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ORIGINAL ARTICLE

Hypoviral-regulated HSP90 co-chaperone p23 (*CpCop23*) determines the colony morphology, virulence, and viral response of chestnut blight fungus *Cryphonectria parasitica*

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Abstract

We previously identified a protein spot that showed down-regulation in the presence of Cryphonectria hypovirus 1 (CHV1) and tannic acid supplementation as a Hsp90 co-chaperone p23 gene (*CpCop23*). The *CpCop23*-null mutant strain showed retarded growth with less aerial mycelia and intense pigmentation. Conidia of the *CpCop23*-null mutant were significantly decreased and their viability was dramatically diminished. The *CpCop23*-null mutant showed hypersensitivity to Hsp90 inhibitors. However, no differences in responsiveness were observed after exposure to other stressors such as temperature, reactive oxygen species, and high osmosis, the exception being cell wall-disturbing agents. A severe reduction in virulence was observed in the *CpCop23*-null mutant. Interestingly, viral transfer to the *CpCop23*-null mutant from CHV1-infected strain via anastomosis was more inefficient than a comparable transfer with the wild type as a result of decreased hyphal branching of the *CpCop23*-null mutant around the peripheral region, which resulted in less fusion of the hyphae. The CHV1-infected *CpCop23*-null mutant exhibited recovered mycelial growth with less pigmentation and sporulation. The CHV1-transfected *CpCop23*-null mutant demonstrated almost no virulence, that is, even less than that of the CHV1-infected wild type (UEP1), a further indication that reduced virulence of the mutant is not attributable exclusively to the retarded growth but rather is a function of the *CpCop23* gene. Thus, this study indicates that *CpCop23* plays a role in ensuring appropriate mycelial growth and development, spore viability, responses to antifungal drugs, and fungal virulence. Moreover, the *CpCop23* gene acts as a host factor that affects CHV1-infected fungal growth and maintains viral symptom development.

KEYWORDS
co-chaperone, *Cryphonectria parasitica*, heat shock protein 90, hypovirulence

Yo-Han Ko and Jeesun Chun contributed equally to this work.

1 | INTRODUCTION

Chestnut blight fungus, *Cryphonectria parasitica*, devastated American chestnut forests in the early 20th century (Van Alfen, 1982). Notably, strains harbouring a single-stranded, positive-sense (+ss) RNA mycovirus Cryphonectria hypovirus 1 (CHV1) showed characteristic symptoms of decreased virulence, a phenomenon referred to as hypovirulence (Anagnostakis, 1982; Nuss, 1992; Van Alfen et al., 1975), including various but specific hypovirulence-associated symptoms including reduced sporulation, pigmentation, oxalate accumulation, laccase production, and female fertility (Elliston, 1985; Havir & Anagnostakis, 1983; Rigling et al., 1989). Systemic analysis of transcripts and proteins of both virus-free and virus-infected isogenic strains of *C. parasitica* has revealed that these phenotypic changes are attributable to hypoviral modulations in the fungal gene expression profiles (Allen & Nuss, 2004; Allen et al., 2003; Chun et al., 2020; Deng et al., 2007; Kang et al., 1999; Kazmierczak et al., 1996, 2012; Kim et al., 2012; Wang et al., 2013). Among the several fungal pathways regulated by the hypovirus infection, the stress-response pathway, which includes specific heat-shock proteins, has been shown to be one of the specific targets perturbed by the hypovirus infection (Allen & Nuss, 2004; Allen et al., 2003; Baek et al., 2014; Lim et al., 2010). Our previous studies using a growth condition supplemented with tannic acid (TA), which is abundant in the bark of chestnut trees and is assumed to be the major defence material against pathogen infection, demonstrated that a gene encoding a co-chaperone of heat-shock protein 90 is specifically regulated by the presence of hypovirus CHV1 and TA supplementation (Kim et al., 2012). As a follow-up, the current study is focused on the biological function of the cloned Hsp90 co-chaperone gene.

All organisms, including bacteria, fungi, plants, and animals, respond to extreme (but sublethal) temperatures by reducing transcription and translation activity but inducing the synthesis of massive quantities of heat-shock proteins (Hsps) (Lindquist & Craig, 1988; Plesofsky, 2004). Hsps are molecular chaperones that prevent the aggregation of partially denatured proteins and promote their return to native conformations after favourable conditions are restored. These proteins are highly conserved and can also be induced by other stresses, which allow living organisms to adapt and survive under challenging conditions. Moreover, many prominent Hsps are essential and are constitutively expressed to contribute to cellular metabolism during normal growth (Lindquist & Craig, 1988; Plesofsky, 2004). Hsps have been classified into several classes or families according to their molecular weight, and a cell may express multiple members of the same family (Walter & Buchner, 2002). Five major families of Hsps exist (Buchner, 1996; Richter et al., 2010), four of which—Hsp100s, Hsp90s, Hsp70s, and Hsp60s—consist of ATP-dependent high molecular mass heat-shock proteins, while the fifth family—the small heat-shock proteins (sHsps)—consists of ATP-independent low molecular mass Hsps ranging in size from 12 to 42 kDa (Jaya et al., 2009) as well as a conserved sequence in their C-terminus called the α-crystalline domain (Buchner et al., 1997; de Jong et al., 1993; Narberhaus, 2002). Of these, Hsp70 and Hsp90 are two major molecular chaperones that exist in eukaryotic cytosol under normal growth conditions (Fang et al., 1998; Röhl et al., 2013). Characterization of Hsp90 chaperone complexes has revealed the presence of a number of associated proteins, referred as co-chaperones, which are thought to be essential and directly participate in the chaperoning process (Felts & Toft, 2003). The core co-chaperones of Hsp90 include Cdc37, Sti1/Hop, peptidyl-prolyl cis-trans isomerases (PPIases), Aha1, and p23/Sba1 (Gu et al., 2016).

The p23 protein was first discovered as part of the Hsp90 complex with the progesterone receptor (Johnson et al., 1994). In addition to being involved in steroid receptors, p23 has been found to be associated via Hsp90 with several other proteins, including transcription factors, protein kinases, viral proteins, and mature client structures (Echtenkamp et al., 2016; Hu et al., 1997; Nair et al., 1996; Xu et al., 1997). Moreover, recent studies have observed that p23 can associate independently with proteins and have additional functions of enzyme activity and more of independent of chaperoning (D'Alessandro et al., 2019; Felts & Toft, 2003). The presence of p23 is ubiquitous and highly conserved from yeast to humans (Garcia-Ranea et al., 2002). Although a chaperone role for fungal Hsp90 in sensing temperature and orchestrating drug resistance has been suggested (Cowen & Lindquist, 2005; Cowen et al., 2009; Lamoth et al., 2013), only a few functional studies on p23 taken from filamentous fungi have been performed (Gu et al., 2016) and the functional studies of p23 are still obscure.

