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Communication

A *th-1* Mutant of *Arabidopsis thaliana* Is Defective for a Thiamin-Phosphate-Synthesizing Enzyme: Thiamin Phosphate Pyrophosphorylase[1]

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ABSTRACT

We have examined the activity of the thiamin phosphate pyrophosphorylase in *Arabidopsis thaliana* wild type and in a mutant (*th-1*) which requires exogenous thiamin for growth. Mutant and wild-type plants grown in 1×10^{-7} molar thiamin were used for the examination of the production of thiamin and thiamin monophosphate (TMP) using 4-methyl-5-hydroxyethylthiazole phosphate and 2-methyl-4-amino-5-hydroxymethylpyrimidine pyrophosphate as substrates. While the wild-type strain formed both thiamin and TMP, the *th-1* mutant did not. When TMP was added to the extracts, the *th-1* mutant, as well as wild type, produced thiamin. Accordingly, it was concluded that the *th-1* mutant was defective in the activity of TMP pyrophosphorylase. Some of the characteristics of the enzyme from the wild-type plant were examined. The optimum temperature for the reaction is 45°C, and the K_m values for the substrates are 2.7×10^{-6} molar for 4-methyl-5-hydroxyethylthiazole phosphate and 1.8×10^{-6} molar for 2-methyl-4-amino-5-hydroxymethylpyrimidine pyrophosphate.

Arabidopsis thaliana has recently become the focus of much attention as an experimental material in plant molecular biology (2, 7). Although more than 300 mutants of this plant have been accumulated during 30 years of experimentation (5), the only known auxotrophic mutants are those which require thiamin[2] and its precursors. Much discussion from various standpoints has been presumed in a search for the reason for this puzzling phenomenon (10). Thiamin-requiring auxotrophic mutants may be useful as materials for plant molecular biology, for example (a) in the study of genetic control of metabolic pathway in plants, (b) as a marker in gene transfer experiments (11), (c) as the starting gene for "gene walking," and (d) as a model for the breeding of artificial plants which produce excess amounts of thiamin. In order to exploit these possibilities, we have initiated a study of a mutant of *A. thaliana* that requires thiamin.

The mutants of *A. thaliana* that require thiamin for growth have been mapped to three loci (5). The mutants are designated *py*, *tz*, and *th-1*, and they require OMP[3], TH, and thiamin, respectively, for their growth. Auxotrophic mutants of *Escherichia coli* requiring thiamin have been designated to *thiC*, *thiA*, and *thiB*, and require OMP, TH, and thiamin, respectively, in the same way. The final step of the biosynthetic pathway of thiamin backbone is catalyzed by thiamin monophosphate pyrophosphorylase (EC. 2.7.4.7) (1, 4). This enzyme catalyzes the formation of TMP from two direct precursors, OMP-PPi and TH-P. Thus, it seems likely that a *th-1* mutant of *A. thaliana* corresponds to an *E. coli thiB* mutant (1, 4). *E. coli thiB* mutants are known to be defective in the enzyme TMP pyrophosphorylase (1, 4). The enzymatic activity was, therefore, examined for the purpose of analyzing and utilizing the *th-1* mutant. This report is the first to examine the activity of TMP pyrophosphorylase in both the wild type and a *th-1* mutant of *A. thaliana*.

MATERIALS AND METHODS

Culture of Plants. Columbia wild type and a *th-1* mutant (6) (kindly supplied by Prof. G. P. Rédei, University of Missouri, Columbia) of *Arabidopsis thaliana* were grown in synthetic agar medium, which contained 3 mM KNO_3, 1.75 mM NaH_2PO_4, 2 mM $Ca(NO_3)_2$, 1.5 mM $MgSO_4$, 75 μM Na_2EDTA, 30 μM H_3BO_4, 8.6 μM $FeSO_4$, 1 μM $MnSO_4$, 0.1 μM $CuSO_4$, 0.1 μM $ZnSO_4$, 24 μM $(NH_4)_6Mo_7O_{24}$, 12.5 μM $CoCl_2$, 2% glucose, 0.8% agar, and 1×10^{-7} M thiamin.

Chemicals. TH-P and OMP-PPi were kindly donated by H. Nakayama. All other reagents were of analytical grade.

