Biosci. Biotechnol. Biochem., **65** (11), 2561-2564, 2001

Note

Induction and Secretion of RNA-degrading Enzymes by Phosphate Deficiency in *Pholiota nameko*

Toshio JOH,[1] Yuji TASAKI,[2] Takashi HARA,[1] and Toshiro HAYAKAWA[1]

[1]*Department of Applied Biological Chemistry, Faculty of Agriculture, and* [2]*Department of Biosystem Science, Graduate School of Science and Technology, Niigata University, Ikarashi 2-8050, Niigata 950-2181, Japan*

Received May 18, 2001; Accepted July 28, 2001

Changes in activity of RNA-degrading enzyme and amounts of phosphorus in mycelia and culture filtrate during P_i-sufficient and -deficient cultures of *Pholiota nameko* were investigated. The results showed that the intracellular and extracellular activities are controlled by the P_i concentration in the medium. Moreover, the induction and secretion of RNA-degrading enzymes under the Pi-deficient condition were analyzed by activity staining.

Key words: *Pholiota nameko*; phosphate deficiency; RNA-degrading enzyme; activity staining

Phosphorus is one of the essential elements for all organisms. Because plants and microorganisms can incorporate phosphorus only as P_i, it is apt to be deficient for them. Many plants and microorganisms have been considered to have adaptive mechanisms for P_i deficiency, although to varying adaptive abilities. The mechanisms are called P_i-starvation-inducible rescue system,[1,2] pho regulon,[3-5] etc., in which multiple genes have been found to be involved. However, the functions of the gene products are still only partially understood.

Ribonucleases (RNases) are involved in various processes of RNA metabolism such as processing, turnover, and degradation of RNAs. In plants, it has been shown that induction of RNases is controlled by a variety of developmental and environmental stimuli including seed germination and maturation, water stress, wounding, pathogen infection, xylogenesis, and senescence.[6,7] Nurnberger *et al.*[8] also showed that RNases are induced and secreted by P_i deficiency in cultured tomato cells and proposed that the secreted RNase is a component of the P_i-starvation-inducible rescue system. On the other hand, in microorganisms, although the characterization and the structure of RNases have been studied vigorously, little is known about the regulation.

We previously reported that three acid phosphatase isozymes are induced and secreted during P_i-deficient conditions in mycelia of *Pholiota nameko* (T. Ito) S. Ito et Imai in Imai.[9-11] In this study, to discover the relationship between the regulation of RNases and P_i, we examined changes in RNA-degrading enzyme activity of mycelia and culture filtrate in P_i-sufficient (P^+) and P_i-deficient (P^-) cultures of *P. nameko* and indicated that the intracellular and extracellular activities are controlled by the P_i concentration in the medium. Moreover, the RNA-degrading enzymes were analyzed by activity staining.

Mycelia of the strain N114 of *P. nameko* (Tohoku Shiitake) were cultured in P^+ and P^- media containing phosphate at the concentrations of 550 and 50 mg/l as KH_2PO_4, respectively, as described previously.[10] Under these conditions, the rate of increase of mycelial dry weight was lower in the P^- culture than in the P^+ culture from day 10 of cultivation, and the dry weight in the P^- culture was 73% of that in the P^+ culture after cultivation for 25 d.[10] After cultivation for various periods, the mycelia were collected by centrifugation at $8,000 \times g$ for 10 min at 4°C and washed three times with 10 mM sodium acetate buffer (pH 5.5). The four supernatants were combined, dialyzed against the buffer, and then used as a crude enzyme from the culture filtrate. The washed mycelia were homogenized in the same buffer with a Polytron homogenizer (Model K; Kinematica) at about 13,000 rpm for 2 min at 4°C and centrifuged at $8,000 \times g$ for 10 min at 4°C. The supernatant was dialyzed and used as a crude enzyme from mycelia. RNA-degrading activity was assayed essentially as described by Abel and Glund.[12] The reaction was done at 37°C for 10 min in a total volume of 150 μl containing 50 mM succinic acid/NaOH (pH 5.0), 2.5 mM EDTA, 250 μg yeast RNA (Boehringer Mannheim), and an appropriate amount of enzyme. After incubation, 1.5 ml of precipitating reagent containing 50 mM acetic acid/NaOH (pH 5.5), 50% (v/v) ethanol, 10 mM magnesium acetate, and 0.8 mM lanthanum nitrate was added to the reaction mixture. After chilling at -20°C for 1 h and centrifugation at $11,000 \times g$ for 20 min, the absorbance at 260 nm of the supernatant was measured against a blank taken at zero time. One unit of the enzyme activity was de-

