Evidence suggesting protein tyrosine phosphorylation in plants depends on the developmental conditions

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Abstract Protein tyrosine phosphorylation plays a central role in a variety of signal transduction pathways regulating animal cell growth and differentiation, but its relevance and role in plants are controversial and still largely unknown. We report here that a large number of proteins from all plant subcellular fractions are recognized by recombinant, highly specific, anti-phosphotyrosine antibodies. Protein tyrosine phosphorylation patterns vary among different adult plant tissues or somatic embryo stages and somatic embryogenesis is blocked in vivo by a cell-permeable tyrosyl-phosphorylation inhibitor, demonstrating the involvement of protein tyrosine phosphorylation in control of specific steps in plant development.

Key words: Anti-phosphotyrosine; Plant development; Plant phosphoprotein; Somatic embryogenesis; Tyrosine phosphorylation

1. Introduction

Reversible protein phosphorylation catalyzed by protein kinases and phosphatases [1] regulates fundamental biological processes. In animals protein tyrosine kinases (PTKs) and phosphatases (PTPs) play a central role in a variety of signal transduction pathways regulating cell growth and differentiation [2]. Protein tyrosine phosphorylation occurs also in yeast, which however, does not contain PTKs; thus, yeast phosphotyrosine proteins are phosphorylated by dual-specificity (serine/threonine and tyrosine) kinases [3], such as MAPK kinases [4].

In higher plants several serine/threonine-specific protein kinases and phosphatases have been identified so far [5,6], but the presence of PTKs and PTPs remains controversial. However, in recent years dual specificity has been demonstrated for *Arabidopsis thaliana* kinases ADK1 [7], and MAPK kinase [8], the product of the plant oncogene *rolB* has been found to be endowed with PTP activity [9], and a PTP1C-like cytoplasmic PTP has been cloned and characterized in *Arabidopsis* [10].

In animals and yeast the activity of MAP kinases is regulated by mitogen stimulation or environmental stress, and it is mediated by reversible threonine-tyrosine phosphorylation of a conserved amino acid motif [11–13]. Recently, several plant MAP kinases have been identified and their regulatory system seems to be conserved with respect to the animal and yeast counterparts [8,14–16]; thus, at least for this transduction pathway, involvement of reversible tyrosine phosphorylation is demonstrated also in plants.

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However, data obtained so far relate to a limited number of proteins and signalling pathways [8,14–17] and the only conclusion we may draw is that reversible protein tyrosine phosphorylation is involved in plant responses to environmental stimuli such as physical, chemical and biological stress signals [10].

Further evidence is needed to support the existence also in plants of a complex protein tyrosine phosphorylation system, and as yet, no clear experimental evidence has been obtained for a large number of plant proteins being phosphorylated on tyrosine residues. Moreover, the involvement of tyrosine phosphorylation in controlling transduction events crucial to plant growth and development as it occurs in animals, remains to be demonstrated.

In order to investigate this topic we have used a high-sensitivity protocol for the immunodetection of proteins phosphorylated on tyrosine (PYP) in cell homogenates. The data on an extensive protein tyrosine phosphorylation in plants and its involvement in developmental control are reported here.

2. Materials and methods

2.1. Plant cell culture, somatic embryogenesis, tissue explants

Carrot cells of the embryogenic cell line A+t3 were grown as reported [9]. *A. thaliana* cells of cell line E157 (kindly provided by Dr. S.C. De Vries, Wageningen, The Netherlands) were grown in the same conditions but in MS medium (Duchefa) supplemented with 3% sucrose. Induction of carrot somatic embryogenesis and purification of embryo stages were performed as reported [18]. Cells or embryo stages were collected by filtration, washed with distilled water and frozen with liquid nitrogen. For tissue explantation adult plants were washed with tap water, then with distilled water; after cutting, tissue explants were immediately frozen with liquid nitrogen.

