Functional Analysis of a Tannic-Acid-Inducible and Hypoviral-Regulated Small Heat-Shock Protein Hsp24 from the Chestnut Blight Fungus *Cryphonectria parasitica*

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A small heat-shock protein gene, *CpHsp24*, of *Cryphonectria parasitica* was selected based on its expression pattern, which showed that it was tannic acid inducible and that its induction was severely hampered by a hypovirus. The predicted protein sequence of *CpHsp24* consisted of a hallmark α -crystalline domain flanked by a variable N-terminal and a short C-terminal region. Disruption of *CpHsp24* resulted in a slow growth rate under standard growth conditions. The *CpHsp24*-null mutant showed enhanced sensitivity to heat shock, which was consistent with Northern and Western analyses displaying the heat-shock induction of the *CpHsp24* gene and protein, respectively. Virulence tests on the excised bark revealed a severe decrease in the necrotic area of the *CpHsp24*-null mutant. When the hypovirus was transferred, virus-containing *CpHsp24*-null progeny displayed severely retarded growth patterns with hypovirulent characteristics of reduced pigmentation and sporulation. Because the tannic-acid-inducible and hypoviral-suppressible expression and the severely impaired virulence are also characteristics of the laccase3 gene (*lac3*), *lac3* expression in the *CpHsp24*-null mutant was also examined. The resulting *lac3* induction was severely affected in the *CpHsp24*-null mutant, suggesting that *CpHsp24* is important for *lac3* induction and that *CpHsp24* may act as a molecular chaperone for the *lac3* protein.

Cryphonectria parasitica (Murrill) M. E. Barr, the causal agent of chestnut blight, has devastated both European and American chestnut forests in the first part of the 20th century (Van Alfen 1982). A recent survey in Korea, where chestnut blight was believed to cause only mild problems, indicated that disease severity has become more pronounced; more than 30% of necrotic lesions of chestnut trees in Korea were infected with *C. parasitica* (Ju et al. 2002) and variations in disease severity were observed among currently available chestnut varieties that were previously considered to be disease-resistant (Lee et al. 1999). However, fungal strains infected with a single-stranded (ss)RNA virus, *Cryphonectria hypovirus 1* (CHV1), exhibited the characteristic symptoms of lowered virulence, a

phenomenon referred to as hypovirulence (Anagnostakis 1982; Nuss 1992; Van Alfen et al. 1975), with diverse hypovirulence-associated phenotypic changes such as reduced sporulation, pigmentation, female fertility, laccase production, and oxalate accumulation (Elliston 1985; Havir and Anagnostakis 1983; Rigling et al. 1989). The molecular basis for these phenotypic changes was attributed to the hypoviral modulations in host transcriptional and translational profiles (Allen and Nuss 2004; Allen et al. 2003; Deng et al. 2007; Kang et al. 1999; Kazmierczak et al. 1996, 2012; Kim et al. 2012; Wang et al. 2013).

Systemic analysis of transcriptional and translational changes regarding the virulence of forest fungal pathogens, such as chestnut blight fungus, to their native hosts has been considered to be more difficult than the analysis of grass pathogens (Chung et al. 2008). However, supplementation with tannic acid (TA), which is abundant in the bark of chestnut trees and is assumed to be one of the major barriers against pathogen infection, is believed to be successful in imitating wood induction in *C. parasitica* and to address some, not all, of the fungal changes that occur in its native host for aspects of pathogenesis and fungal gene regulation by the hypovirus (Chung et al. 2008; Rigling et al. 1989). Moreover, a recent study demonstrated the feasibility of the proteomic analysis of *C. parasitica* grown in the presence of TA, which is known to precipitate protein products (Kim et al. 2012). Therefore, the systemic analysis of transcriptional and translational products in the presence of TA has been established and is expected to foster in-depth understanding of plant–fungus–mycovirus interactions.

Among the several fungal regulatory pathways perturbed by hypovirus infection, the stress-response pathway has been of interest because of its specificity in hypoviral perturbation (Allen and Nuss 2004; Allen et al. 2003). However, few studies have been conducted on the biological function of the components of the stress-response pathway in this fungus (Lim et al. 2010).

Heat-shock response is a molecular reaction to stressful but sublethal temperature and is characteristic of all living organisms, including bacteria, fungi, plants, and animals (Lindquist and Craig 1988; Plesofsky 2004). During the initial stage of the heat-shock response, normal activities of transcription and translation are drastically reduced and massive quantities of heat-shock proteins (Hsp) are produced. Hsp, a group of molecular chaperones found in all organisms, accumulate upon shifts from a physiological temperature to higher temperatures. These proteins are also induced by other stresses, allowing cells to adapt and survive under challenging conditions. The heat-shock response is ubiquitous and prominent Hsp are

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highly conserved among all groups of organisms. Many Hsp are essential proteins that contribute to cellular metabolism during normal growth but are strongly induced during specific stages of organelle biogenesis, protein folding and transport, transcriptional activation mechanisms, stress resistance, and differentiation (Lindquist and Craig 1988; Plesofsky 2004).

According to their molecular weight, Hsp have been classified into several classes or families, and a cell may express multiple members of the same family (Walter and Buchner 2002). There are five major families of Hsp (Buchner 1996; Richter et al. 2010); four of them—Hsp100s, Hsp90s, Hsp70s, and Hsp60s—consist of ATP-dependent high molecular mass Hsp, while the fifth family—the small Hsp (sHsp)—consists of ATP-independent low molecular mass Hsp with sizes of 12 to 42 kDa (Jaya et al. 2009) and a conserved sequence in their C-terminus called the α -crystalline domain (de Jong et al. 1993; Garstel et al. 1997; Narberhaus 2002). Unlike other families of Hsp, sHsp show extensive sequence variation and evolutionary divergence. However, compared with the high molecular mass Hsp, fewer sHsp have been identified and functional studies of sHsp are considerably limited.

