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Protein Kinases in Zucchini[1]

Characterization of Calcium-Requiring Plasma Membrane Kinases

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Using an in situ phosphorylation assay with zucchini (*Cucurbita pepo* L. cv Dark Green) seedling tissue, we have identified numerous polypeptides that are capable of acting as protein kinases. Total protein preparations from different organs contain different kinase profiles, but all are within the range of 55 to 70 kD. At least four kinases are associated with highly purified plasma membranes from etiolated zucchini hypocotyls. The major phosphorylated polypeptides from plasma membranes range in apparent molecular mass from 58 to 68 kD. The plasma membrane kinases are activated by micromolar concentrations of calcium and phosphorylate serine, and, to a lesser extent, threonine residues. These characteristics are similar to those of a soluble calcium-dependent protein kinase that has been purified to homogeneity from soybean suspension cultures. Three of the zucchini plasma membrane kinases share antigenic epitopes with the soluble soybean kinase. The presence of kinase activity at different apparent molecular masses may be indicative of separate kinases with similar characteristics. The zucchini hypocotyl protein kinases are not removed from plasma membrane vesicles by 0.5 M NaCl/5 mM ethylenediaminetetraacetate or by detergent concentrations below the critical micelle concentration of two types of detergent. This indicates that the plasma membrane protein kinases are tightly associated with the membrane in zucchini seedlings.

Protein phosphorylation, mediated by protein kinases (EC 2.7.1.37), is one of a small number of readily reversible covalent protein modifications available for intracellular control of structure, activity, and targeting of enzymes and other proteins. Protein kinases have been found in virtually every cellular compartment and serve myriad functions, from post-translational modification of proteins to involvement in signal transduction and amplification (for review, see Hunter, 1991). In plants, polypeptides capable of acting as protein kinases have been identified in the cytosol and in nuclei, mitochondria, and plastids, as well as the PM (for review, see Ranjeva and Boudet, 1987). Activities of several plant enzymes are affected by phosphorylation-dephosphorylation, including pyruvate dehydrogenase (Rao and Randall, 1980); quinate:NAD oxidoreductase (Refeno et al., 1982); Suc-P synthetase (Huber and Huber, 1990); and pyruvate, Pi dikinase (Ranjeva and Boudet, 1987). The PM proton ATPase is a target of phosphorylation, presumably via a PM protein kinase (Zocchi, 1985; Schaller and Sussman, 1987).

Although 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 extracellular stimuli. Examples of this include responses of tomato tissue culture cells to elicitor (Felix et al., 1991), of soybean to *Phytophthora* infection (Feller, 1989), and of tomato to citrus exocortis viriod infection (Vera and Conejero, 1990), as well as responses to tomato proteinase-inhibiting factor (Farmer et al., 1989) and syringomycin treatment (Bidwai and Takemoto, 1987).

A predominant protein kinase activity of plant PMs is stimulated by calcium (Schaller and Sussman, 1987; Ladror and Zielinski, 1989; Klimczak and Hind, 1990). Several Ca^{2+}-stimulated, PM-bound protein kinases have been identified in plants. Among these are two kinases from silver beet leaf (Klucis and Polya, 1988), one from pea epicotyl (Blowers et al., 1985), one from barley leaf (Klimczak and Hind, 1990), and one from oat root (Schaller et al., 1992). The oat root PM enzyme bears immunological homology to soybean CDPK (Harmon et al., 1987). Soybean CDPK is a soluble enzyme that contains a C-terminal calmodulin-like Ca^{2+}-binding domain contiguous with an N-terminal kinase catalytic domain (Harper et al., 1991; for reviews see Roberts and Harmon, 1992; Roberts, 1993).

In the course of our studies of Ca^{2+}-dependent phosphorylation in zucchini (*Cucurbita pepo* L. cv Dark Green), we have identified several protein kinases that are present to different degrees in total protein preparations from roots, hypocotyls, hooks, cotyledons, and plumules of dark- and light-grown seedlings. In addition, we have identified at least four species of PM-bound CRPKs with apparent molecular masses between 58 and 68 kD. Similar to the soluble 55-kD soybean CDPK, these zucchini hypocotyl PM protein kinases all require micromolar concentrations of Ca^{2+} for activity, and they phosphorylate Ser and, to a lesser extent, Thr. Like the oat PM enzyme, three of the zucchini PM protein kinases are

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Abbreviations: CDPK, calcium-dependent protein kinase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CRPK, calcium-requiring protein kinase; PKA, protein kinase A; PKC, protein kinase C; PM, plasma membrane; PVDF, polyvinylidene difluoride.

recognized by monoclonal antibodies to the soluble soybean CDPK.