2.2. Treatment with tyrphostin

Cell-permeable Tyrphostin A25 (Sigma-Aldrich) is solubilized in dimethylsulfoxide (DMSO) at 50 mM concentration just before each set of experiments and stored in the dark at −20°C. Tyrphostin is added at various concentrations after the onset of somatic embryogenesis and then every 4 days. Treated and control cultures are grown in the dark. Viability is assessed by vital staining with trypan blue (Sigma-Aldrich).

2.3. Preparation of homogenates and subcellular fractionation

Plant material is reduced to powder in a mortar with liquid nitrogen and resuspended in two volumes of freshly prepared, cold extraction buffer (50 mM Tris-HCl, pH 7.4; 250 mM sucrose; 10 mM Na_3VO_4; 10 mM NaF; 1 mM Na-tartrate; 1 mM phenylmethylsulfonyl fluoride (PMSF); 5 μg/ml leupeptin; 5 μg/ml antipain). All subsequent steps are performed at 4°C. After 30′ mixing, the homogenate is centrifuged 15′ at $2000 \times g$. Supernatant (Spn) is separated from the pellet (P1) and used as 'total extract' or centrifuged 30′ at $8000 \times g$. Spn is separated from the pellet (organelles, P2) and centrifuged again 1 h at $100\,000 \times g$. Spn (cytosol) is recovered and the pellet (microsomes) is resuspended in EB. P1 and P2 are resuspended in 100 mM $CaCl_2$ and after 4 h under continuous shaking, suspensions are centrifuged 30′ at

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3000×*g*, precipitating proteins from Spns with cold ethanol. Washed organelles (pellet from P2 suspension) are resuspended in EB. Protein concentrations are determined using the Bio-Rad assay, following manufacturer's instructions. Protein samples are denatured just after preparation by boiling in standard sample buffer for SDS-PAGE.

2.4. Electrophoresis and immunoblotting

SDS-PAGE is performed as reported [19], on 10% polyacrylamide/0.24% bisacrylamide gels. Correct normalization of protein concentrations in electrophoresed samples is assessed by loading in double series aliquots of each sample, staining one series by silver nitrate while the other one is further processed for immunoblotting. Electrophoresed gel is washed 15′ in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for SDS removal, and proteins are transferred at 4°C onto 0.2 μm nitrocellulose filter (Pharmacia-Hoefer) by a 4 h electroblotting run at 360 mA in a Transblot apparatus (Bio-Rad). Correct protein transfer is checked by staining and destaining filters with Ponceau S. The filter is blocked for 14 h at 4°C plus 2 h at room temperature in a blocking solution: TBS (10 mM Tris-HCl pH 7.5, 100 mM NaCl) with 1% bovine serum albumin (BSA, Sigma-Aldrich) and 0.1% Tween-20 (Bio-Rad), and then incubated for 2 h at room temperature with horseradish peroxidase-conjugated, recombinant anti-phosphotyrosine antibody RC20 (Transduction Labs), at 1:2500 dilution in the blocking solution. The filter is washed 2×10′ in TBS with 0.05% Nonidet NP-40 (Sigma-Aldrich), 10′ in TBS and then incubated in ECL reagent (Amersham) for enhanced chemiluminescent detection of bands on Hyperfilm-ECL film (Amersham), following the manufacturer's instructions.

2.5. On-blot tyrosine dephosphorylation

Filter with blotted proteins is incubated 30′ at 37°C in dephosphorylation solution (20 mM Tris-HCl pH 6.8, 150 mM NaCl, 0.1% β-mercaptoethanol, 1 mg/ml BSA, 2 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml antipain) with 1.5 units/ml of commercial *Yersinia* PTP (Boehringer Mannheim) and acid phosphatase (Sigma-Aldrich).

3. Results

Several polyclonal and monoclonal anti-phosphotyrosine antibodies and several different immunodetection methods were used, all of which showed specific recognition of several tyrosine-phosphorylated proteins (PYPs) in plant extracts; however, combined use of RC20 and enhanced chemiluminescence (see Section 2) showed the highest sensitivity. As shown in Fig. 1, several PYPs are recognized by RC20 antibodies in 'total extracts' from *Arabidopsis* and carrot cells. Their number was even higher when performing subcellular fractionation: Fig. 2 shows the presence of PYPs in all fractions and the enrichment of particular bands in each fraction; when films were overexposed, the overall number of bands was over 100 (data not shown).

