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A 67-kDa plasma-membrane-bound Ca^{2+}-stimulated protein kinase active in sink tissue of higher plants

Laurence D.P. Barker[1,2], Matthew D. Templeton[1], Ian B. Ferguson[1]

[1]Horticulture and Food Research Institute of New Zealand, Mt. Albert Research Centre, Private Bag 92-169, Auckland, New Zealand
[2]School of Biological Sciences, University of Auckland, Auckland, New Zealand

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Abstract. A novel 67-kDa protein kinase ($p67^{cdpk}$) was identified in the microsomal membrane fraction of apple (*Malus domestica* Borkh. cv. Braeburn) suspension cultures and subsequently found to be active in sink tissues. Microsomal proteins were blotted onto Nylon or polyvinylidenedifluoride membranes, and $p67^{cdpk}$ assayed by in situ-labelling renatured proteins with [γ-^{32}P]ATP; thin-layer electrophoresis/thin-layer chromatography of acid hydrolysates of the ^{32}P-labelled protein band indicated that serine and threonine, but not tyrosine residues were phosphorylated. A detailed analysis of the ion-dependency of $p67^{cdpk}$ revealed that it was a Ca^{2+}-stimulated, Mg^{2+}-dependent protein kinase. However, $p67^{cdpk}$ was ten times more active in the presence of 10 mM Mn^{2+}, and these assay conditions were used routinely to increase the sensitivity of the assay. Activity of $p67^{cdpk}$ was found at high levels in the plasma membrane, and solubilisation experiments with a number of detergents suggested that $p67^{cdpk}$ is an integral membrane protein. A homologous protein kinase with similar biochemical properties was also present in cell-suspension cultures of pear and maize. In maize (*Zea mays* L.) plants, sink tissues, such as young expanding leaves of both light-grown and etiolated plants, mature etiolated tissue and roots all had high levels of $p67^{cdpk}$ activity. However, mature light-grown (source) tissues had barely detectable levels. In etiolated maize leaves and coleoptiles the kinase activity was highest in expanding tissue and decreased as the cells expanded. When etiolated maize plants were exposed to light, the activity of $p67^{cdpk}$ was reduced to background levels after 8 h. Although $p67^{cdpk}$ has biochemical properties similar to those of other plant calcium-dependent protein kinases, this is the first identification of a membrane-bound calcium-dependent protein kinase which is specifically active in sink tissues.

Key words: Calcium-dependent protein kinase – *Malus* (protein kinase) – Plasma membrane – Sink tissue – *Zea* (protein kinase)

Introduction

During growth and differentiation, a plant is required to respond to a variety of environmental and developmental signals, and protein kinases play a key role in the transduction of these signals (Ma 1993). Protein kinases have been found in virtually every cellular compartment and serve many functions, from metabolic regulation to involvement in signal transduction and amplification (for review, see Hunter 1991). The activities of several plant enzymes are affected by protein kinases, including sucrose-phosphate synthase (McMichael et al. 1995) and nitrate reductase (Kaiser and Huber 1994). The plasma-membrane (PM) H^+-ATPase is a target of phosphorylation by a calcium-dependent protein kinase (CDPK), although the pump has yet to be demonstrated to be regulated by the kinase in vivo (Sussman 1994). Although the involvement of protein kinases in signal transduction across the PM has not been established in plants, changes in protein kinase activity or protein phosphorylation have been reported to correlate with the application of extra-cellular stimuli, such as light (Fallon and Trewavas 1994), or the hormones gibberellic acid (Abo-El Saad and Wu 1995), cytokinin (Kakimoto 1996) and ethylene (Bleecker and Schaller 1996). Extracellular elicitors may alter protein phosphorylation in a number of species (Felix et al. 1991).

A large number of plant protein kinases are dependent on Ca^{2+} for activity. The soluble CDPK purified by Putnam-Evans et al. (1990) was shown to directly bind Ca^{2+}, as expected for a protein containing

Abbreviations: CDPK = calcium-dependent protein kinase; PEG = polyethyleneglycol; PM = plasma membrane; PVDF = polyvinylidenedifluoride; $p67^{cdpk}$ = 67-kDa calcium-dependent protein kinase; TLE = thin-layer electrophoresis

Correspondence to: M.D. Templeton;
E-mail: Matt.Templeton@marc.cri.nz; Fax: 64 (9) 8154201;
Tel.: 64 (9) 8154231

EF-hand sequences. This enzyme was the first to be identified in a new group of plant protein kinases, termed the CDPKs for Calcium-Dependent Protein Kinases (as opposed to calmodulin-like domain protein kinases), which do not require calmodulin; they contain EF-hand sequences with homology to calmodulin, which interact directly with Ca^{2+}. A number of other protein kinase activities have now been associated with this family (Schaller et al. 1992; Verhey et al. 1993), based on biochemical and immuno-cross-reactivity data. We have identified a 67 kDa protein kinase by blot-renaturation assay which has biochemical properties similar to other plant protein kinases; however, it appears to be specifically active in sink tissues.