MATERIALS AND METHODS

Chemicals and Radiochemicals

Radiolabeled ATP ([γ-^{32}P]ATP; specific activity, 3000 Ci mmol^{-1}) was obtained from New England Nuclear (Boston, MA). The divalent cation-chelating resin Chelex and reagents used for SDS-PAGE (acrylamide, bisacrylamide, SDS, *N,N,N′,N′*-tetra-methylethylenediamine, ammonium persulfate, molecular mass standards) were obtained from Bio-Rad. Biotrace NT nitrocellulose was acquired from Gelman, Inc. (Ann Arbor, MI). Anti-CDPK monoclonal antibody (Putnam-Evans et al., 1990) was the generous gift of Dr. Alice Harmon (University of Florida, Gainesville, FL). Goat anti-mouse alkaline phosphatase-conjugated secondary antibody was from Promega (Madison, WI). Unless otherwise noted, all other chemicals were obtained from Sigma.

Plant Material

Seeds of zucchini (*Cucurbita pepo* L. cv Dark Green) and oat (*Avena sativa* L. cv Aberdeen) were obtained from the Chas. A. Lilly Company (Portland, OR). Seeds of soybean (*Glycine max* L.) were obtained from First Alternative (Corvallis, OR). After surface sterilization for 10 min with 20% (v/v) bleach containing a few drops per liter of Tween-20, seeds were rinsed with tap water and sown directly on approximately 3 cm of moist vermiculite (*Avena*) or on moist vermiculite covered with several layers of unbleached absorbant paper (Kimtowels; Kimberly-Clark, Rosewell, GA) (*Cucurbita* and *Glycine*). After 5 d at 27°C in either dark ("dark-grown") or fluorescent-lighted ("light-grown," 35 μM m^{-2} s^{-1}) growth chambers, seedlings were harvested and processed as described below.

Preparation of Membrane Vesicles

All steps were performed on ice or at 3°C. Microsomal membrane vesicles were prepared by homogenizing dark-grown zucchini hypocotyls, using a Brinkman (Westbury, NY) Polytron at setting 8 for 20 to 30 s, with an equal volume of ice-cold buffer 1 (250 mM Suc, 10 mM Tris-Cl [pH 7.5], 1 mM EDTA, 1 mM DTT, 0.1 mM $MgSO_4$) plus protease inhibitors (0.2 mM PMSF, 1 μg mL^{-1} each leupeptin and pepstatin). The brei was filtered through four layers of cheesecloth, and the retentate was rehomogenized with an additional volume of buffer 1. The rehomogenized brei was filtered and the filtrates were pooled. After a 15-min centrifugation at 1000*g* (Beckman GPR, GH3.7 rotor) to remove cell walls and undisrupted cells, microsomes were pelleted by a 30-min centrifugation at 150,000*g* (Beckman L5–65, Ti50.2 rotor). Microsomal pellets were resuspended in buffer 2 (5 mM KPi [pH 7.8], 250 mM Suc, 4 mM KCl), flash frozen in liquid N_2, and stored at −70°C until use.

PM vesicles were prepared by aqueous two-phase (dextran/PEG) separation of microsomal membrane preparations from zucchini seedlings as described by Hicks et al. (1989). Protease inhibitors were replenished after each centrifugation. After the final centrifugation, PM vesicles were solubilized as described below or resuspended in a small volume of buffer 1 plus protease inhibitors, flash frozen in liquid N_2, and stored at −70°C. Marker enzyme assays indicate that zucchini hypocotyl PMs prepared in this manner are >95% pure (Hicks et al., 1989). Protein concentrations were estimated using either the method of Schaffner and Weissmann (1973), which eliminates interference by detergents, or a kit from Bio-Rad based on the Bradford assay. BSA was used as the standard for protein assays.