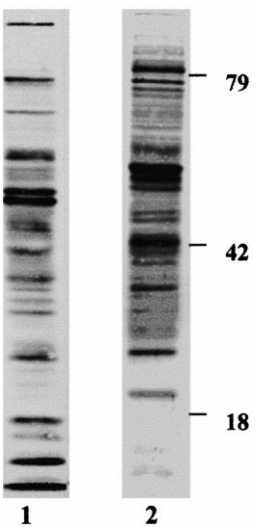

Fig. 1. PYP patterns of 'total extracts' (50 μg proteins/lane) from *A. thaliana* (lane 1) and *D. carota* (lane 2) 7 day old suspension-growing wild-type cells. Molecular weight markers are indicated.

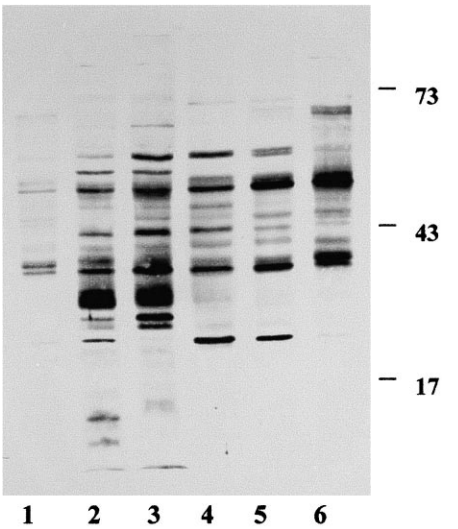

Fig. 2. PYP patterns of subcellular fractions (50 μg proteins/lane) from 7 day old suspension-growing wild-type carrot cells. Lane 1: 'total extract'. Lane 2: $CaCl_2$-extracted proteins from P1. Lane 3: $CaCl_2$-extracted proteins from P2. Lane 4: $CaCl_2$-non-extracted proteins from P2. Lane 5: microsomes. Lane 6: cytosol.

In order to assess the specificity of recognition, blotted PYPs were displaced for antibody binding with 1 mM phosphotyrosine, which could displace recognition of all detected bands. Also blocking the filter with defatted milk which is known to interfere with the recognition of animal PYPs [20], produced loss of bands. Further, on-blot dephosphorylation was performed to get the final demonstration that recognition of detected bands is mediated by phosphotyrosine epitopes. Protein samples were blotted in double series and stained with Ponceau S; then one series was tyrosine-dephosphorylated with tyrosine phosphatases and a control series was treated with the same buffer but phosphatase-free. Filters were then washed and restained with Ponceau S in order to exclude the removal of proteins. After immunoblotting of both filters a normal number of bands was detected only in the control series, whereas the PTP-treated series showed complete loss of bands. Data from phosphotyrosine displaced or dephosphorylated immunoblots are not shown since filters are completely blank.

In order to check for the presence of PYPs in adult plants young and adult leaves, stems and roots were explanted from *Daucus carota* plants. Fig. 3 shows that several PYPs are detected in total extracts from all tissues, and that protein tyrosine phosphorylation is tissue-specific.

Thus, carrot somatic embryogenesis was adopted as a model system to assess the involvement of protein tyrosine phosphorylation also in early plant developmental stages. Suspension-growing unorganized callus cells, pro-embryogenic masses (PEM), globular, heart- and torpedo-shaped embryos, and plantlets were purified from a wild-type embryogenic cell line and their PYP patterns compared. As shown in Fig. 4, a

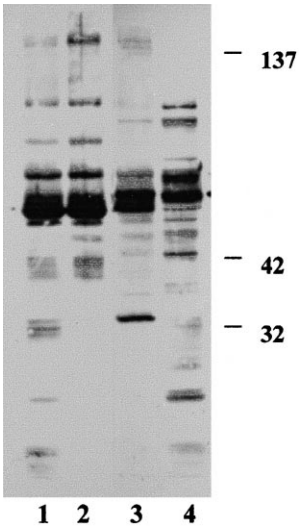

Fig. 3. Immunoblotting with RC20 of 'total extracts' (50 μg proteins/lane) from carrot tissues. Lane 1: young leaves. Lane 2: adult leaves. Lane 3: shoots. Lane 4: roots.

