Plant Physiol. (1995) 108: 295–302

Lack of Control in Inorganic Phosphate Uptake by *Catharanthus roseus* (L.) G. Don Cells

Cytoplasmic Inorganic Phosphate Homeostasis Depends on the Tonoplast Inorganic Phosphate Transport System?

Katsuhiro Sakano*, Yoshiaki Yazaki, Kiyoshi Okihara, Tetsuro Mimura[1], and Seiichiro Kiyota

National Institute of Agrobiological Resources, Department of Applied Physiology, 2–1–2 Kannondai, Tsukuba, Ibaraki 305, Japan (K.S., Y.Y., K.O., S.K.); and Faculty of Science, Himeji Institute of Technology, Harima Science Park City, Hyogo 678–12, Japan (T.M.)

Inorganic phosphate (Pi) uptake by *Catharanthus roseus* (L.) G. Don cells was studied in relation to its apparent uncontrolled uptake using ^{31}P-nuclear magnetic resonance spectroscopy. Kinetics of Pi uptake by the cells indicated that apparent K_m and V_m were about 7 μM and 20 μmol g^{-1} fresh weight h^{-1}, respectively. Pi uptake in Murashige-Skoog medium under different Pi concentrations and different initial cell densities followed basically the same kinetics. When supplied with abundant Pi, cells absorbed Pi at a constant rate (V_m) for the first hours and accumulated it in the vacuole. As the endogenous pool expanded, the rate of Pi uptake gradually decreased to nil. Maximum Pi accumulation was 100 to 120 μmol g^{-1} fresh weight if cell swelling during Pi uptake (about 2-fold in cell volume) was not considered. Results indicated that (a) the rate of Pi uptake by *Catharanthus* cells was independent of initial cell density and was constant over a wide range of Pi concentrations (2 mM to about 10 μM) unless the cells were preloaded with excess Pi, and (b) there was no apparent feedback control over the Pi uptake process in the plasma membrane to avoid Pi toxicity. The importance of the tonoplast Pi transport system in cytoplasmic Pi homeostasis is discussed.

In general, plants are able to control the rate and extent of absorption of mineral nutrients according to their nutritional status. In phosphate nutrition, plants deprived of Pi absorb it at an enhanced rate upon its resupply (Ullrich-Eberius et al., 1981; Clarkson and Scattergood, 1982; Lefebvre and Glass, 1982; Cogliatti and Clarkson, 1983; Drew et al., 1984; Lee, 1988; Lee et al., 1990; Mimura et al., 1990). In addition, the uptake rate decreases as Pi accumulates in the cell (e.g. see Lee et al., 1990; Dunlop and Gardiner, 1993).

Recently, we found that growth of *Catharanthus roseus* (L.) G. Don cells was severely inhibited when they were cultured at low ICD or at normal ICD but with high Pi concentration in medium (Sakano et al., 1995). Under both conditions, cell morphology was similar: (a) starch granules disappeared from the cytoplasm and (b) cells swelled extensively with frequent bursts. The observed growth inhibition at low ICD, however, was completely suppressed when Pi concentration in medium was reduced proportionally to ICD. These results strongly suggested that an excess intake of Pi occurred under low ICD, which in turn led to the growth inhibition.

In the present study, we examined the characteristics of Pi uptake by *Catharanthus* cells and the mechanism of cytoplasmic Pi homeostasis, which was apparently lost from the cells. Results indicate that Pi accumulates to a toxic level in the cell under the conditions of low ICD or high Pi level. Results of ^{31}P-NMR studies, together with those of previous studies (Sakano et al., 1992), suggest that the Pi transport system(s) in the tonoplast predominate over the cytoplasmic Pi homeostasis.

MATERIALS AND METHODS

Plant Materials

Catharanthus roseus (L.) G. Don cells (B-strain) were cultured in MS medium (Murashige and Skoog, 1962) supplemented with 4.5 μM 2,4-D and 3% (w/v) Suc as described previously (Sakano, 1990). In the routine culture, 2.5 mL of the cell suspension was transferred every 7 d to 25 mL of fresh medium in a 100-mL Erlenmeyer flask. The ICD was about 4×10^5 cells mL^{-1} (equivalent to about 0.8 g fresh weight $flask^{-1}$). The flask was shaken to 120 rpm on a rotary shaker at 26 ± 1°C. When ICD was reduced to one-half (1/2C), one-quarter (1/4C), and one-eighth (1/8C) of the control (1C, 4×10^5 cells mL^{-1}), the inoculum cell suspension was properly diluted with fresh MS medium before subculture. When Pi concentration in the medium was raised to two times (2P), four times (4P), and eight times (8P) the control level (1P: 1.25 mM), appropriate amounts of 1.0 M sodium phosphate solution (pH 5.8) were added to the medium. All the time-course experiments were repeated at least once (mostly twice) with similar results that are presented in this paper.

[1] Present address: Biological Laboratory, Hitotsubashi University, Naka 2–1, Kunitachi, Tokyo 186, Japan.

* Corresponding author; e-mail sakanok@abr.affrc.go.jp; fax 81–298–38–8347.

Abbreviations: ICD, initial cell density; MS medium, Murashige and Skoog medium.

Determination of Medium and Intracellular Pi

Under aseptic conditions, 0.5 mL of cell suspension was withdrawn from the culture flask and the cells were separated from the medium by filtration through a glass fiber filter (Whatman GF/A, diameter 10 mm) under suction. The filtrate served as medium sample. The cells collected on the glass fiber filter were washed once with 0.5 mL of distilled water, weighed, and transferred together with the filter to a microcentrifuge tube (1.5 mL volume with lid lock). They were boiled for 1 min after adding 0.5 mL of distilled water and centrifuged (5 min, 15,000 rpm). Pi in 10 to 200 μL of the supernatant fraction (intracellular Pi) and in the filtrate (medium Pi) was determined by the method of Bencini et al. (1983).

