

The apoplastic effector MrB3 from *Moniliophthora roreri* modulates plant defense and interacts with an LRR protein in cacao

Larissa Karen Silva Oliveira

Universidade Estadual de Santa Cruz (UESC)

Taís Araújo Santos

Universidade Estadual de Santa Cruz (UESC)

Maria Eduarda Antunes Lima

Universidade Estadual de Santa Cruz (UESC)

Hermann Vanesca Viana Oliveira

Universidade Estadual de Santa Cruz (UESC)

Carlos Henrique Carvalho

Universidade Estadual de Santa Cruz (UESC)

Keilane Silva Farias

Universidade Estadual de Santa Cruz (UESC)

Janclei Pereira Coutinho

Universidade Estadual de Santa Cruz (UESC)

Raner José Santana Silva

Universidade Estadual de Santa Cruz (UESC)

Éric Roberto Guimarães Rocha Aguiar

Universidade Estadual de Santa Cruz (UESC)

Carlos Priminho Pirovani

`pirovani@uesc.br`

Universidade Estadual de Santa Cruz (UESC)

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Abstract

Moniliophthora roreri is a hemibiotrophic fungus and the causal agent of cacao moniliasis, a major disease affecting cacao production in the Americas. Here, we functionally characterized MrBASIDIN3 (MrB3), a novel candidate effector identified from transcripts differentially expressed in *M. roreri* spores incubated in cacao extract. MrB3 encodes a 256-amino-acid protein with a predicted signal peptide, no transmembrane regions, and features consistent with an apoplastic effector. The protein lacks known conserved domains, and structural analyses predicted a predominantly α -helical structure. Recombinant rMrB3 was heterologously expressed in *Escherichia coli*, purified, and validated by mass spectrometry. The recombinant protein exhibited high thermal stability and induced necrosis and wilting in *Solanum lycopersicum* leaves, accompanied by increased H₂O₂ accumulation. rMrB3 also elicited differential responses among the *Theobroma cacao* genotypes. Affinity capture followed by LC–MS/MS identified an LRR-containing protein from *T. cacao* (TcLRR) as a potential host-associated protein. Molecular docking supported a structurally favorable association between MrB3 and TcLRR. Collectively, these results characterize MrB3 as a novel candidate effector from *M. roreri* with biological activity and features consistent with a secreted effector. The identification of TcLRR as a potential host molecular target provides a basis for investigating the molecular mechanisms underlying *M. roreri*–*T. cacao* interaction and the role of effector-mediated modulation of plant defense.

1 Introduction

The fungus *Moniliophthora roreri* [90], which belongs to the family Marasmiaceae, is the causal agent of frosty pod rot, a devastating disease that affects the fruits of *Theobroma cacao* and *Herrania*, which are crops of major economic importance in several tropical regions. As one of the major threats to cocoa production, frosty pod rot can cause yield losses of up to 100%, as observed during disease outbreaks over time in cocoa-producing areas of Latin America [24, 31, 74, 40].

The earliest historical records associated with frosty pod rot date back to 1817 in northeastern Colombia, particularly in the region corresponding to the present-day department of Norte de Santander [93]. Subsequent genetic evidence identified the upper Magdalena River Valley in Colombia as a probable center of origin and the region with the greatest diversity of *Moniliophthora roreri*. From this region, the pathogen spread to other countries in the Americas, with records reported in Mexico in 2005 and Belize in 2006 [91, 92], Bolivia in 2012 [94], and Jamaica in 2016 [50]. In Brazil, outbreaks have been reported in the states of Acre [10] and Amazonas [11], leading to the implementation of official emergency measures and phytosanitary quarantine due to the risk of pathogen dissemination to other cocoa-producing areas. The geographical expansion of the disease and its impact on cocoa production highlight the need to understand the molecular mechanisms underlying the pathogenicity of *M. roreri*, thereby contributing to the development of disease management and control strategies.

One focus of these approaches is the study of effector proteins, which are molecules secreted by pathogens that modulate host plant physiology, facilitating infection by suppressing or circumventing

plant defense mechanisms [55]. Transcriptomic studies have revealed that genes encoding secreted proteins exhibit distinct expression profiles during the biotrophic and necrotrophic stages of infection, indicating that the fungus adapts its virulence strategy according to the disease stage [75]. During the biotrophic stage, these proteins appear to facilitate the evasion of plant defenses, whereas during the necrotrophic stage, they are associated with the active degradation of plant tissues, promoting necrosis [32]. These findings help elucidate the mechanisms underlying pathogenicity and identify promising targets for disease control.

Effectors constitute a diverse group of molecules that confer adaptive advantages to the organisms that produce them, enabling them to colonize their hosts and complete their life cycles. These molecules are traditionally classified as virulence factors, which promote infection, or avirulence factors, which activate host defense responses [55, 56, 44]. Initially, described in plant–pathogen interactions involving biotrophic fungi, effectors are now widely recognized in hemibiotrophic and necrotrophic fungi [39, 109, 137]. Biotrophic fungi, for example, depend on living hosts and use effectors to evade or suppress plant defense responses and remain undetected [62, 135, 116]. Necrotrophic fungi, in turn, use toxins and plant cell wall-degrading enzymes to kill plant tissues, but they also employ effectors to manipulate the host environment [60, 110, 102].

Several omics approaches have contributed to elucidating the pathogenicity mechanisms of *Moniliophthora roreri*. Meinhardt et al. [75] sequenced the genome of this species, identifying approximately 17,000 genes and characterizing the secretome associated with the biotrophic and necrotrophic stages of infection. Barbosa et al. [7] predicted a broad repertoire of effector proteins and cell wall-degrading enzymes in *Moniliophthora* isolates, highlighting pathogenic specialization and coevolution with their hosts. Reyes et al. [99] identified a high diversity of fungi and bacteria associated with *M. roreri* cultures, particularly members of the genus *Trichoderma*, and detected viral sequences belonging to the family *Narnaviridae*. Zugaib et al. [138] characterized the proteome of *M. roreri* at different developmental stages, revealing proteins associated with metabolism, redox processes, synthesis and folding, and spore germination and infection. More recently, Nascimento et al. [80] performed a comprehensive transcriptional analysis of mycelia and spores grown in media supplemented with *Theobroma cacao* extract, identifying genes with marked differential expression, potential developmental markers, and effector candidates involved in the pathogen–host interaction.

In this study, we identified and characterized a novel effector protein, *MrB3*, which is cysteine rich and was identified from the mRNA_11825 transcript detected in *M. roreri* spores incubated in cocoa extract [80]. A synthetic ORF was cloned and inserted into pET-32a (Symbio Technologies, Cayman) and expressed as a fusion protein with vector-derived thioredoxin. The recombinant protein induced necrosis in the leaves of a model plant, suggesting a potential role during the necrotrophic stage of infection. Structural analysis of the protein revealed a predominance of alpha-helices, which is consistent with characteristics common in effector proteins. Our results identify a novel basidiomycete-specific effector with the potential to modulate host defense mechanisms and contribute to pathogen virulence during frosty pod rot of cocoa.

2 Results

2.1 *In silico* identification and structural characterization of the candidate effector protein *MrB3*

Analysis of the differentially expressed transcripts obtained after 16 h of incubation of *Moniliophthora roreri* spores in media supplemented with cacao fruit extract [80] revealed 59 differentially expressed transcripts (Fig. S1). Following translation of the nucleotide sequences via BLASTx against the NCBI nonredundant (nr) database, 51 transcripts matched proteins from *M. roreri*, seven showed no significant similarity, and one matched a protein from another species. Among the proteins from *M. roreri*, 25 were characterized, and 26 were uncharacterized (Fig. S1). The application of the selection criteria described in Section 2.1 resulted in the identification of eight candidate effector proteins (Fig. S1). Among these, V2YZA0 (UniProt)/ESK97039.1 (NCBI), corresponding to transcript mRNA_11825, was selected for further characterization and subsequently designated *MrBASIDIN3* (*MrB3*).

The *MrB3* protein comprises 256 amino acid residues and has an estimated molecular mass of 26 kDa and a theoretical isoelectric point (pI) of 5.10. An N-terminal signal peptide was predicted with an 83% probability, with a putative cleavage site between residues 19 and 20 (ASA-QL) (Fig. 1A and Fig. S2). Following *in silico* removal of the signal peptide, the mature protein had an estimated molecular mass of 24.23 kDa. Analysis via the NCBI Conserved Domain Database (CDD) did not identify any conserved domains, whereas InterProScan identified a region belonging to the SPARK domain-containing protein family, spanning residues 17–225. No transmembrane regions were predicted via the TMHMM server (Fig. S3).

EffectorP-Fungi 3.0 classified *MrB3* as a candidate apoplastic effector, while DeepLoc 2.0 predicted its subcellular localization to be extracellular (Fig. S1). Analysis via IUPred3 identified an intrinsically disordered region spanning residues Gly196–Asn202, with disorder scores above 0.5 and a maximum score of 0.6220 at Thr200 (Fig. 1C).

Six potential N-linked glycosylation sites were predicted in the mature protein via the NetNGlyc 1.0 server. Four sites (N37, N59, N68, and N152) had high predicted probabilities of glycosylation, with scores above 0.67 and maximum consensus among the prediction algorithms (9/9). Residues N141 and N193 provided moderate support for potential N-linked glycosylation, whereas the N140 motif remained below the prediction threshold established by the server.

The *MrB3* protein sequence was queried against the NCBI nonredundant (nr) protein database via BLASTp. The corresponding protein was subsequently retrieved from the AlphaFold 3 database [126] via the accession numbers V2YZA0 (UniProt) and ESK97039.1 (NCBI). The structural model AF-V2YZA0-F1 was selected for subsequent structural analyses because it corresponded to the *MrB3* sequence investigated in this study (Fig. 1B).

Five potential cysteine pairs capable of forming disulfide bonds were identified in the predicted *MrB3* structure (Fig. 1A). The Cys25–Cys92 and Cys41–Cys76 pairs presented the most favorable geometric and energetic parameters, with bond energies of 0.45 and 0.34, respectively, and χ^3 angles of 85.24° and 91.58°, which are close to the reference value of $\pm 90^\circ$. The Cys71–Cys125 and Cys174–Cys203 pairs also presented energy values compatible with disulfide bond formation, although their geometric parameters were less favorable. In contrast, the Cys133–Cys177 pair presented a bond energy of 3.80, which was greater than those observed for the other pairs.

2.2 Evolutionary relationships of the candidate effector protein *MrB3*

Phylogenetic analysis placed *MrB3* from *Moniliophthora roreri* among homologous proteins from Basidiomycota, which predominantly belong to the class Agaricomycetes (Fig. 2A, Table S1). *MrB3* clustered with the homologous protein from *Moniliophthora perniciosa*, with 97% bootstrap support, and this clade was related to *Paramarasmius palmivorus*, with 95% bootstrap support (Fig. 2A). The homologous protein from *M. perniciosa* shares 80.14% sequence identity with *MrB3*, whereas the protein from *P. palmivorus* shares 70.11% identity. The proteins from *Marasmius crinis-equi* and *M. oreades* formed a strongly supported clade (99% bootstrap support), sharing 52.71% and 48.26% sequence identity with *MrB3*, respectively. The remaining proteins in the ingroup presented sequence identities ranging from 45.80% to 53.81%.

