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# Gene Expression Modulation of Two Biosynthesis Pathways via Signal Transduction in *Cochliobolus heterostrophus*

**Ofir Degani**[1,2]

[1]Tel-Hai College, Tel-Hai, Israel
[2]Migal-Galilee Research Institute, Kiryat Shmona, Israel
Email: d-ofir@bezeqint.net, ofird@telhai.ac.il

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## Abstract

**G-protein-linked pathways have evolved to allow responses to extracellular agonists (hormones, neurotransmitters, odors, chemoattractants, light and nutrients) in eukaryotic cells, ranging from simpler systems, including yeasts, filamentous fungi and slime molds, to more complex organisms, such as mammals. Although the role of G-protein and mitogen-activated protein kinase (MAPK) in filamentous fungi has been studied for over a decade, downstream elements are less known, and the study of target genes has evolved mainly in recent years. Here, we examined the involvement of G-protein subunits and MAPK in controlling the expression of two distinct target genes. These genes were selected from an array database according to their unique expression profile and the role of closely related genes found in other Ascomycetes. One of these genes is *BPH*, which encodes the enzyme responsible for cytochrome P450-dependent benzoate hydroxylation in microsomes. The other gene is *CIPA*, which encodes isoflavone reductase (IfR), an enzyme involved in the synthesis of phytoalexin, which catalyzes an intermediate step in pisatin biosynthesis. The expression profile of these two genes was determined in a series of signaling deficiency mutants that were grown on different media using a DNA microarray. Comparison of the expression profile in the two wild type strains and mutants deficient in the G-protein $\alpha$ or $\beta$ subunits or in MAPK, revealed a unique control mechanism for the *BPH* and *CIPA* genes. The two genes are highly expressed during the infection of the host plant leaves and may associate with the fungal response to the host. Signaling via G-protein or MAPK was shown to be related to cascades that altered the expression of these genes in response to the growth condition. This work demonstrates that signal transduction pathways are controlling genes that, although sharing an environmental dependent response, participate in distinct biosynthesis pathways. Moreover, the transcriptional profile may point to distinct and shared roles of the signaling components.**

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## Keywords

***Cochliobolus heterostrophus*; Cytochrome P450-Dependent Benzoate Hydroxylase; G-Protein; Isoflavone Reductase; Maize; MAPK; Signal Transduction**

## 1. Introduction

The small GTP-binding protein Ras and heterotrimeric G proteins in eukaryotic cells are involved in the transmission of external signals. Filamentous fungi recognize and respond to signals from the environment and host organisms by altering their growth and development. In *Cochliobolus heterostrophus*, the agent of Southern Corn Leaf Blight, G$\alpha$1 (*CGA*1) and G$\beta$ (*CGB*1) subunits, as well as of a mitogen-activated protein kinase (MAPK, *CHK*1), participates in several developmental pathways. Disruption studies of these signaling components results in severe phenotypes, including loss of the normal meandering hyphal growth pattern on hard surfaces, lack of appressorium formation, and defects in mating and virulence [1]-[6]. Both GTP-G$\alpha$ and free G$\beta\gamma$ can activate downstream targets [7], and an epistatic relationship may exist between the two branches of the G-protein pathway [5].

A MAPK module is a known target of G$\beta\gamma$ in budding yeast and mammalian cells [8] [9], and new evidence indicates that some crosstalk may exist between the MAPK and the G-protein pathways in *C. heterostrophus* as well [5]. Data on G-protein subunits and MAPK involvement in the regulation of gene expression in fungal species are constantly growing. Nevertheless, only a few genes have been shown to be regulated by signal transduction in *C. heterostrophus* to date. Among these are two cellulase genes whose expression is modulated by the MAPK pathway [10] [11] and four hydrophobins [12]. In addition, the expression of key genes involved in melanin biosynthesis in two *C. heterostrophus* MAPKs, *CHK*1 and *MPS*1, has been shown to be induced significantly under hyperosmotic conditions compared to invariably high expression in the wild type (WT) [13].

Here, we examined the expression variations resulting from the disruption of G-protein $\alpha$ and $\beta$ subunits and of MAPK in two distinct genes. The two genes were selected from a microarray database according to their unique expression profile and the role of closely related genes found in other Ascomycetes. The first gene, designated *CIPA*, showed high homology in the deduced amino acid sequence (Blastx: Expect = 1e − 54, in 549 bp) to the 2'-hydroxyisoflavone reductase (IfR; EC 1.3.1.45) family protein CipA from *Aspergillus fumigatus*/*nidulans* and to other fungi hypothetical proteins (*Gibberella zeae*, *Neurospora crassa* and *Magnaporthe grisea*). Phytoalexins are low molecular weight antimicrobial compounds synthesized by plants in response to attempted infection by fungal pathogens, exposure to elicitor macromolecules, or other biotic and abiotic stresses [14]-[17]. Isoflavonoid phytoalexins are characteristic of Leguminosae and are very rarely found in other plant families [18]. The pterocarpan medicarpin is the major phytoalexin in the alfalfa forage legume. It is biosynthesized in two pathways, one of them (the isoflavonoid branch pathway) involved specific enzymes that include IfR. This enzyme catalyzes the NADPH-dependent reduction of 2'-hydroxyformononetin to vestitone, which is the penultimate step in the synthesis of medicarpin [19]. IfR protein has been purified and characterized from several legumes, and *IFR* cDNA clones have been obtained from alfalfa [20], chickpea [21] and pea [16]. In unstressed alfalfa plants, transcripts from the single alfalfa *IFR* gene are detected mainly in roots and root nodules, consistent with the accumulation of a medicarpin conjugate, medicarpin-3-O-glucoside-6'-Omalonate, only in these organs [20]. *IFR* transcripts are, however, strongly induced in infected leaves or elicited cell cultures at the onset of medicarpin accumulation [22]. In fungi, CipA is a member of the protein family associated with the response to the antibiotic concanamycin A produced by *Streptomyces* sp. [23]. In *Candida* sp., a *CIP*1 gene with little homology with *IFR* of plants, was proposed to play a crucial role in the establishment of a specific cellular response to stress resulting from the cadmium treatment [24]. These observations may indicate that the specific role of *IFR* in the synthesis of defense-related isoflavonoid compounds may be recruited to defense needs also in fungi.

