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A DnaJ-like Homolog from *Cryphonectria parasitica* Is Not Responsive to Hypoviral Infection but Is Important for Fungal Growth in Both Wild-Type and Hypovirulent Strains

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A DnaJ-like gene, *Cpdj*1, a molecular chaperone and regulator of Hsp70 in *Cryphonectria parasitica*, was characterized. The protein product of *Cpdj*1 gene consists of 379 amino acids with a predicted molecular mass of 40.6 kDa and a pI of 7.79. The deduced protein sequence revealed preservation of the conserved hall-mark J-region and exhibited high homology to all known DnaJ-like proteins. Disruption of the *Cpdj*1 gene resulted in slow growth and produced colonies characterized by retarded growth and a deep orange color. Accordingly, reduced virulence of the *Cpdj*1-null mutant was observed. This reduced growth rate was magnified when the *Cpdj*1-null mutant was cultured under heat-stress conditions. Reduced conidiation was also observed in the *Cpdj*1-null mutant, indicating that *Cpdj*1 gene, although not essential for cell viability, is required for appropriate cellular processes including growth and sporulation. Northern analysis showed that *Cpdj*1 was constitutively expressed, and when the culture was subject to high temperature, a strong induction of the transcript was observed. No significant difference in the expression and induction pattern of *Cpdj*1 was observed between virus-free EP155/2 and virus-infected hypovirulent UEP1 strains. However, further severe defects in mycelia growth and conidiation were observed in the hypovirus-infected *Cpdj*1-null mutant suggesting that the presence of *Cpdj*1 is required for mycelia growth and sporulation of the hypovirus-infected strain.

INTRODUCTION

Cryphonectria parasitica (Murrill) Barr, the causal agent of chestnut blight, was responsible for the virtual disappearance of the chestnut forests in North America in the beginning of the 20th century (Van Alfen, 1982). Furthermore, disease severity has become more pronounced in some areas, including Korea, where chestnut blight was believed to be of minor significance. Recent studies have shown that more than 30% of necrotic lesions of chestnut trees in Korea were caused by *C. parasitica* (Ju et al., 2002), and various levels of susceptibility were observed among current chestnut varieties that were previously considered as resistant (Park et al., 2008). However, strains containing double-stranded (ds) RNA viruses exhibit the characteristic symptoms of hypovirulence, (Anagnostakis, 1982; Nuss, 1992; Van Alfen et al., 1975), and diverse hypovirulence-associated phenotypic changes such as reduced sporulation, pigmentation, laccase production, and oxalate accumulation (Ellistone, 1985; Havir and Anagnostakis, 1983; Rigling et al., 1989). The molecular basis for these phenotypic changes led to alterations in host transcriptional profiles in response to the hypovirus infection (Allen and Nuss, 2004; Allen et al., 2003; Deng et al., 2007; Kang et al., 2000; Kazmierczak et al., 1996). Although specific relationships between each symptom development and a limited set(s) of fungal genes aberrantly expressed in the hypovirulent strain remain to be elaborated, it appears that specific set(s) of fungal genes, including those for stress responses, carbon metabolism, and transcriptional regulation, are perturbed by hypovirus infection. Among these, the stress-responsive pathway was of interest with following reasons. First, microarray studies suggested that, in the case of the stress-response pathway, only selected host genes such as heat shock protein 70 (Hsp70) and glutathione S-transferase (GST) were suggested as another putative target of viral regulation (Allen and Nuss, 2004; Allen et al., 2003). Second, viral regulation on the stress-responsive signal transduction pathway may not be general but rather specific, in that our previous studies on the high osmotic- and stress-related MAP kinases have shown that both genes appear to be important for fungal growth, but only the gene related to high osmotic stress, CpMK1, is specifically modulated by the presence of the hypovirus (Choi et al., 2005; Park et al., 2004). However, few studies on the biological function of the components of the stress-response pathway have been conducted in this fungus.

Heat-shock response is a molecular reaction to stressful, but

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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 are produced. These heat-shock proteins are beneficial to cells, helping them to adapt to the inducing temperature. The heat-shock response is ubiquitous, and the prominent heat-shock proteins are highly conserved among all groups of organisms. Many heat-shock proteins are essential proteins that contribute to cellular metabolism during normal growth but many of them are strongly induced during specific stages of organelle biogenesis, protein folding and transport, transcriptional activation mechanisms, stress resistance and differentiation (Fu et al., 2010; Lindquist and Craig, 1988; Plesofsky, 2004). Among these, it is of interest that the heat-shock protein functions as a molecular chaperone in viral genome replication and chaperone-mediated capsid assembly through the protein-protein interactions between heat-shock protein and viral protein (Chromy et al., 2003; Niewiarowska et al., 1992; Sullivan and Pipas, 2001; Wang et al., 2009).

According to their molecular weight, heat-shock proteins are classified into several classes or families, and a cell may express multiple members of the same family (Walter and Buchner, 2002). DnaJ is a member of the 40 kDa heat-shock protein (Hsp40) family of molecular chaperones and a prototypical member of the J-protein family, which is defined by the presence of a J domain that can regulate the activity of 70 kDa heat-shock proteins (Hsp70). Accordingly, the key functions of DnaJ are to directly interact with Hsp70 to stimulate ATPase activity and to act as a chaperone in conjunction with Hsp70 (Cyr et al., 1994). All DnaJ-like proteins contain a J-domain. Otherwise, members of the DnaJ-like protein family are structurally diverse, containing different combinations of four distinct domains: N-terminal 70 amino acid J-domain, glycine/ phenylalanine-rich region, cysteine-rich zinc finger repeats, and a variable C-terminal region (Johnson and Craig, 2001; Silver and Way, 1993). Functioning with Hsp70 and Hsp90, these DnaJ-like proteins (Hsp40) assist protein folding (Dey et al., 1996; Lu and Cyr, 1998), protein translocation across membranes (Atencio and Yaffe, 1992; Caplan et al., 1992), assembly of macromolecular complexes (Hu et al., 2002), and protein degradation (Lee et al., 1996).