The phylogenetic tree revealed a distinct cluster comprising the three sequences selected as the outgroup, belonging to the genera *Naganishia* and *Saitozyma*. This cluster had 100% bootstrap support, whereas the *Naganishia* sequences formed a subclade supported by 99% bootstrap support. The sequence identities between *MrB3* and *Saitozyma podzolica*, *Naganishia adeliensis*, and *Naganishia albida* were 31.82%, 31.55%, and 30.35%, respectively. Among the sequences included in the ingroup, the lowest sequence identity to *MrB3* was 45.80%, which was observed for *Schizophyllum commune* (Fig. 2A).

In silico characterization revealed that *MrB3* contains a signal peptide and a SPARK domain, which were identified by InterProScan between residues 17–225 (Fig. 2B). The SPARK domain was also predicted in most of the homologous proteins analyzed, whereas no protein family or domain was identified for *M. perniciosa*. Signal peptides were predicted in most proteins but were absent from *M. perniciosa* and *Schizophyllum commune*.

Analysis of intrinsic disorders in the mature protein sequences revealed distinct patterns of regions with a propensity for structural disorder among the proteins analyzed (Fig. S4). Disorder profiles varied among homologs, including outgroup proteins, indicating that the presence and distribution of potentially disordered regions are not restricted to proteins that are phylogenetically closest to *MrB3*.

2.3 Heterologous production, purification, and identity confirmation of recombinant rMrB3

SDS–PAGE analysis revealed a protein band with an apparent molecular mass of approximately 42.61 kDa, corresponding to recombinant rMrB3, which comprises the MrB3 sequence lacking the signal peptide fused to thioredoxin and a histidine tag (Fig. 1D). Most of the recombinant protein was detected in the insoluble fraction. Following purification by nickel affinity chromatography, rMrB3 was recovered from the imidazole-containing elution fractions, in which a predominant band corresponding to the recombinant protein was observed (Fig. 1D).

The identity of rMrB3 was confirmed by LC–MS/MS, which identified seven unique peptides matching the protein sequence of *Moniliophthora roreri* deposited under accession number ESK97039.1. The analysis achieved 30.78% sequence coverage across the 406-amino acid protein and an identification confidence level above 95%. The identified peptides were distributed throughout the protein sequence, covering both the N-terminal and C-terminal regions (Fig. S5A–C, Table S2).

2.4 Assessment of the structural stability of rMrB3 by circular dichroism

Circular dichroism (CD) spectroscopy revealed similar spectral profiles for the recombinant MrB3 protein (rMrB3) at 25°C and 95°C (Fig. 1E). Under both conditions, the spectra exhibited a negative peak at approximately 208–210 nm, followed by a gradual increase in ellipticity at longer wavelengths, indicating a similar spectral profile.

2.5 Functional characterization of the recombinant effector protein rMrB3 in plants

To evaluate the biological activity of recombinant rMrB3, bioassays were conducted using host and nonhost plants of *Moniliophthora roreri*.

In the leaves of *Solanum lycopersicum* cv. Micro-Tom (Fig. 3A), the application of rMrB3 induced progressive phenotypic changes throughout the experimental period, characterized by the development of necrosis, wilting, and leaf tissue collapse. These symptoms intensified over the days following treatment, culminating in pronounced leaf deterioration by day 15.

In the leaves of *Theobroma cacao* cv. Catongo (Fig. 3B), the application of rMrB3 induced mild phenotypic changes, which were mainly localized along the margins of the leaves. These changes were characterized by chlorotic and darkened areas, as well as small, irregular necrotic lesions, particularly in the basal region and along the leaf edges. These symptoms were more evident at a concentration of 3.2 $\mu\text{mol L}^{-1}$, whereas treatment with 2.2 $\mu\text{mol L}^{-1}$ resulted in less pronounced marginal alterations. Despite these changes, no wilting or widespread leaf tissue collapse was observed during the assessment period, and the control leaves remained visually intact.

In contrast, leaves of the CCN 51 genotype showed no visible alterations throughout the assessment period, even when treated with the highest concentrations of *rMrB3* (8.80 and 26.99 $\mu\text{mol L}^{-1}$) (Fig. S6).

2.6 Reactive oxygen species accumulation induced by *rMrB3*

The ability of recombinant *rMrB3* to induce the accumulation of reactive oxygen species was assessed by detecting hydrogen peroxide (H_2O_2) via 3,3'-diaminobenzidine (DAB) staining in *Solanum lycopersicum* leaves (Fig. 4).

The characteristic brown staining resulting from DAB oxidation in the presence of H_2O_2 was observed in leaf discs from plants treated with *rMrB3*, with greater staining intensity than that in the control at the time points evaluated (Fig. 4). An increase in DAB staining intensity over the assessment period was observed in leaves treated with *rMrB3* compared with the control. After three days of treatment with *rMrB3*, the brown staining intensity in the treated leaf discs was approximately 6%, indicating greater H_2O_2 accumulation than that in the control and an intensification of the response over the assessment period.

2.7 Identification of a *Theobroma cacao* protein potentially associated with *rMrB3* and structural prediction of the *MrB3*–*TcLRR* complex

Analysis of the eluted fractions from the affinity capture assay using *rMrB3* immobilized on CNBr-Sepharose revealed a *Theobroma cacao* protein with the potential to interact with *rMrB3* (Fig. 5A; Table S3). The protein was identified as a leucine-rich repeat (LRR) protein (accession A0A061ECS2), with an estimated molecular mass of 52.42 kDa and an isoelectric point (pI) of 5.78 (Fig. 5B).

Identification was based on the detection of the LASLPSLR peptide in two of the three fractions analyzed (LI and LIII), with a charge state of 2+. In fraction LI, the peptide had a score of 6.69 and an intensity of 145,000, whereas in fraction LIII, it had a score of 5.34 and an intensity of 16,600, corresponding to peak values of 75.0% and 64.7%, respectively (Fig. 5C; Table S3).

The refined structural models of *MrB3* and *TcLRR* exhibited good stereochemical quality, as indicated by Ramachandran plot analysis (Fig. S7A–D). Molecular docking generated putative complex models between the two proteins, featuring a well-defined interface and clustering of hotspot residues in the contact region between *MrB3* and *TcLRR* (Fig. 5D). A close-up view of the interface revealed a predicted hydrogen bond between Asn186 of *MrB3* and Ser362 of *TcLRR* (Fig. 5E).

The best-ranked complex had a HADDOCK score of -68.7 ± 2.4 and a Z score of -2.6 , distinguishing it among the clusters obtained. A total of 130 structures were grouped into 12 clusters, corresponding to 65% of the water-refined models. The main cluster comprised 33 structures and exhibited an intracluster RMSD of 0.7 Å. The mean restraint violation was 0.1 ± 0.1 (Table S4).

The complex interface had a buried surface area of $1511.8 \pm 6.3 \text{ \AA}^2$. Independently, PDBePISA estimated an interface area of $731.4 \text{ \AA}^2$ per monomer, corresponding to an approximate total buried surface area of $1462.8 \text{ \AA}^2$, with an interface solvation free-energy gain (ΔiG) of $-7.5 \text{ kcal mol}^{-1}$ (Table S4). PRODIGY estimated a binding free energy of $-12.5 \text{ kcal mol}^{-1}$ and a predicted dissociation constant of $7.4 \times 10^{-10} \text{ M}$. These structural and energetic parameters are summarized in Fig. 5F and detailed in Table S4.

A total of 77 intermolecular contacts were identified at the interface between *MrB3* and *TcLRR*, including contacts involving charged, polar, and nonpolar residues. One hydrogen bond between the proteins was also identified. In addition, the LASLPSLR peptide, previously identified in *TcLRR* by LC–MS/MS, was located in the region corresponding to the predicted interaction hotspot identified by molecular docking (Fig. 5E).

Conservation analysis of the *MrB3* residues involved in the *in silico*-predicted interaction with *TcLRR* revealed that Asn186 (N), Pro190 (P), and Asn194 (N) are conserved between *Moniliophthora roreri* and *M. perniciosa*. The alignment of the *MrB3* sequence with its homologs (Fig. S8) also revealed a conserved pattern in the region corresponding to the interaction residues among *M. roreri*, *M. perniciosa*, *S. scandens*, *P. conissans*, and *T. constricta*, represented by the sequence NxDxPxLxSD.

Within this region, a single amino acid deletion between Pro190 and Asn194 was identified as a characteristic feature of *M. roreri* and *M. perniciosa*. The region corresponding to the residues involved in the predicted interaction with *TcLRR* exhibited lower conservation in sequences from organisms belonging to other taxonomic groups than in those from species of the genus *Moniliophthora*.

In the conservation analysis of *TcLRR*, Ser359, Pro361, and Ser362, which are involved in the predicted interaction with *MrB3*, were conserved among the analyzed sequences from the genus *Theobroma* (Fig. S9). Similarly, the LASLPSLR sequence, which was experimentally identified via LC–MS/MS, was conserved among the *TcLRR* proteins analyzed.

The *TcLRR* protein identified as potentially associated with *rMrB3* lacked a kinase domain in its predicted structure. In addition, a predicted region with features of a lipid anchor was identified, as shown in Fig. S10. These characteristics are consistent with a plasma membrane-associated protein displaying features of a receptor-like protein (RLP).

Identification of *TcLRR* by affinity capture followed by LC–MS/MS, together with molecular modeling of its association with *MrB3*, provided experimental and computational evidence supporting a possible association between the two proteins. In parallel, treatment with *rMrB3* resulted in H_2O_2 accumulation in leaves, indicating the induction of a response associated with oxidative stress.

3 Discussion

3.1 *MrB3* exhibits predictive features consistent with a secreted effector protein from *Moniliophthora roreri*

The identification of effector proteins is a key strategy for understanding the molecular mechanisms employed by phytopathogenic fungi during host colonization. In the present study, selection of the *MrB3* candidate was based on the integration of transcriptomic data and *in silico* analyses, leading to the identification of a protein that exhibits several features commonly associated with fungal effectors, including a relatively low molecular mass, a signal peptide, the absence of transmembrane domains, and the absence of known conserved domains identified by the NCBI Conserved Domain Database (CDD) [130]. Additionally, InterProScan analysis revealed that the region spanning residues 17–225 was associated with the SPARK domain-containing protein family, although this annotation does not establish a specific molecular function for *MrB3*. This combination of features has been widely described in effector proteins secreted by biotrophic [69], hemibiotrophic [62], and necrotrophic fungi [36, 110].

The absence of known conserved domains identified by the NCBI CDD [130] is also consistent with features reported for several effector proteins. Unlike highly conserved metabolic enzymes, effectors often exhibit rapid evolution, high sequence variability, and restricted taxonomic distribution, reflecting the strong selective pressures associated with coevolution with their hosts [22, 115, 69]. Although InterProScan annotation indicates a region associated with the SPARK domain-containing protein family, the absence of known conserved domains in the CDD [130], together with the limited functional characterization of this family, hinders the assignment of a specific molecular function to *MrB3*. This limitation is particularly relevant for fungal effectors, whose identification and functional characterization can be challenging because of low sequence conservation, the absence of well-characterized homologs, and the structural and functional diversity of these proteins [56, 54, 67]. These features may also hinder functional annotation via conventional approaches based solely on sequence similarity [115, 113].

Another relevant finding was the classification of *MrB3* as a probable apoplastic effector by EffectorP-fungi 3.0. Apoplastic effectors play important roles during the early stages of infection by modulating plant immunity, inhibiting defense-related enzymes, and protecting the pathogen cell wall against hydrolytic proteins produced by the host [100, 105]. The presence of a predicted signal peptide further supports this hypothesis and suggests that *MrB3* may be secreted through the conventional endoplasmic reticulum–Golgi pathway before it reaches the extracellular space [100].

