Evaluation of phosphorus starvation inducible bypasses to increase the effieciency of phosphorus utilization in rice

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Abstract

Plants develop strategies to recycle phosphorus so that all organs receive adequate amount of phosphorus, especially new growing organs. To evaluate the metabolic adaptation of rice plant under phosphorus deficient condition, we selected several genes relating phosphorus utilizing efficiency in the cell. Phospho*enol*pyruvate carboxylase, triose phosphate translocator, phospho*enol*pyruvate/inorganic phosphate translocator (PPT), pyruvate kinase, NAD dependent glyceraldehydes-3-phosphate dehydrogenase, NADP dependent glyceraldehydes-3-phosphate dehydrogenase, were selected because of their important role in the phosphorus utilization in the cell and

consisting proposed bypass pathway to save phosphate. Most dramatic change was observed in the expression level of PPT (which tranport phospho*enol*pyruvate (PEP) in the cytosol to chloroplast), thus we consider that PEP may play an important role in maintaining carbon metabolism under phosphate deficient condition.

Introduction

The shortage of phosphorus fertilizer has been focused, indicating to become hard to obtain in the near future (Steen et al., 1998). Thus increasing recycling ability of phosphorus inside a plant may offer one of the solution to give phosphorus resource to a longer life. One of the mechanism to increase phosphorus recycling ability is dependent on the activity of phosphatase, such as acid phosphatase (Duff et al. 1991, 1994) and ribonuclease (Howard et al. 1998) in the cell. The increase of both enzymes is reported and these enzymes are considered to utilize those phosphorus compounds stored in vacuole. The other mechanism is bypassing several metabolic pathway to reduce the usage of phosphorus molecule. Duff et al. (1989, 1991) demonstrated several candidates by using culture cell, among them we are especially interested in the role of bypassing the flow of glycolysis to TCA cycle. Sucrose phosphate synthase (SPS) is key enzyme to synthesize sucrose and it requires releasing phosphorus molecule from sugar phosphate. Both NAD-glyceraldehyde-3-phosphate dehydrogenase (NAD-G3PDH) and NADP-glyceraldehyde-3-phosphate dehydrogenase (NADP-G3PDH) catabolize glucose 3 phosphate in the flow of glycolysis, the former requires NAD and

phosphate and the latter uses NADP instead. By using *Brassica napus* cultre cell, Duff et al. (1989) reported very high increase of the activity of NADP-G3PDH and they conclude the role of NADP-G3PDH is bypassing glycolysis to reduce the usage of phosphate. Under phosphorus deficiency, the activity of NAD-G3PDH decreased in *Achnanthes brevipes* (Guerrini et al. 2000) and *Catharanthus roseus* (Li and Ashihara 1990). Both phospho*enol*pyruvate carboxylase (PEPC) and pyruvate kinase (PK) utilize PEP, the former release phosphate and the latter requires phosphate for the reaction, thus Theodorou et al. (1991) proposed that PEPC might replenish carbon molecule to TCA cycle under phosphorus deficiency.

From our previous study using microarray on phosphorus deficient rice plant, we found that several genes relating carbon and phosphorus metabolism in chloroplast changed their expression level (Wasaki et al. 2003). One of them was phospho*enol*pyruvate/phosphate translocator (PPT), PPT transports PEP into the chloroplast and antiport phosphate (Pi) to cytosol (Hausler et al. 2000), thus the role of PPT under phosphorus deficient condition is suspected to supply substrate for the shikimate pathway. Another Pi transporter; triose phosphate translocater (TPT) is also located on chloroplast membrane, while TPT loads triose phosphate into chloroplast and antiports Pi into cytosol. These two Pi translocators are considered to regulate the Pi level in the chloroplast.

As comprehensive analysis of each pathway using intact plant has not reported, we evaluated by using real-time PCR to determine the expression level of each gene under phosphorus deficient condition. While the expression level of mRNA is not simply representing the activity of those enzymes

corresponding to the gene, obtained information will be useful to consider plant response to phosphorus deficiency.

