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Sugar Phosphates Changes in *Arabidopsis* in Response to Phosphate Nutrition Measured by Improved Ion Chromatography with Pulsed Amperometric Detection Combined with a Titanium Dioxide Column

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Abstract

We have developed a method for comprehensive analysis of sugar phosphates by high performance anion exchange chromatography with pulsed amperometric detection coupled with a titanium dioxide column as a trap-column to remove sample matrices. Levels of sugar phosphates and a nucleotide phosphate from *Arabidopsis thaliana* grown at three different inorganic phosphate (Pi) concentrations in nutrient media or from Pi-related *Arabidopsis* mutants were investigated. Fructose-6-P, Galactose-1-P, Glucose-1-P, Glucose-6-P and Mannose-6-P apparently increased in proportion to increases in the *in vivo* level of Pi in wild type plants. In contrast, levels of Sucrose-6-P and UDP-Glucose decreased as the *in vivo* Pi levels increased. Responses of the former sugar phosphates except Mannose-6-P to the *in vivo* Pi levels in shoots of the mutants were similar in the wild type plants. However, Sucrose-6-P and UDP-Glucose responded differently between the wild type and mutant plants.

Key words: *Arabidopsis*, HPAE-PAD, Sugar phosphates, Titanium dioxide column.

Abbreviations

Frc-6-P, Fructose-6-phosphate; Gal-1-P, Galactose-1-phosphate; Glc-1-P, Glucose-1-phosphate; Glc-6-P, Glucose-6-phosphate; HPAE-PAD, High performance anion exchange chromatography with pulsed amperometric detection; Man-6-P, Mannose-6-phosphate; Pi, Inorganic orthophosphate; Suc-6-P, Sucrose-6-phosphate; UDP-G, UDP-Glucose.

Introduction

Phosphorus is one of the most important elements in biological organisms. The inorganic orthophosphate (Pi), which is taken up from soil by plants is incorporated into various phosphoric compounds, for example, sugar phosphates, nucleotide phosphates or phospholipids (Mimura, 1999). Since phosphate-related metabolites are not only the metabolic substrates, but also the regulators of metabolic reactions, it is important to analyze the metabolic status of phosphoric compounds in plants. Many studies on the metabolism of Pi and phosphate-related metabolites have been done (Foyer and Spencer, 1986; Delhaize and Randall, 1995; Hurry *et al*, 2000). In most of those studies, target analytes have been measured individually. In post-genome approaches to understand whole metabolic patterns in both wild and genetically modified organisms, it is necessary to provide comprehensive analyses of metabolites, so called metabolomics. A number of analytical systems has been used for simultaneous measurement of a range of phosphate-related metabolites. These include NMR (Lauer *et al.*, 1989; Tu *et al.*, 1990), liquid chromatography (LC) (Ashihara *et al.*, 1987; Bhattacharya *et al.*, 1995; Sawada *et al.*, 2003), liquid chromatography coupled with mass spectrometry (LC-MS) (Buchholz *et al.*, 2001) or gas chromatography coupled with mass spectrometry (GC-MS) (Roessner *et al.*, 2000; Chen *et al.*, 2002). The sensitivity of NMR is relatively low for phosphorous, and the structural isomers are not distinguishable by MS

