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A Gene Encoding Phosphatidyl Inositol-specific Phospholipase C from *Cryphonectria parasitica* Modulates the Hypoviral-modulated Laccase1 Expression

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Summary

Hypovirus infection of the chestnut blight fungus *Cryphonectria parasitica* is a useful model system to study the hypoviral regulation of fungal gene expression. The hypovirus is known to downregulate the fungal laccase1 (*lac*1), the modulation of which is tightly governed by the inositol triphosphate (IP_3) and calcium second messenger system in a virus-free strain. We cloned the gene *cplc*1 encoding a phosphatidyl inositol-specific phospholipase C (PLC), in order to better characterize the fungal gene regulation by hypovirus. Sequence analysis of the *cplc*1 gene indicated that the protein product contained both the X and Y domains, which are the two conserved regions found in all known PLCs, with a 133 amino acid extension between the 2nd β-strand and the α-helix in the X domain. In addition, the gene organization appeared to be highly similar to that of a δ type PLC. Disruption of the *cplc*1 gene resulted in slow growth and produced colonies characterized by little aerial mycelia and deep orange in color. In addition, down regulation of *lac*1 expression was observed. However, temperature sensitivity, osmosensitivity, virulence, and other hypovirulence-associated characteristics did not differ from the wild-type strain. Functional complementation of the *cplc*1-null mutant with the *PLC*1 gene from *Saccharomyces cerevisiae* restored *lac*1 expression, which suggests that the cloned gene encodes PLC activity. The present study indicates that the *cplc*1 gene is required for appropriate mycelial growth, and that it regulates the *lac*1 expression, which is also modulated by the hypovirus Although several PLC genes have been identified in various simple eukaryotic organisms, the deletion analysis of the *cplc*1 gene in this study appears to be the first report on the functional analysis of PLC in filamentous fungi.

Introduction

The chestnut blight fungus *Cryphonectria parasitica* virtually eliminated the American chestnut tree in the early 20th century. However, strains that contain the double-stranded (ds) RNA virus

Cryphonectria hypovirus1 (CHV1) display characteristic symptoms of reduced virulence, i.e., hypovirulence (Van Alfen *et al.*, 1975; Anagnostakis, 1982), as well as diverse hypovirulence-associated traits, such as reduced pigmentation, sporulation, laccase production, and oxalate accumulation (Havir & Anagnostakis, 1983; Elliston, 1985; Rigling *et al.*, 1989). Molecular basis for these symptoms revealed the changes in the host transcriptional profile response to hypovirus infection (Kazmierczak *et al.*, 1996; Allen *et al.*, 2003; Allen & Nuss, 2004). 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 there are broad but specific fungal genes modulated by hypovirus infection. These virus-regulated and functionally analyzed fungal genes include: the cutinase gene (Varley *et al.*, 1992); *lac1*, which encodes an extracellular laccase (Rigling &Van Alfen, 1991; Kim *et al.*, 1995); *Crp*, which encodes an abundant tissue-specific cell-surface hydrophobin; and *Mf2/1* and *Mf2/2* which encode pheromone precursors that are involved in poor sporulation in the presence of the hypovirus (Zhang *et al.*, 1993; Zhang *et al.*, 1998). Since the phenotypic changes in the fungal host are pleiotropic, albeit co-ordinated and specific, it has been suggested that the hypovirus disturbs one or several regulatory pathways (Nuss, 1996). Accordingly, several studies have revealed the hypoviral perturbation of the fungal signal transduction pathway as a means of co-ordinating the control of specific set(s) of fungal genes during viral symptom development (Chen *et al.*, 1996; Gao & Nuss, 1996; Kasahara & Nuss, 1997; Kim *et al.*, 2002; Park *et al.*, 2004).

It has been reported that *C. parasitica* produces two different laccases, the extracellular laccase LAC1 and the intracellular laccase LAC2. It has been demonstrated that the virus affects the activities of both enzymes (Rigling & Van Alfen, 1993). In addition, studies of the *lac*1-null mutant suggest the presence of an additional tannic acid-inducible extracellular laccase, which is also likely to be regulated by the hypovirus (Kim *et al.*, 1995). Larson *et al.* (1992) have postulated that the virus affects the expression of the extracellular laccase by affecting signal transduction pathways that regulate *lac*1 gene expression. Since the *lac*1 transcript levels are down regulated by the presence of the hypovirus, this attenuation of gene expressions may serve as a model system for understanding the origin of many hypovirulence-associated traits. Two different and opposing regulatory pathways appear to govern the accumulation of the *lac*1 transcript in the virus-free strain. First, a stimulatory pathway is dependent on both inositol trisphosphate (IP_3) and the calcium second messenger systems. Second, a negative regulatory pathway that requires ongoing protein synthesis is active. Larson *et al.* (1992) have postulated that of these two pathways, the hypovirus interferes with the transduction of an IP_3-calcium-dependent signal that is required to stimulate *lac*1 gene transcription.

The phosphatidyl inositol-specific phospholipases C (PLCs) are relevant to cellular metabolism, including the biosynthesis and degradation of membrane lipids. In addition, the PLCs are known to hydrolyze polyphosphoinositides, which have been implicated in one of the major pathways of signal

transduction during the response to extracellular signals. The PLCs are responsible for the production of two-second messenger molecules: diacylglycerol (DAG), which is an activator of protein kinase c (PKC); and inositol 1,4,5-triphosphate (IP_3), which causes the release of Ca^{2+} from the endoplasmic reticulum. Based on enzymatic characteristics and amino acid sequence similarities, PLCs can be divided into three types: PLC-β, PLC-γ, and PLC-δ (34). Each type of PLC also contains several subtypes. At least 10 subtypes have been identified in mammalian cells. All the subtypes of the three main families of PLCs contain a series of common modules that facilitate the enzymatic reaction. The modular structure consists of an N-terminal pleckstrin homology (PH) domain, an EF-hand calcium-binding domain, an active site that contains two conserved regions, termed the X and Y boxes, and a C-terminal C2 lipid-binding domain (James, 1998; Katan, 1998).

PLC genes have been identified in diverse eukaryotic organisms other than mammals, including the slime mold (Drayer & Haasstert, 1992) and yeast (Yoko-o *et al.*, 1993; Fankhauser *et al.*, 1995; Bennette *et al.*, 1998). In addition, there is growing evidence to implicate phosphoinositides in the signal transduction pathway of filamentous fungi (Gadd, 1995). Moreover, putative full-length and partial genes of PLC have been identified in filamentous fungi, such as *Botryotinia fuckeliana*, *Aspergillus nidulans*, and *Neurospora crassa*, although their actual cellular functions have not been analyzed experimentally (Jung *et al.*, 1997). Recently, a comparative genomic analysis of the calcium signaling machinery in *N. crassa, Magnaporthe grisea*, and *Saccharomyces cerevisiae* predicted the presence of four, four, and one PLC's, respectively (Zelter *et al.*, 2004). Therefore, viral interference with the transduction of the IP_3-calcium-dependent signal for *lac*1 transcription prompted us to clone the PLC gene from *C. parasitica*, in order to investigate the cellular function(s) of PLC and concomitant regulation of *lac*1 expression, and thereby to come to a better understanding of viral regulation of the fungal gene.