Materials and Methods

Plant cultivation

Rice (*Oryza sative* L. ssp. japonica cv. Michikogane) were germinated for 3 days in tap water then precultured hydroponically with 6 µM phosphorus containing complete nutrient solution. Phosphorus treatment started after 14 days of precultivation by transferring plants to the nutrient solution with (32 µM Phosphorus) or without phosphorus. Sampling was performed at 0, 14, 15, 19, and 21 days after with and without phosphorus treatments started.

Sample preparation

Samples were harvested at 0, 14, 15, 19, 21 days after transplanting, then leaves were cut from the leaf base. For RNA extraction, about 500mg of leaf blades were frozen in liquid nitrogen immediately after sampling, then stored at –80℃ until analysis. For the determination of total phosphorus content in the leaf, leaves were oven dried at 80 ˚C for 72 hrs.

RNA extraction and preparation for real-time PCR

Total RNA was extracted by SDS-phenol method (Palmiter 1974). The level of mRNA of each gene was determined by real-time PCR with manufacturer's instruction (Roche) with specific primers (Table 1). The expression level was designated as relative level to sampling at 0 day after with or without

phosphorus treatments. To make sure that no genomic DNA was carried over, 20µg of total RNA was treated by DNase (Nippon Gene).Then residual protein was removed by phenol/chloroform extraction. After protein removal, 0.5µg of total RNA was reverse transcribed using 1st strand cDNA synthesis kit for RT-PCR(AMV) (Roche Diagostics). Synthesized first strand cDNA was diluted 1:10 by RNase free water for real-time PCR analysis.

Primer design

The examined genes were as follows: sucrose phosphate synthase (SPS), NAD-glyceraldehyde-3-phosphate dehydrogenase (NAD-G3PDH), NADP-glyceraldehyde-3-phosphate dehydrogenase (NADP-G3PDH), phospho*enol*pyruvate carboxylase (PEPC), cytosolic pyruvate kinase (PKc), triose phosphate translocator (TPT), and phospho*enol*pyruvate/phosphate translocator (PPT).The primer sets were designed using 3'-sides of each gene to avoid the amplification of the isozyme. For the genes whose 3'-side sequence were not known, we run 3'-race PCR using 3'-fll RACE core set according to the manufacturer's instruction (TaKaRa). Also primer sets are chosen to become the PCR product size was about 200bp. The primer sequence and reference data base are shown in Table 1.

Real-time PCR

The real-time PCR was performed by the LightCycler™ system (Roche Diagnostics) with the LightCycler DNA Master SYBR Green I kit for PCR (Roche Diagnostics), and the TaqStart™ antibody (Clontech) were used for the

repression of unspecific amplification. Each reaction includes prepared first stranded cDNA as described above. The reaction program was: 1 min of preincubation at 95 ˚C followed by 45 cycles for 0 s at 95 ˚C, 5 s at 63 to 68 ˚C (depending on the gene), 7 or 8 s (depending on the gene) at 72 ˚C. Fluorescence was monitored (excitation at 470 nm and emission at 530 nm) at the end of annealing phase. The specificity of amplification was checked by melting curve analysis. Calibration curve was drawn with two replications based on the specific plasmid for each cDNA. The results obtained are standarized by the amount of total RNA used for cDNA synthesis. All experiments are replicated three times, and statistica analysis was performed by SPSS (SPSS 6.0 Macintosh version).

Analysis of total phosphorus

Total phosphate was determined by molybdate yellow method (Watanabe et al., 1998) after digestion with H_2SO_4 (Mizuno and Murakami 1980).

Results and Discussion

Plant phosphorus level

Phosphorus level was gradually decreased when grown without phosphorus (Fig .1). On the other hand, when phosphorus was supplied sufficiently the phosphorus content increased dramatically after 2 weeks.

Expression of mRNA

Using real time PCR methods, we compare the relative expression patterns of genes involved in carbon patitioning in phosphorous deficient and sufficient rice leaves. The results are standardized with the amount of total RNA used for cDNA preparation and the expression patterns were determined through out 3weeks phosphorous treatment. To evaluate increase and decrease results evenly, we used semilogarithmic graph in the figure.