only. High performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) is a type of LC that has been used for the analysis of sugar phosphates in simple materials such as cultured cells or yeast (Taha *et al.*, 1994; Smits *et al.*, 1998; Jensen *et al.*, 1999; Groussac *et al.*, 2000). HPAE-PAD is highly sensitive, and highly selective for sugar phosphates, and is able to analyze sugar phosphates directly without derivatization (for example methylation for GC). But when applied to higher plants, unknown peaks arise and there is interference with the peaks of some sugar phosphates. To overcome these problems, we have developed a new system for HPAE-PAD analysis in which a titanium dioxide column is used as a pre-treatment column to improve the selectivity for sugar phosphates (Sekiguchi *et al.*, in press). Titanium dioxide is selective for phosphate compounds (Matsuda *et al.*, 1990) and therefore it gives a good separation from the plant metabolite matrix. In the present study, we employed this method to determine the relationship between inorganic phosphates and sugar phosphates in *Arabidopsis*. There are several advantages in using *Arabidopsis*, whose genome has now been completely sequenced. These include its rapid life cycle and its well-dissected gene functions. Additionally, there are mutants available with alterations in their patterns of phosphate distribution. The *pho1* mutant has low Pi level in its shoot due to a defect in xylem loading (Poirier *et al.*, 1991; Hamburger *et al.*, 2002), whereas the *pho2* mutant accumulates excess Pi in its shoot for reasons that are not well understood (Delhaize and Randall, 1995). The normal and mutant genotypes allow the analysis of plants with low, normal and high levels of Pi. There have been several other studies in which wild type, *pho1*, and *pho2* mutants have been used to examine the relationship between Pi levels and starch metabolism (Ciereszko *et al.*, 2001) or cold tolerance (Hurry *et al.*, 2000). In this study, we are interested to see how different levels of Pi affect the pattern of sugar phosphates. To do this, first we need to improve the HPAE-PAD method to give adequate separation and detection of sugar phosphates in higher plants.

Materials and Methods

Chemical materials

Deionized water with a specific resistance over 18 MΩ from a Milli-RO/ Milli-Q system (Millipore, Bedford, MA, USA) was degassed by aspiration and used to prepare all eluent and standards. Sodium hydroxide was purchased from Fisher Scientific Inc. (Hampton, NH, USA) as 50% solution, and sodium acetate and acetic acid were purchased from Wako Pure Chemicals (Osaka, Japan). Standards for metabolites were purchased from Sigma (St. Louis, MO, USA). Fructose-6-phosphate (Frc-6-P), galactose-1-phosphate (Gal-1-P), glucose-1-phosphate (Glc-1-P), glucose-6-phosphate (Glc-6-P), mannose-6-phosphate (Man-6-P), sucrose-6-phosphate (Suc-6-P) and UDP-glucose (UDP-G) were selected as standards for sugar phosphate analysis by HPAE-PAD. The stock solutions (0.5 mM each) of the target analytes were prepared in deionized water and then stored in a refrigerator for future use. Working standard solutions were prepared daily by appropriate dilution of the stock solutions.

Plant materials

Arabidopsis thaliana (L.) Heynh. (ecotype Columbia), *pho1-2* mutant and *pho2-1* mutant were used. Seeds of mutant *Arabidopsis* (*pho1-2* and *pho2-1*) were obtained from the Arabidopsis Biological Resource Center (ABRC) (Ohio State University, OH, USA). Seeds were sterilized by soaking in 70% ethanol for 5 min, and in a solution of 1% Triton-X and 0.4% sodium hypochlorite for 10 min successively, and then the seeds were washed four times with sterilized water. Seeds of wild type were spread on 0.2% gellan gum plates containing Murashige and Skoog culture solution containing three different Pi concentrations; 12.5 μM, 125 μM, and 1250 μM. Seeds of *pho1* and *pho2* mutants were spread on the plate containing Murashige and Skoog culture medium supplemented with 125 μM Pi. Plates were placed at 4℃ for 2 days, transferred into a growth cabinet continuously illuminated at 50 μmol m^{-2} s^{-1} at 22±1℃ for 2 weeks, and then replanted in the same media in culture bottles for 6 weeks until required.

Plant extraction

A whole *Arabidopsis* plant was divided into a shoot and root. Each part was frozen in liquid nitrogen and lyophilized for 4 h in a freeze dryer (FREEZONE 1L, LABCONCO, Kansas city, MO, USA), and stored at -80℃ until use. The sample was crushed into small pieces and placed in a 2.0 ml eppendorf tube. Boiling deionized water (0.5 ml) was added and the extract was then immediately irradiated with microwave at 600 W for 15 s. After cooling on ice, the extract was centrifuged at 20,400*g* at 4℃ for 10 min. The supernatant was filtered through a 0.45 μm filter (EKICRODISC AcroLC, Gelman Sciences Japan) and applied to the HPAE-PAD.

