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ORIGINAL ARTICLE

Immune recognition of the secreted serine protease ChpG restricts the host range of *Clavibacter michiganensis* from eggplant varieties

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Abstract

Bacterial wilt and canker caused by *Clavibacter michiganensis* (Cm) inflict considerable damage in tomato-growing regions around the world. Cm has a narrow host range and can cause disease in tomato but not in many eggplant varieties. The pathogenicity of Cm is dependent on secreted serine proteases, encoded by the *chp/tomA* pathogenicity island (PI), and the pCM2 plasmid. Screening combinations of PI deletion mutants and plasmid-cured strains found that Cm-mediated hypersensitive response (HR) in the Cm-resistant eggplant variety Black Queen is dependent on the *chp/tomA* PI. Singular reintroduction of PI-encoded serine proteases into $Cm^{\Delta PI}$ identified that the HR is elicited by the protease ChpG. Eggplant leaves infiltrated with a *chpG* marker exchange mutant (CmΩ*chpG*) did not display an HR, and infiltration of purified ChpG protein elicited immune responses in eggplant but not in Cm-susceptible tomato. Virulence assays found that while wild-type Cm and the CmΩ*chpG* complemented strain were nonpathogenic on eggplant, CmΩ*chpG* caused wilt and canker symptoms. Additionally, bacterial populations in CmΩ*chpG*-inoculated eggplant stem tissues were c.1000-fold higher than wild-type and CmΩ*chpG*-complemented Cm strains. Pathogenicity tests conducted in multiple Cm-resistance eggplant varieties demonstrated that immunity to Cm is dependent on ChpG in all tested varieties, indicating that ChpG-recognition is conserved in eggplant. ChpG-mediated avirulence interactions were disabled by alanine substitution of serine231 of the serine protease catalytic triad, suggesting that protease activity is required for immune recognition of ChpG. Our study identified ChpG as a novel avirulence protein that is recognized in resistant eggplant varieties and restricts the host range of Cm.

KEYWORDS
avirulence protein, bacterial wilt and canker of tomato, *Clavibacter*, eggplant, host specificity, plant immunity, serine protease

1 | INTRODUCTION

Clavibacter is a genus of plant-associated actinobacteria (Li et al., 2018). The genus is composed of multiple pathogenic species, which usually possess high host specificity and are restricted to a small number of host plant species (Nandi et al., 2018). Diseases caused by *Clavibacter* spp. include bacterial wilt and canker of tomato (*C. michiganensis*), potato ring rot (*C. sepedonicus*), Gross's wilt of maize (*C. nebraskensis*), bacterial canker of pepper (*C. capsici*), bacterial wilt of alfalfa (*C. insidiosus*), bacterial mosaic of wheat (*C. tessellarius*), bean leaf yellowing (*C. phaseoli*), and leaf brown spot of barley (*C. zhangzhiyongii*) (Nandi et al., 2018; Tian et al., 2021). In particular, *Clavibacter*-mediated diseases significantly affect the tomato, maize, and potato industries, resulting in substantial yield losses worldwide (Hukkanen et al., 2005; Jackson et al., 2007; Peritore-Galve et al., 2021). Despite their economic importance, pathogenicity and host specificity determinants of these bacteria are not well characterized and resistance loci that confer immunity to *Clavibacter* spp. have yet to be identified.

Bacterial wilt and canker caused by *C. michiganensis* (Cm) is one of the most important diseases of tomato (*Solanum lycopersicum*). The disease is characterized by the appearance of spreading canker lesions in the main stem and secondary branches, leaf wilting, leaf tissue necrosis, and the appearance of "bird's-eye" spotting on fruit (Peritore-Galve et al., 2021). Vascular collapse, which results in death, is frequent in heavily infected plants (Peritore-Galve et al., 2021). Latent infection, which enables further spread into neighbouring plants and contaminated seeds, is common as well (Gitaitis et al., 1991). Cm spreads throughout the plant by systemically colonizing host xylem vessels and bacteria can reach 10^{10} colony-forming units (cfu) per gram plant tissue (Chalupowicz et al., 2012), which results in vascular collapse and leaf wilting due to blockage of water-conducting elements. Cm bacteria are usually introduced into fields or greenhouses through contaminated seed lots and spread to neighbouring plants through water splash or human-based transfer of Cm-contaminated guttation fluid released by the hydathodes (Sharabani et al., 2013; Tsiantos, 1987). Because no commercial Cm-resistant tomato varieties are available, the disease is mainly controlled by horticultural practices and extensive screening for seed contamination (Blank et al., 2016; de León et al., 2011).

Multiple wild tomato varieties and other nonhost solanaceous plants exhibit resistance or high tolerance to Cm. Resistance quantitative trait loci (QTLs) were identified in *Solanum peruvianum* and *Solanum habrochaites* (Coaker & Francis, 2004; van Heusden et al., 1999). However, backcross attempts into domesticated tomatoes only conferred partial resistance to the pathogen (Coaker & Francis, 2004; van Heusden et al., 1999). A recent study aimed at characterizing eggplant (*Solanum melongena*) susceptibility to Cm identified that numerous eggplant varieties demonstrate moderate to high resistance to Cm (Boyaci et al., 2021). In addition, reciprocal crosses between susceptible and resistant varieties followed by segregation analyses identified that resistance to Cm is a dominant trait that follows Mendelian-like segregation, indicating that eggplant resistance to Cm is likely to be linked to a single locus (Boyaci et al., 2021).

The main virulence determinants encoded by Cm are localized in the 129 kb *chp/tomA* pathogenicity island (PI) and two plasmids, pCM1 (c.27 kb) and pCM2 (c.70 kb) (Gartemann et al., 2008; Meletzus & Eichenlaub, 1991). The *chp/tomA* PI is composed of two genomic regions: the 79 kb *chp* subregion has a low gene density (putative coding capacity of 45.3%) and mainly encodes secreted hydrolases such as proteases, pectinases, and glycosyl hydrolases (Gartemann et al., 2008). The 50 kb *tomA* subregion has a high gene density (putative coding capacity of 96.3%) and mostly encodes genes associated with perception and uptake of metabolites and carbohydrates (Gartemann et al., 2008). Two independent clones harbouring a deletion of the entire *chp/tomA* PI were identified in Cm NCPPB 382 (Chalupowicz et al., 2010; Gartemann et al., 2008). Both clones are unable to properly colonize or cause symptoms in tomato (Chalupowicz et al., 2010; Gartemann et al., 2008). Curing of pCM1 or pCM2 does not significantly affect localized colonization at the inoculation site but has a major effect on distal colonization and symptom development, which indicates that pCM1- and pCM2-encoded virulence factors are required for xylem spread of the bacterium (Chalupowicz et al., 2012; Meletzus et al., 1993). Multiple genomic studies identified that the *chp/tomA* PI and pCM1-like plasmid occur in all currently sequenced pathogenic Cm clones, further validating their importance of virulence (Méndez et al., 2020; Thapa et al., 2017). Cm lacks any known translocation apparatus and mainly depends on Sec-dependent secreted carbohydrate-active enzymes and serine proteases to facilitate pathogenic interaction with the host (Chalupowicz et al., 2017; Jahr et al., 2000; Thapa et al., 2017). A conserved family of putative secreted serine proteases that share homology to the pCM2-encoded Pat-1 protein play an important role in the virulence of Cm. Pat-1 homologs are of c.280 amino acids and harbour an N-terminal signal peptide and a C-terminal serine proteases trypsin-type domain (Stork et al., 2008). Cm NCPPB 382 encodes seven functional Pat-1 homologs: three are encoded in the pCM2 plasmid (*Pat-1*, *PhpA*, *PhpB*) and four (*ChpC*, *ChpE*, *ChpF*, *ChpG*) are encoded by the *chp* subregion of the *chp/tomA* PI (Gartemann et al., 2008). Pat-1 homologs have been found to be enriched in the secretome of Cm grown in rich and xylem-mimicking media, which indicates they are indeed secreted proteins (Savidor et al., 2012). In addition, Pat-1 homologs are transcriptionally and translationally induced in planta and in xylem-mimicking media and additively contribute to the virulence of Cm (Chalupowicz et al., 2010, 2017; Savidor et al., 2012, 2014). Pat-1 and the Pat-1 homolog ChpC are particularly important for the virulence of Cm. A *chpC* inactivation mutant is impaired in host colonization and is unable to cause disease symptoms (Stork et al., 2008) while *Pat-1* is the main pCM2-encoded virulence factor and is required for symptom development and systemic colonization of tomato (Chalupowicz et al., 2012; Dreier et al., 1997). Pat-1 homologs function as virulence factors in other *Clavibacter* spp. and ChpG of *C. capsici* and Chp-7 *C. sepedonicus* play a significant role in pathogenesis on pepper and eggplant (Hwang et al., 2020; Nissinen et al., 2009). Besides their role in virulence,

Pat-1 homologs have been reported to elicit localized cell death in nonhost plants. Specifically, Chp-7 of *C. sepedonicus* and ChpG of Cm elicit hypersensitive response (HR)-like cell death on *Nicotiana tabacum* and *Mirabilis jalapa* (Lu et al., 2015; Nissinen et al., 2009; Stork et al., 2008). This indicates that these proteases potentially play a role in host specificity through their recognition in nonhost plants.