In this study, we report the characterization of a TA-inducible and hypoviral-regulated sHsp from *C. parasitica*. By construction of the corresponding null mutant, we demonstrated the biological function of the cloned sHsp and suggested how the protein product of the cloned *sHsp* gene might be involved in the virulence of this fungus.

RESULTS

Cloning and characterization of a TA-responsive and hypoviral-regulated *C. parasitica* Hsp 24 gene.

RNA differential display using the GeneFishing method, a polymerase chain reaction (PCR)-based differential display technique, resulted in more than 30 PCR amplicons showing the expression pattern in response to TA and hypovirus infection. Among them, a 401-bp PCR amplicon exhibiting an expression pattern of induction by TA that was alleviated by hypovirus infection was selected and further characterized. Northern blot analysis was conducted to verify the PCR-based expression pattern. The accumulation of transcript in virus-free EP155/2 was highly induced by the TA supplement (Fig. 1). However, its induction pattern was significantly decreased in virus-infected UEP1. These Northern blot analyses of the hypovirus-regulated TA-induced pattern were in a good agreement with the findings from the GeneFishing technique.

Sequence analysis of the cloned PCR amplicon revealed a high homology with known *sHsp* genes. The highest sHsp homolog was the sHsp30 of *Metarhizium acridum*, which shared 37% identity at the amino acid level. By inspection of the draft genome sequence and sequence comparison with other known sHsp, a 4,047-bp fragment, which was expected to contain a full-length *sHsp* gene, was amplified using genomic PCR. 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). In addition, based on the genomic sequence analysis, a near full-length cDNA clone was obtained using reverse-transcription (RT)-PCR with the primer pair *C. parasitica* Hsp 24 (CpHsp24)-cF1 and CpHsp24-cR1 at nucleotide positions 1 to 21 and 628 to 648 (relative to the start codon), respectively. The resulting 648-bp amplicon was cloned into the pGEM-T Easy Vector (Promega Corp., Madison, WI, U.S.A.). A sequence comparison with the corresponding genomic sequence revealed that the cloned gene appeared to be intron-less, which is common in other fungal sHsp.

Sequence analysis in the promoter region of the *CpHsp24* gene revealed canonical CAAT and TATAA boxes at –191 and –149 nucleotides (nt), respectively (Supplementary Fig. S1A). The minimum consensus sequence of heat-shock element nGAAnnTTCnnGAAn (Tachibana et al. 2002) was not found up to 5.0 kb upstream of the start codon. However, within 2.0 kb, there appeared to be four nonconventional heat-shock response element (HSE) with a 5-bp insertion between two repeating pentameric units, nGAAn-(5-bp)-nGAAn, at –94, –1,378, –1,738, and –1,876 nt from the start codon (Tachibana et al. 2002). In addition, analysis of the promoter region revealed the presence of two stress response element (STRE) motifs (core sequence AGGGG at –1,896 and CCCCT at –1,406), a DNA element present in many stress-induced genes (Martinez-Pastor et al. 1996). The sequence around the first ATG was in good agreement with Kozak's consensus sequence in that the nucleotide –3 position was the A in AAAGATG.

The deduced protein product of the cloned gene consisted of 215 amino acids (aa), with an estimated molecular mass of 24.4 kDa and an isoelectric point of 6.52 (the GenBank accession number for *CpHsp24* is KF201573). Analysis of the deduced amino acid sequence revealed the presence of the conserved hallmark domain of sHsp, an α -crystalline domain between 73 and 195 aa, which was flanked by a variable hydrophobic N-terminal region and a short C-terminal extension. Homology searches using the deduced amino acid sequence indicated that the protein product of the cloned gene is related to the fungal sHsp from *Neosartorya fischeri* (35%), *Talaromyces marneffeii* (35%), *Aspergillus niger* (33%), *Magnaporthe grisea* (30%), *Neurospora crassa* (26%), *Trichoderma reesei* (26%), and *Saccharomyces cerevisiae* (24%). Multiple alignment of closely related sHsp revealed that the cloned gene maintained the conserved amino acids in the α -crystalline domain

Fig. 1. Northern blot analysis of *CpHsp24* in response to tannic acid (TA) supplementation. Total RNA was extracted from the virus-free wild-type (EP155/2) and its isogenic virus-containing hypovirulent strain (UEP1). The identification of the strains is shown at the top of the lanes. ME and ME+TA, indicated above the panel, represent the absence and presence of TA supplementation, respectively. Equal loading of RNA samples was demonstrated in the bottom panels by a parallel blot hybridized with a glyceraldehyde-3-phosphate dehydrogenase (*Gpd*) probe as an internal control and the ethidium bromide-stained gel (rRNA).

and there were two 6-aa extensions at the variable N-terminal region. In addition, the resulting phylogram indicated that the protein product of the cloned gene fell into a different cluster from the other containing those from *N. crassa*, *T. reesei*, *M. grisea*, *Talaromyces marneffe*, *Neosartorya fischeri*, and *A. niger*, with a high bootstrap value suggesting a genuine evolutionary relationship (Henikoff and Henikoff 1992). Together with the presence of the hallmark sequence of the α -crystalline domain within the amino acid sequence, the significant homology to the known fungal sHsp, and the predicted molecular mass of the cloned gene, we referred to the cloned gene as *CpHsp24*.

Regulation of the expression of *CpHsp24*.

