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NOTE

Asexual reproductive organ-specific expression of the *glyceraldehyde-3-phosphate dehydrogenase 2* gene of *Pilobolus crystallinus*

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Abstract *Pilobolus crystallinus* has three putative *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*) genes (*pcgapdh1*, *pcgapdh2* and *pcgapdh3*). The results of this study demonstrate that expression of *pcgapdh2* was increased by irradiation and that this increased expression was correlated with the formation of asexual reproductive organs (trophocysts). Interestingly, expression of *pcgapdh2* was restricted to trophocysts. The formation of trophocysts was likely promoted by light, and the expression of *pcgapdh2* was increased as a result of trophocyst formation. This is the first report that shows the regulation of a *gapdh* gene in an organ-specific manner in fungi.

Keywords GAPDH · Light · Mucorales · Trophocyst

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC1.2.1.12) is a key enzyme involved in glycolysis and gluconeogenesis. Although it is encoded by a single-copy gene in many fungi (Hirano et al. 1999; De Maeseneire et al. 2008; Van Bogaert et al. 2008), some yeasts, mushrooms, and filamentous fungi possess several slightly different *gapdh* genes. *Saccharomyces cerevisiae* expresses three *gapdh* genes (McAlister and Holland 1985), *Agaricus bisporus* Lange possesses two *gapdh* genes (*gpd1* and *gpd2*), although transcripts have only been detected for one of these genes (Harmsen et al. 1992), and *Mucor circinelloides* Schipper and *Pilobolus crystallinus* (Wiggers) Tode each have three *gapdh* genes (*gpd1*, *gpd2* and *gpd3*, and *pcgapdh1*, *pcgapdh2* and *pcgapdh3*, respectively) (Wolff and Arnau 2002; Kubo 2011). A phylogenetic tree constructed based on the amino acid sequences of several fungal GAPDH proteins demonstrated that PCGAPDH1, PCGAPDH2 and PCGAPDH3 are homologous to GPD3, GPD1 and GPD2, respectively, and that these three different GAPDHs arose before *Pilobolus* and *Mucor* diverged (Kubo 2011). Transcripts from *gpd1* have been detected during vegetative growth, but neither the *gpd2* nor *gpd3* transcripts have been detected in *M. circinelloides* (Wolff and Arnau 2002). In contrast, all three *pcgapdh* genes are expressed at a high level in *P. crystallinus*.

Although the expression of *gapdh* genes is constitutive and high in many yeasts and fungi, there are several reports of *gapdh* expression being mediated by carbon sources. In *Kluyveromyces marxianus* Van der Walt, *gap1* and *gap2* are expressed in the presence of glucose, but the expression of *gap2* is strongly reduced in medium in which ethanol is substituted as a carbon source (Fernandes et al. 1995). The promoter activity of the *Pichia pastoris* Phaff *gapdh* is affected by carbon sources (Waterham et al. 1997). *gpd1* expression is promoted by glucose in *M. circinelloides* (Wolff and Arnau 2002), whereas *pcgapdh2* expression is downregulated by sodium acetate and glucose in *P. crystallinus* (Kubo 2011). However, to date, no studies have been reported that demonstrate the regulation of *gapdh* genes by other environmental factors or the regulation of organ-specific expression in fungi. In the study reported here, the organ-specific expression of *pcgapdh2* is demonstrated.

P. crystallinus (strain NBRC 8561) obtained from the NITE Biological Resource Center, Kisarazu, Japan was used in this study. Sporangiospores were inoculated in liquid MYC medium [1% (w/v) malt extract, 0.2% (w/v) yeast extract, 0.2% (w/v) casamino acids]. Mycelia were grown at 22°C in the dark or under continuous white light

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(7.2 W/m^2) provided by fluorescent tubes (FL20SD; Toshiba, Tokyo, Japan).