Preparation of Enzyme Extracts. Plants were frozen in liquid nitrogen and lyophilized. The dry material was powdered in a coffee mill and was further crushed in a mortar with a pestle. The extraction buffer (200 mM K-phosphate buffer, pH 7, 1 mM 2-mercaptoethanol) was added to the resulting powder at a rate of 5 mL per gram dry weight. The mixture was extracted by blending with a Polytron (Kinematika, Switzerland) at its top speed for 5 min at 0°C. The extract was centrifuged at 10,000*g* for 10 min at 2°C. The supernatant solution was used as the crude preparation of enzyme.

Enzyme Assay. The TMP pyrophosphorylase was assayed by measuring the amount of thiamin and TMP produced from two direct precursors, TH-P and OMP-PPi (4). The thiamin, TMP,

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[2] Although the word "thiamine" is generally used, "thiamin," which is recommended by IUPAC, is employed in this article.

[3] Abbreviations: OMP, 2-methyl-4-amino-5-hydroxymethylpyrimidine; TH, 4-methyl-5-hydroxyethylthiazole; TMP, thiamin monophosphate.

and thiamin PPi carried over in crude extracts were removed by passing through a Sephadex G25 (Pharmacia, Upsala, Sweden) column (0.5 × 7 cm). Standard reaction conditions for TMP pyrophosphorylase were: Tris-HCl (pH 7.4); 125 mM; $MgCl_2$, 3.75 mM; TH-P, 32 mM; OMP-PPi, 31 mM; and protein (in the enzyme extract), 0.28 mg, in a total volume of 80 μL. The reaction was carried out at 37°C for 1 h with gentle shaking unless otherwise described. TMP phosphatase was assayed by measuring the amount of thiamin produced from TMP. The conditions for the reaction were: Tris-HCl (pH 7.4), 125 mM; $MgCl_2$, 3.75 mM; TMP, 49 mM; and protein (in the enzyme extract), 0.28 mg, in a total volume of 80 μL. Reactions were terminated by addition of 100 μL of 20% (w/w) cold TCA, and the extracts were kept in ice for 5 min. The mixture was centrifuged at about 8000*g* for 2 min (0°C). The supernatant was extracted with ethyl ether three times. The aqueous phase was recovered, was neutralized by addition of 5 N NaOH to raise the pH to 6 or 6.5, and was freeze dried. The material was stored at −20°C until use. A 50 mM Na-acetate buffer (pH 5) (96 μL), 0.3 M BrCN (1.8 mL), and 20% NaOH (0.1 mL) were added to the dried material successively and the resulting thiochrome derivatives were measured by the method of Ishii *et al.* (3). Initial (at time 0) amounts of thiamin, TMP, and thiamin PPi were subtracted in each reaction as nonenzymic products.

HPLC. The thiochrome derivatives were measured with high performance liquid chromatograph LC-6A (Shimadzu, Tokyo, Japan) on a 4 × 250 mm Hibar prepacked column of LiChrosorb-NH_2 (5 μm) (Cica-Merck, Tokyo, Japan). The solvent used was acetonitrile and 90 mM potassium phosphate buffer (pH 8.4), 6:4 (v/v). The flow rate was 1.3 mL/min. Detection was carried out with a fluorescence HPLC monitor RF530 (Shimadzu, Tokyo, Japan) at λ_{ex} of 375 nm and λ_{em} of 430 nm. Recording and determination were done with Chromatopack C-RIA (Shimadzu, Tokyo, Japan).

Gel Filtration Chromatography. Sephadex G-100 (superfine) column (1.2 × 80 cm) was equilibrated with 20 mM potassium phosphate buffer (pH 7.0) containing 2 mM mercaptoethanol, 1 mM phenylmethylsulfonylfluoride, and 20% glycerol. The crude extract (9.9 mg protein) was applied on the column and eluted with the same buffer at the rate of 2.8 mL/h. One-milliliter fractions were collected.