Preparation of Liquid N_2-Ground Tissues

For some experiments, to minimize exposure to protease activity, organs were harvested directly into liquid N_2 and stored at −70°C until they could be used. Each sample was subsequently ground with liquid N_2 in a mortar and pestle. The frozen powder was dropped, slowly and with stirring, into a small beaker containing SDS-PAGE loading buffer and 100 mM DTT. The small beaker was placed inside a boiling water bath, which maintained the contents of the small beaker at a temperature of at least 65°C. Several aliquots of each organ extract were frozen at −70°C before analysis on SDS-PAGE. A fresh aliquot was prepared just before each analysis by thawing and centrifuging in a microcentrifuge for 5 min. The supernatant was reheated to 95°C for 2 min before the gel was loaded. The amount of material needed for similar protein loads was determined empirically on minigels.

Electrophoresis

Protein electrophoresis was carried out according to instructions furnished with the Bio-Rad Protean II electrophoresis unit. Before SDS-PAGE, samples were heated at 95°C for 2 min in Laemmli sample buffer containing 100 mM DTT and electrophoresed on 10% (w/v) polyacrylamide gels (0.75 mm × 15 cm) unless noted otherwise.

In Situ Phosphorylation on Nitrocellulose

In situ phosphorylation on nitrocellulose blots was carried out as described by Celenza and Carlson (1986), with the following modifications. Subsequent to SDS-PAGE, proteins were electroblotted onto nitrocellulose in an LKB semidry blotting apparatus. The blotting buffer contained 12.5 mM Tris/35 mM Gly (pH 8.3). Nonfat dry milk (5% w/v) was used as the blocking agent. Renaturation buffer contained 50 mM bis-Tris-propane/Hepes (pH 6.8), 100 mM NaCl, 2 mM DTT, 1 mM EGTA, 0.1% (v/v) Nonidet P-40, 5 mM $MgCl_2$, 1.055 mM $CaCl_2$, and 0.25% (w/v) nonfat dry milk; renaturation was carried out at 4°C for at least 9 h. Phosphorylation buffer contained 50 mM bis-Tris-propane/Hepes pH 6.8, 1 mM EGTA, 5 mM $MgCl_2$, 1.055 mM $CaCl_2$, and 0.25% (w/v) nonfat dry milk. The labeling reaction was started by the addition of 20 μCi of [γ-^{32}P]ATP (specific activity 3000 Ci mmol^{-1}) 10 mL^{-1} of phosphorylation buffer (0.7 nM ATP) and continued for 60 min. No unlabeled (carrier) ATP was used. After incubation, the filter was washed with several changes of 10% (w/v) TCA/1 M H_3PO_4 and then with several changes of double-distilled water. Bands were visualized by

autoradiography; exposures were usually for 16 h without an intensifier screen.

Immunostaining

Following autoradiography, blots were incubated with anti-CDPK monoclonal antibody and then with goat anti-mouse alkaline phosphatase secondary antibody as described by Sambrook et al. (1989).

Protein Kinase Solubilization

Pelleted PMs were resuspended at 2 mg of protein mL^{-1} in buffer 3 (10 mM Tris [pH 8.5], 50 mM NaCl, 1 mM DTT, 10% [v/v] glycerol) containing 500 mM NaCl/5 mM EDTA or 0 to 3% (w/v) detergent and held on ice for 30 min. Detergents were CHAPS (Research Organics, Cleveland, OH) or Triton X-100. After centrifugation at 140,000*g* (Beckman TL-100, rotor TLA 100.3) for 5 min, the supernatants were recovered. The pellets were taken up in the appropriate solubilization buffer, and the volumes were adjusted to match those of the corresponding supernatants. Aliquots were then analyzed by SDS-PAGE, followed by in situ labeling on nitrocellulose.

Phosphoamino Acid Analysis

Following SDS-PAGE, PM proteins were blotted to PVDF and labeled in situ as described for nitrocellulose, except nonfat dry milk was omitted. After localization by autoradiography, bands were excised and hydrolyzed by treatment for 2 h with 6 N HCl at 105°C in microcentrifuge tubes. HCl was evaporated under a stream of air, and the hydrolysate was taken up in a small volume of chromatography buffer (15 mM KPi, pH 3.8) filtered, and analyzed by isocratic HPLC (Swarup et al., 1981). A 4.6- × 250-mm Beckman SAX anion-exchange column and internal phosphoamino acid standards were used for HPLC. Following addition of scintillation fluid (Dupont Formula 989), fractions were analyzed for radioactivity by scintillation counting.

