

Phapa-4574965: A multifunctional effector of *Phakopsora pachyrhizi* targeting multiple host metabolic components

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ABSTRACT

Asian soybean rust (ASR), caused by *Phakopsora pachyrhizi*, severely constrains soybean yield. Characterization of the effector candidate Phapa-4574965 was performed to elucidate virulence mechanisms. *In silico* analyses of the amino acid sequence revealed a conserved Gamma-Interferon-Inducible Lysosomal Thiol reductase (GILT) domain and a high-confidence 3D structural model. Comparative genomics placed Phapa-4574965 within a rust-wide cluster enriched for GILT-containing proteins. In soybean leaves, expression was induced early and peaked at 48 h post-inoculation. In *Nicotiana benthamiana*, Phapa-4574965 suppressed pattern-triggered immunity—reducing reactive oxygen species and callose—and blocked hypersensitive cell death triggered by *Pseudomonas syringae*, indicating interference with effector-triggered immunity in this heterologous system. Confocal microscopy showed predominant nuclear localization with partial cytosolic and mitochondrial signals, suggesting multi-compartment activity. Pull-down mass spectrometry from soybean leaves identified eight putative targets, including lipoxygenase, aminomethyltransferase, fructose-bisphosphatase, and proteins linked to carbohydrate metabolism and redox homeostasis. Together, these results suggest that Phapa-4574965 is a multifunctional candidate effector capable of modulating plant immune responses and host metabolic processes, supporting its potential role during soybean–*P. pachyrhizi* interactions and providing targets for resistance breeding and biotechnological interventions.

1. Introduction

The obligate biotrophic fungus *Phakopsora pachyrhizi* (Sydow & Sydow) is the causal agent of Asian Soybean Rust (ASR), a disease that severely affects soybean plants (*Glycine max*) and hampers their growth. Under favorable weather conditions and high disease pressure, ASR can reduce soybean yields by up to 80%, resulting in substantial economic losses [1,2]. To mitigate these losses, field management strategies include a combination of methods such as keeping a host free period before the growing season, applying fungicides and cultivating ASR-resistant soybean varieties [3]. At present, seven genetic loci,

named *Rpp1* through *Rpp7*, have been identified as providing resistance to ASR [4–10]. However, these control measures are not long-lasting due to the genetic diversity and rapid adaptation of *P. pachyrhizi*. Consequently, ongoing research efforts aim to develop and refine strategies to improve ASR management [2,3].

Research into plant-pathogen interactions is one of the key areas of study, as it helps to uncover the molecular details of pathogen attack, host defenses, and the dynamic processes of interaction and co-evolution between host and pathogen [11]. Plants rely on their innate immune system, which involves two distinct but connected biological processes, to protect themselves from biotic threats [12]. One of these defense

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mechanisms is the detection of Microbial- or Pathogen-Associated Molecular Patterns (MAMPs or PAMPs), molecules released by microbes or pathogens. These are recognized by Pattern Recognition Receptors (PRRs) in the extracellular environment, which activates PAMP-Triggered Immunity (PTI) [12–14]. As an obligate fungal pathogen, *P. pachyrhizi* utilizes a specialized structure called the haustorium to extract nutrients from host cells without killing them. In addition, it releases effector molecules that alter the physiology of soybean cells to facilitate infection, leading to Effector-Triggered Susceptibility (ETS).

The other defense pathway is triggered when the host can produce R proteins, which recognize avirulence effectors and activate Effector-Triggered Immunity (ETI) [13,15–17]. The host's resistance persists until it encounters a pathogen strain that produces an effector capable of bypassing both defense pathways, a hallmark of the co-evolutionary process between the pathogen and host [14,18].

To better understand the interaction between *P. pachyrhizi* and *Glycine max*, studying effector molecules is crucial, as these proteins help the pathogen manipulate the host to facilitate infection. Effector candidates have been identified through transcriptome [19,20], secretome [21], and genome analyses [22], and characterization studies are key to revealing their roles in virulence and host interaction. Several *P. pachyrhizi* effectors have been identified and some of their host targets uncovered. For example, PpEC23 interacts with the transcription factor *GMSPL12L*, likely suppressing host immunity [23]. Other effectors, such as PpEC48 and Small Secreted Cysteine-Rich Proteins (SSCRP), interact with soybean cells, with PpEC48 possibly acting as a “metaeffector” [24]. Bueno et al. [25] showed that Phapa-7431740 interacts with Gm β GLU, a key protein in soybean basal defense. More recently, PpEC15 was found to interact with peptide-chain 31 release factor (PCRF), the NAC83 transcription factor, and 3-deoxy-7-phosphoheptulonate (DAHP) synthase, all of which may positively regulate host defense [26].

By combining laser capture microdissection, RNA sequencing, and bioinformatics, a predicted secretome of 851 proteins was identified, including the effector candidate Phapa-4574965, validated in the present study. This effector exhibits typical characteristics of effectors, such as being small and cysteine-rich protein [21]. The role of Phapa-4574965 was confirmed by demonstrating its ability to suppress both ETI (effector-triggered immunity) and PTI (pathogen-associated molecular pattern-triggered immunity) when transiently expressed in *Nicotiana benthamiana* leaves. Pull-down assays and mass spectrometry revealed eight potential targets of this effector, which include proteins with diverse functions: one catalyzes oxygen addition to fatty acids, another facilitates amino group transfer for nitrogen metabolism, and a third is involved in carbohydrate metabolism and cell wall synthesis. Additionally, one enzyme recycles Calvin cycle inhibitors, another supports photorespiration, and a protease contributes to plant immunity by degrading pathogens and inducing cell death.

The results enhance the understanding of the effector Phapa-4574965, shedding light on its potential functions and mechanisms that may contribute to the virulence of *P. pachyrhizi*. This information may guide future characterization studies of this effector, contributing to a better understanding of ASR and potentially supporting improved disease management strategies.

2. Material and methods

2.1. In silico characterization of Phapa-4574965

The transcribed sequence of effector candidate de_novo_3326 [21] was analyzed by significant similarity (70% identity and 50% coverage) with the protein sequences of three reference genomes of *P. pachyrhizi* (MT2006 v0.1, UFV02 v2.1, K8108 v2.0), available at the JGI MycoCosm database (<https://mycocosm.jgi.doe.gov/mycocosm/home/releases?ft=phakopsora>) (accessed February 02, 2024). The gene model with the highest similarity to de_novo_3326 effector candidate in each genome was Phapa-4574965 (MT2006 v0.1), PpacPPUFV02-1581871

(UFV02 v2.1), and PhapaK8108-3103294 (K8108 v2.0). The three-dimensional structure of the protein encoded by Phapa-4574965 was predicted using AlphaFold2 (Colab version).

2.2. Comparative study of Phapa-4574965 in pucciniales

To investigate the relationship of Phapa-4574965 to genes in different rust species, the TribeMCL software [27] was used to cluster the predicted proteins from the genomes of the following rust species: “*Cronartium quercuum* f. sp. *fusiforme* G11 v1.0FilteredModels1”, “*Melampsora americana* R15-033-03 v1.0FilteredModels2”, “*Melampsora lini* CH5ExternalModels”, “*Melampsora larici-populina* v2.0FilteredModels1”, “*Melampsora medusae* f. sp. *deltoideae* Mmd05TRE539 v1.0FilteredModels1”, “*Puccinia graminis* f. sp. *tritici* v2.0BroadModels”, “*Puccinia striiformis* f. sp. *tritici* 104 E137 A-ExternalModels”, “*Puccinia striiformis* f. sp. *tritici* PST-130 ExternalModels” and “*Puccinia triticina* 1-1 BBBD Race 1BroadModels”; and three different isolates of *P. pachyrhizi*: “*Phakopsora pachyrhizi* MG2006 v1.0”, “*Phakopsora pachyrhizi* PPUFV02 v1.0FilteredModels1” and “*Phakopsora pachyrhizi* K8108 v1.0FilteredModels1”, available at JGI MycoCosm (<https://mycocosm.jgi.doe.gov/clm/run/Phakopsora-comparative1.4168;LLi6g8?organism=Phapa1>) (accessed February 02, 2024).