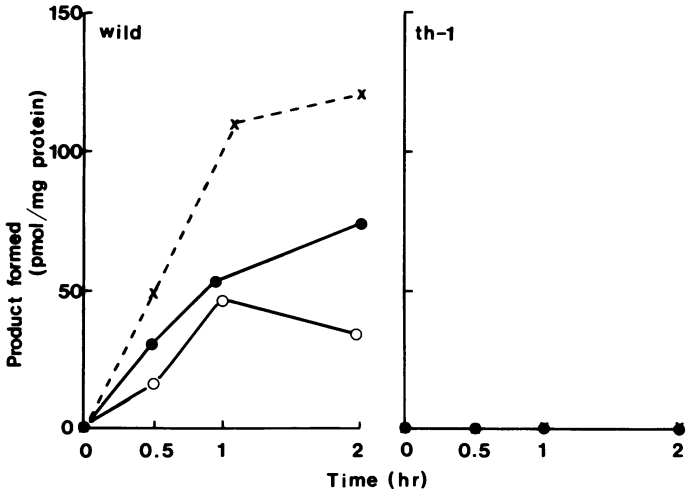

FIG. 1. Time course of thiamin and TMP formation by a *th-1* and wild type strains of *A. thaliana*. The reaction conditions and the examination of the products are described in "Materials and Methods." (●——●) Thiamin produced; (○——○) thiamin phosphate produced; (×-----×) sum of thiamin and thiamin phosphate produced.

Table I. *TMP Phosphatase Activity of th-1 and Wild-Type Strains of A. thaliana*

Reactions were carried out under conditions described in "Materials and Methods" at indicated TMP concentrations.

Strain	Exp.	TMP Added	TMP Phosphatase Activity
		pmol	*pmol thiamin/mg·h*
Wild	1	234	143
Wild	2	1170	339
th-1	1	234	112
th-1	2	1170	286

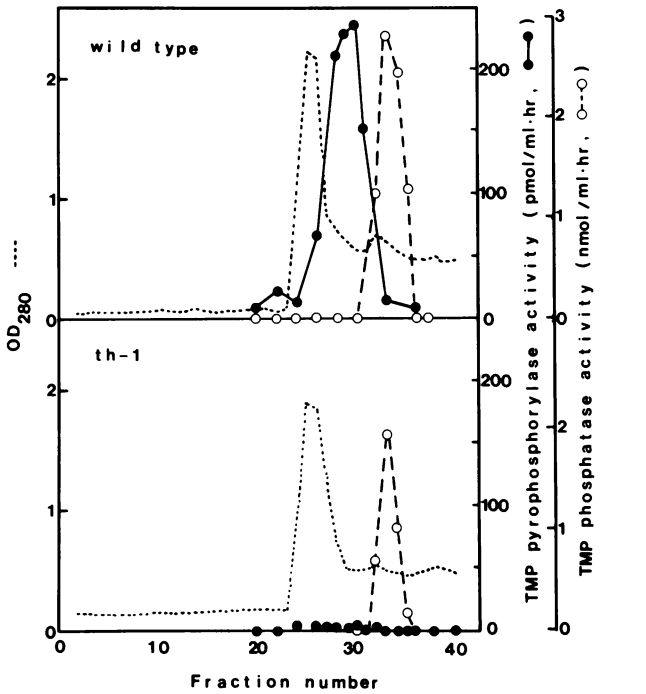

FIG. 2. Gel filtration profiles of extracts from wild type and a *th-1* mutant of *A. thaliana*. Alternate fractions were examined for the activities of thiamin phosphate pyrophosphorylase (●) and of thiamin phosphate monophosphatase (○) as described in "Materials and Methods." Each activity is presented by the amount of the products.

RESULTS AND DISCUSSION

Production of Thiamin and TMP. First, we measured the time-dependent production of thiamin and TMP in crude extracts of wild type and a *th-1* mutant from two substrates OMP-PPi and TH-P (Fig. 1). The amount of TMP increased with the duration of the incubation for 60 min in the extract from Columbia wild type. The quantity of thiamin also increased (Fig. 1). The production of thiamin PPi could not be detected (results not presented) in the reaction mixtures, which contained no added pyrophosphate donor needed for the pyrophosphorylation of free thiamin and no external phosphate donor needed for the pyrophosphorylation of TMP. These results demonstrated that the crude extracts from wild plants contained the two enzymes that catalyzed the condensation of TH-P and OMP-PPi to TMP and the dephosphorylation of produced TMP to thiamin. Then, the activity of the former enzyme, TMP pyrophosphorylase, is measured by amounts of TMP and thiamin produced in our reaction system. On the other hand, we could not detect significant amounts of thiamin and TMP in reactions with extracts from the *th-1* mutant. Thus, the *th-1* mutant is defective for both activities or for the activity responsible for TMP production.