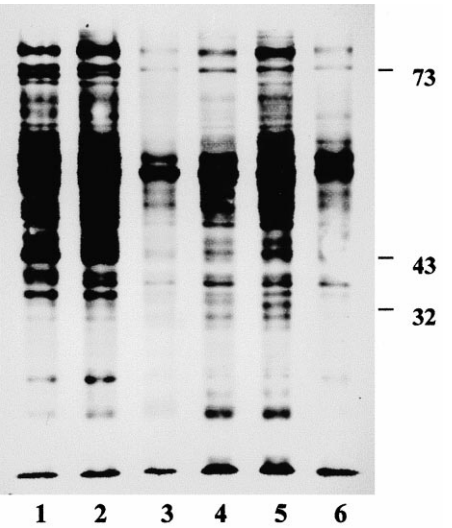

Fig. 4. PYP patterns of 'total extracts' (50 μg proteins/lane) from different developmental stages from a wild-type carrot embryogenic culture: lane 1, suspension-growing undifferentiated cells; lane 2, pro-embryogenic masses; lane 3, globular embryos; lane 4, heart-shaped embryos; lane 5, torpedo-shaped embryos; lane 6, plantlets. Molecular weight markers are indicated.

strong variation in the pattern of protein tyrosine phosphorylation can be observed among the different developmental stages. Fig. 5 shows that also aging of cells is relevant to the modulation of protein tyrosine phosphorylation in plants.

In order to get direct evidence of the relevance of protein tyrosine phosphorylation for plant development, tyrphostin A25, a cell-permeable PTK inhibitor of tyrphostin series [21] was used at various concentrations for interfering in vivo with tyrosine phosphorylation in embryogenic cell cultures. Because of the photolability of tyrphostins, the experiment was performed in the dark, and because of its short half-life [21], tyrphostin was added again every 4 days. As shown in Fig. 6, 10 μM or 50 μM tyrphostin A25 completely blocked carrot somatic embryogenesis, less complete inhibition is observed at 2 μM concentration (data not shown). The tyrphostin solvent DMSO, had an effect as shown in the controls. The embryogenic block was not dependent on cell death, as determined by vital staining of blocked cultures (data not shown).

4. Discussion

In animals, reversible protein tyrosine phosphorylation plays a central role in regulation of growth, development and physiological responses [2]. A similar role has not yet been demonstrated in plants as to date evidence of tyrosine phosphorylation concerns only a limited number of plant proteins, such as the MAP kinases [8,14–17], a tyrosine-specific plant protein kinase has not been identified yet, and data obtained so far suggest that in plants, as well as in yeast, reversible protein tyrosine phosphorylation might only mediate physiological responses to environmental stimuli and stress signals [10].

Instead, our data suggest that in plants tyrosine phosphorylation concerns a relevant number of protein species and that in addition, it is strongly involved in the regulation of plant embryogenesis and tissue differentiation. In fact, a relevant number of tyrosyl-phosphorylated protein (PyP) bands, ranging from low to high M_r, is reproducibly recognized by anti-phosphotyrosine antibodies in cells from plant species as different as carrot and *Arabidopsis*, in all subcellular fractions, in homogenates from suspension-growing undifferentiated cells as well as from PEM, embryos, somatic plantlets and tissues from adult zygotic plants. Recognition of the phosphotyrosine epitope is not only reproducible, but it is also strongly specific, as shown by phosphotyrosine competition and PTP-mediated disappearance of PYP bands.

PYP patterns undergo extensive modulation in plant somatic embryogenesis and they are tissue-specific in adult plants. Further, protein tyrosine phosphorylation is not simply 'associated' to plant development, as it is 'crucial' at least to early embryogenesis, which is completely blocked when embryogenic cultures are treated with inhibitors of tyrosine-specific protein kinases. It should also be noted that an inhibitor specific for tyrosine kinases involved in the control of animal development is also capable of interfering with plant embryogenesis.