The predicted sequences of the members of each cluster were grouped and aligned using ClustalW. Protein sequences containing a predicted signal peptide and a GILT domain (PF03227) were retrieved, and only complete sequences were retained for the comparative analysis. Comparative phylogenetic trees were constructed using MEGA X with the Neighbor-Joining method (1000 bootstrap replicates). The similarity between the effector gene models in the three different *P. pachyrhizi* genomes was verified, and only one representative sequence was used to construct the phylogenetic tree.

2.3. Expression profile of Phapa-4574965

The expression profile of Phapa-4574965 was analyzed over time course in BRS184 (susceptible soybean genotype) inoculated with *P. pachyrhizi* isolate LPF16.1M. The experiment was performed in a completely randomized design with three replications. The soybean plants were grown in 0.5-L plastic pots and were kept in a climatic growth chamber (Fitotron). When the plants reached the V3 stage, the first and second trifoliate leaves of each plant were inoculated, they were collected at 0, 6, 12, 24, and 48 hpi. Inoculated and mock-inoculated soybean leaves were ground with liquid nitrogen and the total RNA was extracted by the Trizol method (Invitrogen®, Carlsbad, CA, USA). The RNA was resuspended in 35 μ l of ultrapure water and was stored at -80°C . RNA concentrations were quantified on a NanoDrop 1000 spectrophotometer (Thermo Scientific®, Waltham, MA, USA) and the integrity was analyzed on a 1% agarose gel. To eliminate the genomic DNA, 5 μ g of RNA was treated with DNase I (Invitrogen®, Carlsbad, CA, USA) and visualized on a 1% agarose gel to confirm the efficiency of the treatment. Subsequently, cDNA was synthesized using the SuperScript III first-strand kit (Invitrogen®, Carlsbad, CA, USA), following manufacturer instructions. Amplification of the cDNA was confirmed and the cDNA was stored at -20°C .

Primer3Plus was used to design primers to amplify Phapa-4574965 amplicons, and serial cDNA dilutions and linear regression were used to determine the efficiency of each primer-pair. Gene expression over a time course of 6, 12, 24 and 48 h post-inoculation was determined by qPCR using *CytB* [28] as the normalizing gene and samples collected at 0 h post-inoculation as a calibrator. qPCR was performed in biological and technical triplicate on the 7900HT Fast Real-Time PCR system (Applied Biosystems®, Foster City, CA, USA), using the Platinum® SYBR® Green qPCR SuperMix-UDG kit (Invitrogen®, Carlsbad, CA, USA). The amplification conditions were 95°C for 10 min, 40 cycles of 94°C for 15 s, and 60°C for 1 min, followed by melting curve analysis. The expression levels of the target genes were determined by relative

quantification, using the REST® software [29].

2.4. PTI and ETI suppression analysis

N. benthamiana plants were grown in a growth chamber at 24 °C with 50–55% humidity and a 12-h photoperiod. For PTI suppression analysis (ROS detection and callose deposition), the *Pseudomonas fluorescens* Pf0-1 (EtHAn) carrying pEDV6 expressing the *Phapa-4574965* coding sequence was grown on KB medium with chloramphenicol (30 µg mL⁻¹) + gentamicin (50 µg mL⁻¹) for 48 h at 28 °C. To analyze ETI suppression, the *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) carrying pEDV6 expressing the *Phapa-4574965* coding sequence was grown on Kings B (KB) solid medium with rifampicin (100 µg mL⁻¹) + gentamicin (75 µg mL⁻¹) for 48 h at 28 °C. The bacterial colonies were resuspended in Milli-Q water and centrifuged at 3000×g for 15 min. The pellets were resuspended in 2 mL of 10 mM MgCl₂ and the optical density at 600 nm (OD₆₀₀) was adjusted to 0.5 for the PTI suppression experiment and to 0.2, 0.02 and 0.004 for the ETI suppression experiment. The abaxial surface of leaves of 5-week-old *N. benthamiana* plants was infiltrated with bacterial suspensions using a needleless syringe. The 10 mM MgCl₂ solution was infiltrated in *N. benthamiana* leaves as negative control, EtHAn containing the pEDV6 empty vector was the positive control in PTI suppression assays, and DC3000 containing the pEDV6 empty vector was the negative control in ETI suppression assays. The experiment was conducted in a completely randomized design, with nine replicates and one plant per replicate. For ETI suppression analysis, the *N. benthamiana* leaves were photographed at 48 h post-infiltration.

To detect ROS at 24 h post-infiltration, *N. benthamiana* infiltrated leaf discs were collected and stained overnight with 3,3'-diaminobenzidine under gentle stirring at room temperature. Leaf discs were washed with distilled water and destained for 15 min in a warmed solution of ethanol/glycerol/acetic acid (3:1:1). The leaf discs were mounted in 50% glycerol and the images were acquired using Motic Images Plus 2.0 (Motic China Group Co., Ltd., Xiamen, China).

To detect callose deposition at 72 h post-infiltration, *N. benthamiana* infiltrated leaf discs were collected and destained overnight in 95% ethanol under agitation at 37 °C, followed by several washes in 70% ethanol. The leaf discs were stained for 1 h with aniline blue (150 mM potassium phosphate, pH 9.0) in the dark under agitation at room temperature. The leaf discs were washed in water, imaged using fluorescence microscopy, and the images were analyzed by the Zeiss Axio-Vision 3.0 software.

The 10 × and 5 × objective lenses were used to take the ROS and callose deposition images, respectively. The images were acquired from the central region, avoiding the edges of the leaf discs that suffer greater stress and keeping the region of the infiltration point in the upper left corner. The quantification of image pixels was used to obtain the ROS and callose deposition values, and the values of the negative controls were used as a calibrator in the calculations. Statistical differences following image analysis were performed using the *t*-test.

2.5. Subcellular localization of *Phapa-4574965*

N. benthamiana plants were grown in a growth chamber at 22 °C with 80% humidity and a 16-h photoperiod. The *Agrobacterium tumefaciens* strain GV3101 with *Phapa-4574965* coding sequence in pSITE vector (Martin et al., 2009) carrying GFP (Green Fluorescent Protein) and different strains with subcellular localization marker protein (plastid, peroxisome, mitochondria, and nucleus) carrying mCherry FP (Fluorescent Protein) were used for co-transient expression in *N. benthamiana* leaves. *A. tumefaciens* clones were grown in Yeast Peptone Extract (YEP) medium with the appropriate antibiotics at 28 °C overnight with shaking. The bacterial cultures were washed three times by centrifuging at 7000×g for 5 min and resuspended in 2 mL of 10 mM MgCl₂, and the OD₆₀₀ was adjusted to 0.3. The abaxial surface of leaves of 5-week-old *N. benthamiana* plants was co-infiltrated with the GV3101 containing

Phapa-4574965 and the *A. tumefaciens* containing the marker protein using a needleless syringe. *N. benthamiana* leaves were infiltrated with 10 mM MgCl₂ as negative control and GV3101 containing the pSITE empty vector and eGFP as positive control. The experiment was conducted in a completely randomized design, with three replicates and one plant per replicate. At 72 h post-infiltration, the leaf discs were imaged using a confocal microscope, LSM 510 META (Zeiss, Jena, Germany).

2.6. Soybean protein identification by pull-down assay and mass spectrometry

Total proteins were extracted from leaves of the PI200487 soybean line, which carries the *Rpp5* resistance gene. Plants were grown in a controlled growth chamber under a day/night temperature cycle of 28 °C/25 °C, with 50% relative humidity and a 12-h photoperiod. The experiment followed a completely randomized design with three replicates.