HPAE-PAD Analysis

A DX-500 ion chromatography system (Dionex, Sunnyvale, CA, USA) consisting of a GP50 gradient pump, LC30 chromatography oven, and ED50A electrochemical detector was used in this study. Dionex Chromeleon software version 6.5 was used for data processing. Dionex CarboPac PA1 (4x250 mm) and PA1 guard (4x50 mm) columns packed with anion-exchange resin were used as the separation columns, and two (4.6x50 mm) Titansphere TiO columns (GL Sciences Inc., Tokyo, Japan) packed with titanium dioxide resin, were used as the inline sample pre-treatment column for analysis of sugar phosphates. The Titansphere TiO columns were connected to a six-port column switching valve, which was placed after the injection valve in the autosampler. After sample loading in the injection loop, the injection valve was switched to the inject position and then the sample was passed from the injection loop into the TiO column by pumping with deionized water. The sample matrices were rinsed away and phosphate compounds were retained in the TiO column. The column switching valve was then switched to the path through the TiO column, in order to desorb phosphate compounds.

Eluents were prepared as follows, 75 mM sodium hydroxide as eluent A, and 75 mM sodium hydroxide and 500 mM sodium acetate as eluent B. Eluent A was flowed for the first 20 min. The sodium acetate gradient was increased from 0 to 500 mM between 20 and 50 min. It remained at 500 mM sodium acetate/ 75 mM sodium hydroxide for 10 min and then returned to the initial solution for 5 minutes. Sample was loaded to the TiO column by using deionized water, and then sample matrices were removed by flowing deionized water at 1.5 ml min^{-1} for 5 min. The loaded sample was eluted from the TiO column by 75 mM sodium hydroxide for 15 minutes after switching the flow line on the six-port valve connected to the TiO column. Data acquisition was started from 20 min.

The detection was carried out by a pulsed amperometry cell equipped with a working gold electrode and a combined pH-Ag/AgCl reference electrode. The following potential-time sequence was used: 0.05 V (0 to 0.4 s), 0.75 V (0.41 to 0.6 s), and -0.15 V (0.61 to 1.0 s). The electrical current was measured and integrated during 0.2 - 0.4 s, resulting in an output signal expressed in nano-coulombs.

In all analyses, 25 μl of sample was injected onto the column by a Dionex AS50 autosampler. The AS50 sample tray was cooled to 4℃ by a tray cooler during analysis. The analysis was performed at 35℃ with the flow rate set at 1.0 ml min^{-1}.

Pi was separated from other anions by using

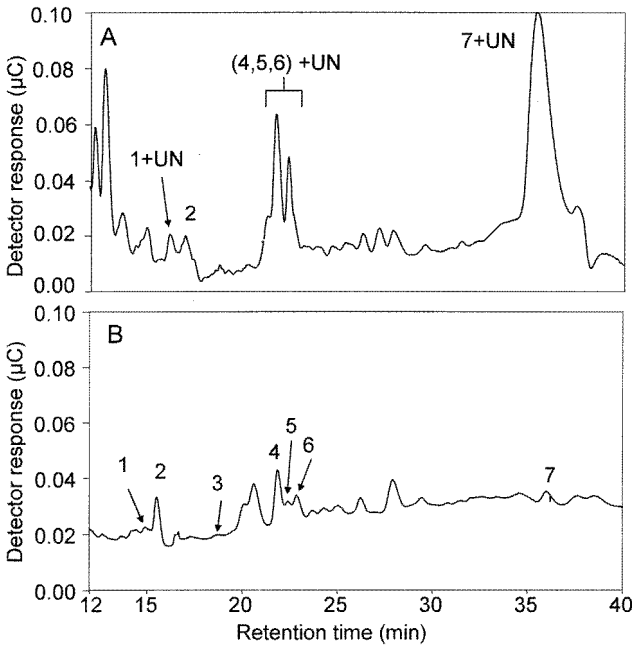

Fig. 1 Comparison of chromatograms of *Arabidopsis* extracts analyzed by HPAE-PAD without (A) or with (B) the titansphere TiO column. *Arabidopsis* shoot extracts (wild type grown at 125 μM Pi medium) analyzed by HPAE-PAD without (A) and with (B) TiO column. Peaks: 1. galactose-1-phosphate; 2. glucose-1-phosphate; 3. sucrose-6-phosphate; 4. glucose-6-phosphate; 5. mannose-6-phosphate; 6. fructose-6-phosphate; 7. UDP-glucose; UN, unknown peak