IUPred3 analysis identified residues with a propensity for disorder distributed throughout the protein sequence. However, no continuous segments consistent with extensive intrinsically disordered regions were observed using the long-disorder mode, which is optimized for predicting global structural disorder. Therefore, these results were interpreted as evidence of local disorder propensity rather than the presence of clearly delineated intrinsically disordered regions [29].

These regions are frequently observed in effector proteins because of the conformational plasticity they confer, potentially facilitating recognition of multiple molecular partners and enabling rapid adaptation to

the different conditions encountered during plant–pathogen interactions [115, 105]. This structural flexibility may represent an important adaptive advantage for effector proteins from *M. roreri*.

Additionally, the identification of multiple potential N-glycosylation sites suggested that *MrB3* may undergo posttranslational modifications during secretion. In phytopathogenic fungi, N-glycosylation is frequently associated with increased structural stability, protection against proteolytic degradation, and maintenance of the biological activity of secreted proteins, potentially influencing their persistence in the apoplastic environment [42].

Collectively, these features indicate that *MrB3* has characteristics consistent with those of secreted effector proteins and support its relevance as a candidate involved in the molecular mechanisms underlying the pathogenicity of *M. roreri*.

3.2 Evolutionary relationships suggest conservation of *MrB3* features

Phylogenetic analysis revealed that *MrB3* was among the homologous proteins from Basidiomycota, which are predominantly Agaricomycetes, with the closest relationships to proteins from *Moniliophthora perniciosa* and *Paramarasmius palmivorus*. The high sequence identities observed for these homologs, particularly those from *M. perniciosa* (80.14%) and *P. palmivorus* (70.11%), together with the high support values for the corresponding clusters, indicate sequence conservation among closely related phylogenetic lineages. In contrast, the proteins from *Naganishia adeliensis*, *Saitozyma podzolica*, and *Naganishia albida*, which were used as the outgroup, presented the lowest sequence identities to *MrB3* (30.35–31.82%) and formed a distinct clade with 100% bootstrap support, which was consistent with their greater divergence from the ingroup.

The occurrence of *MrB3* homologs in species with different lifestyles, including phytopathogenic and predominantly saprotrophic fungi, suggests that this protein family is not restricted to pathogenic organisms [7]. This distribution may indicate the conservation of ancestral features associated with broader functions that were subsequently modified or recruited in different ecological contexts [22, 69]. In phytopathogenic fungi, the diversification of proteins involved in host interactions may occur through several evolutionary processes, including sequence diversification and functional changes associated with selective pressures imposed by host interactions [115, 104–105]. However, the presence of homologs in saprotrophic species does not, by itself, allow an ancestral function or specific recruitment for pathogenicity to be inferred.

The predicted signal peptide in *MrB3* and most of the homologs analyzed suggest that secretion-associated features are conserved in parts of this protein family. However, the absence of this feature in the proteins from *Moniliophthora perniciosa* and *Schizophyllum commune* indicates that signal peptides are not universal among the identified homologs. Thus, the data support partial conservation of secretion-associated features but do not establish extracellular secretion as an ancestral property shared by the entire family.

The identification of the SPARK domain by InterProScan in *MrB3*, most of its homologs, and proteins from the outgroup suggest that this structural feature is conserved across different Basidiomycota lineages [52, 73]. This pattern may reflect structural or functional constraints during evolution, whereas other regions of effector proteins may have undergone greater diversification [113]. However, the specific molecular function of the SPARK domain in fungal effector proteins has not yet been established. Therefore, its identification provides information about the structural organization of the proteins analyzed but does not, by itself, allow their biological function or direct involvement in plant–pathogen interactions to be inferred [52, 73, 69].

The presence of residues with a propensity for structural disorder in proteins exhibiting different levels of divergence suggests that this feature may contribute to the structural diversity of the family, although its relationship with the function of *MrB3* cannot be established from this analysis alone.

Collectively, these results indicate that *MrB3* belongs to a family of homologous proteins distributed across different Basidiomycota lineages, with partial conservation of sequence, domain, and secretion-associated features among its homologs. The phylogenetic proximity of *MrB3* to proteins from *M. perniciosus* and *P. palmivorus*, together with the recurrent occurrence of the SPARK domain and signal peptides among homologs, suggests that certain protein features were maintained during the diversification of this family. In particular, the occurrence of the SPARK domain in outgroup proteins further supports the hypothesis that this domain represents a conserved feature of the family, regardless of phylogenetic proximity to *MrB3*.

Conversely, the occurrence of homologs in fungi with different lifestyles, the absence of signal peptides in some members, and variation in disorder profiles indicate diversification of their molecular features. The structural and functional diversity of fungal effectors, including differences in secretion signals and conserved domains, has been described in several plant–fungus systems [22, 69]. Furthermore, intrinsically disordered regions may contribute to the structural and functional variability of effector proteins [67].

These findings suggest that certain structural features are conserved in *MrB3* but do not directly establish an effector function or a specific role in pathogenicity for its homologs. Domain annotation and the prediction of structural features provide hypotheses regarding protein organization and potential function, but experimental validation is needed to confirm biological activity [52, 73]. This caution is particularly relevant for fungal effector proteins, whose functions may vary depending on the pathogen and host context [109].

3.3 Heterologous production and experimental validation of r*MrB3* reveal activity consistent with that of necrotrophic effectors

The production of recombinant effector proteins represents an important step in the functional characterization of factors involved in the virulence of phytopathogenic fungi, enabling the investigation

of their effects on plant cells and the identification of potential host targets [54]. However, the heterologous expression of these proteins often presents an experimental challenge, primarily because of the diversity of their structural and biochemical properties, the presence of cysteine residues, and the requirement for proper folding to maintain protein stability and biological activity [113, 54, 104, 105].

In the present study, the use of a synthetic open reading frame (ORF) optimized for *Escherichia coli*, which was cloned and inserted into the pET-32a vector in fusion with thioredoxin, enabled the production of sufficient amounts of recombinant rMrB3 for subsequent functional assays. Fusion proteins are commonly used as a strategy for the heterologous expression of fungal proteins and may improve the solubility, stability, and subsequent purification of recombinant proteins [85]. Cysteine residues are found in several classes of fungal effectors, particularly secreted proteins, in which these residues may participate in disulfide bond formation, contributing to structural stability and the maintenance of protein function [18, 54, 67]. In this context, thioredoxin fusion was employed as a strategy to facilitate the recovery of rMrB3 during heterologous expression, helping to obtain recombinant protein under conditions suitable for subsequent functional assays and biochemical analyses.

Although most of the rMrB3 was recovered from the insoluble fraction, solubilization with urea followed by its subsequent removal enabled the recovery of soluble protein after nickel-affinity chromatography. This property allowed the protein to be used in bioassays and subsequent biochemical analyses, indicating that it remained soluble after processing. Similar results have been reported for recombinant proteins expressed in *Escherichia coli*, in which inclusion body formation does not necessarily preclude the recovery of soluble protein following appropriate recovery procedures [113].

Experimental confirmation of the identity of rMrB3 by LC–MS/MS represents another relevant aspect of this study. Unlike the simple confirmation of molecular mass by SDS–PAGE, mass spectrometry provides direct evidence of the composition of the expressed protein through the identification of peptides distributed along its sequence. The sequence coverage of approximately 30%, together with the identification of multiple unique peptides and high statistical confidence scores, meets internationally accepted criteria for proteomic validation, providing strong confidence in the identity of the protein used in the experiments [87, 86].

In addition to validating the recombinant protein, the agreement between the transcriptomic and proteomic data further supports the robustness of the strategy used to identify MrB3. The integration of RNA-seq, heterologous production, and mass spectrometry substantially reduces the likelihood of annotation errors and provides a solid experimental basis for subsequent functional assays. This integrative approach has been increasingly used in the characterization of effector proteins because it combines independent evidence of gene expression and protein presence, thereby increasing the reliability of biological inference [28].

In this context, the experimental validation of rMrB3 establishes a robust platform for investigating its function during the interaction between *M. roreri* and *T. cacao*, allowing the effects observed in bioassays

to be attributed to the recombinant protein produced rather than to contaminants derived from the expression system.

3.4 rMrB3 exhibits high thermal stability and preserves its secondary structure

The high degree of overlap between the circular dichroism spectra obtained at 25°C and 95°C indicates that the recombinant protein rMrB3, expressed as a thioredoxin fusion, retained its spectral profile after heating. Although minor differences in signal intensity were observed, particularly between 190 and 210 nm, no substantial changes in the overall spectral shape were detected, suggesting that the increase in temperature did not induce significant alterations in the protein's secondary structure [41].

The experimentally observed thermal stability is consistent with the structural analyses, which predicted two structurally favorable disulfide bonds between the Cys25–Cys92 and Cys41–Cys76 residues. These pairs presented lower bond energies and χ^3 angles close to 90°, which are parameters that are compatible with the expected geometry of disulfide bonds [20]. Considering that disulfide bonds can contribute to the stabilization of the three-dimensional structure of proteins, particularly secreted proteins, these interactions may be associated with the maintenance of the structural integrity of rMrB3 under the evaluated temperature conditions.

3.5 r Mr B3 triggers differential responses in host and nonhost plants, highlighting its role in modulating plant defense responses

Bioassays performed with the recombinant protein rMrB3 demonstrated that its biological activity depends on the genetic background of the plant host, as evidenced by the contrasting responses observed in *Solanum lycopersicum*, *Theobroma cacao* cv. Catongo (Fig. 3A–B), and *Theobroma cacao* cv. CCN 51 (Fig. S6). The induction of severe necrosis in tomato, together with the absence or substantial reduction in symptoms in cacao genotypes with different levels of resistance, indicates that MrB3 may be involved in molecular mechanisms related to the recognition and modulation of plant immunity.

The response observed in *S. lycopersicum* is consistent with the activation of defense mechanisms following the recognition of pathogen-associated molecules. In nonhost species, effector proteins may be recognized directly or indirectly by the plant and trigger immune responses, including the production of reactive oxygen species, the activation of defense-related genes and, under certain conditions, localized cell death [97, 136]. However, the intensity and outcome of these responses may vary depending on effector function and pathogen lifestyle. Whereas some effectors from biotrophic pathogens suppress immunity but may be recognized as elicitors in incompatible interactions [115], effectors from necrotrophic pathogens may also induce changes in cellular homeostasis and cell death, potentially favoring the colonization of host tissues [136]. Therefore, these responses should be interpreted in the specific context of the plant–pathogen interaction.

The progressive increase in hydrogen peroxide accumulation observed following *rMrB3* application further supports the hypothesis that this protein triggers responses associated with plant defense. Reactive oxygen species production is an early response of plant immunity and involves cell signaling and other mechanisms that may contribute to restricting pathogen development [26]. In addition to exerting direct antimicrobial effects, H_2O_2 contributes to cell wall strengthening, the activation of mitogen-activated protein kinase (MAPK)-dependent signaling cascades, and the induction of the hypersensitive response, which is frequently associated with limiting the colonization of plant tissues [104]. Thus, the substantial H_2O_2 accumulation in tomato leaves suggests that *rMrB3* is efficiently perceived by the immune system of this species, resulting in the activation of defense mechanisms associated with the observed necrosis.

