Plant Cell Reports (1983) 2:140–143

Plant Cell Reports

Rapid Effect of an Elicitor on Uptake and Intracellular Distribution of Phosphate in Cultured Parsley Cells

H. Strasser[1], K. G. Tietjen[1], K. Himmelspach[2], and U. Matern[1]

[1] Institut für Biologie II, Lehrstuhl für Biochemie der Pflanzen, Albert-Ludwigs-Universität Freiburg, Schänzlestr. 1, D-7800 Freiburg, FRG

[2] Max-Planck-Institut für Immunbiologie, Stübeweg 51, D-7800 Freiburg, FRG

Received April 7, 1983

Communicated by H. Kleinig

ABSTRACT

Cell suspension cultures of parsley (Petroselinum hortense) grown in synthetic medium take up most of the inorganic phosphate supplied with the medium within the initial 5 days after transfer. Nuclear magnetic resonance spectra of intact parsley cells from this growth stage revealed that approximately half of the phosphate was located within the vacuoles, whereas after 7 days of growth phosphate content of the vacuoles was relatively low. At both times, addition of an elicitor preparation from Alternaria carthami, which is not toxic to the cells, led to a temporary increase of vacuolar phosphate at the expense of cytoplasmic phosphate, even when excess phosphate was added to the medium. The rapid decrease of cytoplasmic phosphate might play a role in the redirection of phenylpropanoid metabolism reported for elicitor-treated parsley cells.

ABBREVIATIONS

Ac elicitor: elicitor preparation from cell walls of Alternaria carthami Chowdhury
Pmg elicitor: elicitor preparation from cell walls of Phytophthora megasperma Drechsler f.sp. glycinea Kuan and Erwin

INTRODUCTION

Plants, as well as plant cell cultures, produce antibiotics upon challenge with either fungi or fungal elicitor preparations (Albersheim and Valent 1978, Ebel et al. 1976). Conceivably, these antibiotics serve in the defense of plants and have been termed phytoalexins. Several coumarin derivatives have been isolated as phytoalexins from suspension-cultured parsley cells after treatment with elicitor preparations derived from cell walls of either Alternaria carthami (Ac elicitor) or Phytophthora megasperma f. sp.glycinea (Pmg elicitor) (Tietjen et al. 1983). Elicitor-induced increases in the enzymatic activities of phenylalanine ammonia-lyase (EC 4.3.1.5), 4-coumarate:CoA ligase (EC 6.2.1.12), and an enzyme specifically involved in coumarin biosynthesis, dimethylallyl pyrophosphate:umbelliferone dimethylallyl transferase, have been determined in these cells (Tietjen and Matern 1983). De novo enzyme synthesis and a rapid induction of mRNA synthesis for phenylalanine ammonia-lyase and 4-coumarate:CoA ligase have been demonstrated in response to elicitor treatment of parsley cells (Hahlbrock et al. 1981, Kuhn DN, Chappell J, Boudet AM and Hahlbrock K, unpublished results).
Phytoalexin accumulation may be generally induced in plants by a number of different fungal elicitors (West 1981), as well as by a great many other compounds including heavy metal ions (Hargreaves 1979), detergents (Hargreaves 1981) and endogeneous elicitors derived from plant cell walls (Hahn et al. 1981). The molecular mechanism by which all of these compounds bring about the induction of similar biosynthetic pathways in one plant is unknown. It seems reasonable that a common signal is transmitted to the plant's genome after recognition of any kind of elicitor by the plant cell, thus activating a distinct pattern of defense reactions. To date, however, the chemical or physiological nature of such a common signal has not been defined.

In this report we describe the general effect of an elicitor on the subcellular phosphate distribution in parsley cells. The effect is rapid and reversible, and may be involved in the modulated pattern of secondary metabolites produced by elicitor-induced parsley cells.