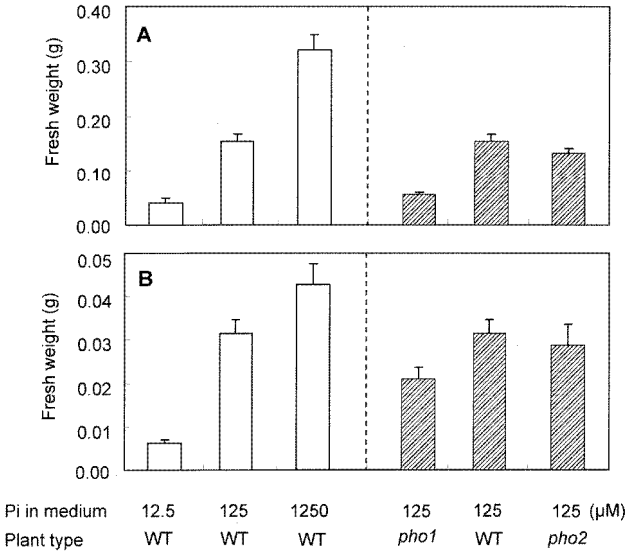

Fig. 2 Growth of shoots (A) and roots (B) of wild type *Arabidopsis* grown in different Pi concentration in the nutrient media, and of mutants grown in 125 μM Pi. The wild type was grown in the Murashige-Skoog nutrient medium which included 12.5, 125 and 1250 μM Pi for eight weeks. The *pho1* and *pho2* were grown in 125 μM Pi medium for eight weeks.

IonPac AS11-HC analytical column and AG11-HC guard column with potassium hydroxide eluent from 5 to 50 mM gradient at 1.4 ml min-1 flow rate. Pi was then detected by a Dionex ED50 conductivity detector with ASRS-ULTRA suppressor.

Results

The effect of TiO column on analysis of sugar phosphates in Arabidopsis

The TiO column was employed because it is known to have specific binding for phosphorylated compounds. A comparison between conventional HPAE-PAD method without the TiO column and the new optimized method with the TiO column for plant extracts was made (**Fig. 1**). Without the TiO column, there was an unknown peak that interfered with Frc-6-P, Gal-1-P, Glc-6-P and Man-6-P, and UDP-G was included in the large unknown peaks (**Fig. 1A**). When the TiO column was used as a trap column, the unknown peaks were greatly diminished resulting in improvement of the resolution of the known peaks (**Fig. 1B**).

Growth and in vivo Pi levels of plants grown at different Pi levels

Wild type *Arabidopsis* was grown in three different Pi concentrations; 12.5 μM as Pi-deficiency, 125 μM as Pi-standard and 1250 μM as Pi-excess. Results are shown in **Fig. 2.** The fresh weight of shoots of the wild type increased in proportion to the increase in Pi levels in the nutrient medium. The fresh weight of *pho2* shoots grown in 125 μM Pi was close to that of the wild type, and was larger than the *pho1* shoots whose fresh weight was similar to that of shoots grown in 12.5 μM Pi (**Fig. 2A**). A similar effect of increasing the medium's Pi level to the fresh weight was observed in roots. The fresh weight of *pho2* roots was close to that of the wild type root, and was larger than the *pho1* roots (**Fig. 2B**). At 125 μM Pi, the weight of the shoots of *pho1* were similar to the wild type grown at 12.5 μM, whereas the root weights were quite different, with *pho1* being much bigger than in the deficient wild type.