In contrast, the responses observed in the *T. cacao* genotypes suggest that the effects of *rMrB3* may vary according to the host's genetic background. The Catongo genotype, which is associated with greater susceptibility to frosty pod rot [104], also exhibits differences in the modulation of defense-related proteins during infection by *Moniliophthora perniciosa* compared with genotypes displaying greater resistance to witches' broom disease [104]. In this genotype, *rMrB3* application caused mild alterations, predominantly along the leaf margins, even at the highest concentration tested. This pattern indicates that the phenotypic response to the effector may be influenced by both the host's genetic characteristics and the specificity of the plant–pathogen interaction.

Conversely, the absence of severe symptoms and the moderately resistant phenotype observed in CCN 51, even at the highest *rMrB3* concentrations tested, suggest that this genotype may possess more effective mechanisms for perceiving, neutralizing, or tolerating the effects triggered by the effector (Fig. S6). Recent studies have shown that CCN 51 has a greater capacity to activate defense-related genes, increased efficiency in producing antimicrobial metabolites, and faster physiological responses to infection by *Moniliophthora* spp., traits that contribute to its partial resistance to disease [123]. The CCN 51 genotype is recognized for its moderate resistance to witches' broom disease (WBD), which is caused by *Moniliophthora perniciosa* [13]. Although the molecular mechanisms underlying this resistance remain incompletely understood, the results obtained in this study indicate that *rMrB3* may be involved in an early stage of this differential interaction.

Effector proteins perform distinct functions depending on the pathogen's lifestyle and the compatibility of the interaction established with the host. In compatible interactions, these proteins frequently suppress components of plant immunity and promote colonization [104–105]. In incompatible interactions or in nonhost species, effector proteins may be recognized directly or indirectly by plant receptors, triggering defense responses and changes in cellular homeostasis that may result in localized cell death. However, the significance of this cell death depends on the type of plant–pathogen interaction: it may contribute to resistance against biotrophic pathogens or favor colonization by necrotrophic pathogens [105]. This behavior has been described for effector proteins from *Phytophthora infestans*, *Cladosporium fulvum*, and *Magnaporthe oryzae*, whose activities vary according to the host's genetic background [97, 115, 136].

The use of effectors as complementary tools for genotype discrimination was also proposed by Santos et al. [104], who suggested evaluating *MrB1* as a potential susceptibility marker in cacao breeding programs. In this context, *MrB3* could be investigated as part of an effector panel, together with *MrB1* and other candidate proteins from *M. royeri*. The combined application of these effectors to contrasting genotypes and segregating materials from breeding programs may help identify phenotypic patterns associated with different host responses. However, this possibility requires further experimental validation through the assessment of a larger number of genotypes, standardization of application concentrations and conditions, and comparison of effector-induced responses with independent data on resistance to frosty pod rot.

Taken together, these results indicate that *rMrB3* may trigger differential responses in host and nonhost plants, involving changes in cellular homeostasis, H_2O_2 accumulation, and necrosis development. The intensity of these responses varied between the *S. lycopersicum* and *T. cacao* genotypes evaluated, indicating that the effects of the protein may depend on the host's genetic background. However, the data do not support the conclusion that *rMrB3* acts exclusively as a virulence determinant or that its presence is directly associated with greater susceptibility to frosty pod rot. Future studies evaluating *rMrB3* as part of an effector panel and across contrasting *T. cacao* genotypes may clarify its potential as a complementary tool for characterizing defense responses and identifying plant materials of interest for breeding programs.

3.6 The effector protein *MrB3* potentially interacts with the *Theobroma cacao* protein *TcLRR*

The identification of host proteins that directly interact with effector proteins is one of the most relevant approaches for elucidating the molecular mechanisms underlying the pathogenicity of phytopathogenic fungi. In this study, an affinity chromatography strategy followed by mass spectrometry identified a *Theobroma cacao* protein containing a leucine-rich repeat (LRR) domain as a potential interaction partner of the *MrB3* effector protein. Subsequent structural validation through molecular docking provided independent evidence supporting this proposed interaction, strengthening the hypothesis that this protein may participate in the early events of host colonization.

LRR domain-containing proteins constitute one of the major classes of receptors involved in the perception of pathogen-associated signals in plants [8, 117]. These domains are characterized by leucine-rich repeats that form a structurally versatile surface for molecular recognition, enabling specific interactions with molecules derived from microorganisms or with effector proteins secreted during infection [26, 84]. Owing to their structural organization, LRR domain-containing proteins may function as plasma membrane-localized receptors or as intracellular components of effector-triggered immunity [8, 117].

The structural model of the *MrB3*–*TcLRR* complex, supported by the HADDOCK score, interface area, binding affinity, and dissociation constant, indicates that complex formation is energetically favorable. Furthermore, the agreement among the results obtained via different computational tools, including

HADDOCK, PDBePISA, and PRODIGY, increases confidence in the proposed interaction. The overlap between the predicted interface and the LASLPSLR peptide previously identified by LC–MS/MS provides additional evidence for the biological plausibility of this structural model, as it integrates independent experimental findings with computational analyses.

Although the experiments performed do not establish that *TcLRR* is a resistance receptor, the proposed interaction suggests that this protein may participate in processes associated with pathogen recognition or the modulation of defense responses during infection by *M. roreri*. In several plant–pathogen systems, effector proteins interact with LRR domain-containing proteins to alter their stability, interfere with their recognition capacity, or modify signaling cascades associated with plant immunity [8, 117]. Thus, the proposed interaction involving *MrB3* represents a biologically plausible mechanism that is consistent with the virulence strategies described for other phytopathogenic fungi.

Another aspect supporting this hypothesis is that the *TcLRR* protein investigated in this study was experimentally identified in leaves of the Catongo genotype, which is known to be susceptible to frosty pod rot. Although susceptibility to this genotype results from the interplay of multiple genetic and physiological factors, structural or functional differences in proteins involved in pathogen recognition may influence the effectiveness of the immune response. In this context, the interaction between *MrB3* and *TcLRR* may represent one of the molecular components associated with the establishment of compatibility between the fungus and its host. This hypothesis should be investigated in future studies involving different *T. cacao* genotypes.

The integrated use of affinity chromatography, proteomic identification, and structural modeling represents one of the main methodological advances of this study. Whereas computational approaches alone can predict only potential interaction partners, prior experimental identification of the target protein substantially narrows the search space and increases the robustness of subsequent structural analyses. This strategy enhances the reliability of biological inference and demonstrates the potential of integrating experimental proteomics with molecular modeling to identify targets of effector proteins in plant pathogens.

Taken together, these results indicate that *TcLRR* is a strong candidate target protein of *MrB3* during the interaction between *M. roreri* and *T. cacao*. The experimental and structural evidence obtained in this study provides a consistent basis for proposing this molecular model and expands our understanding of the mechanisms underlying the pathogenicity of the causal agent of frosty pod rot.

3.7 A mechanistic model for the role of *MrB3* during the interaction between *Moniliophthora roreri* and *Theobroma cacao*

The results obtained in this study support the proposal of a mechanistic model for the role of the effector protein *MrB3* during the interaction between *Moniliophthora roreri* and *Theobroma cacao*. The combination of transcriptomic analyses, structural characterization, heterologous production, bioassays,

experimental identification of a target protein, and molecular modeling provides complementary evidence supporting the involvement of this protein in the early stages of fungal pathogenicity.

The high expression of the *mrna_11825* transcript during spore germination in media supplemented with the cacao extract suggested that *MrB3* is produced during the early stages of pathogen development, a period considered critical for infection establishment. During this phase, hemibiotrophic fungi secrete effector proteins capable of modifying the plant extracellular environment, reducing the effectiveness of immune responses and facilitating the colonization of plant tissues [75, 105]. The presence of a functional signal peptide and the predicted apoplastic localization are consistent with this secretion strategy.

The bioassays demonstrated that the recombinant protein exhibits biological activity when directly applied to plant tissues, inducing distinct responses depending on the genotype evaluated. Whereas *Solanum lycopersicum* developed extensive necrosis accompanied by increased reactive oxygen species production, *Theobroma cacao* leaves presented responses that depended on the genetic material tested. These differences indicate that *MrB3* activity is closely associated with the host's capacity to recognize and respond to the protein, supporting the concept that effector proteins perform specific functions in compatible and incompatible interactions [115, 97].

The experimental identification of an LRR domain-containing protein by affinity chromatography and LC-MS/MS represents one of the main findings of this study. In plants, proteins belonging to this family play a central role in perceiving pathogen-associated signals and activating defense pathways. The predicted structural interaction between *MrB3* and *TcLRR*, supported by the results obtained via HADDOCK, PDBePISA, and PRODIGY, suggests that this protein may represent an important component of the mechanisms involved in the recognition or modulation of plant immunity.

Although the available data do not establish the functional outcome of this interaction, it is possible to hypothesize that *MrB3* alters the activity of *TcLRR* or of components associated with its signaling pathway. In different plant–pathogen systems, effector proteins alter the stability, subcellular localization, or interaction capacity of proteins that regulate immunity, resulting in the suppression or redirection of defense responses to the pathogen's benefit [55, 44, 117]. The high predicted affinity of the *MrB3*–*TcLRR* complex is consistent with this type of mechanism.

Another relevant aspect is that *MrB3* has no functionally characterized homologs in public databases. Its phylogenetic distribution among Basidiomycota, together with the absence of known conserved domains identified by the NCBI CDD, suggests that this protein represents a previously undescribed effector that may be associated with mechanisms specific to the interaction between *M. roreri* and species of the genus *Theobroma*. Although InterProScan associated the region spanning residues 17–225 with the SPARK domain-containing protein family, this annotation alone does not establish a specific molecular function for *MrB3*. This characteristic increases the biotechnological interest of the protein, as poorly characterized effectors may represent novel targets for studies of genetic resistance, the development of diagnostic tools, and disease control strategies.

The results of this study also demonstrate the potential of integrating transcriptomics, experimental proteomics, and structural biology to identify novel effectors and their potential molecular partners. Traditionally, studies of effector proteins have focused on gene expression analyses or computational predictions. In the present study, however, the sequential use of RNA-seq, recombinant protein production, affinity chromatography, mass spectrometry, molecular docking, and structural modeling enabled the construction of an evidence framework based on independent and complementary approaches, substantially strengthening the biological inferences.

Accordingly, we propose that *MrB3* is secreted during the early stages of the interaction between *M. roreri* and *T. cacao* and reaches the apoplastic space, where it interacts with an LRR domain-containing host protein. This interaction may contribute to the modulation of plant defense pathways and facilitate infection establishment, eventually leading to tissue colonization and the development of frosty pod rot. The results presented here provide a consistent experimental basis for future investigations into the molecular mechanisms underlying the pathogenicity of *M. roreri*.

The conservation of the *MrB3* residues Asn186, Pro190, and Asn194 between *M. roreri* and *M. perniciosa* suggests that this protein region is conserved within the genus *Moniliophthora*. The presence of the NxPxLxSD motif in the analyzed sequences further supports this conservation, whereas the deletion of one amino acid between Pro190 and Asn194 observed in *M. roreri* and *M. perniciosa* represents a shared feature of these species.

Conversely, the lower conservation of this region in organisms outside the genus *Moniliophthora* may indicate that sequence-specific features in the region of *MrB3* are involved in the predicted association with *TcLRR*. Similarly, the residues Ser359, Pro361, and Ser362 of *TcLRR*, as well as the experimentally identified LASLPSLR sequence, are conserved among proteins from the genus *Theobroma*. Taken together, these conservation patterns in the *Moniliophthora* and *Theobroma* proteins may be associated with the specific features of their proposed associations and, potentially, with the coevolutionary history of this pathosystem.