In our previous study on transcriptional profiling of virus-free and -containing strains of *C. parasitica* using RNA differential display (Kang et al., 2000), a DNA fragment with a high similarity to known fungal DnaJ genes was obtained. Therefore, to understand the relationship between heat-shock response and hypovirus infection at the molecular level, we characterized the DnaJ-like gene (*Cpdj*1) from *C. parasitica*, determined the biological function of the cloned *Cpdj*1gene, and examined how the *Cpdj*1 gene was related to the hypovirus infection.

MATERIALS AND METHODS

Fungal strains and growth

The CHV1-713 containing hypovirulent *C. parasitica* strain UEP1 and its isogenic virus-free strain EP155/2 (ATCC 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). Radial growth on plates was assessed by measuring the diameter of the colonies. The mycelium was collected and lyophilized as previously described, until use (Powell and Van Alfen, 1987).

Cloning and characterization of a DnaJ-like gene, *Cpdj*1

RNA differential display with ordered differential display using RT-PCR (ODD-PCR) resulted in a cloned 235-bp PCR amplicon exhibiting a high homology with all known DnaJ genes (Kang et al., 2000). The cloned amplicon was radioactive-labeled and used as a probe to screen a genomic λ library of *C. parasitica* to obtain a full-length genomic clone according to standard procedures (Sambrook et al., 1989).

In order to obtain the cDNA clone of *Cpdj*1, RT-PCR was performed with primers Cpdj-mF1 (forward) 5′-ATGGTCAAAG AAACGAAGCTG-3′ and Cpdj1-mR1 (reverse) 5′-TTTGTCGCT ATGTAAACA-3′. The cDNA was sequenced using the dideoxynucleotide method with synthetic oligonucleotide primers.

To identify the transcriptional initiation site, a primer extension experiment was performed using the reverse primer 5′-GCCTTTCTTGATCGCGTC-3′ according to a standard protocol (Sambrook et al., 1989).

Southern blot and Northern blot analysis

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

The temporal expression pattern of C*pdj*1 was examined by Northern blot analysis using RNA extracted from cultures at 1, 2, 3, 5 and 7 days after inoculation, as previously described (Kim et al., 2002). *C. parasitica* glyceraldehyde-3-phosphate dehydrogenase (*Gpd*) was used as an internal control for gene expression (Choi and Nuss, 1990).

Heterologous expression of *Cpdj*1 in *E. coli*

The full-length *Cpdj*1 protein product CpDJ1 was expressed in *E. coli* as a hexahistidine fusion protein and purified by nickel affinity chromatography according to the manufacturer's directions (Novagen; Merck, Darmstadt, Germany). cDNA encoding the full-length CpDJ1 was amplified by PCR using the primers 5′-GCCATATGGTCAAAGAAACGAAGCTG-3′ (forward) and 5′-ATGCGGCCGCTTACAGAATCTCTTT-3′ (reverse). The primers were modified to incorporate the restriction sites (underlined) for *Nde*I and *Not*I, respectively. The full-length *Cpdj*1 (1137 bp) was inserted into the *Nde*I/*Not*I sites in the expression vector pET24 (Novagen). The resulting recombinant plasmids were transformed into *E. coli* strain BL21. The induction, purification and confirmation of the recombinant CpDJ1 using the anti-hexahistidine antibody were conducted according to the manufacturer's directions (Novagen). The *E. coli*-derived inclusion body consisting of the recombinant CpDJ1 was solubilized and then refolded by the step-wise dilution dialysis of denaturants (Sambrook et al., 1989).

The anti-CpDJ1 antibody was obtained by injecting 100 μg of purified full-length CpDJ1 into an 8-week old BALB/c mouse, which was boosted with the same amount of the CpDJ1 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 et al., 1989).

Construction of a replacement vector and fungal transformation

The replacement construct pDDj1, which was designed to favor double homologous recombination events, was constructed as follows: a 3.1-kb *Bam*HI/*Apa*I fragment containing the full-length *Cpdj*1 ORF was ligated into *Sal*I-inactivated pBluescriptII SK (+), and the resulting plasmid was used as template for

inverted PCR using the primers 5′-GGGTCGACATTGGCAG TCTGTACTGC-3′ and 5′-GGGTCGACATGCAGTGCCTCAGC GATC-3′, which incorporate the restriction site for *Sal*I (underlined). The PCR amplicon was digested with *Sal*I and re-ligated. The resulting plasmid was further digested with *Sal*I and fused with a 2.4-kb *Sal*I fragment of pDH25 (Cullen et al., 1987) carrying the hygromycin phosphotransferase gene cassette (*hph*). In the replacement vector pDDj1, the *hph* cassette was inserted between sites 1 and 1337 of the *Cpdj*1 gene relative to the start codon, and was flanked by approximately 780 bp and 1357 bp of 5′ and 3′ sequences, respectively. *Bam*HI-digested linear pDDj1 was then used to transform the virus-free EP155/2 strain.

Functional complementation of *Cpdj*1-null mutant using a wild-type allele was performed. The complementing construct pCDj2 was constructed by insertion of a 2.6-kb blunt-ended *Sal*I fragment of pSV50 containing the benomyl resistance cassette (Orbach et al., 1986) into blunt-ended *Kpn*I-digested pCDj1 carrying a 5.5-kb *Apa*I fragment containing the full-length *Cpdj*1 gene. The resulting vector was then used to transform the *Cpdj*1-null mutant.

Protoplast preparation and transformation were performed as described previously (Kim et al., 2002; Park et al., 2004). Transformants were selected from agar plates that were supplemented with 150 μg/ml hygromycin B (Calbiochem; Merck, Germany) or 1.5 μg/ml benomyl (DuPont), as appropriate, passaged three to four times on selective media, and single-spore isolated, as described previously (Kim et al., 2002). PCR and Southern blot analysis was conducted with genomic DNA from the transformants to confirm the replacement and *in-trans* complementation of the *Cpdj*1 gene.