In this study we report the characterization of TA- and hypoviral-regulated Hsp90 co-chaperone p23 from *C. parasitica*. By constructing a corresponding null mutant, we demonstrated the biological function of the cloned Hsp90 co-chaperone p23. On the basis of this observation, we then suggest how the protein product of the cloned Hsp90 co-chaperone p23 gene might be involved in the fungal responses to abiotic as well as biotic stressors, and in determining its virulence.

2 | RESULTS

2.1 | Cloning and characterization of a tannic acid-responsive and hypoviral-regulated Hsp90 co-chaperone p23 (*CpCop23*) gene

Our previous studies using proteomics identified 30 protein spots that were significantly changed in response to CHV1 infection and TA supplementation (Kim et al., 2012). One of the spots, tentatively identified by liquid chromatography-tandem mass spectrometry as a heat-shock protein 90 co-chaperone p23 orthologue, was selected for further analysis. A genomic sequence that encoded the corresponding amino acid residues was obtained using the *C. parasitica* genome database (http://genome.jgi-psf.org/Crypa2/Crypa2.home.html) (Crouch et al., 2020). PCR amplification of the sequence harbouring the full-length genomic DNA resulted in a 5031-bp amplicon, and subsequent sequence analysis revealed the highest level of homology with known Hsp90 co-chaperone *Sba1* of *Diaporthe batatas*

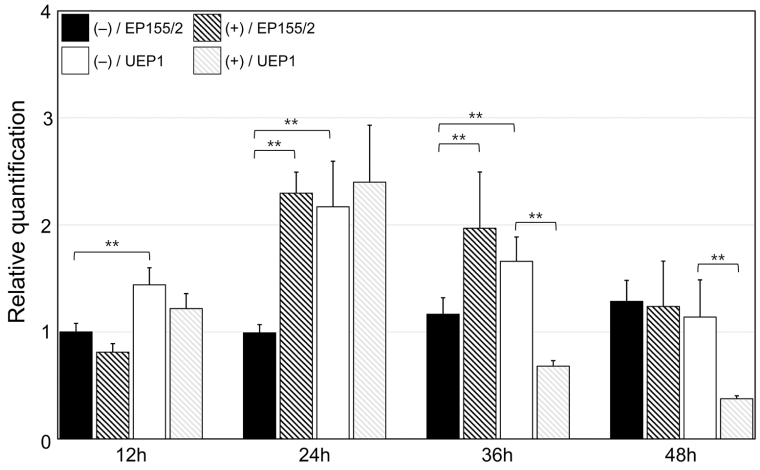

FIGURE 1 Expression of *CpCop23* transcript in response to tannic acid (TA) supplementation and hypovirus infection. The accumulated transcript levels of *CpCop23* relative to levels of *gpd* (glyceraldehyde-3-phosphate dehydrogenase) were assessed by reverse transcription-quantitative PCR for wild-type EP155/2 and its isogenic CHV1-infected UEP1 strains cultivated on media with (+) or without (–) TA supplementation. The relative quantification of *CpCop23* transcript for EP155/2 cultivated without TA supplementation is normalized as "1". Data are represented as the mean ± *SD* of three independent replicates, significance was calculated by Student's *t* test, $^{**}p < 0.01$.

(86.41% shared identity at the nucleotide level). A Joint Genome Institute genome database search using the cloned gene nucleotide sequence, and Southern blot analysis of restriction enzyme-digested *C. parasitica* genomic DNA, indicated that the cloned PCR amplicon was present as a single-copy gene in the *C. parasitica* genome (data not shown).

Based on the genomic sequence analysis, a near full-length cDNA clone was obtained using reverse transcription (RT)-PCR with a primer pair CpCop23-cF1 at nucleotide positions (nt) −7 to 17 and CpCop23-cR1 at positions nt 587 to 604 (relative to the start codon). The resulting 611-bp amplicon was cloned into the pGEM-T Easy vector (Promega). A sequence comparison of the cloned amplicon with the corresponding genomic sequence revealed that the cloned gene consisted of four exons, with three intervening sequences (26 to 182, 577 to 637, and 779 to 906), which differed from two instead of three annotated by the computer prediction. Sequence analysis in the promoter region of the *CpCop23* gene revealed canonical CAAT and TATAA boxes at −60 and −16 nt, respectively (Figure S1a). The sequence around the first ATG was in good agreement with Kozak's consensus sequence insofar as the nt −3 position was the A in AAAGATG. The putative poly(A) signal GATAAA was observed at 168 nt downstream of the stop codon (Lusk et al., 2021).

The deduced protein product of the cloned gene consisted of 200 amino acids, with an estimated molecular mass of 21.9 kDa and a pI of 4.38 (the GenBank accession number for *CpCop23* is XP_040777266.1). Homology searches using the deduced amino acid sequence demonstrated that the protein product of the cloned gene is closely related to the fungal p23/Sba1 orthologues from *D. batatas* (KAG8160577.1; 81.52% identity), *Purpureocillium lilacium* (XP_018177014.1; 77.58% identity), *Torrubiella hemipterigena* (CEJ79895.1; 71.89% identity), *Moelleriella libera* (OAA32634.1; 68.53% identity), *Fusarium longipes* (RGP72698.1; 64.95% identity), *Neurospora crassa* (XP_956470.2; 64.76% identity), *Aspergillus nidulans* (XP_664525.1; 61.82% identity), *Aspergillus fumigatus* (XP_753264.1; 54.69% identity), and *Saccharomyces cerevisiae* (NP_012805.1; 40.37% identity). Multiple alignment of closely related p23 revealed that the protein encoded by the cloned gene maintained the conserved N-terminal IbpA domain containing the WPRLL/TKE signature residues, which have previously been recognized as a p23 signature (Felts & Toft, 2003), at positions 99–103 amino acids, as well as a hallmark domain of an α-crystalline between 8 and 124 amino acids (Figure S1b). A phylogenetic analysis indicated that the protein product of the cloned gene was clustered with other fungal p23 but fell into a different clade containing that from *D. batatas*, with a high bootstrap value suggesting a genuine evolutionary relationship (Figure S1c). Based on the predicted molecular mass of the protein, the presence of the hallmark domains within the amino acid sequence, and the significant homological and phylogenic relationship to the known fungal p23/Sba1, we referred to the cloned gene as *CpCop23* (*C. parasitica* co-chaperone p23).