On the basis of these results, we propose that *MrB3* may be perceived by a *T. cacao* membrane receptor represented by the protein *TcLRR*, triggering a defense response associated with the accumulation of reactive oxygen species. This hypothesis is supported by the experimental identification of *TcLRR* as a protein potentially associated with *rMrB3* and by the observed accumulation of hydrogen peroxide following treatment with the recombinant protein.

The *TcLRR* protein may be classified as a receptor-like protein (RLP), as it lacks a kinase domain. RLPs are frequently described as membrane receptors related to receptor-like kinases (RLKs) but lack a cytoplasmic kinase domain [82]. In the case of *TcLRR*, the absence of a kinase domain, together with the presence of a predicted lipid anchor-like region (Fig. S10), may be associated with its localization at the plasma membrane.

Given these characteristics, we hypothesize that TcLRR may act as a coreceptor in the perception of MrB3, participating in the signaling process leading to ROS production and H₂O₂ accumulation. This hypothetical mechanism may be similar to the mechanism described for REXG1 in *Nicotiana benthamiana* [129]. A representation of this model is provided in the Supplementary Material (Fig. S10).

4 Conclusion

This study contributes to the functional characterization of a novel effector protein, MrB3, from the phytopathogenic fungus *Moniliophthora roreri*, the causal agent of frosty pod rot in cacao. The integration of transcriptomic approaches, *in silico* analyses, heterologous production, biological assays, experimental proteomics, and structural modeling demonstrated that MrB3 features are consistent with secreted effector proteins, including the presence of a signal peptide, the absence of transmembrane domains, intrinsically disordered regions, and predicted apoplasmic localization.

Recombinant expression and experimental validation of the protein by mass spectrometry provided a reliable basis for functional studies. In addition, circular dichroism spectroscopy demonstrated that the recombinant protein retained its spectral profile even after heating to 95°C, indicating high thermal stability and supporting the structural predictions obtained through *in silico* analyses.

The bioassays demonstrated that rMrB3 is biologically active, inducing distinct responses in different plant species and genotypes. The induction of necrosis and wilting, together with increased reactive oxygen species production in *Solanum lycopersicum* and the differential responses observed among the *Theobroma cacao* genotypes indicate that protein activity depends on the host's genetic background and support its potential role in modulating plant defense responses.

One of the main advances of this study was the experimental identification of an LRR domain-containing *T. cacao* protein as a potential interaction partner of MrB3 through affinity chromatography coupled with mass spectrometry. The strong agreement between the experimental results and molecular docking analyses provided independent and complementary evidence supporting the proposed interaction between these proteins, establishing a consistent molecular model to explain some of the biological effects observed in the bioassays.

Phylogenetic analyses indicated that MrB3 belongs to a group of poorly characterized proteins apparently restricted to Basidiomycota, suggesting that this effector may represent an evolutionary innovation associated with the adaptation of *M. roreri* to its host. The absence of functionally characterized homologs and known conserved domains identified by NCBI CDD, together with the InterProScan annotation of a region associated with the SPARK domain-containing protein family spanning residues 17–225, further highlights the limited characterization of this protein and increases its potential relevance for studies on the evolution of pathogenicity, plant–pathogen interactions, and the identification of novel targets for plant breeding programs.

Taken together, these results expand our understanding of the molecular mechanisms underlying the interaction between *M. roreri* and *T. cacao*, identifying *MrB3* as a novel effector and *TcLRR* as a potential molecular target in the host. The integrative strategy employed in this study demonstrates the potential of combining transcriptomics, experimental proteomics, and structural biology for the discovery and functional characterization of effector proteins in phytopathogenic fungi.

Although these data provide consistent evidence supporting the interaction between *MrB3* and *TcLRR*, further studies are needed to validate this model under physiological conditions. Protein–protein interaction assays, functional analyses in plants, assessment of defense-related gene expression, and reverse genetics approaches may clarify the molecular mechanisms through which *MrB3* modulates plant immune responses and contributes to the pathogenicity of *M. roreri*. These investigations may support the development of novel strategies for managing frosty pod rot, including the identification of molecular markers for resistance and potential targets for disease control.

This study presents the first functional characterization of the effector protein *MrB3* from *Moniliophthora roreri* and provides the first experimental and structural evidence of its interaction with an LRR domain-containing protein from *Theobroma cacao*. The demonstration of its high thermal stability, together with evidence of its biological activity, structural predictions, and identification of a potential molecular target in the host, advances our understanding of the mechanisms underlying *M. roreri* pathogenicity and establishes a solid foundation for future investigations into virulence factors, plant–pathogen interactions, and the development of innovative strategies for frosty pod rot management and genetic improvement.

5 Experimental procedures

5.1 *In silico* identification and characterization of *MrB3*

The sequence of the candidate effector protein was selected from the transcriptome of *Moniliophthora roreri* spores obtained via RNA-seq after 16 h of incubation in a medium containing cacao extract [80]. Initially, differentially expressed transcripts were translated into protein sequences via BLASTx, with the NCBI nonredundant (nr) protein sequence database used as the reference, enabling the identification of the corresponding proteins.

Uncharacterized proteins were then filtered according to sequence length, and those containing fewer than 300 amino acid residues ($n = 10$) were selected because their size was consistent with that of the effector proteins described in phytopathogenic fungi [75]. Subsequently, predictive criteria for fungal effector identification were applied, including the presence of a signal peptide, the absence of transmembrane and conserved domains, and the presence of cysteine residues. The selection was guided by data previously reported by Meinhardt et al. [75] concerning the differential expression of genes encoding secreted proteins during the biotrophic and necrotrophic phases of infection.

The presence of a signal peptide and its corresponding cleavage site was analyzed via SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP>), whereas transmembrane domains were assessed via TMHMM 2.0 (<https://services.healthtech.dtu.dk/service.php?TMHMM-2.0>). Potential disulfide bonds in *MrB3* were predicted via disulfide by the Design v2.13 server (<http://cptweb.cpt.wayne.edu/DbD2/index.php>) on the basis of structural analysis of cysteine residues [20].

Potential N-glycosylation sites in *MrB3* were predicted via the NetNGlyc 1.0 server, with the mature protein sequence obtained after removal of the signal peptide. This method is based on the identification of the consensus motif N-X-S/T and neural network algorithms to estimate the probability of glycosylation [42].

The identification of conserved domains was performed via InterProScan (<https://www.ebi.ac.uk/interpro/search/sequence/>) [52] and the NCBI Conserved Domain Database (CDD; <https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) [130]. The physicochemical properties were predicted via ProtParam (<https://web.expasy.org/protparam/>). Protein sequences were analyzed via EffectorP-fungi 3.0 (<https://effectorp.csiro.au/>) to predict effector-associated features [113], and the subcellular localization of *MrB3* was predicted via DeepLoc 2.0 (<https://services.healthtech.dtu.dk/services/DeepLoc-2.0/>) [121].

The predicted structural model of *MrB3* was identified in the AlphaFold 3 database (<https://alphafold.ebi.ac.uk/>) [126] following a BLASTp search against the nonredundant (nr) protein database via sequence alignment. The amino acid sequence of the protein was retrieved in FASTA format from the NCBI and UniProt databases [120]. The predicted structure was then refined and validated in PDB format via MolProbity (<http://molprobity.biochem.duke.edu/>) [17] to identify potential adjustments to torsion angles and eliminate steric clashes. This procedure improved the reliability of the structural model, enabling its use in functional and molecular interaction studies.

The secondary structure was visualized via PyMOL v3.1.8 [108] (<https://www.pymol.org/>). Intrinsically disordered regions were predicted via IUPred3 (<https://iupred3.elte.hu/>), with residues exhibiting disorder scores greater than 0.5 considered potentially disordered [29].

To visualize conserved amino acid residues among the sequences, the alignment used for phylogenetic analysis was imported into Jalview v2.11.5.2 [131]. To investigate the potential interaction of *MrB3* with LRR domain-containing proteins from other species, homologous sequences were searched for in different *Theobroma cacao* genotypes. Additional searches were performed for homologous sequences from *Theobroma grandiflorum*, *Theobroma umbraticum*, *Nicotiana tabacum*, *Solanum tuberosum*, *Solanum lycopersicum*, and sugarcane (*Saccharum_hybrid_R570*). The sequences were aligned via Clustal Omega (ClustalO) [111], and the resulting alignment was visualized via Jalview.

5.2 Phylogenetic analysis

A set of hypothetical proteins was selected via the accession sequence ESK97039.1, corresponding to *MrB3*, as the query sequence in BLASTP (<https://sky-blast.com/blast/p>). The organisms corresponding to the selected sequences were identified via the NCBI Taxonomy Browser (<https://www.ncbi.nlm.nih.gov/datasets/taxonomy/browser/>), and their complete taxonomic classifications were obtained according to Schoch et al. [107]. Protein sequences were retrieved from the NCBI Protein (<https://www.ncbi.nlm.nih.gov/protein/?term=>) and UniProt (<https://www.uniprot.org/>) databases [120].

For the phylogenetic analysis, 22 amino acid sequences were used, including the *MrB3* sequence, 18 homologous proteins from Basidiomycota, and three sequences selected as the outgroup. The ingroup comprised *MrB3* and 18 homologous proteins from Basidiomycota, primarily representatives of the class Agaricomycetes, selected on the basis of sequence similarity and phylogenetic proximity to *MrB3*. To root the phylogenetic tree, three Basidiomycota proteins belonging to the genera *Naganishia* and *Saitozyma*, classified within the class Tremellomycetes, were selected. These sequences were used as the outgroup on the basis of their taxonomic positions and the phylogenetic clustering observed relative to the Agaricomycetes sequences included in the ingroup.

The mature sequences of the 22 proteins were used for phylogenetic analyses after removal of their predicted signal peptides. Multiple sequence alignment of the mature proteins was performed via the MAFFT algorithm [57], and the alignment was visualized and edited via AliView [65]. After alignment, 218 positions were retained following partial deletion of positions with less than 95% coverage.

The phylogenetic tree was constructed via MEGA X v.10.2.6 [64], employing the maximum likelihood method, the JTT evolutionary model [51], and a gamma distribution to model rate heterogeneity among sites. The robustness of the inferred clades was assessed via 1,000 bootstrap replicates [34]. Among the trees evaluated, the tree with the highest log-likelihood value (– 6663.13) was selected. The final phylogenetic analysis comprised 22 amino acid sequences and 218 positions in the analyzed dataset.

5.3 Heterologous expression and purification of recombinant *MrB3*

A synthetic open reading frame (ORF) corresponding to the gene encoding the candidate effector protein *MrB3* was synthesized with codons optimized for expression in *Escherichia coli* and cloned and inserted into the NcoI and XhoI restriction sites of the pET-32a vector, which contains an ampicillin resistance gene and the thioredoxin coding sequence, which was used to express r*MrB3* as a fusion protein (<https://fastbio.com.br/proteina-recombinante/>). The recombinant pET-32a/*MrB3* construct was introduced into competent *E. coli* Rosetta (DE3) cells via heat-shock transformation [104]. Colonies carrying the recombinant plasmid were inoculated into Luria–Bertani medium, and protein expression was subsequently induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mmol L⁻¹.