Urediniospores of *P. pachyrhizi* (LPF16.1M) were resuspended in 0.01% (v/v) Tween 20 to a final concentration of 4×10^5 spores mL⁻¹. PI200487 leaves at the V₂ growth stage were inoculated with the spore suspension [30]. Leaves were collected at 0, 6, 12, 24, 48, 72, and 96 h post-inoculation for protein extraction.

Leaf samples (200–500 mg) were ground in 750 µL of protein extraction buffer containing 50 mM Tris (pH 7.5), 250 mM sucrose, 10 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1 mM dithiothreitol (DTT), and protease inhibitor cocktail (2 µL mL⁻¹). The extract was incubated on ice for 15 min, followed by centrifugation at 16 000×g for 20 min at 4 °C. The supernatant was transferred to a new tube, centrifuged under the same conditions, and stored at –80 °C.

The interacting partners of *Phapa-4574965* were identified using Pierce™ NHS-Activated Magnetic Beads (ThermoFisher®, Waltham, MA, USA). Eluted proteins were resolved on 12% acrylamide/bis-acrylamide (37.5:1) tris-tricine SDS-PAGE mini gels (Mini-Protein III apparatus, Bio-Rad, Hercules, CA, USA) and visualized with Coomassie blue staining. Gel-separated proteins were excised, transferred to 1.5-mL microtubes, and stored at 4 °C.

In-gel digestion was performed according to the protocol of the Laboratório de Espectrometria de Massas (LNBio), Universidade Federal de Viçosa, Viçosa, Brazil [31]. High-resolution mass spectrometry analysis (Q-ToF MAXIS 3G, Bruker Daltonics, Bremen, Germany) was carried out at the Centro Analítico de Instrumentação – Central Analítica, Universidade de São Paulo, São Paulo, Brazil. The raw data were processed by the Núcleo de Análise de Biomoléculas, Universidade Federal de Viçosa, Viçosa, Brazil.

3. Results

3.1. Analysis of the *Phapa-4574965* sequence

The de novo_3326 transcript sequence was analyzed using BLASTx against the reference genomes of *P. pachyrhizi*. In the MT2006 v1.0 genome, the *Phapa-4574965* protein was identified with 99.6% identity and 100% coverage. This protein is encoded by a gene with a genomic length of 1361 nucleotides (including exons and introns), comprising five exons and four introns. The coding sequence encodes a 265-amino-acid protein with a predicted 13-amino-acid signal peptide, approximately 4.2% cysteine content, and an estimated molecular mass of ~30 kDa. An identical sequence was found in the K8108 genome v2.0 with the protein ID *PhapaK8108-3103294*. In the UFV02 v2.1 genome, the protein *PpafUFV02-1581871* was identified with 99.6% identity and 100% coverage. This protein is encoded by a gene with a genomic length of 1414 nucleotides (including exons and introns), comprising five exons and four introns. The corresponding coding sequence encodes a 285-amino-acid protein with a predicted 13-amino-acid signal peptide, a cysteine content of 4.2%, and an estimated molecular mass of approximately 32 kDa.

The predicted proteins in all three genomes lack the first 45 nucleotides, resulting in the loss of part of their signal peptides. In the Phapa-4574965 protein predicted from the MT2006 v1.0 genome, two synonymous nucleotide variations were detected in the 1st and 3rd exons, and one nonsynonymous variation in the 5th exon. This altered isoleucine in de_novo_3326 (278th amino acid) to asparagine in Phapa-4574965 (263rd amino acid) (Fig. 1A).

The PhapaK8108-3103294 protein, predicted in the K8108 v2.0 genome, and PpacUFV02-1581871 from the UFV02 v2.1 genome, showed only one synonymous substitution in the fifth exon. The PpacUFV02-1581871 protein had a longer sequence compared to the others, while PhapaK8108-3103294 exhibited the highest similarity to the de_novo_3326 transcript, which was initially predicted in the *Phakopsora-soybean* secretome by Carvalho et al. [21].

Sequence comparison revealed that the proteins encoded by Phapa-4574965, PpacPPUFV02-1581871 (UFV02 v2.1), and PhapaK8108-3103294 (K8108 v2.0) share more than 99% amino acid identity and over 99% nucleotide sequence similarity. Due to this high level of conservation, Phapa-4574965 was selected as the representative sequence for downstream structural prediction and functional analyses. The high sequence conservation among these proteins is shown in [Supplementary Figs. S1 and S2](#), which present the nucleotide and amino acid sequence alignments.

Additionally, the protein contains two domains: GAMMA-INTERFERON INDUCIBLE LYOSOMAL THIOL REDUCTASE (GILT) (PF03227) between amino acids 79-163, and interferon-induced GILT (IPR004911) spanning positions 66-258. In the protein PpacUFV02-1581871, the latter domain is positioned slightly differently, from amino acids 67-256. These domains are linked to oxidoreductase activity, specifically targeting sulfur groups with disulfide as acceptors (GO:0016671).

The three-dimensional structure of the effector was predicted using AlphaFold 2 - Colab (Fig. 1B). Based on AlphaFold's accuracy parameters, the structural model exhibits distinct features of confidence and evolutionary support across different regions of the protein. The regions with high sequence coverage (positions 50-230) show strong evolutionary support, indicating that these areas of the target sequence are well-represented in multiple sequence alignments (MSA). In contrast, the termini of the sequence display low coverage, which may suggest the presence of unstructured domains or regions with low evolutionary conservation. These low-coverage areas are likely associated with greater uncertainty in the structural prediction.

The Predicted Aligned Error (PAE) matrices (Fig. 1B) provide further insight into the reliability of the spatial relationship between residue pairs. Regions close to the diagonal exhibit low PAE values (blue), reflecting high confidence in local structural predictions. Conversely, areas farther from the diagonal (red) indicate uncertainty in the relative orientation of different domains. These patterns suggest that the protein is composed of well-defined domains interspersed with more flexible or independent regions, as observed in the highest-confidence model.

To assess the reliability of the structural prediction generated by AlphaFold2, the multiple sequence alignment (MSA) coverage and predicted confidence metrics for the Phapa-4574965 effector were analyzed ([Supplementary Fig. S3](#)). The sequence coverage plot (Fig. S3A) shows that the central portion of the protein, between amino acid positions 50 and 230, exhibits high coverage and sequence identity, indicating strong evolutionary support for this region. In contrast, both the N- and C-terminal regions display lower coverage, suggesting the presence of unstructured or variable domains.

The predicted pLDDT scores (Fig. S3B) provide a quantitative measure of confidence for each residue in the sequence. Central regions (positions 50-230) consistently exhibit pLDDT values above 70, indicating high confidence in these structural predictions. In contrast, terminal regions (positions 1-50 and 230-250) display pLDDT values below 50, which is consistent with characteristics of disordered or flexible regions. Overall, these results suggest that the structural

prediction is reliable for the central domains of the protein, while the sequence termini are more uncertain, potentially representing intrinsically disordered regions.

The combination of high sequence coverage, low PAE values, and elevated pLDDT scores in the central regions demonstrates strong consistency in the structural prediction, reinforcing confidence in the accuracy of the predicted structured domains. Notably, these stable core regions overlap with the predicted GILT domain, supporting its structural stability and functional relevance. This finding suggests that the GILT domain forms the structural core of Phapa-4574965, likely contributing to its redox activity and role in modulating host immune responses during infection.