In this study, we identified that the Pat-1 homolog ChpG functions as a host specificity determinant that restricts Cm from colonizing certain eggplant varieties through elicitation of host-specific immune responses.

2 | RESULTS

2.1 | The *chp/tomA* PI is required for immune recognition of Cm in eggplant

The host range of Cm is mostly restricted to tomato and pepper; the bacteria cannot cause disease in potato and in numerous varieties of eggplant (Boyaci et al., 2021; Yim et al., 2012). We speculated that immune recognition plays a role in determining the host range of Cm. To test this, we examined whether Cm induces selective HR localized cell death, a hallmark of immune activation, in eggplant. Tomato (cv. Moneymaker), pepper (cv. Early California Wonder), and eggplant (cv. Black Queen) leaves were infiltrated with suspensions of Cm and monitored for the appearance of HR and disease symptoms. HR was not observed as of 72 h postinoculation (hpi) in tomato and pepper (Table 1 and Figure S1), and infiltrated tissues developed chlorotic symptoms that later became necrotic between 5 and 10 days postinoculation (dpi), similar to the leaf symptoms reported by Chalupowicz et al. (2017). On the other hand, eggplant leaves inoculated with Cm developed HR-like localized cell death between 36 and 72 hpi (Table 1 and Figure S1).

Host-specific immune responses are usually facilitated through recognition of secreted virulence effectors. Most secreted virulence effector proteins of Cm are encoded within the *chp/tomA* PI and the plasmids pCM1 and pCM2 (Gartemann et al., 2008). Therefore, we hypothesized that these genomic regions are likely to encode an avirulence effector. To test this, eggplant leaves were infiltrated with a series of Cm mutant strains, carrying combinations of deletions of the *chp/tomA* PI and curing of pCM1 or pCM2 plasmids (Table 1 and Figure S1), and monitored for HR. The occurrence of pCM1 or pCM2 did not affect HR induction in eggplant. On the other hand, two independent deletion mutant strains of the *chp/tomA* PI failed to elicit HR in eggplant (Table 1 and Figure S1). Similar to the wild-type Cm strain, none of the mutants affected HR elicitation in tomato or pepper (Table 1 and Figure S1). The analyses suggests that immunity of eggplant to Cm is mediated by recognition of a *chp/tomA* PI-encoded protein.

2.2 | The protease effector ChpG induces host-specific HR in eggplant

The *chp* region of the *chp/tomA* PI encodes a large number of secreted hydrolases that potentially could be recognized in eggplant as immune elicitors. In particular, it encodes four secreted proteases of the Pat-1 family (*chpC*, *chpE*, *chpF*, and *chpG*; Figure 1a), of which specific members were previously reported to elicit HR-like cell death in certain nonhost genotypes (Lu et al., 2015; Nissinen et al., 2009; Stork et al., 2008). Elicitation of an HR in eggplant by the four *Pat-1* homologs was examined by singular introduction of each homolog into the *chp/tomA* PI deletion strain CMM30-18 ($Cm^{\Delta PI}$) and using marker exchange mutant strains of the *Pat-1* homologs in the background of CMM101 (referred to throughout the manuscript as Cm). The four Pat-1 homologs along with their putative promoter regions (250–1000 bp upstream of the start codon) were amplified from Cm, cloned into the pHN216 *Escherichia coli–Clavibacter* shuttle vector (Laine et al., 1996), and introduced into $Cm^{\Delta PI}$. Transcript accumulation of the introduced *Pat-1* homologs in $Cm^{\Delta PI}$ was confirmed by reverse transcription (RT)-PCR (Figure S2). Infiltration of eggplant leaves with $Cm^{\Delta PI}$ carrying a plasmid encoding *ChpG* restored the ability to elicit an HR (Figure 1b). $Cm^{\Delta PI}$ carrying *ChpC*, *ChpE* or *ChpF* failed to elicit an HR in eggplant in a similar manner to $Cm^{\Delta PI}$ (Figure 1b). Eggplant leaves infiltrated with *chpC*, *chpE* and *chpF* marker-exchanged mutants developed an HR in the same intensity and kinetics as the wild-type Cm (Figure 1c) while eggplant leaves infiltrated with the *chpG* marker-exchanged mutant (CmΩ*chpG*) failed to develop an HR by 72 hpi (Figures 1c and 2a). In addition, CmΩ*chpG* infiltrated leaves exhibited chlorotic symptoms

TABLE 1 Induction of hypersensitive response (HR) on solanaceous crops by *Clavibacter michiganensis* mutant strains

Genotype	Strain name	pCM1	pCM2	PI	Tomato	Pepper	Eggplant
Cm	NCPPB 382	+[a]	+	+	N[b]	N	HR[b]
Cm^{-pCM2}	CMM101	+	−	+	N	N	HR
Cm^{-pCM1}	CMM102	−[a]	+	+	N	N	HR
$Cm^{-pCM1,-pCM2}$	CMM100	−	−	+	N	N	HR
$Cm^{\Delta PI,-pCM2}$	CMM30-18	+	−	−	N	N	N
$Cm^{\Delta PI}$	CMM27	+	+	−	N	N	N

[a]+ or − indicates the presence/absence of the pCM1, pCM2, and *chp/tomA* pathogenicity island (PI) in the strain.

[b]HR, localized cell death was observed within 72 h after infiltrating OD_{600} = 0.1 of the indicated strain; N, no response was observed.

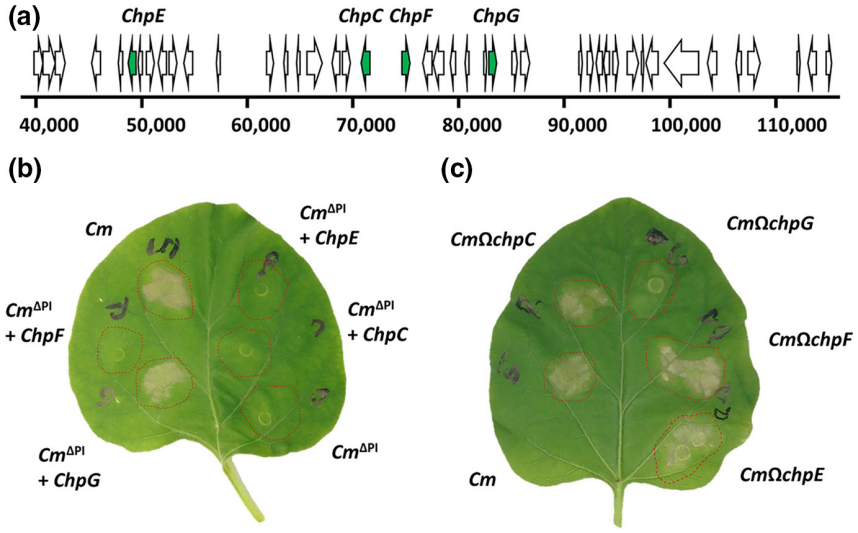

FIGURE 1 Hypersensitive response (HR) in eggplant is mediated by a pathogenicity island (PI)-encoded *Pat-1* homolog. (a) Physical map of the *chp* genomic island of Cm NCPPB 382 (38,000–117,000 region). Open reading frames encoding *Pat-1* homologs are marked in green. (b) *Clavibacter michiganensis* (Cm) CMM30-18 ($Cm^{\Delta PI}$), a *chp/tomA* island deletion strain, carrying pHN216 plasmids encoding for the indicted *Pat-1* homologs were syringe infiltrated (10^8 cfu/ml) into Black Queen eggplant. Pictures were taken 72 h later. (c) CMM101 (Cm) strains harbouring insertional inactivation of the indicated *Pat-1* homologs were syringe infiltrated (10^8 cfu/ml) into Black Queen eggplant. Pictures were taken 72 h later. Data was repeated in three independent experiments. Five plants were used for each experimental repeat

that later became water-soaked and necrotic around 6–10 dpi (Figure S3). HR cell death was further validated by quantification of ion leakage (Figure 2b).

