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Note

L-Phenylalanine supplementation increases the production of phenylalanine ammonia-lyase and methyl cinnamate in the mycelia of *Tricholoma matsutake*

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ABSTRACT

We investigated changes in the expression of *Tmpal1* and *Tmpal2*, that encode phenylalanine ammonia-lyases (PALs), during the growth of *Tricholoma matsutake* NBRC 30605 mycelia, and the effect of exogenously added L-phenylalanine (L-Phe), a substrate of PALs, on *pal* expression and methyl cinnamate production in the mycelia. Expression patterns of *Tmpal1* and *Tmpal2* differed during mycelial growth. Transcript levels of *Tmpal2*, PAL activity, and methyl cinnamate content increased markedly in cultures supplemented with 4–6 mM L-Phe. These results indicate that L-Phe supplementation increases PAL activity that is mainly responsible for *Tmpal2* expression; consequently, methyl cinnamate is abundantly produced from L-Phe.

Phenylalanine ammonia-lyase (PAL) catalyzes the non-oxidative deamination of L-phenylalanine (L-Phe) to *trans*-cinnamic acid in plants, fungi, and a few prokaryotes (MacDonald & D'Cunha, 2007). Because this enzyme is the first key member of the phenylpropanoid pathway in plants, which is responsible for producing a large number of secondary metabolites such as flavonoids and anthocyanins (Dixon et al., 2002), plant PALs have been studied extensively. In microorganisms, several PALs from yeasts and filamentous fungi have been characterized. In addition, PALs from several yeast *Rhodotorula* species have been thoroughly characterized because their commercial applications include the production of L-Phe (MacDonald & D'Cunha, 2007). PAL is induced by the addition of L-Phe, a substrate of PAL, to the culture medium in only a few species (Hattori, Nishiyama, & Shimada, 1999; Kane & Fiske, 1985). This induction is regulated at the level of transcription in *Neurospora crassa* and *Rhodosporidium toruloides* (Gilbert, Stephenson, & Tully, 1983; Sikora & Marzluf, 1982).

Tricholoma matsutake, commonly known as the pine-mushroom, is a representative mushroom species that emits a characteristic and pleasant aroma. The major volatile aromatic compounds of *T. matsutake* fruiting bodies have been identified as 1-octen-3-ol and methyl cinnamate (Cho, Choi, & Kim, 2006). 1-Octen-3-ol is the most abundant in many mushrooms, while methyl cinnamate is unusually dominant in *T. matsutake*. Methyl cinnamate is typically abundant in the gills of the mature fruiting body (Cho et al., 2006), but this compound has been little studied in the mycelia.

Methyl cinnamate is also produced at high levels in various plant tissues (Kapteyn et al., 2007). These authors demonstrated the biosynthesis of methyl cinnamate through the phenylpropanoid pathway in basil (*Ocimum basilicum*), wherein cinnamate was formed from L-Phe by PAL and converted to methyl cinnamate by a cinnamate/*p*-coumarate carboxyl methyltransferase. In addition, a recent study using isotope tracer techniques revealed that *T. matsutake* synthesized methyl cinnamate from L-Phe via cinnamate, similarly to basil, and that cinnamate was likely formed through two pathways (Hattori et al., 2016). In the case of *T. matsutake*, two *Tmpal* genes encoding PALs have been characterized (Tasaki & Miyakawa, 2015; Yoon et al., 2013). The transcript level patterns of *Tmpal1* and *Tmpal2*, PAL activity, and methyl cinnamate content in the 4 parts of the fruiting body (pileus, gills containing the spores, stipe, and base) indicate that PAL coded by *Tmpal2* may be most responsible for generating methyl cinnamate of *T. matsutake* (Tasaki & Miyakawa, 2015). However, the biosynthetic pathways leading to the formation of methyl cinnamate, and

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their relationship with *pal* expression in *T. matsutake,* remain poorly understood.

In the current study, we examined the effect of exogenously added L-Phe on *pal* expression and methyl cinnamate production for further understanding of the biosynthetic mechanism of methyl cinnamate production in the mycelia of *T. matsutake*.

Changes in mycelial dry weight and transcript levels of *Tmpal1* and *Tmpal2* during cultivation were examined. The *T. matsutake* dikaryotic strain NBRC 30605 (Biological Resource Center, NITE, Tokyo, Japan) was used in this study. Mycelia were cultured for 30 d at 20 °C on Hamada's agar medium (Hamada, 1964). Three mycelial agar disks (7 mm in diam) were cut from the leading edge of colonies grown on the agar media and then inoculated into 20 mL of liquid medium (2% glucose, 0.15% yeast extract, and 0.15% Bacto Soytone; Terashita et al., 1991) in a 100 mL Erlenmeyer flask. The mycelia were then grown statically at 20 °C in darkness. For real-time quantitative polymerase chain reaction (PCR) analysis, mycelia were harvested by filtration using a stainless steel filter mesh (40 mesh), and washed with distilled water. The washed mycelia were immersed immediately in liquid nitrogen and stored at −80 °C until use. For dry weight measurements, mycelia were harvested by filtering through Whatman No. 3 filter paper (GE Healthcare, Chicago, Illinois, USA), washed with distilled water, and dried at 105 °C for 16 h. Mycelial dry weight and transcript levels of *Tmpal1* and *Tmpal2* were measured at intervals of 15 d of cultivation. At least 3 replicates were applied in this study for each measurement.