3.2. Phapa-4574965 is similar to effector proteins found in other rust fungi

The Phapa-4574965 belongs to cluster 2,312, generated by MCLCluster [32]. The DNA sequence from various rust species in the cluster, including *P. pachyrhizi*, were used to generate a comparative phylogenetic tree (Figura 1C). These species include one from *Cronartium quercuum* f. sp. *fusiforme*, three from *Melampsora americana*, one from *Melampsora lini*, two from *Melampsora larici-populina*, three from *Melampsora medusae* f. sp. *deltoidae*, one from *Puccinia graminis* f. sp. *tritici*, one from *Puccinia striiformis* f. sp. *tritici* PST-130, two from *Puccinia striiformis* f. sp. *tritici* 104 E137, one from *Puccinia triticina* 1-1 BBBD Race 1, and one each from *Phakopsora pachyrhizi* MG2006, *Phakopsora pachyrhizi* PPUFV02, and *Phakopsora pachyrhizi* K8108.

Out of the 19 tree members, 13 contain the GILT domain, accounting for about 73.68% of the sequences. The members from different isolates of *P. pachyrhizi* grouped together into an independent subgroup, with the GILT domain identified in all members. This subgroup clustered closely with members from the Melampsoraceae family, particularly species from the *Melampsora* genus, which also carry the GILT domain.

3.3. Phapa-4574965 effector affects PTI and ETI responses

In response to environmental stress or pathogen attack, plants enhance callose accumulation as a defense mechanism [33]. Besides callose deposition, in plants, reactive oxygen species (ROS) are crucial for sensing abiotic and biotic stresses, integrating environmental signals, and activating stress-response pathways, thereby strengthening defense mechanisms and resilience [34]. To investigate the effects of the Phapa-4574965 effector, the *Pseudomonas fluorescens* Pf0-1 (EtHAN) strain carrying the Phapa-4574965 coding sequence in the pEDV6 plasmid was infiltrated into *N. benthamiana* leaves. Microscopy was used to assess callose deposition and ROS accumulation. The results showed that Phapa-4574965 exhibited significantly lower callose fluorescence compared to the positive control (EtHAN empty vector) ($p \leq 0.01$) (Fig. 2A). For ROS detection, tissue was stained with DAB, revealing reduced pigmentation in the effector-inoculated leaves compared to the positive control ($p \leq 0.01$) (Fig. 2B). Quantitative analysis showed that Phapa-4574965 led to more than a 50% reduction in ROS and callose deposition compared to the EtHAN empty vector. The negative control (10 mM $MgCl_2$) showed no DAB staining or callose fluorescence. These findings indicate that the Phapa-4574965 effector is capable of inhibiting plant defense responses, effectively undermining the plant's primary defense mechanism, known as Pattern-Triggered Immunity (PTI).

Regarding the suppression of defense responses associated with ETI, it was observed that the left side of the *N. benthamiana* leaf infiltrated with *Pst* DC300 carrying the empty pEDV6 vector exhibited hypersensitive response (HR), as expected. In contrast, the region infiltrated with the Phapa-4574965 effector did not show any signs of HR at any of the three tested bacterial dilutions (Fig. 2C).

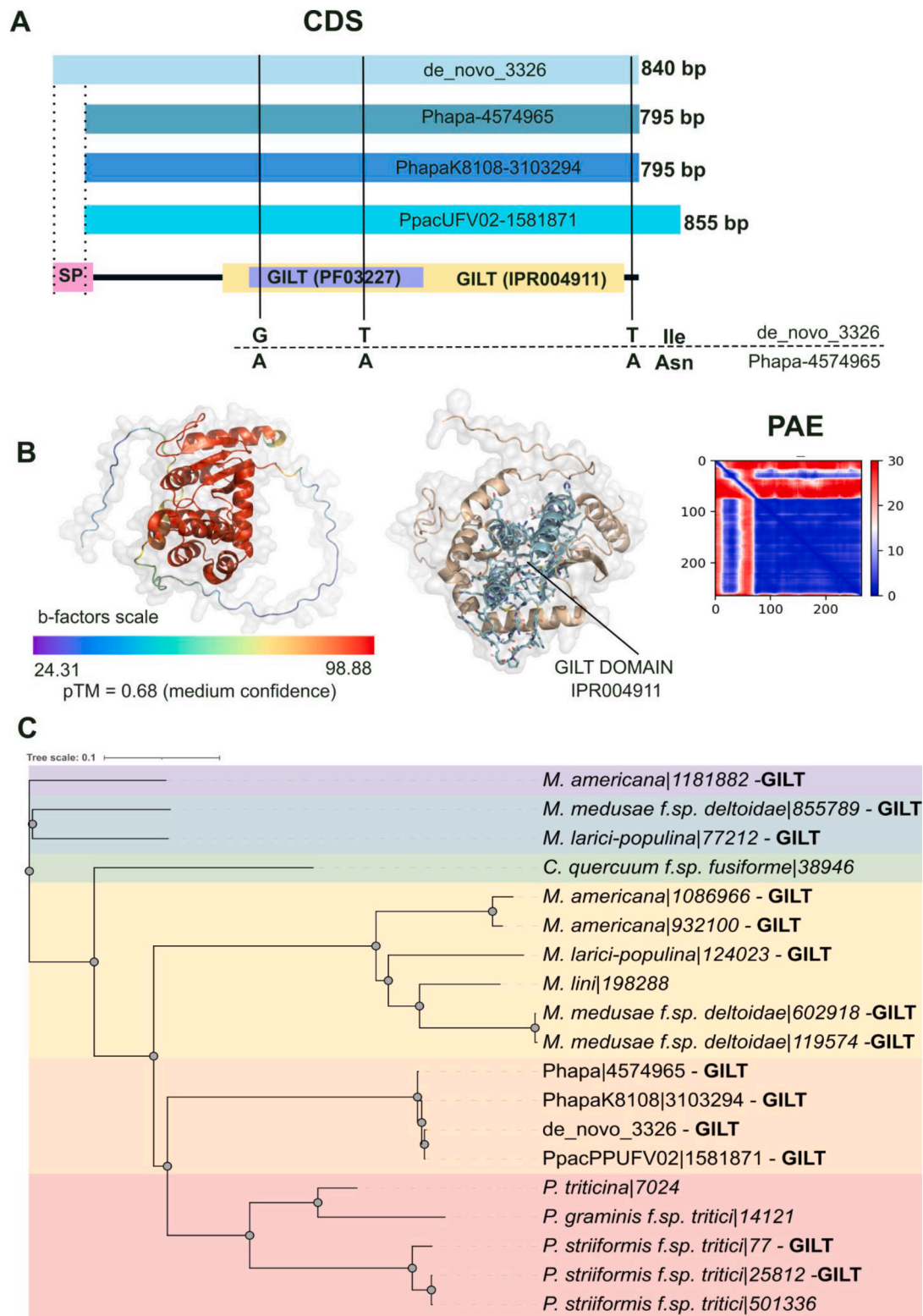


Fig. 1. Sequence analysis and structure of the Phapa-4574965 protein and phylogenetic comparison with other rust genera. **A:** Amino acid sequence motifs of the de_novo_3326, Phapa-4574965, PhapaK8108-3103294, and PpacUFV02-1581871 genes. The GILT domain (PF03227) and GILT (IPR004911) are indicated in lilac and yellow, respectively. SP: signal peptide. Two synonymous substitutions (G → A and T → A) and one non-synonymous substitution (T → A), resulting in the replacement of an Isoleucine (Ile) with an Asparagine (Asn) between the de_novo_3326 and Phapa 4574965 sequences. **B:** Structural modeling of Phapa-4574965 predicted using AlphaFold 2 - Colab and visualized with the PyMOL Molecular Graphics System. The B factor scale ranges from blue to red, with a predicted Template Modeling (pTM) score of 0.68, indicating medium confidence. The GILT domain is highlighted in blue. The PAE graph shows predicted alignment error, where red represents low confidence and blue represents high confidence in the relative positioning of residues. **C:** Phylogenetic tree among the de_novo_3326 sequence, the three corresponding sequences from the three Phakopsora genomes (MT2006, UFV02, and K8108), and other sequences from the 2312 cluster generated by MCLCluster, compared with other rust genera (JGI, 2024). Sequence alignment was performed using Clustal Omega in the Snapgene software, and the tree was generated using iTOL. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