RNA was isolated as previously described (Kubo 2009). Briefly, mycelia were ground with extraction buffer [4.2 M guanidine thiocyanate, 0.5% (w/v) *N*-lauroylsarcosine sodium salt, 25 mM sodium citrate], extracted with phenol:chloroform:isoamyl alcohol (25:24:1) four times, precipitated using 2 M LiCl, suspended in RNase-free water and used for reverse transcription (RT)-PCR. To isolate RNA from trophocysts, the trophocysts were first separated from mycelial hyphae using forceps under a stereomicroscope. Total RNA was extracted from the trophocysts and the remaining mycelial hyphae separately. Approximately 50 trophocysts were used for the preparation of total RNA.

First-strand cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase with an oligo-d(T)$_{15}$ primer and 0.5 μg of total RNA as the template. For the RNA obtained from trophocysts, the entire isolated RNA sample was used for the RT-PCR analysis because the amount of RNA was too low for its concentration to be measured. PCR was performed with gene-specific primer sets as previously described (Kubo 2011), except for the PCGPD2Fwd primer, which was replaced with 5′-TAG AATCCACTGGTGTGTTC-3′. Each sample was amplified for 24 PCR cycles for all of the primer sets. PCR products were loaded onto an agarose gel, electrophoresed and stained with ethidium bromide. The density of the obtained bands was quantified using a densitometer (Densitograph, Atto, Tokyo, Japan), and the expression level was shown relative to the density of the tubulin band. All steps were conducted quantitatively. The number of trophocysts was counted in photographic images of mycelia.

Light induces various photo-responses in *P. crystallinus* (Page 1956; Kubo and Mihara 1986; 1996). Therefore, the effect of light on the expression of *pcgapdh* genes was examined in *P. crystallinus*. Figure 1a shows the time course of the photo-induction of trophocyst formation; Fig. 1b and c show the time course of the expression of the three *pcgapdh* genes after the initiation of irradiation. Mycelia were grown for 2 weeks under dark conditions and then irradiated with continuous white light. Total RNA was extracted from whole mycelia between 0 and 72 h after the initiation of irradiation, and the expression of the three *pcgapdh* genes was examined by RT-PCR. *pcgapdh2* expression did not increase until 24 h after the beginning of irradiation and slightly increased at 36 h, reaching its maximum level at 48–72 h. The expression of *pcgapdh1* and *pcgapdh3* was not affected by irradiation.

The expression of *pcgapdh* genes was then examined at various growth stages. After mycelia were grown for 5–8 days under continuous white light, the number of trophocysts was counted and the expression of *pcgapdh* genes examined. As shown in Fig. 2a, although the mycelia

