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Phosphate as a Limiting Factor for the Cell Division of Tobacco BY-2 Cells

Toshio Sano, Yoshiki Kuraya [1], Shin-ichi Amino [2] and Toshiyuki Nagata

Department of Biological Sciences, Graduate School of Science, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-0033 Japan

The re-addition of phosphate to tobacco BY-2 cells deprived of phosphate for 3 d induced cells to semi-synchronously re-enter the cell cycle from a static state. Though the addition of auxin to auxin-starved tobacco BY-2 cells also induced cell division (Ishida et al. 1993), some major differences were observed between these two systems. BY-2 cells lost the ability to re-enter the cell cycle after prolonged periods of auxin deprivation, but in contrast retained this ability after longer periods in the absence of phosphate. By differential cDNA screening we identified a phosphate-induced gene *phi-1*. *phi-1* was rapidly induced by the addition of phosphate with transcript levels starting to decrease by the start of DNA synthesis. *phi-1* does not share any significant homology with any gene with known functions over its full length. However, the N-terminus shared some homology with plasma membrane ATPases suggesting that it may be involved in some process of phosphorylation. Immunolocalization of the phi-1 gene product revealed that it rapidly accumulated in the cytoplasm prior to the start of plastid and nuclear DNA synthesis. These results are discussed in relation to the role of phosphate in inducing plant cell division.

Key words: *Nicotiana tabacum* BY-2 cells — Phosphate-starvation — Phosphorylation — Plastid development — Synchronous cell division.

Phosphate is a major plant nutrient required for the biosynthesis of macromolecules, such as nucleic acids and phospholipids. In fungi, phosphate utilization by the PHO system has been well characterized. In this system, acid and alkaline phosphatase activities are repressed at high phosphate concentrations and induced upon depletion of phosphate in the culture media (Oshima 1991). In contrast, however, phosphate utilization by higher organisms is poorly understood. The presence of high and low affinity systems for phosphate uptake in cultured plant cells has been shown by kinetic studies (Furihata et al. 1992, Schachtman et al. 1998). However, the fate of phosphate taken up into these cells has not been examined in detail. Several genes up-regulated under phosphate-limiting conditions have been identified. Acid phosphatases induced under phosphate-limiting conditions, were secreted into the soil to facilitate the remobilization of phosphate (Goldstein et al. 1988). RNases have also been reported to play a role under phosphate-limiting conditions (Bariola et al. 1994). Phosphate transporters have been identified in *Arabidopsis thaliana* roots cultured under phosphate-limiting conditions (Muchhal et al. 1996, Smith et al. 1997). The over-expression of these phosphate transporter genes in tobacco BY-2 cells resulted in increased phosphate uptake and a subsequent increase in growth rate (Mitsukawa et al. 1997). Phosphate is a limiting factor for the growth of *Catharanthus roseus* cells in culture. The re-addition of phosphate to the medium induced cell division in these cells (Amino et al. 1983). A correlation was found between the phosphate concentration at the start of culture and the number of cells attained at the stationary phase.

The growth, cell biology and biochemistry of tobacco BY-2 cells have been more extensively studied than in any other established plant cell line. In addition, the BY-2 cell cycle can be synchronized to a high level using the chemical inhibitors aphidicolin and propyzamide, making it particularly suitable for cell cycle studies (Nagata et al. 1992). The affect of withdrawing and subsequently re-adding auxin to tobacco BY-2 cell division has been previously described (Ishida et al. 1993). The removal of auxin resulted in cells arrested in static state and the subsequent re-addition of auxin resulted in a semi-synchronous re-entry of cells back into the cycle. An auxin-responsive gene, *arcA*, up-regulated upon the re-addition of auxin has been isolated (Ishida et al. 1993).

The consumption of phosphate by BY-2 cells from the culture medium is exceptionally rapid compared to other major inorganic constituents and sucrose (Kato et al. 1977). However, little is known about the role of phosphate in the proliferation of tobacco BY-2 cells at either the cellular or molecular level. In this study, we show that the addition of phosphate to phosphate-starved cells induced them to semi-synchronously re-enter the cell cycle. We

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; MI, mitotic index; PBS, phosphate-buffered saline; PIPES, piperazine-*N*,*N*′-bis (2-ethanesulfonic acid).

The nucleotide sequence reported in this paper has been submitted to the DDBJ, EMBL and GenBank under accession number AB018441.

[1] Present address: Plant Breeding and Genetics Research Laboratory, Japan Tobacco Inc., 700 Higashihara, Toyoda-cho, Iwata-gun, Shizuoka, 438-0802 Japan.

[2] Present address: Department of Bioscience and Technology, School of Engineering, Hokkaido Tokai University, 5-1-1 Minamizawa, Minami-ku, Sapporo, 005-8601 Japan.

also identified a gene, *phi-1*, which was rapidly induced by the addition of phosphate to phosphate-starved cells. These results are discussed in terms of resolving the relationship between phosphate availability and plant cell division.