1. Gene encoding key enzyme of sucrose synthesis

SPS exsists in cytosol of mesophyll cell and the combined reaction of SPS and sucrose phosphate phosphatase is the main route for sucrose synthesis. During sucrose synthesis, one molecule of Pi is leberated from sucrose phosphate. Thus the synthesis of sucrose will liberate Pi from intermediate compounds, it is expected that the level of mRNA expression of SPS increased with -P treatment. The relative expression of SPS in rice leaves was slightly increased with –P treatment (Fig. 2). The –P treated plants first uses Pi stored in vacuole but after they used up all Pi in vacuole, cytosolic Pi content became lower. Then the plants with lower Pi concentration may facilitate sucrose synthesis and excrete Pi from sugar phosphate to keep up the Pi concentration in cytosol of mesophyll cell. Sucrose content in phosphate starved plant varys with species. In bean and sugar beet, leaf sucrose content increased by phosphate deficiency (Rao et al., 1990; Rao and Terry 1995; Ciereszko et al., 2000), although in leaves of soybean it decreased (Qui and Islael 1994). Our result may suggest rice will

facilitate sucrose synthesis in phosphate deficiency to some extent.

2. Genes encoding candidates for glycolytic bypass enzymes

NADP G3PDH instead of NAD G3PDH, and PEPC instead of PK are expected to play alternative pathways to regulate carbon flow of glycolysis under phosphorus deficient condition. While we could not find any relative incresae of so-called bypassing enzyme's coding gene mRNA expressioon. It seems rather increasing original metabolic pathways is more important to alleviate phosphorus deficient condition. Which will lead higher phosphorus recycling under phosphorus deficient condition.

In rice leaves under phosphorous deprivation, we did not see any increase in relative expression of NADP-G3PDH, which is known as phsphate stavation inducible bypass enzyme for NAD-G3PDH in *B.nigra* (Duff et al., 1989). On the contrary, NAD-G3PDH relative expression was significantly high in –P plants after 21days of treatment (Fig. 2). In the level of gene expression argument, this result may suggest that NADP-G3PDH is not working as glycolitic bypass at least in rice plant. The lack of induction of nonphopholylating pathway were also seen in other plant species, such as *S. minutum* (Theodorou et al., 1991) and *A. brevipes* (Guerrini et al., 2000).

Also PEPC and PK has the relation of gycolytic bypass enzyme induced under phosphate deficiency (Li and Ashihara 1990). Even though PEPC were thought to be catalyse the alternative pathway of PK under phosphate deficiency (Li and Ashihara 1990), both of the enzyme relative expression were rised by –P treatment.

3. Genes encoding chloroplast membrane transporters

Precise value of Pi concentration in cytosol and chloroplast is not known while it has been reported that it was between 10 to 15 mM in cytosol (Mimura et al. 1999) and 20 to 35 mM in chloroplast (Diez et al. 1984). This indicates that higher requirement for maintaining Pi level in chloroplast rather than in cytosol, and low phosphorus condition is expected to increase the relative mRNA expression level of TPT against PPT. While as shown in Fig. 3, the expression level of TPT was not changed by phosphorus deficient condition. On the other hand, the expression level of PPT increased dramatically. As one molecule of phosphorus is transported into chloroplast as a constitute of PEP with exportng one Pi, thus when incorporated PEP is decomposed there is no net change in the meaning of phosphorus nutrition. Thus we assumed that the role of PPT is increasing the PEP metabolism and makes a cycle from primary photosynthate synthesized in chloroplast and metabolized in cytosol with glycosis then re-enter chloroplast with PEP then decomposed to release Pi in the chloroplast (Fig. 4). From the analysis of rice microarray (Wasaki et al. 2003), PKp (plastid type PK) and shikimate kinase expression were enhanced under phosphorus deficient condition. These results indicate that physiological adaptation to incorporate PEP into chloroplast to support photosynthetic carbon flow and synthesis of second metabolic compounds. Recently it is reporeted that another type of Pi transportor (PHT2; 1, which has high homology with Na+/Pi sympoter of fungi) is reported (Versaw et al. 2002). It should be evaluated how these Pi transporters are operated to regulate Pi flux between cytosol and chloroplast.