2.2 | Regulation of *CpCop23* expression

Compared to the constitutive expression of its homologue *Sba1* in *S. cerevisiae* (Fang et al., 1998), the cloned *CpCop23* gene showed regulated expression.

Consistent with a previously performed proteomic analysis, the expression of the cloned *CpCop23* gene was examined using RT-quantitative real-timePCR (RT-qPCR) when the cultures were transferred on TA-supplemented solid medium (Kim et al., 2012). As shown in Figure 1, wild-type EP155/2 showed significant induction of the transcript levels of the *CpCop23* gene 24 and 36 h after the transfer to TA-supplemented media. No further induction was observed 48 h after the transfer. Compared to the wild type, CHV1-infected UEP1 showed significantly increased transcription of the

CpCop23 gene up to 36 h after the transfer, which was consistent with the results of proteomic analysis (Kim et al., 2012). No additional increase in transcription of the *CpCop23* gene was observed in UEP1 as a result of TA supplementation. In fact, significantly lowered transcription of the *CpCop23* gene was observed 36 h after the transfer to the TA-supplemented medium (Figure 1). These results suggest that *CpCop23* expression is tightly regulated by TA supplementation and CHV1 infection, and that *CpCop23* induction by TA supplementation is affected by CHV1 infection.

2.3 | Construction of the *CpCop23*-null mutant

The biological function of the *CpCop23* gene was examined by comparing a parental wild-type strain to the *CpCop23*-null mutant, which was constructed by site-directed gene replacement during integrative transformation of the wild type. After a transformation using the corresponding *CpCop23*-deletion vector, a total of 95 transformants were screened by PCR for the replacement of the *CpCop23* gene by the disrupted *CpCop23* gene. Among 95 stable transformants, one transformant showed a PCR amplicon of the expected size, 3.5 kb, corresponding to the disrupted allele of the *CpCop23* gene (Figure S2b). The putative *CpCop23*-null mutant (TdCop23-1) was further confirmed by Southern blot analysis (Figure S2c). As shown in Figure S2c, the hybridization pattern of the *Xho*I-digested genomic DNA of the three single-spored clones of *CpCop23*-null mutant with the probe prepared using the 430-bp PCR amplicon of the *CpCop23* gene (Probe A) differed from that of the wild type. The hybridizing band of the wild type was the expected 2.9 kb and that of the *CpCop23*-null mutant was 5.5 kb, which corresponded to the expected size of the replaced allele. These hybridizing patterns and PCR analysis results confirmed that the *CpCop23* gene was replaced with part of the transforming vector.

2.4 | Characteristics of the colony morphology, conidiation, and spore viability of the *CpCop23*-null mutant

The *CpCop23*-null mutant showed severely retarded colonial growth with an irregular margin and less than 40% of the typical growth rate was observed (Figure 2a). A densely pigmented and extensively interwoven hyphal mat with almost no aerial hyphae was exposed (Figure 2a) and conidia production was severely hampered, that is, only a small number of conidia (fewer than 6×10^6 conidia per plate, equal to 1000 times less than the wild type) were observed despite the prolonged incubation (Figure S3). The conidia of the *CpCop23*-null mutant were characteristically hyaline, aseptate, and cylindrical-shaped, with no changes to conidia morphology observed (Figure 2a). However, conidia viability, as measured by the number of colony-forming units on the selective medium, had dramatically decreased, as fewer than 20% of plated conidia had germinated (Figure 2b). In addition, no signs of germination of resting conidia, such as swelling, were observed even after a prolonged incubation in liquid medium. These results indicate that the viability of the conidia had significantly decreased (Figure 2b). The viability of harvested conidia further diminished as the conidia aged in the conidial suspension. After 30 days at 4°C, fewer than 5% of the conidia were viable; therefore, it appears that the presence of the *CpCop23* gene affects the viability and longevity of conidia.

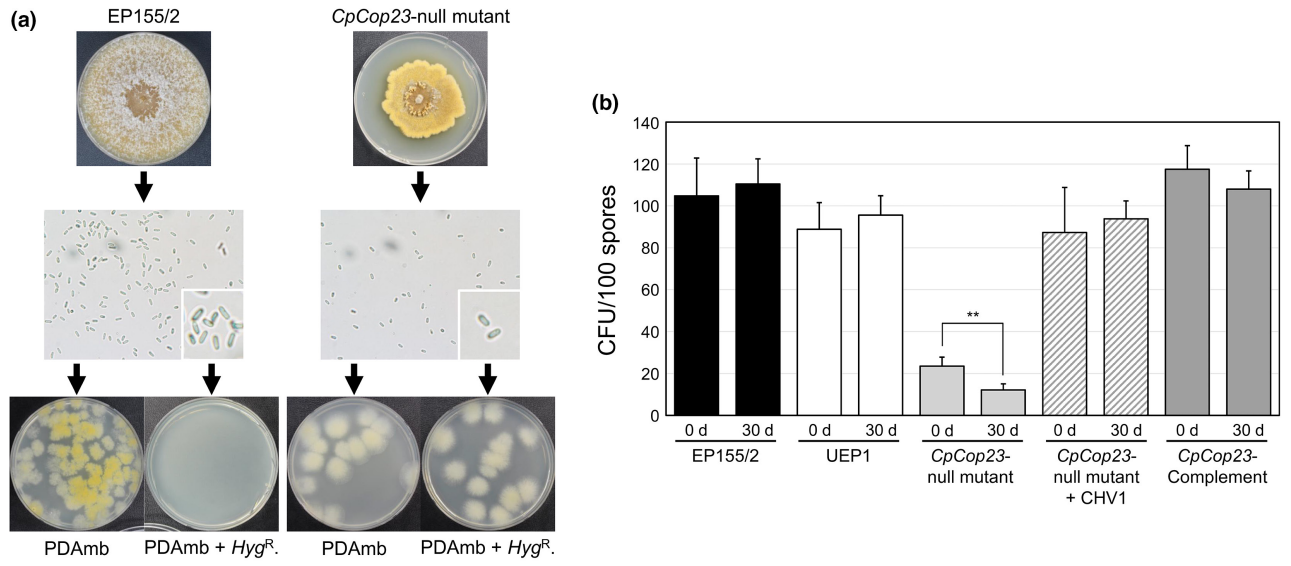

FIGURE 2 Conidia production and viability of wild-type EP155/2, *CpCop23*-null mutant, and CHV1-transferred mutant strains. (a) After conidia were harvested from each plate, the number of conidia was measured, diluted to 100 conidia, and spread on potato dextrose agar with methionine and biotin (PDAmb) plates with or without hygromycin B. Enlarged image of conidia is provided as an inset. (b) Conidial viability was measured by counting the colony-forming units on the corresponding media. Note that the viability of the conidia of the virus-free mutant strain dramatically decreased, and continued to decrease, as the conidia were stored.