RESULTS

We surveyed the activity of protein kinases from several different light-grown and etiolated organs of zucchini, as well as from dark-grown roots of oat and hypocotyls of soybean. Whole plant material was ground in liquid N_2 and extracted with hot SDS-PAGE-loading buffer to minimize protease action. Total proteins so prepared were separated on one-dimensional SDS-PAGE, blotted to nitrocellulose, and incubated with [γ-^{32}P]ATP following blocking of nonspecific binding sites with nonfat dry milk (in situ phosphorylation). As can be seen in Figure 1, lanes loaded with protein from etiolated zucchini hooks contained the greatest number of kinases active under these conditions, a total of eight bands between approximately 55 and 68 kD (Fig. 1A, lane 3). Lanes containing protein from light- and dark-grown zucchini cotyledons showed the least activity, with only two to three bands visible (Fig. 1A, lanes 4 and 7, respectively). Plumules contained a kinase profile most similar to etiolated hooks (Fig. 1, cf. lanes 3 and 8). In general, more activity was present in lanes loaded with protein from etiolated organs than from their light-grown counterparts.

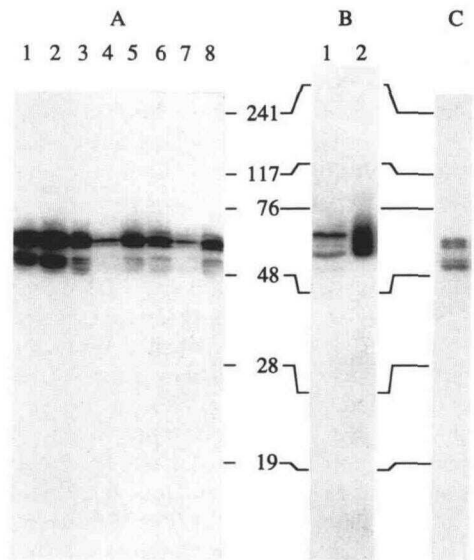

Figure 1. Size and diversity of protein kinases in zucchini, soybean, and oat. Proteins were separated by SDS-PAGE and electroblotted to nitrocellulose. After the blots were blocked and renatured, they were incubated with 2 μCi mL^{-1} of [γ-^{32}P]ATP for 1 h, then washed with 10% TCA/1 M H_3PO_4, and subjected to autoradiography. Results from three autoradiograms are shown. A, Zucchini organs that were frozen and ground in liquid N_2 before loading. Lane 1, Dark-grown roots; lane 2, etiolated hypocotyls; lane 3, etiolated hooks; lane 4, etiolated cotyledons; lane 5, light-grown hypocotyls; lane 6, apical 5 mm of light-grown hypocotyls; lane 7, light-grown cotyledons; lane 8, light-grown plumule. Lanes contain similar amounts of protein. B, Similar amounts of protein from etiolated soybean hypocotyls (lane 1) and dark-grown oat roots (lane 2) prepared as described for A. C, Forty micrograms of zucchini hypocotyl PM protein prepared by aqueous two-phase (dextran/PEG) partitioning. Autoradiography was for 16 h without an intensifying screen.

Total protein from etiolated zucchini hypocotyls yielded radiolabeled bands at six molecular masses, between approximately 58 and 68 kD (Fig. 1A, lane 2). Lanes loaded with protein from soybean hypocotyls contained five labeled bands, with apparent molecular masses ranging from approximately 54 to 67 kD (Fig. 1B, lane 1). Roots from etiolated plants displayed a kinase profile similar to that of hypocotyls (Fig. 1A, lane 1). Lanes loaded with protein from oat roots contained only two labeled bands, at approximately 57 and 61 kD (Fig. 1B, lane 2). None of the soybean or oat bands had molecular masses identical with any zucchini band (Fig. 1, cf. A and B).