Conclusion

At least from the analysis of mRNA expression level of rice leaf under different phosphorus levels, several proposed bypass pathways to use phosphorus more efficiency in plant did not work. The most enhanced expression was observed in PPT. As PEP incorporation into chloroplast indicates that recycling use of phosphorus, and not only phosphorus but also recycling use of carbon seems to be enhanced. Further evidence based on biochemical analysis is progressing.

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LEGENDS

Fig. 1 Leaf phosphorus content (mg gDW-1) of rice. Plants were precultured with low phosphorus condition (6 µM) for 3 weeks, then transplanted to solution with phosphorus (32 µM) and without phosphorus. Bars in the figure indicate standard error (n=3).

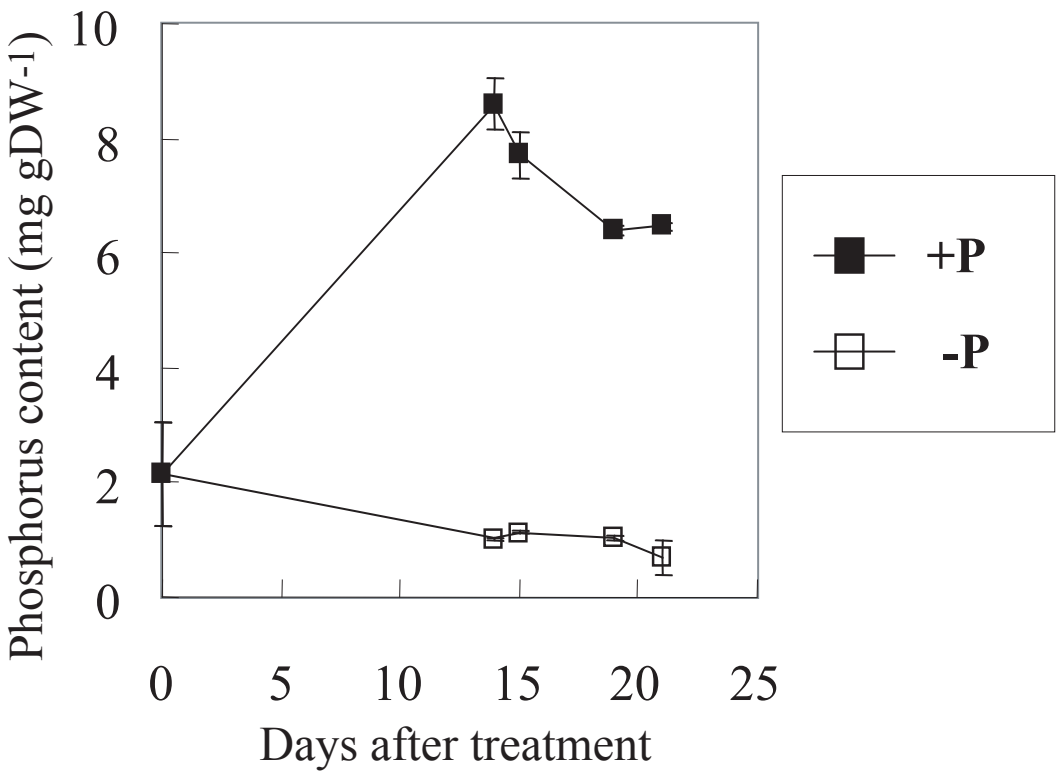
Fig. 2 Successive change of mRNA expression level of enzymes relating to sucrose synthesis and glycolytic bypass pathways. Expression level was determined by real-time PCR. The value was expressed as the relative value to the expression level of each mRNA at the beginning of with or without phosphorus treatment. Bars in the figure indicate standard error (n=3).

Fig. 3 Successive change of mRNA expression level of translocators located in the chloroplast membrane. Expression level was determined by real-time PCR. The value was expressed as the relative value to the expression level of each mRNA at the beginning of with or without phosphorus treatment. Bars in the figure indicate standard error (n=3).