Materials and methods

Chemicals and radiochemicals. Hybond N^+ membranes and [γ-^{32}P]ATP (specific activity > 111 TBq/mmol^{-1}) were obtained from Amersham International (Bucks., UK) and polyvinylidenedifluoride (PVDF; Immobilon-P) was from Millipore Corp. (Bedford, Mass., USA). Chemicals for electrophoresis, including prestained molecular-mass markers, were from Bio-Rad Laboratories (Hercules, Calif., USA). Phosphoamino acids, phenylmethylsulfonylfluoride (PMSF) and BSA (A4503 or A3059) were purchased from Sigma-Aldrich Pty (St. Louis, Mo., USA). Protease inhibitors leupeptin, antipain and apoprotinin were obtained from Boehringer Mannheim (Mannheim, Germany). All other chemicals were of AnalaR grade or better.

Plant material. Cell-suspension cultures were derived from the cortical tissue of apple (*Malus* × *domestica* cv. Braeburn) or pear (*Pyrus communis* L. cv. Passe Crassane) fruit in a liquid medium with 4.5 μM 2,4-dichlorophenoxyacetic acid and no cytokinin, as described by Codron et al. (1979). Plants of maize (*Zea mays* cv. Furio) were grown from seed in vermiculite, either in the dark, or under a 16-h photoperiod, at 23 ± 2 °C with an irradiance of 300 μmol · m^{-2} · s^{-1} at plant level.

Isolation of membrane fractions. Cell-suspension cultures in the early logarithmic phase of growth were harvested by filtration and resuspended (1 g cells · mL^{-1}) in extraction buffer (Schaller and Bleecker 1993), in which the 20% glycerol was replaced with 0.33 M sucrose. The protease inhibitors included in the buffer were 1 mM PMSF, with apoprotinin, antipain and leupeptin at 1 μg · mL^{-1} each. Cells were disrupted with a Polytron blender (Kinematica, Luzern, Switzerland) for up to 15 min. Whole plant tissue was also prepared as described by Schaller and Bleecker (1993). Cell walls and larger organelles were removed by centrifugation at 10 000 *g* for 15 min. Microsomal membranes were then pelleted by centrifugation at 100 000 *g* for 30 min (OTD combi, rotor TFT 50.38; Sorval, Norwalk, Conn., USA), and resuspended in extraction buffer and stored in aliquots at −80 °C until use. When thawed, samples were used only once then discarded. Freeze-thawing or holding preparations at temperatures above −20 °C resulted in significant loss of p67^{cdpk} activity. Protein was estimated with the BCA protein assay reagent (Pierce, Rockford, Ill., USA).

To provide a purified PM fraction, freshly prepared microsomal membranes were fractionated in a 6.1% (w/v) aqueous two-phase [polyethyleneglycol/(PEG) Dextran] system (Kjellbom and Larsson 1984), with the protease inhibitors used in cell breakage added to the sucrose/phosphate resuspension buffer. Cytochrome *c* reductase and oxidase were assayed according to Hodges and Leonard (1979), with assay volumes reduced from 3 to 1 mL. Rates of cytochrome *c* oxidation or reduction were determined from the initial linear rates and the amount of cytochrome *c* utilised established using the extinction coefficient for cytochrome of 18.5 mM^{-1} · cm^{-1} at 550 nm. 1,3-β-glucan synthase (glucan synthetase II) was assayed according to the method of Palmgren et al. (1990). Chlorophyll was estimated by the method of Lichtenthaler (1987).

Renaturation and in-situ labelling. Standard techniques (Laemmli 1970; Verhey et al. 1993) were used for SDS-PAGE and semi-dry blotting. Blotted proteins were denatured, labelled and washed as described by Ferrell and Martin (1991). Blots were dried at 80 °C for 15 min and autoradiography performed. For the experiments in which the cation dependency was determined, the BSA used in the renaturation and blocking solution was passed through a Chelex 100 column (Bio-Rad) to remove contaminating calcium. Membranes were also washed in 1 mM EGTA followed by two rinses in 30 mM Tris-HCl (pH 7.2) prior to assaying. Individual, blotted samples were assayed in separate containers. The final free concentration of ions present in the assay solution was calculated by a computer program, BAD (Bound And Determined) based on the method of Brooks and Story (1992).

Results

Renaturation of protein kinase activity. Preliminary in-vitro phosphorylation experiments established that protein kinase activity, as seen by the extent and number of radiolabelled protein bands, was present in the microsomal membrane fraction isolated from cultured apple cells. This level of phosphorylation appeared to be greater when assayed in the presence of 10 mM $MnCl_2$ than 10 mM $MgCl_2$ (Fig. 1).

Apple microsomal membrane proteins were resolved by SDS-PAGE, transferred to PVDF, denatured, renatured and assayed for kinase activity. A single protein kinase activity with an apparent molecular mass of 67 kDa (hereafter referred to as p67^{cdpk}) was observed (Fig. 2). Greatest activity was detected in the presence of

