF1 protein structure. DUF3455, domain of unknown function 3455; SP, signal peptide; UvDUF1, *Ustilagoideae vires*; DUF domain-containing protein 1. (B) Functional validation of the UvDUF1 SP using a yeast invertase secretion assay. The SP sequence of UvDUF1 was fused in-frame to the invertase gene in the pSUC2 vector and transformed into the invertase-deficient yeast strain YTK12. Yeast transformants expressing the *Phytophthora sojae* Avr1b SP (Avr1b-SP) served as the positive control, whereas those carrying the empty pSUC2 vector (pSUC2 EV) served as the negative control. The experiment was repeated three times with similar results. (C) Maximum likelihood phylogenetic analysis of UvDUF1 and its homologs from representative fungal species. The bootstrap consensus tree (1000 replicates) illustrates the inferred evolutionary relationships among the analyzed taxa. Branches supported by less than 50% bootstrap values were collapsed.

3.2 | *UvDUF1* is essential for *U. virens* infection

To investigate the potential role of *UvDUF1* during infection, we examined its transcript levels in *U. virens* at different stages of rice infection. *UvDUF1* exhibited very low expression at 0 dpi but was significantly induced following host infection. Its expression peaked at 3 dpi, reaching more than a 15-fold increase than 0 dpi, and remained elevated at 6 dpi before declining thereafter. From 9 to 20 dpi, *UvDUF1* transcript levels gradually returned to near-basal levels (Figure 2A). These results indicate that *UvDUF1* is specifically upregulated during the early stages of host colonization, suggesting a role in infection establishment.

To further assess the contribution of *UvDUF1* to *U. virens* pathogenicity, three independent knockout mutants ($\Delta UvDUF1$ -1, -2, and -3) were generated via

homologous recombination, and a complementation strain expressing the wild-type *UvDUF1* were subsequently obtained (Figure S2). When inoculated onto the susceptible rice cultivar Wanxian-98, the $\Delta UvDUF1$ mutants produced significantly fewer false smut balls than the wild type, whereas complementation of *UvDUF1* restored virulence and markedly increased smut ball formation (Figure 2B,C). These findings demonstrate that *UvDUF1* contributes to the pathogenicity of *U. virens*. To determine whether the reduced virulence resulted from defects in fungal growth or development, we examined colony morphology and measured colony diameters of the transformants (Figure S3). Neither deletion nor complementation of *UvDUF1* affected fungal growth characteristics, indicating that the contribution of *UvDUF1* to virulence is independent of fungal development and instead likely reflects its role as an effector that modulates plant immunity.