To assess which kinases were PM associated, we examined kinase activity in zucchini hypocotyls in greater depth. When PM proteins isolated by aqueous two-phase (dextran/PEG) partitioning were labeled as described above, four bands at 58.6, 60.5, 65.7, and 67.9 kD were most heavily labeled (Fig. 1C). These four bands matched the most heavily labeled bands seen in experiments involving total protein from etio-

lated hypocotyls (cf. Fig. 1A, lane 2). No labeled bands were seen on blots incubated with [α-^{32}P]ATP (data not shown).

Calcium dependence of the PM kinases was examined by in situ phosphorylation on nitrocellulose, using Chelex-treated BSA as a calcium-free blocking agent and Chelex-treated solutions during blot preparation and labeling. Renaturation was in the presence of 87 μM free Ca^{2+}. As shown in Figure 2, all four kinases were active in 11 μM free Ca^{2+}, whereas virtually no activity was present in the same region following incubation of blots with [γ-^{32}P]ATP in the presence of 1 mM EGTA (Fig. 2, A and B). Increasing free calcium to 87 μM had no further effect on labeling (Fig. 2C). Similar results were obtained when blots were renatured in the absence of Ca^{2+} (data not shown).

The amino acid specificity of phosphorylation reactions is useful in classification of protein kinases (Krebs, 1986). To determine amino acid specificity, the four zucchini hypocotyl PM kinases were labeled with [γ-^{32}P]ATP after blotting to PVDF. The individual bands were excised and hydrolyzed with HCl, and labeled amino acids were identified by HPLC and scintillation counting. As can be seen in Figure 3, all four kinases autophosphorylated on both Ser and, to a lesser extent, Thr residues (Fig. 3).

To assess the antigenic relatedness of the zucchini PM kinases to soybean CDPK, western blot analysis was carried out following in situ phosphorylation. As shown in Figure 4, anti-CDPK monoclonal antibodies (Putnam-Evans et al., 1990) cross-reacted with three major bands having molecular masses identical with those labeled by [γ-^{32}P]ATP. An additional, somewhat larger band, not labeled by [γ-^{32}P]ATP in this experiment, was also recognized by the monoclonal antibodies.

Finally, as shown in Figure 5, we examined the extent to which the PM-associated zucchini enzymes were associated with the membrane. No detectable kinase activity was removed from PM by washing the membranes with 0.5 M NaCl/5 mM EDTA (Fig. 5, lanes 1). Relatively high detergent concentrations were required to remove the proteins from the PM: only at CHAPS (Fig. 5A) or Triton X-100 (Fig. 5B) concentrations greater than the critical micelle concentration (0.49% [w/v] and 0.02% [w/v], respectively) of each detergent was significant kinase activity released into the supernatant. Although the largest kinases were solubilized at lower detergent concentrations, all four kinases were soluble in concentrations of >0.3% (w/v) Triton X-100 under these conditions. Further experiments demonstrated that all four kinases could also be solubilized by 1% (w/v) CHAPS; similar results were obtained with 1% (w/v) octyl glucoside (data not shown). Following solubilization, removal of CHAPS during gel filtration led to aggregation of the protein kinases (data not shown).

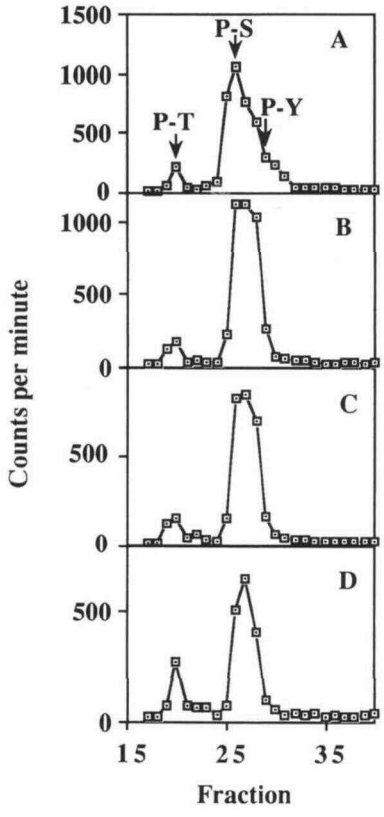

Figure 3. Phosphoamino acid analysis. Labeled bands were excised from PVDF blots and hydrolyzed in microcentrifuge tubes with 6 N HCl. After lyophilization, the residue was taken up in chromatography buffer, spiked with phosphoamino acid standards, and chromatographed on HPLC anion exchange. Arrows indicate elution fractions of internal standards (P-S, phosphoserine; P-T, phosphothreonine; P-Y, phosphotyrosine). A to D represent results from largest through smallest PM kinases, respectively.