Elicitation of immune responses that result in HR usually correlates to the restriction of bacterial spread (Balint-Kurti, 2019), therefore we monitored leaf bacterial colonization in eggplant and identified that CmΩ*chpG* populations were c.50-fold higher at 6 dpi compared to the wild-type Cm strain (Figure 2c). Introduction of pHN216:*ChpG* complementation vector into CmΩ*chpG* reduced bacterial leaf colonization to the levels of the wild-type Cm strain and restored HR elicitation in eggplant (Figure 2a–c). To confirm that the data represent a host-specific avirulent interaction, leaf infiltration tests were repeated in susceptible tomato. The experiments showed that inactivation of *chpG* affected neither HR elicitation nor leaf colonization in tomato (Figure 2a–c). The data suggest that ChpG is an elicitor of host-specific immune responses in eggplant.

2.3 | Cm *chpG* mutant is pathogenic on eggplant

Immune recognition of secreted virulence effectors is common in resistant plants. However, effector recognition is not the only factor that determines host range, which can be dictated by a combination of numerous factors such as host physiology, metabolic state, production of secondary chemical compounds, structural variations in target tissues, and altered host physical barriers (van den Bosch et al., 2020; Favaro et al., 2014; Pizarro et al., 2020; Stice et al., 2020; Sun et al., 2011).

We aimed to decipher whether ChpG recognition is the main determinant that restricts Cm from causing disease in eggplant. To do so, eggplant and susceptible tomato plants were inoculated with Cm, CmΩ*chpG*, and the complemented strain and monitored for disease development and systemic colonization. Four-leaf stage eggplant and tomato plants were inoculated with the aforementioned Cm strains by puncturing the main stem area between the cotyledons with a wooden toothpick soaked in Cm suspensions (5×10^7 cfu/ml). Inoculated plants were monitored for a duration of 10 days and quantified for the severity of wilting (according to the percentage of wilted/leaf blotched leaves), canker development (according to the speared of the canker lesion), and stem bacterial colonization.

Tomato plants inoculated with the three strains developed wilt and canker symptoms, and stem bacterial populations 1 cm above the puncher site reached 10^9–10^{10} cfu/g stem at 10 dpi (Figure S4). No significant difference was observed in the severity of disease symptoms and bacterial colonization between the *chpG* mutant and the wild-type strain in tomato (Figure S4).

Wild-type Cm and the *ChpG*-complemented strain (CmΩ*chpG* + ChpG) were nonpathogenic on eggplant and bacterial colonization only reached c.10^6 cfu/g stem (Figure 3a,b). The strains did not cause any significant wilting or leaf blotch symptoms (Figure 3c,d), and canker lesions were localized to the inoculated areas and did not significantly spread throughout the main stem (Figure 3e,f). On the other hand, eggplants inoculated with CmΩ*chpG* developed wilt and leaf blotch symptoms in 50%–100% of the leaves (Figure 3a,c,d), large expanded canker lesions (Figure 3e,f), and eruptions of independent canker lesions on the upper stem regions and leaf petioles. In addition, CmΩ*chpG* populations in the stem area 1 cm above the puncher site were around c.1000-higher than the wild-type and complemented strains and reached 5×10^8–10^{10} cfu/g stem (Figure 3b).

Taken together, the results demonstrate that *ChpG* is a bona fide avirulence gene that restricts the host range of Cm on eggplant.

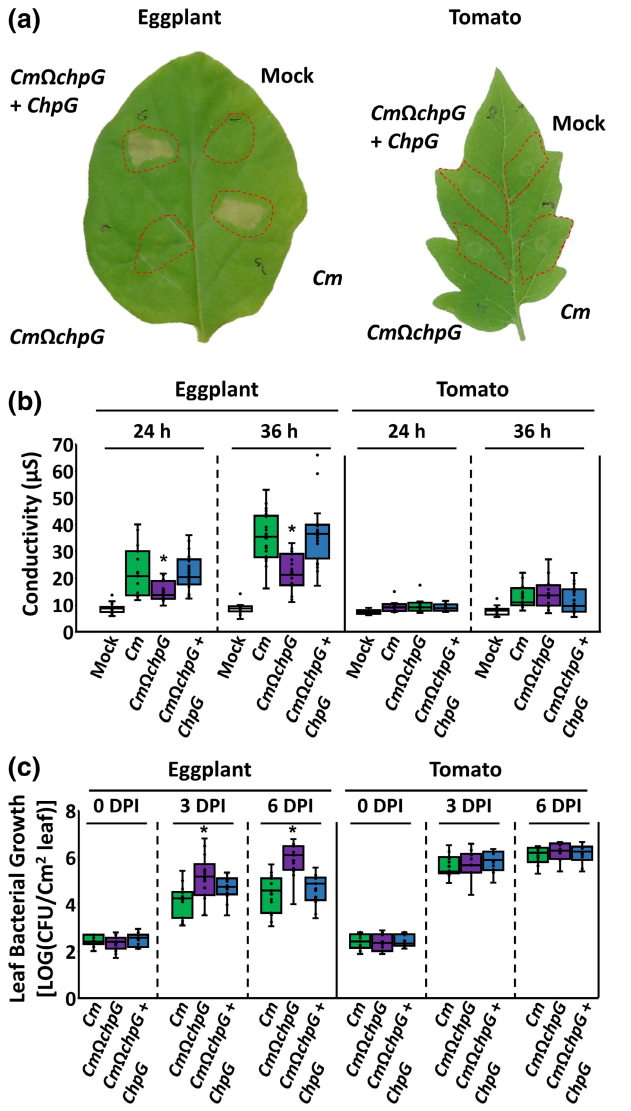

FIGURE 2 ChpG is required for elicitation of hypersensitive response (HR) in eggplant. Black Queen eggplant and Moneymaker tomato leaves were infiltrated (10^8 cfu/ml for a and b, 10^4 cfu/ml for c) with *Clavibacter michiganensis* wild-type CMM101 (Cm), CMM101 *chpG* inactivation mutant (CmΩ*chpG*), and CmΩ*chpG* carrying pHN216:*ChpG* (CmΩ*chpG* + *ChpG*). (a) Leaves were photographed 72 h after infiltration. (b) Cell death was quantified by ion leakage at 24 and 36 h postinfiltration. Lower and upper quartiles are marked at the margins of the boxes. Central lines and "o" represent medians and data points of at least 15 (for eggplant) or 10 (for tomato) biological repeats collected from two (tomato at 24 h) or three independent experiments. * indicates significant difference (Mann–Whitney *U* test, $p \leq 0.05$) from Cm. (c) Leaf apoplast bacterial populations of the indicated Cm strains were quantified at 0, 3, and 6 days postinoculation (dpi). Lower and upper quartiles are marked at the margins of the boxes. Central lines and "o" represent medians and data points of 15 (for eggplant) or 10 (for tomato) biological repeats collected from two (tomato) or three (eggplant) independent experiments. * indicates significant difference (Mann–Whitney *U* test, $p \leq 0.05$) from Cm

2.4 | Immune recognition of ChpG is dependent on its catalytic activity

Recognition of avirulent proteins is usually associated with plant immune receptors that can directly recognize the effector through protein–protein interaction or indirectly recognize it through its activity on a guardee or a decoy target (Kourelis & van der Hoorn, 2018). The following experiments aimed to identify the mode of ChpG-mediated immune recognition in eggplant. First, recognition of ChpG as a standalone elicitor was examined. *ChpG* was fused to a maltose-binding protein (MBP) tag and purified from *Escherichia coli* (Figure 4a, western blot validation is presented in Figure S5). Eggplant and tomato leaves were infiltrated with purified MBP or MBP-ChpG and monitored for elicitation of immune responses through visual inspection of localized cell death, quantification of cell death by ion leakage, and quantification of secreted peroxidase (POX) activity, which was previously reported to be induced in response to innate immune activation in plants (Daudi et al., 2012; Felix et al., 1999; Seto et al., 2020). MBP-ChpG-infiltrated but not MBP-infiltrated eggplant leaf areas developed localized cell death 24–48 h postinfiltration (Figure 4b), which was accompanied by increased ion leakage and POX activity (Figure 4c,d). No difference was observed between MBP and MBP-ChpG infiltrated regions in tomato (Figure 4b–d). Next, we tested whether ChpG recognition is mediated by its catalytic activity. Sequence-based function and structure modulation analyses conducted using NCBI conserved domain (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and SWISS MODEL (https://swissmodel.expasy.org/) predicted ChpG to be a trypsin-like serine protease that harbours an Asp/His/Ser catalytic triad, which was conserved among the other six Cm-encoded Pat-1 homologs (Figure S6). To inactivate the putative catalytic activity of ChpG, the conserved catalytic Ser231 residue was substituted with alanine, and protein was purified (Figure 4a) and infiltrated into eggplant and tomato leaves. The S231A substitution abolished immune elicitation by ChpG and MBP-$ChpG_{S231A}$ neither caused cell death nor promoted POX activity (Figure 4b–d), which indicates that catalytic activity is required for immune recognition of ChpG.