MATERIALS AND METHODS

All chemicals were of analytical grade. Methylenediphosphonic acid was from Sigma,München. Deuterium oxide (100%) was purchased from EGA Chemie, Steinheim. Sodium dihydrogen [^{32}P] orthophosphate (7.4 TBq/mol) was from Amersham-Buchler, Braunschweig.
Parsley cell cultures were continuously dark-grown in B5 medium (Gamborg et al. 1968). Cells were grown in manganese-free medium for 2 weeks prior to spectral analysis.
Elicitor was prepared from cell walls of either Alternaria carthami (Ac elicitor) or Phytophthora megasperma f.sp.glycinea (Pmg elicitor) as reported previously (Tietjen et al. 1983, Ayers et al. 1976).
^{31}P-Nuclear magnetic resonance spectroscopy of intact parsley cells was carried out in a Bruker WM 300 spectrometer. NMR-Tubes of 1 cm internal diameter were used. Deuterium oxide (0.5 ml) was added to 3.5 ml of packed pars-

ley cells in these tubes, and spectra were acquired under the conditions described by Martin et al. (1982). Methylenediphosphonic acid was used as an external standard. The cells were not aerated during spectroscopy (900 scans, 30 minutes), but remained viable as determined by oxygen uptake subsequently. Definition of chemical shifts in all spectra was based on the reference signal set at 1,925 Hz (+ 15.8427 ppm). The calibration of chemical shifts with respect to pH of the solution was achieved according to Martin et al. (1982) using either 50 mmol/l potassium phosphate buffers, pH 4.5 to 6.4, containing 20 mmol/l KCl, 30 mmol/l $CaCl_2$, 30 mmol/l $MgCl_2$, 30 mmol/l citrate and 10 mmol/l malate or 50 mmol/l potassium phosphate buffers, pH 6 to 8, containing 100 mmol/l KCl, 1 mmol/l $CaCl_2$ and 1 mmol/l $MgCl_2$.
Radioactivity in the cells and the growth medium was determined by liquid scintillation counting in dioxane containing 100 g naphthalene and 5 g diphenyloxazole per liter. Cells were separated from the medium by filtration and washed extensively with fresh growth medium on the filter prior to liquid scintillation counting.

RESULTS

Preliminary evidence for different degrees of toxicity of Pmg and Ac elicitors has been reported previously (Tietjen and Matern 1983). The effects of both elicitors on the parsley cell growth were determined by transferring portions (2 g fresh weight, equivalent to 0.7 g after filtration) of 7-day-old cells into fresh medium (40 ml), which contained between 0.001 and 0.25 mg/ml dry weight of either Ac or Pmg elicitor. Cells were propagated in the dark and harvested after an additional 13 days by filtration. While the increase in cell fresh weight was reduced considerably with respect to control cells in the presence of as little as 0.025 mg/ml Pmg elicitor

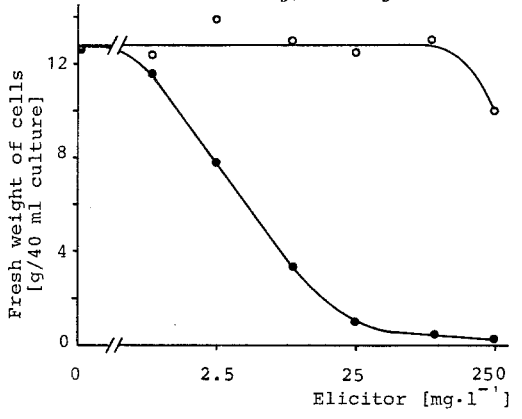

Fig.1. Effect of increasing amounts of Ac elicitor (o) or Pmg elicitor (●) on the fresh weight of parsley cells 13 days after inoculation of the cultures.

(Fig.1), only a slight reduction in the cell fresh weight increase was noticed after treatment with the highest concentration of Ac elicitor (Fig.1). In all further experiments, a concentration of 0.25 mg/ml of Ac elicitor was employed for maximal induction.