Because the cloned gene appeared to encode the sHsp, we examined the expression pattern of the gene at the protein level under the heat-shock condition. Western blot analysis of the protein product (CpHSP24) of the *CpHsp24* gene revealed the major antigen-antibody reacting band at 37 kDa in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. The absence of the corresponding band at 37 kDa in the *CpHsp24*-null mutant indicated that the band at 37 kDa was specific to the protein product of the *CpHsp24* gene. The molecular weight deduced from the DNA sequence of the gene was 24.4 kDa while the specific antigen-antibody reacting band ran at 37 kDa in SDS-PAGE gels. Considering the fact that there is no conventional glycosylation site Ser/Thr-X-Asn

in the deduced sequence of CpHSP24, the reason for these differences in molecular size estimates is currently unknown.

Western blot analysis indicated that the expression of CpHSP24 was induced, remained until 12 h after the heat shock, and then decreased at 24 h (Fig. 2). These induction patterns in response to heat-shock stress demonstrated that, indeed, the cloned gene encoded the Hsp. Interestingly, the induction pattern of CpHSP24, although not as distinctive as in TA induction, was hampered by the hypovirus, as suggested by the expression pattern of CpHSP24 from the hypovirus-infected UEP1 strain under the heat-shock condition.

Construction of the *CpHsp24*-null mutant.

To determine the biological function of the *CpHsp24* gene, the *CpHsp24*-null mutant was constructed by site-directed recombination during integrative transformation. In total, 150 single-spored transformants with the disrupted *CpHsp24* gene designed for gene replacement were screened by PCR using the outer gene-specific and inner hygromycin phosphotransferase (*hph*) primers, which corresponded to -1,360 to -1,340 and 1,222 to 1,241 (relative to the start codon of *CpHsp24* and *hph*, respectively). One transformant showed a PCR amplicon of the expected size, 2.9 kb, corresponding to the disrupted alleles of the *CpHsp24* gene. The putative *CpHsp24*-null mutant (TdHsp24-1) was further confirmed by Southern blot analysis (Supplementary Fig. S2). The hybridization pattern of *SacI*-digested genomic DNA of the three single-spored clones of *CpHsp24*-null mutant with the probe prepared using the 619-bp *SacI*-digested *CpHsp24* fragment (probe A) differed from that of the wild type. In addition, the hybridization pattern of *Bam*HI-digested genomic DNA of the three clones probed with the 1,350-bp *Bam*HI-digested *CpHsp24* fragment (probe B) differed from that of the wild type. These results suggested that the transforming vector had integrated at the *CpHsp24* locus by site-directed homologous recombination. Moreover, the *SacI*- and *Bam*HI-digested genomic DNA of three clones of *CpHsp24*-null mutant had the hybridizing bands at 4.8 and 1.2 kb, respectively, corresponding to the expected size of the replaced allele. These hybridizing patterns along with PCR confirmation indicated that

Fig. 2. Western blot analysis of CpHSP24 in response to heat shock conditions. **A**, Coomassie blue-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis of cell-free protein extract of *Cryphonectria parasitica*. **B**, Antigen-antibody reaction of the corresponding gel. The arrowhead indicates the CpHSP24 protein. Strains designated above the line are the wild-type (EP155/2) and its isogenic hypovirulent strain (UEP1). Numbers at the top of the lanes refer to hours after heat shock. For each lane, 30 μ g of protein extract was loaded.

Fig. 3. Colony morphology of the *CpHsp24*-null mutant strain. Colonies after 10 days of culturing on potato dextrose agar supplemented with methionine and biotin are shown. Strains used, indicated above the panel, are the wild-type EP155/2, hypovirulent UEP1, *CpHsp24*-null mutant (TdHsp24-1), and the *CpHsp24*-complemented (TcHsp24-1) strains.

Table 1. Characteristics of null mutants compared with the wild type and hypovirulent strains^a

Strains	Characteristic	Number of conidia/ml ^b
EP155/2	Wild-type	$3.8 \times 10^8 \pm 2.3 \times 10^8$
TdHsp24-1	<i>CpHsp24</i> -null mutant	$3.1 \times 10^8 \pm 2.5 \times 10^8$
TcHsp24-1	<i>CpHsp24</i> -complemented strain	$3.5 \times 10^8 \pm 2.7 \times 10^8$
UEP1	Hypovirulent	$2.5 \times 10^6 \pm 1.1 \times 10^6$

^a Culture conditions were described previously (Kim et al. 1995).

^b Conidia from each plate were harvested with 10 ml of sterile water and the number of conidia per plate was determined using a hemacytometer. Four replicates of each strain were used and the experiment was repeated three times. Values are means $\pm$ standard deviation.

the *CpHsp24* gene was replaced with part of the transforming vector rather than just disrupted, as was expected.

Phenotypic characteristics of the *CpHsp24*-null mutant.

The *CpHsp24*-null mutant had a retarded growth rate, as measured by the diameter of colonies on potato dextrose agar (PDA) supplemented with methionine and biotin (PDAMB), with less than 80% of the typical growth rate observed (Fig. 3). Pigmentation of the interwoven hyphal mat was observed as the wild-type EP155/2 (Fig. 3) and no difference in the conidiogenesis was observed (Table 1).

Because the heat-shock response of the cloned *CpHsp24* gene was confirmed, we measured the sensitivity of the *CpHsp24*-null mutant to different temperatures (Fig. 4). When the *CpHsp24* mutant was exposed to the heat-shock conditions, it showed further defects in its growth rate. Interestingly, the *CpHsp24*-null mutant exhibited more severely retarded

growth defects due to cold shock (20°C) than heat shock (30°C). However, when the *CpHsp24*-null mutant strain was placed under other stresses such as oxidative and osmotic stresses, the responsiveness determined by the ratios between the growth rates in the presence or absence of menadione and high osmotic stress, respectively, were not changed. These results indicated that *CpHsp24* contributed to adaptation to temperature stress but not to oxidative or osmotic stresses.