We further validated our results by the introduction of catalytically inactive $ChpG_{S231A}$ into CmΩ*chpG* and monitoring complementation through HR elicitation, symptom development, and stem bacterial colonization. *ChpG* and $ChpG_{S231A}$ fused to HA-tag under the control of the *pCMP1* promoter, which was previously reported to confer strong constitutive expression in Cm (Chalupowicz et al., 2012; Stevens et al., 2021), were introduced into CmΩ*chpG*. Western blot analysis validated that $ChpG^{HA}$ and $ChpG^{HA}_{S231A}$ were expressed and accumulated in similar amounts in CmΩ*chpG* (Figure 5a). As expected, the introduction of $ChpG^{HA}$ into CmΩ*chpG* restored the ability to elicit HR upon infiltration into eggplant leaves similarly to wild-type Cm whereas the introduction of $ChpG^{HA}_{S231A}$ failed to do so (Figure 5b,c). Additionally, expression of $ChpG^{HA}$ but not $ChpG^{HA}_{S231A}$ in CmΩ*chpG*

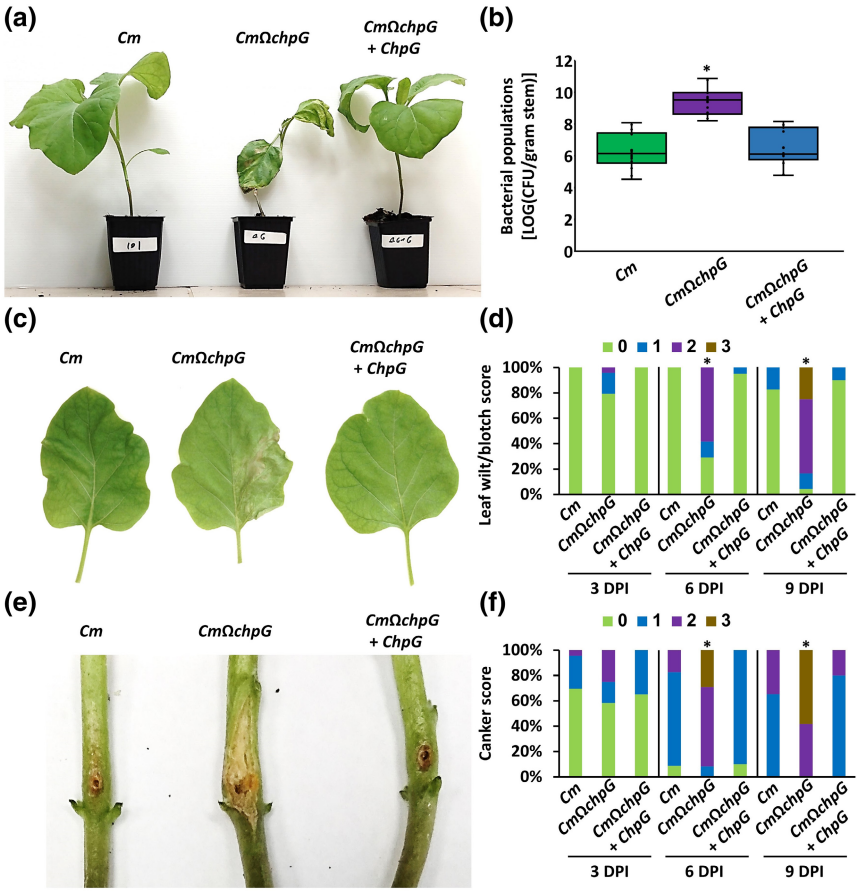

FIGURE 3 *chpG* inactivation mutant is pathogenic on eggplant. Four-leaf stage Black Queen eggplants were inoculated with *Clavibacter michiganensis* wild-type CMM101 (Cm), CMM101 *chpG* inactivation mutant (CmΩ*chpG*), and CmΩ*chpG* carrying pHN216:*ChpG* (CmΩ*chpG* + *ChpG*) by puncturing the stem area between the cotyledons with a wooden toothpick soaked in Cm solution (5×10^7 cfu/ml). (a) Picture was taken 10 days postinoculation (dpi). (b) Stem bacterial populations 1 cm above the inoculation site at 10 dpi. Lower and upper quartiles are marked at the margins of the boxes. Central lines and "o" represent medians and data points of 14 biological repeats collected from four independent experiments. * indicates significant difference (Mann–Whitney *U* test, $p \leq 0.05$) from Cm. (c) Leaves from the second rosette were photographed at 10 dpi. (d) Leaf wilt and blotch symptoms in each plant were scored according to the percentage of symptomatic leaves (0 = no leaf wilt/blotch, 1 = 1%–25%, 2 = 26%–50%, 3 = 51%–100%) at 3, 6, and 9 dpi. Bar charts represent the distribution of scores of 23 (Cm), 24 (CmΩ*chpG*) or 20 (CmΩ*chpG* + *ChpG*) plants pooled from four experimental repeats. * indicates the score distribution is different from Cm (Pearson's chi-squared test, $p \leq 0.05$). (e) Canker lesions were photographed at 10 dpi. (f) Canker symptoms of each plant were scored according to length of the canker lesion (0 = no canker, 1 = <0.1 cm, 2 = 0.1–1 cm, 3 = >1 cm) at 3, 6, and 9 dpi. Bar charts represent the distribution of scores of 23 (Cm), 24 (CmΩ*chpG*) or 20 (CmΩ*chpG* + *ChpG*) plants pooled from four experimental repeats. * indicates the score distribution is different from Cm (Pearson's chi-squared test, $p \leq 0.05$)

hindered its ability to cause disease symptoms and colonize eggplant on stem inoculations (Figure 5d–f). Similar to the overexpression experiments, introduction of $ChpG_{S231A}$ expressed under its native promoter into CmΩ*chpG* failed to elicit an immune response, and bacteria did not cause an HR on leaf infiltration (Figure S6a,b) and were as pathogenic as CmΩ*chpG* on stem puncher inoculations (Figure S6c–e).

These experiments show that the protease activity of ChpG is required for its recognition in eggplant.

2.5 | Immune recognition of ChpG is conserved in multiple eggplant varieties

A recent screen conducted by Boyaci and colleagues identified that numerous eggplant varieties demonstrate moderate to high Cm resistance and classified only three out of 46 screened varieties as susceptible to the bacteria (Boyaci et al., 2021). We hypothesized that resistance to Cm, which is abundant in eggplants, is mediated through immune recognition of ChpG. To test this, we monitored HR elicitation and disease development by either wild-type Cm or CmΩ*chpG* in 12 eggplant varieties (Figure 6). These included 10 heirloom varieties and two commercial varieties (Tudla and Aragon). On leaf infiltration, wild-type Cm elicited an HR in all tested varieties within 36–72 hpi (Figure 6a). An HR was not observed in CmΩ*chpG*-infiltrated leaves, which demonstrated weak to moderate chlorosis within 72–96 hpi (Figure 6a). Next, we inoculated eight varieties with wild-type Cm or CmΩ*chpG* using stem puncture and monitored plants for disease symptoms and bacterial colonization (Figure 6b,c). Seven out of the eight eggplant varieties inoculated with wild-type Cm did not develop significant wilting or leaf blotch symptoms while

