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An increase in cytosolic calcium ion concentration precedes hypoosmotic shock-induced activation of protein kinases in tobacco suspension culture cells

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Abstract Hypoosmotic shock induced a transient increase in cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$) and subsequent activation of 50-, 75- and 80-kDa protein kinases in tobacco (*Nicotiana tabacum* L.) suspension culture cells. Depletion of external calcium suppressed both the elevation of $[Ca^{2+}]_{cyt}$ and the activation of protein kinases in response to hypoosmotic shock, indicating that the elevation of $[Ca^{2+}]_{cyt}$ is prerequisite for the activation of protein kinases. Pharmacological analysis indicated that the hypoosmotic shock-activated protein kinases were activated by phosphorylation, suggesting that the activities of these protein kinases are regulated by putative protein kinases. These results suggest that the hypoosmotic signal is transduced to protein kinase cascades which are triggered by $[Ca^{2+}]_{cyt}$ elevation.

Key words: Hypoosmotic shock; Protein kinase; Cytosolic Ca^{2+}; Aequorin; *Nicotiana tabacum*

1. Introduction

Protein phosphorylation in response to external stimuli is an important biochemical process in eukaryotic cells. In plants, it has been reported that phytohormones and external stimuli, such as light, cold shock, fungal elicitor and wounding, induce the phosphorylation of specific proteins and activate protein kinases [1–7]. Transient elevation of cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$) has been reported to be caused by external stimuli such as cold shock, fungal elicitor and wounding as well [8,9]. Calcium ions in the cytosol function as a primary second messenger in signal transduction [10,11] and have been shown to modify a number of physiological processes [12,13]. The above stimuli were supposed to activate protein kinases via calcium signal. In plant cells, Ca^{2+}-dependent or calmodulin-like domain protein kinase (CDPK) has been well characterized; however, protein kinase C whose roles have been established in Ca^{2+}-mediated signal transduction in animal cells has not been identified.

The maintenance and regulation of turgor pressure is essential for many aspects of plant growth, development and movement. Hypoosmotic shock induces an increase in turgor pressure dependent upon the osmotic potential of the cell. After shock, plant cells can usually recover their original osmotic potential [14], a phenomenon termed hypoosmotic regulation. To accomplish this, plant cells must have mechanisms for both sensing hypoosmolarity and transducing this information to effect response leading to hypoosmotic regulation. We previously reported that protein phosphorylation might be involved in osmoregulation in a halotolerant green alga *Dunaliella tertiolecta* and that Ca^{2+}-dependent process(es) might be associated with the hypoosmotic shock-induced phosphorylation of proteins [15]. It was suggested that a CDPK which was already purified and characterized [16] involved in the above process and several other protein kinases seem likely to participate in the mechanism of hypoosmotic signaling in *Dunaliella*. These protein kinases were assumed to compose a protein kinase cascade(s) [17]. In order to elucidate the roles of Ca^{2+} in the signal transduction pathway in plant cells we have genetically transformed tobacco suspension culture cells to express aequorin and measured cytosolic calcium as calcium-dependent luminescence of aequorin. Hypoosmotic shock-induced transient $[Ca^{2+}]_{cyt}$ elevation was observed in the transformed tobacco cells [18]. In the present study, we examined the involvement of protein kinases in hypoosmotic signaling as monitoring $[Ca^{2+}]_{cyt}$ with aequorin luminescence and protein kinase activities with an in-gel protein kinase assay.

2. Materials and methods

2.1. Materials

Dephosphorylated casein, histone type III-S (histone H1), MBP were purchased from Sigma (St. Louis, MO). [γ-^{32}P]ATP (148 TBq/mmol) was obtained from ICN (Cost Mesa, CA). Anti-phosphotyrosine antibody (PY-20) was purchased from Transduction Laboratories (Lexington, KY). Prestained SDS-PAGE standards and protein A–Sepharose CL-4B were products of Bio-Rad (Hercules, CA) and Pharmacia (Uppsala, Sweden), respectively. K-252a and calyculin A were purchased from Seikagaku Kogyo Co. (Tokyo, Japan) and Sigma, respectively.

2.2. Plant materials and hypoosmotic shock treatment

The transgenic tobacco (*Nicotiana tabacum* L. cv bright yellow 2) suspension culture cells expressing apoaequorin which had been prepared with *Agrobacterium tumefaciens* LBA4404 containing pMAQ2 [8,18] were used. Three-day-old tobacco suspension culture cells in the mid-log phase of growth were washed with and suspended in a fresh culture medium containing 3% sucrose (200 mOsm) and used after incubating over 30 min. The cells were subjected to hypoosmotic shock by diluting with 2 volumes of the culture medium depleted of sucrose (90 mOsm). This operation changed the extracellular osmolarity from 200 to 125 mOsm.