To verify that the phenotypic changes of the *CpCop23*-null mutant were a result of the loss-of-function mutation in the *CpCop23* gene, *in trans* complementation of the *CpCop23*-null mutant with a wild-type allele of the *CpCop23* gene was performed, and the resulting geneticin-resistant transformants were compared to the parental wild type. The growth abnormalities of the *CpCop23*-null mutant previously observed under the standard growth and stress conditions no longer existed, and colony growth and observed responses to stress conditions were then similar to those of the wild type (Figures 3, 4, and S4). These results suggest that phenotype changes in the *CpCop23*-null mutant were a result of mutation in the *CpCop23* gene.

2.5 | Sensitivity of the *CpCop23*-null mutant to known Hsp90 inhibitors

Because the Hsp90 complex is known to regulate adaptive responses to antifungal drugs, we examined the responses of the *CpCop23*-null mutant to an antifungal polyene drug (amphotericin B), an Hsp90-related antifungal azole drug (ketoconazole), and a natural Hsp90 inhibitor (geldanamycin). As shown in Figure 3, no indication of sensitivity was observed in response up to 1 μg/ml of amphotericin B. However, 1 and 2 μg/ml of ketoconazole, an azole inhibitor of fungal specific ergosterol biosynthesis, prompted a significant inhibition in growth across all tested strains. Although ketoconazole inhibited the fungal growth of all tested strains and very limited growth of the *CpCop23*-null mutant was observed at 2 μg/ml, it was difficult to observe discernible differences in the sensitivity among them. However, when geldanamycin, a specific inhibitor of Hsp90, was administered to the culture medium, all tested strains proved to be sensitive, and the *CpCop23*-null mutant began to show more hypersensitivity than the others. In fact, the *CpCop23*-null mutant began to show hypersensitivity in response to an even lower concentration (0.2 μg/ml) and almost no (or only superficial) growth was observed at 1 μg/ml, whereas the growth of other strains showed was less reduced. Comparing to the responses to ketoconazole, other strains showed a similar or lower level of sensitivity to geldanamycin, but the *CpCop23*-null mutant revealed more severely reduced growth in response to geldanamycin. That Hsp90 inhibition reduced azole resistance (Cowen & Lindquist, 2005; Gu et al., 2016; Zhang et al., 2013), in conjunction with the observed hypersensitivity of the mutant to a known azole antifungal drug and Hsp90-specific inhibitor, strongly suggests that the cloned *CpCop23* gene is indeed a core co-chaperone of Hsp90 and that it plays an important role in Hsp90 activity. Accordingly, the absence of the *CpCop23* gene showed phenotypic changes resulting from the dysfunction of Hsp90.

2.6 | Stress responses of the *CpCop23*-null mutant

Because the heat-shock response of the Hsp90 co-chaperone p23 gene has been reported in other fungi, we measured the sensitivity of the *CpCop23*-null mutant to different temperatures (Figure 4a). After the *CpCop23* mutant was exposed to various nonstandard temperatures (15, 20, 32, and 35°C, with 25°C as the baseline), we detected no significant defects in its growth rate. These results demonstrated that the *CpCop23*-null mutant was not sensitive to temperature stress.

We also measured the response of the *CpCop23*-null mutant to other stressors, including oxidative, osmotic, and cell-wall inhibiting factors (Figures 4b and S4). No significant change in colony growth was observed when the *CpCop23*-null mutant was placed under oxidative stress using menadione (Figure S4a). Interestingly, once the medium was supplemented with up to 1 M sorbitol as an osmoticum, the colony growth of the *CpCop23*-null mutant on the PDAmb medium was enhanced (Figure 4b). The growth of the *CpCop23*-null mutant returned as it was at 1.5 M sorbitol, and above which (>2 M) the *CpCop23*-null mutant showed severely retarded growth as the wild type. When we changed the osmotic stabilizer from sorbitol

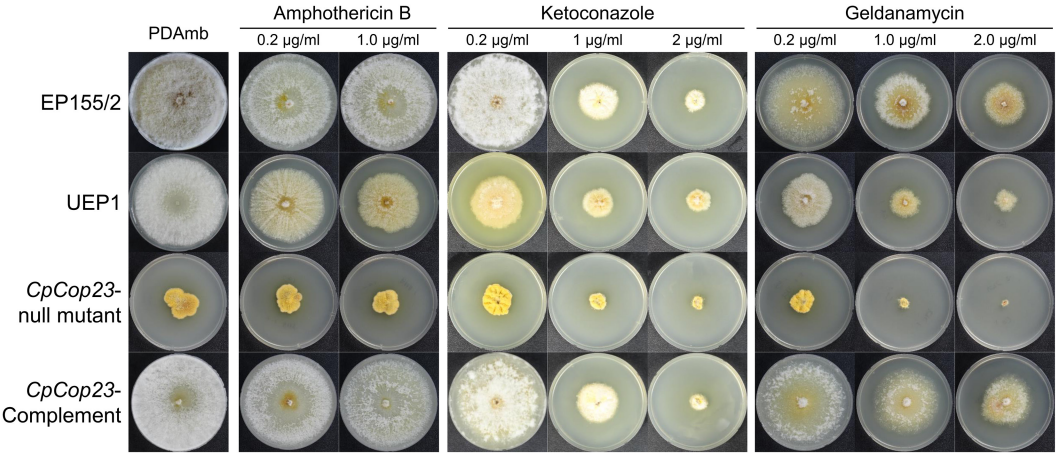

FIGURE 3 Effects of antifungal agent (amphotericin B) and Hsp90 inhibitors (ketoconazole and geldanamycin) on colony morphology. Colony morphologies of wild-type EP155/2, virus-infected UEP1, *CpCop23*-null mutant, and *CpCop23*-complemented strains on potato dextrose agar with methionine and biotin (PDAmb) and in the presence of antifungal agents or Hsp90 inhibitors are shown after 7 days of cultivation in the dark.