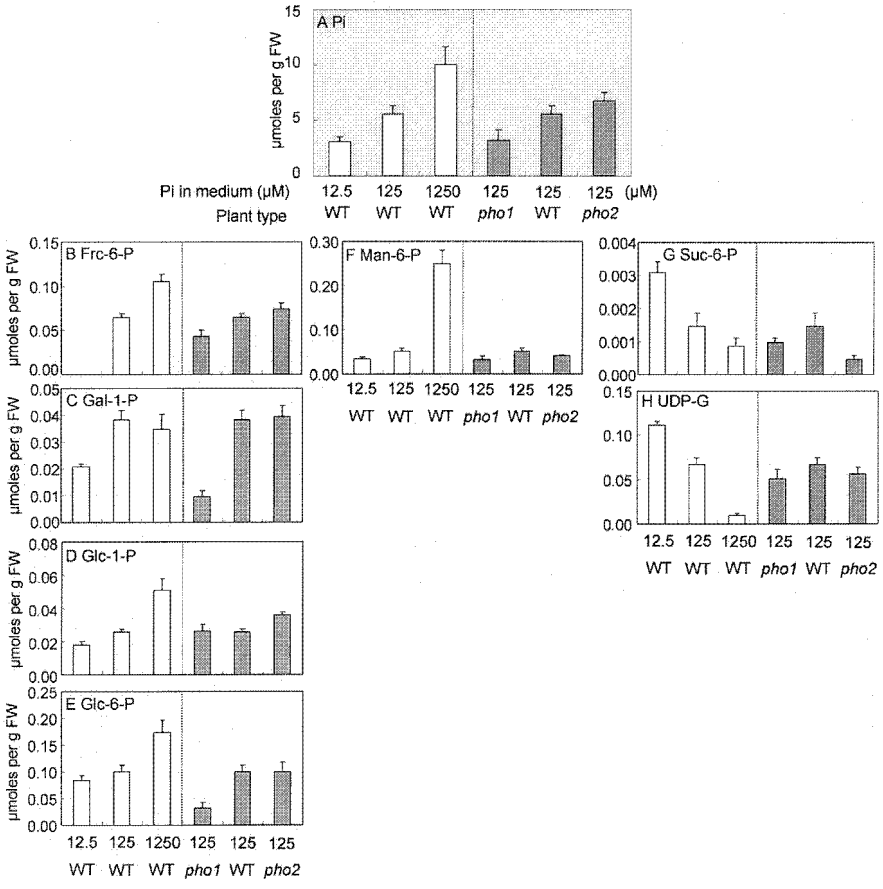

Fig. 3 The levels of *in vivo* Pi and sugar phosphates in shoots of wild type plants grown in different Pi concentrations, and those of mutants grown in 125 μM Pi. The wild type was grown in the nutrient medium including 12.5, 125 and 1250 μM Pi for eight weeks. The *pho1* and *pho2* mutants were grown in 125 μM Pi medium for eight weeks. Sugar phosphates and UDP-G were analyzed simultaneously by HPAE-PAD method with a TiO column.