Characteristics of the *Cpdj*1- null mutant

The phenotypic and molecular characteristics of the *Cpdj*1-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 and Van Alfen, 1987). Virulence test using excised chestnut tree bark was conducted according to Lee et al. (1992). Laccase activity was gauged by growing the strains on Bavendamm's medium (0.05% tannic acid, 1.5% malt extract, 2.0% agar) and assessing the resulting coloration of the medium (Rigling et al., 1989).

To examine the acute heat-shock response, ten 0.5-cm diameter agar plugs that contained actively growing young hyphae were inoculated on cellophane that was layered on top of PDAmb medium and incubated until the mycelia reached the end of the Petri plate. The cellophane and the actively growing *C. parasitica* were then transferred to a plate that has been maintained in an incubator at 35°C and incubated further for a 24 h. Northern blot and Western blot analyses were conducted at 6-h intervals to examine accumulation of transcript and protein product of the *Cpdj*1 gene, respectively. The assessment of response to chronic heat stress was based on the level of hyphal growth on PDAmb incubated at 30°C.

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 placed on PDAmb medium adjacent to mycelial plugs of virus-free recipient transformants. After 7 days of coculture, putatively fused mycelia at the border between 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 et al. (2008). The UEP1 and dsRNA-transmitted transformants were grown on a cellophane membrane overlaying PDAmb for 7 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, 2 mM EDTA], 2% SDS, 1% sodium bisulfate) using a bead beater (Biospec Products, Inc., USA). Following two successive phenol extractions, dsRNA was isolated by affinity chromatography using CF11 resin. Isolated dsRNA was analyzed by electrophoresis in a 0.8% agarose gel.

RESULTS

Characteristics of the DnaJ gene

RNA differential display with ordered differential display using RT-PCR (ODD-PCR) resulted in a cloned 235-bp PCR amplicon exhibiting a high homology with known DnaJ genes (Kang et al., 2000). The highest homolog for this cloned PCR amplicon was the heat-shock protein DnaJ domain protein of *Methylocella silvestris*, which shared 44% identity at the nucleotide level. Southern blot analysis indicated that the cloned PCR amplicon was present as a single-copy gene in the *C. parasitica* genome (data not shown). The cloned amplicon was radioactive-labeled and used as a probe to screen a genomic λ library of *C. parasitica* to isolate a clone harboring the full-length DnaJ gene (Kim et al., 2002). Among 30,000 plaques screened, three showed strong hybridizing result. A 3.3-kb *Apa*l-*Bam*HI digested λ DNA fragment containing the full-length DnaJ was selected and sub-cloned into pBSSKII for further analysis. Based on the genomic sequence analysis, a near full-length cDNA clone was obtained using RT-PCR with the primer pair Cpdj-mF1 and Cpdj-mR1 at nucleotide positions (nt) 1 to 21 and nt 1634 to 1651 (relative to the start codon), respectively, and the resulting 1451-bp amplicon was cloned into a pGEM-T easy vector. A sequence comparison with the corresponding genomic sequence revealed that the gene referred as *Cpdj*1 consisted of three exons, with two introns of 120 bp and 80 bp. A primer extension experiment revealed that the transcription start site of the *Cpdj*1 was located 96 bp upstream of the putative start codon. We discovered canonical CAAT and TATA boxes at -192 nt and -295 nt, respectively, in the promoter region of *Cpdj*1. The minimum consensus sequence of heat-shock element nGAAnnTTCnnGAAn was found 472-bp upstream of the start codon (Tachibana et al., 2002). The sequence around the first ATG was in good agreement with Kozak's consensus sequence in that the nt -3 position was the A in CAATATG. The 5′-end, 3′-end, and the internal consensus sequence of the introns were compared to *S. cerevisiae*, *N. crassa,* and *C. parasitica* consensus intron splice signals (Choi et al., 1992; Kim et al., 2002; Roberts et al., 1988). The 5′-ends and 3′-ends of both intron structures closely matched the known consensus sequences, 5′-GTRRGT-3′ and 5′-YAG-3′ of *C. parasitica*, respectively (Choi et al., 1992; Zhang et al., 1994). The internal consensus sequence of 5′-NCTRAC-3′ was found 16 bp and 12 bp upstream of the 3′-end of both intron 1 and 2, respectively, with the exception of T at the 3′-end of intron 1 (Choi et al., 1992; Gurr et al., 1987; Roberts et al., 1988). In addition, the poly(A) site was located 437 bp downstream of the stop codon TAA, and the putative poly(A) signal AATAAT at 21

