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Diacylglyceryltrimethylhomoserine content and gene expression changes triggered by phosphate deprivation in the mycelium of the basidiomycete *Flammulina velutipes*

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ABSTRACT

Diacylglyceryltrimethylhomoserines (DGTS) are betaine-type lipids that are phosphate-free analogs of phosphatidylcholines (PC). DGTS are abundant in some bacteria, algae, primitive vascular plants and fungi. In this study, we report inorganic phosphate (P_i) deficiency-induced DGTS synthesis in the basidial fungus *Flammulina velutipes* (Curt.: Fr.) Sing. We present results of an expression analysis of the *BTA1* gene that codes for betaine lipid synthase and two genes of PC biosynthesis (*CHO2* and *CPT1*) during phosphate starvation of *F. velutipes* culture. We demonstrate that Fv*BTA1* gene has increased transcript abundance under phosphate starvation. Despite depletion in PC, both *CHO2* and *CPT1* were determined to have increased expression. We also describe the deduced amino acid sequence and genomic structure of the *BTA1* gene in *F. velutipes*. Phylogenetic relationships between putative orthologs of BTA1 proteins of basidiomycete fungi are discussed.

1. Introduction

Betaine lipids are non-phosphorous glycerolipids that are structurally similar to the phospholipid PC. Both phospho- and betaine lipids have positively charged trimethylammonium group and similar phase transition temperatures (Sato and Murata, 1991). Diacylglyceryl-N,N,N-trimethylhomoserines (DGTS) are the most widespread class of betaine lipids. DGTS are abundant in several groups of bacteria (Benning et al., 1995; Geiger et al., 1999), green algae (Eichenberger, 1982; Sato and Furuya, 1985; Vaskovsky et al., 1996; Künzler and Eichenberger, 1997), primitive vascular plants such as mosses (Sato and Furuya, 1985; Künzler and Eichenberger, 1997), lycophytes and ferns (Künzler and Eichenberger, 1997; Rozentsvet et al., 2000; Rozentsvet, 2004), as well as lichens (Künzler and Eichenberger, 1997) and fungi (Künzler and Eichenberger, 1997; Dembitsky, 1996; Vaskovsky et al., 1998; Kotlova and Popov, 2005).

Biosynthesis of DGTS has been studied in detail only in bacterial, algal and yeast cells. Two enzymes named BtaA and BtaB are required for DGTS production in bacteria (Klug and Benning, 2001; Riekhof et al., 2005). BtaA transfers a four-carbon backbone from S-adenosylmethionine to the diglyceride moiety, forming the intermediate diacylglycerylhomoserine. BtaB catalyses a three-step N-methylation of the amino group on the intermediate to form the final DGTS product. The green algae *Chlamydomonas reinhardtii* has a single polypeptide, BTA1, containing BtaA- and BtaB-like domains that carry out all steps in DGTS biosynthesis

Abbreviations: AdoMet, S-adenosylmethionine; CDP, cytidine diphosphate; DAG, diacylglycerols; DGTS, diacylglyceryltrimethylhomoserines; DGTA, diacylglyceryl-hydroxymethyltrimethylalanines; DGCC, diacylglycerylcarboxyhydroxymethyl-cholines; DPG, diphosphatidylglycerols; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ITS, internal transcribed spacer; P_i, inorganic phosphate; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PI, phosphatidylinositols; P_iC, inorganic phosphate containing (medium); P_iF, inorganic phosphate-free (medium); PS, phosphatidylserines; RACE, Rapid Amplification of cDNA Ends; RT-qPCR, real time quantitative polymerase chain reaction.

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consecutively (Riekhof et al., 2005). Analyses of whole-genome sequences have revealed that Cr*BTA1* orthologs are abundant among eukaryotic organisms, and their distribution correlates with the distribution of DGTS. However, eukaryotic DGTS synthases, aside from BTA1 of *C. reinhardtii*, have been functionally studied only in the ascomycete yeast *Kluyveromyces lactis* (Riekhof et al., 2014). The amino acid sequence of KlBta1 was shown to be similar to CrBTA1 and contains conserved residues that have been implicated in the binding of AdoMet.

Distribution of DGTS in basidiomycete fungi has been demonstrated to be heterogeneous. In certain fungal taxons, such as *Agaricales, Polyporales* and *Russulales*, there are species that synthesize and species that do not synthesize DGTS that belong to the same order or even family (Dembitsky, 1996; Vaskovsky et al., 1998). The unstable presence of betaine lipids in some groups of Basidiomycetes suggests a regulatory mechanism for the synthesis of DGTS in fungi. Phosphate deficiency is considered to be one a condition that triggers the synthesis of DGTS. The ability to compensate for reduced phospholipid content by producing phosphorus-free betaine lipids during P_i starvation has been shown in the photosynthetic bacteria *Rhodobacter sphaeroides* (Benning et al., 1995), the symbiotic soil bacteria *Sinorhizobium meliloti* (Geiger et al., 1999; López-Lara et al., 2003; Zavaleta-Pastor et al., 2010), the mycelial ascomycete *Neurospora crassa* and in the yeast *K. lactis* (Riekhof et al., 2014).

It should be noted that several authors have suggested that there is a negative correlation between the presence and abundance of betaine lipids and PC (Eichenberger, 1982; Sato, 1992; Benning et al., 1995; Dembitsky, 1996; Vaskovsky et al., 1998). However, the mechanism behind the reciprocity between DGTS and PC remains unclear.