The second gene chosen for this study, *BPH*, showed high homology in the amino acid sequence (BLASTX: Expect = 9e − 177, in 1605 bp) to cytochrome P450 benzoate 4-monooxygenase (Benzoate-para-hydroxylase, BpH, EC 1.14.13.12.) from *A. fumigatus* and other fungi (*N. crassa*, *Phanerochaete chrysosporium* and *Rhodotorula minuta*), and to other hypothetical fungi proteins (*G. zeae*, *Ustilago maydis* and *M. grisea*). Cytochromes

P450 (CYP) are a super family of enzymes crucial for the oxidative, peroxidative and reductive metabolism of a diverse group of compounds, including endobiotics, such as steroids, bile acids, fatty acids, prostaglandins and leukotrienes, and xenobiotics, including most of the therapeutic drugs and environmental pollutants [25]. CYP enzymes are expressed ubiquitously in different life forms: they have been found in animals, plants, fungi and bacteria [25]. They seem to be indispensable for eukaryotic species, but not for prokaryotes, since some bacteria lack CYP enzymes [27]. Eukaryotics need CYPs for the biosynthesis of sterols, which are constituents of plasma membrane [26]. Eukaryotic CYP enzymes are membrane-bound, mostly localized to the endoplasmic reticulum, but some CYPs are also present in mitochondrial inner membranes. In order to function, cytochrome P450s require an electron transfer chain. In the endoplasmic reticulum, this source is NADPH-cytochrome P450 reductase, previously called NADPH-cytochrome C reductase [26]. In mitochondria, electrons are transferred from NADPH by redoxin reductase to redoxin and then to CYP [28]. Despite their occasionally minimal sequence similarity, all CYPs have a similar structural fold with a highly conserved core [29].

P450rm has been well conserved during the evolution of fungi as a benzoate 4-hydroxylase in the dissimilation pathway starting from L-phenylalanine [30]. The enzyme catalyzed the reaction: Benzoate + NADPH + $H^+$ + $O_2$ = 4-hydroxybenzoate + $NADP^+$ + $H_2O$ and requires P450 reductase (CprA) as the mediator in electron donation from NADPH. In the endoplasmic reticulum of the filamentous fungus *A. niger*, a cytochrome P450 enzyme system is present that is capable of the para-hydroxylation of benzoate [31]. The *A. niger* gene called *CYP*53 was defined to be a member of the cytochrome P450 sub family, P450LIII (*CYP*53 family). The expression of the two genes encoding the components of this system, the cytochrome P450 gene encoding benzoate para-hydroxylase A (BpHA) and the gene encoding cytochrome P450 CprA, is inducible by benzoate [31]. The *BPHA* orthologous gene from *A. nidulans*, *BZUA*, was localized in the endoplasmic reticulum and was identified as being required for the utilization of benzamide as the sole nitrogen source [32]. The *BZUA*1 mutation prevents the use of benzoate as a carbon source, and intracellular accumulation of benzoate results in growth inhibition on benzamide. *BZUA* is strongly benzoate inducible and subject to CreA-mediated carbon catabolite repression and a probable inactivation of benzoate induction by glucose [32]. A cytochrome P450rm was also isolated from the basidiomycete yeast *R. minuta* as a bifunctional enzyme with isobutene-forming and benzoate 4-hydroxylase activities. The gene product shared 48% amino acid sequence identity with *CYP*53*A*1 from *A. niger*, indicating that the gene belongs to a novel subfamily of *CYP*53, *CYP*53*B* [30].

Here, we used microarray as a means to isolate these two genes, whose expression is upregulated during plant infection, and to identify their transcriptional profile in *C. heterostrophus* WT and signal deficiency strains. The results may define the unique contribution of each signaling unit to the regulation of distinct biosynthesis pathways. Moreover, the transcriptional profile may point to distinct and shared roles of the signaling components.

## 2. Materials and Methods

### 2.1. Strains

The strains used are listed in **Table 1**.