Kinetics of Pi Uptake by *Catharanthus* Cells

Kinetic parameters (K_m and V_m) of Pi uptake by *Catharanthus* cells were determined at about 25°C under conditions similar to those described previously (Sakano, 1990). Cells were collected on a filter paper and washed once with 10 mM $CaCl_2$ solution. Five grams of fresh weight of the washed cells were suspended in 50 mL of the medium composed of 10 mM $CaCl_2$, 1 mM KCl, and 2% Glc in a Lucite vessel (45 mm diameter, 70 mm height, internal volume about 110 mL). The lid of the vessel was equipped with an inlet (with aseptic filter, pore size 0.22 μm) and an outlet for aeration (20 mL min^{-1}) and a port (with stopper) for Pi injection and sampling. The vessel was shaken at 140 rpm on a rotary shaker to keep cells suspended and to facilitate gas exchange.

About 120 min after adding cells into the medium (when medium pH dropped to about 3.8 and stabilized), 10 μmol of Pi (sodium phosphate solution, pH 4.0) was injected into the cell suspension (final concentration 0.2 mM). At 1-min intervals, 0.5 mL of the cell suspension was removed and put into a microcentrifuge tube with a membrane filter (Millipore, UFC30GV00) and centrifuged. Submicromolar concentrations of Pi in the filtrate were determined by ion chromatography (Yokogawa Electric, Tokyo, Japan, IC-7000 M).

^{31}P-NMR Study

Time courses of changes in Pi level and pH of the cytoplasm and the vacuole, as well as those in external medium, were studied by ^{31}P-NMR spectroscopy. The methods were similar to those described previously (Sakano et al., 1992). In short, 0.36 g (fresh weight) of d-4 cells were put into an NMR tube (diameter 10 mm) and suspended in 3.6 mL of medium (10 mM $CaCl_2$, 1 mM KCl, and 2% Glc, unbuffered). To keep cells aerobic, the cell suspension was bubbled with pure oxygen gas during the experiment (3 mL min^{-1}).

^{31}P-NMR spectra were obtained with a Varian VXR-500S spectrometer, operating at 202.3 MHz with 45° pulses at a repetition rate of 0.2 s, at 26°C. Total accumulation time for each spectrum was 16 min (4096 transients). Methylene diphosphonate in Tris buffer, pH 8.9, in a capillary tube was used as an external reference. Heights of peaks assigned to Pi in each cell compartment relative to that of external reference were taken as the measure of their Pi contents. The pH of each cell compartment was estimated from the chemical shift values as described previously (Sakano et al., 1992).

RESULTS

Long-Term Time-Course Studies of Pi Uptake

Figure 1, a and b, shows the long-term time courses of Pi accumulation (a) and Pi uptake by *Catharanthus* cells (b) under different ICDs. Medium Pi was almost completely exhausted by d 1 in 1C (control) and 1/2C and these very low-medium Pi levels were maintained thereafter. In contrast, when ICD was further reduced to 1/4C and 1/8C, medium Pi was never exhausted during the culture period. On the contrary, the increase of medium Pi took place on or before d 2 and lasted for several days in both low ICDs. Intracellular content of Pi (Fig. 1a) was the highest on d 1 under all ICD conditions, and they were roughly equal to

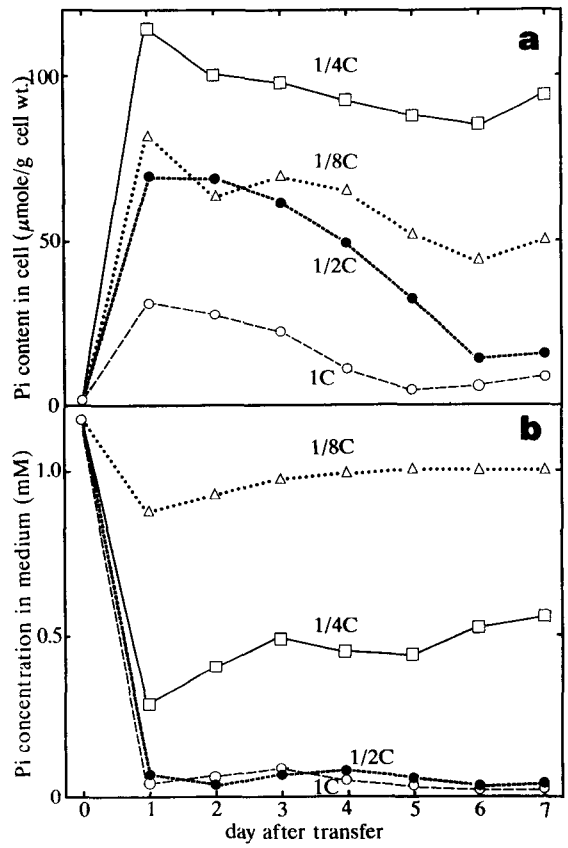

Figure 1. Long-term time courses of Pi accumulation (a) and Pi uptake by *C. roseus* cells (b) in MS medium under low ICDs at constant Pi concentration (1.25 mM). 1C (control) indicates ICD of 4 × 10^5 cells mL^{-1}, and 1/2C, 1/4C, and 1/8C mean one-half, one-quarter, and one-eighth of the control ICD, respectively. The ordinates for a and b show Pi content in cells (on a cell weight basis) and Pi concentration in the medium, respectively; the abscissa show time (d) after the inoculum cells (cultured for 7 d in MS medium) were transferred to each medium.

the amount of Pi that disappeared from each medium (Fig. 1b). In 1/8C, the rate of uptake and hence the rate of accumulation in cells were lower than those in 1/4C.