PC biosynthesis in mushrooms has been extensively studied. In ascomycete yeasts, as in most eukaryotes, two pathways for PC synthesis have been found. One method for PC synthesis is by the methylation of phosphatidylethanolamine (PE), where PE is converted to PC by a three-step S-adenosylmethionine (AdoMet)-dependent methylation reaction. The first methylation reaction is catalyzed by the *CHO2*-encoded PE methyltransferase (Kodaki and Yamashita, 1987; Summers et al., 1988) and the final two methylations are catalyzed by the *OPI3*-encoded phospholipid methyltransferase (Kodaki and Yamashita, 1987; McGraw and Henry, 1989). When choline is present in the growth media, PC may also be synthesized by the Kennedy pathway from CDP-choline that reacts with DAG in reactions catalyzed by the CPT1-encoded choline phosphotransferase (Hjelmstad and Bell, 1987, 1990).

Previous studies have suggested that the contribution of the methylation pathway for PC synthesis in *Saccharomyces cerevisiae* is more important but that the Kennedy pathway for PC synthesis assumes a critical role when the enzymes in the CDP-DAG pathway are defective or repressed (Carman and Henry, 1989; Greenberg and Lopes, 1996). However, it is not clear what the relative contributions of the CDP-DAG and Kennedy pathways in basidiomycetes are and whether their balance changes during adaptation to phosphate starvation.

Basidiomycete xylotrophic fungus *Flammulina velutipes* (Curt.: Fr.) Sing. is an edible and medicinal mushroom commercially cultivated all over the world. According to the early data, fruit bodies of *F. velutipes* do not contain DGTS (Vaskovsky et al., 1998). In a previous report, we demonstrated that surface cultures of *F. velutipes* do synthesize DGTS when they are deprived of a complex of nutrients, including phosphorus, nitrogen, potassium, and some trace elements (Senik et al., 2012). The present study provides evidence that phosphorus deficiency alone induces DGTS synthesis by this fungus.

This study focuses on mechanisms of reciprocity between DGTS and PC in fungi during P_i starvation. We report changes in expression of the BTA1 gene and two PC biosynthesis genes during phosphate starvation of *F. velutipes* culture. We describe the deduced amino acid sequence and genomic structure of the Fv*BTA1* gene coding for DGTS synthase in *F. velutipes*. We show that the Fv*BTA1* gene has increased transcript abundance under phosphate starvation. Despite PC depletion, expression of both PC biosynthesis genes was determined to increase. Phylogenetic relationships between putative orthologs of the BTA1 gene are also discussed.

2. Results

2.1. Strain verification

We verified our strain by a ribosomal DNA internal transcribed spacer (ITS)1, 5.8S and ITS2 (rDNA ITS) amplification using the basidiomycete-specific primers ITS1-F and ITS4-B (Gardes and Bruns, 1993). The partial nucleotide sequence of the 18S-ITS1-5.8S-ITS2-28S region (921 bp) was obtained (GenBank accession number KM668876) (Fig. S1). A BLAST search of this fragment against the whole GenBank database revealed 100% identity with *F. velutipes* (e.g., GenBank accession number EU191062.1 and FJ889514.1).

2.2. Changes in membrane lipid content in F. velutipes culture under phosphate starvation

To analyze the effect of phosphate deprivation on the lipid content of *F. velutipes*, we compared cultures that were grown on inorganic phosphate-free (P_iF) media with control cultures grown on media containing phosphate salts (P_iC media).

As shown in Fig. 1, PC and PE were the primary membrane glycerolipids of *F. velutipes* grown on P_iC media. Minor phospholipids included phosphatidic acids (PA), phosphatidylserines (PS), phosphatidylinositols (PI), and diphosphatidylglycerol (DPG). During growth of the culture on P_iC media, the level of PA increased from 3% to 12% of the total membrane glycerolipids.

The culture grown under phosphate-limiting conditions exhibited a lower biomass accumulation rate and a lesser density of the aerial mycelium compared with control colonies (data not shown). These changes were accompanied by induction of DGTS synthesis and its accumulation up to 42% of the total membrane lipids. DGTS accumulation was concomitant with PC depletion from 31% to 14%. Special attention is given to the fact that in culture starved for phosphorus PS was elevated 2-fold compared with one growing on complete medium. The culture grown under phosphate starvation showed a similar but less pronounced trend than that observed for the P_iC culture with PA increasing from 0.5% to 6% of the total membrane glycerolipids.

2.3. Amplification and analysis of BTA1 gene ortholog coding by genome of F. velutipes

The full-length sequence (2954 bp) of the Fv*BTA1* gene was found to contain an open reading frame of 2241 bp encoding a protein product of 747 amino acids (GenBank accession number KM668875) (Fig. S2). A comparison of the genomic and cDNA sequences of Fv*BTA1* indicated the presence of 14 introns varying in size from 47 to 56 bp with splicing junctions adhering to the GT-AG rule (Padgett et al., 1984). The deduced amino acid sequence of the Fv*BTA1* gene product revealed a 37% identity and 58% similarity with a previously characterized BTA1 protein from *K. lactis* (NCBI Accession # XM_456071.2), with the regions of highest conservation being observed in predicted methyltransferase motifs (Fig. 2). We found that regions corresponding to AdoMet binding sites predicted by means of a Conserved Domain