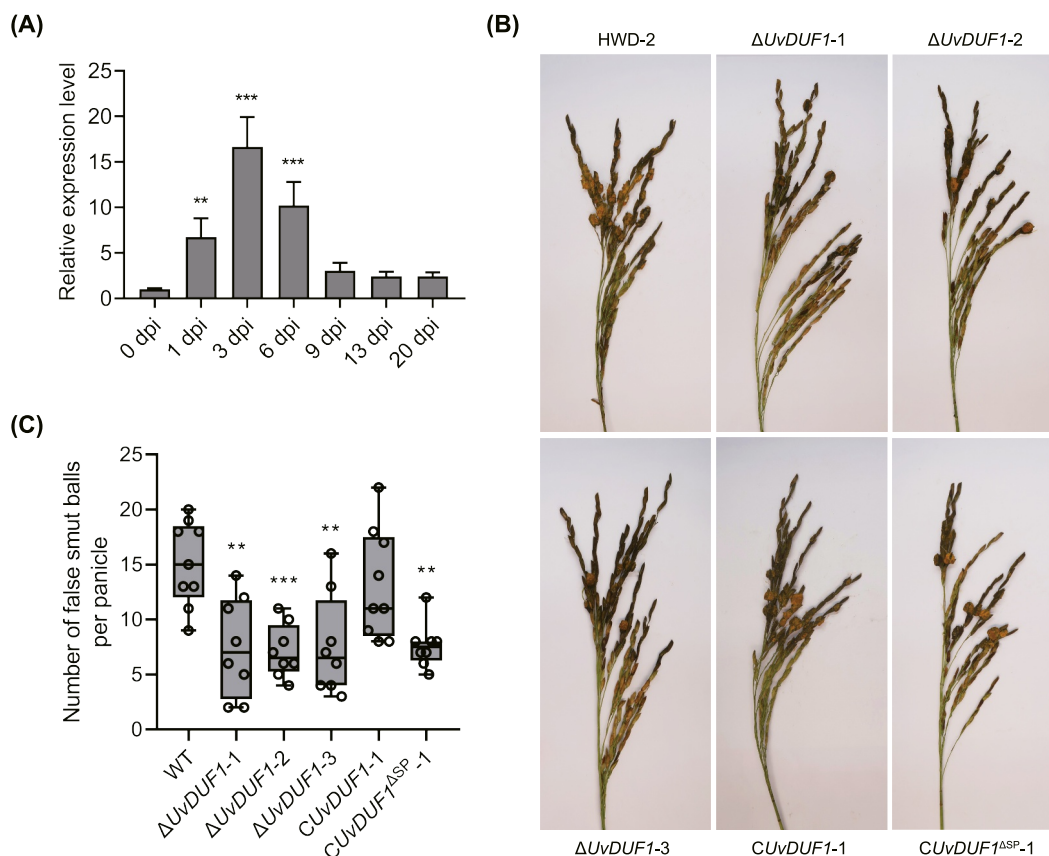


FIGURE 2 *UvDUF1* contributes to the virulence of *Ustilago virens*. (A) Expression profile of *UvDUF1* during *U. virens* infection. Transcript levels were quantified at different infection stages (0–20 days post-inoculation, dpi) by RT-qPCR. Gene expression levels were normalized to those at 0 dpi using *Tubulin* as an internal reference. Data represent the mean $\pm$ SD of three biological replicates. Statistical significance was determined by Dunnett's test (**p < 0.01 and ***p < 0.001). (B) Disease symptoms on rice panicles inoculated with the wild-type and mutant strains. *UvDUF1* knockout mutants ($\Delta UvDUF1$ -1, $\Delta UvDUF1$ -2, and $\Delta UvDUF1$ -3) and complementation strains ($CUvDUF1$ -1 and $CUvDUF1^{\Delta SP-1}$) were analyzed. Plants of the susceptible rice cultivar "Wanxian-98" were inoculated at the late booting stage, and false smut balls were photographed at 25 dpi. (C) Quantification of false smut balls per panicle. Data were obtained from three independent experiments. Statistical significance was determined by Dunnett's test (**p < 0.01; ***p < 0.001). RT-qPCR, reverse transcription quantitative PCR; *UvDUF1*, *U. virens* DUF domain-containing protein 1.

3.3 | The signal peptide is required for the pathogenic function of UvDUF1

To test whether UvDUF1 functions as a secreted effector, we generated a secretion-deficient complementation strain lacking the SP (*CUvDUF1^{ΔSP}-1*) and evaluated its roles in pathogenicity and fungal development. Unlike the full-length *UvDUF1* complementation strain, which restored the reduced virulence of the $\Delta UvDUF1$ mutant, *CUvDUF1^{ΔSP}-1* failed to rescue pathogenicity (Figure 2B,C). In addition, colony morphology and the growth rate of *CUvDUF1^{ΔSP}-1* were comparable to those of the wild-type strain (Figure S3). These results indicate that secretion of UvDUF1 is essential for its pathogenic function.

3.4 | UvDUF1 induces plant immune responses and enhances disease resistance in rice

To evaluate whether UvDUF1 modulates plant immune responses, we first tested whether recombinant UvDUF1 protein could induce ROS production and activate defense-related genes in rice. The recombinant protein was expressed in a prokaryotic system and subsequently purified for functional assays (Figure S4). To measure ROS burst, 1 μ M UvDUF1 was applied to rice stems, and ROS production was measured using a luminol-based detection system. UvDUF1 treatment triggered a robust ROS burst, whereas the EV control failed to elicit a detectable response (Figure 3A,B).