To ensure that the phenotypic changes attributed to the *CpHsp24*-null mutant were due to gene replacement of *CpHsp24*, we complemented the *CpHsp24*-null mutant in trans with a wild-type allele of the *CpHsp24* gene. The resulting geneticin-resistant transformants were compared with examined growth rate. No growth defects under standard growth conditions as well as heat-shock conditions were observed in the *CpHsp24*-complemented transformant (Fig. 3), which unequivocally confirmed that the phenotype changes in the *CpHsp24*-null mutant were due to the disruption of the *CpHsp24* gene.

The oxidase activity of phenolic compounds by the strains measured by the browning of the plates containing TA was examined. Compared with the wild type, the *CpHsp24*-null mutant produced a brown-colored area with a size and intensity comparable with that of the wild type (Fig. 5). Considering the fact that the *CpHsp24*-null mutant showed reduced growth rates under standard growth conditions, no further reduction in the size and intensity of the browning area suggested that phenol oxidase production was not affected by the mutation of *CpHsp24*. Interestingly, even if the *CpHsp24* gene was strongly induced by TA supplementation, no further changes in the growth rate were observed on TA-containing malt extract Bavendamm's media. However, when the *CpHsp24*-null mutant was placed on nutrient-poor minimal media supplemented with TA, further growth retardation (>10% more) was observed (Fig. 5), which might account for a significant induction of the *CpHsp24* transcript by TA supplementation.

Effect of hypoviral infection on the colony morphology of the *CpHsp24*-null mutant.

In order to examine the biological functions of the *CpHsp24* gene related to hypovirus infection, we compared the pheno-

Fig. 4. Growth rate of the *CpHsp24*-null mutant strain under **A**, standard temperature (25°C) conditions; **B**, cold shock (20°C) conditions; and **C**, heat-shock (30°C) conditions. Strains used, indicated above the panel, are the wild-type EP155/2, hypovirulent UEP1, *CpHsp24*-null mutant (TdHsp24-1), and hypovirus-transferred *CpHsp24*-null mutant (TdHsp24-1-V1) strains. Vertical bars represent the standard deviation.

Fig. 5. Phenol oxidase activity of the *CpHsp24*-null mutant strain. Colonies after 10 days of culturing on **A**, potato dextrose agar supplemented with methionine and biotin; **B**, malt extract media; and **C**, malt extract media with tannic acid (TA) supplementation are shown. Strains used, indicated above the panel, are the wild-type EP155/2, hypovirulent UEP1, *CpHsp24*-null mutant (TdHsp24-1), and hypovirus-transferred *CpHsp24*-null mutant (TdHsp24-1-V1) strains.

typic changes between the virus-free and virus-containing isogenic transformants. Following co-culturing of the recipient *CpHsp24*-null mutant with UEPI, at least three putative virus-containing recipient mycelia sections were independently transferred onto a new hygromycin B-containing PDAMB plate and single spored to ensure nuclear homogeneity on the hygromycin B-containing medium. The single-spored strains from different sections were checked for the presence of double-stranded (ds)RNA by agarose gel electrophoresis before further comparisons (data not shown). When the hypovirus CHV1-713 was transferred to the *CpHsp24*-null mutant, colonial growth of the virus-containing *CpHsp24*-null mutant was severely restricted compared with the virus-free *CpHsp24*-null mutant (Fig. 4). Further growth inhibition of the virus-containing *CpHsp24*-null mutant was observed at 20 and 30°C, suggesting the sensitivity of the mutant to the temperature shock stress in the presence of the hypovirus (Fig. 4). In addition, reduced pigmentation and asexual sporulation, characteristic phenotypic changes caused

by hypovirus infection, were observed in the virus-containing *CpHsp24*-null mutant (Fig. 5). These phenotypic characteristics of the virus-containing *CpHsp24*-null mutant were stably maintained during the successive subculture of the virus-containing null mutant, which suggests that the presence of the *CpHsp24* gene is required for the appropriate response of *C. parasitica* to hypoviral infection but is not required for the maintenance of the hypovirus.

Virulence assay.

A virulence assay on the excised bark from a chestnut tree demonstrated that the size of the necrotic area induced on excised bark by the *CpHsp24*-null mutant was significantly smaller than that induced by the wild-type EP155/2, indicating that the *CpHsp24*-null mutant is less virulent than the wild type. In addition, the size of the necrotic area of the *CpHsp24*-null mutant was even smaller than that of a hypovirulent UEPI strain (Fig. 6). Statistical analysis using multiple range test at $P = 0.05$ indicated that the virulence of the *CpHsp24*-null mutant differed significantly from that of the wild type and even differed from that of the UEPI. Although it is known that pathogenicity tests are tightly correlated with the growth defects of the mutant, the remarkable reduction in the size of the necrotic area of the *CpHsp24*-null mutant suggested that the *CpHsp24* gene is involved in determining the virulence of this fungus.

Molecular characteristics of the *CpHsp24*-null mutant.