Mycelial dry weight reached a maximum at 75 d of cultivation (Fig. 1A). *Tmpal1* and *Tmpal2* transcript levels were examined using the real-time quantitative PCR with total RNAs isolated from frozen mycelia as described previously (Tasaki & Miyakawa, 2015). The *T. matsutake* NBRC 30605 *ras* gene (DDBJ accession no. LC216924) was used as an internal reference. Relative expression was determined as a ratio of the target and reference genes. The transcript levels of *Tmpal1* increased temporarily at 45 d, then decreased, and increased again (Fig. 1B). In contrast, *Tmpal2* transcript levels decreased until 45 d, remained constant, and then increased at 90 d of cultivation. Thus, the expression patterns of *Tmpal1* and *Tmpal2* differed during mycelial growth, but both transcript levels increased during 60–90 d of cultivation when the mycelial growth rate decreased (the stationary and death phases).

We also compared transcript levels of *Tmpal1* and *Tmpal2* in mycelia harvested at 15 and 45 d, respectively, using the threshold cycle (Ct) values obtained from the real-time PCR analyses. Average Ct values for *ras* expression subtracted from the expression of *Tmpal1* and *Tmpal2* were 3.74 at 15 d and 0.18 at 45 d, respectively, for 3 independent experiments. With an ideal amplification efficiency of 1, a difference in 1 cycle indicated a 2-fold difference in the starting template amount. Therefore, these results suggest that the starting template amount of *Tmpal2* was much higher than that of *Tmpal1* and, furthermore, that *Tmpal2* was expressed more abundantly than *Tmpal1* throughout cultivation of the mycelia. Therefore, PAL activity in the mycelia could be primarily responsible for *Tmpal2* expression.

We investigated the effect of exogenously added L-Phe on *pal* expression and methyl cinnamate production in the mycelia of *T. matsutake*. Transcript levels of *Tmpal1* and *Tmpal2*, soluble protein and methyl cinnamate contents, and PAL activity were measured in mycelia cultured for 45 d in the same medium described above, and then supplemented with 0.5–6 mM L-Phe and grown for a further 15 d. Mycelia cultured without L-Phe supplementation for 60 d in the medium were used as a control sample. Crude extracts were prepared from the mycelia harvested for enzymatic, protein, and methyl cinnamate assays as described previously (Tasaki & Miyakawa, 2015). PAL activity was assayed by measuring the rate of *trans*-cinnamic acid formation as increasing absorbance at 290 nm ($\varepsilon 290 = 10{,}000\ M^{-1}\ cm^{-1}$) (Zimmermann & Hahlbrock, 1975).

Mycelial dry weight decreased gradually with increasing L-Phe concentrations (Fig. 2A). The mycelial dry weight at 6 4mM added L-Phe decreased by 68% in the control sample. These results indicate that higher concentrations of L-Phe inhibit the mycelial growth of *T. matsutake*. The transcript levels of *Tmpal1* did not change markedly with L-Phe supplementation (Fig. 2B). In contrast, the transcript levels of *Tmpal2* increased greatly in cultures supplemented with 4–6 mM L-Phe (Fig. 2C). In addition, the expression patterns of *Tmpal1* and *Tmpal2* differed during mycelial growth (Fig. 1B) and in the presence of added L-Phe (Fig. 2B and C). These results suggest that the expression of *Tmpal1* and *Tmpal2* is controlled by different regulatory mechanisms and support a previous suggestion that they may have different biological functions in *T. matsutake* (Tasaki & Miyakawa, 2015). Moreover, the increase in transcript levels of *Tmpal1* and *Tmpal2* during the stationary and death phases suggest that the gene products are involved in adaptations to nutrient limitation because PAL was induced by limiting carbon or nitrogen in the cultures of *Rhodotorula glutinis* and *Phanerochaete chrysosporium* (Hattori et al., 1999; Kane & Fiske, 1985).

The soluble protein content on a dry weight basis tended to increase gradually in cultures supplemented with 1–4 mM L-Phe (Fig. 2D). PAL specific activity increased in cultures supplemented

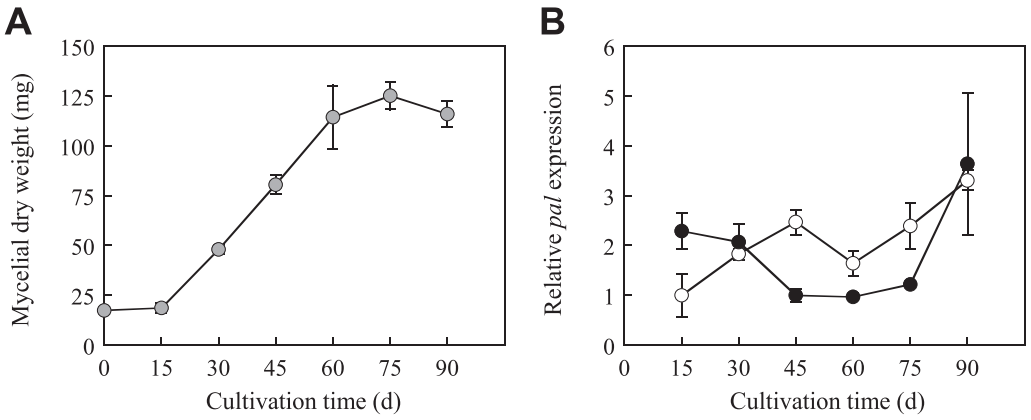

Fig. 1. Dry weight and *pal* expression during the cultivation of *Tricholoma matsutake* NBRC 30605 mycelia. Data are presented as means ± standard deviations. A: Mycelial dry weight. B: Relative expression of *Tmpal1* (white circles) and *Tmpal2* (black circles). The expression of *Tmpal1* and *Tmpal2* in the mycelia harvested at 15 and 45 d, respectively, was arbitrarily taken as one.