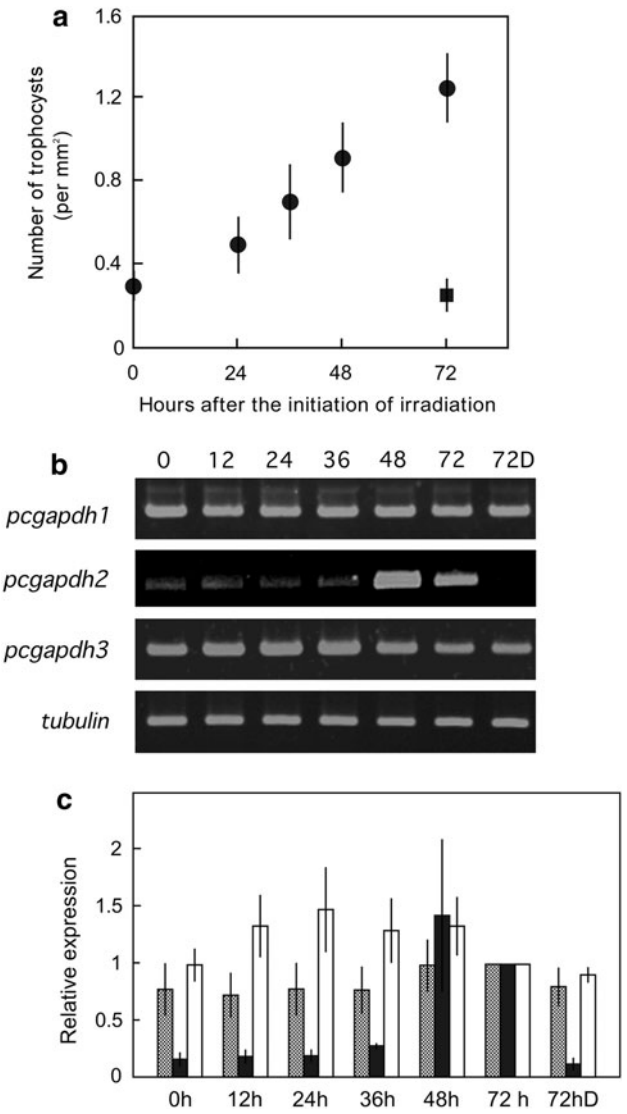
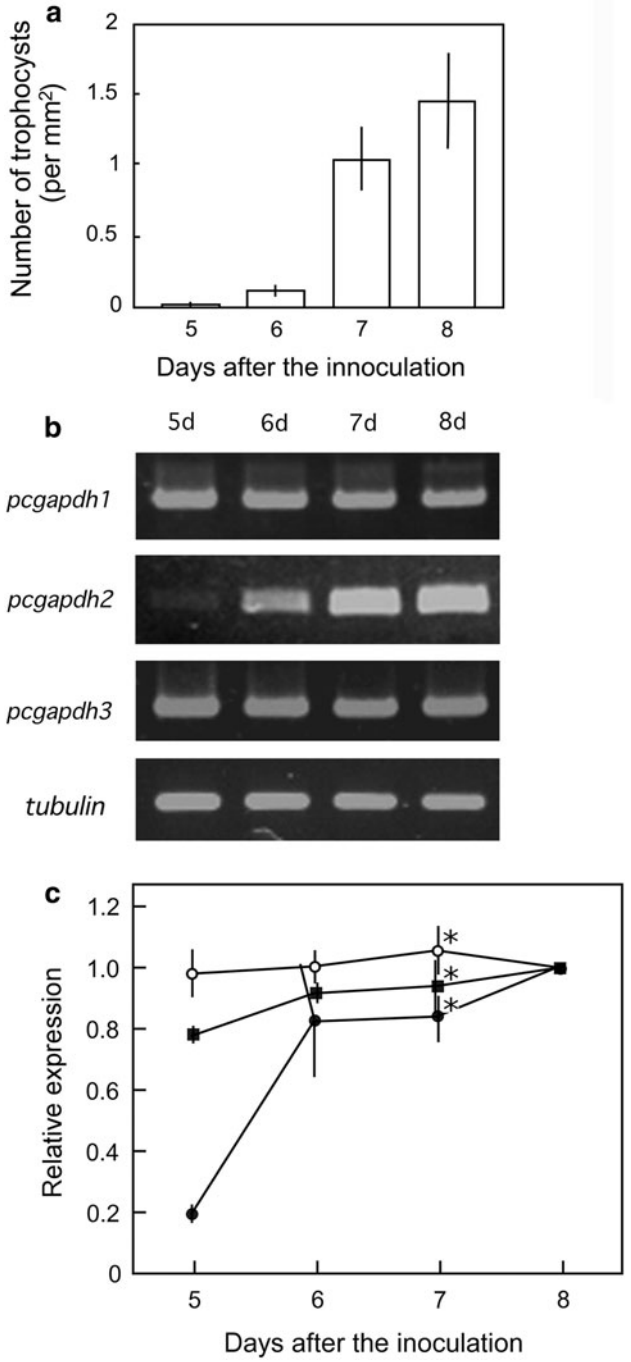


Fig. 1 Time course of the formation of trophocysts and the expression of three putative *Pilobolus crystallinus glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*) genes (*pcgapdh1*, *pcgapdh2* and *pcgapdh3*) after the initiation of irradiation. **a** Mycelia were grown for 2 weeks under dark conditions and then irradiated with continuous white light for 0–72 h (*filled circles*) or kept in the dark for 72 h (*filled square*). The number of trophocysts was counted in photographic images of mycelia. *Bars* standard error (SE). **b** Time course of the expression of the three *pcgapdh* genes after the initiation of irradiation. Mycelia were grown for 2 weeks under dark conditions and then irradiated with continuous white light for 0–72 h or kept in the dark for 72 h (*72D*). Total RNA was extracted from mycelia at specific time points between 0 and 72 h after the initiation of irradiation. The expression of the three *pcgapdh* genes was examined by semiquantitative reverse transcription (RT)-PCR, and a representative result is shown. *Tubulin* was used as a control. **c** The level of expression normalized to that of tubulin between 0 and 72 h after the initiation of irradiation relative to that at 72 h. *Shaded bar pcgapdh1*, *black bar pcgapdh2*, *white bar pcgapdh3*. Each value is the average and SE of three to four PCR amplifications