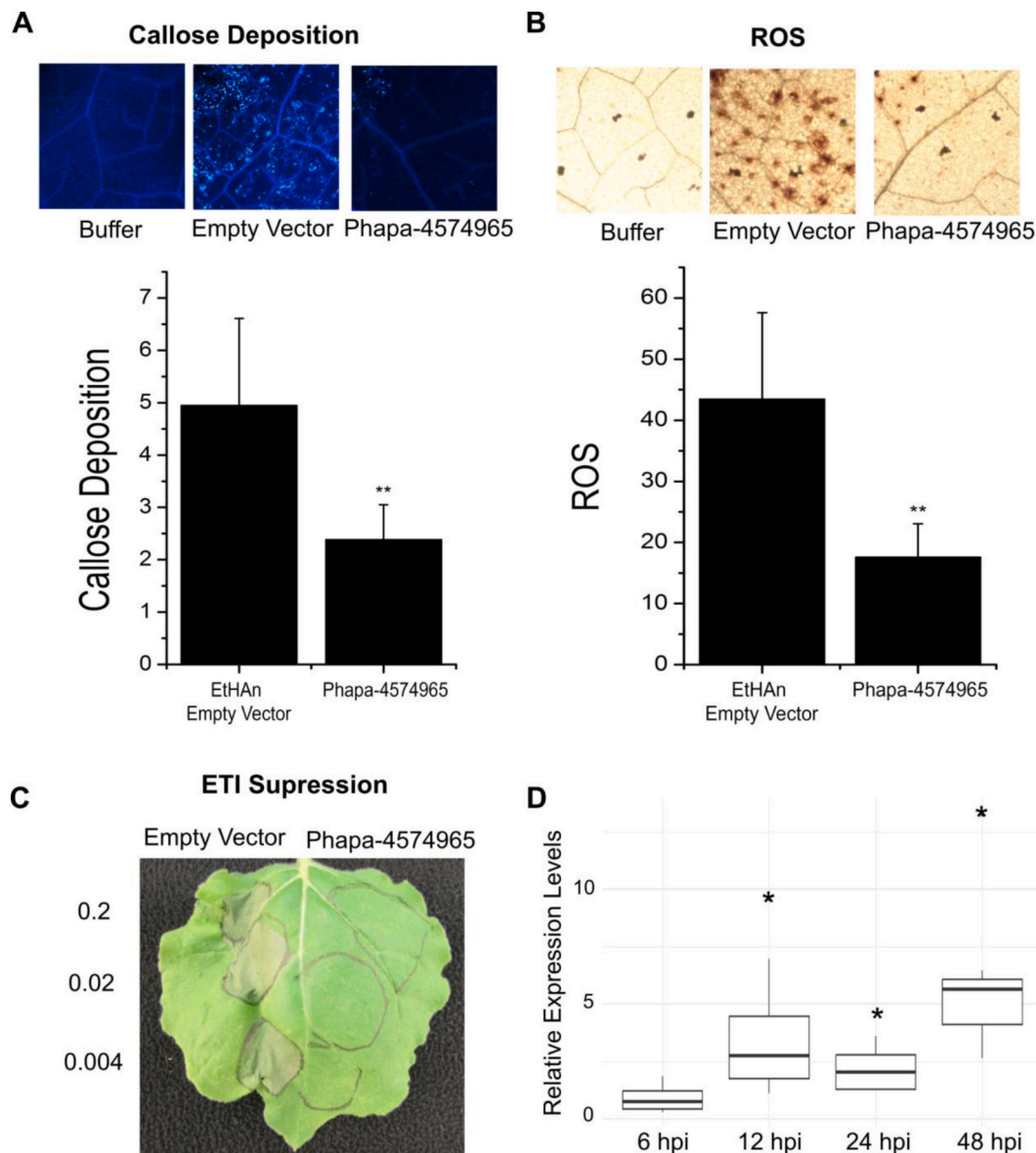


Fig. 2. Functional Analysis of Phapa-4574965. A. Quantification of aniline blue staining for callose deposition in *N. benthamiana* leaves infiltrated with negative control (10 mM MgCl₂), positive control (EtHAn carrying empty pEDV6), and EtHAn expressing Phapa-4574965 from pEDV6. Images were captured using a 5× objective. B. Quantification of DAB staining for ROS accumulation in *N. benthamiana* leaves. Comparisons were made between EtHAn carrying empty pEDV6 and EtHAn expressing Phapa-4574965. Images were captured with a 10× objective. Significant differences ($p \leq 0.01$) are marked by two asterisks (**), as determined by the *t*-test. C. *N. benthamiana* leaves infiltrated with positive control (*Pst* DC300 carrying empty pEDV6) (left) and *Pst* DC300 expressing Phapa-4574965 from pEDV6 (right) at three different concentrations (OD₆₀₀ = 0.2, 0.02, 0.004). D. Relative expression of *Phapa-4574965* mRNA transcripts during the infection cycle (6, 12, 24, and 48 h post inoculation) in the *P. pachyrhizi* LPF16.1M isolate in a susceptible soybean genotype (BRS184 cultivar). The *CytB* gene was used as an endogenous control, and 0 hpi expression was used as the calibrator. Gene expression levels are relative to the 0 hpi expression. *Values significantly different from the control ($p < 0.01$), according to the Randomization Test of Fixed Pairwise Reallocation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. *Phapa-4574965* gene is induced during the infection in soybean leaves

Expression of *Phapa-4574965* is upregulated during infection in soybean leaves, suggesting it plays a crucial role in the pathogen's ability to infect and manipulate the host. It was observed that the mRNA transcripts of *Phapa-4574965* increases up to 12 h post-inoculation (hpi), followed by a decline at 24 hpi, and then reaches its peak expression at 48 hpi, indicating its active involvement in the early stages of infection (Fig. 2D). The increased expression of *Phapa-4574965* during infection highlights its potential as a key factor in the virulence of the pathogen.

3.5. The *Phapa-4574965* effector demonstrates subcellular localization primarily in the nucleus of *Nicotiana benthamiana* cells

To facilitate subcellular localization of *Phapa-4574965*, it was fused to GFP and mCherry fused to marker proteins for different organelles. The *Phapa-4574965*-GFP construct was transiently co-expressed with each marker protein fused to mCherry in *N. benthamiana* leaves. Following transient co-expression, confocal microscopy images were obtained and analyzed to identify the subcellular localization of the effector.

In all transient co-expression experiments, *Phapa-4574965* was strongly detected in the nucleus and partially in the cytosol of

N. benthamiana cells (Fig. 3). Specifically, Phapa-4574965 was prominently localized in the nucleoplasm (Fig. 3C). Among all tested combinations, subcellular co-localization was observed between Phapa-4574965 and the nuclear protein, confirming its nuclear localization in plant cells (Fig. 3C), as well as co-localization with a mitochondrial protein (Fig. 3B). Leaf disks infiltrated with the infiltration solution showed no fluorescence, while those infiltrated with the empty pSITE vector exhibited intense GFP fluorescence in the cytoplasm and nucleus, as expected in tissues expressing the free eGFP.