Results were similar when medium Pi was raised (Fig. 2). In 1P (control) and 2P medium Pi was almost completely exhausted within 6 h. However, in 4P and 8P it was never exhausted during the culture period and, again, a Pi release lasting for about 2 d, as seen in 1/4C and 1/8C (Fig. 1b), started after about 10 h.

Short-Term Time-Course Studies of Pi Uptake

Figure 3, a and b, show the short-term time courses of Pi accumulation (a) and Pi uptake by *Catharanthus* cells (b) under different ICD conditions. In control (1C), the rate of Pi uptake (Fig. 3b) was a linear function of time (about 20 μmol g^{-1} fresh weight h^{-1}) and the medium Pi decreased below 0.1 mM within about 2 h after transfer. Endogenous Pi content (Fig. 3a) increased from about 1 to 31 μmol g^{-1} fresh weight within the first 2 h and did not change appreciably thereafter.

In 1/4C, Pi uptake (Fig. 3b) continued linearly for about 6 h at a rate (21 μmol g^{-1} fresh weight h^{-1}) similar to that of control. The rate gradually decreased to nil for the next few hours, then a Pi release into medium started. In contrast, Pi accumulation in the cell (Fig. 3a) was a linear function of time (about 16 μmol g^{-1} fresh weight h^{-1}) until the maximum level (about 117 μmol g^{-1} fresh weight) was attained after 7 h. The content decreased to 105 μmol g^{-1} fresh weight in 24 h due to the Pi release.

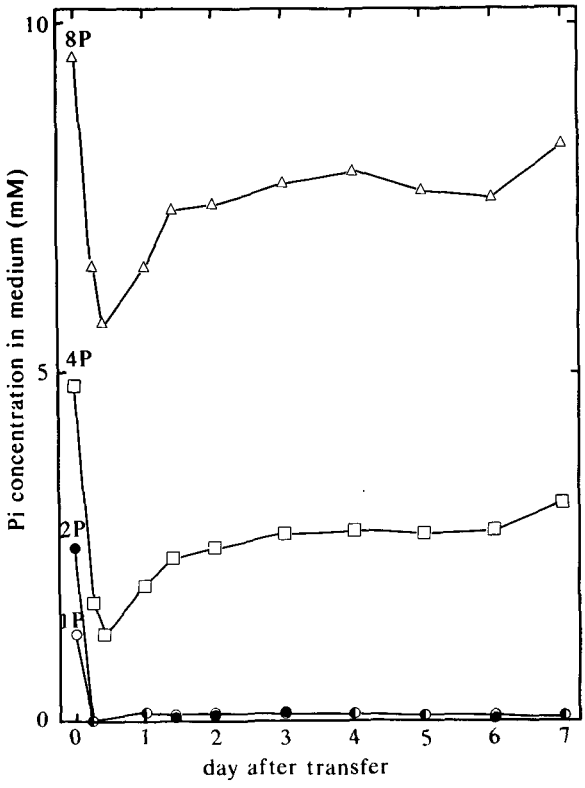

Figure 2. Long-term time courses of Pi uptake by *C. roseus* cells in MS medium with high Pi concentrations at a constant ICD (4×10^5 cells mL^{-1}). 1P (control) indicates that Pi concentration in the medium is 1.25 mM, and 2P, 4P, and 8P are two times, four times, and eight times the control Pi concentration, respectively. The ordinate and abscissa are as for Figure 1b.

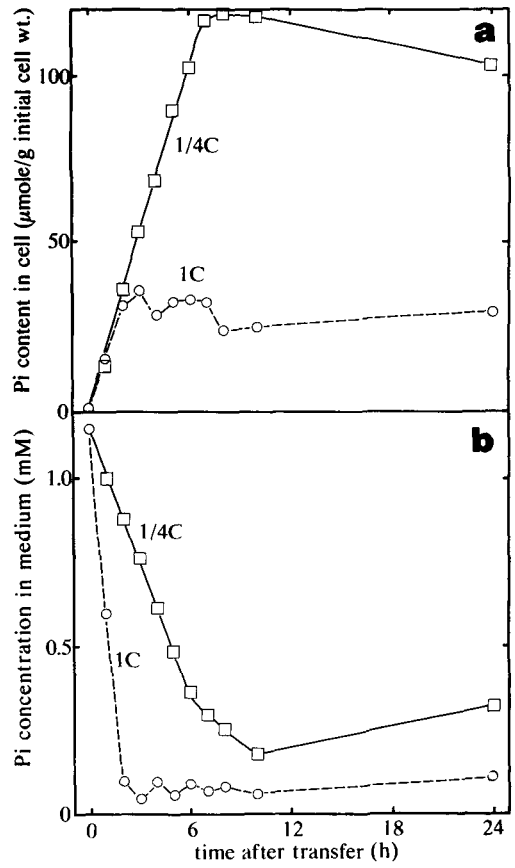

Figure 3. Short-term time courses of Pi accumulation (a) and Pi uptake by *C. roseus* cells (b) in MS medium under low ICD. 1C, 1/4C, and ordinates are as for Figure 1, except that the value for Pi content in cell a is on the initial cell weight basis. The abscissa shows time (h) after the inoculum cells (cultured for 7 d in MS medium) were transferred to each medium.

The difference between the initial rates of uptake (22–23 μmol g^{-1} fresh weight h^{-1}) and accumulation (15–16 μmol g^{-1} fresh weight h^{-1}) in both 1C and 1/4C may be the rate of conversion of Pi into organic phosphates in the cell (Rebeille et al., 1982).

The results were similar when Pi concentration was raised (Fig. 4, a and b). In 4P, medium Pi (Fig. 4b) was never exhausted, and the uptake rate gradually decreased until 9 h, and again, Pi release followed thereafter. The rates of Pi uptake and accumulation (22 and 12 μmol g^{-1} fresh weight h^{-1}, respectively), as well as the maximum Pi accumulation (105 μmol g^{-1} fresh weight) were comparable to those in 1/4C.