Following protein expression induction at 37°C for 4 h, cells from approximately 200 mL of culture were transferred into 50-mL Falcon tubes and harvested by centrifugation at 5,000 rpm for 40 min at 4°C. After the supernatant was discarded, the cell pellets were stored at – 20°C until purification. To obtain the soluble fraction, pellets corresponding to approximately 100 mL of culture were resuspended in 10 mL of 1× binding buffer (pH 7.4) without urea, prepared from an 8× binding buffer stock solution and supplemented with 100 µL of lysozyme (50 µM mL⁻¹). The suspension was incubated at room temperature for 30 min and subjected to sonication using an ultrasonic processor (Ultrasonic Processor Gex 130, 130 W) for 20 min, with cycles of 10 s ON and 25 s OFF at 70% amplitude, using a 2-mm probe.

The lysate was then centrifuged at 5,000 rpm for 40 min at 4°C, and the supernatant was collected as the soluble fraction, whereas the pellet was used to obtain the insoluble fraction. To obtain the insoluble fraction, the pellet was resuspended in 10 mL of 1× binding buffer containing 6 M urea supplemented with 100 µL of lysozyme. The suspension was sonicated via the same ultrasonic processor (Ultrasonic Processor Gex 130, 130 W) for 5 min, with cycles of 10 s ON and 30 s OFF at 70% amplitude, using the same 2 mm probe. After sonication, the sample was centrifuged at 4,200 rpm for 40 min at 4°C, and the supernatant was used in the subsequent affinity chromatography purification step. The pellets containing inclusion bodies (insoluble fraction) and the supernatant containing soluble protein were collected and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), followed by staining with 0.08% colloidal Coomassie blue [104].

Recombinant proteins were purified by affinity chromatography via a HisTrap FF crude nickel column (Cytiva) and eluted with elution buffer containing imidazole concentrations ranging from 50 to 500 mmol L⁻¹. The proteins were subsequently dialyzed via a 10-kDa molecular weight cutoff dialysis membrane (Spectra/Por) against Tris–HCl buffer at concentrations of 5 or 10 mmol L⁻¹, depending on the subsequent experiment. Protein concentrations were determined via the Bradford method [9] and confirmed via densitometric analysis of SDS–PAGE bands compared with a bovine serum albumin (BSA) standard stained with Coomassie blue. For densitometric analysis, gels were scanned and analyzed via ImageJ software and the “Gel Analysis” tool, as described by Schneider et al. [106]. The theoretical molecular weight of *rMrB3* was determined via the ProtParam tool (ExPASy) and used to confirm the expected band position on the gel and support the correct identification of the protein of interest [106].

5.4 Mass spectrometry analyses

Mass spectrometry analyses were performed on the recombinant protein *rMrB3* to confirm its identity, and protein fractions were obtained after affinity capture assays were performed via protein extracts from *Theobroma cacao* fruit husks to identify proteins potentially associated with *rMrB3*. Sample preparation was performed via tryptic digestion via a protocol adapted from [127], following strategies previously employed for protein identification in plant–pathogen interaction studies [96, 25].

To confirm the identity of *rMrB3*, a sample of the purified recombinant protein was analyzed. The resulting peptides were subjected to ultrahigh-performance liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) via a 1290 Infinity II UHPLC system coupled to a 6545 LC/Q-TOF mass

spectrometer (Agilent Technologies, Singapore). MS/MS spectra were processed via the Spectrum Mill package integrated into the MassHunter Workstation (Agilent Technologies; revision B.07.08.214) and searched against the NCBI (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) and UniProt (<https://www.uniprot.org/>) databases [120]. For recombinant protein identification, the *Moniliophthora roreri* sequences corresponding to NCBI protein ID ESK97039.1 and UniProt accession V2YZA0 were used.

Recombinant protein identification was assessed on the basis of sequence coverage, the number of unique peptides identified, and protein identification confidence. Relative peptide intensities and their respective individual contributions to sequence coverage were also obtained. The mapping of identified peptides, sequence coverage and relative intensity values, and a summary of LC–MS/MS identification parameters are presented in Fig. S5A–C and Table S3.

To identify *T. cacao* proteins potentially associated with rMrB3, fractions obtained from the affinity capture assay using the recombinant protein were analyzed according to the protocol described by Pirovani et al. [95] (Fig. 5A). The resulting spectra were processed and searched against the NCBI (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) and UniProt (<https://www.uniprot.org/>) databases [120], considering the *Theobroma cacao* protein sequences (Table S3). The identified proteins were classified as potential proteins associated with rMrB3.

5.5 Circular dichroism spectroscopy

The circular dichroism (CD) spectra of the recombinant protein MrB3 (rMrB3) were recorded via a Jasco J-815 spectropolarimeter after the samples were dialyzed against 5 mmol/L Tris–HCl buffer. Measurements were performed over the spectral range of 190–250 nm, with a resolution of 1 nm, using a quartz cuvette with a 1.0-mm optical path length at temperatures of 25°C and 95°C. Each spectrum was obtained by averaging six independent scans to improve the signal-to-noise ratio and data reliability [41].

5.6 Bioassays in model and host plants for functional characterization of the candidate effector

Solanum lycopersicum (tomato) and *Theobroma cacao* (Catongo cultivar, susceptible to *Moniliophthora roreri*) plants were used in the bioassays. In tomato plants, compound leaves consisting of three leaflets were sprayed with 1 mL of recombinant MrB3 protein (rMrB3) at a concentration of 3.2 $\mu\text{mol L}^{-1}$ or with 5 mmol L^{-1} Tris–HCl buffer, pH 7.4 (control). The plants were monitored for 15 days following treatment.

For *T. cacao* cv. Catongo, leaves were treated with purified protein at concentrations of 2.2 and 3.2 $\mu\text{mol L}^{-1}$. A volume of 5 mL of purified protein mixture was applied by submerging the leaves in the mixture. The control leaves were treated with 5 mmol/L Tris–HCl buffer, pH 7.4. Symptoms were evaluated over 20 days.

In both assays, the plants were monitored periodically for wilting, necrosis, chlorosis, and other phenotypic alterations to assess the potential effector activity of the candidate protein rMrB3.

5.7 H₂O₂ accumulation assay

Leaves of *Solanum lycopersicum* were sprayed with recombinant rMrB3 at a concentration of 3.5 µmol/L, whereas control leaves were treated with 5 mmol/L Tris–HCl buffer (pH 7.4). After 24, 48, and 72 h of treatment, leaf discs measuring 1 cm in diameter were collected from treated and control leaves.

The discs were immersed in a 1 mg mL⁻¹ solution of 3,3'-diaminobenzidine (DAB) prepared in distilled water acidified with HCl to pH 3.8. The discs were then vacuum infiltrated for 30 min and subsequently incubated in the same DAB solution for 24 h at room temperature in the dark.

At the end of each incubation period, the discs were boiled in 96% ethanol for 20 min and subsequently washed with 50% ethanol to remove leaf pigments. The formation of a brown precipitate resulting from DAB oxidation in the presence of H₂O₂ was recorded via a Leica EZ4 stereomicroscope at 4× magnification and was used as an indicator of H₂O₂ accumulation.

The intensity of brown staining was subsequently quantified via image analysis via Fiji (ImageJ) software, allowing H₂O₂ accumulation to be compared between treatments and across the different DAB incubation periods.

5.8 Identification of *Theobroma cacao* cv. Catongo target proteins associated with rMrB3 via CNBr-Sepharose affinity capture and LC–MS/MS

To identify the *Theobroma cacao* cv. Catongo proteins potentially associated with recombinant rMrB3, an affinity capture assay was performed in which the recombinant protein was covalently coupled to CNBr-activated Sepharose resin. Total soluble proteins were extracted from approximately 1 g of young *T. cacao* cv. Catongo fruit husks according to the protocol described in [95] and applied in studies identifying proteins associated with *Moniliophthora* effectors [25].

Coupling of rMrB3 to the resin, incubation with the protein extract, washing, and elution were performed according to protocols previously described by Pirovani et al. [96] and Do Carmo Santos et al. [25], with specific adaptations for the recombinant protein used in this study. The eluted fractions were collected and subjected to LC–MS/MS analysis, as described in Section 5.4, “Mass spectrometry analyses,” to identify *T. cacao* proteins potentially associated with rMrB3.

The identified proteins were considered potential proteins associated with rMrB3 since affinity capture followed by LC–MS/MS alone does not confirm direct interactions between the proteins.

5.9 Molecular docking of MrB3 with the *Theobroma cacao* LRR protein (TcLRR)

Structural modeling of the interaction between the candidate effector protein *MrB3* from *Moniliophthora roreri* and the leucine-rich repeat (LRR)-type protein from *Theobroma cacao* (A0A061ECS2) was performed via protein–protein molecular docking with the HADDOCK 2.4 server (High Ambiguity Driven protein–protein DOCKing), a platform widely used for data-driven docking [27, 125].

The three-dimensional protein structures were obtained from models predicted via AlphaFold 3 [126, 53]. For structural preparation, signal peptides previously predicted via SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP>) were removed to represent the mature forms of the proteins. The structures were then prepared via UCSF ChimeraX [89], with water molecules, ions, and other heteroatoms removed, followed by the addition of hydrogen atoms to prepare the structures for molecular docking.

The stereochemical quality of the refined structural models of *MrB3* and *TcLRR* was subsequently assessed via the MolProbity platform (<https://molprobity.biochem.duke.edu/>) through analysis of Ramachandran plots and molecular geometry. The percentages of residues located in favored, allowed, and disallowed regions of the Ramachandran plots were evaluated, along with the overall structural quality parameters provided by the platform, to assess model reliability before molecular docking analyses [133]. The Ramachandran plots and structural validation statistics for the models used in this study are presented in Fig. S7A–D.

The active residues used for docking were defined on the basis of experimental evidence obtained by mass spectrometry (LC–MS/MS). For *MrB3*, residues 186, 190, and 194 were selected, corresponding to experimentally detected and structurally accessible regions. For the LRR protein, residue selection was based on the LASLPSLR peptide, which was experimentally identified via LC–MS/MS and subsequently mapped onto the structure to identify the corresponding region involving residues 359, 361, and 362.

Passive residues were automatically defined by HADDOCK on the basis of the spatial proximity of the selected active residues. Docking was performed using the platform's default parameters. The generated models were clustered according to structural similarity and ranked on the basis of the HADDOCK score, Z score, restraint violation energy, and buried surface area (BSA) [125].

The resulting structures were subsequently analyzed via PyMOL v3.1.8 [108] for molecular visualization, inspection of the protein–protein interface, and identification of residues involved in polar contacts and hydrophobic interactions.

Declarations

Competing interests

The authors declare that a patent application related to the recombinant effector protein *MrB3* described in this study was filed by the Universidade Estadual de Santa Cruz (UESC), Brazil, on 15 September 2026 under application number BR 10 2026 023156 8. The patent application is pending and relates to the

protein, its recombinant production and purification, and its potential application in plant defense modulation. The inventors listed in the patent application are Larissa Karen Silva Oliveira, Carlos Priminho Pirovani, Eric Guimarães Aguiar, Keilane Silva Farias, Taís Araújo Santos, Hermanna Vanesca Viana de Oliveira, and Maria Eduarda Antunes Lima.