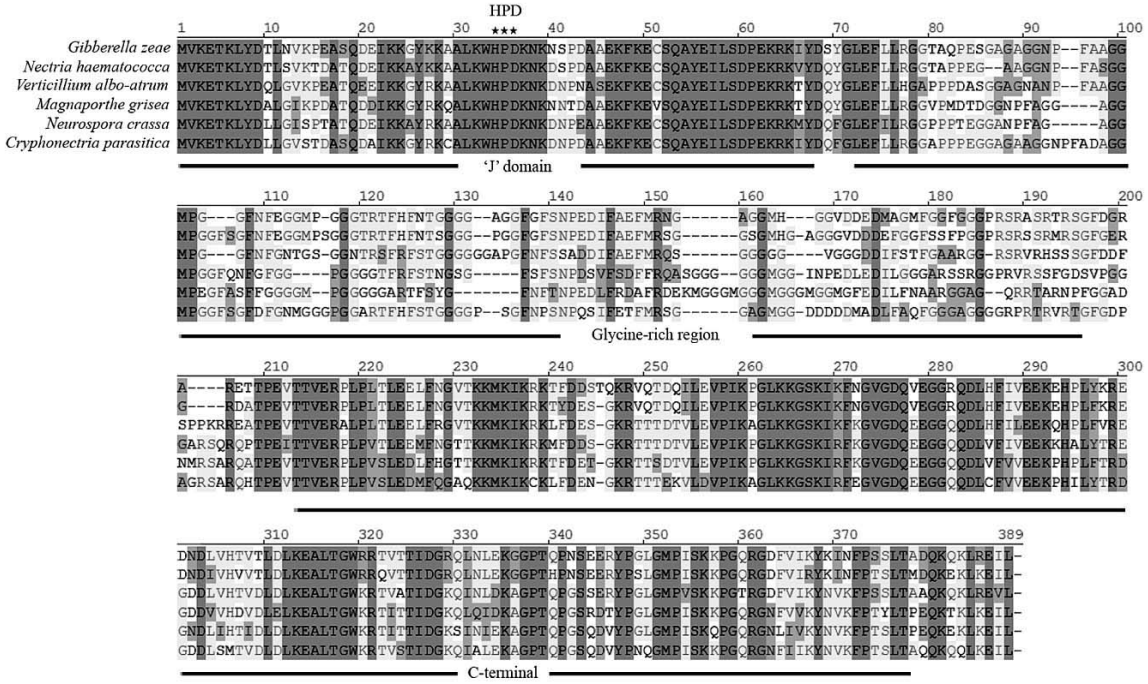

Fig. 1. Alignment of amino acid sequences of the predicted *Cpdj*1 gene product with other DnaJs of filamentous fungi. Identical amino acids are highlighted in dark gray, and similar amino acids are shaded. Dashes indicate gaps in the alignments. The asterisks indicate the hallmark triptych HPD sequence, which is essential for interaction of the protein with Hsp70. Numbering of the residues from the N-terminus of the whole protein is shown at the top. J domain, glycine-rich domain, and C-terminal domain are indicated below the sequence. The GenBank accession numbers for other DnaJs are *M. grisea* (XP_362597), *V. albo-atrum* (EEY14098), *G. zeae* (XP_ 385309), *N. haematococca* (EEU44807), *N. crassa* (XP_961508), and *C. parasitica* (GU175984).

bp upstream of the poly(A) site.

The deduced *Cpdj*1 protein (CpDJ1) consisted of 378 amino acids, with an estimated molecular mass of 40.6 kDa and a pI of 7.79 (the GenBank accession number for *Cpdj*1 is GU175984). Analysis of the deduced CpDJ1 amino acid sequence revealed the presence of an N-terminal J-domain including the hallmark triptych HPD sequence, which is essential for the interaction of the protein with Hsp70 (Greene et al., 1998; Walsh et al., 2004). Glycine/phenylalanine-rich regions (38 glycine/13 phenylalanine residues out of 121 amino acid residues between aa73-193) followed by the C-terminal domain were also observed (Fig. 1). Homology searches using the deduced amino acid sequence indicated that the *Cpdj*1 protein is related to fungal DnaJ proteins from *Neurospora crassa* (69%), *Verticillium albo-atrum* (68%), *Gibberella zeae* (67%), *Nectria haematococca* (67%), and *Magnaporthe grisea* (65%). Multiple alignment of closely related DnaJ proteins indicated that the cloned *Cpdj*1 protein clustered into the group containing those from *V. albo-atrum*, *G. zeae*, *N. haematococca*, and *N. haematococca*. But the DnaJ gene from *M. grisea* and *N. crassa* clustered distantly from the others.

Regulation of *Cpdj*1 gene expression

Northern blot analysis using RNA samples from *C. parasitica* grown in EP complete media revealed that *Cpdj*1 is constitutively expressed without heat induction (Fig. 2A). No significant difference in the expression level of *Cpdj*1 was observed between the *C. parasitica* strain EP155/2 and its isogenic hypovirulent strain UEP1 (Fig. 2A). *Cpdj*1 gene expression was further analyzed based on the response to acute heat stress conditions. When both strains were subjected to acute heat

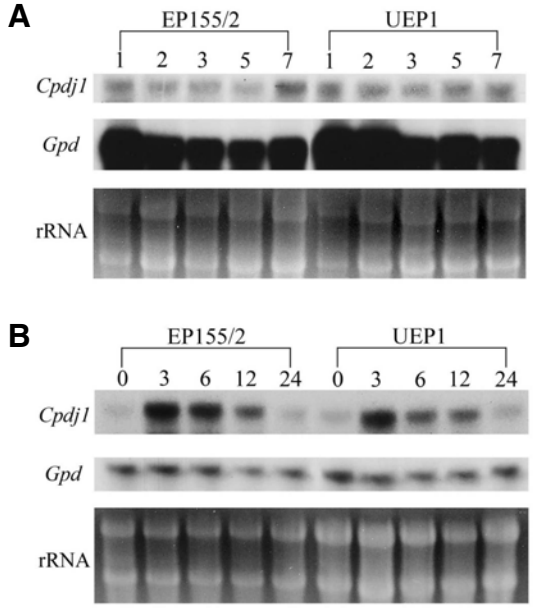

Fig. 2. Northern blot analysis of *Cpdj*1 under standard growth conditions (A) and acute heat induction (B). Equal loading of RNA was confirmed by a parallel blot hybridized with a *Gpd* probe as an internal control and the ethidium bromide-stained gel. The strains used, indicated above the panel, were the virus-free wild-type (EP155/2) and its isogenic virus-containing hypovirulent strain (UEP1). Numbers at the top of lanes refer to the days after inoculation (A) and hours after heat induction (B).