Morphological Changes

Microscopic observation (data not shown) indicated morphology of cells under condition of low ICD or high

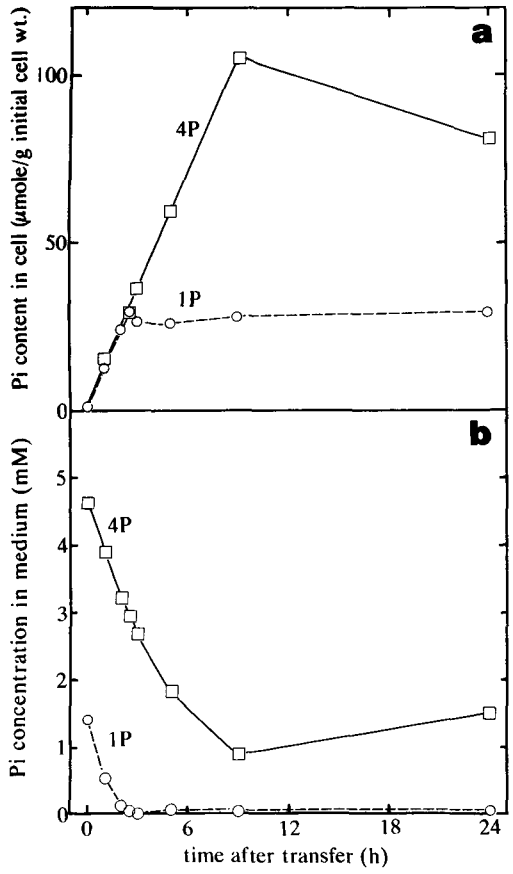

Figure 4. Short-term time courses of Pi accumulation (a) and Pi uptake by *C. roseus* cells (b) in MS medium with high Pi concentration at control ICD (4×10^5 cells mL^{-1}). 1P and 4P are as for Figure 2, and the ordinates and abscissa are as for Figure 3.

medium Pi concentrations became abnormal by the time maximum Pi accumulation was attained (after 7–9 h): cells were extremely swollen and had lost most of the starch granules they had initially in the cytoplasm. Indeed, as shown in Table I, the average cell volume increased more than 100% after 9 h in the Pi-enriched medium (4P), although the corresponding volume increase in control was only 10%. The increment was mainly due to increase in cell diameter. If these changes in the cell volume are taken into account, the average intracellular concentrations of Pi initially and at 9 h in 1P and 4P media are 0.98, 27.1, and 52.2 mM, respectively. In the present paper, however, all the values for the short-term studies (Figs. 3 and 4) are expressed on the basis of initial fresh weight of the cell at the beginning of each experiment, because we could not determine the changes in the average cell size throughout the time-course experiments.

Table I. *Swelling of C. roseus cells during uptake of Pi*

Change in cell size was determined under a microscope immediately and 9 h after transfer to MS media of different Pi concentrations. Data are from the same experiment as in Figure 4. ICD was 4×10^5 mL^{-1}. Pi concentration was 1.25 mM in control (1P) and 5 mM in 4P. Values are averages of 20 or 21 cells with SDs. Cell volume was calculated on the assumption that the cell shape was a cylinder. Values in parentheses are cell volumes relative to that of initial cells (100).

Cell	Length	Diameter	Volume	No. of Cells Determined
	μm	μm	pL	
Initial	126 ± 17	31 ± 8	95 (100)	20
9 h				
1P (1.25 mM)	129 ± 35	32 ± 7	104 (109)	20
4P (5.0 mM)	137 ± 35	44 ± 9	208 (219)	21

Kinetics of Pi Uptake by *C. roseus* Cells

Figure 5 shows a time course of Pi uptake by *Catharanthus* cells. In this experiment, we used, instead of MS medium, a simple medium composed of $CaCl_2$, KCl, and Glc as described previously (Sakano et al., 1992). This was to avoid interference by other ions in the determination of sub-micromolar concentration of Pi by ion chromatography. The rate of Pi uptake in this medium was almost the same as that in MS medium (see below).

Pi uptake by the cells was a linear function of time until Pi concentration declined to about 10 μM, at which point

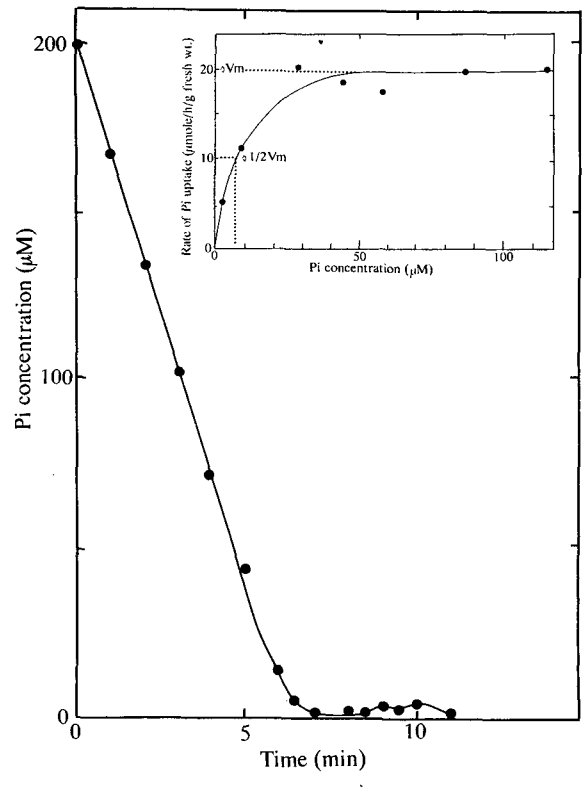

Figure 5. Rate of Pi uptake as a function of Pi concentration in the medium. Ten micromoles of sodium phosphate were added to the cell suspension of *C. roseus* (5 g fresh weight in 50 mL of medium) at time 0. At intervals, samples were taken and Pi concentration in the medium was determined as described in "Materials and Methods." Inset, Michaelis-Menten plot of the uptake rate-Pi concentration relationship from the same experimental data, indicating V_m = 20 μmol g^{-1} fresh weight h^{-1}, and K_m = about 7 μM.