To further determine whether UvDUF1 activates defense-related gene expression, we examined the transcript levels of four immune marker genes in rice leaves at different time points following treatment with recombinant UvDUF1. As shown in Figure 3C–F, UvDUF1 significantly induced the expression of *mitogen-activated protein kinase 3* (*OsMPK3*), which is associated with jasmonic acid (JA) signaling,²² *WRKY transcription factor 70* (*OsWRKY70*), which is associated with both salicylic acid (SA) and JA signaling,²³ *ent-kaurene synthase-like 4* (*OsKS4*), which is involved in phytoalexin biosynthesis,²⁴ and *Phenylalanine ammonia-lyase 1* (*OsPAL1*), which is related to SA signaling²⁵ at 1 h posttreatment (1 hpt) compared with the EV control. Notably, *OsKS4* expression remained significantly higher in the treatment group than in the control group at 2 hpt, whereas the other three genes showed no significant differences compared with the EV control. At later time points (4, 8, and 12 hpt), no significant differences in expression of these four genes were observed between the UvDUF1-treated and EV groups (Figure 3C–F). Collectively, these results indicate that UvDUF1 rapidly and transiently activates multiple defense-associated genes in rice.

Given the role of UvDUF1 in inducing immune responses in rice, we next tested whether it enhances resistance to rice blast disease. Leaves of the susceptible variety CO39 were sprayed with 1 μ M purified UvDUF1 or EV protein and inoculated with 5 mL of a conidial suspension of *M. oryzae* strain Guy11 4 h later. The results show that UvDUF1 treatment significantly reduced blast disease severity compared to EV control (Figure 4A). Quantification of lesion area and relative fungal biomass further supported this observation (Figure 4B,C). To determine whether UvDUF1 directly inhibits fungal growth, Guy11 was cultured on a medium containing 1 μ M UvDUF1 protein. Compared to the EV protein control, colonies grown on a UvDUF1-supplemented medium exhibited no observable differences in morphology or the growth rate (Figure S5). These findings demonstrate that exogenous application of UvDUF1 markedly enhances rice resistance to *M. oryzae*, likely through activation of host immunity rather than direct inhibition of pathogen growth.

3.5 | UvDUF1 induces immune responses and enhances disease resistance in *N. benthamiana*

To determine whether UvDUF1 can activate defense responses in nonhost plants, we examined ROS accumulation and the expression of defense-related marker genes in *N. benthamiana* following treatment with purified UvDUF1 protein. As shown in Figure 5A,B, UvDUF1 treatment triggered a pronounced ROS burst in *N. benthamiana*. In addition, UvDUF1 significantly induced the early expression of PTI-related genes. Specifically, *WRKY transcription factor 22* (*NbWRKY22*), which is typically induced during PTI to activate defense genes,²⁶ and *Cytochrome P450 71D20* (*NbCYP71D20*), which is involved in antimicrobial compound synthesis,²⁷ were rapidly induced, with their expression levels peaking at 1 h and remaining elevated at 2 h posttreatment compared with the EV control (Figure 5C,D). In addition, *Avr9/Cf-9 rapidly elicited protein 31* (*NbAcre31*), a putative calcium-binding protein,²⁸ was strongly induced from 1 to 4 h posttreatment, whereas *pathogenesis-related genes transcriptional activator PTI5-like* (*NbPia5*), a transcription factor that induces defense gene expression,¹⁸ showed a modest but significant induction at 4 h (Figure 5E). These results demonstrate that UvDUF1 also activates multiple defense-associated genes in *N. benthamiana*.

We next examined whether UvDUF1 enhances disease resistance in *N. benthamiana*. Leaves were sprayed with purified UvDUF1 protein prior to inoculation with the oomycete pathogens *P. parasitica* and *P. capsici*. As shown in Figure 6A and C,