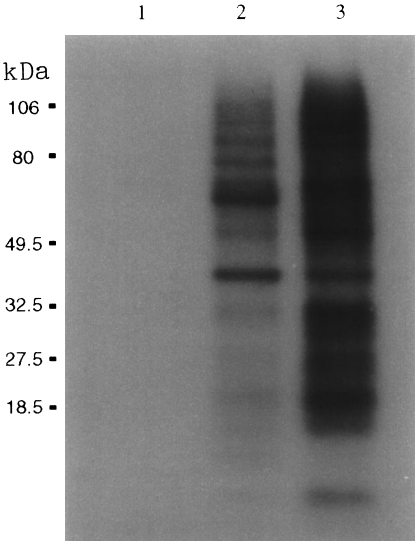

Fig. 1. In-vitro protein kinase activity from apple microsomal membranes. Membrane protein was assayed in the presence of 10 mM of either EDTA (*lane 1*), $MgCl_2$ (*lane 2*) or $MnCl_2$ (*Lane 3*). The reaction was stopped after 30 min with the addition of an equal volume of gel loading buffer and labelled proteins were separated by SDS-PAGE (10%) (w/v). Equal amounts of total protein (10 μg) were loaded per lane

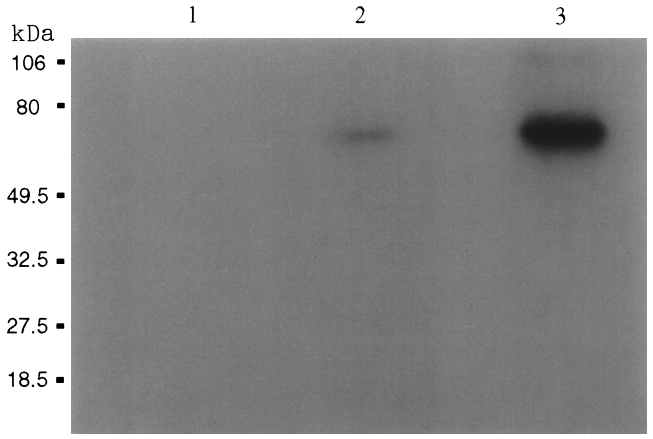

Fig. 2. Renaturation of protein kinase activity from apple microsomal membranes. Microsomal proteins (40 μg) were resolved by SDS-PAGE and blotted onto Hybond N^+. Blotted proteins were denatured, renatured and assayed for kinase activity. The blot was cut into strips and assayed in the presence of 10 mM of either EDTA (*lane 1*), $MgCl_2$ (*lane 2*) or $MnCl_2$ (*lane 3*)

10 mM $MnCl_2$ (Fig. 2, lane 3), while a lower level of activity was seen with 10 mM $MgCl_2$ (Fig. 2, lane 2). No activity was detected with 1 mM EDTA alone (Fig. 2, lane 1). This pattern of protein kinase activity corresponded to that detected in the in vitro experiments (Fig. 1). As $p67^{cdpk}$ was most active in the presence of 10 mM $MnCl_2$, these assay conditions were used routinely. The $p67^{cdpk}$ renatured equally well on the hydrophilic, positively charged membrane Hybond N^+ or on the hydrophobic PVDF-based support Immobilon-P. A lower background was obtained after assaying on PVDF, and this membrane was therefore routinely used for the blot-renaturation experiments.

Cation dependency. Protein kinases use Mg^{2+} or Mn^{2+} ions as part of their substrate, together with the triphospho-nucleotide, for catalytic activity, but many are also stimulated by low levels of Ca^{2+} (≈10 μM), which acts as a positive effector molecule (Roberts and Harmon 1992). Following the detection of kinase activity by blot-renaturation in the presence of 10 mM $MnCl_2$ and a lower level of activity with 10 mM $MgCl_2$, an investigation into the cation dependency of this detected activity was carried out.

The activity of $p67^{cdpk}$ was assayed over the concentration range 0.05–10 mM for both Mn^{2+} and Mg^{2+}, in the presence of 50 μM Ca^{2+}. Activity steadily increased between 0.5 and 5 mM with a maximum at 10 mM in the presence of either Mn^{2+} or Mg^{2+}. Activity in the presence of Mn^{2+} was greater than with Mg^{2+} (data not shown). Therefore, the following experiments were performed with 10 mM Mn^{2+} or Mg^{2+}.

In the presence of Mn^{2+}, no difference in the level of $p67^{cdpk}$ activity was detected regardless of whether Ca^{2+} and/or EGTA had been added (as indicated by analysis in a Storm Phosphoimager) (Fig. 3A). In the presence of Mg^{2+}, a radiolabelled protein with the same apparent molecular mass was observed, but protein kinase activity was approximately ten fold lower (Fig. 3B). Inhibition of Mg^{2+}-dependent $p67^{cdpk}$ activity occurred in the presence of EGTA whether or not Ca^{2+} (50 μM) had been added (Fig. 3B, treatments 2 and 4): EGTA (1 mM) was sufficient to chelate the 50 μM Ca^{2+}, but not to significantly alter the Mg^{2+} concentration. However, if 2 mM Ca^{2+} was added, together with Mg^{2+} and EGTA (resulting in a free Ca^{2+} concentration of 1 mM), $p67^{cdpk}$ was again detected (Fig. 3B, treatment 5). Of the three treatments in which $p67^{cdpk}$ was detected, no difference in the level of activity was found among them, as indicated by phosphoimager analysis. These results indicate that $p67^{cdpk}$ is more active with Mn^{2+}-ATP as a substrate than with Mg^{2+}-ATP, and that the latter activity is dependent on the presence of Ca^{2+}. Whether or not the activity detected using Mn^{2+}-ATP as the substrate is dependent on Ca^{2+} cannot be determined under these assay conditions, due to the relative critical stability constants of Mn^{2+}-EGTA and Ca^{2+}-EGTA (see *Discussion*).