To whom correspondence should be addressed. Toshio JOH, Tel & Fax: +81-25-262-6640; E-mail: joh@agr.niigata-u.ac.jp

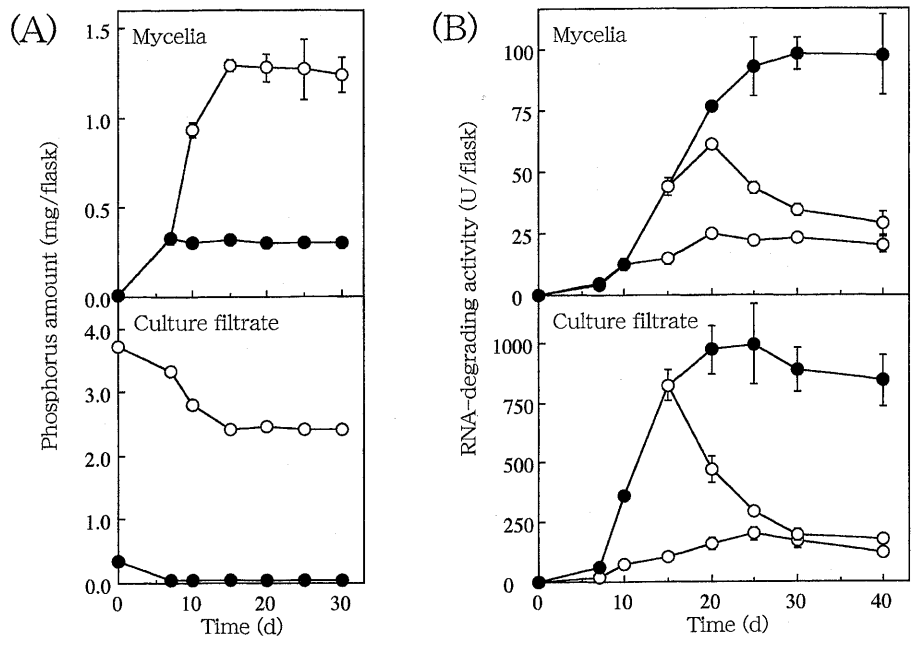

Fig. 1. Changes in Phosphorus Amount (A) and RNA-degrading Activity (B) in Mycelia and Culture Filtrate during Cultivation.
Symbols: ○, P^+ culture; ●, P^- culture. KH_2PO_4 solution (pH 6.5) was added to the P^- culture at a final concentration of 500 mg/l on day 15 in Fig. 1(B). Data are means and SE of three replicates.

fined as the amount of enzyme increasing 1.0 of absorbance at 260 nm per min under the assay conditions.[13] Phosphorus in the mycelia and the culture filtrate was measured using the mycelial homogenate and the culture filtrate containing the washings of mycelia, respectively, as samples. After the samples were heated with equal volumes of 60% perchloric acid for 1 h by a Microkjeldahl nitrogen degradation apparatus (Shibata), the P_i produced was measured by the method of Nakamura[14] and calculated as the amount of phosphorus.