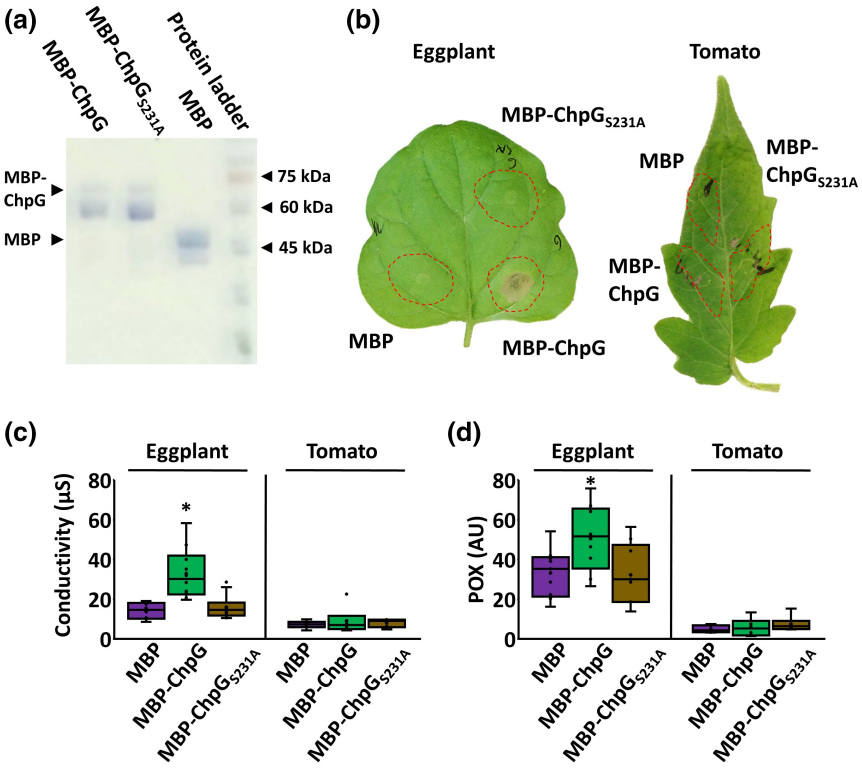

FIGURE 4 The catalytic activity of ChpG is required for its immune recognition. (a) Wild-type and catalytic site mutant (S231A) ChpG variants were fused to a maltose-binding protein (MBP) tag, purified from *Escherichia coli*, and visualized by SDS-PAGE. (b–d) Purified proteins (0.02 µg/ml) were infiltrated into Black Queen eggplant and Moneymaker tomato leaves. (b) Leaves were photographed 72 h postinfiltration. (c) Cell death was quantified by ion leakage at 36 h. (d) Peroxidase (POX) activity was quantified at 36 h. (c, d) Lower and upper quartiles are marked at the margins of the boxes. Central lines and "o" represent medians and data points of 10 (for eggplant) or 6 (for tomato) biological repeats collected from two (tomato) or three (eggplant) independent experiments. * indicates significant difference (Mann–Whitney *U* test, $p \leq 0.05$) from MBP control

CmΩ*chpG*-inoculated plants showed wilting/leaf blotch symptoms in 50%–100% of the leaves (Figure 6b). Some cv. Japanese Finger eggplants inoculated with wild-type Cm developed wilting symptoms and significantly larger canker lesions compared to other varieties (not shown). This phenomenon was not consistent between biological repeats. Symptom severity caused by CmΩ*chpG* ranged dramatically between different varieties. However, it is difficult to assess if these differences occurred due to immune elicitation or to plant vigour because varieties also demonstrated differential growth rates that might affect symptom development. When monitored for stem bacterial colonization all varieties inoculated with CmΩ*chpG* had significantly higher stem bacterial titre compared to wild-type Cm within the same variety (Figure 6c). Stem bacterial populations of wild-type Cm differed between eggplant varieties. In particular, stem colonization of the five oval fruit eggplant varieties used in this study (Aragon, Baladi, Bianca White Snowy, Black Queen, Tudla) ranged from 5×10^4 to 10^6 cfu/g and demonstrated lower variance between biological repeats (Figure 6c). In contrast, bacterial populations of wild-type Cm were higher and more variable in the three tested slender fruit varieties (Japanese Finger, Little Finger, Thai Long Green) and ranged from 10^5 to 5×10^7 cfu/g (Figure 6c). These data suggest that immune recognition of ChpG is conserved in different eggplant varieties. However, the quantitative contribution of ChpG recognition to Cm resistance varies between varieties.

3 | DISCUSSION

Most plant bacterial diseases are associated with specific genera or species, such as *Xanthomonas*, *Pseudomonas syringae*, *Ralstonia*, *Erwinia*, and *Clavibacter*. While bacteria that belong to these genera cause disease in diverse plant hosts, the majority of subspecies harbour a very narrow host range and are confined to a small number of crops. In the majority of cases, the bacterial and plant factors that dictate host range are unknown. Such interactions are likely to be multifaceted and depend on host tissue structure, secondary metabolites, and elicitation or avoidance of host immune responses. With that in mind, immune recognition of specific secreted bacterial effectors was found to be the sole determinant that controls host range in several pathosystems (Leach & White, 1996; Saur & Hückelhoven, 2021). This study identified the protease ChpG of Cm as the sole determinant through its immune recognition in resistant eggplant varieties.

Clavibacter spp. rely on Sec-dependent hydrolases to promote disease. Accordingly, Pat-1 homologs play a central role in the virulence of these bacteria and specific proteases were found to be crucial for the pathogenicity of different *Clavibacter* spp. on tomato, pepper, and eggplant (Dreier et al., 1997; Hwang et al., 2020; Nissinen et al., 2009; Stork et al., 2008). While relatively conserved as a protein family, specific Pat-1 homologs demonstrate differential distribution between *Clavibacter* spp. (Figure 7), which suggests that these proteases function in a host-specific manner. In particular, *ChpG* is conserved in most Cm and *C. capsici* genomes, but is absent from *C. sepedonicus* (Figure 7). Moreover, previous studies have reported that eggplants are susceptible to *C. sepedonicus* whereas a large number of eggplant varieties are resistant to Cm (Bishop & Slack, 1987; Boyaci et al., 2021; Nissinen et al., 2001) and that ChpG contributes to virulence on pepper, which serves as a main and alternative host of *C. capsici* and Cm, respectively (Hwang et al., 2020; Oh et al., 2016; Yim et al., 2012). Altogether, these findings