Author Contribution

Conceptualization: L.K.S.O., R.J.S.S., E.R.G.R.A., C.P.P.; Methodology: L.K.S.O., T.A.S., M.E.A.L., H.V.V.O., C.H.C.N., K.S.F.; Validation: K.S.F., J.P.C., R.J.S.S., E.R.G.R.A.; Investigation: L.K.S.O., T.A.S., M.E.A.L.; Data curation: L.K.S.O., R.J.S.S., E.R.G.R.A.; Writing - original draft preparation: L.K.S.O., T.A.S.; Writing - review and editing: C.P.P., E.R.G.R.A.; Visualization: L.K.S.O., T.A.S., R.J.S.S.; Supervision: C.P.P., E.R.G.R.A.; Resources, Project administration and Funding acquisition: C.P.P. All authors reviewed and approved the final manuscript.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figures

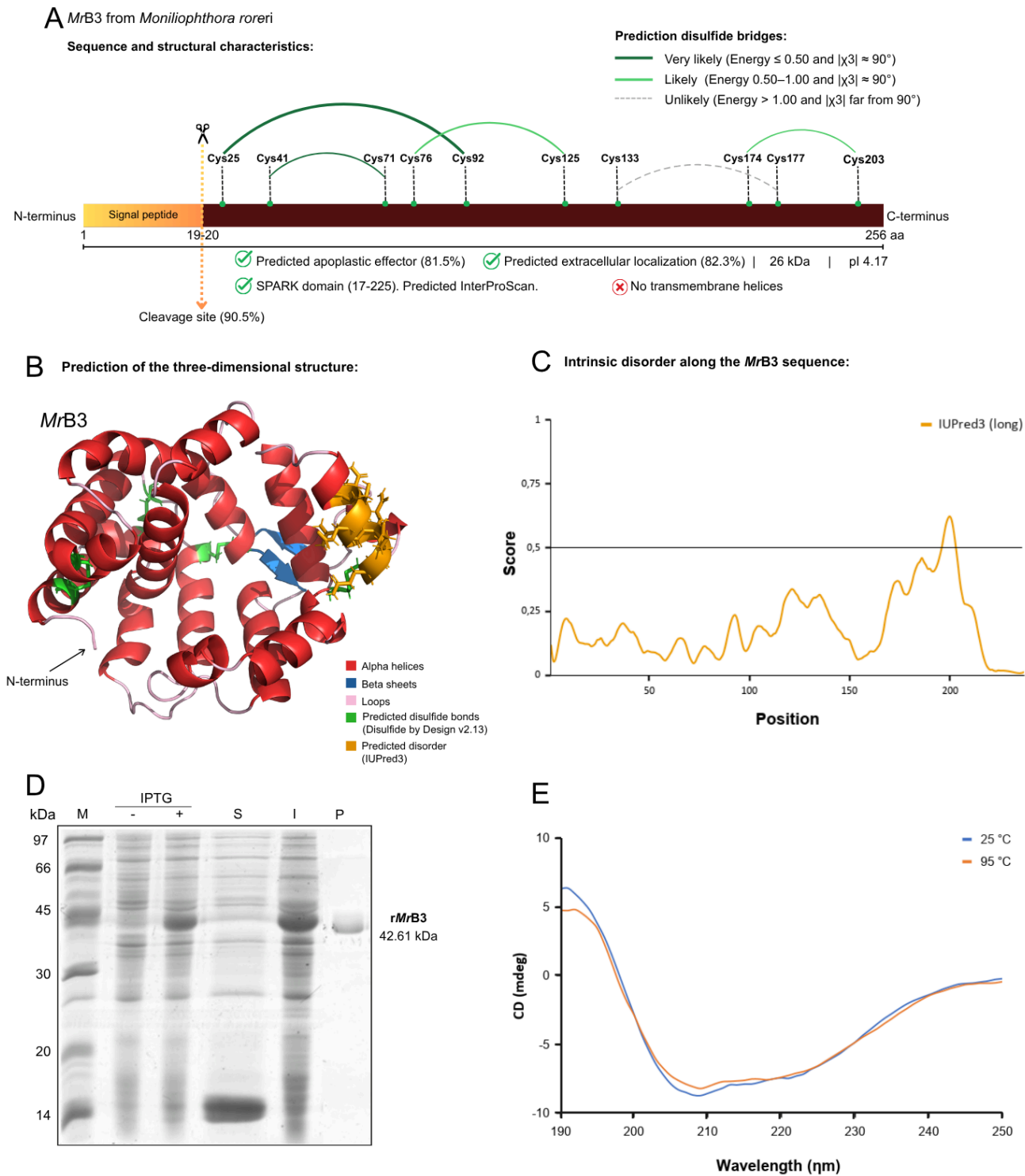


Figure 1

In silico and experimental characterization of the candidate effector protein *MrB3* from *Moniliophthora roreri*. **(A)** Sequence features of the *MrB3* protein, including prediction of the signal peptide, disulfide bonds, extracellular localization, potential activity as an apoplastic effector, the presence of a region belonging to the SPARK domain-containing protein family identified by InterProScan between residues 17–225, and the absence of transmembrane helices. **(B)** Predicted three-dimensional structure of the

mature protein generated by AlphaFold and refined by energy minimization, showing 13 α -helices, two β -sheets, loops, and cysteine residues belonging to the predicted pairs involved in disulfide bond formation (Cys25–Cys92, Cys41–Cys76, Cys71–Cys125, and Cys174–Cys203). **(C)** Prediction of intrinsic disorder in the mature protein via IUPred3. Residues with disorder scores >0.5 were considered intrinsically disordered and corresponded to the Gly196–Asn202 region. **(D)** Expression profile of recombinant rMrB3 analyzed by SDS–PAGE, highlighting the purified band corresponding to the recombinant protein (42.61 kDa). M, molecular mass marker; (–), total extract without IPTG induction (negative control); (+), total extract after IPTG induction (pET-32a:MrB3); (S), soluble fraction; (I), insoluble fraction; (P), purified protein. **(E)** Circular dichroism (CD) spectra of rMrB3 obtained at 25 °C (blue) and 95 °C (orange), demonstrating the thermal stability of the protein.

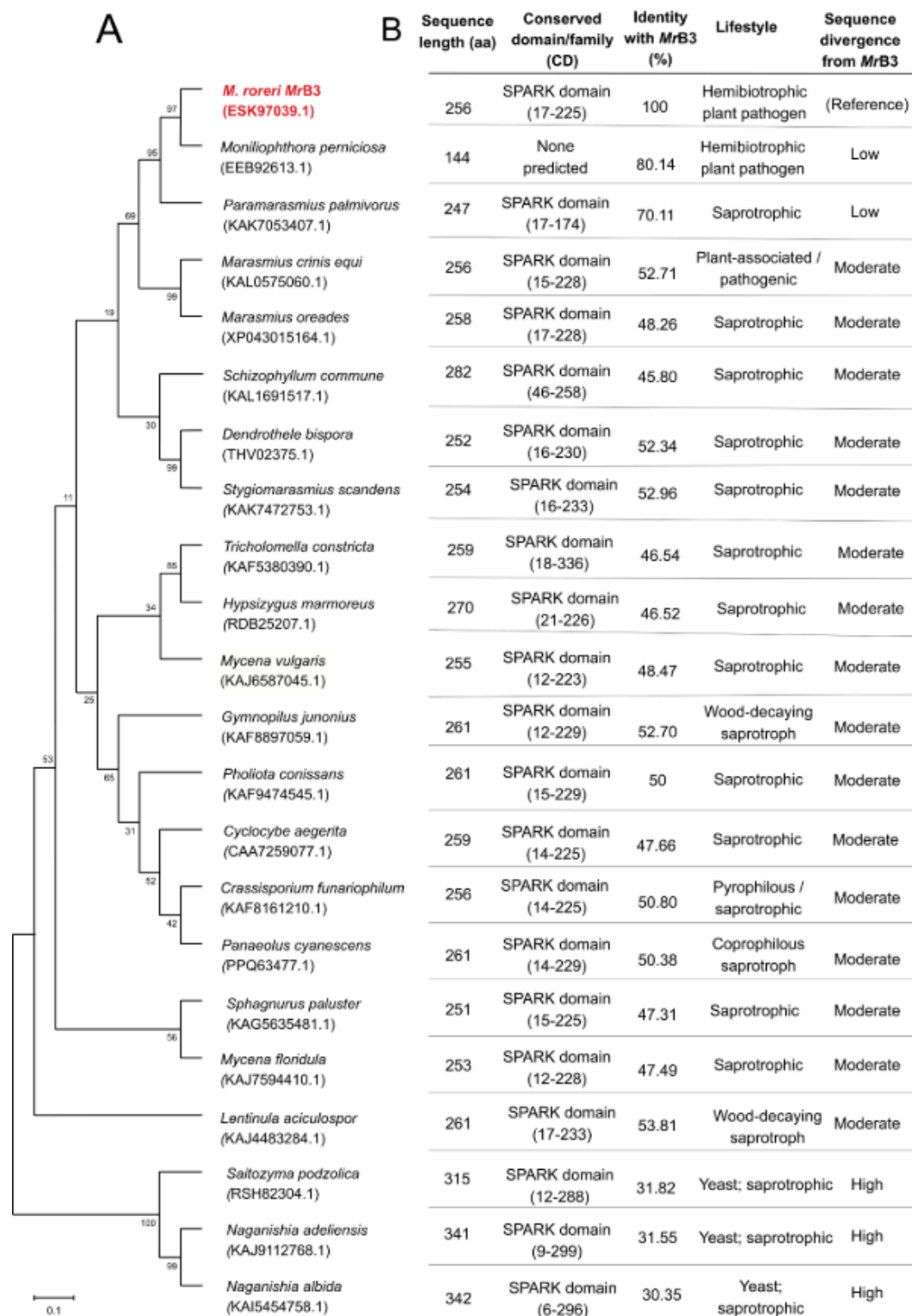


Figure 2

Phylogenetic analysis and *in silico* characterization of proteins homologous to MrBASIDIN3 (MrB3) from *Moniliophthora roreri*. **(A)** Phylogenetic tree inferred via the maximum likelihood method with the JTT model and 1,000 bootstrap replicates. The values at the nodes indicate bootstrap support, and the scale bar represents the number of substitutions per site. Mature sequences were used after removal of the predicted signal peptides. The ingroup comprises MrB3 and homologous proteins from Basidiomycota,

whereas *Saitozyma podzolica*, *Naganishia adeliensis*, and *N. albida* were used as the outgroup to root the tree. **(B)** Predicted protein features, including sequence length, protein domain/family identified by InterProScan, sequence identity to *MrB3* determined by BLASTp, lifestyle, and sequence divergence from *MrB3*. *MrB3* is highlighted in red.