Because the expression pattern of the *CpHsp24* gene (i.e., induction by TA supplementation that was hampered by hypovirus) was similar to that of the laccase3 gene (*lac3*) gene, an important virulence factor of *C. parasitica* (Chung et al. 2008), and the virulence of the *CpHsp24*-null mutant was severely reduced, we examined *lac3* expression in the *CpHsp24*-null mutant using quantitative real-time RT-PCR (Fig. 7). Interestingly, the accumulation of *lac3* transcript in response to TA supplementation was hampered in the *CpHsp24*-null mutant. Therefore, it is suggested that the reduced *lac3* induction may at least partially account for the *CpHsp24*-null mutant's virulence attenuation. Although the similar expression pattern and the impaired virulence do not necessarily mean the interaction of two gene products, it would be of interest to examine whether the protein product of the *CpHsp24* gene plays a part as a molecular chaperone for the LAC3 protein.

DISCUSSION

In order to understand fungal gene expression during pathogenic conditions, it is better to study the gene expression in the natural infection site; for example, the chestnut tree. However, in planta analyses of the pathogenesis of forest fungal pathogens have been considered to be more difficult than those of grass pathogens. In the case of chestnut blight by *C. parasitica*, supplementation with TA, which has been found in high concentrations in chestnut bark and is known to be a natural barrier against pathogen infection, was suggested as an alternative for mimicking host conditions. For example, the Baven-damm assay, which measures the degree of oxidizing activity on TA-supplemented plates, has been considered to be one of the best alternatives for measuring virulence (Rigling et al. 1989). Second, TA supplementation induced the expression of the *lac3* gene, which has been known to be a fungal virulence factor. Thus, it was believed that TA supplementation could create conditions more reflective of natural infection. The current study demonstrated that the specific *Hsp* gene *CpHsp24* is influenced by TA supplementation and appears to be important for fungal virulence. This can be used as another example for validating the use of TA supplementation for analyzing viru-

Fig. 6. Virulence assay using excised chestnut tree bark as previously described (Lee et al. 1992). The strains used were the wild-type EP155/2, hypovirulent UEPI, *CpHsp24*-null mutant (TdHsp24-1), and hypovirus-transferred *CpHsp24*-null mutant (TdHsp24-1-V1) strains. **A**, Representative figure of excised tree barks 1 week after inoculation. **B**, Lesion measurement values are shown as the means $\pm$ standard deviation (square millimeters). Three replicates for each strain were used, and each experiment was repeated twice. Different letters indicate significant differences between treatments according to Duncan's multiple range test at $P = 0.05$.

lence in this host–parasite system. Recent transcriptome and proteome analysis of mycelia grown under the presence of TA revealed that several genes are affected by TA and hypovirus infection (Kim et al. 2012). Thus, functional analysis of these affected genes could provide more information on the fungal response in the presence of natural host barriers.

The heat responsiveness of the accumulated transcript and protein products of the cloned *CpHsp24* gene indicated that the *CpHsp24* gene belongs to a member of Hsp. Moreover, the presence of a conserved region with the signature sequence of the α -crystalline superfamily domain and the estimated molecular size of the protein product of the *CpHsp24* gene suggested that the cloned *CpHsp24* gene from *C. parasitica* encoded an sHsp that was a member of the Hsp23 family of molecular chaperones. In silico analysis of the promoter region 2.0 kb upstream of the start codon identified four nonconventional HSE motifs and two STRE motifs. These functional elements explain the heat-shock response and the TA induction of the *CpHsp24* gene. However, not all types of stress induced the expression of the *CpHsp24* gene and this induction was affected by hypovirus infection, suggesting the presence of a more complicated regulatory hierarchy in this promoter.

The biological function of sHsp in pathogenic microorganisms is largely unknown (Mayer et al. 2012). In yeast, multiple sHsp, including Hsp12, Hsp26, and Hsp30, exhibit different induction patterns in response to different stresses (Fu et al. 2012). The requirements for growth and survival of these proteins under a variety of stress conditions as well as the requirements for the maintenance of normal cell morphology have been assessed (Welker et al. 2010). In addition to stress adaptation, the involvement of these Hsp in the virulence of eukaryotic pathogens (Mayer et al. 2012) and intracellular signaling in viral infections has been suggested (Rajaiya et al. 2012). In this study, Northern blot analysis demonstrated that the accumulation of the *CpHsp24* gene transcript was significantly induced by TA supplementation and temperature stress. In addition, severe growth defects were observed when the *CpHsp24*-null mutant was placed under stress conditions such as temperature and TA supplementation. These results clearly indicated that the *CpHsp24* gene is involved in stress adaptation. Although sHsp, unlike the high molecular mass Hsp, have been shown to be mainly expressed under stress conditions, Western blot analysis has indicated that the protein product of the *CpHsp24* gene was found in considerable concentrations even in the absence of any stress, suggesting a physiological role under standard growth conditions. These results might explain the growth retardation of the *CpHsp24*-null mutant under standard growth conditions.

A severely reduced area of necrosis was observed on the excised bark of chestnut trees, which suggested that the *CpHsp24* gene is involved in the pathogenicity of *C. parasitica*. Considering that pathogenicity tests of the loss-of-function mutant of the Hsp DnaJ, the first characterized Hsp gene of *C. parasitica* at the molecular level, appeared to be the mere reflection of the growth defect of the mutant (Lim et al. 2010), the dramatically reduced pathogenicity (even smaller than that of the hypovirulent UEP1) of the *CpHsp24*-null mutant appears to be specific. This is very interesting because only a few studies have described the role of sHsp in microbial pathogenicity; two bacterial (Stewart et al. 2005; Tsai et al. 2009) and one fungal (Mayer et al. 2012) pathogenicity. Based on the high induction pattern of the *CpHsp24* gene by TA supplementation and the limited pathogenicity of the *CpHsp24*-null mutant, it can be assumed that the protein product of the *CpHsp24* gene is implicated in chaperone activity of the virulence factors. sHSP have been shown to bind non-native proteins in an ATP-independent manner, maintaining them in a folding-competent