Materials and Methods

Plant materials and culture conditions—Tobacco BY-2 cells (*Nicotiana tabacum* L. cv. Bright Yellow 2) were maintained by weekly subculture in modified Linsmaier and Skoog medium (1965), as described (Nagata et al. 1992).

Phosphate-starvation was attained by incubating 10 ml aliquots of 8 d-old cell suspension in phosphate-free medium for 3 d. Subsequently, concentrated KH_2PO_4 (100 mg ml^{-1}, pH 5.8) was added to the culture medium to give a final concentration of 370 mg $liter^{-1}$, while control cells were continually cultured in the phosphate-free medium.

Auxin starvation and re-addition was carried out as described by Ishida et al. (1993). In brief, 10 ml of 8 d-old cell suspension was washed with 10-volumes of culture medium without 2,4-D and incubated in medium without 2,4-D for 3 d. Subsequently, 2,4-D was re-added to the culture medium to give a final concentration of 0.2 mg $liter^{-1}$.

Synchronization of tobacco BY-2 cells was carried out as described by Nagata et al. (1992). In brief, 7 d-old cell suspension was transferred to fresh medium containing 5 μg ml^{-1} of aphidicolin (Sigma Chemical Co., St. Louis, U.S.A.) and incubated for 24 h. After washed with fresh medium, cell synchrony starting from S phase was obtained.

Determining DNA synthesis and cell division of the BY-2 cells—Cell division percentages were determined by counting MI after staining the cells with 4′,6-diamidino-2-phenylindole (DAPI) and viewing under a fluorescence microscope (BH-2, Olympus Optical Co., Tokyo, Japan), as described in Yasuda et al. (1988).

DNA synthesis was detected after the incorporating of 5-bromo-2′-deoxyuridine (BrdU) into cells for 30 min using a Cell Proliferation Kit, RPN20 (Amersham, Buckinghamshire, U.K.), by staining the cells with an antibody against BrdU (Amersham) under fluorescence microscopy (Miyake et al. 1997). After incorporating BrdU, cells were fixed with 3.7% formaldehyde in PMEG buffer (50 mM PIPES, 2 mM $MgSO_4$, 5 mM EGTA and 2% glycerol, pH 6.8) for overnight at 4°C. After fixation, cells were digested in an enzyme solution [1% Cellulase Onozuka RS (Yakult Honsha Co., Tokyo, Japan), 0.1% Pectolyase Y-23 (Seishin Co., Tokyo, Japan) and 0.4 M mannitol in PMEG buffer] for 5 min and then incubated with 1% Nonidet P-40 (Sigma) in phosphate-buffered saline (PBS) (20 mM Na-phosphate and 150 mM NaCl, pH 7.0)] for 15 min. After treatment with a blocking solution (0.1 mM glycine and 1% bovine serum albumin in PBS) for 10 min, cells were incubated with an anti-BrdU monoclonal antibody (Amersham) for 1 h, according to the manufacture's protocol. After washing with PBS, cells were incubated with a fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG antibody (Sigma) for 30 min. The cells in which all the cell nucleus was stained were scored as S phase cells.

Isolation of RNA—Cells were harvested by centrifugation (1,000 × *g* for 1 min) and frozen in liquid nitrogen. Cell pellets were ground to a fine powder with a pestle and a mortar, which was suspended in a denaturing solution (4 M guanidium thiocyanate, 100 mM Tris, 0.5% Sarcosyl and 140 mM 2-mercaptoethanol, pH 7.0) and mixed. Total RNAs were extracted with phenol-chloroform-isoamylalchol (25 : 24 : 1) and ethanol precipitated. Poly(A)$^+$ RNAs were prepared using Oligo(dT) Cellulose columns according to the manufacture's protocol (Life Technologies, Gaitherburg, U.S.A.).

Differential plaque hybridization—Double stranded cDNAs were synthesized from poly(A)$^+$ RNA isolated from cells cultured for 2 h after the re-addition of phosphate using a cDNA synthesis kit according to the manufacture's protocol (Amersham). A cDNA library was then constructed in the λgt10 vector (Amersham). Differential screening of this cDNA library was carried out on Nylon membranes (Biodyne B, Pall BioSupport, East Hills, U.S.A.), which were hybridized with ^{32}P-labeled cDNA probes prepared from poly(A)$^+$ RNA isolated from cells cultured for 2 h in the presence or absence of phosphate. Putative clones were subcloned into pUC18 vector and sequenced with the Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP according to the manufacture's protocol (Amersham).

RNA gel blot analysis—Total RNAs were separated on 0.8% formaldehyde-agarose gels, blotted on to Biodyne B membranes and hybridized to ^{32}P-labeled cDNA probes in 6 × SSC and 0.1% SDS at 65°C for 16 h. Membranes were washed for 30 min three times with 2 × SSC and 0.1% SDS at 65°C and exposed to an X-ray film (X-OMAT, Kodak, Rochester, U.S.A.).