### 2.2. Sample Preparation for Microarray

Wild type and signaling mutants were grown in different liquid media: complete medium with xylose (CMX, 48 h) or maltose (CMM, 48 h), minimal medium with xylose (MMX, 72 h) and also inoculated on maize cv. Grand Jubilee (from Pop Vriend Seeds B.V., Andijk, The Netherlands, supplied by Eden Seeds, Reut, Israel) plants (described previously by [11]). The leaves of the plants were dipped into homogenized mycelial suspension in order to obtain widespread lesions. Infected plants were incubated for different periods of time according to virulence of mutant strains. Plants infected with WT or G-protein $\alpha$2 subunit gene disrupted mutant (*cga*2) were incubated for 24 hours; during this time, the plant tissue was completely destroyed. Plants infected by *chk*1, *cgb*1 and *cga*1 were incubated for 48 hours. *cga*1 and *cgb*1 mutants caused a similar degree of infection: a large portion of maize leaf area became necrotic. The *chk*1 mutant caused the least severe symptoms: leaves acquired yellow coloring and small necrotic lesions appeared. The portion of fungal RNA in samples isolated from infected tissue varies from about 23% (inoculation by *chk*1) to 50% (inoculation by wild type). Ethidium bromide staining of gel-electrophoresed RNA enables relative estimation of plant RNA portion in each sample: the intensity of bands representing plant chloroplast rRNA varies significantly between samples.

**Table 1.** Strains used.

| Strain | Genotype | Reference or Source | Comments |
|---|---|---|---|
| WT C4 | *MAT*1-2; *Tox*1$^+$ ATCC 48331 | Leach *et al*. (1982) | Wild type strains obtained after six backcrosses and are nearly isogenic. |
| WT C5 | *MAT*1-1; *Tox*1$^-$ ATCC 48332 | Leach *et al*. (1982) | |
| *cga*1 | *MAT*1-1; *Tox*1$^-$ ATCC 48332 | Horwitz *et al*. (1999) | G-protein $\alpha$1 subunit disrupted mutant created by insertion of the hygromycin cassette into the coding region combined with an 18-bp deletion. |
| *cga*2 | *MAT*1-2; *Tox*1$^+$ ATCC 48331 | Giloh M.Sc. thesis (cited in Ganem *et al*., 2004) | G-protein $\alpha$2 subunit disrupted mutant having no known phenotypes. |
| *cgb*1 | *MAT*1-2; *Tox*1$^+$ ATCC 48331 | Ganem *et al*. (2004) | G-protein $\beta$ subunit disrupted mutant created by insertion of the hygromycin cassette into the coding region combined with a 473 bp deletion. This strain contains additional mutations but its phenotypic traits are similar in almost every aspect to the *cgb*1 that contains only one mutation. |
| *chk*1 | *MAT*1-2; *Tox*1$^+$ ATCC 48331 | Lev *et al*. (1999) | MAPK disrupted mutant created by replacing the coding region with the hygromycin resistance cassette. |

### 2.3. Normalization of Fungal RNA Quantities

A *GAPDH* gene (encoding glyceraldehyde-3-phosphate dehydrogenase) fragment generated by PCR with primers 5' CCCTCGCCTGACGCCCCCAT 3' and 5' CGAGGACACGGCGGGAGTAA 3' was used to quantify fungal RNA. Total RNA of mutants and WT (with plant RNA added) infected leaves were the starting material for mRNA isolation using the polyATtract kit (Promega).

### 2.4. Microarray Search and Results Processing

The two selected genes, *BPH* and *CIPA*, were chosen from the array database (Syngenta Biotechnology Inc., NC USA, Affymetrix Chip containing 25,215 sequences based on 13,309,801 bases) according to their unique expression profile in the various signaling mutants. The array search parameters aimed at finding fungal genes that are upregulated in plant infection in comparison to liquid media growth, *i.e*., genes with low expression levels in all strains and all media, except for the corn leaves infection experiment. We also defined the search parameter to isolate genes that differ in their expression in one or more mutant strains in comparison to the WT in at least two-fold normalized expression intensity. The selected genes were identified by homology search against the *C. heterostrophus* genome sequence database (genome sequences of *C. heterostrophus* strains C4 and C5 are now available publicly from the JGI website at http://www.jgi.doe.gov/) using TBLASTN [33] and by BLASTX against the gene bank database using the NCBI (National Center for Biotechnology Information, MD, USA) BLAST sequence analysis services at http://www.ncbi.nlm.nih.gov/BLAST/.

### 2.5. Phylogenetic Relationships

Protein sequences were aligned using ClustalX 1.83 (http://www.clustal.org) [34]. Alignment was done using bootstrap N-J tree (1000) and exclude positions with gaps. The TreeView program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) was used to generate a phylogenetic tree; default options were chosen.

## 3. Results

We used two new isolated genes *BPH* and *CIPA* to study the role of the G-protein $\alpha$ and $\beta$ subunits and of MAPK (Chk1) in regulating distinct biosynthetic pathways and in controlling target gene expression. These two genes were chosen from the array database (Affymetrix Chip) according to their unique expression profile in the various signaling mutants. The *BPH* and *CIPA* genes are upregulated in plant infection in comparison to liquid media growth and are differ in their expression in one or more mutant strains in comparison to the WT in at least two-fold normalized expression intensity.

### 3.1. *CIPA* Regulation by *C. heterostrophus* Signal Transduction G-Protein and MAPK Pathways

Comparison of the deduced amino acid sequence of the *C. heterostrophus* CipA protein with the sequences

present in the databank (BLASTX, NCBI BLAST sequence analysis services) indicated a significant similarity to IfR family protein of *A. fumigatus* (BLASTX: Expect = 1e − 54, in 549 bp). *C. heterostrophus* CipA share high homology with other IfR proteins from bacteria, fungi and plants (**Figure 1**). Phylogenetic analysis of 61 known *IFR* genes products (including the *C. heterostrophus* CipA) revealed the well-conserved distribution of the IfRs into three branches according to the kingdoms with apparently common ancestor (**Figures 1** and **2**). *C. heterostrophus CIPA* is located in the phylogenic tree in the fungi branch and is most closely related to the *A. fumigatus IFR* gene (in 869 out of 1000 predicted trees).