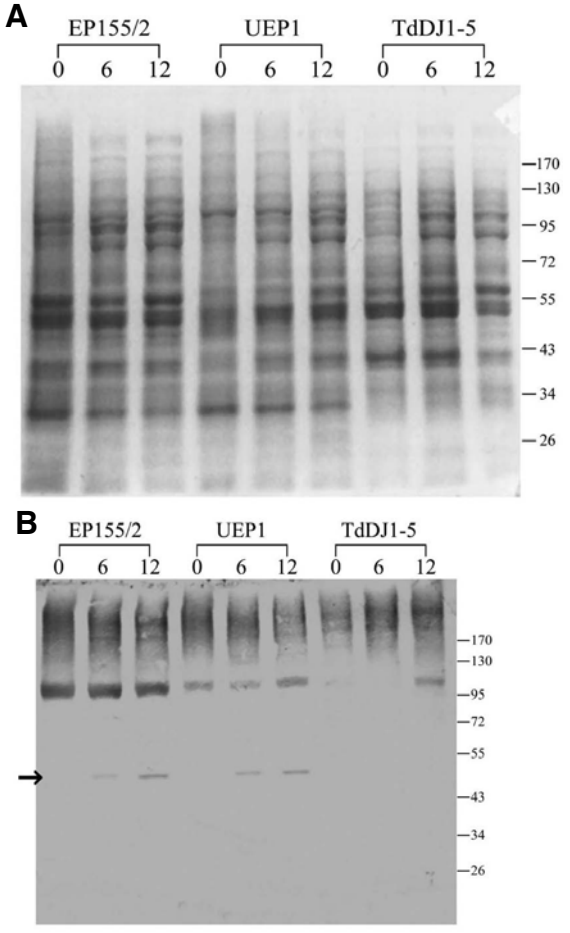

Fig. 3. Western blot analysis of the protein product of *Cpdj*1 gene in response to acute heat-shock conditions. (A) Coomassie blue-stained SDS-PAGE of cell-free protein extract of *C. parasitica*. (B) Antigen-antibody reaction of the corresponding gel. The arrowhead indicates the CpDJ1 protein. The strains are designated above the line, and numbers at the top of lanes refer to hours after heat induction. For each lane, 20 μg of protein extract was loaded. No antigen-antibody reaction was observed in the *Cpdj*1-null mutant (TdDJ1-5) due to the absence of the protein product of the *Cpdj*1 gene as expected.

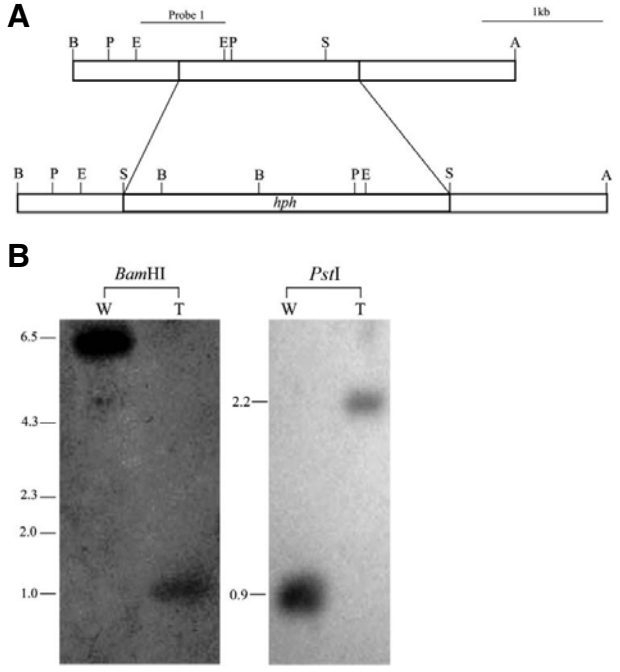

Fig. 4. Restriction map and Southern blot analyses of the *Cpdj*1-null mutant (TdDJ1-5) and the wild-type EP155/2. (A) Restriction map of the *Cpdj*1 genomic region and the gene replacement vector pDdj1, which contains 780-bp and 1,357-bp fragments as the 5′- and 3′-flanking regions of *Cpdj*1 gene, respectively. (B, *Bam*HI; P, *Pst*I; E, *Eco*RI; S, *Sal*I; A, *Apa*I) (B) Southern blot analysis of the wild-type EP155/2 strain (lane W) and the *Cpdj*1-null mutant TdDJ1-5 (lane T). Enzymes used to digest the DNA samples are indicated above the line. The blots were hybridized with the probe 1 indicated in the restriction map in the upper panel (A). The TdDJ1-5 transformant has undergone the desired replacement at *Cpdj*1, as indicated by the expected changes in size of the bands that hybridized with the probe 1.

stress conditions by transferring cellophane that was layered with actively growing mycelia onto a new PDAmb plate maintained at 35°C, the *Cpdj*1 gene transcript was induced in both strains. Accumulation of the *Cpdj*1 gene transcript peaked at 3 h after induction and gradually declined after that (Fig. 2B). No significant difference in the induction pattern of the *Cpdj*1 transcript was observed in the EP155/2 and UEP1 strains (Fig. 2B). Western blot analysis was also conducted to examine the induction of the *Cpdj*1 protein. As shown in Fig. 3, the protein product of *Cpdj*1, with a calculated molecular weight of 48.5 kDa, was induced in both EP155/2 and UEP1 strains by the acute heat shock. No difference in induction of the protein product was observed in the two strains which is in agreement with Northern blot analysis. The temporal expression pattern of the *Cpdj*1 protein did not correlate well with that of the *Cpdj*1 transcript. Although further studies are required to answer this question, it appears that the protein product of *Cpdj*1 is more stable than the *Cpdj*1 transcript in response to acute heat-shock conditions.

Construction of *Cpdj*1-null mutant

To explore the effects of deletion of the *Cpdj*1 gene, the *Cpdj*1-null mutant was constructed by gene replacement during integrative transformation. A *Bam*HI-digested linear plasmid pDDj1 that contained the disrupted *Cpdj*1 gene was used to transform the virus-free *C. parasitica* EP155/2 strain (Fig. 4A). A total of 150 single-spored transformants were screened by PCR using the outer and inner primers, which corresponded to nucleotide positions (nt) -1 to -18 and 2476 to 2493 (relative to the start codon), respectively. All but one showed both 2.5-kb and 3.6-kb fragments expected from the wild-type and disrupted alleles of the *Cpdj*1 gene, respectively, which indicated transformants were ectopic resulting from random integrations of the transforming vector. However, one transformant did not have the wild-type 2.2-kb amplicon but showed only the 3.4-kb fragment of the disrupted allele of *Cpdj*1, suggesting that the disruption construct replaced the wild-type allele (data not shown). The putative *Cpdj*1-null mutant was further confirmed by Southern analysis (Fig. 4). The hybridization pattern of *Bam*HI-digested genomic DNA of the putative *Cpdj*1-null mutant with the probe prepared using the 0.7-kb *Eco*RI-digested *Cpdj*1 fragment differed from the wild type, suggesting that the disruption construct had integrated at the *Cpdj*1 locus by site-directed ho-