state ready for refolding by other ATP-dependent components of protein quality control systems (Basha et al. 2012). Although some sHSP interact with a whole suite of denaturing proteins, many sHSP have specific substrates in vivo. Thus, further studies are needed to determine whether *CpHsp24* is the major chaperone during the infection process or has specific protein partners involved in pathogenicity. It would be of great interest to clarify how the protein product of the *CpHsp24* gene functions in vivo and what the major in vivo substrates of the CpHSP24 protein are. Considering the similarities in the expression profiles of the *lac3* gene, an important virulence factor of *C. parasitica* (Chung et al. 2008), and the *CpHsp24* gene under TA induction and hypovirus infection, as well as the abnormal expression of the *lac3* gene in the *CpHsp24*-null mutant, it is tempting to speculate that *CpHsp24* might function as a molecular chaperone for the protein product of the *lac3* gene. Therefore, further studies on the *CpHsp24*-*lac3* interaction could provide information on the former's in vivo function as well as point the way for further specificity analysis of the *CpHsp24*.

In addition to reduced pigmentation and sporulation, which are characteristic hypovirulence-associated phenotypic changes of the CHV1-infected hypovirulent strain, viral transfer to the *CpHsp24*-null mutant resulted in severe growth retardation compared with the virus-free *CpHsp24*-null mutant. These results suggested that the presence of *CpHsp24* is required for the appropriate fungal response to a biotic stress such as hypovirus infection. This growth retardation was further increased when the virus-infected *CpHsp24*-null mutant was cultured under temperature stress conditions. *C. parasitica* must adapt to a variety of stresses and it is likely that some of these stresses occur simultaneously. These results suggest that there appear to be two different stress-response pathways: one for abiotic stress such as temperature and another for biotic stress such as hypovirus infection. The presence of *CpHsp24* is required for appropriate adaptation of the fungus to both abiotic and biotic stresses. Although the Hsp has been implicated as a molecular chaperone for viral replication and assembly (Sullivan and Pipas 2001), persistent growth retardation of the virus-infected *CpHsp24*-null mutant without reverting phenotypes, such as rapidly growing sectors shown in the virus-infected *pro1*-null mutant (Sun et al. 2009), provided evidence of the stable maintenance of CHV1-EP713 in the *CpHsp24*-

Fig. 7. Molecular changes in the accumulation of the *lac3* gene transcript in response to tannic acid (TA) supplementation. Total RNA was extracted before and 24 h after TA induction. The expression level of the *CpHsp24*-null mutant (TdHsp24-1) was compared with the wild-type EP155/2, hypovirulent UEP1, and complemented *CpHsp24*-null mutant (TcHsp24-1) strains. Quantitative real-time reverse-transcription polymerase chain reaction analysis of *lac3* transcript levels relative to the levels of *gpd*. Values on the y-axis were normalized to the transcript levels of the corresponding gene in the 5-day cultured EP155/2 strain, with standard deviations, based on three independent measurements of two independent RNA preparations, indicated by the error bars.

null mutant and, thereby, indicated that the *CpHsp24* gene may not be required for hypovirus replication. Bioassay data using the *CpHsp24*-null mutant suggested that the presence of *CpHsp24* is important for fungal growth in hypovirus infections. Transcriptional analysis, however, revealed that the induction of *CpHsp24* by TA supplementation has been hampered by the hypovirus. Considering that the *CpHsp24* gene is not required for the hypoviral replication, it is suggested that the presence of the *CpHsp24* gene is more beneficial to the fungal biology. Accordingly, the disturbance of *CpHsp24* induction by the hypovirus may result in a fungal condition favoring viral persistence. Therefore, it would be of interest to examine the antiviral activity of *CpHsp24*, a trait that has been observed in other sHsp (Arockiaraj et al. 2012; Rajaiya et al. 2012).

The current study represents the first detailed description of an sHsp in *C. parasitica* and shows an sHsp contributing to the stress adaptation and virulence of a eukaryotic pathogen. In addition, this study suggests the putative partner protein of the CpHSP24 chaperone, which could be an interesting model for studying the function of molecular chaperones during pathogenesis.

MATERIALS AND METHODS

Fungal strains and growth.

The CHV1-713 containing hypovirulent *C. parasitica* strain UEP1 and its isogenic virus-free strain EP155/2 (American Type Culture Collection 38755) were maintained on PDAMB plates under constant low light at 25°C (Kim et al. 1995). The culture conditions and methods for preparation of the primary inoculum for liquid cultures have been previously described (Kim et al. 1995). TA (Sigma-Aldrich, St Louis) was supplemented to the media as previously described (Rigling and Van Alfen 1993). TA induction of EP155/2 and UEP1 was performed according to Chung and associates (2008). Radial growth on plates was assessed by measuring the diameter of the colonies. The mycelium was collected and lyophilized as previously described until its use (Powell and Van Alfen 1987).

RNA differential display analysis using the PCR-based GeneFishing technique.