Apparently the ancestor carrying the *IFR* gene for the first time was a bacterium, and this gene was then spread to other bacteria, fungi and plants species in a nonlinear way. Although variations exist in the roots of the phylogenic tree, as expected (for example, 211 out of 1000 predictions in one root that split into bacteria and plant branches, or 149 out of 1000 predictions in the root of the *C. heterostrophus IFR* gene branch), an obvious similarity exists within the gene families (usually over 600 of the 1000 predictions) (**Figure 1**). A phylogenic analysis based on the NCBI BLASTX high homology sequences revealed a much more conserved tree (**Figure 2**). To date, only several *IFRL* genes have been identified in fungi members, including *A. fumigatus*, *E. nidulans*, *Candida* sp. and *N. crassa*. Nevertheless, hypothetical proteins with high homology have been identified in other fungi as well.

The *CIPA* expression profile was examined by microarray analysis (**Figure 3**). The microarray is a sensitive tool enabling us to compare the amount of transcript levels between the different media and the different signaling disrupted mutant strains. *CIPA* showed a basal, relatively low, expression level on all four media examined by the microarray analysis, except for in plant infection (**Figure 3**). RNA samples taken from infected leaves indicate significant differences (four-fold) in the expression levels of the *CIPA* gene between *C. heterostrophus* strains. High expression levels of *CIPA* were observed in WT C4, *cga*2 and *chk*1 strains in comparison to *cga*1 and *cgb*1 strains. The similar consequence of disruption *cga*1 or *cgb*1 separately may imply a common role for both signaling pathways.

### 3.2. *BPH* Regulation by *C. heterostrophus* Signal Transduction G-Protein and MAPK Pathways

Comparison of the deduced amino acid sequence of the *C. heterostrophus* second protein tested here, *BPH*, against the sequences present in the databank (BLASTX, NCBI BLAST sequence analysis services) indicated a significant similarity to the superfamily of cytochrome P450 IfR enzymes. *C. heterostrophus BPH* is a high homolog (BLASTX: Expect = 9e − 177, in 1605 bp) of an *A. niger* P450 gene, *CYP*53, which belongs to the P450LIII family [35] [36]. The gene shares high homology with other fungal *BPH* genes such as *E. nidulans*, *N. crassa*, *P. chrysosporium*, *R. minuta* and *Nectria haematococca BPH*s (**Figure 4**). Moreover, many hypothetical proteins in other fungi also show high homology to this gene product (including *G. zeae*, *U. maydis* and *M. grisea*).

Phylogenetic analysis of 55 known cytochrome P450 genes products (including the *C. heterostrophus* BpH) revealed the distribution of the BpHs into several branches according to the organisms' kingdoms with apparently a common bacterial ancestor (**Figure 4**). It appears that the cytochrome P450 gene was then spread to other bacteria, fungi, plants and animal species in parallel pathways. *C. heterostrophus* BpH is located in the phylogenic tree in the fungi branch and is most closely related to *Aspergillus* sp. BpH (in 845 out of 1000 predicted trees). It also has a common root with *N. crassa*, *Rhodotorula* spp. and *U. maydis BPH* genes (in 100% of the predicted trees). The fungi branch is rooted together with part of the animal cytochrome P450 branch in 301 out of 1000 predicted trees and this shared root is attached to the plant's cytochrome P450 root (in 118 out of 1000 predicted trees) (**Figure 4**).

Microarray analysis of *C. heterostrophus BPH* expression profile exhibits significant upregulation of this gene when fungi grow on maize plant leaves (in comparison to modified liquid media) (**Figure 5**). In comparison to the WT, *BPH* expression on plant leaves increased in the *cga*1 strain and decreased in both the *cga*2 and *cgb*1 strains. Under these conditions, *chk*1 showed normalized *BPH* expression intensity levels similar to the WT.

## 4. Discussion

Deciphering the signaling mechanisms that lead to fungal development and pathogenicity has been subject to intensive study over the past decade. Although a considerable amount of data exists on the key player in these conserved pathways, the G-protein-encoding genes and the MAPK, less is known about downstream effectors,