Different temperatures (25°C and 37°C) were employed for

Table II. *Comparison of the properties of TMP pyrophosphorylase in A. thaliana and E. coli*

	A. thaliana	*E. coli*
Molecular weight	100,000	17,000
Optimum temperature	45° C	40° C
K_m value for TH-P	2.7×10^{-6} M	4.0×10^{-7} M
K_m value for OMP-PPi	1.8×10^{-6} M	8.5×10^{-7} M

the assay of TMP pyrophosphorylase activity in extracts of mutant and wild type. However, the activity did not demonstrate any temperature-sensitive phenotype in either extract.

TMP Phosphatase Activity. TMP phosphatase activities of the *th-1* and wild-type strains were investigated. As shown in Table I, TMP phosphatase activities were found to be the same level in both *th-1* and wild-type strain. Thus, this result suggests that the enzymic lesion in the *th-1* mutant occurs prior to the TMP phosphatase reaction and is consistent with the evidence presented above that the *th-1* mutant has a defect in TMP production.

Gel Filtration Chromatography. In order to confirm the above results, these enzymic activities were examined after separation of proteins by gel filtration chromatography. Figure 2 shows the pattern of gel-filtration chromatography (Sephadex G-100 superfine) of each enzymic activity. The pattern clearly shows that two enzymic activities, TMP pyrophosphorylase and TMP phosphatase, were separated from each other by gel filtration. The M_r of the major peak of TMP pyrophosphorylase is estimated to be about 100 kD, while that of TMP phosphatase is about 10 kD (calculation not shown). The *th-1* mutant was deficient in the peak that corresponded to the activity required for the production of TMP. The TMP phosphatase activities were found in both *th-1* mutant and Columbia wild type.

Accordingly, the results of the present study show the production of TMP and thiamin from the substrates of TMP pyrophosphorylase. The results conformed to the suggested pathway of the production of thiamin PPi in many organisms (8). Figure 3 depicts schematically the biosynthetic pathway suggested by this study.

Properties of the Enzyme. Some properties of TMP pyrophosphorylase were examined with crude extracts from wild type plants. From the results described above, the activity of TMP pyrophosphorylase in the crude extract was represented as the total amount of thiamin and TMP produced. The optimum temperature of the reaction was 45°C. The K_m values for the substrates were calculated (Table II). Earlier reports by Mitsuda *et al.* (8, 9) presented evidence for the existence of the enzyme in some higher plants, such as cabbage, Japanese radish, rape, spinach, and turnip. Summarizing the results in this study, Table II describes the comparison of the TMP pyrophosphorylase of *A. thaliana* to the *E. coli* enzyme. The value of the M_r represents preliminary data, since Sephadex G-100 superfine was used for the filtration medium.

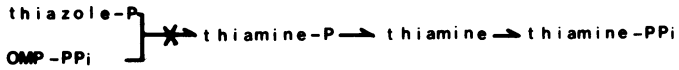

FIG. 3. Schematic pathway of the production of thiamin pyrophosphate from the two precursors. The site of the enzymic defect in the *th-1* mutant is denoted with an ×.

Results presented here indicate that a *th-1* mutant of *A. thaliana* is defective in the activity of TMP pyrophosphorylase. Thus, it is inferred that the gene product of the wild-type counterpart of the *th-1* allele is responsible for the activity of the enzyme. We propose to redesignate the wild-type allele as *THI1* and the mutant *th-1* as *thi1-1* for clarification. The product of the *THI1* gene should have one of the following functions: (a) the *THI1* gene may be the structural gene for the enzyme itself; (b) the *THI1* gene product may be responsible for the existence of mRNA specific for the enzyme, but may itself have a regulatory function; (c) the *THI1* gene product may be necessary for the existence of the active enzyme, but its function may involve the maturation of the enzyme. The results of the present study do not allow us to choose among these possibilities. The analysis of *th-1*^{ts} mutants will give us insight into these problems, but those materials are currently unavailable.

Since the *THI1* gene is now known to be responsible for the activity of thiamin phosphate pyrophosphorylase, the corresponding *E. coli thiB*$^+$ gene might be used for the complementation of the *Arabidopsis th-1* defect. We are currently trying this experiment (11).

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