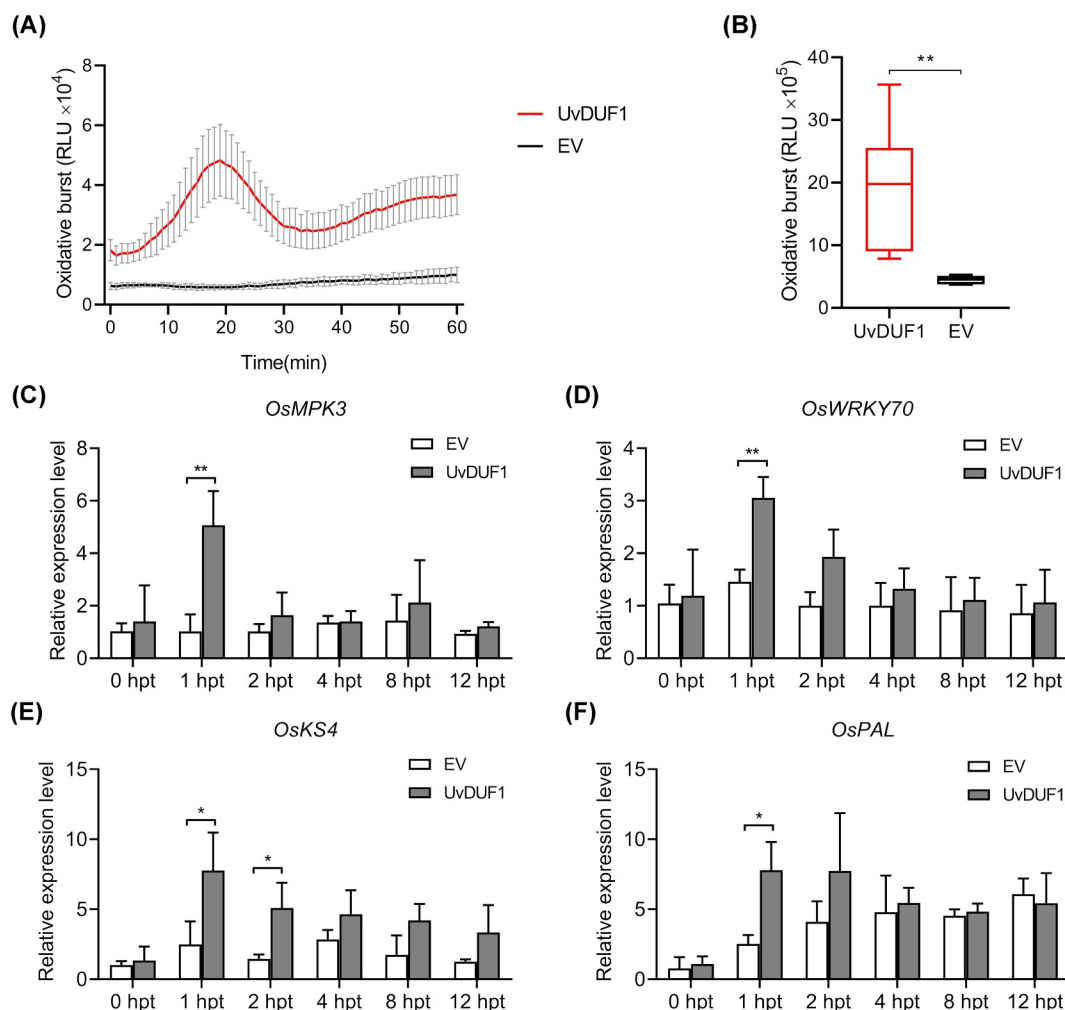


FIGURE 3 UvDUF1 induces immune responses in rice. (A) Reactive oxygen species (ROS) production in rice treated with purified UvDUF1 protein (1 μ M) or the negative control (EV). Data represent the mean $\pm$ SEM ($n = 6$). The experiment was repeated three times with similar results. (B) Statistical analysis of ROS production shown in panel (A). Data were calculated as the total relative light units over the entire measurement period. Statistical significance was determined by Student's t -test (** $p < 0.01$). (C–F) Relative transcript levels of four defense-related marker genes in rice leaves at 0, 1, 2, 4, 8, and 12 h after treatment with the indicated proteins. Gene expression levels were analyzed by RT-qPCR. Data represent the mean $\pm$ SD of three independent biological replicates. Asterisks indicate significant differences based on Student's t -test (* $p < 0.05$; ** $p < 0.01$). RT-qPCR, reverse transcription quantitative PCR; UvDUF1, *U. vires* DUF domain-containing protein 1.

UvDUF1-treated leaves developed significantly smaller lesions than control treatments. Quantitative analysis further confirmed that the average lesion area was markedly reduced in UvDUF1-treated leaves compared with EV-treated leaves (Figure 6B and D), indicating enhanced resistance to oomycete infection. To determine whether UvDUF1 directly affects pathogen growth, the oomycetes were cultured on a medium supplemented with UvDUF1 protein. No observable differences in colony morphology or the growth rate were detected compared with the EV protein control (Figure S5). Collectively, these findings demonstrate that exogenous application of UvDUF1 induces immune responses and enhances disease resistance not only in its host, rice, but also in the nonhost plant *N. benthamiana*.