Fig. 3A,B. Cation-dependency of renatured protein kinase activity. Microsomal membrane protein (40 μg) was resolved by SDS-PAGE (12%) and assayed for $p67^{cdpk}$ activity. After blotting to PVDF, the blot was washed in 1 mM EGTA to remove any contaminating Ca^{2+}, followed by two washes in 30 mM Tris-HCl (pH 7.2) to remove the remaining EGTA. The blot was then cut into strips. Each strip was incubated with [γ-^{32}P]ATP in a buffer that contained: **A** *lanes*: 1, 10 mM $MnCl_2$; 2, 10 mM $MnCl_2$, 1 mM EGTA; 3, 10 mM $MnCl_2$, 50 μM $CaCl_2$; 4, 10 mM $MnCl_2$, 1 mM EGTA, 50 μM $CaCl_2$; **B** *lanes*: *1*, 10 mM $MgCl_2$; *2*, 10 mM $MgCl_2$, 1 mM EGTA; *3*, 10 mM $MgCl_2$, 50 μM $CaCl_2$; *4*, 10 mM $MgCl_2$, 1 mM EGTA, 50 μM $CaCl_2$; *5*, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM $CaCl_2$

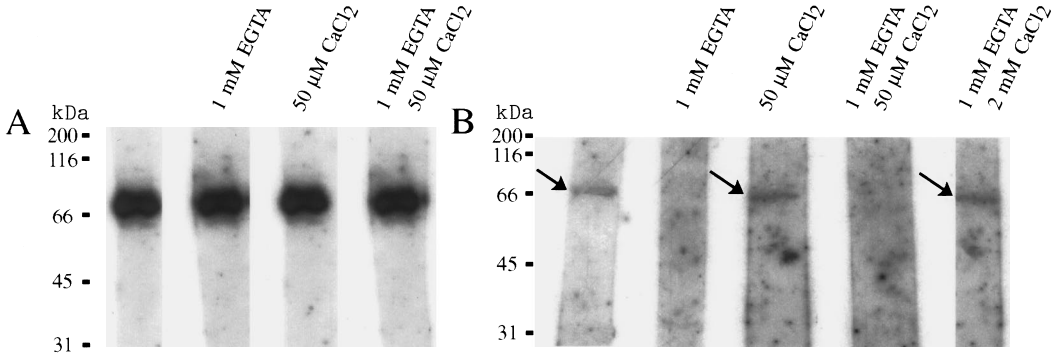

Table 1. Marker-enzyme analysis of membrane vesicles prepared by aqueous two-phase partitioning. Microsomal membranes were separated by aqueous two-phase partitioning, yielding two fractions, an upper PM-enriched fraction and a lower fraction containing intracellular membranes. Both fractions, together with the parent microsomal membrane fraction, were assayed for 1,3-β-glucan synthase (PM), NADPH-cytochrome *c* reductase (ER) and cytochrome *c* oxidase (mitochondrial) activity. These activities are also quoted as a percentage (in parentheses) of the original microsomal membrane fraction

Fraction	Specific activity (U · (mg protein)$^{-1}$)		
	1,3-β-glucan synthase	NADPH-cytochrome *c* reductase	Cytochrome *c* oxidase
Microsomal membranes	0.0105 (100%)	0.0267 (100%)	0.117 (100%)
Intracellular membranes	0.0045 (43%)	0.0274 (100.4%)	0.0858 (73%)
Plasma membranes	0.0494 (471.3%)	0.0038 (14.4%)	0.0203 (17%)

Sub-cellular localisation of p67^{cdpk}. Aqueous two-phase partitioning was used to purify PM vesicles from the microsomal preparation. Marker-enzyme analysis was then used to determine the purity of the isolated PMs.

1,3-β-Glucan synthase is the most reliable enzyme marker for the PM (Fredrikson and Larsson 1989). When assayed in the presence of the detergent digitonin (the enzyme active site is on the cytoplasmic side of the PM; Fredrikson and Larsson 1989), 1,3-β-glucan synthase activity was 4.7-fold greater in the PM fraction than the parent microsomal membrane fraction (Table 1). The activity of cytochrome *c* oxidase, located exclusively in the inner mitochondrial membrane (Widell and Larsson 1990), and NADPH-cytochrome *c* reductase, located primarily in the ER, indicated that only a very low level of contamination of the PM fraction was present. Together, these marker-enzyme data indicate that aqueous two-phase partitioning had provided an enriched PM fraction of high purity. This was confirmed by comparison of the PM fraction polypeptide pattern resolved by SDS-PAGE with that of the other two membrane fractions (data not shown).

The isolated membrane fractions were assayed for p67^{cdpk} activity, which revealed that the PM fraction (top, PEG phase) possessed the highest kinase activity, with the lower Dextran phase which contains the other cellular membranes plus any inside-out PM vesicles also having activity (Fig. 4). Phosphoimager analysis indicated that the upper PEG phase was enriched approximately 12-fold over the lower Dextran phase for p67^{cdpk} activity.