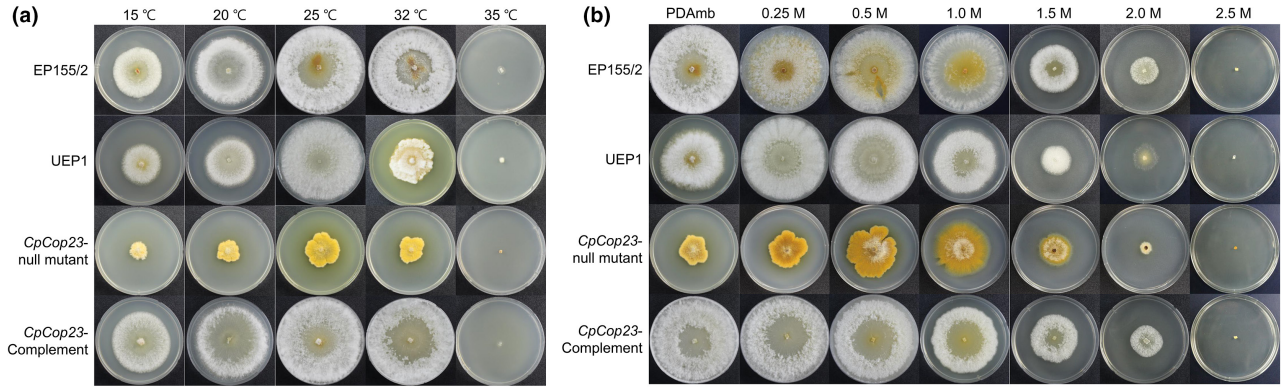

FIGURE 4 Effect of temperature and osmotic stress on colony morphology. Colony morphologies of wild-type EP155/2, virus-infected UEP1, *CpCop23*-null mutant, and *CpCop23*-complemented strains after 7 days of cultivation on potato dextrose agar with methionine and biotin (PDAmb) (a) under different temperature conditions and (b) with different concentrations of sorbitol.

to sucrose, no difference in growth patterns was observed in the *CpCop23*-null mutant (data not shown). The fact that supplementation of medium concentrations of osmotic stabilizer enhanced the growth of the *CpCop23*-null mutant suggests that loss of the *CpCop23* function was in part responsible for intracellular osmotic stress, which can be complemented by extracellular supplementation. As osmotic stress was observed in the *CpCop23*-null mutant, we measured the responses of the mutant to cell-wall inhibiting agents such as sodium dodecyl sulphate (SDS), Congo red, and Calcofluor white (Figure S4b). The *CpCop23*-null mutant showed hypersensitivity to all three agents, suggesting that the cell-wall integrity of the *CpCop23*-null mutant was affected.

2.7 | Effect of hypoviral infection on the colony morphology of the *CpCop23*-null mutant

The biological functions of the *CpCop23* gene relevant to hypovirus infection were examined by comparing phenotypic changes in the virus-free and virus-containing isogenic transformants. Following coculturing of the recipient *CpCop23*-null mutant with donor UEP1 (Figure 5a), at least three putative virus-containing recipient mycelial areas were selected and transferred onto a new hygromycin B-containing PDAmb plate. The putative CHV1-transferred *CpCop23*-null mutant progenies were single-spored for nuclear homogeneity on the selection medium. The single-spored progenies from different sections were examined for the presence of double-stranded (ds) RNA by agarose gel electrophoresis prior to comparison (data not shown). The complete loss of the *CpCop23* gene in the CHV1-transferred null mutant strain was confirmed again by PCR (Figure S5). Colony growth with relatively vigorous aerial mycelia recovered to nearly normal levels in the virus-transferred *CpCop23*-null mutant, but not in the virus-free *CpCop23*-null mutant (Figure 5b). However, reduced pigmentation and asexual sporulation (characteristic phenotypic changes caused by CHV1 infection) were observed in the CHV1-transferred

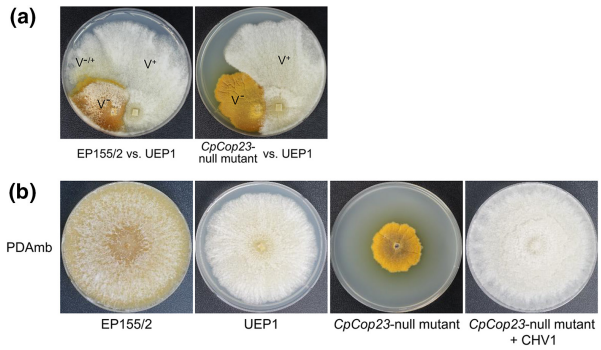

FIGURE 5 Effects of CHV1 infection on fungal growth. (a) Transmission of CHV1 through hyphal anastomosis in paired cultures with CHV1-infected UEP1 are shown. V^+, V^-, and $V^{-/+}$ indicate hypovirus donor, hypovirus recipient, and putative hypovirus-transferred recipient, respectively. (b) Colony morphology of CHV1-free (EP155/2 and TdCop23) and -infected strains (UEP1 and TdCop23 + CHV1) on potato dextrose agar with methionine and biotin (PDAmb) medium.

CpCop23-null mutant (Figures 5b and S3). No further reduction of conidiation was observed in the *CpCop23*-null mutant after the CHV1 infection (Figure S3). Considering the down-regulation of the *CpCop23* gene by CHV1 infection, the *CpCop23* gene appears to be a direct target for the viral regulation of symptom development including reduced conidiation. Such phenotypic changes were stable and maintained throughout the successive subculture of the CHV1-containing null mutant. RT-qPCR revealed that there was no difference in the virus titres of the CHV1-infected wild type (UEP1) and the CHV1-transferred *CpCop23*-null mutant (Figure S6). In addition, we estimated the conidial viability of the CHV1-transferred *CpCop23*-null mutant. Interestingly, the viability of conidia from the CHV1-transferred *CpCop23*-null mutant recovered to reach the same level as the wild type or CHV1-infected wild type (UEP1) (Figure 2b). Thus, these results suggest that the *CpCop23* gene is important for mediating the appropriate response of *C. parasitica* to hypoviral infection.