Fig. 4 Proposed model of metabolites flow in the photosynthetic organ under phosphorus deficient condition. The width of the arrow indicate the expression level observed under phosphorus deficient condition relative to phosphorus sufficient condition. We suppose that under phosphorus deficient condition in rice leaf, there exists recycling system of primary photosynthate back into the chloroplast by using PPT.

Table 1. Examined genes and primer sequences for real time PCR.

Name of gene	Accession number	Sequence (5'-3')
TPT	AU108940	TGGTTTCTGTGAATATGGCAAGG
		ACATTTGGGCTCTCTCGACAATC
SPS	U33176	TGCCTGCTGGGAAGCCTAATA
		TGAGCCAACAGTAGCATCAGCA
NAD-G3PDH	AU165583	ACCCAGTAGAATCCTTTTGCTTGC
		CAGGTCCATATCATCAGCATCGTT
NADP-G3PDH	AU032835	AACATGATGACCAAGGTGAAGAGC
		AGAACTGTGGTGCAAATAGCGTAC
PEPC	obtained in this experiment	AAGCTGAACCCGACCAGTGA
		CCTCTGCATCGGCAAAATTAAA
cytosolic PK	3' RACE was performed with E1439	TCAGCTGTACAGAAGCAGTTGTGC
		AAATTGTCCCCCAAAGATGGACTA
PPT	AF372834	CTCTTGCCGGAGTTTTCCTGTACT
		CGATACACACAGCAGCATCAGTTC



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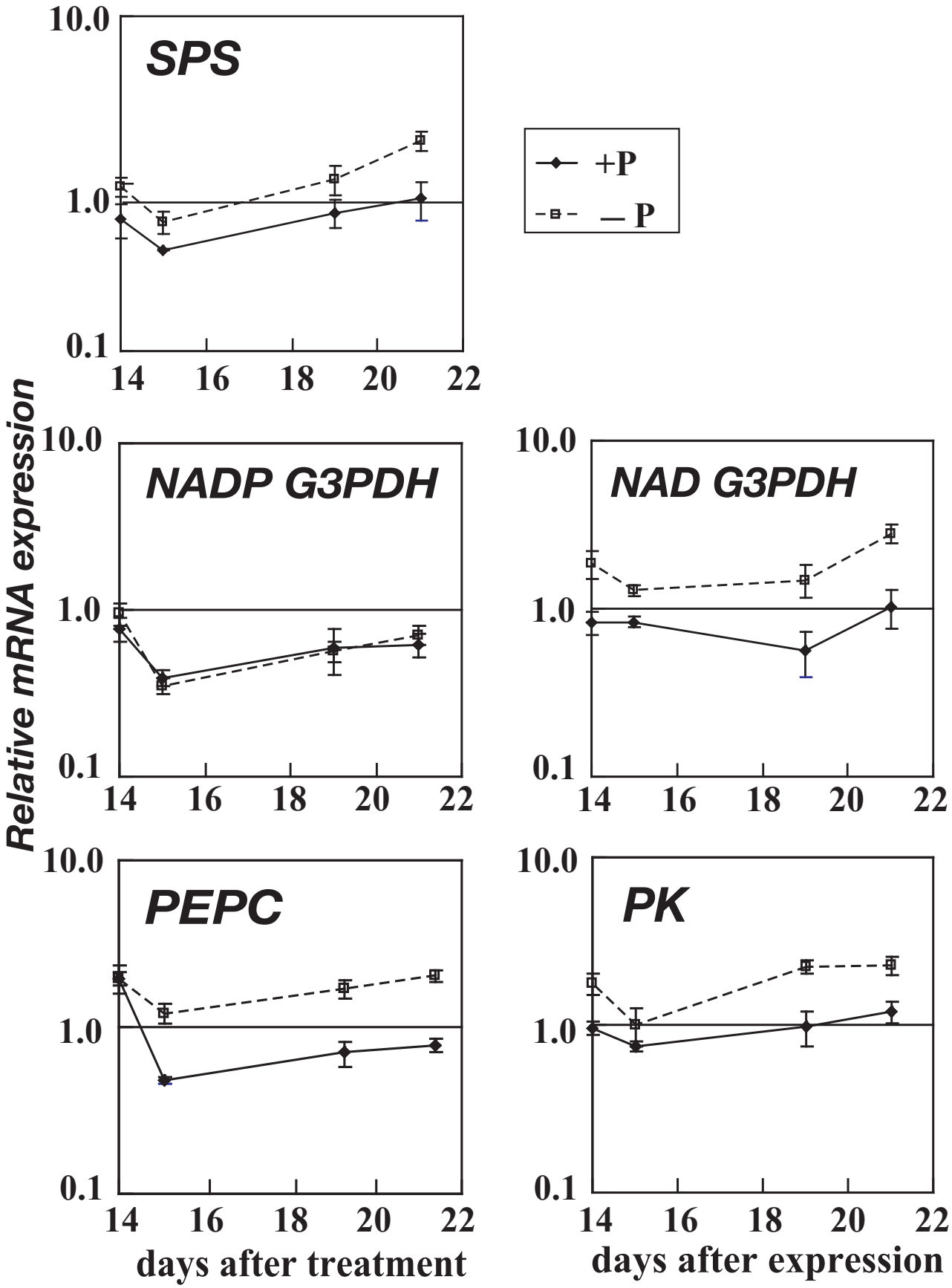