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Abbreviations: BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; $[Ca^{2+}]_{cyt}$, cytosolic free calcium ion concentration; MBP, myelin basic protein; TCA, trichloroacetic acid

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2.3. Methods

An in-gel protein kinase assay and immunoprecipitation with an anti-phosphotyrosine monoclonal antibody were performed as reported previously [17]. Radioactive bands on the gels were visualized by a Bioimaging Analyzer BAS 2000 (Fuji Photo Film Co., Tokyo, Japan).

Luminescence intensity and osmotic pressure of the medium were measured with a CHEM-GROW photometer (American Instrument Co., MD) and an Advanced Osmometer, model 3D3 (Advanced Instruments, MA), respectively as described previously [18].

3. Results

3.1. Activation of protein kinases in response to hypoosmotic shock

When the tobacco cell suspension in the 200 mOsm medium was diluted with 2 volumes of the culture medium depleted of sucrose (90 mOsm; Fig. 1A), a 50-kDa protein kinase was transiently activated 2–10 min after shock with a maximum at 5 min. A 75-kDa protein kinase was activated 2–15 min after shock and remained activated for 30 min. Activation of a 80-kDa protein kinase was observed 2–15 min after shock. These changes in activities of protein kinases were not observed in the cells treated with the ordinary medium (200 mOsm; Fig. 1B) and also in the cells treated with 200 mM mannitol solution (200 mOsm; data not shown). Therefore the observed activation of these protein kinases was caused by hypoosmotic shock induced by reducing the extracellular osmolarity but not by the lack of sucrose as a nutrient. Similar changes in protein kinase activities were detected in a histone gel but scarcely detected in a casein gel (data not shown). When an in-gel protein kinase assay was performed in a SDS-PAGE gel that contained no substrate, protein kinase activities were scarcely detected (data not shown). These results indicate that the protein kinases detected on the gel prefer MBP as substrates to other proteins examined and their autophosphorylation activities were very low.

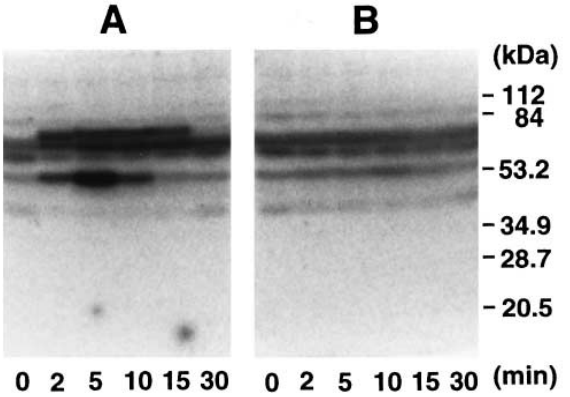

Fig. 1. The effect of hypoosmotic shock on the protein kinase activities in tobacco suspension culture cells. Tobacco suspension culture cells were killed by 10% TCA at indicated times after addition of hypoosmotic medium (A) or isoosmotic medium (B). The cell extracts were subjected to SDS-PAGE followed by in-gel protein kinase assays. The reaction mixture contained 40 mM HEPES–KOH (pH 7.6), 10 mM $MgCl_2$, 0.5 mM EGTA, 1 mM DTT and 25 μM [γ-^{32}P]ATP (37 kBq/ml). A: Hypoosmotic shock (200–125 mOsm). B: Isoosmotic condition (constant osmolarity at 200 mOsm). Molecular masses of standard proteins are indicated at the right of the gel.