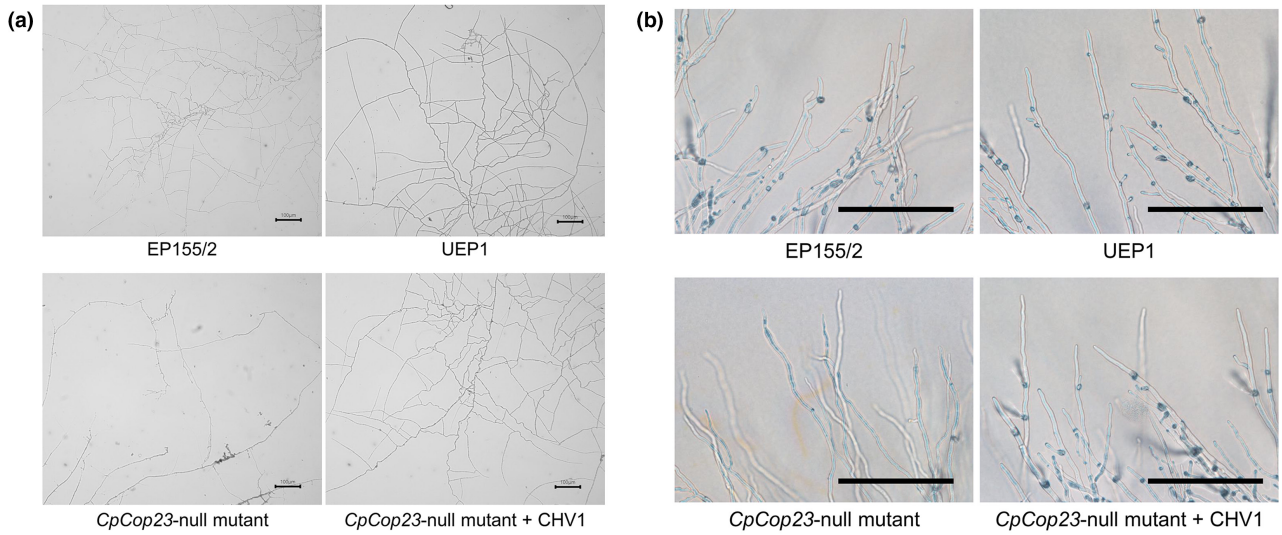

FIGURE 6 Morphology of hyphae. (a) Micrographs of germinated conidia after 24 h growth on EP complete medium. (b) Micrographs of hyphal tips after 24 h growth on potato dextrose agar with methionine and biotin. Scale bar = 100 μm.

2.8 | Hyphal fusion analysis

Because the frequency of successful viral transfer via hyphal fusion was very low (Figure 5a shows a near complete absence of area with a different growth pattern indicated by "$V^{-/+}$" following coculturing of the recipient *CpCop23*-null mutant with donor UEP1), microscopy-based analyses were then performed to examine intracolonial hyphal fusion within the young germinated mycelia and actively growing colonies, that is, whether hyphal fusion was affected by the mutation of the *CpCop23* gene. To confirm the connected fusion hyphae, cytoplasmic continuity was verified by observing the flow of cytoplasmic organelles under the microscope. In the wild type, hyphal fusion, represented by the typical "H" form indicating fusion hyphae, was readily observed in young, germinated hyphae from 24 h after germination of conidia. However, the *CpCop23*-null mutant showed less mycelial growth as well as the rarely observed connected hyphal fusion with cytoplasmic flow in the subapical region (Figure 6a), that is, almost no hyphal fusion was observed in the young, germinated hyphae (Figure 6a). Interestingly, active mycelial growth, including lateral hyphal fusion of the geminated conidia, was observed in the CHV1-infected *CpCop23*-null mutant.

Mycelial growth at the colony margin was also compared (Figure 6b). Dense and well-branched apical hyphae were distinctive in the wild type while fewer and rarely branched hyphal growth was discerned in the *CpCop23*-null mutant. Apical mycelial growth in the CHV1-infected *CpCop23*-null mutant was very similar to that in the wild type (Figure 6b). Active mycelial growth and hyphal fusion of the CHV1-infected *CpCop23*-null mutant were further verified by viral transfer from the CHV1-infected *CpCop23*-null mutant to the virus-free wild type via hyphal fusion. Following coculturing of the CHV1-infected *CpCop23*-null mutant with the virus-free wild type, successful viral transfer from the donor CHV1-infected *CpCop23*-null mutant to the recipient virus-free wild type was easily detectable (Figure S7).

2.9 | Virulence assay

A virulence assay using the excised bark from a chestnut tree demonstrated that the virulence of the *CpCop23*-null mutant was significantly reduced based on the smaller size of the necrotic area induced on excised bark compared to that induced by the wild type EP155/2. The size of the necrotic area produced by the *CpCop23*-null mutant was comparable to that resulting from a hypovirulent UEP1 strain (Figure 7). Statistical analysis indicated that the virulence of the *CpCop23*-null mutant was significantly different from that of the wild type but was similar to that of the UEP1. The complemented strains showed a similar level of virulence as the wild type. Interestingly, almost no virulence was observed in the CHV1-transferred *CpCop23*-null mutant. Because the CHV1-transferred *CpCop23*-null mutant showed a more recovered growth rate than its parental CHV1-free *CpCop23*-null mutant (which was itself similar to that of the wild type), we suggest that the significantly decreased virulence of the *CpCop23*-null mutant was not merely the result of the impaired growth rate but rather a consequence of the fact that the *CpCop23* gene is involved in determining the virulence of *C. parasitica*.

3 | DISCUSSION

Eukaryotic Hsp90, which is important for the maintenance of protein homeostasis, functions with numerous co-chaperones that modulate its activity, while bacterial Hsp90 is with no accompanying co-chaperones. Co-chaperone p23, a central component of the Hsp90 complex that stabilizes the conformation of Hsp90, inhibits the ATPase activity and contributes to client maturation (Biebl et al., 2021). Although p23 is essential to regulatory interactions, it is only Hsp90 (and not Hsp90 complexed with an accompanying co-chaperone such as p23) that is absolutely essential for viability in budding and fission