3.6. Identification of soybean proteins that potentially interact with Phapa-4574965

To identify interaction partners of the Phapa-4574965 effector, an *in vitro* protein-protein interaction assay was performed using a pull-down method. The Phapa-4574965 protein plus an N-terminal histidine tag was expressed from the pET30a vector in *E. coli*. After purification, the His-Phapa-4574965 protein was incubated with Pierce™ NHS-Activated Magnetic Beads to allow covalent coupling of the protein to the beads. The beads were then blocked and subsequently incubated with soybean leaf protein extracts, followed by pull-down assays. Four soybean leaf extracts were used: one from a susceptible genotype (cultivar BRS184) and one from a resistant genotype (Rpp5), each including inoculated and non-inoculated samples. A negative control with PBS 1X buffer was employed during the effector-bead binding step to eliminate soybean proteins that non-specifically bound to the beads. The recovered proteins were run on a polyacrylamide gel to verify the presence of the effector, and the samples were digested with trypsin for mass spectrometry analysis. A total of eight soybean proteins were identified as potential interaction partners (Table 1). The selection criterion for these

proteins was based on their absence in the negative control (PBS). Only those proteins detected in samples from the Phapa4574965 pull-down assay with extracts from resistant (*Rpp5*) and/or susceptible soybean leaves, both inoculated with *P. pachyrhizi*, were considered for further analysis.

The identified proteins were annotated as lipoxygenase, alanine transaminase, NAD-dependent epimerase/dehydratase domain-containing protein, CBBY-like protein, aminomethyltransferase, fructose-bisphosphatase, cysteine proteinase, and linoleate 9S-lipoxygenase-4. Among these, lipoxygenase (LOX) is a dioxygenase enzyme that catalyzes the addition of oxygen to polyunsaturated fatty acids, such as linoleic and linolenic acids, producing hydroperoxides. In plants, LOX isoforms are expressed in different tissues and phases and are classified into 9-LOX (e.g., linoleate 9S-lipoxygenase-4) and 13-LOX, based on their positional specificity [35,36].

Alanine aminotransferase (AAT) is a pyridoxal phosphate-dependent enzyme that facilitates the reversible transfer of amino groups, contributing to nitrogen-use efficiency and enhancing stress tolerance under low nitrogen conditions [37,38]. NAD-dependent epimerase/dehydratase (NAD) plays a crucial role in carbohydrate metabolism and cell wall biosynthesis, particularly in the formation of extracellular polysaccharides and rRNA processing [39–41]. Together, these enzymes are integral to maintaining essential metabolic processes in plants, especially under stress conditions.

CbbY is a phosphatase from the Calvin-Benson-Bassham (CBB) cycle, conserved in plants and photosynthetic organisms. It converts XuBP, an inhibitor of RuBisCO, into Xu5P, which is then recycled into RuBP, thereby enhancing RuBisCO function [42,43]. Similarly, aminomethyltransferase (AMT), a mitochondrial protein essential for photorespiration, plays a key role in the glycine cleavage system, which

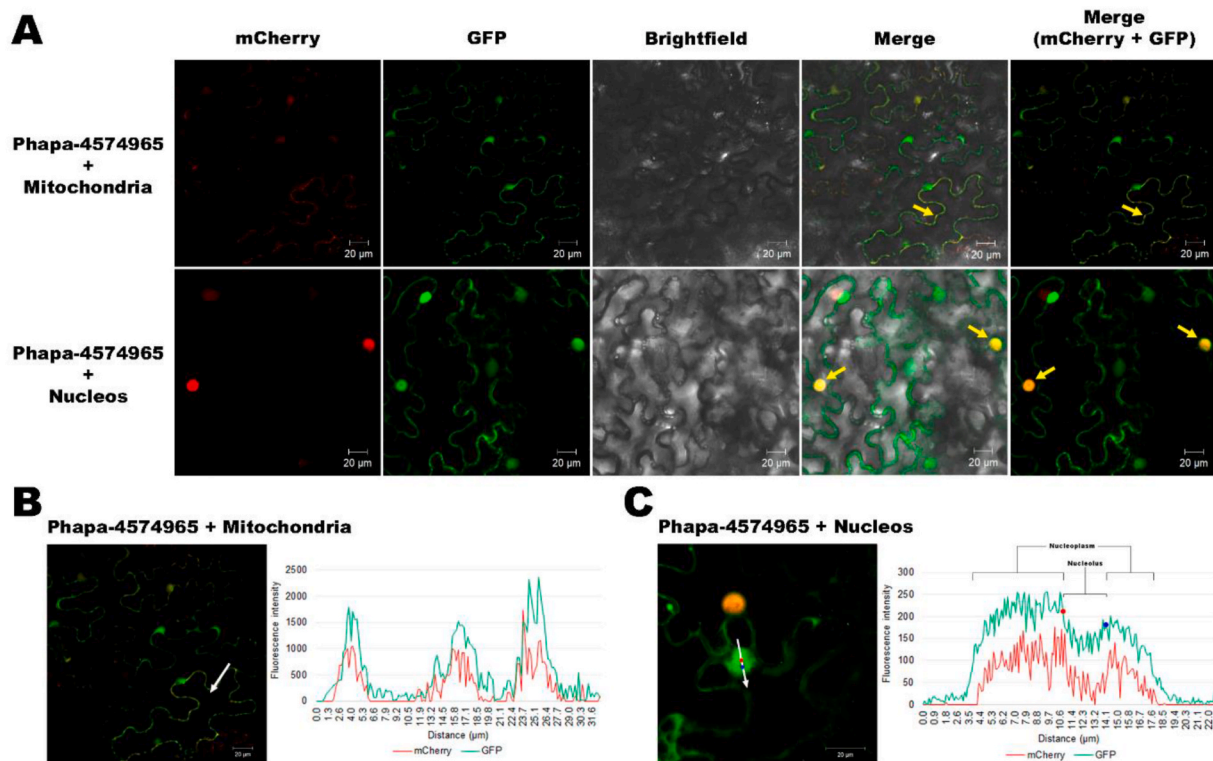


Fig. 3. Subcellular localization of Phapa-4574965. Confocal images of *N. benthamiana* leaves transiently expressing GFP-fusion of Phapa-4574965 and mCherry fusion of specific organelle proteins. **A.** The co-localization between Phapa-4574965 and mitochondrial or nuclear protein is indicated by yellow arrows. **B, C.** Plots of the profile (right) indicated by white arrow in the confocal image (left) show mCherry (red line) and GFP (green line) fluorescence. **C.** There is less fluorescence in the nucleolus (delimited by the red and blue points in the graph) than the nucleoplasm. The mCherry was excited at 543 nm, and GFP and chlorophyll (magenta) fluorescence were collected at 580–620 nm, 505–525 nm and 680–700 nm, respectively. Confocal images were acquired using a 20 × objective for panels A and B and a 40 × objective for panel C. Scale bar: 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1

Characteristics of soybean proteins identified as potential interactors with Phapa-4574965 by pull-down assay and mass spectrometry.

Phapa-4574965 + BRS Inoc ^{a,c}	Phapa-4574965 + Rpp5 Inoc ^{b,c}	Protein name	Size (amino acids)	Gene Ontology (Cellular component)
A0A0R0G554 A0A0R0G554_SOYBN	A0A0R0G554 A0A0R0G554_SOYBN	Lipoxygenase (EC 1.13.11.-)	853	cytoplasm [GO:0005737]
I1JR38 I1JR38_SOYBN	A8IKE9 A8IKE9_SOYBN I1JR38 I1JR38_SOYBN	Alanine transaminase (EC 2.6.1.2)	480	
I1KC82 I1KC82_SOYBN	I1KC82 I1KC82_SOYBN	NAD-dependent epimerase/dehydratase domain-containing protein	378	cytosol [GO:0005829]
	I1MR41 I1MR41_SOYBN	CBBY-like protein	335	
	I1N2R6 I1N2R6_SOYBN	Aminomethyltransferase (EC 2.1.2.10) (Glycine cleavage system T protein)	407	mitochondrion [GO:0005739]
		Fructose-bisphosphatase (EC 3.1.3.11) (D-fructose-1,6-bisphosphate 1-phosphohydrolase)	410	cytoplasm [GO:0005737]; cytosol [GO:0005829]
K7MNM4 K7MNM4_SOYBN	K7MNM4 K7MNM4_SOYBN	Cysteine proteinase	446	
	P38417 LOX4_SOYBN	Linoleate 9S-lipoxygenase-4 (EC 1.13.11.58) (Lipoxygenase-4) (L-4) (VSP94)	853	cytoplasm [GO:0005737]

^a Interaction between Phapa-4574965 and protein extract from BRS 184 soybean (susceptible) inoculated with *P. pachyrhizi*.