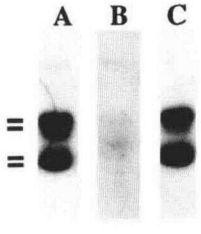

Figure 2. Calcium requirement of the PM kinases. Blots were prepared as described, blocked with Chelex-treated BSA, and incubated with Chelex-treated buffers. Both blots were incubated in the same renaturation buffer containing 87 μM Ca^{2+} and rinsed with the appropriate phosphorylation buffer before labeling. Autoradiograms (16 h, no intensifying screen) are shown of blots labeled in the presence of 11 μM Ca^{2+} (A), 1 mM EGTA (B), or 87 μM Ca^{2+} (C). Calcium concentration was controlled using an EGTA/Ca^{2+}/Mg^{2+} buffer, and free Ca^{2+} was calculated using a computer program (Perrin and Sayce, 1967) with constants from Martell and Smith (1974). Bars indicate major polypeptides that require Ca^{2+} for activity.

DISCUSSION

In situ SDS-PAGE phosphorylation, either in gels or on nitrocellulose blots, has been used in the analysis of purified protein kinases from animals (Geahlen et al., 1986), yeast (Celenza and Carlson 1986), and plants (Blowers et al., 1985; Harmon et al., 1987; Klimczak and Hind, 1990). We used this approach to identify and characterize protein kinase

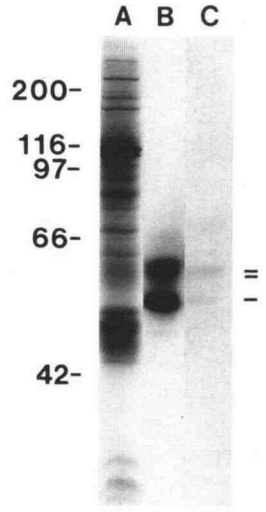

Figure 4. Western blot analysis: antigenic similarity of the zucchini PM kinases to soybean CDPK. After labeling and autoradiography as described for Figure 1, the blot was incubated with a mixture of three anti-CDPK monoclonal antibodies. Immunodecorated proteins were located with alkaline phosphatase-conjugated goat anti-mouse antibody. A, Coomassie-stained gel. B, Autoradiogram (16-h exposure, no intensifying screen). C, Western analysis of blot used to create B. Bars indicate the major phosphorylated and immunoreactive bands.

activity in zucchini with respect to organ distribution, subcellular localization, molecular mass, calcium requirement, amino acid specificity, and immunological similarity to other kinases.

We examined total protein extracted from dark-grown roots, hypocotyls, hooks, and cotyledons, as well as protein from light-grown hypocotyls, opened hooks, cotyledons, and plumules (Fig. 1A). We also analyzed dark-grown soybean hypocotyls and *Avena* roots (Fig. 1B). In zucchini, hooks contained the largest diversity of kinases. Eight polypeptides exhibiting protein kinase activity, with molecular masses ranging from approximately 52 to 67 kD, were present in hook extracts. It is interesting that, although the hooks had opened in light-grown seedlings, the kinase pattern from the apical 5 mm of light-grown hypocotyls more closely resembled that of etiolated hooks than that of the lower part of light-grown hypocotyls. Cotyledons, both light and dark grown, contained the fewest protein kinases active under these conditions. Intermediate numbers and activities of kinases were present in the other zucchini organs. Although SDS-PAGE should separate the kinases from potential inhibitors, such as phenolics, which may be present in higher concentrations in light-grown material or in cotyledons, this explanation for the difference in activity between light-grown and etiolated tissues cannot be completely ruled out.