A rapid uptake of the extracellular phosphate by parsley cells was anticipated based on work with other cultured plant cells (Knobloch et al. 1981). Uptake of [^{32}P]orthophosphate into suspension-cultured cells was monitored

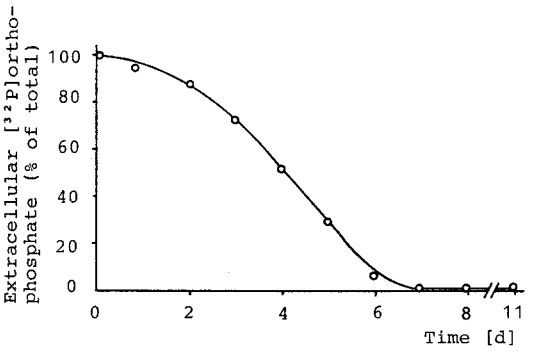

Fig.2. Uptake of phosphate, as determined by the decrease of phosphate in the medium, by cultured parsley cells. About 2.6 g fresh weight of cells was transferred into fresh B5 medium (40 ml) containing [^{32}P]orthophosphate (50 µmol , approximately 11 GBq/mol). Cells were propagated in the dark, and the radioactivity present in the medium was determined for 11 days as percent of total.

for 11 days (Fig.2). The cells were carefully removed by filtration, and the radioactivity present in the supernatant was determined. Results were corrected for the decay rate of [^{32}P]orthophosphate during the 11-day growth period, and data were expressed as percentage of the amount of orthophosphate present in the growth medium at the beginning of the experiments (Fig.2). After approximately 7 days of growth, almost all of the phosphate had been taken up by the cells, and the amount of radioactivity in the medium (approximately 0.8% of the initial radioactivity) did not decrease any further. At this growth stage, the division rate of parsley cells diminishes while the cells begin to enlarge (Hahlbrock et al. 1980). No distinction was made with respect to a possible incorporation of the residual radioactivity into other compounds, although the presence of organic phosphate in the growth medium of viable cells appears unlikely.
The phosphate uptake rate by 8-day-old parsley cells was monitored in the presence and absence of Ac elicitor (Fig.3). The pH of the growth medium of these cultures was determined to be 6.2. In the presence of elicitor, phosphate uptake was inhibited considerably.

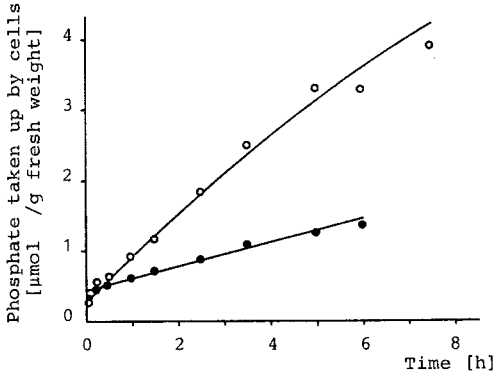

Fig.3. Inhibition of phosphate uptake by Ac elicitor. The amount of phosphate in the cells was monitored for several hours after addition of either (o) 1 ml of sodium [^{32}P]phosphate buffer pH 6.2 (50 µmol , 3.7 GBq/mol) or (●) 1 ml of this buffer plus Ac elicitor (10 mg) to 40 ml portions of an 8-day-old parsley suspension culture.

Treatment of 8-day-old parsley cultures, which had taken up all of the extracellular

phosphate, with Ac elicitor and phosphate of high specific radioactivity (Fig.4) revealed that the inhibitory effect of the elicitor on the phosphate uptake was rapid. Moreover, when the elicitor was added to the cultures later than the phosphate, the uptake rate was also reduced almost completely within 10 minutes (Fig.5).