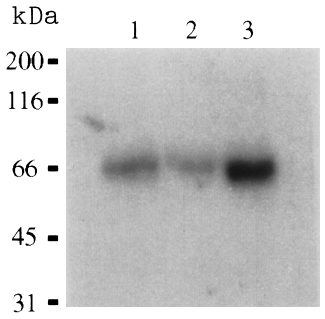

Fig. 4. Subcellular localisation of p67^{cdpk}. The microsomal membrane preparation was fractionated using aqueous two-phase partitioning. Membrane protein (20 μg) was resolved by SDS-PAGE (10%) and p67^{cdpk} assayed by blot-renaturation, in the presence of 10 mM $MnCl_2$. *Lanes*: *1*, microsomal preparation; *2*, Dextran phase; *3*, PEG-4000 (PM) phase

Solubilisation of PM-bound p67^{cdpk} activity. In order to characterise the association of p67^{cdpk} to the PM, a variety of reagents were tested for their ability to solubilise p67^{cdpk}. Sodium Chloride (0.4 M) or EDTA (10 mM) had little effect (Fig. 5A). Triton X-100 (1%) was more effective, and Triton X-114 (1%) was the most successful solubilising reagent for p67^{cdpk}. Upon heat-induced Triton X-114 phase separation, p67^{cdpk} partitioned primarily to the upper aqueous phase, although a small proportion was present in the lower detergent-rich phase.

The kinase activity solubilised from the PM did not bind to concanavalinA-agarose (Fig. 5B), distinguishing it from the Mn^{2+}-dependent kinase activities from

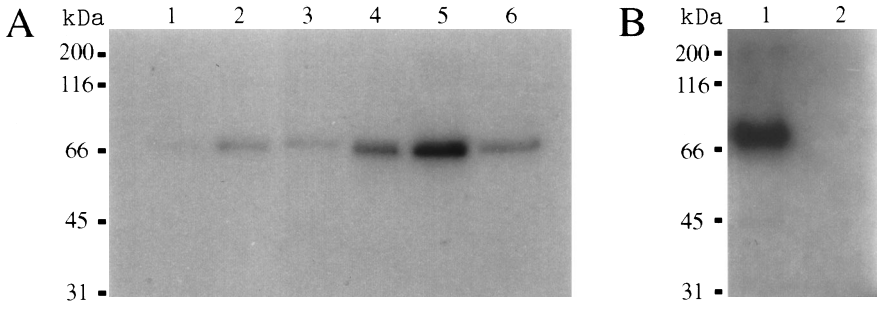

Fig. 5A,B. Solubilisation of p67^{cdpk}. Solubilised protein (10 μg) was resolved by SDS-PAGE (10%) and assayed by blot-renaturation for p67^{cdpk} activity. A portion of the PM fraction was incubated with Concanavalin A (ConA)-agarose and thoroughly washed (Schaller and Bleecker 1993). **A** *Lanes*: *1*, cytosolic fraction; *2*, 0.4 M NaCl; *3*, 10 mM EDTA; *4*, 1% Triton X-100; *5*, 1% Triton X-114 (upper aqueous phase); *6*, 1% Triton X-114 (lower detergent-rich phase). **B** *Lanes*: *1*, ConA-agarose supernatant; *2*, ConA-agarose-bound fraction

Arabidopsis leaves characterised by Schaller and Bleecker (1993), the latter also having higher molecular mass.

Identification of amino acid residues phosphorylated by p67^{cdpk}. A number of amino acid residues can be phosphorylated by protein kinases including serine, threonine, tyrosine and histidine. To determine the residue(s) phosphorylated by p67^{cdpk}, two-dimensional thin-layer electrophoresis/thin layer chromatography. (TLE/TLC) of acid hydrolysates of the excised labelled band from the microsomal fraction (Fig. 4, lane 1) and PM fraction (Fig. 4, lane 3) was performed. The results of both of these hydrolyses were identical; serine was the predominant amino acid phosphorylated with lower amounts of phosphothreonine also present (Fig. 6). Phosphotyrosine, however, was not detected. Phosphohistidine is acid-labile, and thus would not be detected under these conditions (Wei and Matthews 1991). Thus p67^{cdpk} appeared to be a serine/threonine protein kinase, as are the vast majority of plant protein kinases characterised to date (Stone and Walker 1995).

Activity of p67^{cdpk} in other cell-suspension cultures. Cell-suspension cultures derived from apple leaf (cv. Royal Gala) and fruit (cv. Braeburn) and pear fruit (cv. Passe Cassane) were assayed for p67^{cdpk} activity. Cultures were harvested during the logarithmic phase of growth and microsomal membranes prepared. All three cell lines were found to contain a highly active p67^{cdpk} protein (Fig. 7A). The same protein was also identified in a maize cell line (cv. Black Mexican Sweet) (Fig. 7B, lane 1). This suggests that p67^{cdpk} is ubiquitously present in cultured plant cells grown on MS medium (Murashige and Skoog 1962) with sucrose as a carbon source.

Activity of p67^{cdpk} in whole-plant tissue. The presence of p67^{cdpk} in whole tissue from several plants was investigated. Preliminary experiments showed that the kinase could not be detected in mature light-grown leaves from apple, *Arabidopsis*, or pea (data not shown). Maize plants were chosen to assay p67^{cdpk} during growth and development. The tissues with the most highly active p67^{cdpk} were etiolated leaves and roots (Fig. 7B, lanes 3 and 4). These results suggest that dividing and expanding cells possess p67^{cdpk} activity and confirm the data from cultured apple cells. The high activity of p67^{cdpk} in etiolated tissue warranted further analysis of the distribution of p67^{cdpk} in the developing plant.