Changes in the amount of phosphorus and RNA-degrading activity per flask in the mycelia and the culture filtrate during cultivation were investigated. The amount of phosphorus in the mycelia of P^+ culture increased quickly up to day 15 but then remained constant despite the rest of available P_i in the medium (Fig. 1(A)). On the other hand, in the P^- culture, phosphorus in the medium was exhausted during the first 7 d and phosphorus in the mycelia did not increase after that. RNA-degrading activity in the mycelia and the culture filtrate of P^+ culture increased only gradually (Fig. 1(B)). However, in P^- culture, the activity increased considerably in the mycelia and the culture filtrate from day 10 to 25 and from day 7 to 20, respectively. RNA-degrading activity in the mycelia and the culture filtrate of P^- culture was 4.3-fold and 5.0-fold higher than those of P^+ culture on day 25 of cultivation. When P_i was added to the culture fluid of a P^- culture on day 15, RNA-degrading activity in the mycelia and the culture filtrate decreased to the same level as the P^+ culture on day 30 to 40 of cultivation. These results suggest that in *P. nameko* the RNA-degrading enzyme is induced and secreted by deficiency of available P_i in the medium. A nutrient starvation-mediated growth inhibition may be another reason for the increase in RNA-degrading activity. However, since the secretion of RNA-degrading enzyme was stimulated even at an early time of cultivation (day 7 to 10) when mycelial growth was not depressed in spite of exhaustion of P_i in the medium,[10] it is considered to be a specific response to P_i deficiency. RNA-degrading activity in the culture filtrate instantly responded to the exhaustion or the addition of P_i, while the response of the activity in the mycelia to them was slow. From this result, it can be presumed that the regulatory mechanism of expression by P_i is different between the intracellular and extracellular RNA-degrading enzymes or that some regulatory mechanism other than the induction and secretion exists, e.g., a degradation of RNA-degrading enzymes by protease. RNA-degrading activity in the culture filtrate was about 10-fold higher than that in the mycelia in both of the P^+ and P^- cultures, that is, a large portion of the enzyme was released into the culture medium. It has been reported in cultured tomato cells that 60–80% of the overall RNA-degrading activity was found in the culture medium.[8,12] As compared with the cultured tomato cells, the ability to secrete the enzyme in *P. nameko* is very high. This probably is because *P. nameko* is a kind of wood-rotting fungus that obtains available nutrients by degrading components of wood.

After the mycelia were cultured for 25 d under P^+ and P^- conditions, RNA-degrading enzymes in the

mycelia and the culture filtrate were analyzed by the activity staining method of Abel and Glund[12] after native-polyacrylamide gel electrophoresis (PAGE). As shown in Fig. 2(A), in the mycelia, 5 active bands (IR1–5) could be detected and, among them, only one band (IR2) was specific to P^- culture. The zymogram was scanned and analyzed by NIH Image (National Institute of Health). The result showed that the band intensity of IR4 was almost equal between the P^+ and P^- cultures but those of IR1, IR3, and IR5 were higher in the P^- culture (Table 1). Since no difference in the mycelial protein amount per flask on day 25 of cultivation was found between the P^+ and P^- cultures,[10] these suggest that P_i deficiency induces the production of IR2 and stimulates the productions of IR1, IR3, and IR5 but has no influence on the production of IR4. In *Arabidopsis thaliana*, three RNase genes were isolated and the effect of P_i starvation on their expressions was investigated: *RNS1*, which is highly inducible by P_i starvation; *RNS2*, which is expressed at a fairly high level in the presence of P_i but further inducible by P_i starvation; and *RNS3*, which is relatively insensitive to P_i starvation.[15,16] Therefore, the expression of RNA-degrading enzymes, IR1–5, in *P. nameko* may be also controlled individually in the same manner as those in *Arabidopsis*. In the culture filtrate, 4 major active bands (ER1–4) could be detected (Fig. 2(B)). The band intensities of ER1 and ER2 were higher in the P^- culture than in the P^+ culture, while a significant difference in those of ER3 and ER4 was not found between the P^+ and P^- cultures (Table 1). The protein amount per flask in the culture filtrate of the P^- culture on day 25 of cultivation was two times that in the P^+ culture.[10] Therefore, when considered with total activity per flask, this result suggests that P_i deficiency stimulates the secretion of all of the four extracellular RNA-degrading enzymes. Both in the mycelia and the culture filtrate, the sum of each band intensity in the P^- culture was 1.6 times higher than that in P^+ culture. The value is too low for the increase in the RNA-degrading activity by P_i deficiency, even if the difference in the protein amount between the P^+ and P^- cultures is considered. This is probably attributable to the limitation in sensitivity of the activity staining. By the activity staining, multiple RNA-degrading enzymes were detected in the mycelia and the culture filtrate. When the mobility of each band was compared between the mycelia and the culture filtrate, IR1, IR3, and IR4 coincided with ER1, ER2, and ER3, respectively. Koch *et al.*[17] investigated the structures of the extracellular RNase LE and the intravacuolar RNase LV3 that are inducible by P_i starvation in cultured tomato cells. However, no structural difference was found between them in spite of the different cellular location, and the RNases are suggested to be encoded by one or two closely related genes, the products of which are sent to the vacuole and to the outside of the cell. Accordingly, the intracellular and extracellular enzymes having the same mobility in *P. nameko* may be an identical protein that is encoded by the same gene, though their detailed structures was not examined.