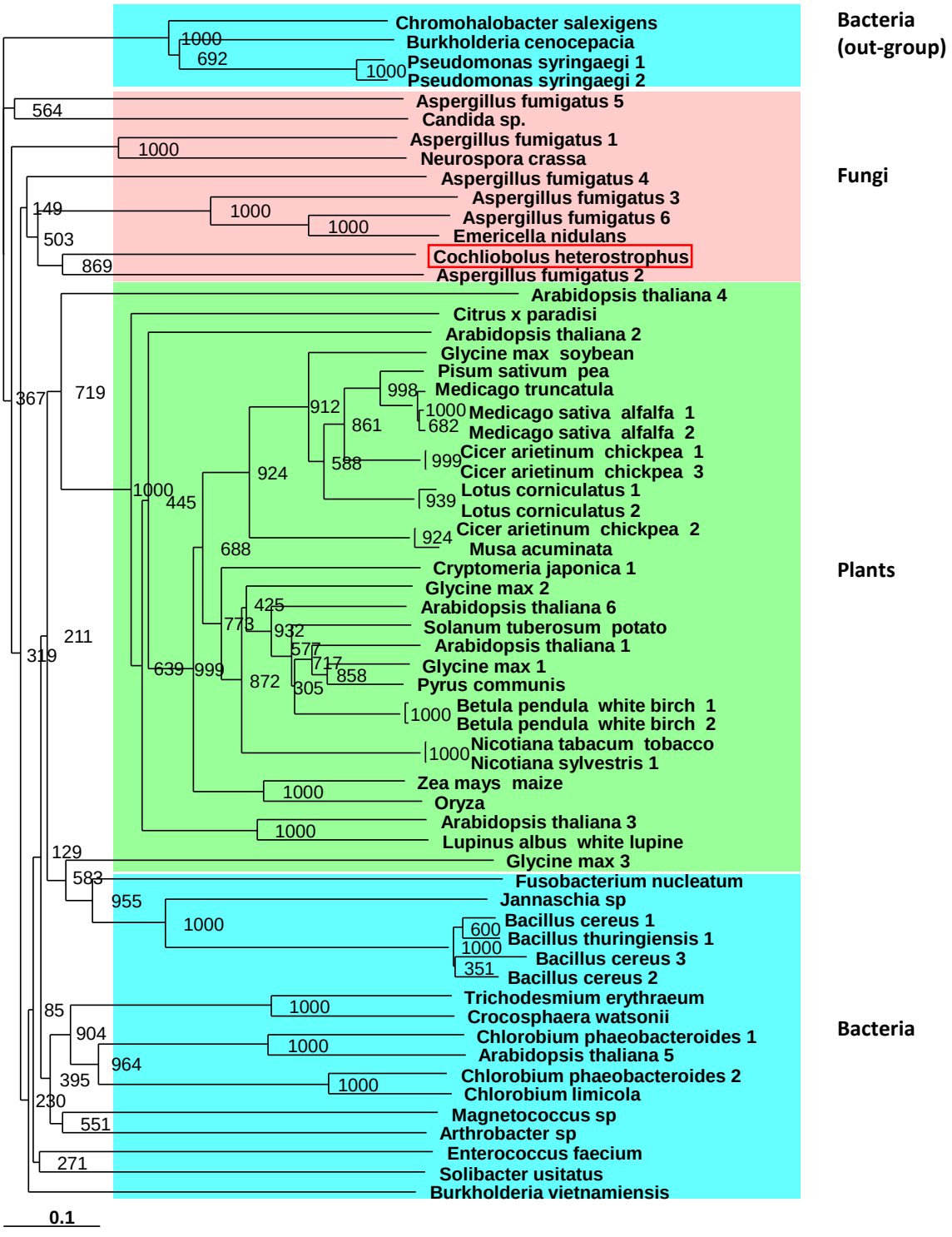


**Figure 1.** *C. heterostrophus* 2'-hydroxyisoflavone reductase (IfR, CipA) phylogenic relationship. The IfR enzyme involved in the synthesis of phytoalexin, which catalyzes an intermediate step in pisatin biosynthesis. Protein sequences were aligned using CLUSTALX 1.83 (Web site: http://www.clustal.org) [34]. Alignment was done using bootstrap N-J tree (1000). The TreeView program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) was used to generate a phylogenetic tree; default options were chosen. Square red frame indicates the *C. heterostrophus* CipA deduced protein. Numbers indicate bootstrap values for each node point. Few bacteria species were defined to be an out group and the root of this tree.