4 | DISCUSSION

U. vires infects rice stamens and induces the formation of false smut balls. As a biotrophic fungus that depends on intimate host interactions, it secretes effectors that are essential for successful infection.² Although its genome encodes more than 600 predicted secreted proteins,¹ only a small number have been functionally characterized. Here, we report that UvDUF1, a secreted protein containing a DUF3455 domain, promotes *U. vires* pathogenicity. Notably, UvDUF1 also triggers immune responses in both its host, rice, and the nonhost *N. benthamiana*. Furthermore, pretreatment with UvDUF1 enhances rice resistance to *M. oryzae* and *N. benthamiana* resistance to

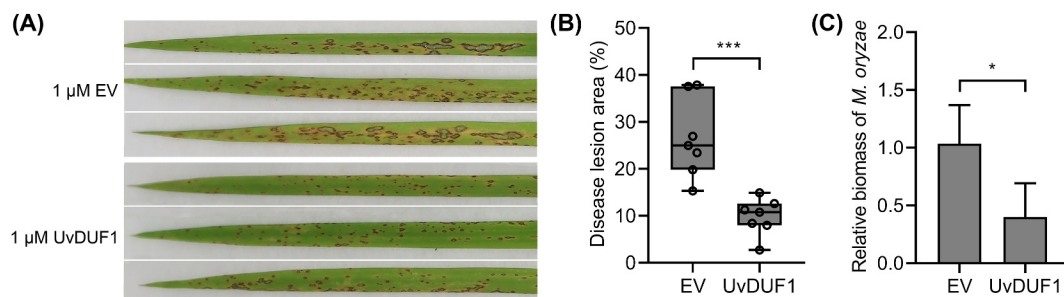


FIGURE 4 Exogenous application of UvDUF1 enhances resistance to *Magnaporthe oryzae* in rice. (A) Disease symptoms on rice leaves inoculated with *M. oryzae* strain Guy11 at 7 days postinoculation (dpi). Two-week-old rice seedlings (cv. CO39) were pretreated with 1 μ M UvDUF1 or EV protein for 4 h prior to spray inoculation with a conidial suspension of *M. oryzae*. (B) Quantification of diseased lesion area. Data represent the mean $\pm$ SD. Statistical significance was determined by Student's *t*-test (** p < 0.01). (C) Fungal biomass in infected leaves shown in panel (A). The relative biomass of *M. oryzae* was quantified by real-time quantitative PCR using *MoPot2* and *OsUbi* as internal references. Data are presented as the mean $\pm$ SD from four independent experiments. Statistical significance was determined by Student's *t*-test (* p < 0.05). UvDUF1, *U. vires* DUF domain-containing protein 1.