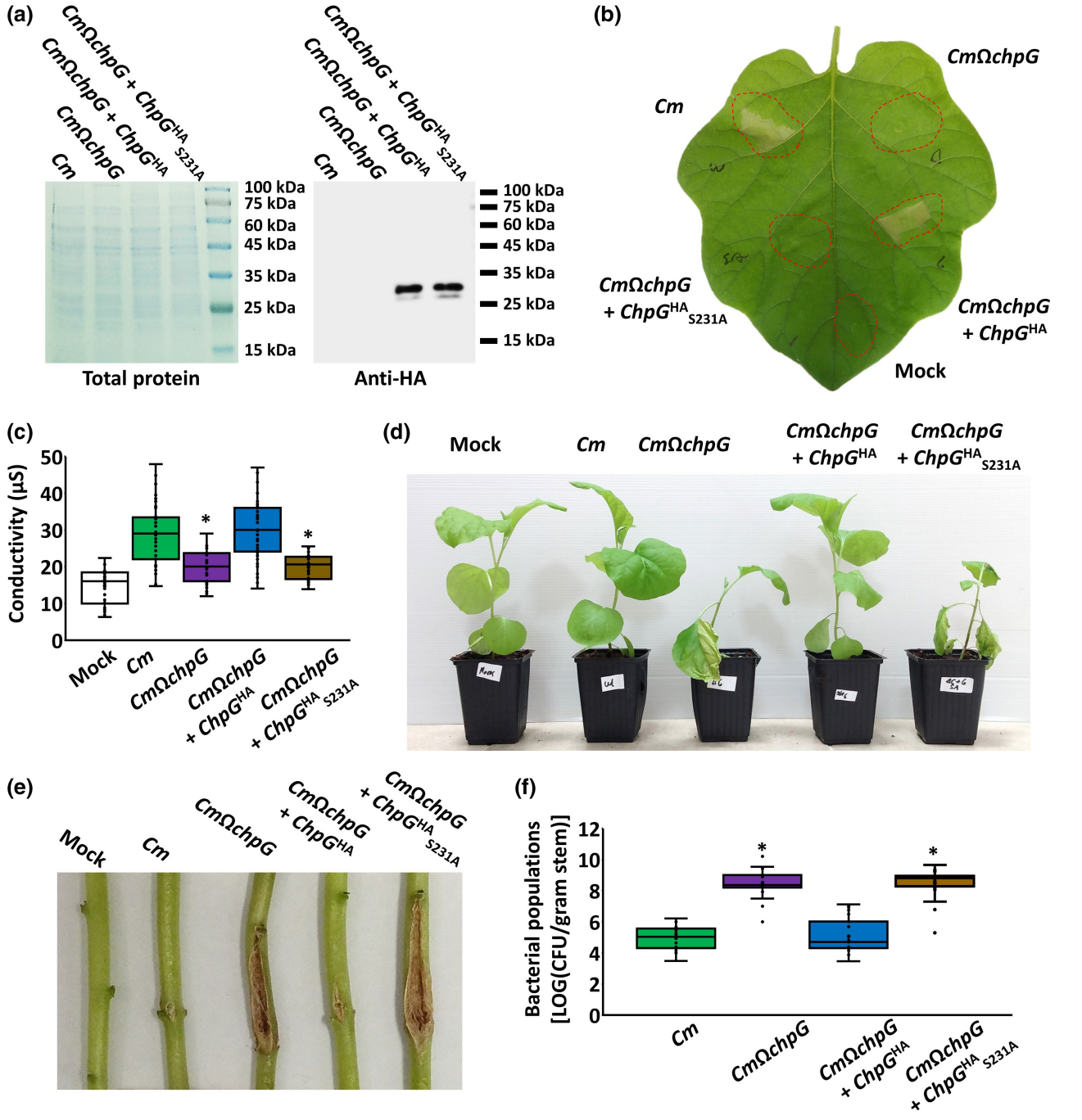

FIGURE 5 Immune recognition of ChpG expressed by *Clavibacter michiganensis* (Cm) is dependent on its catalytic activity. (a) Total protein was extracted from CMM101 (Cm), CmΩ*chpG*, and CmΩ*chpG* carrying pHN216 plasmids expressing *ChpG* or $ChpG_{S231A}$ fused to HA-tag under the control of the *pCMP1* promoter. Samples were separated on SDS-PAGE and gels were either stained with Coomassie brilliant blue (left panel) or immunoblotted with anti-HA antibody (right panel). (b, c) The indicated bacterial cultures (10^8 cfu/ml) were infiltrated into Black Queen eggplant leaves. (b) Picture was taken 48 h after infiltration. (c) Cell death was quantified by ion leakage 36 h postinfiltration. Lower and upper quartiles are marked at the margins of the boxes. Central lines and "o" represent medians and data points of at least 34 biological repeats collected from four independent experiments. * indicates significant difference (Mann–Whitney *U* test, $p \leq 0.05$) from Cm. (d–f) Four-leaf stage Black Queen eggplant plants were inoculated with the indicated Cm strains or water control (mock) by puncturing the stem area between the cotyledons with a wooden toothpick soaked in Cm solution (5×10^7 cfu/ml). Plants (d) and canker lesions (e) were photographed at 12 days postinfiltration (dpi). (f) Stem bacterial populations 1 cm above the inoculation site at 12 dpi. Lower and upper quartiles are marked at the margins of the boxes. Central lines and "o" represent medians and data points of at least 19 biological repeats collected from four independent experiments. * indicates significant difference (Mann–Whitney *U* test, $p \leq 0.05$) from Cm

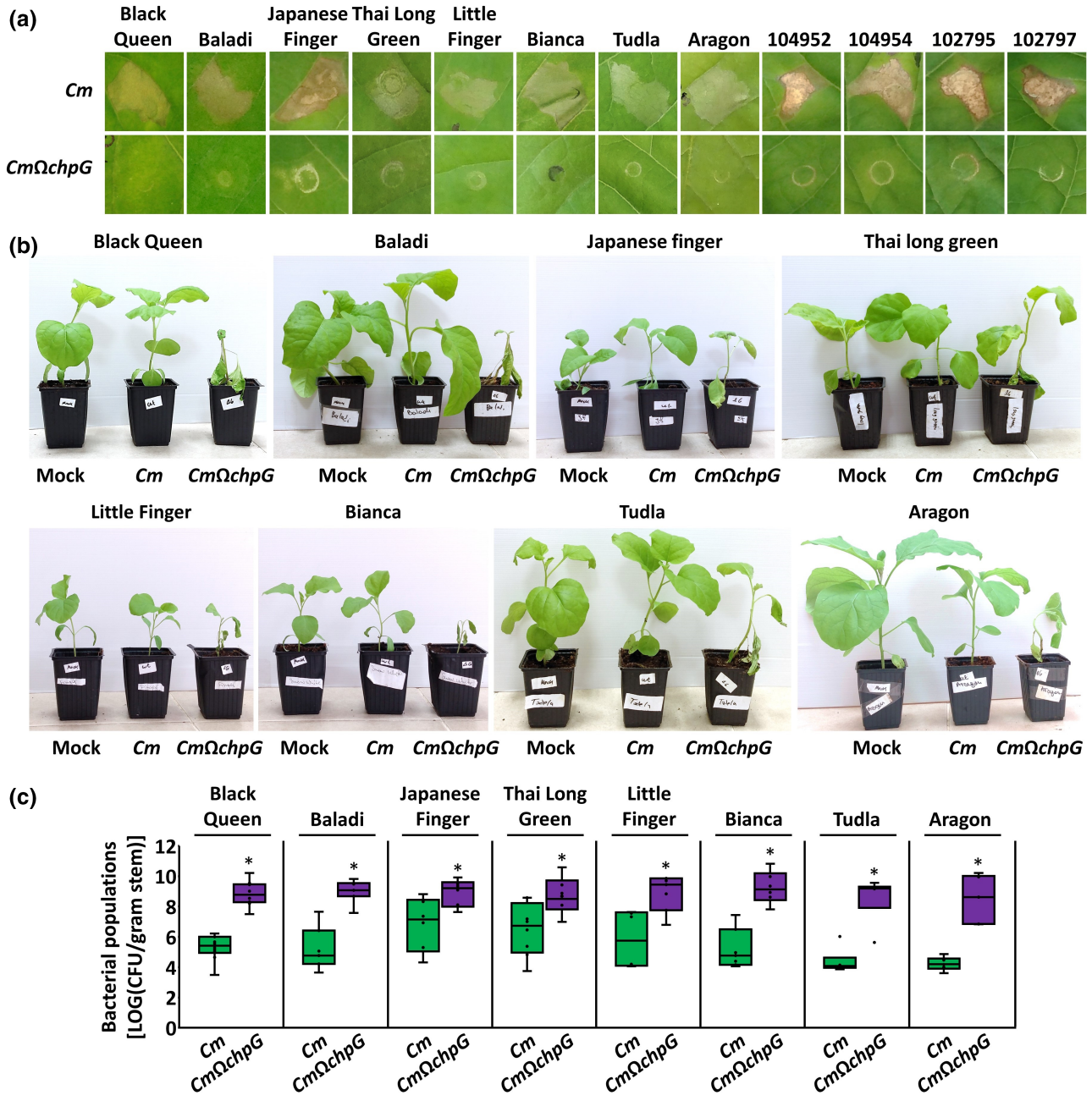

FIGURE 6 ChpG functions as an avirulence factor in multiple eggplant varieties. (a) Leaves of the indicated eggplant varieties were infiltrated (10^8 cfu/ml) with *Clavibacter michiganensis* wild-type CMM101 (Cm) and CMM101 *chpG* inactivation mutant (CmΩ*chpG*). Pictures were taken 96 h later. Representative pictures were taken out of at least 10 biological repeats conducted in two independent experiments. (b, c) The indicated eggplant varieties were inoculated with Cm, CmΩ*chpG* or water control (mock) by puncturing the stem area between the cotyledons with a wooden toothpick soaked in Cm solution (5×10^7 cfu/ml). (b) Pictures were taken 14 days postinoculation (dpi). (c) Stem bacterial populations 1 cm above the inoculation site at 12 (Black Queen) or 14 dpi. Lower and upper quartiles are marked at the margins of the boxes. Central lines and "o" represent medians and data points of at least six biological repeats collected from two independent experiments. * indicates significant differences (Mann–Whitney *U* test, $p \leq 0.05$) from Cm in the same eggplant variety

suggest that loss and/or acquisition of *ChpG* in the genome through horizontal gene transfer plays a key role in the differentiation of these bacteria into host-specialized pathogens. Interestingly, even though this recognition was not directly linked to virulence, ChpG has been reported to specifically trigger an HR in the nonhost plants *N. tabacum* and *M. jalapa* (Lu et al., 2015; Stork et al., 2008). This indicates that ChpG is immune recognized in a number of plant species, which suggests that its role in determining the host range of *Clavibacter* pathogens might extend beyond restricting Cm from eggplant varieties.