the uptake slowed abruptly. In this time course, Pi concentration and uptake rate (slope of the curve) changed simultaneously with time. Therefore, we obtained several sets of data (Pi concentration versus uptake rate) along the curve and plotted according to the Michaelis-Menten equation (inset in Fig. 5). V_m and K_m were 20 μmol g^{-1} fresh weight h^{-1} and about 7 μM, respectively. Although V_m varied with the cell age (10–24 μmol g^{-1} fresh weight h^{-1}), the linearity of the curve to about 10 μM was always observed when the initial Pi concentrations were 2 mM and below (data not shown).

Intracellular Accumulation of Pi Observed by ^{31}P-NMR

Catharanthus cells used in this experiment were the d-4 cells that were in the state of "Pi-starvation," since, in addition to exhaustion of medium Pi, resonance assigned as neither Pi in the cytoplasm nor Pi in the vacuole was detected in the ^{31}P-NMR spectrum. However, this may not mean that the *Catharanthus* cells had completely run out of intracellular Pi, because the presence of Pi in an NMR-invisible form in *Catharanthus* cells has been reported (Brodelius and Vogel, 1985).

On application of the first 5 μmol of Pi (at time B in Fig. 6), the cytoplasmic and vacuolar Pi resonance peaks appeared and their heights increased. The external (medium) Pi peak also appeared, but its height decreased rapidly. It is noteworthy that the increase in the cytoplasmic Pi level was temporary and soon leveled off at a "higher level," whereas the increase in the vacuolar Pi level continued throughout the Pi uptake period. However, as soon as external Pi was exhausted, the cytoplasmic Pi level decreased to a "lower level" (about one-half to one-third of the higher level) and the vacuolar Pi increase leveled off. These observations are consistent with those reported previously (Sakano et al., 1992). In the present paper, the lower level is designated as the regular cytoplasmic Pi concentration of the cells that are not starved of Pi. On the other hand, the higher level is the transient, two to three times higher cytoplasmic Pi concentration compared with the lower level seen, usually, only while the cells are absorbing Pi.

The second application of Pi (18 μmol, at time C in Fig. 6) induced similar changes at the beginning: the cytoplasmic Pi level stepped up to the higher level and the vacuolar level increased continuously. As Pi uptake proceeded, however, the cytoplasmic Pi and external Pi peaks in the spectrum overlapped each other (data not shown), because these two peaks were located close to each other in the NMR spectrum and the proton/Pi co-transport mechanism brought them closer through simultaneous acidification of the cytoplasm and alkalinization of the external medium (Sakano, 1990; Sakano et al., 1992). Therefore, further changes in cytoplasmic Pi level could not be followed (Fig. 6). The vacuolar Pi level increased almost linearly until a maximum level was attained about 330 min after application. External level decreased linearly for the first 120 min but its rate decreased gradually to nil.

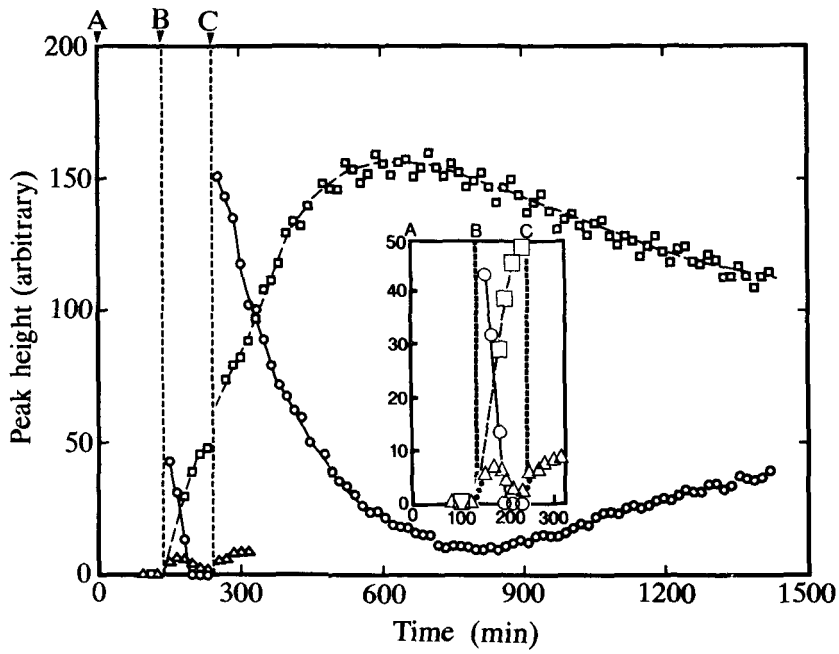

Figure 6. Changes in the relative content of Pi in cytoplasm (△), vacuole (□), and external medium (○) during uptake of Pi by *C. roseus* cells. Day-4 cells (0.36 g fresh weight) were suspended in 3.6 mL of the medium as described in "Materials and Methods." Bubbling with oxygen gas was started at time A. First application of Pi (5 μmol) was at about 130 min after initiation of oxygen bubbling (time B). Second application of Pi (18 μmol) was about 100 min after the first application (time C). Resonance peaks for the cytoplasmic and external Pi overlapped each other soon after the second application, and no further data for cytoplasmic Pi level were obtainable. See inset for the details of the early stage of the time course.