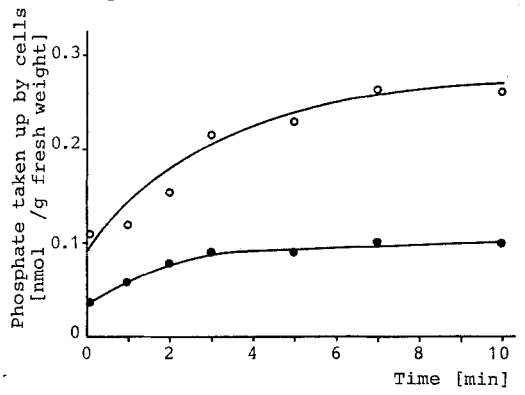

Fig.4. Inhibition of phosphate uptake by Ac elicitor. The amount of phosphate in the cells was monitored for 10 minutes after addition of either (o) sodium [^{32}P]orthophosphate (18.5 KBq, 7.4 TBq/mol) or (●) this amount of [^{32}P]phosphate plus Ac elicitor (10 mg) to 40 ml portions of an 8-day-old parsley suspension culture.

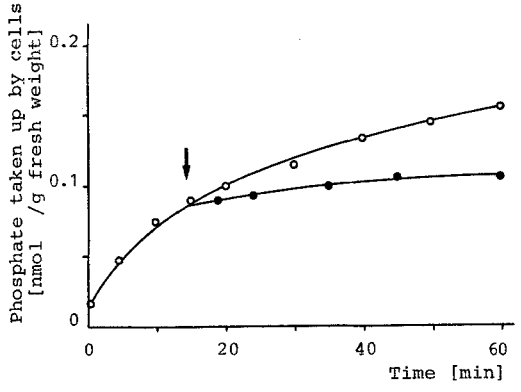

Fig.5. Inhibition of phosphate uptake by Ac elicitor. Sodium [^{32}P]orthophosphate (18.5 KBq, 7.4 TBq/mol) was added to an 8-day-old parsley cell culture, and the amount of phosphate accumulating in the cells (o) was monitored. The arrow indicates the time when Ac elicitor (0.25 mg/ml) was added to a portion of the culture. Further phosphate uptake by these cells was monitored separately (●).

An experiment was devised in order to determine whether the inhibitor of the phosphate uptake can be removed again from the parsley cells after inhibition had been induced. Parsley cells were exposed to Ac elicitor for 10 minutes, and subsequently the cells were extensively washed on a filter with fresh medium. Half of the cells was transferred into fresh growth medium, the other half was transferred into fresh medium containing Ac elicitor. Control cells derived from the same culture received the same treatment except that no elicitor was added to the cells either before or after the washing (Fig.6). While the non-elicitor-treated cells and the cells from which the elicitor had been washed out took up phosphate at about the same rate, the uptake by cells propagated in the presence of elicitor was greatly reduced (Fig.6).

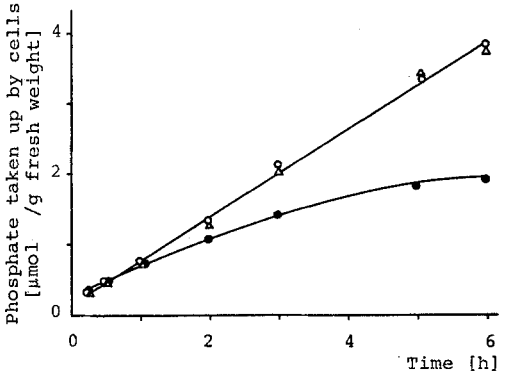

Fig.6. Reversal of Ac elicitor inhibition of phosphate uptake in 8-day-old parsley cells. Cells were treated with Ac elicitor (10 mg/40 ml culture) for 10 minutes, washed carefully and transferred into either (Δ) B5 medium or (●) B5 medium containing Ac elicitor. Control cells (o) were treated similarly except that at no time was elicitor added. Sodium [^{32}P]orthophosphate (7.4 TBq/mol , 37 KBq/40 ml culture) was added and uptake of phosphate by the cells was monitored.