Our work was not aimed at characterizing a particular PTK, PTP, PYP or tyrosine phosphorylation pathway, and a lot more work will be needed for elucidating the relevance

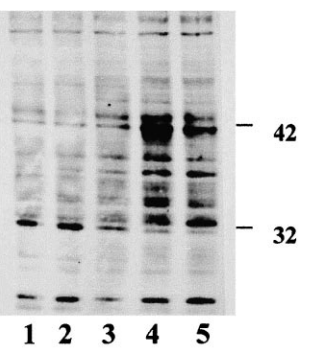

Fig. 5. PYP patterns of 'total extracts' (50 μg proteins/lane) from wild-type carrot cells collected after 1 (lane 1); 4 (lane 2); 7 (lane 3); 14 (lane 4) and 21 days (lane 5) of subcultivation. Molecular weight markers are indicated.

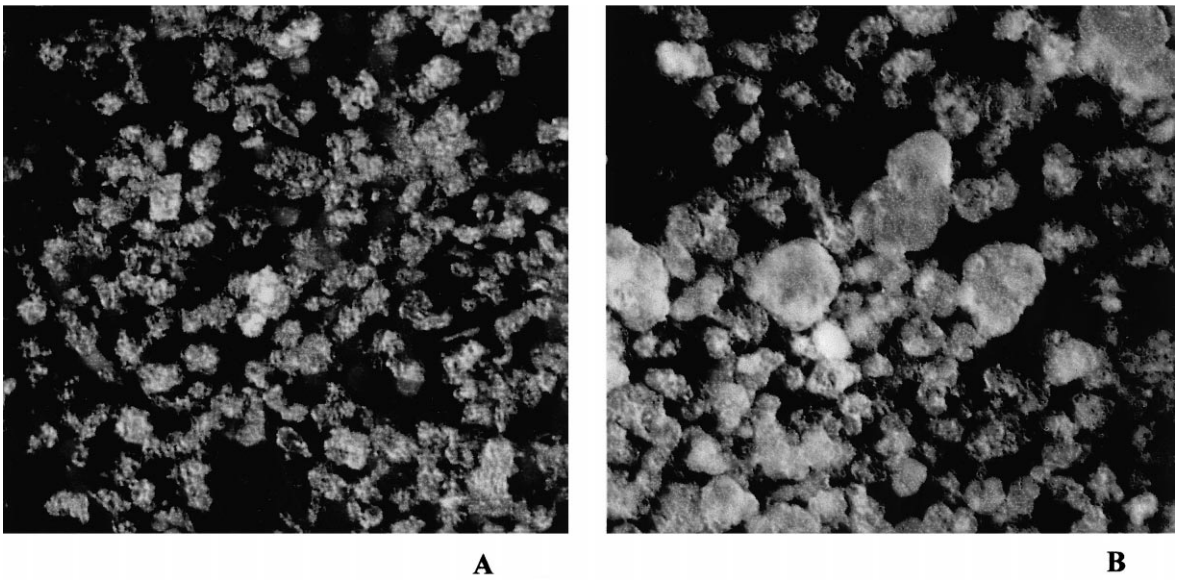

Fig. 6. Wild-type embryo cultures after 14 days from induction of embryogenesis. A: Culture treated with 10 μM tyrphostin (50 μM is not different): embryogenesis is completely blocked and no embryo stage is apparent. B: 'DMSO control' (not different from untreated control): somatic embryo stages are apparent.

and role of protein tyrosine phosphorylation in plants. However, our results clearly suggest that tyrosine phosphorylation in plants is not simply restricted to a limited number of protein species and to stress-related responses, but as well as in animals it concerns many other proteins from all subcellular fractions and also with developmental control, strongly suggesting that a complex protein tyrosine phosphorylation system might exist in plants too.

Thus, the analysis of such system via the identification and characterization of plant PTK, PTP and their substrates will be probably of great help for shedding light on one of the main themes of plant biology.

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