Preparation of polyclonal antibodies—The *phi-1* cDNA was subcloned into the pET30 vector (Novagen Inc., Madison, U.S.A.). To do this, an *Eco*RI site was created at position of 388 by PCR using the primer: 5′-GGGAATTCCTTTCACTCTATTTGGGC-3′ and a *Sal*I site was created at position 1074 using the primer:5′-GGGTCGACATTCACTACACTAGATTA-3′. The resulting 700 bp PCR product was ligated into the pET30 vector and the protein was expressed in *E. coli* strain BL21 (DE3). The protein was purified with an Ni-NTA-agarose column (Qiagen, Hilden, Germany) and injected into a mouse to produce anti-phi-1 antiserum.

Affinity-purification of antiserum was carried out according to the method described in Olmsted et al. (1981) with some modifications. The purified phi-1 protein described above was separated on 10% polyacrylamide gel and it was transferred to a PVDF membrane. The transferred portion of the protein was cut out and incubated with anti-phi-1 antiserum for 30 min at 30°C. After washed with PBS containing 0.5% Tween-20 for 5 min five times, the antibody was eluted by incubating with 0.2 M glycine buffer (pH 2.6) for 2 min. The purified antibody was used after neutralization with 2 M Tris-HCl (pH 8.0).

Immunoblotting and immunolocalization—Cells were harvested by centrifugation, frozen by liquid nitrogen and homogenized to a fine powder with a pestle and a mortar. This powder was suspended in 50 mM Tris-HCl buffer (pH 7.0) containing 10% glycerol and 2% SDS, mixed and centrifuged for 10 min at 10,000 × *g*. The resulting supernatant was considered to be the whole protein extract. The whole protein extract was separated on 10% polyacrylamide gel and transferred to a PVDF membrane (Pall BioSupport). The blotted membrane was blocked with 5% skim milk and washed with Tris-bufferd-saline. After incubated with the affinity-purified anti-phi-1 antibody for overnight followed by alkaline phosphatase-conjugated anti-mouse antibody (Tago Inc., Burlingame, U.S.A.) for 1 h, phi-1 protein was detected using an alkaline phosphatase substrate kit (Vector Laboratories Inc., Burlingame, U.S.A.).

To examine the localization of the phi-1 protein, cells were pretreated with 250 μM *m*-maleimidobenzoyl-*N*-hydroxysuccimide ester (MBS) (Pierce Chemical Co., Rockford, U.S.A.) in

PMEG buffer prior to fixation for 30 min. After treatment with the enzyme solution to degrade the cell walls, Nonidet P-40 and the blocking solution as described above, cells were incubated with the affinity-purified anti-phi-1 antibody for 1 h followed by FITC-conjugated sheep anti-mouse IgG antibody (Sigma) for 30 min.

Results

Phosphate re-addition to phosphate-starved tobacco BY-2 cells induces cell division—When 8 d-old stationary BY-2 cells were transferred to phosphate-free medium, an increase of MI ($4.8 \pm 0.7\%$) was observed 1 d after transfer. By 3 d after transfer, however, the MI was almost 0%. Microscopic observation of 3 d-old cells revealed that they were in a static state. At this stage, a percentage of cells ($8.9 \pm 0.9\%$) were binucleate suggesting that phosphate starvation caused aberrant cell plate formation. Thus, it took 3 d of culture in phosphate-free medium for tobacco BY-2 cells to become static state.

After the re-addition of phosphate to phosphate-starved BY-2 cells changes in MI were followed by staining the cells with DAPI and viewing them under fluorescence microscopy. As shown in Fig. 1, MI began to increase 12 h after the addition of phosphate, reaching a maximum of $26.0 \pm 1.5\%$ at 16 h. To determine at what stage cells were in the cell cycle under phosphate-limiting conditions, DNA synthesis was examined by measuring the incorporation of BrdU into nuclei, which was assessed by staining with an antibody against BrdU and viewed under fluorescence microscopy. Four hours after the addition of phosphate, S-phase cells began to be observed reaching a maximum of 18.3% 8 h after the addition of phosphate (Fig. 1). This result showed that the addition of phosphate to the phosphate-starved cells resulted in cells re-entering the cell cycle from static state and semi-synchronously progressing through S-phase into mitosis.

Comparison of phosphate-starved cells with auxin-starved cells—We have previously shown that the addition of auxin to auxin-starved BY-2 cells induced a semi-synchronous cell division (Ishida et al. 1993). In this case, DNA synthesis also preceded mitosis (Nagata et al. 1998). Thus, like the addition of phosphate to phosphate-starved cells, the addition of auxin to auxin-starved BY-2 cells caused to progress through the cell cycle from static state to mitosis. Although both these semi-synchronous cell division systems are similar, we went to characterize the differences between the two.