Total protein preparations from etiolated zucchini hypocotyls yielded six radiolabeled bands with molecular masses between 58 and 78 kD. In comparison to zucchini, etiolated soybean hypocotyls were found to contain approximately five labeled bands, with molecular masses between approximately 54 and 67 kD. The 54-kD band from soybean hypocotyls is similar in size to a 55-kD CDPK that has been purified from soybean suspension-cultured cells (Putnam-Evans et al., 1990). Oat roots contained only two kinases active in our in situ phosphorylation assay, at approximately 57 and 61 kD. Schaller and co-workers (1992) described three oat root polypeptides, with molecular masses of 57, 61, and 79 kD, that cross-react with a mixture of monoclonal antibodies raised against soybean CDPK. Although we did not detect phosphorylation products in the 79-kD size range identified by immunostaining of oat root proteins (Schaller et al., 1992), Schaller et al. also observed only very limited in vitro phosphorylation of the 79-kD immunoreactive polypeptide of oat (E. Schaller, personal communication).

A potential trivial explanation for the presence of multiple kinase bands is posthomogenization proteolytic activity, which might convert one or more higher molecular mass kinases into a series of smaller ones. Evidence suggesting that this can occur in plants has been presented (Schaller et al., 1992). However, the same zucchini kinase bands were seen in total protein prepared by freezing and grinding harvested hypocotyls directly in liquid N_2 and then dropping the frozen powder into hot SDS-loading buffer before electrophoresis, blotting, and labeling (Fig. 1A). Heating at high temperatures with SDS inactivates plant PM proteases (North, 1989). Thus, the presence of kinase activity at different apparent molecular masses may be indicative of the existence of separate kinases with similar characteristics. We cannot, however, rule out the possibility that any of the kinase bands are derived in vivo from a larger kinase or kinases via endogenous protease activity.

Examination of zucchini PM proteins via in situ phosphorylation on nitrocellulose blots led to the identification of

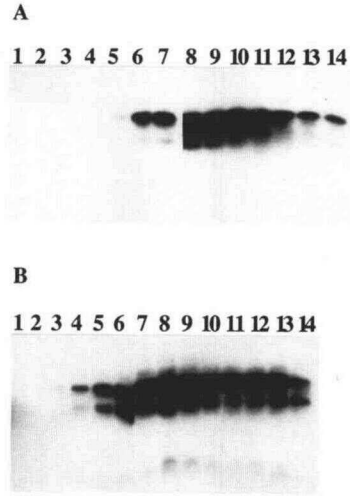

Figure 5. PM kinase solubilization. Autoradiograms from blots of gels loaded as follows. Lanes 1 to 7, Supernatants; lanes 8 to 14, the corresponding pellets. A, CHAPS; B, Triton X-100. Treatments: Lanes 1 and 8, 500 mM NaCl/5 mM EDTA; lanes 2 and 9, 0.01% detergent; lanes 3 and 10, 0.03% detergent; lanes 4 and 11, 0.1% detergent; lanes 5 and 12, 0.3% detergent; lanes 6 and 14, 1% detergent; lanes 7 and 13, 3% detergent. Exposures were for 16 h without an intensifying screen.

at least four major PM-bound protein kinases (Fig. 1C). The molecular masses of these polypeptides are identical with those of the four most heavily labeled bands found among total protein from hypocotyl (cf. Fig. 1A, lane 2). Thus, the majority of protein kinases active under these conditions are evidently PM localized.

Using in situ phosphorylation on nitrocellulose, we determined that Ca^{2+} is not required for effective renaturation of the protein kinases. The zucchini PM protein kinases require Ca^{2+} for autophosphorylation, however, at about the same concentration as does soybean CDPK. CDPK displayed 100-fold activation in the presence of 10 μM free Ca^{2+} (Harmon et al., 1987; Putnam-Evans et al., 1990). The zucchini PM kinases were virtually inactive in the presence of 1 mM EGTA but were active in the presence of 11 μM free Ca^{2+} (Fig. 2).

Phosphoamino acid analysis suggests another similarity between CDPK and the zucchini kinases. The zucchini kinases were phosphorylated on Ser and, to a lesser extent, Thr residues (Fig. 3). Soybean CDPK autophosphorylates primarily Ser but also Thr (Putnam-Evans et al., 1990).