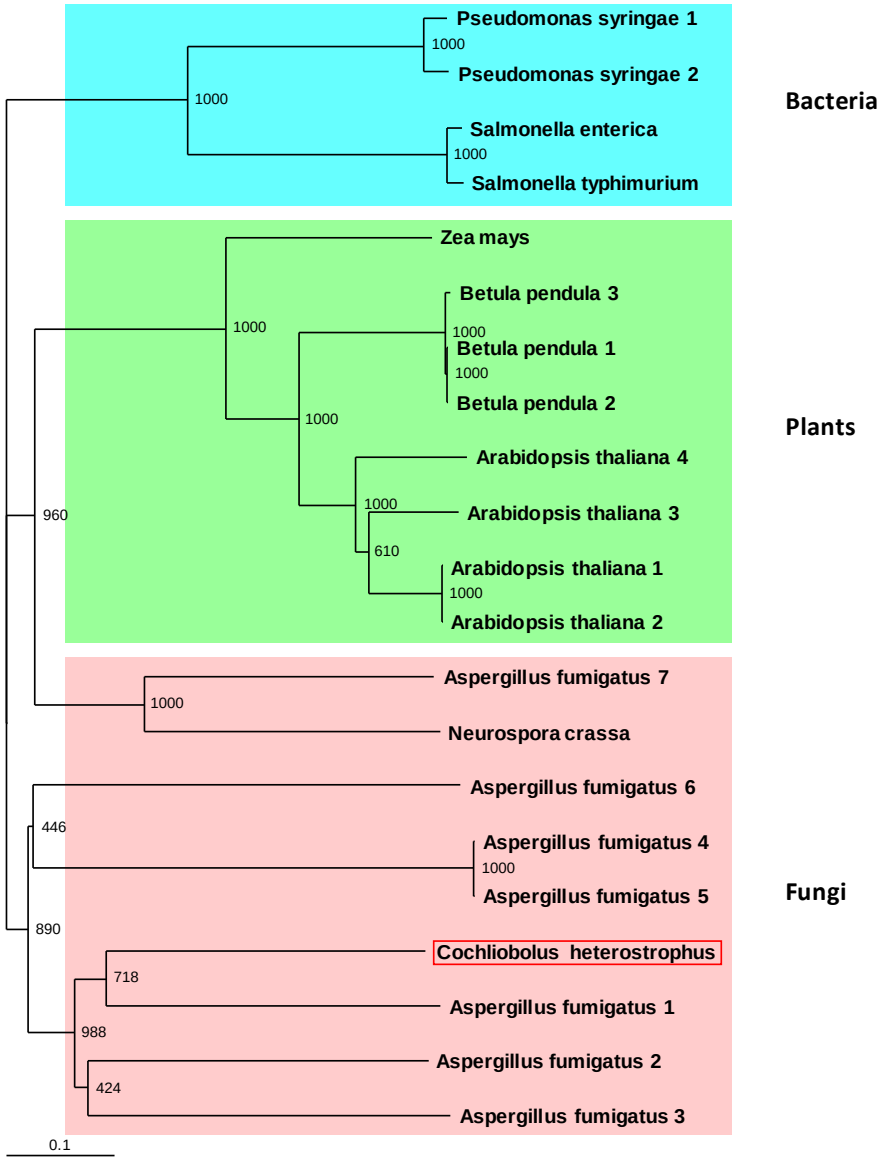


**Figure 2.** *C. heterostrophus* CipA phylogenic relationship to closely related sequences. Protein sequences that showed high homology in the BLASTX of *C. heterostrophus* IfR against the database were aligned using CLUSTALX 1.83, and a phylogenetic tree was generated using the TreeView program as described in **Figure 1**. Square red frame indicates the *C. heterostrophus* CipA deduced protein. Numbers indicate bootstrap values for each node point.

and the remaining components of these pathways still need to be revealed [37]. At the output of these cascades are sets of target genes, whose study of expression has begun in recent years. We previously identified roles for the individual G-protein G$\alpha$ and G$\beta$ subunits [1]-[3] [5] [6] and for MAPK [4] [11] in *C. heterostrophus* development, reproduction and pathogenicity (summarized in [5]). To date, little knowledge exists on downstream signaling elements of these cascades in this species. The influence on target genes expression was demonstrated in only a few instances [11]-[13].

Here, we identified two genes controlled by the *C. heterostrophus* signal transduction pathway that are key players in biosynthetic processes. These processes are involved in response to environmental conditions that may include the fungal response to the host as is discussed in detail below. Indeed, the two genes encode for *IFRL* and *BPH*, and are highly expressed during the infection of the host plant leaves (**Figures 3** and **5**).

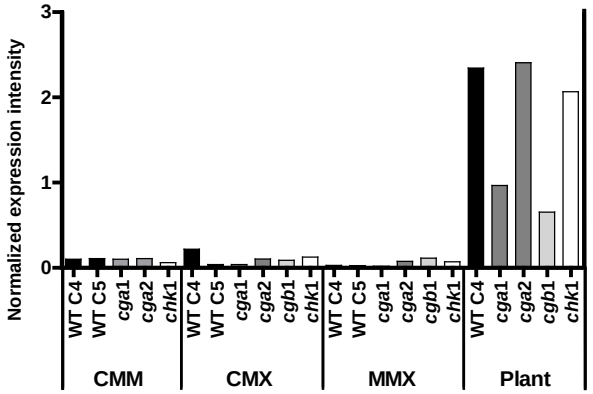


**Figure 3.** *C. heterostrophus CIPA* expression profile. Microarray (Affymetrix Chip) normalized expression results indicate transcript levels of WT (C4, C5) and the signal deficiency mutant strains: *cga*1 (Δ*Gα*1); *cga*2 (Δ*Gα*2); *cgb*1 (*ΔGβ*1); *chk*1 (Δ*MAPK*1). CMM (complete medium with maltose); CMX (complete medium with xylose); MMX (minimal medium with xylose); Plant (plant inoculation).

IfR is an NADPH-dependent reductase involved in the biosynthesis of isoflavonoid phytoalexins in legumes [19]. It is known that plants respond to pathogen attack by activating defense reactions, including changes in ion flux, generation of reactive oxygen species, production of phytoalexins, production of pathogenesis-related proteins and enzymes, and reinforcement of the cell wall [38]. Isoflavonoid phytoalexins are low molecular weight anti-microbial compounds produced by plants in response to pathogen attack, elicitor molecules, or other biotic and abiotic stresses [15]. Obligate mycorrhizal fungi that form mycorrhizae with alfalfa roots apparently suppressed certain host defense reactions. For example, *Glomus versiforme* suppressed the host defense response in two *Medicago* spp. by lowering the relative mRNA ratios (standardized to rRNA) of *IFR* after 40 days [39]. Volpin *et al.* [40] also suggested that suppression of the normal host isoflavonoid phytoalexin defense response is mediated by changes in RNA transcription. Suppression of the plant defense response by a penetrating microorganism has also been reported in bacterial systems [41]. Bacteria reduces their exposure to isoflavonoids by catabolization of flavonoids [42] and isoflavonoids [43] [44] or by isoflavonoid efflux pumps, as found in *rhizosphere bacterium* [45].