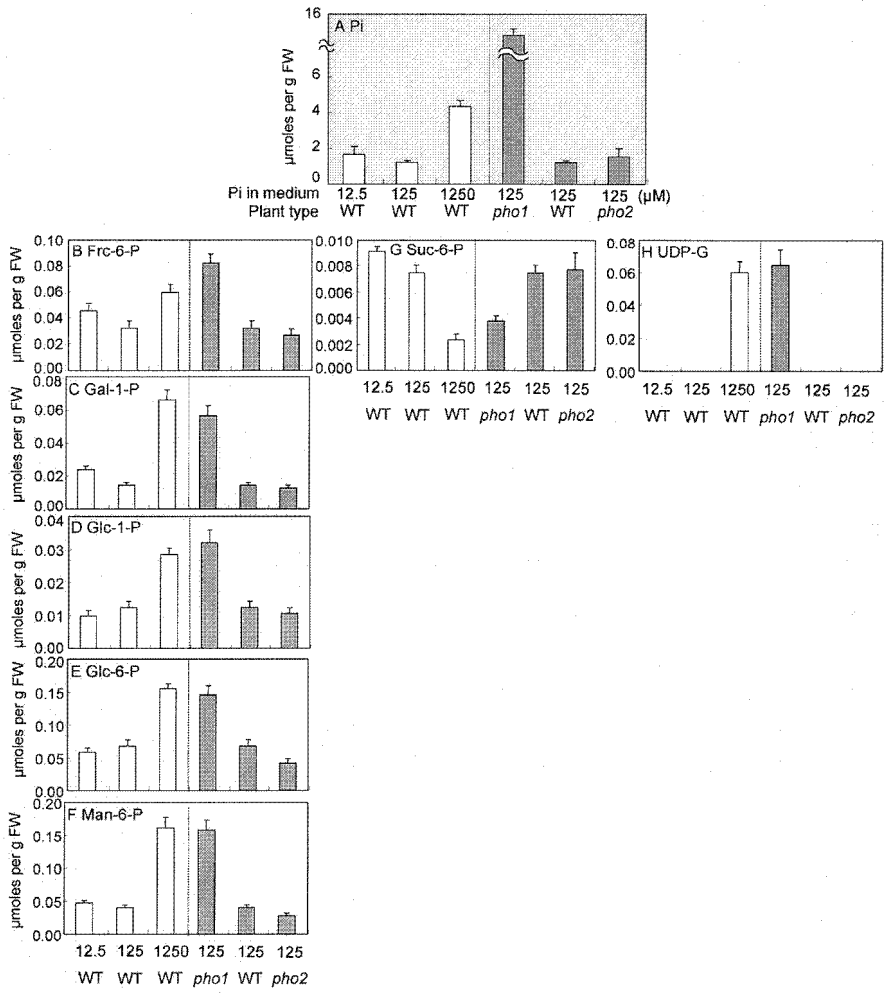

Fig. 4 The levels of *in vivo* Pi and sugar phosphates in roots of wild type plants grown in different Pi concentrations, and those of mutants grown in 125 μM Pi.Growth conditions and analytical conditions are the same as in **Fig. 3**.

The Pi levels in wild type shoots increased in proportion to the increase of Pi levels in culture media (**Fig. 3A**). Compared to the wild type the Pi level of *pho1* was lower and Pi level of *pho2* was higher when grown in 125 μM Pi medium (**Fig. 3A**). In roots of the wild type plants, the difference in the Pi levels between 12.5 μM and 125 μM Pi of the culture media was small, but increased significantly at 1250 μM (**Fig. 4A**). At 125 μM, the Pi level of *pho2* was almost the same as that of a wild plant root, while the Pi level of *pho1* was almost 10-fold higher than the wild type plant. These results are consistent with those obtained in other studies (Poirier *et al.*, 1991; Delhaize and Randall, 1995).

Relationship between Pi and sugar phosphate levels in shoots and roots

By the present method, we simultaneously detected Frc-6-P, Gal-1-P, Glc-1-P, Glc-6-P, Man-6-P, Suc-6-P and UDP-G in *Arabidopsis* plants. **Fig. 3B-H** shows sugar phosphate and UDP-G levels in shoot of wild type plant grown in different Pi concentrations, and shoot of *pho1* and *pho2* plant grown at 125 μM Pi. In shoot of wild type plants, levels of Frc-6-P, Glc-1-P, Glc-6-P and Man-6-P increased with increasing *in vivo* Pi level. Gal-1-P levels in wild type plants grown in 125 and 1250 μM medium Pi were almost equal and higher than that in wild type grown in 12.5 μM medium Pi. On the contrary, levels of Suc-6-P and UDP-G decreased with increasing *in vivo* Pi level (**Fig. 3B-H**).