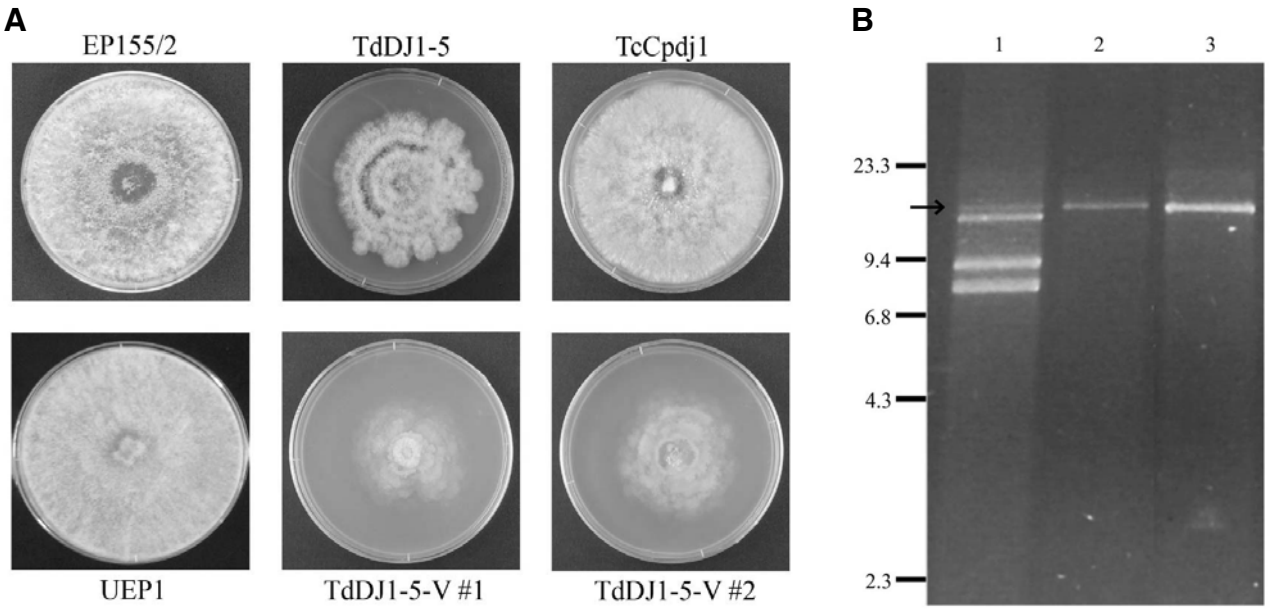

Fig. 5. Colony morphology of the *Cpdj*1-null mutant strain. (A) Colonies after 10 days of culturing are shown. The strains used, indicated above or below the panel, are the wild-type EP155/2, hypovirulent UEP1, *Cpdj*1-null mutant (TdDJ1-5), the Cpdj1-com-plemented strain (TcCpdj1), and two hypovirus-transferred *Cpdj*1-null mutant strains (TdDJ1-5-V1 and -V2). (B) Agarose gel electrophoresis of dsRNA from virus-containing transformants. An arrow indicates the location of the viral genome. Lanes 1-3 contain dsRNA preparations from UEP1, TdDJ1-5-V1, and -V2, respectively. Numbers on the left indicate the size markers in kb.

mologous recombination. Moreover, the *Bam*HI-digested genomic DNA of the putative *Cpdj*1-null mutant had the hybridizing band at 1.0 kb, corresponding to the expected size of a replaced allele, and the corresponding band also hybridized to the 0.4-kb *Sal*I/*Bam*HI-*hph* fragment. In addition, the hybridization pattern of *Pst*I-digested genomic DNA of the *Cpdj*1-null mutant with the 0.7-kb probe showed the hybridizing band at 2.2 kb instead of 0.9 kb in the wild type (Fig. 4), which is in good agreement with the results of *Bam*HI digestion, indicating that the *Cpdj*1 gene was replaced with part of the transforming vector rather than disrupted, as expected.

Phenotypic characteristics of *Cpdj*1-null mutant

The *Cpdj*1-null mutant displayed a defect in growth rate, as measured by the diameter of colonies on PDAmb (Fig. 5A). The *Cpdj*1-null mutant had a retarded growth rate, or approximately 70% of the radial growth observed in the wild-type. The mutant was further distinguished from the wild-type strain by irregular outgrowth of the dense aerial mycelia and indentation of the medium surface at the irregular colony margin. Pigmentation of the interwoven hyphal mat was observed as the wild-type EP155/2, but the color was deep orange rather than bright yellow. As the cultivation was prolonged, fewer numbers of pigmented pycnidial structures were observed in the aerial mycelia.

The laccase activity of the strains was examined on plates containing tannic acid. Compared to the wild type, the *Cpdj*1-null mutant had colonies with reduced growth in diameter, but it produced a brown-colored area of comparable intensity, suggesting that laccase production was not affected by the mutation of *Cpdj*1 (data not shown). The reduced colored area was likely due to the growth defect of the *Cpdj*1-null mutant.

Chronic temperature sensitivity was examined at the elevated temperature of 30°C. Compared with growth under the standard culture conditions at 25°C, more than 30% inhibition of radial growth was observed in both the wild-type and hypovirulent strains when the temperature was shifted to 30°C. When the *Cpdj*1-null mutant was cultured at 30°C, the growth was less than 1.5 cm in diameter, which suggested that the loss-of-function mutation in *Cpdj*1 resulted in the severely retarded growth defect at the elevated temperature compared to that of the wild type. However, when the mutant strain was placed under oxidative stress, the growth rate decreased, but compared to that of the wild type under oxidative stress, the responsiveness determined by the ratio between the growth rates in the presence or absence of menadione was not changed, indicating that *Cpdj*1 was not involved in the response to oxidative stress. Osmotic stress was also applied to the *Cpdj*1-null mutant, but no significant changes in growth pattern were observed (data not shown).