The findings presented in this manuscript show that immune recognition of ChpG in certain eggplant varieties restricts them from being a host of Cm, which classifies it as an avirulence gene under Flor's gene-for-gene model (Flor, 1971). Numerous secreted

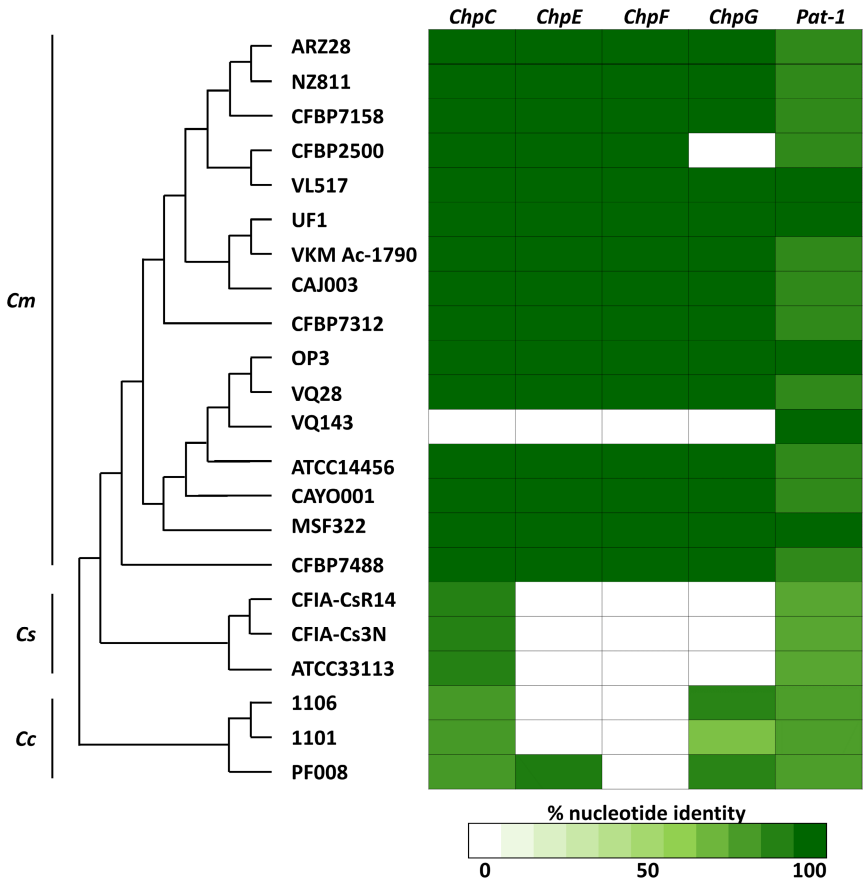

FIGURE 7 Distribution of Pat-1 homologs among *Clavibacter* spp. Figure states the distribution of *Pat-1* homologs of *Clavibacter michiganensis* (Cm) NCPPB 382 [*ChpC* (CMM_0052), *ChpE* (CMM_0039), *ChpF* (CMM_0053), *ChpG* (CMM_0059), and *Pat-1* (pCM2_0054)] within NCBI genome deposits of Cm, *C. sepedonicus* (Cs), and *C. capsici* (Cc). Left, multilocus sequence typing phylogenetic analysis conducted by AutoMLST (https://automlst.ziemertlab.com/analyze); right, percentage nucleotide identity according to NCBI blastn suite (https://blast.ncbi.nlm.nih.gov/Blast.cgi, cut off set at E-value of e−6) of the five *Pat-1* homologs in the Cm, Cs, and Cc genomes marked by green colour intensity according to the scale located at the bottom of the figure

apoplastic effectors or hydrolases have been classified as avirulence proteins in fungi and oomycetes (Tyler & Rouxel, 2012). However, with the exception of RaxX in rice (Pruitt et al., 2015), all reported avirulence factors that define the host range of plant-pathogenic bacteria are translocated type III secretion system effectors (Khan et al., 2016; Leach & White, 1996). Nevertheless, secreted apoplastic hydrolases have been characterized as immune elicitors. Such immune elicitation is hypothesized to be mainly mediated through recognition of damage-associated molecular patterns (Hou et al., 2019; Sinha et al., 2013). Specific secreted hydrolases such as *Xanthomonas* XagP, *Pseudomonas cichorii* HrpW, and *Clavibacter* Pat-1 protease homologs promote nonhost-specific HR but inactivation of these elicitors does not expand the host range of these pathogens (Kaewnum et al., 2006; Kajihara et al., 2012; Lu et al., 2015; Nissinen et al., 2009; Stork et al., 2008). Unlike the aforementioned examples, recognition of ChpG dictates the host range of Cm and restricts it from causing disease in numerous eggplant varieties. This suggests that immune recognition of apoplastic hydrolases such as ChpG, in addition to recognition of translocated type III secretion system effectors, plays a role in determining the host range of plant-pathogenic bacteria.

Eggplant genes associated with immunity to Cm are yet to be identified. A recent screen identified that a large number of eggplant varieties are resistant to Cm and that this resistance is likely to be associated with a single dominant locus (Boyaci et al., 2021). Considering that ChpG is conserved in most Cm strains (Figure 7), and that all eggplant varieties used in this study displayed ChpG-dependent immune elicitation that resulted in reduced disease symptoms and bacterial colonization (Figure 6), it is reasonable to hypothesize that the putative aforementioned resistance locus facilitates the recognition of ChpG. If that is indeed the case, the locus is likely to encode a transmembrane receptor that mediates immunity in the presence of ChpG. Therefore, ChpG can be used as a screening marker for future backcross lines, which will greatly assist in the identification of the immune locus that recognizes it.

Immune recognition of ChpG was abolished when its catalytic active site was mutated. Therefore, its recognition is likely to be indirect and dependent on catalytic modification of a plant protein. Such recognition could be mediated through modification of a specific guarded or decoy target by ChpG, which is later recognized by a transmembrane receptor-like kinase or a receptor-like protein in a similar manner to the Avr2-RCR3-Cf2 resistance model (Rooney et al., 2005; Shabab et al., 2008). Alternatively, ChpG might target structural cell wall components, which could result in accumulation of a damage-associated molecular pattern elicitor recognized by an receptor-like kinase in a similar manner to the oligogalacturonides-WAK1 model (Brutus et al., 2010). Because immune recognition is based on the unique perception of a single Pat-1 homolog but not by other family members, the decoy-type model is more plausible. The biological functions and plant cleavage targets of ChpG, or any other Pat-1 homolog, are unknown. Elucidating the molecular mechanisms that facilitate the virulence contribution of ChpG and other Pat-1 homologs will provide further insights into the mechanisms of perception. Additionally, further functional analyses of the involvement of plant immune components in the perception of ChpG, such as the

BAK1 and SOBIR1 co-receptors (Gust & Felix, 2014), will pinpoint if this recognition is indeed mediated by a transmembrane receptor or through an unknown mechanism.

In summary, this study identified the protease ChpG as a novel avirulence protein that functions as a host specificity determinant by prohibiting Cm from colonizing and causing disease in eggplant varieties. In addition, the study indicates that immune recognition of ChpG in eggplant is dependent on its catalytic activity, which suggests that perception of ChpG by a potential immune receptor is indirect. This is the first evidence of secreted extracellular bacterial hydrolase that functions as a sole host-specificity factor in plant-pathogenic bacteria.

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial strains and plant material

Bacterial strains and plasmids used in the study are listed in Table S1. Cm and *E. coli* were grown in Luria Bertani (LB) broth at 28 or 37°C, respectively. When required, media were supplemented with 10 μg/ml nalidixic acid, 10 μg/ml chloramphenicol, 50 μg/ml neomycin, 50 μg/ml kanamycin or 100 μg/ml ampicillin.

Plant cultivars used in this study were tomato Moneymaker, pepper Early California Wonder, eggplant heirloom varieties Baladi, Bianca White Snowy, Black Queen, Japanese Finger, Little Finger, Thai Long Green, 104952, 104954, 102796, and 102797, and commercial eggplant varieties Aragon and Tudla. Varieties 104952, 104954, 102796, and 102797 were supplied by the Israel Plant Gene Bank (IGB, https://igb.agri.gov.il/web/?lang=en&page=0). Plants were grown in a 25°C temperature-controlled growth room under a 16 h light/8 h dark day cycle.