When phosphate was added to the phosphate-starved cells cultured in phosphate-free medium for 3 d, we could see the semi-synchronous cell division. When the phosphate-starvation was prolonged for up to 13 d, a semi-synchronous cell division upon the re-addition of phosphate was still observed (Fig. 2). However, the start of mitosis was delayed. When the phosphate-starvation was prolonged for 20 d, though MI increased to 4%, no apparent semi-synchronous cell division was observed. In contrast, when auxin-starvation was prolonged for more than 4 d, the re-addition of auxin did not recover the increase of MI and under this condition, the cells became deteriorated. Therefore, periods of auxin-deprivation longer than 4 d was detrimental to cells.

To examine the morphological differences between the phosphate and auxin-starved cells, cells were stained with DAPI and viewed under fluorescence microscopy (Fig. 3). In phosphate-starved cells, although 50 to 60 plastids could be observed in a cell, the fluorescence intensity of the plastid nucleoids was very weak, indicating a low amount

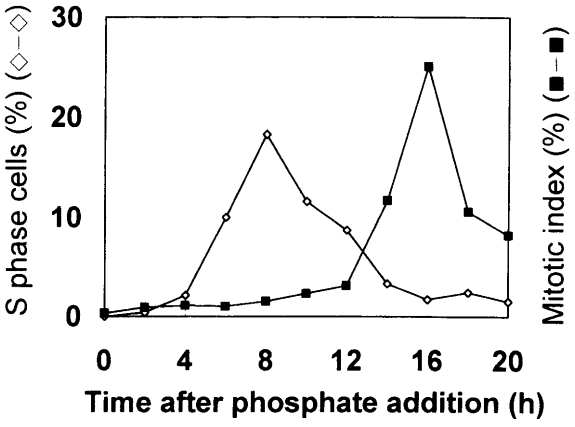

Fig. 1 After the addition of phosphate to phosphate-starved cells, MI (–■–) was determined by fluorescence microscopy after staining with DAPI and the percentages of S phase cells (–◇–) were calculated after incorporation of BrdU into nuclei was assessed under fluorescence microscopy by staining with anti-BrdU antibody.

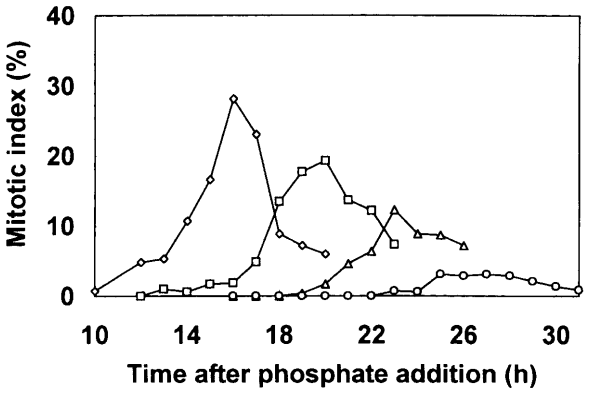

Fig. 2 Effect of different phosphate-starvation periods on the change in MIs after the addition of phosphate. After 8 d-old BY-2 cells were transferred to the culture medium without phosphate for 3 d (–◇–), 7 d (–□–), 13 d (–△–) and 20 d (–○–), phosphate was added to the culture medium and the changes in MIs were followed after staining with DAPI.