^b Interaction between Phapa-4574965 and protein extract from Rpp5 soybean (resistant) inoculated with *P. pachyrhizi*.

^c Protein Access on UniProtKB (<https://www.uniprot.org>).

oxidizes glycine and produces ammonia [44,45]. Additionally, fructose-bisphosphatase (FBP) is crucial in the Calvin Cycle, converting fructose-1,6-bisphosphate into fructose-6-phosphate [46]. Finally, cysteine proteinase (CP) cleaves peptide bonds and is vital in plant immune responses, detecting pathogens and triggering cell death by degrading pathogenic effectors [47,48].

4. Discussion

The plant-pathogen interaction involves a continuous co-evolutionary process in which the pathogen develops mechanisms to infect, while the plant enhances its defense responses [49,50]. Effector proteins, which act as pathogenicity factors, play a crucial role in this process, becoming important targets in the development of strategies to control biotic stress in agricultural crops [51,52]. Researching these proteins provides a better understanding of the biological mechanisms involved in infection and plant response.

The availability of *P. pachyrhizi* genomes from JGI has enabled the identification and characterization of effectors, such as Phapa-4574965, with potential influence on pathogen virulence and adaptation. Approximately 93% of the *P. pachyrhizi* genome is composed of transposable elements, suggesting that this high genetic variability may be a strategy for the pathogen to adapt to different hosts and environmental conditions [22]. Despite the advances in genomic data, the *Glycine max*-*P. pachyrhizi* pathosystem remains highly complex, mainly due to the difficulty in manipulating this phytopathogen in the laboratory [53]. As a result, researching the molecular mechanisms, particularly the effectors that drive infection and adaptation, is crucial for understanding this disease. Recent investigations have begun to shed light on *P. pachyrhizi* effectors [23–26,53], emphasizing the importance of further investigation to develop more effective control strategies for Asian soybean rust.

Phapa-4574965 shares common structural characteristics with other fungal effectors, such as small size, high cysteine content (4.2%), and the presence of a signal peptide—features typical of secreted proteins that manipulate host immunity [21]. In addition, Phapa-4574965 contains a GILT domain (PF03227), an oxidoreductase-related domain that acts on sulfur-containing substrates with disulfide bonds as acceptors (GO:0016671). Notably, proteins carrying this domain were also identified in homologous gene models from several rust fungi in the JGI MycoCosm database, including species of *Melampsora*, *Puccinia*, and *Cronartium*, indicating that GILT-like sequences commonly occur in a subset of rust effectors. Although its functional role in fungal pathogenesis remains unclear, the recurrent presence of this domain across rust genomes suggests that it may contribute to conserved mechanisms linked to redox modulation during host interaction.

The GILT domain, extensively studied in the animal kingdom but rarely described in plant and fungal organisms, may be related to the immune system, participating in the denaturation, renaturation, and reduction of disulfide bonds. In humans, GILT is part of a group of redox factors that facilitate pathogen entry through endocytic routes, promoting plasma membrane invagination [54]. Moreover, the GILT domain is linked to the maintenance of reactive oxygen species (ROS) homeostasis and the expression of Superoxide Dismutase 2 (SOD2) [55–58]. In *Populus trichocarpa*, the gene PtOSH1, with structural motifs similar to human GILT, was identified. Under prolonged cadmium (Cd) stress, it promoted disulfide bond reduction, helping repair antioxidant enzymes and regulate ROS levels, thus maintaining the stability of plant cells [58]. Therefore, it is possible that through its GILT domain, the effector Phapa-4574965 manipulates the host, especially during critical moments of pathogen penetration and development within plant tissues. Further functional analyses, such as domain-specific mutagenesis or deletion of the GILT domain, will be important to determine its precise role in the suppression of host immune responses.

This role is further supported by the significant expression of the Phapa-4574965 gene within the first 48 h post-inoculation (hpi) in soybean leaves, reaching its peak expression during this period. This pattern suggests that the effector plays a crucial role in the early stages of infection, especially when the fungus penetrates the host plant cells. This behavior aligns with what has been observed in other rust pathogen effectors, which also show high expression during the critical stages of pathogen penetration and establishment [21].

During the early infection process of *P. pachyrhizi*, fungal structures involved in host penetration develop sequentially. The germ tube and appressorial structures form during the initial hours after inoculation, enabling the pathogen to breach the epidermis. Haustorium formation—responsible for nutrient uptake—occurs between 24 and 48 hpi within the mesophyll cells [59]. The two expression peaks observed at 12 and 48 hpi correspond to these critical infection stages: epidermal penetration and subsequent intracellular development.

These data suggest that the Phapa-4574965 gene is activated during key phases of plant tissue invasion, playing an essential role in the infection process. The movement of pathogenic effectors within host cells allows them to interact with targets in different cellular compartments, influencing multiple defense pathways. Nuclear-localized effectors, known as nuclear effectors, reprogram plant defense genes, while others act in organelles such as mitochondria, affecting stress signaling pathways [50,51,60].

The subcellular localization of Phapa-4574965, predominantly in the nucleus and partially in the cytoplasm of *N. benthamiana* cells supports the idea that this effector can modulate the transcription of plant defense genes. This is consistent with other studies on nuclear effectors that

directly regulate host gene expression [26,61]. The additional presence of Phapa-4574965 in the mitochondria suggests potential interference with stress signaling pathways, given the central role of mitochondria in the generation of ROS and in the plant immune response [62,63]. Thus, the expression of the Phapa-4574965 gene during critical infection stages and its subcellular localization suggest multifunctional roles in manipulating plant cells.

Research with other pathogens, such as *Magnaporthe oryzae* and *Melampsora larici-populina*, have shown that effectors localized in the nucleus or organelles can significantly impact plant defense by modulating key immune pathways [62]. In *Melampsora larici-populina*, 19 effector candidates were identified in both the cytoplasm and nucleus. The effector MLP124017 interacted with the TOPLESS protein, a co-repressor involved in plant defense processes, while MLP107772 was localized to the chloroplast and mitochondria, interacting with the CTP1 (Chloroplast-Targeted Protein 1) protein [64–66]. In *Puccinia striiformis* f. sp. *tritici*, it was reported that the effector PEC6, located in both the cytoplasm and nucleus, interacts with adenosine kinase (TaADK) in wheat (*Triticum aestivum*) cells [67]. Additionally, the PstGSRE1 effector was described as being present in both the cytoplasm and nucleus, and it also interacts with the transcription factor TaL2, inhibiting its translocation to the nucleus and preventing its action in ROS-mediated hypersensitive response (HR) [61].

Recent investigations also demonstrated that the effector PstSIE1 interacts with TaSGT1, a cofactor of Nucleotide-Binding Leucine-Rich Repeat (NB-LRR) proteins involved in effector recognition and activation of effector-triggered immunity, in both the cytoplasm and nucleus of wheat cells [68]. In *Puccinia graminis* f. sp. *tritici*, the AvrSr50 effector, present in both the cytoplasm and nucleus, is recognized by the NB-LRR protein Sr50 in the cytosol was identified, triggering effector-triggered immunity [69]. Another example is that 18 effector candidates from *Uromyces appendiculatus* were localized in both the cytoplasm and nucleus of *N. benthamiana* cells [70]. Similarly, several *P. pachyrhizi* effector candidates were found in both the cytoplasm and nucleus of plant cells [21,23]. The effector PpEC15 from *P. pachyrhizi* was strongly localized in the nucleus and of *N. benthamiana* cells, but it is also present in cytoplasm and chloroplasts where it interacts with DAHP synthase (3-deoxy-7-phosphoheptulonate synthase) [26].