To ensure that any phenotypic changes in the *Cpdj*1-null mutant were due to the gene replacement event, we complemented the mutant with a wild-type allele of *Cpdj*1. The benomyl-resistant transformants of the *Cpdj*1-null mutant that had received a wild-type *Cpdj*1 gene showed normal growth rate and abundant conidiation on PDAmb (Fig. 5A). PCR analyses revealed that all of the complemented transformants contained a wild-type allele of *Cpdj*1 (data not shown). Thus, functional complementation using a wild-type *Cpdj*1 gene unequivocally confirmed that the phenotypic changes in the mutant were caused by disruption of *Cpdj*1.

Effect of hypoviral infection on colony morphology of *Cpdj*1-null mutant

In order to examine biological functions of the *Cpdj*1 gene related to hypovirus infection, we compared the phenotypic changes between the virus-free and -containing isogenic transformants. Following co-culturing of the recipient TdDJ1-5 transformant with UEP1, at least three putative virus-containing recipient mycelia sections were independently transferred on to a

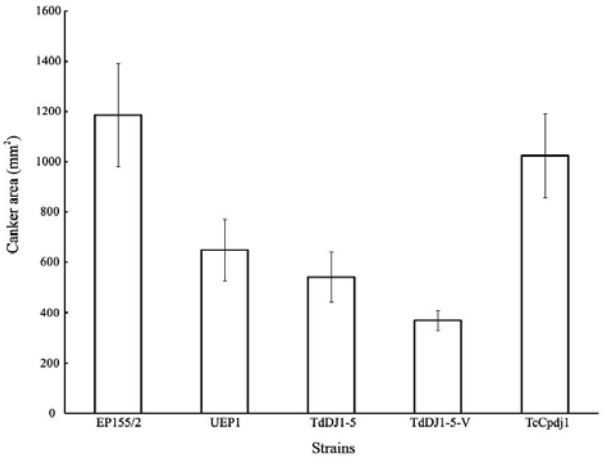

Fig. 6. Virulence assay using excised tree barks as described previously (Lee et al., 1992). The strains used were the virus-free wild type (EP155/2), its isogenic virus-containing hypovirulent strain (UEP1), the *Cpdj*1-null mutant (TdDJ1-5), the virus-containing TdDJ1-5 strain (TdDJ1-5-V1), and the Cpdj1-complemented strain (TcCpdj1). The lesion measurement values are shown as the means ± standard deviation (mm^2). Three replicates for each strain were used, and each experiment was repeated three times.

new hygromycin B-containing PDAmb plate and single-spored on hygromycin B-containing medium. The single-spored strains from different sections were used to purify dsRNA, and the presence of dsRNA in selected strains was confirmed by agarose gel electrophoresis before further comparisons (Fig. 5B). When the hypovirus CHV1-713 was transferred to the *Cpdj*1-null mutant, reduced pigmentation, a characteristic phenotypic change caused by hypovirus infection, was observed in the virus-containing *Cpdj*1-null mutants TdDJ1-5-V#1 and TdDJ1-5-V#2 (Fig. 5A). Compared to the virus-free *Cpdj*1-null mutant, conidiation of TdDJ1-5-V#1 and -V#2 was almost abolished. The most dramatic change appeared to be retarded colony growth. All of the virus-containing *Cpdj*1-null progeny displayed characteristics of thinner invasive feeding hyphae, near-absence of the typical mycelial mat on the surface, and sparse aerial hyphae. In addition, the phenotypic characteristics of the virus-containing *Cpdj*1-null mutant were maintained throughout the successive subculturing of the virus-containing null mutant. These phenotypic differences between the virus-free and -containing *Cpdj*1-null mutants suggest that, although the aberrant expression of *Cpdj*1 in the hypovirulent UEP1 was not observed under our experimental conditions, the presence of the *Cpdj*1 gene is required for the appropriate response of *C. parasitica* to the hypoviral infection.

Virulence assay

Pathogenicity tests performed using bark excised from a chestnut tree demonstrated that the size of the necrotic area induced by the *Cpdj*1-null mutant was smaller than that induced by the wild-type EP155/2, indicating that the *Cpdj*1-null mutant is less virulent than EP155/2 (Fig. 6). In addition, the size of the necrotic area of the hypovirus-containing *Cpdj*1-null mutant was even smaller than that of a hypovirulent UEP1 strain (Fig. 6). Pathogenicity tests of either the virus-free or -containing *Cpdj*1-null mutant were tightly correlated with the growth defect. Therefore, if pathogenic determinants are defined as factors that affect only pathogenicity, *Cpdj*1, which, when disrupted, results in defective mycelial growth, may not be considered as a specific pathogenic determinant following this strict definition, but rather an important factor for normal fungal growth on host surface.

DISCUSSION

Because phenotypic changes in fungal hosts induced by hypoviral infection are polymorphic, as demonstrated by hypovirulence and its associated symptoms, such as reduced sporulation, laccase production, oxalate accumulation, and pigmentation, molecular mechanisms associated with these phenotypes appear to be the result of aberrant expression of specific fungal genes in a coordinate and specific manner (Allen and Nuss, 2004; Allen et al., 2003; Kang et al., 2000; Kazmierczak et al., 1996). In order to understand the impact of hypoviral infection on fungal gene expression, several studies have demonstrated the hypoviral perturbation of fungal signal transduction pathways during viral symptom development, explaining a sophisticated but coordinated mode of gene regulation. For symptom-related genes, several genes, such as laccase, cutinase and cryparin gene, were characterized at the molecular level, but genes associated with pigmentation, conidiation, and oxalate accumulation have not been analyzed to date. Moreover, no molecular characterization of hypovirus infection on fungal stress-responsive genes has been performed, nor have studies on the biological function of well-known components in the molecular chaperone been conducted in *C. parasitica*.