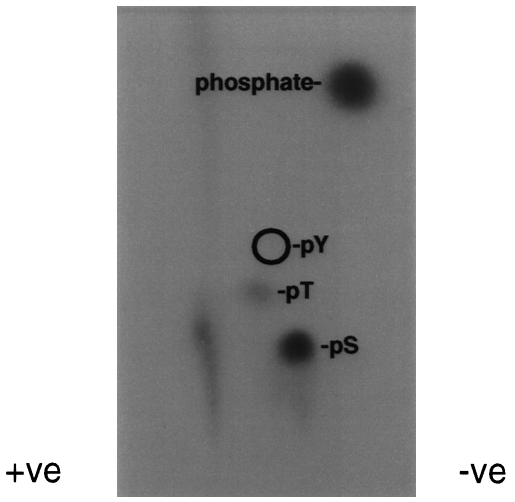

Fig. 6. Analysis of phosphoamino acids by two-dimensional TLE/TLC. The labelled band from Fig. 2, lane 3, was excised and hydrolysed in 6 M HCl for 1.5 h at 110 °C. HCl was removed under vacuum over conc. H_2SO_4 and NaOH overnight. The pellet was resuspended in 10 µl H_2O and centrifuged at 12 000 *g*. Phosphoamino acid standards (2 µg each) were added to the supernatant and the mixture was applied to a 20 cm × 20 cm cellulose TLC plate, and TLE was performed at pH 2 for 20 min. The plate was rotated 90°, eluted in butanol-acetic acid-water-pyridine (5:1:4:4, by vol.), dried, autoradiographed and sprayed with ninhydrin. The three standards had R_f values of 0.3 (*pY*, phosphotyrosine), 0.26 (*pT*, phosphothreonine) and 0.22 (*pS*, phosphoserine) allowing identification of each of the same three standard compounds in the two-dimensional pattern

Distribution of p67^{cdpk} in etiolated and light-grown maize plants. Maize plants were grown in the presence and absence of light, and various tissues extracted and assayed for p67^{cdpk} activity. In tissues such as the growing tip and expanding leaves, p67^{cdpk} was highly active in both dark- and light-grown tissue (Fig. 8A, lanes 1–4). Expanding etiolated coleoptiles also possessed active p67^{cdpk}, but levels of activity were markedly reduced in mature etiolated coleoptiles (Fig 8A,

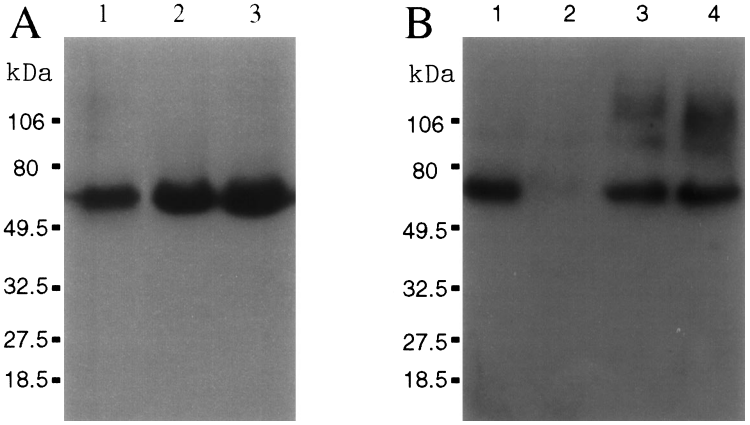

Fig. 7A,B. Activity of p67^{cdpk} in plant cell-suspension cultures and in whole plant tissue. Microsomal membranes (70 µg) were prepared and p67^{cdpk} assayed by blot-renaturation. **A** *Lanes*: *1*, pear fruit cells; *2*, apple leaf cells (cv. Royal gala); *3*, apple fruit cells (cv. Braeburn). **B** *Lanes*: *1*, maize cell suspension cultures; *2*, light-grown maize leaves; *3*, etiolated maize leaves; *4*, maize roots