◄ **Fig. 2** Time course of the formation of trophocysts and the expression of *pcgapdh1*, *pcgapdh2* and *pcgapdh3* after inoculation of sporangiospores into liquid MYC medium. **a** Mycelia were grown for 5–8 days under continuous white light, and the number of trophocysts was counted in photographic images of mycelia. *Bars* SE. **b** Time course of the expression of the three *pcgapdh* genes was examined after inoculation. RNA was extracted from mycelia grown under continuous white light for 5–8 days after inoculation. The expression of the three *pcgapdh* genes was examined by semiquantitative RT-PCR, and a representative result is shown. *Tubulin* was used as a control. **c** The level of expression normalized to that of tubulin 5–7 days after inoculation relative to that on the 8th day. *Open circle pcgapdh1*, *filled circle pcgapdh2*, *closed square pcgapdh3*. Each value is the average and SE of four PCR amplifications, except for the data marked with *stars*, which show the average of two PCR amplifications and the maximum and the minimum values

began to grow, they formed few trophocysts until the 6th day post-inoculation, with the number of trophocysts increasing greatly on post-inoculation days 7–8. *pcgapdh1* and *pcgapdh3* showed full expression on days 5–6 post-inoculation, but *pcgapdh2* was not expressed, even under light conditions, until the 6th day after inoculation, reaching its maximum level on days 7–8 post-inoculation (Fig. 2b, c).

Figures 1 and 2 show that the time course of *pcgapdh2* expression correlated with the formation of trophocysts. Therefore, the expression of *pcgapdh2* was examined in trophocysts and residual mycelial hyphae separately. Mycelia were grown for 8 days under continuous white light. Trophocysts and mycelia were separated, and total RNA was extracted from each sample. Expression of *pcgapdh1* and *pcgapdh3* was observed in both trophocysts and residual mycelial hyphae, but *pcgapdh2* expression was observed only in trophocysts (Fig. 3a, b). This result showed that the expression of *pcgapdh2* was asexual reproductive organ-specific.

As shown in Fig. 1, *pcgapdh2* expression was induced by irradiation. However, the induction of *pcgapdh2* may not have been a direct effect of light on the activation of the *pcgapdh2* gene because *pcgapdh2* was not expressed in young mycelia, even when grown under light. Rather, the expression of *pcgapdh2* may have increased as a result of the photo-induced formation of the trophocysts.

To the best of my knowledge, this is the first published study to show that a member of the GAPDH family is expressed in an organ-specific manner in fungi. Although GAPDH is indispensable for all living cells, it is encoded by a single-copy gene in most filamentous fungi (Hirano et al. 1999; De Maeseneire et al. 2008; Van Bogaert et al. 2008). *M. circinelloides* possesses three *gapdh* genes (*gpd1*, *gpd2* and *gpd3*), but only *gpd1* is known to be expressed (Wolff and Arnau 2002). Nevertheless, it is possible that the other *gpd* genes are expressed because the expression of the three *gpd* genes has not been examined in various growth stages and organs (Wolff and Arnau 2002). It would be interesting to examine whether *M. circinelloides* possesses a *gapdh* gene specific to an asexual reproductive organ.