^{31}P-Nuclear magnetic resonance (^{31}P-NMR) spectroscopy has been employed to distinguish the inorganic phosphate in the cytoplasm and in the vacuole of intact plant cells (Martin et al. 1982). The chemical shift of the corresponding signals is largely dependent on the pH of the phosphate microenvironment, and allows in turn the determination of the pH of vacuole and cytoplasm with reference to standard salt solutions (Martin et al. 1982).

When 5- or 7-day-old parsley cells were subjected to ^{31}P-NMR spectroscopy, two signals between approximately 0 and + 2 ppm were observed and assigned to the inorganic vacuolar and cytoplasmic phosphate, respectively (Fig. 7). An almost equal distribution of the phosphate between both cell compartments was determined after 5 days of growth. After 7 days of growth, the residual vacuolar phosphate gave rise to only a small signal, as compared to the large cytoplasmic phosphate signal (Fig.7B).

A vacuolar pH of 5.5 was determined at both growth stages. In the cytoplasm, a pH of approximately 7.2 was estimated. It should be noted, however, that the latter value represents the pH in non-aerated cells which is reportedly lower than in aerated plant cells (Martin et al. 1982), while the vacuolar pH does not depend so much on the aeration rate of the cells.

Treatment of either 5- or 7-day-old parsley cells with Ac elicitor induced a rapid change in the relative proportion of cytoplasmic and vacuolar phosphate contents as monitored by ^{31}P-NMR spectroscopy at different time intervals (Fig.7). For technical reasons, spectra from the elicitor-treated cells were not taken between 3.5 and 21 h after elicitor addition. While at 3.5 h a low cytoplasmic phosphate content was still apparent, the phosphate content had once more reached the values seen in non-treated cells 21 h after elicitor addition.

The pH of the vacuoles decreased to approximately 5.1 within 1 h after addition of the elicitor. Within an additional 2 h, this value increased again to 5.4. Apparently, the elicitor effect on the vacuolar pH was as

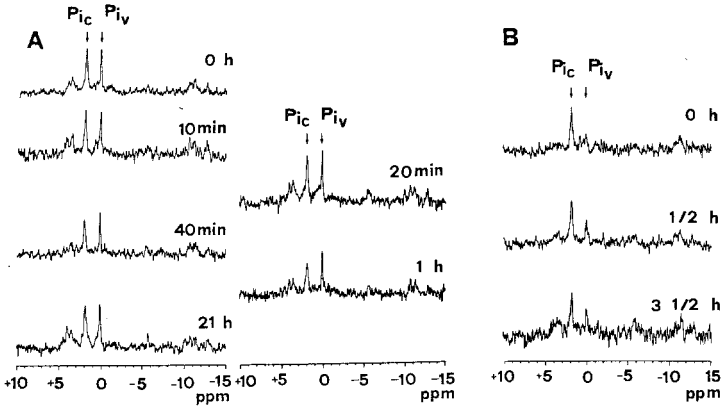

Fig.7. ^{31}P-Nuclear magnetic resonance spectroscopy of intact parsley cells harvested at various times after Ac elicitor treatment. Either 5-day (A) or 7-day (B) old cultures grown in manganese free B5 medium were used for the experiments. Chemical shifts are indicated with reference to Martin et al. (1982) using the methylenediphosphonic acid signal at 1,925 Hz as a standard. Signals assigned to the cytoplasmic inorganic phosphate (P_{ic}) and the vacuolar inorganic phosphate (P_{iv}) are indicated by arrows.

rapid as the effect on the subcellular phosphate distribution, but the former effect was reversed prior to the latter effect (Fig.7).

DISCUSSION

^{31}P-Nuclear magnetic resonance spectroscopy of intact parsley cells revealed that an elicitor preparation from Alternaria carthami induces a rapid redistribution of intracellular phosphate leading to an elevated vacuolar concentration and a reduced cytoplasmic concentration. Concomitantly, the pH of the vacuoles decreased by about 0.5 units within 60 minutes.