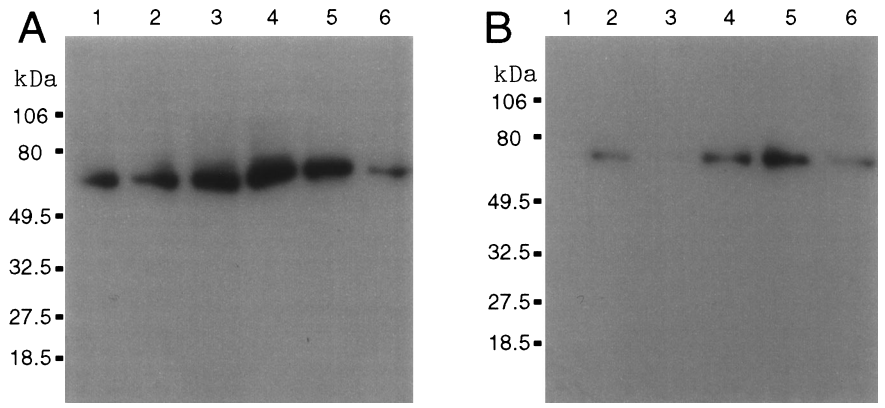

Fig. 8A,B. Distribution of p67^{cdpk} in maize plants. Microsomal membranes (80 μg) were prepared and p67^{cdpk} assayed by blot-renaturation. **A** *Lanes*: *1*, light-grown tip; *2*, etiolated tip; *3*, light-grown very young expanding leaf; *4*, etiolated very young expanding leaf; *5*, expanding etiolated coleoptile; *6*, mature, fully expanded coleoptile. **B** *Lanes*: *1*, light-grown fully expanded primary leaf; *2*, etiolated primary leaf; *3*, light-grown secondary leaf; *4* etiolated secondary leaf tip; *5*, etiolated secondary leaf middle segment; *6*, etiolated secondary leaf bottom segment

lanes 5–6). In mature tissue, such as fully expanded leaves, p67^{cdpk} was only active in etiolated plants and was barely detected in light-grown plants (Fig. 8B). Secondary leaf material had higher levels of activity than primary leaf in both dark- and light-grown tissue; within the etiolated secondary leaf, activity was higher in the bottom half. These results suggest that p67^{cdpk} is most active in dividing and expanding tissue, but that it becomes inactive in light-grown tissue. In etiolated tissue the activity of p67^{cdpk} drops as the tissue ages, but does not reduce to zero.

To investigate the kinetics of p67^{cdpk} inactivation, etiolated plants were exposed to light and the secondary leaf assayed for p67^{cdpk} (Fig. 9). Etiolated maize plants were irradiated by light (at the beginning of a 16-h photoperiod, with irradiance 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at plant level) and secondary leaves were harvested at various times over 3 d. The activity of p67^{cdpk} reduced to background levels between 8 and 10 h (Fig. 9). The reduction in p67^{cdpk} activity occurred before maximum chlorophyll levels were reached. Chlorophyll levels increased steadily during this time from 0.65 to 104 $\mu g \cdot g^{-1}$ fresh weight after 8 h and to 343 $\mu g \cdot g^{-1}$ fresh weight after 76 h.

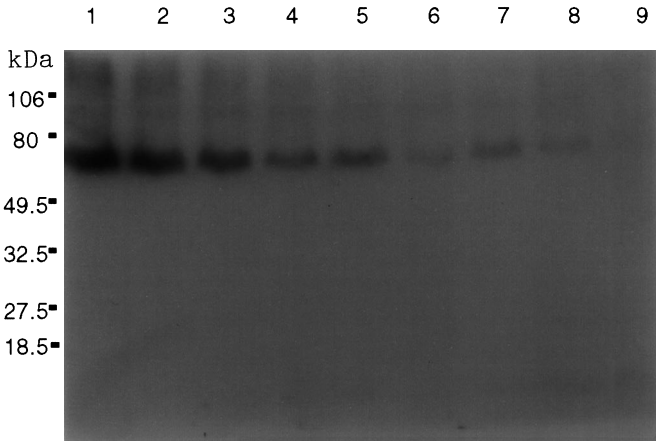

Fig. 9. Effect of light on p67^{cdpk} activity in the secondary leaf of etiolated maize plants. Etiolated plants were grown until the secondary leaf had fully expanded before being irradiated at the beginning of a 16-h photoperiod as described in *Materials and methods*. The secondary leaf was harvested after 0, 2, 4, 6, 8, 10, 26 and 76 h (*lanes 1–8*, respectively). *Lane 9* contained material from light-grown secondary leaf. Microsomal membranes (100 μg) were prepared and p67^{cdpk} assayed by blot-renaturation

Discussion

In-vitro phosphorylation of solubilised microsomal membrane proteins from suspension-cultured apple cells was strongly dependent upon the presence of Mn^{2+}. This finding was corroborated by the identification of the 67-kDa protein kinase (p67^{cdpk}) after blot-renaturation and ^{32}P-labelling. Although the in-vitro labelling pattern with Mg^{2+} was both less intense and different from that observed for Mn^{2+} (Fig. 1), in the blot-renaturation assay, a protein with the same apparent molecular mass was labelled in the presence of Mg^{2+} (Fig. 2). These ion-dependent activities may be catalysed by the same protein with different cation dependencies, or alternatively they might be distinct proteins. Since protein kinases renature with different efficiencies (Ferrell and Martin 1991), it cannot be claimed that p67^{cdpk} is the only kinase active in the microsomal membranes from apple cells. It is more likely that p67^{cdpk} is the only one that renatures under these conditions.

Buffers containing EGTA are frequently used to chelate Ca^{2+}, often without appreciation of the high affinity it has for Mn^{2+}. While EGTA chelates Ca^{2+} 100 000 times more readily than Mg^{2+}, it chelates Mn^{2+} even more readily than Ca^{2+}, by about 100-fold (Martell and Smith 1974). Therefore, even if there was a stimulatory effect by Ca^{2+} on the activity of p67^{cdpk} (Fig. 3A), on treatment with EGTA it would not have been inhibited as the EGTA would have bound Mn^{2+} instead of Ca^{2+}.