The amino acid sequence of PCGAPDH2 shows the highest homology to the GPD1 of *M. circinelloides*. However, because *pcgapdh2* was found to be expressed specifically in an asexual reproductive organ, in contrast to the vegetative organ expression of *gpd1* (Wolff and Arnau

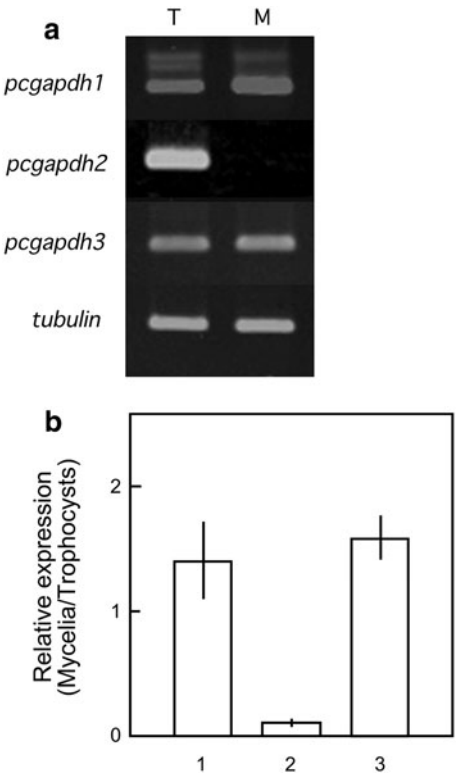


Fig. 3 Expression of *pcgapdh1*, *pcgapdh2* and *pcgapdh3* in trophocysts and mycelia. **a** Expression of the three *pcgapdh* genes was examined in trophocysts (*T*) and mycelia (*M*). Mycelia were grown under continuous white light for 8 days after inoculation of sporangiospores into liquid MYC medium. Trophocysts and mycelia were separated, and total RNA was extracted from each sample. The expression of the three *pcgapdh* genes was examined by semiquantitative RT-PCR, and a representative result is shown. *Tubulin* was used as a control. **b** The level of expression normalized to that of tubulin is shown in mycelia relative to trophocysts. Each value is the average and SE from four PCR amplifications

2002), the roles of the *pcgapdh2* and *gpd1* genes appear to be different.

Although the *gapdh* gene family has not been described in *Phycomyces blakesleeanus* Burgeff, differences in carbohydrate metabolism between reproductive organs and mycelia have been reported. Light promotes the accumulation of glycerate 2-phosphate and phosphoenol pyruvate in sporangiophores, but not in mycelia (Diez et al. 1988). Thus, there could be a different mechanism regulating *gapdh* expression between sporangiophores and mycelia in *P. blakesleeanus*.

The reason that the *pcgapdh2* gene is expressed specifically in asexual reproductive organs is not known. It would appear to be unlikely that *pcgapdh2* regulates the development of asexual reproductive organs via regulation of carbon metabolism because the other two *pcgapdh* genes are constitutively expressed in trophocysts. Rather, PCGAPDH2 may supply energy and materials by glycolysis and gluconeogenesis specifically in asexual reproductive organs. Because sporangiophores are large and their growth rate is rapid, large amounts of energy and materials are likely to be required for their development. Alternatively, PCGAPDH2 might have functions in addition that of glucose metabolism. GAPDH has been shown to be a multifunctional protein (Sirover 2005). In *S. cerevisiae*, three GAPDH (TDH1, TDH2 and TDH3) are distributed in the cytoplasm and the outermost layer of the cell wall (Delgado et al. 2001). In *Candida albicans* Berkhout, cell wall-associated GAPDH activity increases in response to starvation and temperature increases (Gil et al. 2001). In mammalian cells, GAPDH functions in endocytosis (Robbins et al. 1995), vesicular transport (Tisdale 2001), the control of gene expression (Zheng et al. 2003), apoptosis (Ishitani and Chuang 1996), and DNA repair (Krynetski et al. 2001).

In mammals, seven of the ten enzymes in the glycolytic pathway have spermatogenic cell-specific isozymes (Eddy et al. 1994). Phosphoglycerate kinase-2 (PGK2) and glyceraldehyde 3-phosphate dehydrogenase-s (GAPDS), which are highly conserved homologues of constitutively expressed genes, are expressed only in spermatogenic cells (Boer et al. 1987; Welch et al. 1992). In *P. crystallinus*, one of the *gapdh* genes is expressed specifically in asexual reproductive organs, but whether the other genes in the glycolytic pathway have a homologue that is expressed specifically in asexual organs remains to be determined.

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