Decrease in vacuolar Pi and increase in external Pi started simultaneously about 10 h after the second Pi application. These features are consistent with those observed in the experiments shown in Figures 1 to 4. In addition, it is clear that the vacuole is the main site of intracellular Pi storage.

Changes in Intracellular and External pH during Pi Uptake

Figure 7 shows the changes in intracellular and external pH values during uptake of Pi as estimated from the chemical shift values of the Pi resonance peaks assigned. As mentioned above, the d-4 cells used in this experiment did not have enough Pi to be detected by NMR spectroscopy either in the cytoplasm or in the vacuole. Therefore, pH values of neither cytoplasm nor vacuole could be determined for the cells before Pi application. However, it has been established that, after bubbling with oxygen gas for longer than 60 min, pH values for the cytoplasm and vacuole are about 7.5 and 5.2 to 5.3, respectively (Sakano et al., 1992). Thus, upon first application of Pi (at time B in Fig. 7), the cytoplasmic pH was inferred to drop from about 7.5 to about 7.3 and the external pH increased (as a result of proton/Pi co-transport) until external Pi was exhausted. Vacuolar pH was also inferred to drop slightly during the same time.

As soon as external Pi was exhausted, the cytoplasmic pH recovered its normal value (about 7.55) after an over-shoot to about 7.7. A similar over-shoot was also seen during the recovery of vacuolar pH. Probable recovery of medium pH (acidification) could not be confirmed in the NMR spectrum because of Pi exhaustion in the external medium. Such pH responses during and after Pi uptake are consistent with those reported previously (Sakano, 1990; Sakano et al., 1992).

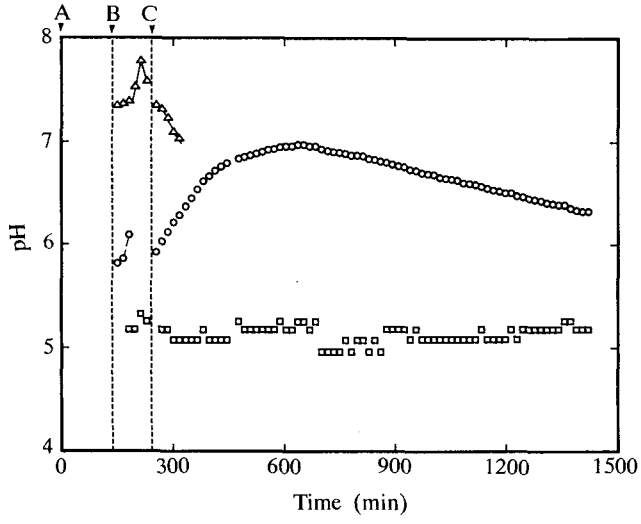

Figure 7. Changes in the pH of cytoplasm (△), vacuole (□), and external medium (○) during uptake of Pi by *C. roseus* cells. Data are from the same experiment as shown in Figure 6. No data were available before the first Pi application, since the d-4 cells used in the experiment had no visible (by NMR) amount of Pi either in the cytoplasm or the vacuole. However, the cytoplasmic and vacuolar pH immediately before the first application of Pi are inferred to be about 7.5 and 5.2 to 5.3, respectively (see "Results").

The second application of Pi (18 μmol, final concentration 5 mM, at time C in Fig. 7) induced pH changes similar to those observed in the first application in all the compartments. However, this was so only for the first few hours: the cytoplasmic pH never recovered the normal value (7.5) because Pi uptake continued (as seen in the prolonged expansion of the vacuolar Pi pool) against the inexhaustible Pi in the external medium (Fig. 6). Further acidification to 7.04 was observed before the cytoplasmic Pi peak overlapped with the external Pi peak (data not shown). The increase of the external pH leveled off about 6 h after the second Pi application, and a slow acidification followed. When the external pH dropped below 6.3 (about 20 h after the second Pi application), the resonance peak that would have corresponded to cytoplasmic Pi could not be detected in the resonance range covering pH 7.5 to 6.7. This suggests that the cytoplasmic pH dropped below 6.7 during Pi uptake. Vacuolar pH decreased slightly during Pi uptake, but not below 5.0.

DISCUSSION

Uptake and Accumulation of Pi in the Cell

The present results indicate that *Catharanthus* cells are equipped with a high-affinity uptake system for Pi (K_m = about 7 μM) with an extraordinarily high V_m (about 20 μmol g^{-1} fresh weight h^{-1}) compared with other plants and cells (0.22–3.9 μmol g^{-1} fresh weight h^{-1}, and 19–24 μmol g^{-1} dry weight h^{-1}; see citations in Lee et al., 1990). These kinetic parameters indicate that the causes of growth inhibition under low ICD and high-medium Pi concentrations (Sakano et al., 1995) are the same: excess Pi is taken up by the cells that have no safety mechanism against excess intake, which results in toxicity.

Under the conditions that induced excess intake, cells were swollen and a lot of cell debris was found in the medium. We have confirmed that the cell viability decreased appreciably within 1 d under both low ICD and high-Pi conditions (Sakano et al., 1995). This was likely brought about by increased intracellular osmolarity that was caused by the accumulated Pi as well as by the enhanced starch phosphorolysis promoted by the elevated Pi level (Preiss and Levi, 1980).

^{31}P-NMR studies also confirmed the excess intake. When Pi was abundantly available, cells continued to absorb Pi from external medium until a large amount of Pi accumulated in the vacuole (Fig. 6), which was estimated to be more than 100 μmol g^{-1} fresh weight (Figs. 1a and 3a). In addition, the cytoplasmic Pi concentration was increased to the higher level and did not recover the regular lower level. Lee et al. (1990) also reported a similar increase in the cytoplasmic Pi concentration (higher than normal level) during prolonged uptake of Pi by Pi-starved maize roots. It is noteworthy that the excess intake took place under the conditions that acidified the cytoplasm below pH 7.04, which was much lower than the corresponding value (about 7.3) of the cells absorbing nontoxic amounts of Pi (Sakano et al., 1992).