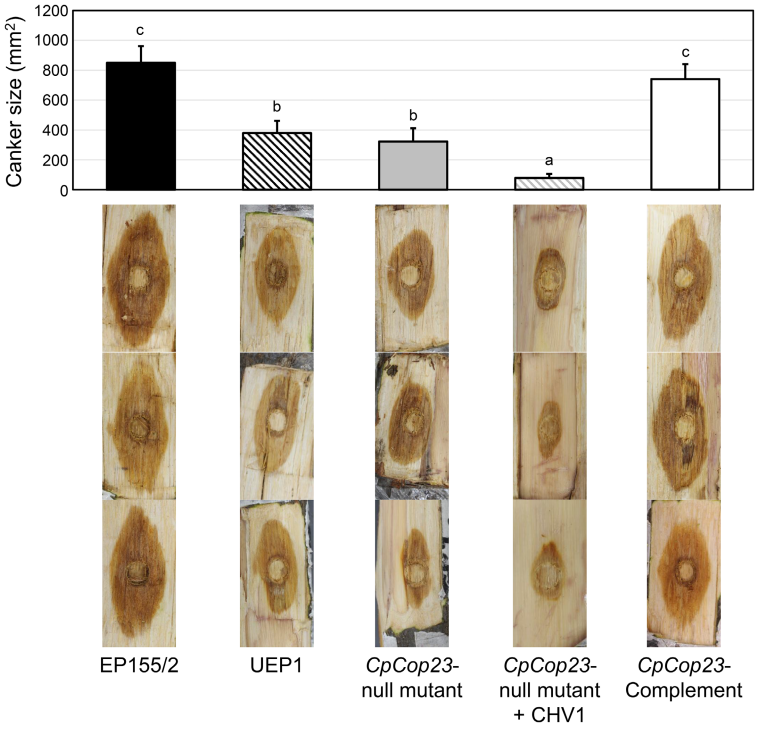

FIGURE 7 Virulence test of *CpCop23*-null mutant on excised chestnut tree bark. EP155/2, virus-infected UEP1, *CpCop23*-null mutant (TdCop23), virus-infected isogenic mutant strain (TdCop23 + CHV1), and the complemented strain of TdCop23 were inoculated onto excised chestnut tree bark. Potato dextrose agar block was used as a control and canker size was measured 7 days after treatment. The *CpCop23*-null mutant showed reduced virulence in comparison with the wild type. In the bar graphs of canker size, the mean ± *SD* of each of the three independent experiments is shown. Different lowercase letters indicate statistically significant differences ($p < 0.01$, one-way analysis of variance).

yeasts, suggesting the existence of a functional diversity of p23 other than Hsp90 modulation (Forafonov et al., 2008). For that reason, it was interesting to observe that only p23, as opposed to Hsp90, was suggested by our previous study as a protein product modulated by the presence of CHV1 and TA supplementation. This result prompted us to analyse the function of p23 in the filamentous fungus *C. parasitica*.

The resulting pure *CpCop23*-null mutant showed various responses depending on the stressors tested. In light of the hypersensitivity of the *CpCop23*-null mutant to cell wall-disturbing agents such as SDS, Congo red, and Calcofluor white, the cloned *CpCop23* gene can be identified as the molecular chaperone functionally involved in that stress response. However, that no dramatic changes were observed in the responses to other tested stress conditions, which included temperature and reactive oxygen species, suggests that the pathway governed by the *CpCop23* gene is specific. The hypersensitivity to the cell wall-disturbing agents, recovery of colony growth after supplementation with some concentrations of osmotic stabilizer (including 0.5 and 1 M of sorbitol), and the hypersensitivity of the *CpCop23*-null mutant to antifungal drugs such as azole (which inhibits the syntheses of ergosterol) suggest that the pathway modulated by the *CpCop23* gene is specific to the cell membrane and its associated cell wall integrity. Although functional redundancy may exist, hypersensitivity to the known Hsp90 inhibitor and Hsp90-related azole drug clearly indicates that the *CpCop23* gene functions as a co-chaperone of Hsp90. In addition, the presence of pure *CpCop23*-null mutant suggests that the co-chaperoning activity of *CpCop23* is important but not essential to the cell wall stress response.

It is interesting that the *CpCop23* gene is involved in conidial viability, as more than 80% of freshly harvested conidia did not germinate and showed no signs of initial germination. This diminished viability continued as the harvested conidia aged. Although there have been several studies of the genes affecting the conidial production in *C. parasitica* (Jo et al., 2019; Ko et al., 2021; Sun et al., 2009), no studies of genes that exert an effect on conidial viability have been reported for this fungus. As Hsp90 is known to govern the cellular activity for morphogenetic transition from yeast to filamentous growth in *C. albicans*, the lack of any sign of conidial germination from the *CpCop23*-null mutant suggests that *CpCop23* is involved in morphogenetic development, that is, germination (from swelling to polar growth) in *C. parasitica*. Given that there was no indication of even initial germination, the *CpCop23* gene appears to be implicated in the production of the initial signal for the germination or conidia-specific biological process. The retarded growth, which lacks aerial hyphae but maintains the mostly invasive mycelial mat of the *CpCop23*-null mutant, suggests that the *CpCop23* gene is important for the differentiation of mycelia as well.

Our previous studies have shown that molecular chaperones are important for fungal virulence (Baek et al., 2014). The severely reduced virulence of the *CpCop23*-null mutant suggests that the *CpCop23* gene plays an important role in determining virulence. As Hsp90 is one of two major chaperones, and recalling the close intermolecular interactions between Hsp70 and Hsp90, we ascribe the phenomenon of fungal virulence via the *CpCop23* gene to the chaperoning activity of the *CpCop23* gene for virulence factor(s), which we assume to be the client protein. In the future, it will be interesting to identify which client proteins of *CpCop23* affect fungal virulence.

Aside from the direct involvement of the *CpCop23* gene in determining fungal virulence, the decreased production of conidia and their impaired viability can be expected to inhibit fungal pathogenicity during the disease cycle, as the conidia of *C. parasitica* are believed to be the primary natural inoculum. In other words, the diminished production and viability of this primary inoculum (conidia) that can be observed when the *CpCop23* gene is absent will impair

the ability of the fungus to disseminate chestnut blight throughout a chestnut tree population.

It is interesting to observe that infection with CHV1 had a dramatic effect on the colony morphology of the *CpCop23*-null mutant, as CHV1 infection over-ruled the mutant phenotypes. Unlike the virus-free *CpCop23*-null mutant, the CHV1-infected *CpCop23*-null mutant showed recovered colonial growth with aerial hyphae, recovered conidial viability, recovered hyphal branching with intrahyphal fusion, and decreased pigmentation, similar to that of the CHV1-infected wild-type strain UEP1. Based on the observed characteristic phenotypic changes associated with the mutation of the *CpCop23* gene, it is highly unlikely that there is a functional redundancy of the *CpCop23* gene. As the phenotypic recovery of the *CpCop23*-null mutant occurred by CHV1 infection and the *CpCop23* gene was down-regulated by CHV1 infection (Kim et al., 2012), it is possible that viral symptom development occurs only under the condition of the repression of the *CpCop23* gene. Therefore, there must be a complex mechanism for the regulation of the *CpCop23* gene and associated symptom development. Many studies have shown that Hsp90 is universally required for the propagation of viruses via protein folding and stabilization (Geller et al., 2012). In light of the recovered mycelial growth and reduced pigmentation observed in the CHV1-infected *CpCop23*-null mutant, which are characteristic symptoms of CHV1 infection, we suggest that Hsp90 has no effect on CHV1 replication. RT-PCR analysis likewise suggested that no significant changes in viral titre occurred in the *CpCop23*-null mutant. CHV1-EP713, the CHV1 strain used in this study, is a type member of hypovirus defined as a capsidless virus unable to form rigid particles. It has been suggested that the viral proteins of CHV1 are relatively simple but not complex structures that are required to be folded by Hsp90 and its core co-chaperones (Blair et al., 2019; Luengo et al., 2018). It is interesting to see that treatment with an Hsp90-specific inhibitor (geldanamycin) impeded growth but caused no changes in pigmentation in the wild type. Likewise, the *CpCop23*-null mutant showed hypersensitivity to the geldanamycin treatment, but retained its abnormal intensive pigmentation, which is characteristic of the mutant strain. In both the wild type and the *CpCop23*-null mutant, pigmentation was significantly reduced by CHV1 infection. Therefore, we regard it as highly likely that the *CpCop23* gene has additional functions in pigmentation, which is under the control of CHV1 infection and more of independent of co-chaperoning of Hsp90.

