Molecular characterization of a cystathionine beta-synthase gene, *CBS1*, in *Magnaporthe grisea*

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Running title: Cystathionine β-synthase of *M. grisea*

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Abstract

CBS1 from *Magnaporthe grisea* is a structural and functional homolog of the cystathionine beta synthase (CBS) gene from *Saccharomyces cerevisiae*. Our studies indicated that *M. grisea* can utilize homocysteine and methionine through a CBS-independent pathway. Results also revealed responses of *M. grisea* to homocysteine that are reminiscent of human homocystinuria.

In the filamentous fungi *Aspergillus nidulans* and *Neurospora crassa*, inorganic sulfur is assimilated directly into either homocysteine (Fig.1, enzyme 5) or cysteine (Fig.1, enzyme 6) (16). The transsulfuration pathways allow the inter-conversion of homocysteine and cysteine with the intermediary formation of cystathionine (Fig. 1) (16). Cystathionine β-synthase (CBS) catalyzes the formation of cystathionine from homocysteine and serine (Fig.1, enzyme 1). Cysteine is synthesized from cystathionine in a reaction catalyzed by cystathionine γ-lyase (Fig. 1, enzyme 2). There is only one existing transsulfuration pathway in mammals, i.e. from homocysteine to cysteine (6). In *A. nidulans*, *N. crassa*, and the yeast *Saccharomyces cerevisiae*, an opposite transsulfuration pathway is present, allowing the conversion of cysteine to homocysteine (Fig. 1, enzymes 3 and 4) (5, 16). CBS has been conserved in eukaryotic evolution and it is directly involved in the removal of homocysteine from the methionine cycle. In human, CBS deficiency results in an elevated level of circulating homocysteine (homocystinuria) which is a risk factor for a number of neurological defects and vascular diseases (17). This disorder is commonly caused by recessive mutations in the human CBS gene (17).

We have initiated a genome-wide effort (7) to study gene functions in *Magnaporthe grisea*, a filamentous fungus that causes diseases in rice and other cereal crops (18). As part of this effort, a cosmid clone from a *M. grisea* (strain Guy11, 15) genomic library (7) was shotgun sequenced as described (8). BLASTX searches (1) of the sequence against the NCBI nr protein database (June 27, 2001) identified a putative gene, *CBS1*, encoding

a CBS-like protein. The coding sequence (GenBank Accession AF422799) is interrupted by an intron of 71 bp which was confirmed by comparison to a cDNA sequence.

The deduced gene product of *CBS1* shares extensive homology with CBS proteins from *S. cerevisiae* (46% identity) and human (45% identity) (Fig. 2). CBS is a pyridoxal phosphate (PLP)-dependent enzyme (10). In human CBS, Lys119 is the PLP binding residue (13) and this residue is conserved in *M. griesa* and yeast (Fig. 2). Similarly, the CBS domain, comprising the human CBS residues 416-419 (2), can be identified in the yeast and *M. grisea* proteins (Fig. 2) by Hidden Markov Model searches against the Pfam database (Nov. 16, 2001). CBS domains are also present in a wide range of unrelated proteins (2). The region containing the CBS domains in human CBS is involved in regulation by S-adenosyl-L-methionine (9). The Cys52 and His65 residues that axially coordinate the iron in the heme group of human CBS (12) are not conserved in *M. grisea* and yeast (Fig. 2). In fact, yeast CBS was recently found to be a non-heme protein (10, 11). It is therefore likely that the *M. grisea* enzyme does not contain a heme group either. In *S. cerevisiae*, biosynthesis of cysteine occurs exclusively through the CBS pathway and CBS null mutants are cysteine auxotrophs (5). We demonstrated that introduction of an expression plasmid containing the *M. grisea CBS1* rescued the growth defect of a CBS-deficient yeast strain in the absence of cysteine (data not shown). These findings indicate that *M. grisea CBS1* is a structural and functional homolog of the yeast CBS gene.

CBS1 is a single copy gene in *M. grisea*, as revealed by genomic Southern analysis (data not shown). We performed *in-silico* hybridization (TBLASTN searches, 1) of the CBS1 amino acid (aa) sequence against our internal *M. grisea* unigene database and the complete *N. crassa* genome database (version 2, Whitehead Institute/MIT Center for Genome Research, www-genome.wi.nit.edu/annotation/fungi/neurospora). There is no evidence of a second gene encoding CBS in either filamentous fungus. The closest matches from both databases were sequences encoding cysteine synthase (CYS)-like proteins. CBS and CYS are related proteins and they both required PLP as a co-factor. The aa sequence identity between *M. grisea* CBS1 and *A. nidulans* CYS (GenBank Accession P50867, the only annotated filamentous fungal CYS in NCBI nr as of Oct. 18, 2001) was 38%. However, CYS proteins are considerably shorter in length (~300 aa) compared to CBS proteins (>500 aa) (5). There are no indications that CYS proteins from different species exhibit CBS activities. Based on these findings, we conclude that *CBS1* is the only gene encoding CBS in *M. grisea*.

CBS1 was deleted from *M. grisea* by replacement with a modified hygromycin phosophotransferase gene (4) following procedures as described (21). The null (*cbs1*$^-$) mutants were found to retain virulence on rice (data not shown). In addition, the *cbs1*$^-$ mutants were not auxotrophic and were able to utilize inorganic sulfate, cysteine, cystathionine, homocysteine, or methionine as sole sulfur sources (data not shown). In the absence of inorganic sulfur sources, the pathway through CBS (Fig. 1) is the only known route for cysteine biosynthesis in filamentous fungi and other microbes (16; Kegg metabolic pathways, http://www.genome.ad.jp/kegg/metabolism.html, Jan 8, 2002).