Mechanism Responsible for Cytoplasmic Pi Homeostasis

It has repeatedly been reported that the cytoplasmic level of Pi in plant cells is under strict control (Rebeille et al., 1983; Lee et al., 1990; Mimura et al., 1990). Using ^{31}P-NMR, Lee et al. (1990) found that the vacuolar content of Pi in maize root tissue changed extensively in response to Pi starvation and refeeding. In contrast, the cytoplasmic content (about 6.5 mol m^{-3}) scarcely changed during the same treatments. It was only after a severe Pi stress that the cytoplasmic Pi level started to decrease.

In *Catharanthus* cells, similar cytoplasmic Pi homeostasis was observed when the cells were supplied with nontoxic amounts of Pi (the first application in Fig. 6; see also figs. 6 and 7 in Sakano et al., 1992). The cytoplasmic Pi concentration stepped up from the ordinary lower level to the transient higher level that was maintained throughout the uptake periods, whereas the vacuolar Pi content continued to increase. When the external Pi was exhausted, the cytoplasmic Pi level decreased to the original, regular, lower level. The results indicate that the transport at the tonoplast plays a central role in the cytoplasmic Pi homeostasis. The transient higher level of cytoplasmic Pi observed during uptake may not be a simple reflection of differences in the rates of Pi transports at the plasma membrane (responsible for import from external medium) and at the tonoplast (responsible for export to the vacuole). It seems logical to assume that the higher level is the signal to activate the tonoplast transport system (exporter to the vacuole).

Also, it is a common observation that a constant cytoplasmic Pi level is maintained at the expense of the vacuolar Pi pool under the condition of Pi deprivation from external medium (Rebeille et al., 1983; Lee et al., 1990). This again indicates that the Pi transport at the tonoplast (importer from the vacuole) plays a critical role in the cytoplasmic Pi homeostasis.

Does the Plasma Membrane Pi Transport System Play Any Role in the Cytoplasmic Pi Homeostasis?

The question arises about whether the plasma membrane system has any regulatory role in the cytoplasmic Pi homeostasis.

In *Neurospora*, coordination of high- and low-affinity uptake systems is known to control the Pi uptake rate constant against varying external Pi concentration (Beever and Burns, 1980). Although similar dual-uptake systems have been reported also in plant cells (Furihata et al., 1992; Schmidt et al., 1992), we detected no second uptake isotherm component with low affinity in the *Catharanthus* cells we used. But, with only the high-affinity uptake system (K_m = 7 μM), a constant uptake rate (V_m) could be shown over external Pi concentration from about 10 μM to at least 2 mM in the *Catharanthus* cells. Although constant, their abnormally high uptake rate caused an excess intake under abundant supply. Thus, controlling Pi uptake at a constant rate makes sense only when the controlled rate meets the requirement for growth, which was not the case in the *Catharanthus* cells.

The importance of both influx and efflux at the plasma membrane for the regulation of net Pi uptake has been suggested (Bieleski and Ferguson, 1983; Elliott et al, 1984). In the present study we measured only net uptake. The uptake was so exhaustive that no significant net Pi efflux into medium could be detected after medium Pi was taken up (e.g. see Fig. 5). Although we have observed that cell bursts took place during and after excess Pi uptake, the possible occurrence of concomitant efflux cannot be excluded. It might explain a part of the observed gradual decrease in the uptake rate, and a part of the Pi release that followed (Figs. 3b and 4b). However, the eventual excess intake of Pi indicates that the efflux, if it occurred, failed to control the net Pi uptake, and therefore, failed to achieve cytoplasmic Pi homeostasis.

These results indicate that, at least in *Catharanthus* cells, no effective mechanism is present in the plasma membrane to adjust the net rate of Pi uptake in accordance with the concentration of Pi in the cytoplasm, and that only the Pi transport system(s) (importer and exporter) in the tonoplast are responsible for the cytoplasmic Pi homeostasis. A similar rationale has been proposed by Rebeille et al. (1983).

Why No Regulation of Pi Uptake in *Catharanthus* Cells?

In many plants, Pi starvation induces enhancement of Pi uptake rate, which has been suggested to accompany a net increase of Pi transporter (coarse control) in the plasma membrane (Anghinoni and Barber, 1980; Lee, 1982; Cogliatti and Clarkson, 1983; Drew et al., 1984; McPharlin and Bieleski, 1987).

During evolution, plants might not have experienced such a Pi-rich environment as to cause an excess intake and, therefore, no fine control mechanism might have been necessary. In other words, coarse control over Pi uptake capacity would have been sufficient for most plants to survive under such an environment (Bieleski and Ferguson, 1983). The maximum number of Pi transporters per cell (V_m) might be determined, from the viewpoint of cell economy, by the importance of Pi uptake relative to that of other cellular processes that require energy as well.

In contrast, *Catharanthus* cells used in the present study have been subcultured for years in MS medium, a rich, nutrient medium from which various nutrients except for Pi were never exhausted during growth of the cells (data not shown). Pi was the primary limiting nutrient in the medium (Amino et al., 1983). We suppose that repeated subcultures of *Catharanthus* cells over years in the "Pi-limited" MS medium may have selected a cell line that adapted to the mode of Pi feeding: only those that could take up the largest amount of Pi immediately after subculture (cells having larger V_m and smaller K_m) could become the major population.

The extraordinarily high Pi uptake rate in *Catharanthus* cells compared with other plants and cells suggests that the Pi transporter gene of the cell may be constitutively expressed under the culture conditions. Our current work is directed toward isolating the Pi transporter in the plasma membrane and identifying its gene to reveal the regulatory mechanism of gene expression (Okihara et al., 1995).