4 | EXPERIMENTAL PROCEDURES

4.1 | Construction of fungal strains and culture conditions

Wild-type *C. parasitica* strain EP155/2 (ATCC 38755) and its isogenic hypovirulent strain UEP1 infected with hypovirus CHV1 were cultured on solid potato dextrose agar (PDA) supplemented with methionine and biotin (PDAmb) or liquid EP complete medium at 25°C under conditions of constant low light as previously described (Kim et al., 1995). To assess the function of the Hsp90 co-chaperone p23 gene, the *CpCop23* null mutant was generated by transforming the EP155/2 strain with a deletion construct carrying the hygromycin phosphotransferase gene. Briefly, the *CpCop23* gene was replaced with the *hph* selective marker by double-joint PCR (Yu et al., 2004). The DNA fragments upstream of *CpCop23* were amplified with primers CpCop23-5FL-F and 5FL-CpCop23-hph-R, while those downstream were amplified with hph-CpCop23-3FL-F and 3FL-CpCop23-R (see Table S1 for primer sequences). The *hph* marker was amplified from plasmid pDH25 with primers 5FL-CpCop23-hph-F and hph-CpCop23-3FL-R. These three fragments were fused and the resulting fused cassette was used to transform the EP155/2 strain by protoplast fusion. Protoplast preparation and transformation were performed as previously described (Chen et al., 1994; Churchill et al., 1990) with the following minor modification: protoplasts were plated in agar supplemented with 150 μg/ml hygromycin B or 150 μg/ml geneticin. Gene replacement was screened by PCR using two pairs of outer gene-specific primers (primers P1/P3 and P2/P4 in Figure S2a), corresponding to nt (−1964 to −1943)/(1222 to 1241) and (466 to 465)/(3046 to 3067), relative to the start codon of *CpCop23* (Figure S2a). Southern blot analysis using a combination of restriction enzyme *Xho*I and a gene-specific probe was applied to confirm the putative *CpCop23*-null mutant (Churchill et al., 1990).

4.2 | Nucleic acid isolation and manipulation

Ten micrograms of DNA was digested with restriction enzymes, blotted onto the nitrocellulose membrane, and then hybridized with radiolabelled probe.

RNA was prepared from mycelial mats grown on cellophane that had been layered on top of appropriate media as previously described (Park et al., 2004).

4.3 | RT-qPCR analysis

The mRNA levels of *CoCop23* and *gpd* were evaluated with quantitative real-time PCR by a GeneAmp 7500 sequence detection system (Applied Biosystems) using a cDNA reverse transcription kit with a SYBR green mixture RT kit (Applied Biosystems) consistent with the manufacturer's protocol. Gene expressions were quantified following the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) and the glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) gene was used as a reference gene. All primer sequences used for gene expression are listed in Table S1.

4.4 | Bioinformatic tools

Multiple alignment of fungal p23/Sba1 orthologue proteins was performed using ClustalX software (Thompson et al., 1997) and a subsequent phylogenetic tree was constructed by maximum-likelihood

(ML) method, with a bootstrap of 1000 replicates using the Jones–Taylor–Thornton (JTT) model of amino acid substitution (Kumar et al., 2018). Identification of orthologue genes and the conserved domain were assessed using BLASTP and Pfam, respectively.

4.5 | Stress tolerance assay

To examine thermal tolerance, wild-type EP155/2, virus-infected UEP1, *CpCop23*-null mutant, and *CpCop23*-complemented strains were each incubated at 20, 25, and 30°C for 7 days. To observe the influence of osmotic stress, wild-type EP155/2, virus-infected UEP1, *CpCop23*-null mutant, and *CpCop23*-complemented strains were treated with varying concentrations (0.25, 0.5, 1, 1.5, 2 or 2.5 M) of sorbitol and incubated for 7 days at 25°C. To determine the response to various cell wall perturbing agents, the culture medium was supplemented with 0.005 to 0.01% SDS, 50 to 100 μg/ml Calcofluor white, or 0.2 to 0.3 mg/ml Congo red. To examine the responses of each to various antifungal agents such as amphotericin B (0.2 or 1 μg/ml), geldanamycin (0.2, 1 or 2 μg/ml), and ketoconazole (0.2, 1 or 2 μg/ml), wild-type EP155/2, virus-infected UEP1, *CpCop23*-null mutant, and *CpCop23*-complemented strains were incubated at 25°C for 7 days.

4.6 | Virulence assay

A virulence assay was conducted by inoculating EP155/2, UEP1, mutant, and virus-infected mutant, and complemented strains on excised bark from a chestnut tree as described previously (Lee et al., 1992). After 7 days, the size of cankers was measured. This experiment was repeated three times.

4.7 | Transmission of CHV1

CHV1 was transmitted through hyphal anastomosis as described previously (Cortesi et al., 2001). Briefly, the virus-containing strain UEP1 was cultured on a PDA plate with a virus-free recipient *CpCop23*-null mutant strain in close proximity and incubated at 25°C. Putatively fused mycelial plugs were randomly taken along the border between the UEP1 and the *CpCop23*-null mutant after 7 days of cocultivation, and then transferred to hygromycin-containing PDAmb. Single-spored isolates manifesting the distinctive CHV1-infected morphology, such as reduced growth or pigmentation, were evaluated for hypovirus transmission. The presence of hypovirus was confirmed by viral genomic dsRNA profiling.

4.8 | Statistical analysis

Statistical differences among the strains were analysed by Student's *t* test and analysis of variance using SPSS v. 18. $p < 0.01$ was considered significant.

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DATA AVAILABILITY STATEMENT

All data sets generated for this study are included in this article or supporting information; further enquiries can be directed to the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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