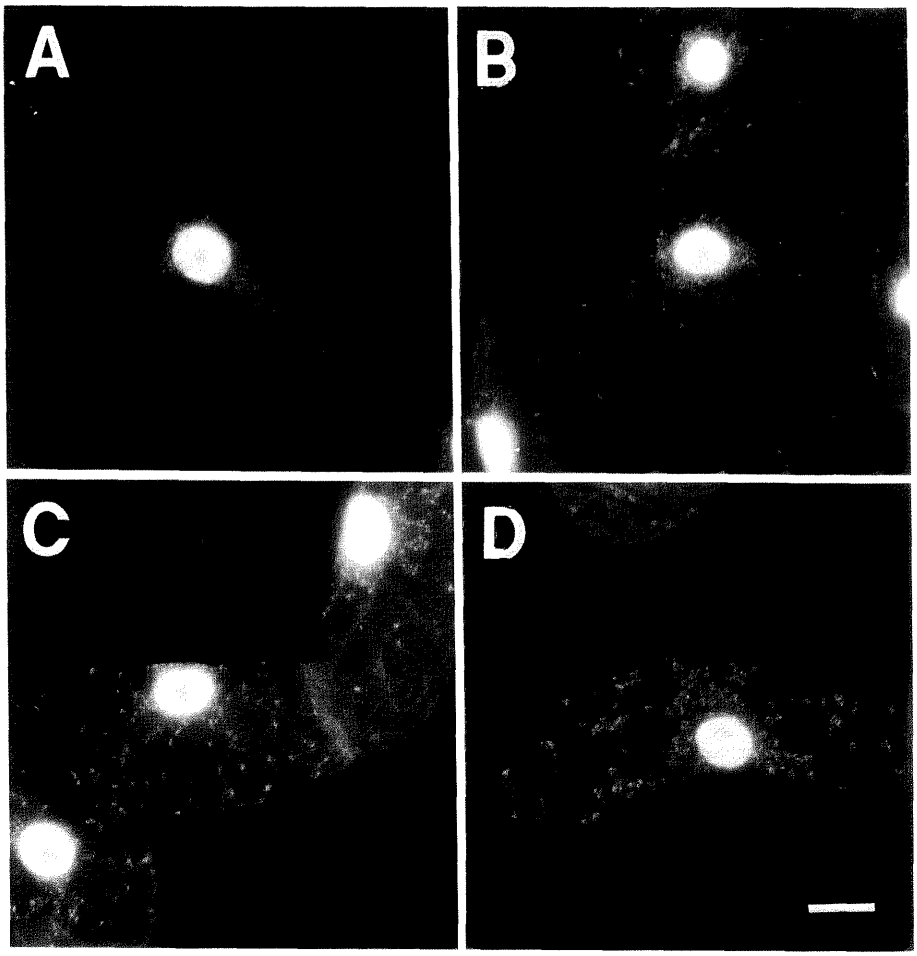

Fig. 3 Morphology of tobacco BY-2 cells under different conditions. Cells were observed under fluorescence microscopy after staining with DAPI. (A) Phosphate-starved cells. (B) Cells 4 h after the addition of phosphate. (C) Cells 10 h after the addition of phosphate. (D) Auxin-starved cells. The scale bar represents 10 μm.

of plastid DNA (Fig. 3A). However, 4 h after the addition of phosphate, an increase in the fluorescence intensity of the plastid nucleoids was observed around the nuclei (Fig. 3B), and by 10 h, plastids with high fluorescence intensities were also observed in the cytoplasmic strands (Fig. 3C). In contrast, the fluorescence intensity of plastid nucleoids in auxin-starved cells was strong not only around the nuclei, but also in the cytoplasmic strands and the size of plastids was larger than in phosphate-starved cells (Fig. 3D). Therefore, although both phosphate and auxin-starved cells were in static state, differences between the physiological states of phosphate and auxin-starved cells were conspicuous at the subcellular level.

Isolation of phosphate-induced genes—To examine the early molecular changes induced by the addition of phosphate to phosphate-starved BY-2 cells, we searched for phosphate-induced genes by differential cDNA screening.

A cDNA library was constructed from mRNA isolated from BY-2 cells 2 h after the re-addition of phosphate. This library was hybridized with cDNA probes prepared from phosphate-starved cells cultured for 2 h with or without phosphate. Ninety cDNA clones were isolated that hybridized preferentially with the cDNA probes from cells cultured with phosphate. After further characterization by repeated Northern hybridization, one cDNA clone was isolated that unambiguously hybridized to RNA from cells cultured with phosphate. Sequencing of this cDNA clone revealed that it was 1,166 bp in length with a putative open reading frame (ORF) 942 bp in length, encoding a protein 314 amino acids long with a molecular mass of 34 kDa.

The gene encoding this cDNA was named phosphate-induced gene (*phi*)-1. The predicted full length phi-1 protein showed no significant homology with anything in the databases, indicating that phi-1 may belong to a novel class of proteins. However, the N-terminal region has some homology with plasma membrane H^+-ATPases of fungi and plants (Fig. 4). The Lys-Gly-Ala sequence at position

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                                        *
tobacco      phi-1   23 SRKLTAL-VQEPENQLLQYHKGALLF  47

N.crassa             455 .K.VV.V-.ES.QGERITCV...P..  479
S.cerevisiae PMA1    455 .K.V..V-.ES..GERIVCV...P..  479
S.pombe      PMA1    452 .K.V..Y-.QA.DGTRITCV...P.W  476
Arabidopsis  AHA1    403 VD.R...TYIDSDGNWHRVS....EQ  428
tobacco      pma4    406 VD.R...TYIDNNNNWHRAS...PEQ  431
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Fig. 4 Comparison of the deduced amino acid sequence of the N-terminal region of phi-1 gene product with the plasma membrane H^+-ATPases of *N. crassa* (Harger et al. 1986), *S. cerevisiae* (Serrano et al. 1986), *S. pombe* (Ghislain et al. 1987), *Arabidopsis* (Harper et al. 1989) and tobacco (*Nicotiana plumbaginifolia*) (Perez et al. 1992). Dots represent identical amino acids. The conserved lysine residue is indicated by an asterisk.

of 42 to 44 of the phi-1 gene product was conserved among the reported plasma membrane H^+-ATPases and the conserved lysine residue at position 42 in the phi-1 gene product is suggested to be the ATP-binding residue of these plasma membrane H^+-ATPases (Farley et al. 1984).

Mode of expression of phi-1 gene—RNA gel blot analysis revealed that *phi-1* expression could be detected 20 min after the addition of phosphate, continuing to increase until 2 h. Six hours after the addition of phosphate, when DNA synthesis was increasing (Fig. 1), *phi-1* expression started to decrease (Fig. 5).