The Mg^{2+}-dependent activity was reduced by treatment with EGTA. In the absence of added EGTA or Ca^{2+}, p67^{cdpk} activity was detected; perhaps Ca^{2+} was present as a contaminant in the solutions of other ions (precautions were taken with all other assay components). In the presence of EGTA (1 mM), Ca^{2+}-stimulated p67^{cdpk} activity could be restored with the addition of 2 mM Ca^{2+}, resulting in a free Ca^{2+} concentration of 1 mM, which would clearly stimulate p67^{cdpk}. In conclusion, p67^{cdpk} has been shown to be a Ca^{2+}-stimulated protein kinase when assayed in the presence of Mg^{2+}.

Unlike most kinases characterised to date, some plant protein kinases show a higher level of autophosphorylation in the presence of Mn^{2+} than Mg^{2+} (Schaller and Bleecker 1993; Horn and Walker 1994; Ohto and Nakamura 1995), as do the animal receptor kinases (Yarden and Ullrich 1988). It has been suggested that Mn^{2+}-ATP is a more efficient substrate than Mg^{2+}-ATP for these protein kinases (Ohto and Nakamura 1995; Johnson et al. 1996). In vivo, however, the substrate is more likely to be Mg^{2+}-ATP, as only trace amounts of free Mn^{2+} exist in the cytoplasm of plant cells and Mg^{2+} can be as high as millimolar concentrations (Marschner 1986).

Aqueous two-phase partitioning provided a top phase of pure PM vesicles (Table 1). The lower Dextran phase however, contained some 1,3-β-glucan synthase activity, suggesting some PM contamination of the lower phase. This is not surprising considering any inside-out PM vesicles, in addition to ER and tonoplast, would partition to this lower phase (Larsson et al. 1984); this might explain the presence of $p67^{cdpk}$ in that phase. Solubilisation data quite clearly indicate that $p67^{cdpk}$ is an integral membrane protein; it was not solubilised at all by NaCl or EDTA treatment, and required at least 1% Triton X-100 for solubilisation. More efficient solubilisation was achieved with 1% Triton X-114. Whether or not it is a transmembrane protein has yet to be determined.

Association of $p67^{cdpk}$ activity with growing tissue was confirmed after analysis of its distribution in maize plants. Mature, fully expanded light-grown leaves of maize had barely detectable $p67^{cdpk}$ activity, whereas the cells of expanding green leaves, etiolated tissues and roots had high levels (Fig. 8). A more detailed study in both light-grown and etiolated maize plants revealed that expanding tissues, such as the growing tip, rapidly expanding leaves, and expanding coleoptiles had particularly high levels of $p67^{cdpk}$ activity (Fig. 8A). Mature etiolated tissue had less activity, but it was still significantly higher than in mature light-grown leaves (Fig. 8B). An inactivating effect of light was confirmed where $p67^{cdpk}$ activity dropped to light-grown levels in etiolated secondary leaves within 8 h of exposure to light (Fig. 9).

Light-dependent loss of in-situ phosphorylation has also been found in calcium-dependent kinase activity from etiolated wheat protoplasts. Fallon and Trewavas (1994) have suggested, that as with a possible mechanism for a similar effect of blue light (Short et al. 1992), the effect of light may be to increase in-vivo levels of phosphorylation, thus reducing potential in-situ phosphorylation. The light effects in these latter studies are very short compared with the effect we found: a few minutes rather than the several hours in maize plants. Higher levels of phosphorylated polypeptides in etiolated as opposed to green tissues have been found before (Verhey et al. 1993), suggesting that the attainment of photosynthetic capacity might be correlated with substantial changes in signalling pathways which involve kinase activity. The rate of light response found in our work suggests that either there is a transcriptional response to light or at least one which is not necessarily associated with primary light reception.

A common factor associated with $p67^{cdpk}$ activity is that it appears to be active in sink tissues. These include cultured cells, etiolated tissues, roots and rapidly expanding cells. Mature light-grown tissue on the other hand had virtually undetectable levels of $p67^{cdpk}$, and these source tissues obviously produce carbon endogenously. Clearly, the metabolic state of cells that require an exogenous carbon source will be different from those that are photosynthetically active.

The properties of $p67^{cdpk}$ are similar to those of a number of characterised protein kinase activities which have been postulated to be members of the calcium-dependent protein kinase (CDPK) family (Roberts and Harmon 1992; Verhey et al. 1993; Ohto and Nakamura 1995). Ohto and Nakamura (1995) identified two renaturable PM-bound kinases which required Ca^{2+} for activity, and were stimulated by sucrose, fructose or glucose. Verhey et al. (1993) reported four PM-bound kinase activities between 58 and 68 kDa that required micromolar free Ca^{2+} in the presence of Mg^{2+} for activity. These kinases phosphorylated on serine and to a lesser extent threonine residues, and were found to be most active in the etiolated as opposed to light-grown organs of zucchini. Whether $p67^{cdpk}$ can be classed as a membrane-bound member of the CDPK protein kinase family, will depend on the results of further biochemical and immuno-cross-reactivity data.

In conclusion, we have demonstrated the presence of a Ca^{2+}-stimulated, Mg^{2+}-dependent, PM-bound protein kinase active in sink tissues of a wide range of plant species, which has biochemical properties similar to those of other plant protein kinases. Determining the role $p67^{cdpk}$ plays in signal perception and transduction will require further biochemical characterisation, and knowledge of its primary amino acid sequence and target protein(s).

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