ACKNOWLEDGMENT

We are grateful to Yokogawa Analytical Systems for its kind cooperation in measuring Pi with its ion chromatographic analyzer (IC-7000 M).

Received October 3, 1994; accepted January 30, 1995.
Copyright Clearance Center: 0032-0889/95/108/0295/08.

LITERATURE CITED

Amino S, Fujimura T, Komamine A (1983) Synchrony induced by double phosphate starvation in a suspension culture of *Catharanthus roseus*. Physiol Plant **58:** 393–396

Anghinoni I, Barber SA (1980) Phosphorous influx and growth characteristics of corn roots as influenced by phosphorus supply. Agron J **72:** 685–688

Beever RE, Burns DJW (1980) Phosphate uptake, storage and utilization by fungi. Adv Bot Res **8:** 127–129

Bencini DA, Wild JR, O'Donovan GA (1983) Linear one-step assay for the determination of orthophosphate. Anal Biochem **132:** 254–258

Bieleski RL, Ferguson IB (1983) Physiology and metabolism of phosphate and its compounds. *In* A Läuchli, RL Bieleski, eds, Encyclopedia of Plant Physiology, New Series, Vol 15A. Springer-Verlag, Berlin, pp 422–449

Brodelius P, Vogel HJ (1985) A phosphorus-31 nuclear magnetic resonance study of phosphate uptake and storage in cultured *Catharanthus roseus* and *Daucus carota* plant cells. J Biol Chem **260:** 3556–3560

Clarkson DT, Scattergood CB (1982) Growth and phosphate transport in barley and tomato plants during the development of, and recovery from, phosphate-stress. J Exp Bot **33:** 865–875

Cogliatti DH, Clarkson DT (1983) Physiological changes in, and phosphate uptake by potato plants during development of, and recovery from phosphate deficiency. Physiol Plant **58:** 287–294

Drew MC, Saker LR, Barber SA, Jenkins W (1984) Changes in the kinetics of phosphate and potassium absorption in nutrient-deficient barley roots measured by a solution-depletion technique. Planta **160:** 490–499

Dunlop J, Gardiner S (1993) Phosphate uptake, proton extrusion and membrane electropotentials of phosphorus-deficient *Trifolium repens* L. J Exp Bot **44:** 1801–1808

Elliott GC, Lynch J, Läuchli A (1984) Influx and efflux of P in roots of intact maize plants. Double-labeling with ^{32}P and ^{33}P. Plant Physiol **76:** 336–341

Furihata T, Suzuki M, Sakurai H (1992) Kinetic characterization of two phosphate uptake systems with different affinities in suspension-cultured *Catharanthus roseus* protoplasts. Plant Cell Physiol **33:** 1151–1157

Lee RB (1982) Selectivity and kinetics of ion uptake by barley plants following nutrient deficiency. Ann Bot **50:** 429–449

Lee RB (1988) Phosphate influx and extracellular phosphatase activity in barley roots and rose cells. New Phytol **109:** 141–148

Lee RB, Ratcliffe RG, Southon TE (1990) ^{31}P NMR measurements of the cytoplasmic and vacuolar Pi content of mature maize roots: relationships with phosphorus status and phosphate fluxes. J Exp Bot **41:** 1063–1078

Lefebvre DD, Glass ADM (1982) Regulation of phosphate influx in barley roots: effects of phosphate deprivation and reduction of influx with provision of orthophosphate. Physiol Plant **54:** 199–206

McPharlin IR, Bieleski RL (1987) Phosphate uptake by *Spirodela* and *Lemna* during early phosphorus deficiency. Aust J Plant Physiol **14:** 561–572

Mimura T, Dietz K-J, Kaiser W, Schramm MJ, Kaiser G, Heber U (1990) Phosphate transport across biomembranes and cytosolic phosphate homeostasis in barley leaves. Planta **180:** 139–146

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol Plant **15:** 437–497

Okihara K, Mimura T, Kiyota S, Sakano K (1995) Furosemide: a specific inhibitor of Pi transport across the plasma membrane of plant cells. Plant Cell Physiol **36:** 53–58

Preiss J, Levi C (1980) Starch biosynthesis and degradation. *In* J Preiss, ed, The Biochemistry of Plants, Vol 3. Academic Press, New York, pp 371–423

Rebeille F, Bligny R, Douce R (1982) Regulation of Pi uptake by *Acer pseudoplatanus* cells. Arch Biochem Biophys **219:** 371–378

Rebeille F, Bligny R, Martin J-B, Douce R (1983) Relationship between the cytoplasm and the vacuole phosphate pool in *Acer pseudoplatanus* cells. Arch Biochem Biophys **225:** 143–148

Sakano K (1990) Proton/phosphate stoichiometry in uptake of inorganic phosphate by cultured cells of *Catharanthus roseus* (L.) G. Don. Plant Physiol **93:** 479–483

Sakano K, Matsumoto M, Yazaki Y, Kiyota S, Okihara K (1995) Inorganic phosphate as a negative conditioning factor in plant cell culture. Plant Sci (in press)

Sakano K, Yazaki Y, Mimura T (1992) Cytoplasmic acidification induced by inorganic phosphate uptake in suspension cultured *Catharanthus roseus* cells. Measurement with fluorescent pH indicator and ^{31}P-nuclear magnetic resonance. Plant Physiol **99:** 672–680

Schmidt M-E, Heim S, Wylegalla C, Helmbrecht C, Wagner KG (1992) Characterization of phosphate uptake by suspension cultured *Catharanthus roseus* cells. J Plant Physiol **140:** 179–184

Ullrich-Eberius CI, Novacky A, Fischer E, Lüttge U (1981) Relationship between energy-dependent phosphate uptake and the electrical membrane potential in *Lemna gibba* G1. Plant Physiol **67:** 797–801