In previous microarray assays, it was found that, among 2,200 fungal gene candidates, hsp70 and GST appeared to be the only two genes in the stress pathway that were significantly affected by the presence of hypovirus among 2,200 fungal gene candidates, suggesting that specific, and not general, components in the stress pathway are the targets of hypovirus infection. Likewise, we characterized the thioredoxin gene implicated in fungal response to oxidative stress conditions but found no viral interference of thioredoxin expression (Kim and Kim, 2006). Therefore, it appeared that not all components in the stress pathway are affected by the hypovirus infection. If hypovirus infection can be considered as a biological stress to a fungal host, it would be of interest to characterize how fungal stress-responsive pathways react to hypovirus infection.

Heat-shock induction of the cloned *Cpdj*1 gene transcript and protein products indicated that the *Cpdj*1 protein belongs to a member of heat-shock proteins. Estimated molecular size of the protein product of the *Cpdj*1 gene and the presence of a conserved region with a signature sequence of J-domain indicate that the cloned *Cpdj*1 gene encoded *C. parasitica* DnaJ, a member of the Hsp40 family of molecular chaperones. Sequence-based classification suggests three types of J proteins. Type I J proteins have all three structurally defined domains of the N-terminal J-domain, a glycine/ phenylalanine-rich region, and central cysteine-rich zinc finger domain. Type II J proteins differ from Type I by the absence of a cysteine-rich zinc-binding domain. Type III J proteins have a J domain but lack the other sequence features found in type I and II members of the family (Walsh et al., 2004). Sequence analysis of the deduced *Cpdj*1 protein revealed the presence of a J domain followed by a glycine/phenylalanine-rich domain. However, neither a cysteine-rich region nor a canonical zinc-finger motif CXXGXGXG (where X denotes a charged or polar amino acid) were found in the C-terminal region of the deduced *Cpdj*1 gene product, indicating that the cloned *Cpdj*1 gene belongs to the type II J proteins (Kelly, 1998; Walsh et al., 2004). In addition, phylogenetic analysis indicated that the deduced *Cpdj*1 protein was distantly

clustered from the *S. cerevisiae mdj1* protein, a representative type I J protein, further suggesting that *Cpdj*1 belongs to type II.

Northern blot analysis demonstrated that the *Cpdj*1 gene is a constitutively expressed gene, suggesting a physiological role under standard growth condition. This might explain why the *Cpdj*1-null mutant showed growth retardation under standard growth conditions. However, lack of significant difference in *Cpdj*1 expression pattern between the virus-free EP155/2 and virus-infected UEP1 strains is of interest because the accumulated Hsp70 transcript was severely affected by the hypovirus infection (Allen and Nuss, 2004; Allen et al., 2003) and molecular chaperone systems comprising of hsp70 family were implicated in viral replication and assembly (Chromy et al., 2003; Niewiarowska et al., 1992; Sullivan and Pipas, 2001; Wang et al., 2009). Multiple isoforms of DnaJ as well as Hsp70 are present in a single organism. Genome-wide sequence analysis suggests at least 22 J proteins and 14 Hsp70 proteins are present in *Saccharomyces cerevisiae* (Plesofsky, 2004; Walsh et al., 2004). Accordingly, selectivity exists in the interactions between J domains and isoforms of Hsp70. In some cases, a specific Hsp70 seems to coexist with many distinct J proteins. At least 15 J-protein genes, including the *Cpdj*1 gene, were identified in the genome of *C. parasitica* by inspection of the draft genome sequence (http://genome.jgi-psf.org/Crypa1/Crypa1.home.html). Therefore, the cloned *Cpdj*1 may not be the specific regulator of the previously known Hsp70.

Viral transfer to the *Cpdj*1-null mutant resulted in severe growth retardation and pigmentation and almost abolished conidiation compared to the virus-free *Cpdj*1-null mutant. Both reduced pigmentation and sporulation are characteristic hypovirulence-associated phenotypic changes in the CHV1-infected hypovirulen strain. However, we were not able to observe any difference in expression patterns of the *Cpdj*1 gene between virus-free and -containing strains. These results suggest that the presence of *Cpdj*1 is required for the appropriate fungal response to hypovirus infection, in that *Cpdj*1 expression is not disturbed by the hypoviral infection. But the absence of the *Cpdj*1 gene resulted in changes in *Cpdj*1-related fungal metabolism, which appears to be a fungal phenotype of hypoviral regulation and therefore makes the fungus severely retarded by hypovirus infection. Although many studies indicated the heat-shock proteins function as a molecular chaperone for viral replication and assembly (Sullivan and Pipas, 2001; Wang et al., 2009), the absence of reverting phenotypes, such as rapidly growing sectors from the virus-infected *Cpdj*1-null mutant, suggests the stable persistence of CHV1-EP713 in the *Cpdj*1-null mutant and thereby indicated that the *Cpdj*1 gene may not be beneficial for the hypovirus replication. These results seem to be consistent with our observed results: no difference in expression pattern between virus-free and -containing strains. Since hypovirus is encapsulated in pleomorphic vesicles without true capsid, it can be presumed that genes involved in regulating viral coat protein assembly are not present. However, it will be of interest to examine why no defective viral genome was observed in the virus-infected *Cpdj*1-null mutant. Contrary to the general rule that molecular chaperones are considered to be beneficial for viral replication (Sullivan and Pipas, 2001; Wang et al., 2009), negative effect on viral replication was also observed (Sohn et al., 2006). Accordingly, recent studies indicate that the RNA silencing antiviral pathway in *C. parasitica* promotes viral RNA recombination to result in defective-interfering (DI) RNAs (Sun et al., 2009). Therefore, it will be on interest to examine the relationship between the *Cpdj*1 gene and components for RNA interference.

Fungal genes known to show aberrant expression patterns in the presence of the hypovirus are referred as viral-regulated genes. Not all viral-regulated fungal genes, however, result in discernable phenotypic changes, and this has been shown in a specific null mutant (Kim et al., 1995). But *Cpdj*1 gene in this study differs in that no changes in expression profile were observed after hypovirus infection, whereas phenotypic changes became obvious when the null-mutant was infected by the hypovirus. Besides the viral-regulated genes, we suggest that another group of genes, which is not as sensitive as viral-regulated genes but is present, is required for the normal response of fungi against virus infection.

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