RNA differential display with the GeneFishing technique was conducted to examine the differential expression gene (DEG) between virus-free EP155/2 and hypovirulent UEP1 with or without TA supplementation. GeneFishing DEG kit (SeeGene Inc., Seoul, Korea) was used according to the manufacturer's instructions. Briefly, for first-strand synthesis, 2 µg of DNA-free total RNA was converted into ssDNA using control primer one (10 µM dT-ACP1), two (dT-ACP2, which prime from polyA tail), dNTP (2 mM each), 2.5 µl of 25 mM MgCl₂, 2 µl of RNase inhibitor (40 U/µl; Promega Corp.), and 1 µl of reverse transcriptase (200 U/µl; Promega Corp.). The volume of the reaction mixture was 20 µl, and it was allowed to react for 60 min at 42°C and then for 15 min at 70°C. For second-strand synthesis, the PCR was performed as one cycle at 94°C for 3 min, followed by 50°C for 3 min and 72°C for 1 min. After the ssDNA synthesis was completed, the second-stage PCR amplification protocol was 40 cycles at 94°C for 40 s, 65°C for 40 s, and 72°C for 40 s; and then a 5-min final extension step at 72°C. In total, 20 different annealing control primer (ACP) pairs were used (each primer's sequence can be found in the SeeGene Inc. protocol). ACP primers were designed for highly specific PCR in second-stage PCR amplification. The differentially expressed bands were cut out of the gel using a QIAquick gel extraction kit (Qiagen, Carlsbad, CA, U.S.A.), directly cloned into pGEM-T Easy vectors (Promega Corp.), and then sequenced.

GeneFishing DEG analysis resulted in a cloned 401-bp PCR amplicon exhibiting a high homology with known fungal small heat-shock genes (Lindquist and Craig 1988; Plesofsky 2004). A genome database of *C. parasitica* (*Cryphonectria parasitica* EP155 v2.0) was screened for a full-length gene, and PCR amplification was performed with the primers CpHsp24-gF1 (forward) 5'-TTCTCAAGCCACCCAAAGTCA-3' and CpHsp24-gR1 (reverse) 5'-CGTACGTTTCACATGCGCGTA T-3'. The resulting 4.0-kb PCR amplicon was cloned into the pGEM-T Easy Vector (Promega Corp.) and sequenced using the dideoxynucleotide method with universal and synthetic oligonucleotide primers.

In order to obtain the cDNA clone of *CpHsp24*, RT-PCR was performed with primers CpHsp24-cF1 (forward) 5'-CGC GGA TCC ATG GCT GAA GAC ATC ATC CAT-3' and CpHsp24-cR1 (reverse) 5'-ACG CGT CGA CTC AGT GGC CCG CAT GTT CGA T-3'. The primers were modified to incorporate the restriction sites (underlined) for *Bam*HI and *Sal*I, respectively. The cDNA was sequenced using the dideoxynucleotide method with synthetic oligonucleotide primers.

Southern blot and Northern blot analysis.

Genomic DNA from *C. parasitica* was extracted using the method described by Churchill and associates (1990). DNA (10 µg) was digested with restriction enzymes *Bam*HI and *Sac*I, blotted onto the nylon membrane, and hybridized with radioactive-labeled probes.

RNA from liquid cultures was extracted as previously described (Kim et al. 2002). RNA from solid cultures was prepared from mycelia mats grown on cellophane that was layered on top of appropriate media, as previously described (Park et al. 2004). The level of CpHsp24 transcript was compared against glyceraldehyde-3-phosphate dehydrogenase (*Gpd*) of *C. parasitica* as an internal control for gene expression (Chio and Nuss 1990).

Heterologous expression of *CpHsp24* in *Escherichia coli*.

The full-length *CpHsp24* protein product CpHSP24 was expressed in *Escherichia coli* as a hexahistidine fusion protein and purified by nickel affinity chromatography according to the manufacturer's directions (Qiagen). The full-length open reading frame of *CpHsp24* (648 bp) was inserted into the *Bam*HI/*Sal*I sites in the expression vector pQE30 (Qiagen). The resulting recombinant plasmids were transformed into *E. coli* strain M15, including the pREP4 repressor vector. The induction, purification, and confirmation of the recombinant CpHSP24 using the anti-hexahistidine antibody were conducted according to the manufacturer's directions (Qiagen). The *E. coli*-derived inclusion body consisting of the recombinant CpHSP24 was solubilized and then refolded by the step-wise dilution dialysis of denaturants (Sambrook and Russell 2001).

The anti-CpHSP24 antibody was obtained by injecting 100 µg of purified full-length CpHSP24 into an 8-week-old BALB/c mouse, which was boosted with the same amount of the CpHSP24 emulsified in incomplete Freund's adjuvant 10 days after the initial injection. Polysera were obtained 5 days after the booster injection and then Western blot analysis was conducted according to the standard procedure (Sambrook and Russell 2001).

Construction of a replacement vector and fungal transformation.

The replacement vector pDHsp24, which was designed to favor double-crossover integration events, was constructed as follows. A 4,047-bp *Not*I fragment containing the full-length *CpHsp24* was ligated into *Sal*I-inactivated pBluescriptII SK

(+), and the resulting plasmid was used as the template for inverted PCR using the primers 5'-ACGCGTCGACATCCAC AATACGCGCGCAC-3' and 5'-ACGCGTCGACCTTGTTGT GTGCGGGTGCG-3', which incorporate the restriction site for *Sall* (underlined). The PCR amplicon was digested with *Sall* and religated. The resulting plasmid was further digested with *Sall* and fused with a 2.4-kb *Sall* fragment of pDH25 (Cullen et al. 1987) carrying the *hph* gene cassette. In the replacement vector pDHsp24, the *hph* cassette was inserted between sites 1 and 648 of the *CpHsp24* gene relative to the start codon, and was flanked by approximately 1,176 and 1,355 bp of the 5' and 3' sequences, respectively. A 4,398-bp PCR fragment containing the *hph* cassette and flanking regions of the *CpHsp24* gene was obtained from pDHsp24 with the primers CpHsp24-gF1 and CpHsp24-gR1, and was then used to transform the virus-free EP155/2 strain.