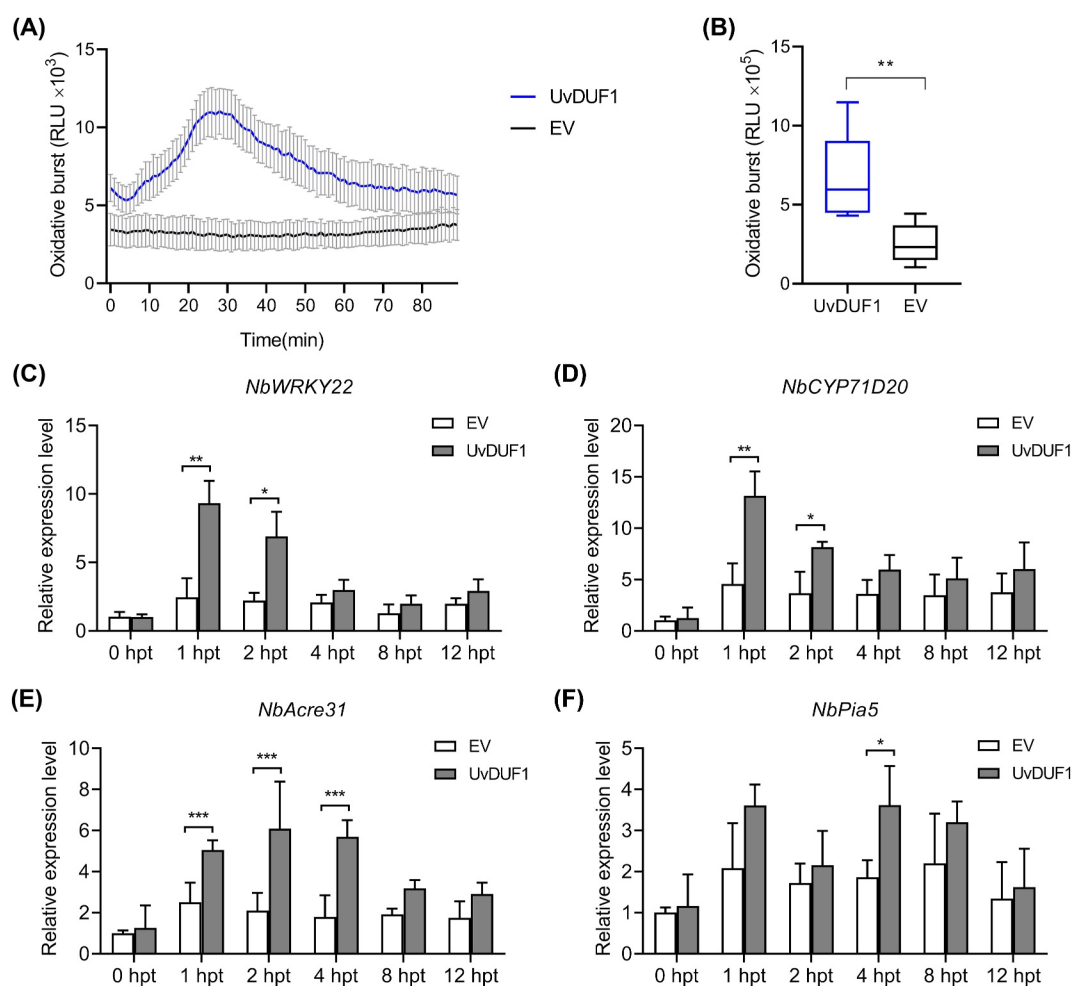


FIGURE 5 UvDUF1 induces immune responses in *Nicotiana benthamiana*. (A) Reactive oxygen species production in *N. benthamiana* leaves treated with purified UvDUF1 protein (1 μ M) or the negative control. Data represent the mean $\pm$ SEM (n = 6). The experiment was repeated three times with similar results. (B) Statistical analysis of reactive oxygen species production shown in panel (A). Statistical significance was determined by Student's *t*-test (** p < 0.01). (C–F) Relative transcript levels of four defense-related marker genes in *N. benthamiana* leaves after treatment with the indicated proteins. Gene expression levels were analyzed by RT-qPCR. Data are presented as the mean $\pm$ SD of three independent biological replicates. Statistical significance was determined by Student's *t*-test (* p < 0.05; ** p < 0.01; *** p < 0.001). RT-qPCR, reverse transcription quantitative PCR; UvDUF1, *U. vires* DUF domain-containing protein 1.

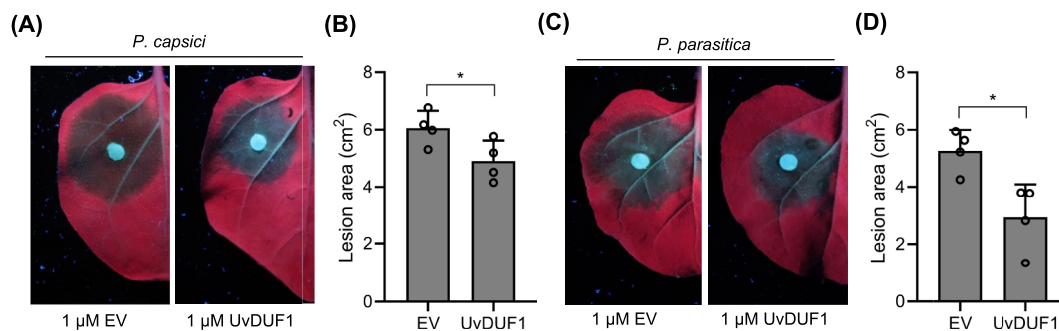


FIGURE 6 Exogenous application of UvDUF1 enhances resistance to *Phytophthora* pathogens in *Nicotiana benthamiana*. (A and C) Representative disease symptoms caused by *Phytophthora capsici* and *Phytophthora parasitica* on *N. benthamiana* leaves at 48 h postinoculation (hpi). Leaves were pretreated with 1 μM UvDUF1 or EV protein for 4 h prior to inoculation with a 5-mm-diameter mycelial plug. (B and D) Quantification of lesion areas corresponding to panels (A) and (C), respectively. Lesion areas were measured using ImageJ software. Data represent the mean ± SD. The experiment was repeated four times. Statistical significance was determined by Student's *t*-test (***p* < 0.01). UvDUF1, *U. virens* DUF domain-containing protein 1.

oomycete pathogens of the genus *Phytophthora*. Collectively, these findings highlight the potential of UvDUF1 as a broad-spectrum, cross-species resistance inducer for crop protection.