To examine whether *phi-1* expression is dependent on cell cycle, RNAs were prepared from cells synchronized by aphidicolin treatment. Although removal of aphidicolin resulted in synchronous progression of cell cycle in BY-2 cells starting from S phase, *phi-1* expression was not significantly changed through the cell cycle progression (Fig. 6).

To determine the relationship between the phosphate-induced gene *phi-1* and the auxin-induced gene *arcA*, *phi-1* expression was examined in auxin-starved cells and *arcA* expression in phosphate-starved cells (Fig. 7). *phi-1* expression was detected at similar levels in both auxin-starved cells and in cells after auxin re-addition (Fig. 7A) and *arcA* expression was also detected in both phosphate-starved cells and cells after phosphate re-addition (Fig. 7B). When cells were deprived of both phosphate and auxin, after the addition of phosphate and auxin, the start of cell division was observed at 21 h, reaching a peak MI of $11.9 \pm 1.4\%$ at 25 h . Though the start of mitosis was delayed compared to a single starvation, *phi-1* was induced at least 2 h after the addition of phosphate and auxin, and *arcA* was induced 4 h (Fig. 8), which was the same expression pattern in auxin-starved condition.

Accumulation and intracellular localization of the

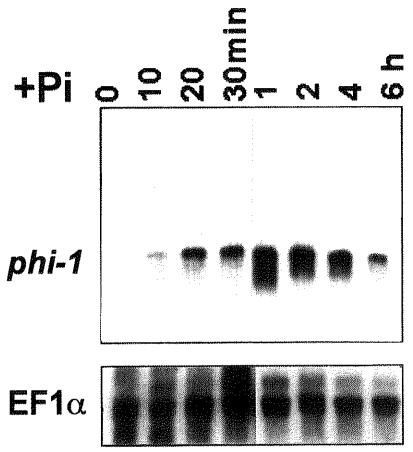

Fig. 5 Time course of *phi-1* gene expression after the addition of phosphate. After the addition of phosphate to phosphate-starved BY-2 cells, RNAs were isolated from cells cultured for 0, 10, 20 and 30 min and 1, 2, 4 and 6 h. Total RNA (20 μg per lane) was separated on 0.8% formaldehyde-agarose gels, transferred to a Nylon membrane and hybridized with radiolabeled *phi-1* and EF1α (Kumagai et al. 1995) cDNA probes. EF1α cDNA probe was used as a control.

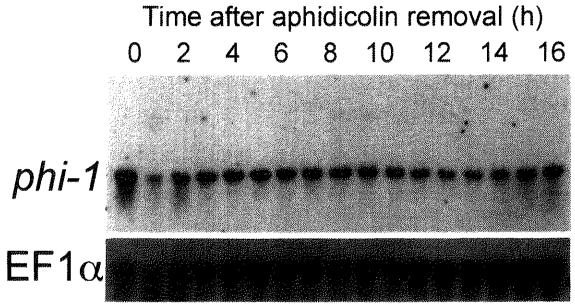

Fig. 6 *phi-1* gene expression in synchronized cells. After tobacco BY-2 cells were synchronized by aphidicolin treatment, cells were collected every hour until 16 h, during which cell cycle progressed from S phase to G1 phase. Total RNA (20 μg per lane) was separated on 0.8% formaldehyde-agarose gels, transferred to a Nylon membrane and hybridized with radiolabeled *phi-1* and EF1α cDNA probes. EF1α cDNA probe was used as a control.

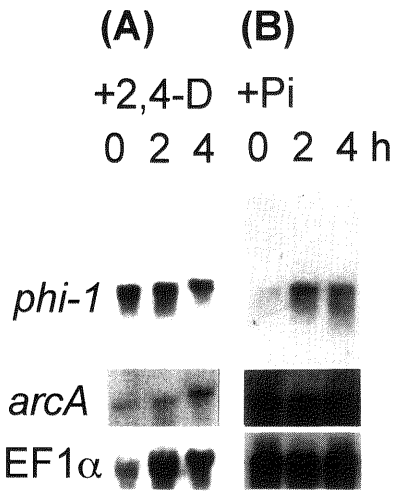

Fig. 7 Effect of auxin (A) and phosphate (B) on *phi-1* and *arcA* gene expression, respectively. After the addition of 0.2 mg liter^{-1} 2,4-D to auxin-starved tobacco BY-2 cells (Ishida et al. 1993) (A), and after the addition of phosphate to phosphate-starved tobacco BY-2 cells (B), RNAs were isolated from cells cultured for 0, 2 and 4 h, respectively. Total RNA (20 μg per lane) was separated on 0.8% formaldehyde-agarose gels, transferred to a Nylon membrane and hybridized with *phi-1, arcA* and EF1α cDNA probes. EF1α cDNA probe was used as a control.

phi-1 protein—Accumulation of the phi-1 gene product was followed by immunoblotting using an affinity-purified antibody raised against the phi-1 polypeptide produced in *E. coli*. The phi-1 gene product was almost undetectable in phosphate-starved BY-2 cells. Levels had increased by 2 h after the addition of phosphate and remained high until 6 h (Fig. 9), when *phi-1* gene expression started to be decreased.