4.2 | Plasmid construction and bacterial transformation

The oligonucleotides used in this study are listed in Table S2. For *Clavibacter*-mediated expression of Pat-1 homologs, DNA fragments carrying *ChpE* (CMM_0039), *ChpF* (CMM_0053), and *ChpG* (CMM_0059) and their upstream regulatory regions (as specified in Table S1) were amplified from genomic DNA of Cm strain CMM101 using Q5 high-fidelity DNA polymerase (NEB) and cloned into the *E. coli–Clavibacter* shuttle vector pHN216 (Laine et al., 1996). Serine231 of the predicted catalytic triad of ChpG was substituted to alanine using the QuikChange II kit (Agilent Technologies, Inc.). For constitutive overexpression of *ChpG* fused to HA-tag, the 801 bp fragment located upstream to *GFP*, which contains the 254 bp *pCMP1* promoter (Chalupowicz et al., 2012), and the open reading frames of *ChpG* or $ChpG_{S231A}$ were amplified from pK2-22, pHN216:*ChpG*, and pHN216:$ChpG_{S231A}$, respectively, and sequentially subcloned into a pBBR1MCS-2 derivative containing an HA tag (kindly provided by Guido Sessa). Next, *pCMP1:ChpG-HA* fragments were amplified as one unit and cloned into pHN216 (Figure S8).

pHN216 derivatives were introduced into $Cm^{\Delta PI}$ (CMM30-18) or CmΩ*chpG* by electroporation as previously described (Kirchner et al., 2001) with slight modifications. Briefly, stationary phase Cm cultures were diluted to $OD_{600} = 0.1$ and grown in 100 ml of LB medium until the mid-log phase was reached ($OD_{600} = 0.5–0.7$) at 25°C on an orbital shaker (120 rpm). Medium was supplemented with 20% glycine solution to a final concentration of 2.5% glycine and bacteria were kept at 25°C on an orbital shaker for 2 h. Bacteria were pelleted at 4°C and washed three times with 25 ml of ice-cold 10% glycerol solution and finally resuspended in 1 ml of ice-cold 15% glycerol. Electroporation was performed in the following conditions: 25 μF, 12.5 kV/cm, and 600 Ω using the Gene Pulser Electroporation System (Bio-Rad). After electroporation, bacteria were supplemented with 1 ml of LB medium, incubated for 5 h at 25°C, and plated on LB agar supplemented with neomycin and incubated for 4 days at 28°C. Neomycin-resistant colonies were validated by PCR using gene-specific primers.

Protein accumulation of ChpG-HA derivatives expressed in Cm under the control of the *pCMP1* promoter was validated by western blot analysis as described: overnight Cm cultures were diluted to $OD_{600} = 0.3$ and lysed by SDS gel-loading buffer followed by 10 min of incubation in 95°C. Equal volumes were separated in parallel on two SDS-PAGE gels: one gel was stained with Coomassie brilliant blue to visualize protein loading. The second gel was used for western blot analysis using anti-HA-tag (F-7) mouse monoclonal antibody (Santa Cruz Biotechnology) as described by Sambrook and Russell (2006) and according to the manufacturer's instructions.

4.3 | RNA purifications and RT-PCR

Bacterial cultures were grown for 24 h in M9 medium supplemented with tomato xylem sap (Savidor et al., 2012). Total RNA was purified from culture pellets using GeneMATRIX Universal RNA Purification Kit (EURx), treated with DNase I (Hylabs), and reverse transcribed to cDNA using a qScript cDNA Synthesis Kit (Quantabio) according to the manufacturer's instructions. *Pat-1* homologs and *gyrA* (positive control) were PCR amplified from 1-μl cDNA or control RNA samples using gene-specific primers (Table S2).

4.4 | Expression and purification of MBP fusion proteins in *E. coli*

The 112–831 bp fragments containing the *ChpG* open reading frame minus its signal peptide-coding region (predicted by SignalP-5.0 Server; http://www.cbs.dtu.dk/services/SignalP/) were amplified from pHN216:*ChpG* and pHN216:$ChpG_{S231A}$, cloned into pMALc2× (NEB) and plasmids were introduced into *E. coli* Rosetta (Merck). Bacterial cultures were grown in an orbital shaker at 37°C to $OD_{600} = 0.4–0.6$, supplemented with 0.1 mM isopropyl β-D-1-thiogalactopyranoside, and incubated for 3 h at 37°C. Bacteria were pelleted and resuspended in ice-cold buffer solution (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4) and lysed using a SONIC-150W ultrasonic processor (MRC

Labs). MBP-fused proteins were purified from supernatants using amylose resin (NEB) according to the manufacturer's instructions. Purified proteins were quantified by Bradford protein assay kit (Bio-Rad) and validated by SDS-PAGE followed by staining with Coomassie brilliant blue (Sambrook & Russell, 2006). Protein accumulation in total bacterial extracts and in the purified fractions was confirmed with western blot using anti-MBP tag (8G1) mouse monoclonal antibody (Cell Signaling) according to the manufacturer's instructions (Figure S5).

4.5 | Leaf infiltrations, ion leakage, and peroxidase activity measurements, and quantification of leaf bacterial populations

Purified proteins or bacterial suspensions were infiltrated to fully expanded upper rosette leaves of the four- to six-leaf stage Black Queen eggplant and six-leaf stage Moneymaker tomato using a needleless syringe. For protein infiltration, purified MBP, MBP-ChpG, and MBP-ChpG$_{S231A}$ proteins were diluted to a concentration of c.0.02 μg/ml in sterile distilled water prior to infiltration. For bacterial suspensions, Cm bacteria were scraped from fresh 2-day-old cultures grown on LB agar and diluted to a concentration of 10^8 (OD_{600} = 0.2, used for HR visualization, ion leakage, and POX activity measurements) or 10^4 cfu/ml (used for quantification of leaf bacterial populations) in sterile distilled water prior to infiltration.

For ion leakage measurements and POX activity, 1.5-cm (for eggplant) or 1-cm (for tomato) diameter leaf disks were sampled from the inoculation sites, transferred to flasks containing 10 ml of distilled water, and incubated on an orbital shaker (50 rpm) for 4 h at room temperature. Electrolyte leakage was quantified in water solutions using a conductivity meter (MRC Labs). Secreted POX activity quantification was modified from Mott et al. (2018). One hundred microlitres of diluted supernatants from each sample was incubated with 100 μl of freshly prepared POX assay solution (1 mg/ml 5-aminosalicylic acid, 0.01% H_2O_2, pH 6.0) and timed. Reactions were stopped with 40 μl of 2 M NaOH once brown pigmentation started to appear and quantified in optical density of 595 nm using a Multiskan FC Microplate Photometer (Thermo Fisher). POX activity was determined in arbitrary units (AU) and calculated as 1000 × (OD_{595}/[time (min)]).

Leaf bacterial population was quantified in each plant in two pooled 0.2-cm diameter leaf disks from different inoculated leaves. Samples were homogenized in 1 ml of sterile water and bacterial numbers were determined per cm^2 leaf areas by plating 10 μl of 10-fold serial dilutions and counting the resulting colonies.

4.6 | Plant inoculations, disease severity assessments, and quantification of stem bacterial populations

Stem inoculations were conducted by a single punctures of the stem areas between the cotyledons of four-leaf stage eggplants or Moneymaker tomatoes with Cm-contaminated toothpicks. Contaminated toothpicks were prepared as follows: Cm bacteria were scraped from fresh 2-day-old cultures grown on LB agar and diluted to 5 × 10^7 cfu/ml in distilled water in 1.5-ml tubes. Wooden toothpicks were soaked in solutions for at least 10 min and each toothpick was then used for a single inoculation. After inoculations, plants were kept at 25°C in a greenhouse and wilt/leaf blotch and canker symptoms were determined and scored at 3, 6, and 9 dpi.

Wilting/leaf blotch symptoms were scored in each plant as the percentage of leaves demonstrating wilting and/or necrotic blotch symptoms according to the following scale: 0 = no wilt or leaf blotch, 1 = 1%–25%, 2 = 26%–50%, 3 = 51%–100%. The length of the canker lesion expanding from the inoculation areas was measured with a ruler and given a score according to the following scale: 0 = no canker, 1 = <0.1 cm, 2 = 0.1–1 cm, 3 = >1 cm. During virulent interactions, additional canker lesions appeared in different areas of the stem and leaf petioles. Due to high plant-to-plant variations in the quantity and size of cankers, these lesions were not included in canker scoring.

Bacterial populations were quantified in 1-mm stem areas taken 1 cm above the inoculation sites. Samples were weighed and supplemented with 1 ml of sterile distilled water. Samples were homogenized and bacterial numbers per gram of tissue were determined by plating 10 μl of 10-fold serial dilutions and counting the resulting colonies.

4.7 | Phylogenetic analysis and determining the distribution of *Pat-1* homologs in *Clavibacter* spp.

Phylogenetic analysis was conducted on available NCBI genome deposits of *C. michiganensis*, *C. sepedonicus*, and *C. capsici* by multilocus sequence typing analysis with AutoMLST online server (Alanjary et al., 2019) (https://automlst.ziemertlab.com/) using the fast alignment mode feature; the genes used are found in File S1. To determine the distribution of *Pat-1* homologs, the nucleotide identity of the closest homologs of Cm NCPPB 382 *ChpC* (CMM_0052), *ChpE* (CMM_0039), *ChpF* (CMM_0053), *ChpG* (CMM_0059), and *Pat-1* (pCM2_0054) found in *Clavibacter* genomes depicted in Figure 7 was determined NCBI blastn suite (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Cutoff was set at E-value of e−6 and minimum coverage of 80%.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interests.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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