Recent cloning of a gene encoding a kinase closely related to CDPK has demonstrated that CDPK is the prototype for a new family of calcium-regulated protein kinases in which kinase activity is modulated by direct binding of calcium. Sequence analysis has shown that CDPK contains a C-terminal calmodulin-like Ca^{2+}-binding domain contiguous with an N-terminal kinase catalytic domain. Southern blot analysis of genomic DNA from soybean and *Arabidopsis thaliana* reveals multiple restriction fragments hybridizing with a polymerase chain reaction-generated CDPK probe (Harper et al., 1991), which is consistent with the possibility that multiple subspecies exist. Because of similarities in terms of Ca^{2+} dependence, amino acid specificity, and molecular mass between the zucchini PM kinases and soybean soluble CDPK, we assessed the immunological relatedness of kinases from the two organisms.

Western blot analysis of zucchini PM proteins following in situ labeling showed that three of the major labeled polypeptides were recognized by a mixture of three monoclonal antibodies raised against CDPK (Fig. 4, bars; Putnam-Evans et al., 1990). Presumably, the smallest labeled band is not immunostained in this experiment because it is more widely diverged than the other three, because it is present in smaller quantity, or for some other reason. An approximately 76-kD band, which was not radiolabeled by [γ-^{32}P]ATP under these conditions, was also recognized by the monoclonal antibodies (Fig. 4). It is interesting that, in oat root, three bands are recognized by anti-CDPK monoclonal antibody (Schaller et al., 1992), but little protein kinase activity is associated with the largest band, at 79 kD (E. Schaller, personal communication). It should be noted that the anti-CDPK monoclonal antibodies recognize epitopes in the nucleotide-binding domain remote from the calmodulin-like Ca^{2+}-binding domain (A. Harmon, personal communication). Thus, binding of the antibodies to a protein kinase does not necessarily demonstrate that the kinase is a member of the CDPK family of kinases; such a demonstration can most reliably come from sequence analysis (Roberts, 1993). To avoid confusion, we will refer to the zucchini kinases as CRPKs.

In contrast to the soluble soybean CDPK, the zucchini PM CRPK are evidently integral membrane proteins or are otherwise tightly associated with the PM. They were not removed from PM by 0.5 M NaCl/5 mM EDTA washes and were only solubilized by ≥1% (w/v) CHAPS or ≥0.3% (w/v) Triton X-100. The larger of the major kinase bands appeared to be somewhat more soluble or more active after solubilization than the smaller kinases under these conditions (Fig. 5). When detergent was removed by gel filtration, the kinases aggregated (data not shown). These results suggest that all of the 58- to 68-kD polypeptides with kinase activity are membrane bound and contain hydrophobic regions. In this sense, they resemble the 79-kD PM-associated calcium-activated protein kinase from *Avena* (Schaller et al., 1992).

Peptide mapping or individual sequence analysis will be necessary to determine whether any of the PM bands represent distinct polypeptides or one or more sets of subspecies of one or more types of protein kinase. Some types of protein kinase, including PKC (Nishizuka, 1988), cAMP-dependent PKA (Dostmann et al., 1990), Ca^{2+}/calmodulin-dependent protein kinase II (Colbran and Soderling, 1990), cGMP-dependent protein kinase (Ruth et al., 1991), and raf (Sithanandam et al., 1990), exist as related subspecies. The PKC family consists of at least seven subspecies, which display variable tissue distribution and subtly different biochemical regulation (Nishizuka, 1988). Different isozymes of some protein kinases, including those of PKC (Nishizuka, 1988; Otte and Moon, 1992) and PKA (Ginty et al., 1992), have been suggested to have particular functions. In other cases, the roles of different forms have not been elucidated. Heterologous molecular masses of subspecies or isozymes arise by mechanisms that include expression of separate genes on different chromosomes (Nishizuka, 1988) and differential mRNA splicing (Francis et al., 1988–1989).

As noted above, although several lines of evidence suggest that the zucchini PM kinases might be membrane-bound members of the CDPK family described by Harper et al. (1991), such a conclusion is premature. Purification and sequence analysis of the zucchini hypocotyl PM CRPK, as well as further characterization of the kinases from other organs, should reveal more information concerning their relationship both to one another and to other classes of kinases. It should also facilitate the generation of reagents useful in establishing the regulation and function of these enzymes.

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