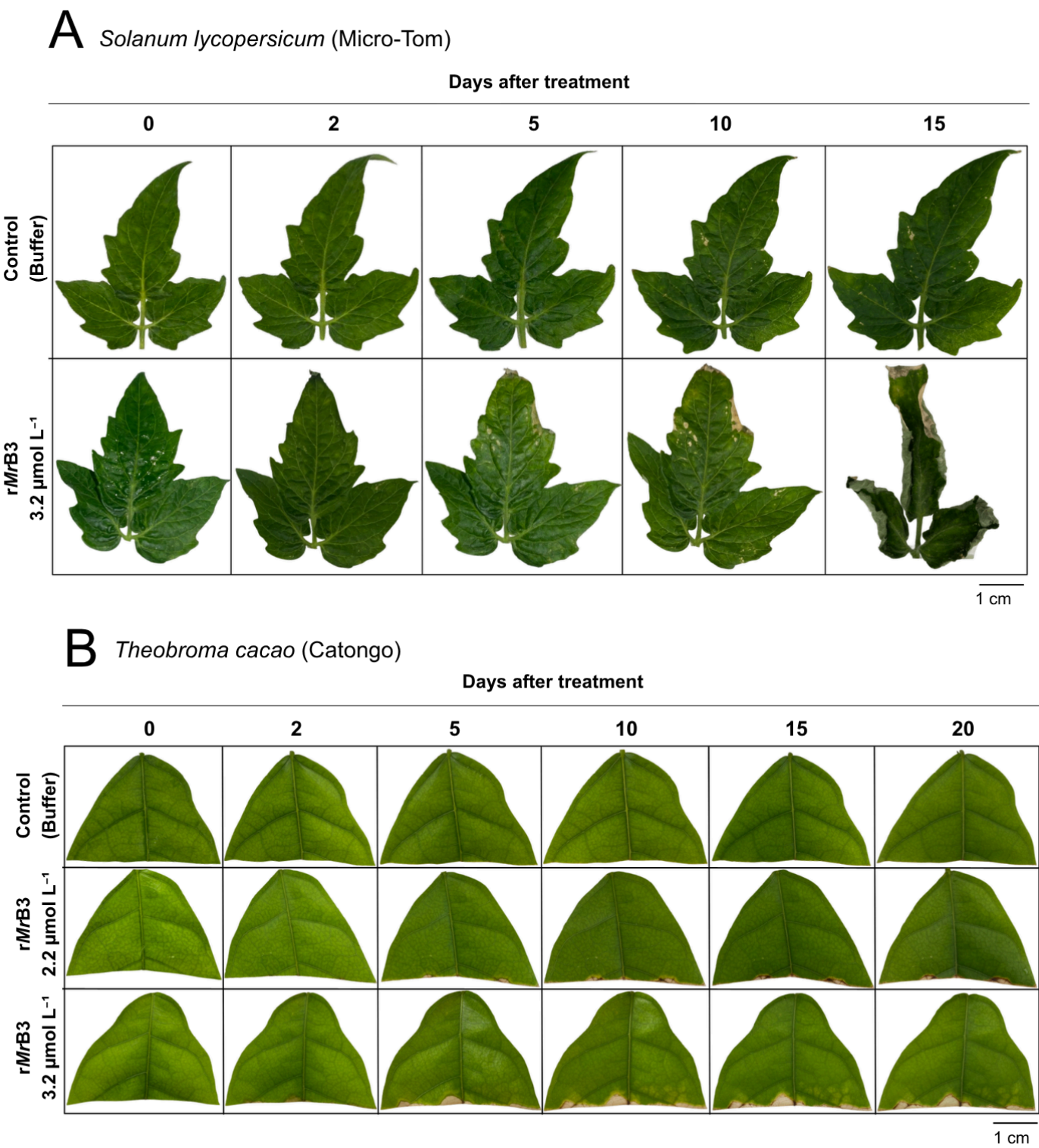


Figure 3

Phenotypic changes induced by rMrB3 in the leaves of host and nonhost plants. **(A)** Leaves of *Solanum lycopersicum*(Micro-Tom) treated with rMrB3 (3.2 $\mu\text{mol L}^{-1}$) or the control were evaluated for 15 days after application. **(B)** Leaves of *Theobroma cacao* (Catongo genotype) treated with rMrB3 (2.2 and 3.2 $\mu\text{mol L}^{-1}$) or the control, showing concentration- and time-dependent symptoms. Scale bars = 1 cm.

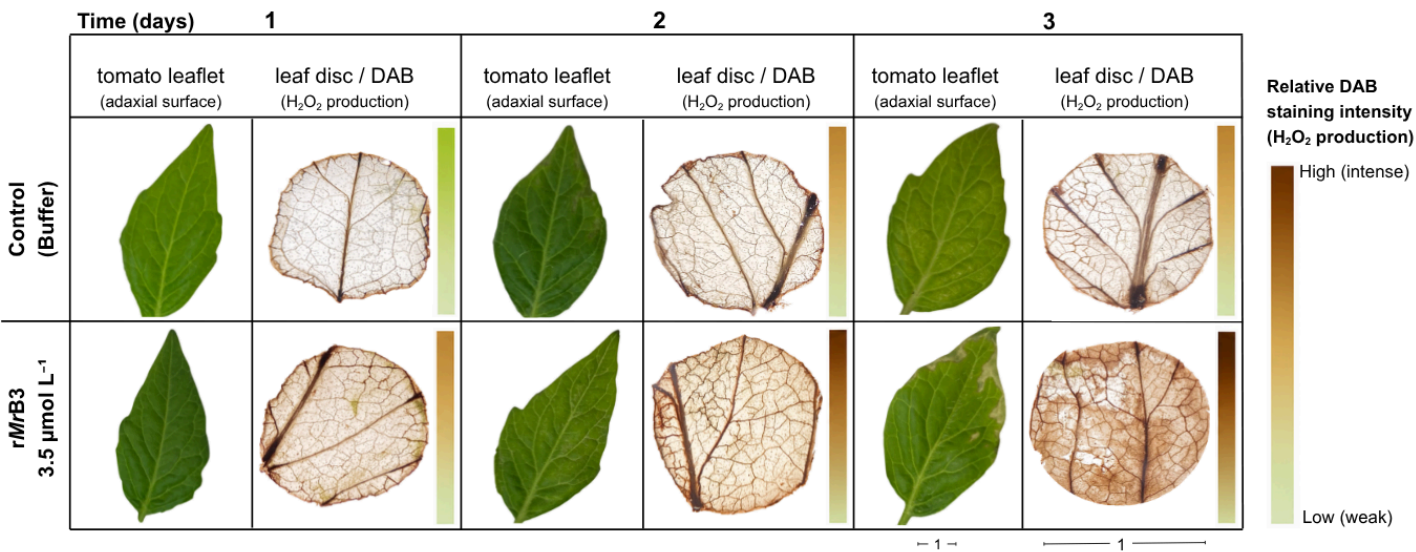


Figure 4

Accumulation of H₂O₂ in *Solanum lycopersicum* leaves following treatment with rMrB3. Leaves treated with rMrB3 (3.5 $\mu\text{mol L}^{-1}$) were compared with those treated with 5 mmol L⁻¹ Tris-HCl buffer (pH 7.4) as the control at 1, 2, and 3 days after treatment. H₂O₂ production was detected by 3,3'-diaminobenzidine (DAB) staining of leaf discs obtained from treated leaves. The intensity of brown staining was used as an indicator of H₂O₂ accumulation and is represented by a visual intensity scale next to each leaf disc. The staining intensity was quantified via Fiji (ImageJ), which revealed greater H₂O₂ accumulation in leaves from plants treated with rMrB3 than in those from control plants throughout the experimental period.

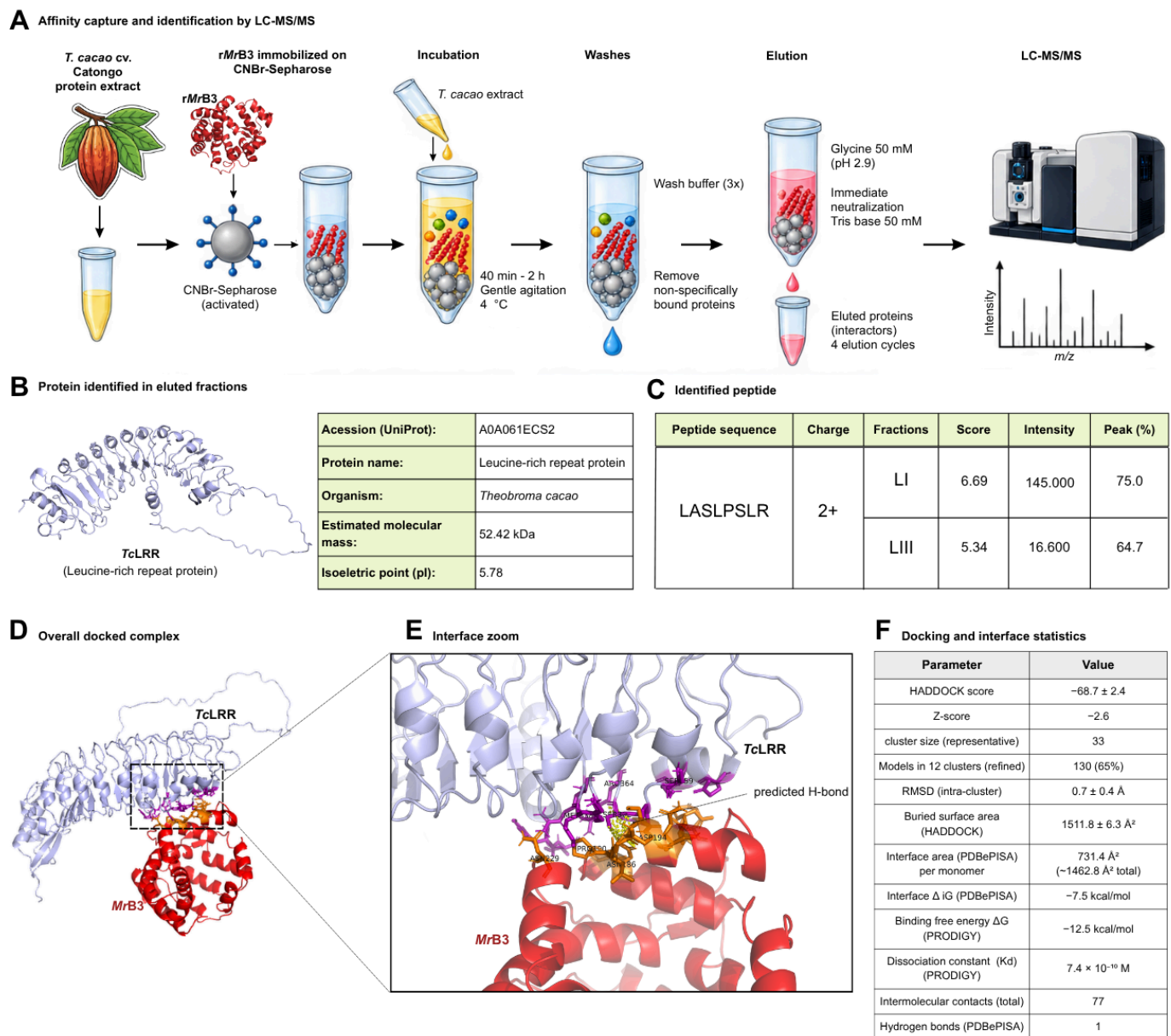


Figure 5

Identification of a *Theobroma cacao* protein associated with rMrB3 and structural prediction of its interaction with MrB3 **(A)** Affinity capture assay of *T. cacao* cv. Catongo proteins using rMrB3 immobilized on CNBr-Sepharose, followed by elution and identification by LC-MS/MS. **(B)** Leucine-rich repeat-containing protein (TcLRR; A0A061ECS2), with an estimated molecular mass of 52.42 kDa and a pI of 5.78. **(C)** LASLPSLR peptide (2+) identified in fractions LI and LIII, with scores of 6.69 and 5.34 and intensities of 145,000 and 16,600, respectively. **(D)** MrB3–TcLRR complex obtained by molecular docking via HADDOCK; MrB3 is shown in red, and TcLRR is shown in light blue. **(E)** Predicted interface highlighting contact residues and the predicted hydrogen bond between Asn186 of MrB3 and Ser362 of TcLRR; the LASLPSLR peptide is located within the interface region. **(F)** Structural and energetic parameters of the complex obtained via HADDOCK, PDBePISA, and PRODIGY. These results identify

TcLRR as a candidate protein associated with *rMrB3* and provide structural support for a possible molecular interaction.

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