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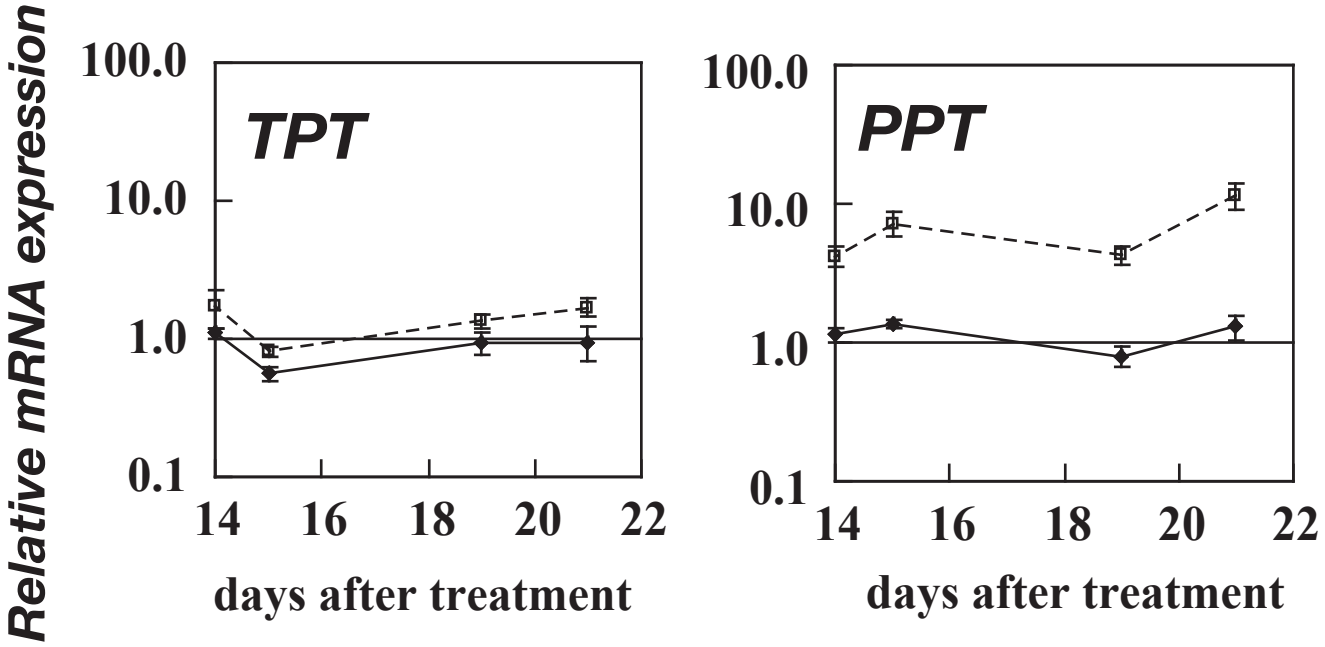