Thus, the diverse subcellular localization of effectors in rust pathogens, such as PstSIE1, AvrSr50, and PpEC15, which operate in both the nucleus and cytoplasm, suggests a common functional pattern among these effectors, allowing them to regulate different plant defense pathways across multiple cellular compartments. Similarly, Phapa-4574965 shows predominant localization in the nucleus, but is also present in the cytoplasm and mitochondria, indicating possible functions in various compartments. This diverse localization suggests that Phapa-4574965, like other effectors, utilizes its multifunctionality to suppress plant defenses and facilitate infection.

This ability to suppress plant defenses was further supported by experiments demonstrating that Phapa-4574965 inhibits basal defense responses, such as callose deposition and the accumulation of ROS, key components of PTI. The inhibition of these responses indicates that the effector efficiently can interfere with early plant immune responses [34]. Additionally, Phapa-4574965 was able to suppress the hypersensitive response (HR) in *N. benthamiana*, preventing necrosis induced by *P. syringae*, suggesting that the effector directly interferes with pathways responsible for activating effector-triggered immunity in this heterologous system. Together, these findings support the idea that Phapa-4574965 functions as an effector capable of suppressing plant immune responses, although its specific role during soybean–*Phakopsora pachyrhizi* interactions remains to be further investigated.

An initial protein-protein interaction investigation using pull-down revealed eight potential soybean proteins that interact with Phapa-4574965. These proteins play important roles in essential metabolic processes, such as fatty acid metabolism, nitrogen fixation, and photosynthesis regulation. This suggests that the effector may manipulate

various metabolic pathways in the plant to facilitate infection [35,43]. The interaction with lipoxygenase (LOX), a key enzyme in fatty acid metabolism, is particularly relevant as this process is closely linked to plant defense responses.

LOX participates in the oxidation of polyunsaturated fatty acids, initiating the synthesis of oxylipins, signaling compounds such as jasmonic acid, which play a role in plant immunity against biotic and abiotic stresses [35,71]. Additionally, 9-LOX pathways are involved in hypersensitive responses (HR), promoting the production of hydroperoxides of free fatty acids and being stimulated by abscisic acid, which is related to stomatal closure during plant defense responses. The involvement of LOX has been observed in various pathosystems, including interactions of plants with both bacterial and fungal pathogens [35,36].

Among the identified proteins, cysteine protease (CP) stands out as another crucial enzyme involved in protein cleavage and immune system function, particularly as PLCP (papain-like cysteine protease). Silencing of PLCP in *N. benthamiana* increased susceptibility to infection by *Phytophthora infestans* [72], while its reduction in tomatoes resulted in heightened sensitivity to fungi, bacteria, and oomycetes, highlighting its role in basal defense against various pathogens [73]. In the wheat–*Puccinia striiformis* f. sp. *tritici* pathosystem, the interaction of the protein TaCP1 with TaTrxh1 promotes disulfide bond reduction, regulating cell death and defense via salicylic acid signaling [74]. This co-evolution of effectors with plant defense mechanisms has turned PLCPs into targets for pathogenic effectors, as seen in maize, where the apoplastic effector Pit2 inhibits apoplastic PLCPs, promoting infection by *Ustilago maydis*. Other pathogens, like *Phytophthora*, possess protease inhibitors such as EPICs, which interact with PLCPs like Rcr3 and PIP1 to suppress plant defense [72].

The remaining proteins identified in the investigation are linked to energy metabolism, such as photorespiration and carbohydrate biosynthesis. The concept of “high-sugar resistance” explains how increased sugar concentration can stimulate plant immunity by inducing the expression of defense genes and promoting resistance to fungal pathogens [75,76]. Sugars such as glucose and fructose, which result from sucrose degradation, also function as signaling molecules in plant defense [46], suggesting a potential functional role for the interaction with Phapa-4574965 in carbohydrate biosynthesis.

Among other proteins, alanine aminotransferase (AAT) has its expression regulated by salicylic acid and ethylene during the plant resistance response [77], while fructose-bisphosphatase (FBP) plays a role in lignin and polysaccharide biosynthesis, which are essential for reinforcing the plant's structural defenses against pathogens [78]. Aminomethyltransferase (AMT), involved in glycine metabolism, is also associated with ROS control and plant defense [79,80], suggesting a potential role in redox balance during infection.

The association of the proteins identified through the pull-down assay with gene expression data and the subcellular localization of the Phapa-4574965 effector reveals a coordinated strategy by the pathogen to manipulate the plant's defenses. The expression peaks of the Phapa-4574965 gene observed within the first 48 h post-inoculation coincide with critical infection stages, such as epidermal rupture and intracellular penetration. This expression profile suggests that the effector is active during the early stages of infection, a period in which the identified proteins, such as those involved in energy metabolism (AAT and FBP), may play crucial roles in pathogen establishment. The interaction with proteins like lipoxygenase (LOX), which participates in oxylipin synthesis and defense responses through ROS regulation, indicates that Phapa-4574965 is manipulating metabolic and defense signaling pathways in the plant to facilitate infection.

The diverse subcellular localization of Phapa-4574965, predominantly in the nucleus but also present in the cytoplasm and mitochondria, suggests that the effector manipulates processes in different cellular compartments. In the nucleus, Phapa-4574965 may be reprogramming defense-related genes, which aligns with its interaction with

proteins involved in the biosynthesis of defense compounds, such as FBP, which participates in lignin synthesis. In the cytoplasm, its interaction with LOX, an enzyme involved in oxylipin production and ROS signaling, indicates that the effector interferes with defense responses associated with HR, reducing the effectiveness of the plant's initial immune response. The effector's presence in the mitochondria suggests potential interference with redox pathways and stress signaling, with aminomethyltransferase (AMT) being a key protein involved in ROS regulation, further supporting Phapa-4574965's role in modulating redox balance during infection.

These findings indicate that Phapa-4574965 employs a multifunctional approach to interfere with plant defenses, both at the nuclear level, where it reprograms gene expression, and at the cytoplasmic and mitochondrial levels, where it affects metabolism and stress responses. Its interaction with proteins related to sugar biosynthesis and ROS regulation suggests that the pathogen is manipulating metabolic pathways to meet its energy needs while simultaneously suppressing critical defense responses. Thus, the diverse subcellular localization and coordinated expression of Phapa-4574965 at crucial infection stages reinforce the idea that it plays a central role in the virulence of *P. pachyrhizi*.

5. Conclusions

In summary, the effector Phapa-4574965 from *P. pachyrhizi* demonstrates a multifunctional capacity to interfere with plant defense mechanisms by acting in various cellular compartments, including the nucleus, cytoplasm, and mitochondria. Its ability to suppress both pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) underscores its role as a critical virulence factor. The identification of proteins interacting with Phapa-4574965, including those involved in metabolic and redox processes, suggests that the pathogen manipulates multiple host pathways to facilitate infection. These insights into the molecular function of Phapa-4574965 provide valuable knowledge for understanding the complexity of the *Glycine max*-*P. pachyrhizi* pathosystem. Moving forward, targeting the interactions between Phapa-4574965 and host proteins could serve as a basis for developing new strategies to enhance soybean resistance to Asian soybean rust, contributing to more sustainable disease management and crop protection practices. Further research should focus on validating these interactions *in planta* and exploring their potential in breeding programs and biotechnological approaches.

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CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pmpp.2026.103271>.

Data availability

All data is available in the article.

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