This study reveals that the effector UvDUF1 plays a dual role in virulence and immunity. Knockout of *UvDUF1* in *U. virens* significantly reduces its virulence on rice panicles, whereas complementation restores pathogenicity to wild-type levels (Figure 2B,C). These results demonstrate that *UvDUF1* is crucial for fungal infection of rice panicles. An elicitor is defined as a microbe-derived molecule that is recognized by plant immune receptors and triggers specific defense responses.²⁹ Notably, UvDUF1 fulfills this definition by inducing a rapid ROS burst and activating defense-related genes in both its host, rice, and the nonhost *N. benthamiana* (Figures 3 and 5). Moreover, pretreatment with UvDUF1 enhances rice resistance against *M. oryzae* and *N. benthamiana* resistance to *Phytophthora* infection (Figures 4 and 6). These combined properties—specific activation of immune responses and priming of broad-spectrum resistance—firmly establish UvDUF1 as a pathogen-derived elicitor. This functional duality raises an intriguing question: How does *U. virens* successfully infect rice panicles while producing an elicitor capable of activating plant immunity? Similar phenomena have been reported for other *U. virens* effectors. For example, UvGHF1, a glycoside hydrolase family effector, exhibits a paradoxical role in panicle infection by promoting virulence while, when expressed in rice, inducing resistance to RFS.⁸ Likewise, the essential microbe-associated molecular pattern elicitor SGP1 selectively induces immunity in leaves but not in panicles, suggesting its involvement in the panicle-specific infection strategy of *U. virens*.⁷ It is possible that UvDUF1-induced immunity is suppressed by other *U. virens* effectors during infection. *U. virens* secretes numerous effectors that facilitate host

colonization, including UgsL and UvGH18.1.^{1,5,6} Further investigations into whether UvDUF1 triggers immune responses during panicle infection will be important for understanding how multiple effectors coordinately modulate plant immunity and ensure successful infection.

UvDUF1 was initially identified in the secretome of *U. virens*⁷ and later experimentally verified as a secreted protein using an yeast secretion assay (Figure 1B). The N-terminal SP of UvDUF1 is functional and sufficient to mediate invertase secretion, enabling the growth of invertase-deficient yeast on a YPRAA medium and facilitating the conversion of TTC into the insoluble red compound TPF. Furthermore, deletion of the SP abolished the ability of UvDUF1 to fully restore the pathogenicity of *U. virens* (Figure 2), confirming that secretion is essential for its virulence function. These findings underscore the critical role of secretion in effector functionality and suggest that targeting effector secretion or trafficking may represent a promising strategy for disease control.

UvDUF1 contains a DUF3455 domain, a domain of unknown function that is predominantly found in ascomycete fungi (Figure S1). Comparative genomics analyses aimed at identifying lifestyle-specific virulence strategies in plant-pathogenic ascomycetes revealed that DUF3455-containing proteins constitute one of the seven core effector families,³⁰ underscoring the fundamental importance of this domain in fungal development and/or virulence. Knockout of *UvDUF1* in *U. virens* reduces virulence on rice panicles, and complementation with the full-length gene restores pathogenicity to wild-type levels (Figure 2B and C), demonstrating that *UvDUF1* is crucial for fungal infection of rice panicles. However, due to the limited understanding of the DUF3455 domain, the molecular mechanism by which it contributes to pathogen virulence remains unclear and warrants further

investigation. The *M. oryzae* homolog MoCEP7, which also contains a DUF3455 domain, has been reported to suppress flg22-triggered ROS production in *N. benthamiana* but is dispensable for full virulence in *M. oryzae*.³¹ These findings suggest that DUF3455-containing proteins can perform divergent biological roles in different fungal pathogens despite sharing conserved structural features. Such functional divergence may result from subtle sequence variation within the core domain, as well as differences in temporal expression patterns during infection—UvDUF1 is induced during the early stages of *U. virens* invasion, whereas MoCEP7 expression increases at later stages of infection. Together, these observations highlight the functional diversity within effector families that share common domains and emphasize the importance of investigating these proteins on a case-by-case basis across distinct plant–pathogen systems.