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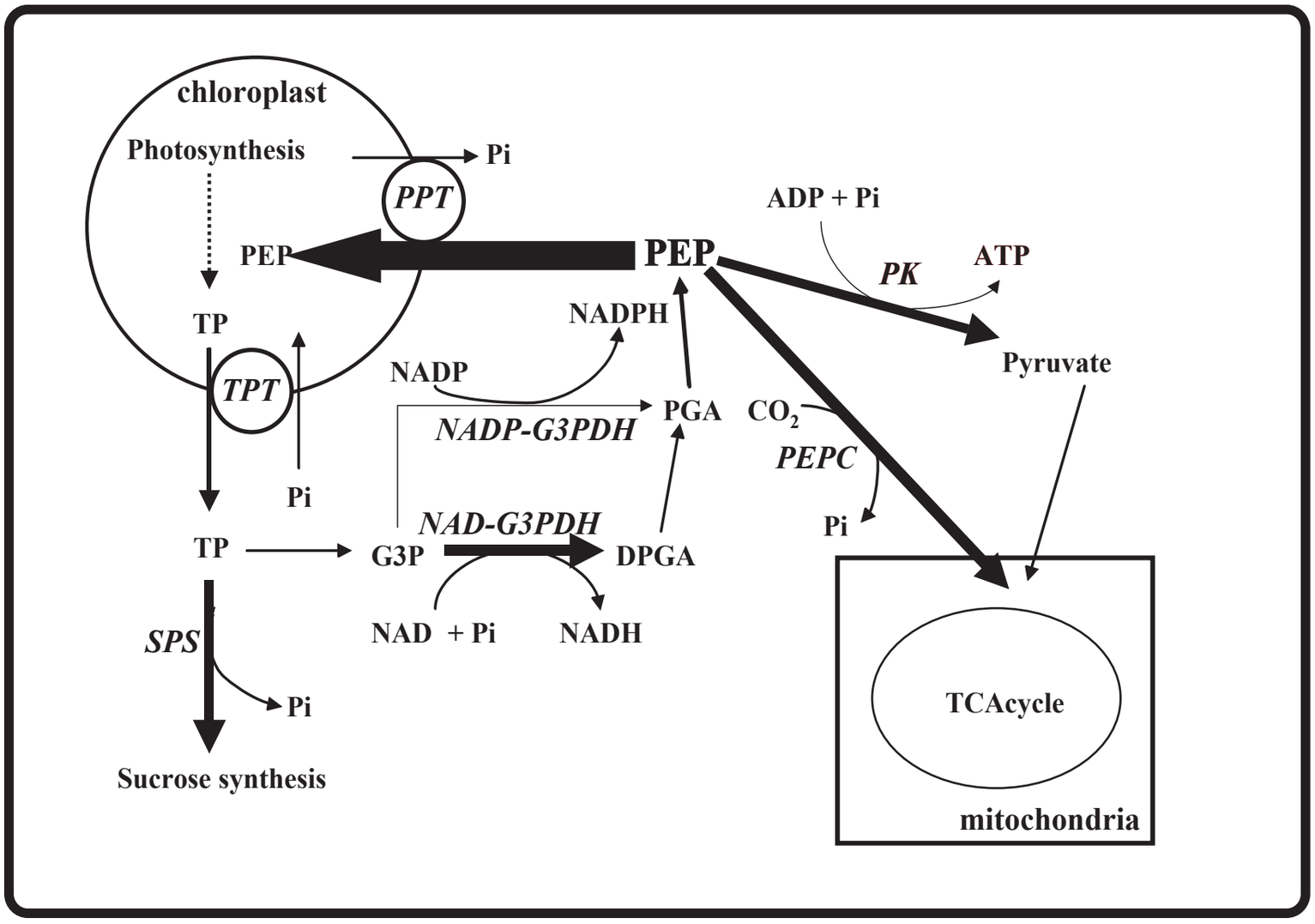

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