Functional complementation of the *CpHsp24* null mutant using a wild-type allele was performed. The complementing vector pCHsp24 was constructed by the insertion of a 1.7-kb blunt-ended *Xho*I fragment of pSilent-Dual1G (pSD1G) containing the geneticin resistance cassette (Nguyen et al. 2008) into *Sac*II/*Spe*I-digested pCHsp24 carrying the 4,047-bp fragment with the full-length *CpHsp24* gene. The resulting vector was then used to transform the *CpHsp24* null mutant.

Protoplast preparation and transformation were performed as previously described (Churchill et al. 1990; Kim et al. 1995). Transformants were selected from agar plates that were supplemented with hygromycin B (Calbiochem, San Diego, CA, U.S.A.) at 150 µg/ml or geneticin (Invitrogen, Carlsbad, CA, U.S.A.) at 150 µg/ml, as was appropriate; passed three to four times on the selective media; and single-spore isolated, as previously described (Kim et al. 2004; Nguyen et al. 2008). PCR and Southern blot analysis was conducted with genomic DNA from the transformants to confirm the replacement and in trans complementation of the *CpHsp24* gene.

Characteristics of the *CpHsp24* null mutant.

The phenotypic and molecular characteristics of the *CpHsp24* null mutant were compared with those of the wild-type EP155/2 and the hypovirulent UEP1 strains. Phenotypic changes in pigmentation and conidiation were measured as previously described (Kim et al. 2002; Powell et al. 1987). A virulence test using excised chestnut tree bark was conducted according to Lee and associates (1992). Phenol oxidase activity was gauged by growing the strains on Bavendamm's medium (0.05% TA, 1.5% malt extract, and 2.0% agar) and assessing the resulting coloration of the medium (Rigling et al. 1989).

To examine the acute wet heat-shock response, the actively growing *C. parasitica* mycelia on cellophane that was layered on top of PDAMB medium were ground, transferred to *Endothia parasitica* (EP) complete media, and incubated at 25°C for 3 days. The resulting mycelia were harvested, dried using paper towels until no further liquid leaked out, transferred to EP complete medium pre-equilibrated to appropriate temperatures, and, finally, incubated further for 24 h. The assessment of the response to chronic temperature stress was based on the level of hyphal growth on PDAMB incubated at appropriate temperatures. Northern blot and Western blot analyses were conducted to examine the accumulation of the transcript and protein product of the *CpHsp24* gene, respectively.

Transmission of dsRNA virus.

Virus transmission was performed as previously described (Kim et al. 2008). Briefly, mycelial plugs of the virus-containing strain UEP1 were co-cultured with adjacent mycelial plugs of virus-free recipient transformants on PDAMB medium. After 5 days of co-culture, putatively fused mycelia at the border be-

tween each pair of strains were transferred to hygromycin-containing PDAMB and examined for the occurrence of a sector that showed different colonial phenotypes, such as reduced growth or pigmentation. Mycelia in the sector were successively transferred to fresh hygromycin-containing media and then strains were single spored to select for the virus-infected recipient transformants. The presence of hypovirus was confirmed by purification of dsRNA from the single-spored isolates.

Isolation of dsRNA from *C. parasitica*.

The dsRNA was isolated according to the modified procedure of Kim and associates (2008). The UEP1 and dsRNA-transmitted transformants were grown on a cellophane membrane overlaying PDAMB for 5 days at 25°C. Mycelia (0.2 g wet weight) were homogenized in extraction buffer (2× STE [0.2 M NaCl, 0.1 M Tris-HCl [pH 8.0], and 2 mM EDTA], 2% SDS, and 1% sodium bisulfate) using a bead beater (Biospec Products, Inc., Bartlesville, OK, U.S.A.). Following two successive phenol extractions, dsRNA was isolated by affinity chromatography using CC41 resin. Isolated dsRNA was analyzed by electrophoresis in a 0.8% agarose gel.

Quantitative analysis of *lac3* transcript accumulation using real-time RT-PCR.

To examine the expression levels of target and internal control genes, quantitative RT-PCR was performed using a GeneAmp 7500 sequence detection system (Applied Biosystems, Foster City, CA, U.S.A.) and a SYBR green mixture RT kit (Applied Biosystems). A single cycle to reverse-transcribe 1 µg of RQ1-treated RNA was followed by real-time PCR using 2 µl of reverse-transcribed cDNA and 150 nM each of forward and reverse primers. Analyses were conducted at least twice in triplicate for each transcript and were from at least two independent RNA preparations with primers specific for *gpd* and the target genes. The *gpd* was amplified with the primers RT-gpd-F (5'-CTCTCAACACGGCAACTTCA-3') and RT-gpd-R (5'-ACCAGTGGACTCGACAATG-3'). The *lac3* transcript was amplified with the primers RT-lac3-F (5'-CACGGAATCCGA CAAAACTTC-3') and RT-lac3-R (5'-CTGCACGTAGAAGT GCGAGT-3'). Transcript abundance relative to the amount of *gpd* in the sample was calculated using the comparative threshold cycle method, as previously described (Parsley et al. 2002).

Statistical analysis of virulence data.

Necrotic areas of the virulence test were analyzed by analysis of variance using SPSS (version 15; SPSS, Chicago), with significant differences between strains determined by the magnitude of the *F* value at *P* = 0.05. When a significant *F* value was obtained, the significance of the effects of the strains was determined by Duncan's multiple range test at *P* = 0.05.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Department of Energy Joint Genome Institute draft genome sequence:
genome.jgi-psf.org/Crypa1/Crypa1.home.html