Plants have evolved sophisticated surveillance mechanisms to recognize pathogen effectors and activate innate immunity. Some fungal apoplastic effectors are perceived by PRRs, thereby triggering plant defense responses.^{32,33} For example, the leucine-rich repeat (LRR) receptor-like protein RXEG1 recognizes the glycoside hydrolase XEG1 in the apoplast through its LRR domain and initiates defense signaling by forming a complex with the co-receptors BAK1 and SOBIR1.²⁷ Similarly, the *U. virens* elicitors SGP1 and UvGHF1 induce cell death in a manner dependent on the membrane-localized receptors BAK1 and/or SOBIR1, although their corresponding immune receptors have not yet been identified.^{7,8} The ROS burst triggered by UvDUF1, together with the rapid induction of defense marker genes such as *OsMPK3* and *OsWRKY70* within 1 h, underscores the sensitivity and temporal precision of plant immune recognition toward this effector (Figures 3 and 5). This rapid activation of defense responses is characteristic of PTI. Further investigation into the putative rice receptor(s) or interacting proteins responsible for UvDUF1 perception will be essential to elucidate its recognition mechanism and downstream signaling pathways.

In the context of increasing fungicide resistance and growing environmental concerns associated with conventional chemical controls, environmentally friendly alternatives such as microbe-derived elicitors offer sustainable strategies for plant protection. Chitosan, for example, functions both as a direct antimicrobial agent—by altering microbial morphology—and as an exogenous elicitor that activates plant defense responses, and it is widely used in agriculture.^{34,35} Our results demonstrate that UvDUF1 treatment enhances resistance to *M. oryzae* in rice and suppresses *Phytophthora* spp. infection in *N. benthamiana*. Notably, UvDUF1 exhibits no direct antimicrobial activity against these three pathogens (Figures 4, 6, and S5). These

findings indicate that UvDUF1-triggered immunity is conserved across phylogenetically distant plant species. This property resembles that of the immune-inducing protein Fg62 from *Fusarium graminearum*, which enhances resistance against multiple pathogens across different plant species.¹⁸ To our knowledge, this study provides the first evidence that a fungal phytopathogen-derived protein containing a DUF3455 domain can induce immunity in both host and nonhost plants. Further exploration of the practical application of UvDUF1—either as a recombinant protein elicitor or through transgenic approaches—may offer a promising eco-friendly strategy to reduce reliance on synthetic pesticides and promote sustainable disease management in agriculture.

5 | CONCLUSION

In this study, we characterized the elicitor UvDUF1 from *U. virens*. UvDUF1 functions not only as a virulence factor that contributes to pathogen infection but also as an elicitor that triggers immune responses in both the host (*O. sativa*) and the nonhost (*N. benthamiana*), including ROS bursts and activation of defense-related genes. Consequently, UvDUF1 enhances rice resistance to *M. oryzae* and *N. benthamiana* resistance to *Phytophthora*. Our work provides the first evidence that a fungal phytopathogen-derived protein harboring a DUF3455 domain can function as an immune elicitor. These findings highlight UvDUF1 as a promising eco-friendly candidate for broad-spectrum plant disease management.

AUTHOR CONTRIBUTIONS

Shuchen Wang: Investigation; funding acquisition; writing—original draft; data curation; visualization. **Wen Zhang:** Investigation; data curation; validation. **Wenyue Zheng:** Investigation; validation. **Qian Zhang:** Investigation; validation. **Mina Yu:** Investigation; validation. **Junjie Yu:** Investigation; validation. **Huijuan Cao:** Investigation; validation. **Xiayan Pan:** Investigation; validation. **Zhongqiang Qi:** Investigation; validation. **Yan Du:** Investigation; validation. **Rongsheng Zhang:** Investigation; validation. **Yunpeng Wang:** Investigation; methodology. **Yongfeng Liu:** Writing—review and editing; resources; supervision. **Tianqiao Song:** Writing—review and editing; funding acquisition; resources; data curation; visualization.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in Supporting Information S1 of this article.

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