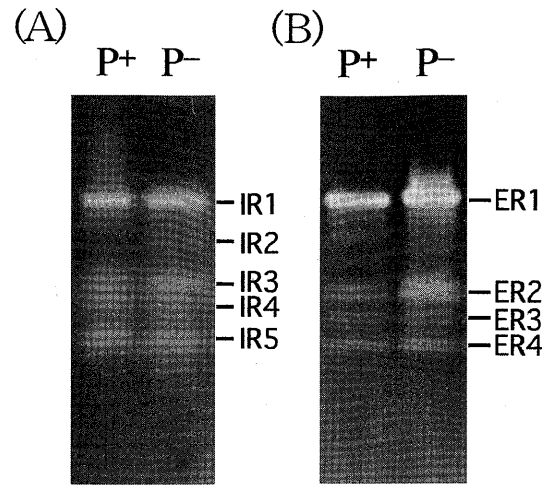

Fig. 2. Zymogram of RNA-degrading Enzymes in P^+ and P^- Cultivations.

Crude enzymes from mycelia (A) and culture filtrate (B) on day 25 of cultivation containing 3 μg of proteins were put through native-PAGE followed by staining for RNA-degrading activity.

Table 1. Measurement of RNA-degrading Enzymes in the P^+ and P^- Cultures

Band	Mycelia: Intensity of band		Band	Culture filtrate: Intensity of band	
	P^+ culture	P^- culture		P^+ culture	P^- culture
IR1	5667±495	8141±552	ER1	7723±50	11812±177
IR2	0±0	395±36	ER2	1530±213	4663±766
IR3	1555±172	3619±182	ER3	905±224	763±366
IR4	847±69	994±8	ER4	1529±54	1475±256
IR5	1662±129	2247±64			

The intensity of each active band in Fig. 2 was measured by densitometry. Data are means and SE of three replicates.

In this study, it was indicated that the intracellular and extracellular RNA-degrading activities are increased by P_i deficiency in *P. nameko* as shown in plants.[15,18] In *P. nameko*, intracellular and extracellular acid phosphatase activities also greatly increased under P_i-deficient conditions.[9,19] From these phenomena, we speculate that RNA-degrading enzymes and acid phosphatases are components of the adaptive mechanism for P_i deficiency in *P. nameko* and the combined actions of these enzymes produce available P_i from RNA in the intracellular and extracellular spaces under P_i-deficient conditions.

References

1) Goldstein, A. H., Baertlein, D. A., and McDaniel, R.