In the shoot of mutant plants, *pho1* and *pho2* compared to wild type plant grown in the same Pi concentration of culture medium (125 μM), changes in levels of Frc-6-P, Gal-1-P, Glc-1-P and Glc-6-P showed the same tendency of the *in vivo* Pi levels, that is, the higher *in vivo* Pi levels correlated to the higher levels of sugar phosphates. However, detailed responses of mutant plants were different from those of wild type plants grown in different Pi concentrations. Glc-1-P level of *pho1* was almost the same as that of wild type plant (125 μM Pi), although the *in vivo* Pi level of *pho1* was lower than that of the wild type plant (12.5 μM Pi). Glc-6-P level of *pho2* was almost the same as that of wild

type plant (125 μM Pi), although the *in vivo* Pi level of *pho2* was higher than that of the wild type plant (125 μM Pi). Levels of Man-6-P, Suc-6-P and UDP-G of mutant plants showed the much different aspects of the *in vivo* Pi levels. In these compounds (Man-6-P, Suc-6-P and UDP-G), there was no distinct relationship to the *in vivo* Pi levels in the mutant plants different from the wild type plant.

In roots, levels of Gal-1-P, Glc-1-P, Glc-6-P and Man-6-P of wild type plants were similar at 12.5 μM and 125 μM Pi in the culture medium, and were higher at 1250 μM Pi (**Fig. 4B-H**). This is similar to the relationship between sugar phosphates and Pi levels in wild type shoots. Although the changing pattern of Frc-6-P level was similar to these sugar phosphates, its level at 1250 μM medium Pi was not high supposed from the *in vivo* Pi level. Suc-6-P again showed an opposite pattern to the *in vivo* Pi and to other sugar phosphates. In mutant plants, *pho1* maintained very high Pi levels in the root, while the Pi level in *pho2* root was almost the same as that of wild type root (**Fig. 4A**). The levels of Frc-6-P, Gal-1-P, Glc-1-P, and Man-6-P in *pho2* were similar to those of the wild type plant, and were clearly lower than in *pho1*. The level of Suc-6-P in *pho1* was much lower than that in the wild type, whereas the level of Suc-6-P in *pho2* was similar to the wild type roots, that is, Suc-6-P level seems to behave in opposite pattern of the *in vivo* Pi level. In roots, the detection of UDP-G was slightly affected by interference from an unknown substance(s), and it was therefore difficult to accurately estimate the level of UDP-G when it was low. The levels of UDP-G of wild type plant grown in 1250 μM Pi and in *pho1* at 125 μM Pi were exceptionally high.

Discussion

HPAE-PAD is a simple yet highly sensitive method for sugar phosphates. In this study we have applied, for the first time, the HPAE-PAD to analyze sugar phosphates and a nucleotide phosphate from a higher plant. Furthermore, we have improved the separation of sugar phosphates with HPAE-PAD by using a titanium dioxide column as a sample pre-treatment column to decrease the influence of sample matrices (**Fig. 1**). This improved method made it possible to determine and analyze various sugar phosphates simultaneously without plant matrices interference. However, there are still many sugar phosphates that are not detectable, even with these improvements because of their low levels in living tissues.

Growth and Pi content in both wild type and mutant plants.

The fresh weight of shoots increases as the Pi levels increase in the shoots (**Figs. 2, 3A**). Similar results have been shown previously with many different plant types (Nieman and Clark, 1976; Ashihara and Tokoro, 1985; Fredeen *et al.*, 1989). The increase of the fresh weight was not proportional to the increase in Pi levels of plants. It is well known that the excess Pi in the cell is stored in the vacuole, and that cytoplasmic Pi is kept at a quasi constant level (Bieleski, 1968). Plants grown in 12.5 μM Pi must have been phosphate deficiency, because of the low fresh weight compared to the higher Pi supply. The increase in fresh weight between 125 μM and 1250 μM Pi was small, suggesting that 125 μM must be approaching the level that gives maximum growth. The growth of *pho1* roots was similar to that of the wild type roots and in this respect was different from that of the shoots in which there was a marked increase with increasing Pi supply (**Fig. 2, 4A**). The lower shoot growth in *pho1* compared to the wild plant correlated with the lower shoot Pi level which supposedly arises due to a defect in xylem loading of Pi in this mutant (Poirier *et al.*, 1991). Pi then accumulated to very high concentrations in the roots but this was not reflected in a higher growth of the roots, which suggests that root growth must be partly under the control of the shoot. In the *pho2* mutant, the fresh weights of both shoots and roots were similar to those of wild type plants, although the Pi level in the shoots was a little higher than in the wild plant (**Figs. 2, 3A, 4A**).