Thus, homocysteine and methionine can be utilized via a CBS-independent pathway in the *M. grisea* mutants. Similarly, *Schizosaccharomyces pombe*, which lacks CBS naturally, is able to convert methionine to cysteine (3). Alternatively, homocysteine or methionine may be degraded through unknown pathways and the resulting sulfide ion assimilated by *M. grisea*.

Our growth studies revealed that homocysteine is toxic to *M. grisea*. Spore suspensions were inoculated in minimal medium (MM, 18) containing different concentrations of homocysteine as described (7). Fungal growth was monitored as increase in absorbance at 600nm. Complete inhibition of growth was observed when the wild-type (WT) strain was grown in homocysteine at a concentration of 0.5 mM or higher (Fig. 3). The *cbs1*$^{-}$ mutants were hypersensitive to exogenous homocysteine. For example, growth of the *cbs1*$^{-}$ mutants was completely inhibited at a concentration of 0.25 mM (Fig. 3). Inhibitory effects on growth were not evident with cysteine, cystathionine, or methionine at all the tested concentrations (data not shown). Toxicity of homocysteine on fungal growth has not been described elsewhere. However, humans with homocystinuria have been known to develop different clinical phenotypes caused by elevated levels of circulating homocysteine (17). This disorder is primarily a consequence of CBS deficiencies. It is possible that *M. grisea* and human CBS proteins share a common physiological function as a detoxification mechanism for homocysteine.

Vitamin or co-enzyme treatments of homocystinuria patients serve to enhance pathways that remove excess circulating homocysteine (19). Interestingly, we

demonstrated that addition of vitamin B_{12} relieved the toxicity of homocysteine in *M. grisea*. As shown in Fig. 4, supplementation of vitamin B_{12} at 50 μM allowed both the WT and *cbs1*$^{-}$ mutant strains to grow on MM containing 1 mM of homocysteine. The vitamin B_{12} response appeared to be concentration-dependent. Thus, the growth of WT and ectopic strains in homocysteine was moderately restored when 10 μM of vitamin B_{12} was supplemented (Fig. 4). Growth of the *cbs1*$^{-}$ mutants, which are more sensitive to homocysteine, remained inhibited at 10 μM of vitamin B_{12} (Fig. 4). In the human body, homocysteine can either be remethylated to methionine by methionine synthase, or undergo the transsulfuration reactions via CBS to form cysteine (6). The remethylation of homocysteine to methionine by methionine synthase is dependent on vitamin B_{12}, betaine, and folate while the CBS-catalyzed reaction requires vitamin B_6 as a co-enzyme (20). In *M. grisea*, methionine synthase (Fig. 1, enzyme 7) activities were likely to be enhanced with the supplementation of vitamin B_{12} to remove homocysteine.

In conclusion, our studies on *CBS1* in *M. grisea* indicate that homocysteine and methionine can be utilized by the fungus through pathways that are independent of CBS. In addition, our results revealed similarities between *M. grisea* and human in their sensitivities to homocystenie and responsiveness to vitamin B_{12} supplementation. The fungus may be exploited as a system to screen for therapeutic agents to relieve homocysteine toxicity.

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FIGURE LEGENDS

FIG. 1. Transsulfuration pathways in the filamentous fungi *A. nidulans* and *N. crassa*. OAH: *O*-acetyl-homoserine; OAS: *O*-acetyl-serine; SAM: *S*-adenosyl-methionine; SAH: *S*-adenosyl-homocysteine. Enzymes: 1, cystathionine β-synthase; 2, cystathionine-γ-lyase; 3, cystathionine-γ-synthase; 4, cystathionine β-lyase; 5, homocysteine synthase; 6, cysteine synthase; 7, methionine synthase.

FIG. 2. Amino acid alignment of *M. grisea* CBS1 (Mg) with *S. cerevisiae* CBS (Sc, GenBank Accession AAC37401) and human CBS (Hs, GenBank Accession A55760). Sequences are aligned using the ClustalW program in Lasergene software (DNASTAR, Madison, WI). Gaps in the alignments are indicated by dashes. Asterisks indicate identical residues in all sequences. Colons indicate conservative substitutions. Dots indicate semi-conserved substitutions. The Lys119 in human CBS is involved in PLP binding and is conserved in *M. grisea* and yeast (arrow). The Cys52 and His65 that axially coordinate the iron in the heme group in human CBS are double underlined. The CBS domains in the C-termini of the sequences are underlined.

FIG. 3. Inhibition of growth in *M. grisea* strains by homocysteine. The fungal strains were grown in minimal medium containing different concentrations of homocysteine (HCY) as sole sulfur sources. Mycelial growth was monitored by absorbance at 600 nm 7 days after inoculation. Growth inhibition is reflected by the lower absorbance value than that obtained in the sulfur-free (S-free) medium. Wild type (WT) strain and ectopic

transformant (162e) were inhibited at 0.5 mM or higher concentration of homocysteine. The *cbs*$^{-}$ mutants, 143 and 153, showed increased sensitivity to homocysteine.

FIG. 4. Effect of vitamin B_{12} on homocysteine sensitivity in *M. grisea* strains. Vitamin B_{12} was supplemented to MM containing homocysteine as the sole sulfur source. Complete inhibition of growth was observed in all strains growing in the presence of 1 mM homocysteine. Mycelial growth was restored in all strains when 50 μM of vitamin B_{12} was supplemented to the medium. At 10 μM of vitamin B_{12}, growth of the WT and the ectopic transformant (162e) was delayed and partially restored. The *cbs*- mutants, 143 and 153, which are hypersensitive to homocysteine, did not respond to vitamin B_{12} at this concentration. Note that vitamin B_{12}, when added to the S-free medium, did not support growth. Fungal strains growing in MM containing inorganic sulfate (SO_4) as the sole sulfur source were used as positive controls.