IfRL proteins have been grouped in a family solely on the basis of their high sequence similarity with IfRs, which catalyze the reduction of *α*,*β*-unsaturated ketones and which are involved in biosynthesis of isoflavonoid phytoalexins in legumes in response to fungal infection [20] [21] [46]. However, IfRL proteins have been identified in plants that do not synthesize isoflavonoid phytoalexins in response to pathogen attack [47], and therefore IfRLs are likely to have broader functions. IfRLs have been implied to function in response to oxidative stress in *Arabidopsis* [48], prolonged sulfur starvation in maize [49], and UV radiation in harvested grapefruit [50]. It has been suggested that all IfRL proteins are oxidoreductases (EC 1.3.1 that act on the CH-CH Group of Donors) utilizing NAD(P)H as a cofactor, which have various substrates that may or may not be related structurally to flavonoids [48]. Evidence from *A. fumigatus*/*nidulans* [23] and *Candida* sp. [24] indicates that the *IFR* genes are upregulated in fungi in response to biotic and abiotic stresses. Together with the findings in this work that point to an upregulation of *IFRL* in response to plant nutritional source (**Figure 3**), it may be possible that IfR recruits general defense responses for the fungus including host defense induced stresses.

Very little is known about the signal transduction pathways inducing *IFRL* genes. Kim *et al.* [51] showed that the induction of *OsIRL* by the fungal elicitor in plants was blocked by calyculin A, but not affected by staurosporine, suggesting that phosphatase activity is necessary for *OsIRL* induction, but not hyperphosphorylation. Elicitor-induced *OsIRL* transcription was also somewhat induced by the calcium ionophore A23187, suggesting that $Ca^{2+}$ ions are involved. Here, we reported that *IFRL* expression is controlled in the plant pathogen *C. heterostrophus* by the G-protein signaling pathway, but not the MAPK. In particular, we discovered that G-protein *α* and *β* subunits are involved in maintaining normal levels of *IFRL* expression. Disruption of this signaling components results in a significant decrease in *IFRL* expression (**Figure 3**). It is likely that this well-known