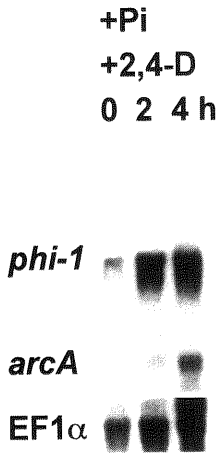

Fig. 8 *phi-1* and *arcA* gene expression after the addition of phosphate and auxin. After the addition of 370 mg liter^{-1} KH_2PO_4 and 0.2 mg liter^{-1} 2,4-D to the cells cultured in a medium deprived both phosphate and auxin for 3 d, RNAs were isolated from cells cultured for 0, 2 and 4 h. Total RNA (20 μg per lane) was separated on 0.8% formaldehyde-agarose gels, transferred to a Nylon membrane and hybridized with radiolabeled *phi-1, arcA* and EF1α cDNA probes. EF1α cDNA probe was used as a control.

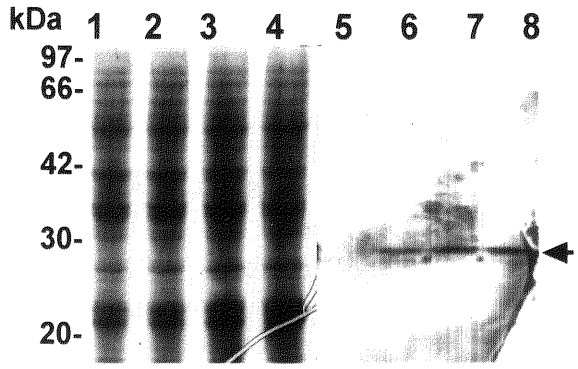

Fig. 9 Immunoblot analysis of phi-1 protein accumulation after phosphate addition. Phosphate-starved tobacco BY-2 cells were cultured for 0 h (lanes 1 and 5), 2 h (lane 2 and 6), 4 h (lanes 3 and 7) and 6 h (lane 4 and 8) after the addition of phosphate. Whole protein extract (50 μg per lane) was separated by SDS-PAGE. The protein was stained with Coomassie brilliant blue (lane 1 to 4) or blotted onto a PVDF membrane and incubated with affinity-purified anti-phi-1 antibody (lanes 5 to 8). The arrow indicates the position of the phi-1 protein and molecular mass markers are indicated on the left.

Staining of the phosphate-starved cells with the phi-1 antibody revealed that there was no specific staining in these cells (Fig. 10A). Six hours after the addition of phosphate, conspicuous staining was detected on the nuclear surface and staining patterns in the cytoplasm revealed a network-like structure (Fig. 10B). When these cells were observed at the mid plane, the staining on the cortex was observed, suggesting that the network was localized on the cortex. Fibrous structures resembling cytoplasmic strands that connected the nucleus with the network in the cytoplasm were also observed (Fig. 10C). Thus, the phi-1 gene product rapidly accumulates in the cytoplasm after the re-addition of phosphate to phosphate-starved cells.

Discussion

It was shown in this study that the addition of phosphate to phosphate-starved tobacco BY-2 cells induces a semi-synchronous mitosis, which was preceded by DNA synthesis. Thus, it has been explicitly demonstrated that phosphate-starvation of stationary-phase cells caused cells to bring static state after 3 d. In this context, it is intriguing to speculate on why under normal batch culture conditions, phosphate-starvation is not attained 4 d after the subculture of cells into fresh culture medium, when phosphate in the culture medium has been shown to be consumed (Kato et al. 1977). This could be explained, by the utilization of phosphate stored in the vacuoles upon