Several metabolic pathways appear to be regulated by phosphate, and the formation of secondary metabolites induced by a limited phosphate supply in the growth medium has been reported for bacteria (Martin 1977), fungi (Krupinski et al. 1976), and also for higher plant cells (Knobloch et al. 1981). In the latter case, phenylpropanoid metabolism in Nicotiana cells was changed in such a way that large quantities of cinnamoyl putrescine were formed. This effect was reversed by addition of excess phosphate to the medium. It is to be expected that, under limited extracellular phosphate supply, the intracellular phosphate concentration decreases, although to date no relevant data are available. The decreased cytoplasmic phosphate concentration which we have demonstrated in elicitor-treated parsley cells may similarly exert its effect on the cell's phenylpropanoid metabolism, leading in turn to the formation of the fungitoxic coumarins described previously (Tietjen et al. 1983).

The rapid inhibition of cellular phosphate uptake by the Ac elicitor preparation may also serve in the regulation of intracellular phosphate levels where excess phosphate is present externally to the cells. The uptake inhibition precludes an experimental reversal of the elicitor-induced phosphate flux by exogeneous phosphate.

An apparent inhibition of phosphate uptake may have possibly been caused by reaction of the medium phosphate with the elicitor. Since the elicitor preparation does not pass through a dialysis membrane (Tietjen et al. 1983), it's molecular weight must be at least 14,000. Thus, the elicitor concentration used in these experiments was no greater than 18 µmol $\cdot l^{-1}$. In contrast, the concentration of phosphate was 1.25 mmol $\cdot l^{-1}$. It appears rather unlikely, therefore, that the elicitor preparation could have bound the entire extracellular phosphate.

All the elicitor effects described above were reversed within 21 h after addition of the elicitor to the cells. Moreover, the elicitor could be washed out from the parsley cells after short time exposure. A reversal of the phytoalexin induction by washing out the elicitor was also observed recently (E. Ziegler, Aachen, personal communication) in soybean tissue treated with Pmg elicitor for short times.

ACKNOWLEDGEMENT

This research was supported by Deutsche Forschungsgemeinschaft, Schwerpunkt "Mechanismen und populationsdynamische Aspekte der Resistenz von Pflanzen gegenüber Schadorganismen".

REFERENCES

Albersheim P, Valent BS (1978)
J Cell Biol 78: 627-643

Ayers AR, Ebel J, Valent BS, Albersheim P (1976) Plant Physiol 57: 760-765

Ebel J, Ayers AR, Albersheim P (1976)
Plant Physiol 57: 775-779

Gamborg OL, Miller RO, Ojima K (1968)
Exp Cell Res 50: 151-158

Hahlbrock K, Lamb CJ, Purwin C, Ebel J, Fautz E, Schäfer E (1981)
Plant Physiol 67: 768-773

Hahlbrock K, Schröder J, Vieregge J (1980)
In: Fiechter A (ed) Adv Biochem Engin 18: 39-60

Hahn MG, Darvill AG, Albersheim P (1981)
Plant Physiol 68: 1161-1169

Hargreaves JA (1979)
Physiol Plant Pathol 15: 279-287

Hargreaves JA (1981)
New Phytol 87: 733-741

Knobloch KH, Beutnagel G, Berlin J (1981)
Planta 153: 582-585

Krupinski VM, Robbers JE, Floss HG (1976)
J Bacteriol 125: 158-165

Martin JF (1977)
In: Ghose TK, Fiechter A, Blakebrough N (eds) Adv Biochem Engin 6: 105-127

Martin JB, Bligny R, Rebeille F, Douce R, Legnay JJ, Mathieu Y, Guern J (1982)
Plant Physiol 70: 1156-1161

Tietjen KG, Hunkler D, Matern U (1983)
Eur J Biochem 131: 401-407

Tietjen KG, Matern U (1983)
Eur J Biochem 131: 409-413

West CA (1981)
Naturwissenschaften 68: 447-457