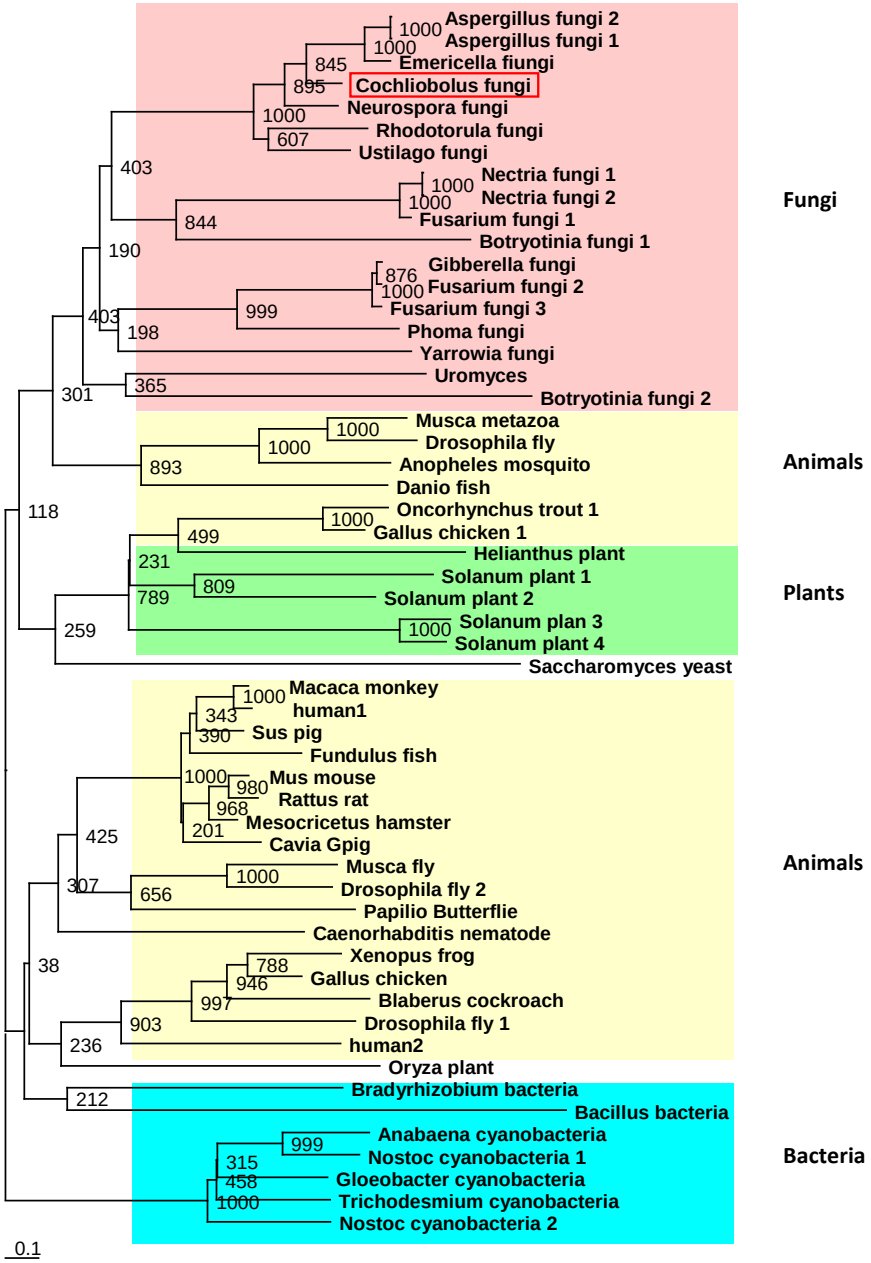


**Figure 4.** Cytochrome P450 benzoate 4-monooxygenase (BpH) family phylogenic tree. Protein sequences were aligned using CLUSTALX 1.83, and the TreeView program was used to generate a phylogenetic tree, as described in **Figure 1**. Square red frame indicates the *C. heterostrophus BPH* deduced protein. Numbers indicate bootstrap values for each node point.

environmental sensing mediation mechanism will be involved if *IFRL* is indeed related to stress response in fungi.

The *BPH* is another gene that may be a target for signaling mediation control. *BPH* is a member of cytochrome P450 enzyme systems that are found throughout nature and are involved in many different, often complex, bioconversions [29]. We identified a high homolog to the *A. niger BPHA* gene in the *C. heterostrophus* genome. The antimicrobial nature of lipophilic weak acids such as benzoate has been widely used in the preservation of foodstuffs [52]. Benzoate induces an energy-consuming stress response in conjunction with the reduction in glucose fermentation, which leads to a drastic net decrease in cellular ATP levels, reducing the rate of growth of

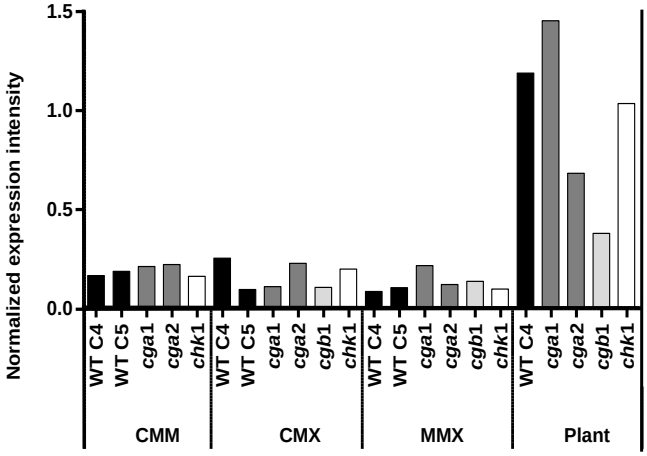


**Figure 5.** *C. heterostrophus BPH* expression profile. Microarray (Affymetrix Chip) normalized expression results indicate transcript levels of WT (C4 and C5) and the signal deficiency mutant strains in different media and in maize plants infected leaves. Abbreviations as in **Figure 3**.

*Saccharomyces cerevisiae* [53]. Mutation in the *BZUA*, an *A. nidulans* ortholog of *BPHA* from *A. niger*, led to sensitivity to benzoate, indicating that BpH plays an essential role in resistance to benzoate stress [32]. In this work, we found an overall induction of *BPH* gene expression in *C. heterostrophus* cultures that grew on the host plan tissue in comparison to cultures in modified liquid media (**Figure 5**). This interesting observation may raise the possibility that the host plant uses some defense mechanism that results in benzoate production. Indeed, infection of tobacco plants with tobacco mosaic virus or elicitation of cells led to a rapid *de novo* synthesis and accumulation of conjugated benzoic acid [54]. It was concluded that benzoic acid is likely to play a role as precursor of salicylic acid biosynthesis in tobacco plants undergoing a hypersensitive response following infection. Salicylic acid (SA, 2-hydroxybenzoic acid) has been known for some time to be a key endogenous component of local and systemic disease resistance in plants [55]. By using a series of signaling deficiency mutants, we identified possible roles for G-protein subunits in mediating *BPH* expression in response to the host plant (**Figure 5**). Microarray analysis indicates induction in *BPH* transcription levels in *cga*1, moderate reduction in *cga*2 and severe reduction in *cgb*1. This observation may hint to a common role for Gα and Gβ in maintaining accurate levels of this enzyme during pathogenesis. Such epistasis relations were observed in the determination of other phenotypic traits [5]. Interestingly, it is the first recognized role for *cga*2 in this organism, and the opposite function in comparison to *cga*1 makes this finding even more inquiring. It will be interesting to see if inoculating these strains on benzoate will support these findings.

## 5. Conclusion

The key players in signal transduction pathways, G-protein and MAPK, are mediators of environmental sensing responses that lead, through as yet unknown cascades, to an alteration of target gene expression. This work has shown that these genes, even though they share an environmental dependent response, participate in the regulation of two distinct biosynthesis pathways key players, CipA—a member of the IfR protein family and *BPH*—a member of cytochrome P450, enzyme systems. Furthermore, the signaling components act as inducers or repressors depending on the target gene and environmental conditions, indicating a complex role. It will be most interesting to understand how the same signal mechanism can fine-tune an enormous number of distinct and sometimes opposite responses and, at the same time, maintain a fine balance with the other signal cascades.

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