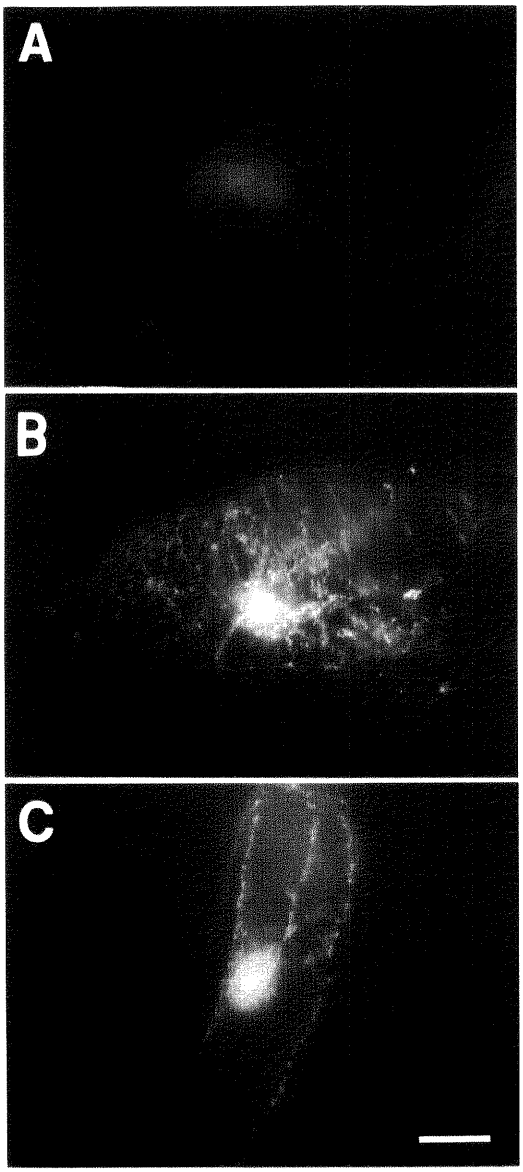

Fig. 10 Intracellular localization of phi-1 protein in a phosphate-starved cell (A) and in a cell 6 h after the addition of phosphate (B, C). The focus was on the cell surface (B) and at the mid plane of the cell (C). The phi-1 protein was detected by staining with affinity-purified anti-phi-1 antibody and observed by fluorescence microscopy. So that fluorescence intensities were comparable, the exposure time was fixed at 3 min. The scale bar represents 10 μm.

depletion of the phosphate in the medium. The storage of phosphate in the vacuoles of sycamore cells (Rebeille et al. 1983) and tobacco BY-2 cells (Nagata et al. 1992) has been shown to occur by ^{31}P-NMR. Therefore, upon transfer of the cells to phosphate-free medium, the stored phosphate takes 3 d to be completely consumed and a state of phosphate-starvation attained.

The *phi-1* gene isolated 2 h after the addition of phosphate to phosphate-starved tobacco BY-2 cells was rapidly expressed after the addition of phosphate, before the onset of plastid and nuclear DNA synthesis. From the expression pattern of *phi-1* gene during the cell cycle progression of BY-2 cells, *phi-1* does not act as a cell cycle regulator but act to cause phosphate-starved cells to re-enter the cell cycle. The search for *phi-1* gene homologues in the data base did not allow us to identify any known function for *phi-1* gene, suggesting that *phi-1* belongs to a novel family of genes. However, N-terminal region of the phi-1 gene product had limited homology with plasma membrane H^+-ATPases of fungi and plants. In this context, Farley et al. (1984) showed that the conserved lysine residue was labeled by FITC, that inhibited the ATP hydrolytic activity completely and irreversibly. This inhibition of activity was prevented in the presence of ATP, suggesting that FITC reacted at the ATP-binding site. Thus, the conserved lysine residue at position 42 of the phi-1 gene product is likely to be an ATP-binding site. Although *phi-1* is not an ATPase in overall structure, *phi-1* may have some role in phosphorylation. Based on the report by Ashihara and Ukaji (1986), the addition of phosphate to phosphate-starved *Catharanthus roseus* cells resulted in a steady increase in ATP levels in cells for up to 4 h. It may be speculated that under accumulated levels of ATP, the phi-1 gene product may play a role in the phosphorylation of certain substrates. Although Wilson et al. (1998) reported the phosphate-induced cell cycle re-entry of tobacco BY-2 cells and showed the transient activation of a MAP kinase activity by re-feeding of phosphate, the significance of their observation to this study remains to be clarified.

Although both the addition of phosphate to phosphate-starved cells and the addition of auxin to auxin-starved cells induces tobacco BY-2 cells to re-enter the cell cycle from static state, several fundamental differences between these two systems were identified in this study. Firstly, a difference was observed at the subcellular level, as the plastid DNA level between the two was conspicuously different. Secondly, in the case of auxin-starved condition, if starvation was prolonged for longer than 3 d, the cells deteriorated, while phosphate-starvation could be prolonged for at least 13 d. Although prolonged phosphate-starvation increased the time taken for cells to reach mitosis. Thirdly, the expression pattern of *phi-1* which was induced by the addition of phosphate, was completely different to that of *arcA* which was induced by the addition of auxin to auxin-starved cells. From these observations, phosphate-starved cells and auxin-starved cells are staying in different physiological conditions in static state and phosphate signaling seems to be different from auxin signaling to re-enter the cell cycle.

In conclusion, the addition of phosphate to the phosphate-starved tobacco BY-2 cells induced cells to semi-

synchronously re-enter the cell cycle from static state. Upon this re-entering of the cell cycle, expression of a novel gene *phi-1* which is supposed to play a role in phosphorylation is involved. Further elucidation of the function of *phi-1* gene and search for other phosphate-regulated genes under this condition should help us in understanding the role of phosphate in inducing cell division of plant cells.

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