G., Phosphate starvation inducible metabolism in *Lycopersicon esculentum*. I. Excretion of acid phosphatase by tomato plants and suspension-cultured cells. *Plant Physiol.*, **87**, 711–715 (1988).
2) Goldstein, A. H., Danon, A., Baertlein, D. A., and McDaniel, R. G., Phosphate starvation inducible metabolism in *Lycopersicon esculentum* II. Characterization of the phosphate starvation inducible-excreted acid phosphatase. *Plant Physiol.*, **87**, 716–721 (1988).
3) Shimogawara, H., Makino, K., Amemura, M., and Nakata, A., Structure and function of the regulatory genes for the phosphate regulon in *E. coli*. In "Phosphate metabolism and cellular regulation in microorganisms", eds. Torriani, A., Rothman, F. G., Silver, S., Wright, A., and Yagil, E., American Society for Microbiology, Washington, DC, pp. 20–25 (1987).
4) Torriani, A. and Ludtke, D. N., The pho regulon of *Escherichia coli*. In "The molecular biology of bacterial growth", eds. Schaechter, M., Neidhardt, F. C., Ingraham, J., and Kjeldgaard, N. O., Jones and Bartlett, Inc., Boston, pp. 224–243 (1985).
5) Yoshida, K., Kuromitsu, Z., Ogawa, N., Ogawa, K., and. Oshima, Y., Phosphate metabolism and cellular regulation. In "Phosphate metabolism and cellular regulation in microorganisms", eds. Torriani, A., Rothman, F. G., Silver, S., Wright, A., and Yagil, E., American Society for Microbiology, Washington, DC, pp. 49–55 (1987).
6) Farkas, G. L., Ribonucleases and ribonucleic acid breakdown. In "Encyclopedia of plant physiology", eds. Parthier, B. and Boulter, D., Springer Verlag, Berlin, **14B**, pp. 224–262 (1982).
7) Green, P. J., The ribonucleases of higher plants. *Annu. Rev. Plant Physiol. Mol. Biol.*, **45**, 421–445 (1994).
8) Nurnberger, T., Abel, S., Jost, W., and Glund, K., Induction of an extracellular ribonuclease in cultured tomato cells upon phosphate starvation. *Plant Physiol.*, **92**, 970–976 (1990).
9) Joh, T., Malick, D. H., Yazaki, J., and Hayakawa, T., Purification and characterization of secreted acid phosphatase under phosphate-deficient condition in *Pholiota nameko*. *Mycoscience*, **37**, 65–70 (1996).
10) Joh, T., Tasaki, Y., Yazaki, J., and Hayakawa, T., Changes in soluble proteins in the mycelia and the culture filtrate of *Pholiota nameko* in a phosphate-deficient culture. *Nippon Kingakukai Kaiho* (in Japanese), **37**, 147–154 (1996).
11) Yazaki, J., Joh, T., Tomida, S., and Hayakawa, T., Acid phosphatase isozymes secreted under phosphate-deficient conditions in *Pholiota nameko*. *Mycoscience*, **38**, 347–350 (1997).
12) Abel, S., and Glund, K., Localization of RNA-degrading enzyme activity within vacuoles of cultured tomato cells. *Physiol. Plant.*, **66**, 79–86 (1986).
13) Wilson, C. M., Plant nucleases. *Ann. Rev. Plant Physiol.*, **26**, 187–208 (1975).
14) Nakamura, M., Colorimetric estimation of phosphorus. *Agric. Chem. Soc. Japan*, **24**, 1–5 (1950).
15) Bariola, P. A., Howard, C. J., Taylor, C. B., Verburg, M. T., Jaglan, V. D., and Green, P. J., The *Arabidopsis* ribonuclease gene *RNS1* is tightly controlled in response to phosphate limitation. *Plant J.*, **6**, 673–685 (1994).
16) Taylor, C. B., Bariola, P. A., DelCardayre, S. B., Raines, R. T., and Green, P. J., RNS2: A senescence-associated RNase of *Arabidopsis* that diverged from the S-RNases before speciation. *Proc. Natl. Acad. Sci. USA*, **90**, 5118–5122 (1993).
17) Kock, M., Loffer, A., Abel, S., and Glund, K., cDNA structure and regulatory properties of a family of starvation-induced ribonucleases from tomato. *Plant Mol. Biol.*, **27**, 477–485 (1995).
18) Glund, K. and Goldstein, A. H., Regulation, synthesis, and excretion of a phosphate starvation inducible RNase by plant cells. In "Control of plant gene expression", eds. Verma, D. P. S., CRC Press, Boca Raton, FL, pp. 311–323 (1993).
19) Malick, D. H., Joh, T., Tasaki, Y., and Hayakawa, T., Identification of *myo*-inositol monophosphates in mycelia of *Pholiota nameko*. *Mycoscience*, **37**, 381–383 (1996).