Changes in sugar phosphates related to the in vivo Pi content.

For many phosphate compounds, it is known that the *in vivo* levels tend to be proportional to the supply of Pi, even when metabolism is strictly regulated (Ashihara *et al.*, 1988; Ciereszko *et al.*, 2001). The levels of Frc-6-P, Glc-1-P, and Glc-6-P nearly corresponded to the *in vivo* Pi level in most materials measured irrespective of plant type, and in both shoots and roots. Levels of Gal-1-P in shoots of wild type and mutant plants also increased with an increase of *in vivo* Pi levels, but they showed a saturation in higher *in vivo* Pi levels. From these results, it seems that Glc-1-P, Glc-6-P, Frc-6-P, and Gal-1-P are positively regulated by the *in vivo* Pi level. Levels of Man 6-P in wild type plants also increased proportional to the *in vivo* Pi levels, although Man 6-P level in mutants differently behaved.

A greater divergence between wild type and

mutants was observed with Suc-6-P and UDP-G. In contrast to the monosaccharide sugar phosphates, the levels of Suc-6-P of both shoots and roots and UDP-G of shoots in wild type plants tended to decrease with increasing *in vivo* Pi. It is well known that under Pi-deficiency, starch synthesis is activated (Paul *et al.*, 1993) which is consistent with the pattern observed here. However, in the shoots of both *pho* mutants, levels of both Suc-6-P and UDP-G were unresponsive to the *in vivo* Pi levels (**Fig. 3A, 3G, 3H**). This may suggest that in the mutants (and may be in the wild type plant), Suc-6-P and UDP-G are controlled not by the *in vivo* Pi level, but by some other factors.

Control of sugar phosphate metabolism.

Metabolism of some phosphate compounds seems to be regulated by *in vivo* Pi level (**Fig. 3, 4**). Although *in vivo* Pi level is kept at a quasi constant level, it still fluctuates depending on the environmental Pi supply. Limiting the supply of Pi induces many Pi deficiency responses. In the present study, we showed that in the wild type plants, levels of Frc-6-P, Gal-1-P, Glc-1-P, Glc-6-P, and Man-6-P were positively related to the *in vivo* Pi levels, and those of Suc-6-P and UDP-G were negatively related to the *in vivo* Pi levels. These patterns were expected from previous studies of metabolic regulation (Bieleski, 1968; Sicher and Kremer, 1988). Here, we tried to compare changes in the levels of these compounds in wild type plants to those in mutants. The *pho* mutants are believed to be able to mimic the Pi status of the wild type shoots under Pi-deficient conditions or Pi-rich conditions, without any changes in Pi supply from culture media (Poirier *et al.*, 1991; Delhaize and Randall, 1995). The observed changes of *in vivo* Pi levels in both mutants were consistent with these previous reports, *i.e.* the shoot of *pho1* becomes Pi-deficient and that of *pho2* does Pi-rich. In these experiments, the mutants were used to try to distinguish between responses emanating from the external supply and responses from the internal Pi status. In the wild type plants, internal Pi was manipulated from the external Pi supply. However, since internal and external Pi changed in the same way, it was not possible to distinguish which level was dictating changes in the levels of sugar phosphates. With the mutant, it was possible to vary the internal Pi level while keeping the external Pi level constant. Comparing the results from wild type and mutant plants, levels of Frc-6-P, Gal-1-P, Glc-1-P, Glc-6-P and Man-6-P in shoots might be directly regulated through the changes in the medium/internal Pi level. While the levels of Suc-6-P and UDP-G in shoot might be regulated by not only internal Pi level, but also any other factors.

We propose two possible explanations for the above phenomena. One is that the mutation of a specific gene may affect other metabolic processes. The other possibility is that Pi-related metabolism is regulated by not only the *in vivo* Pi status, but also by environmental signal(s) related to Pi supply. The mutants have the ability to sense Pi supply. In order to analyze the control mechanism of Pi-related metabolism, we need to measure more detailed regulation of metabolite levels in both wild type and mutant plants.

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