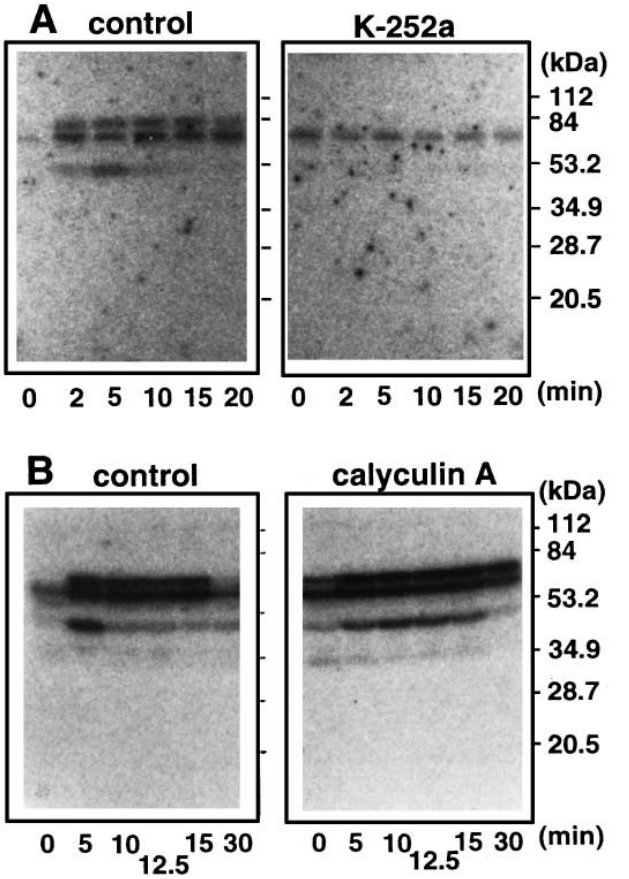

Fig. 2. The effect of K-252a and calyculin A on the activation of protein kinases in the tobacco cells in response to hypoosmotic shock. Tobacco suspension culture cells were preincubated for 5 min with 1 μM K-252a (A) or 0.5 μM calyculin A (B), and subjected to hypoosmotic shock. Controls were treated with 0.01% DMSO. The cells were killed at indicated time after the shock and analyzed by in-gel protein kinase assays.

3.2. Activation of the protein kinases was regulated by protein phosphorylation

Preincubation of cells for 5 min with K-252a, an inhibitor of serine/threonine-type protein kinases, markedly suppressed the hypoosmotic shock-induced activation of 50-, 75- and 80-kDa protein kinases (Fig. 2A). When preincubated for 5 min with calyculin A, a potent inhibitor of protein phosphatase 1 and 2A, the 50-kDa protein kinase activated 5 min after shock was sustained in its activated state for 15 min. The activities of 75- and 80-kDa protein kinases in the calyculin A-treated cells were much higher than the respective controls at $t=0$, increased after shock and kept their activated state for 30 min (Fig. 2B). These results indicate that the protein kinases are up-regulated by protein phosphorylation in response to hypoosmotic shock and suggest that they are down-regulated by dephosphorylation. When extracts of hypoosmotically shocked tobacco suspension culture cells were treated with alkaline phosphatase, protein kinases activities were completely inactivated (data not shown), indicating that the hypoosmotic shock-activated protein kinases detected with an in-gel protein kinase assay were activated by phosphorylation.

3.3. Tyrosine phosphorylation of a 50-kDa protein kinase

Immunoprecipitates with an anti-phosphotyrosine mono-

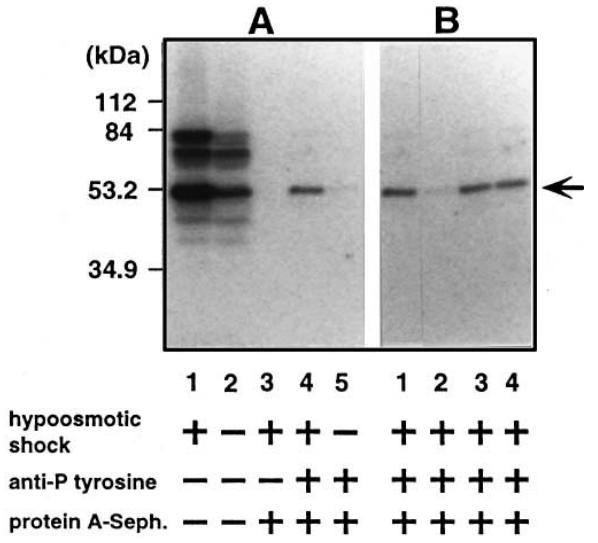

Fig. 3. Activities of protein kinases in immunoprecipitates from cell extracts treated with anti-phosphotyrosine antibody. The tobacco cells were subjected to hypoosmotic treatment and killed after 3 min. The cell extracts were solubilized in Laemmli's sample buffer, and immunoprecipitated with an anti-phophotyrosine monoclonal antibody (PY-20). Hypoosmotic shock + and − represent cells treated hypoosmotically and isoosmotically, respectively. Anti-P-tyrosine + and − represent cell extracts treated with the antibody and preimmune serum, respectively. Protein A-Seph + and − represent cell extracts treated with and without protein A–Sepharose, respectively. B: The antibody was treated in the absence (lane 1), or presence (lane 2) of 10 μM phosphotyrosine, phosphothreonine (lane 3) or phosphoserine (lane 4). The arrow indicates the 50-kDa protein kinases.

clonal antibody were analyzed with an in-gel protein kinase assay (Fig. 3A). The 50-kDa protein kinase was detected in immunoprecipitate from the hypoosmotically shocked cells (lane 4). When the antibody was excluded from the reaction mixture for immunoprecipitation assay, the 50-kDa protein kinase was not detected (lane 3). As shown in Fig. 3B, co-incubation with phosphotyrosine (lane 2) prevented immunoprecipitation of the 50-kDa protein kinase with the anti-phosphotyrosine antibody, but co-incubation with phosphothreonine (lane 3) or phosphoserine (lane 4) did not. These results show that the 50-kDa protein kinase possesses phosphotyrosine residue in its active form, but the 75- and 80-kDa protein kinases do not.

3.4. *Increase in cytosolic free Ca^{2+} concentration precedes activation of protein kinases*

We have observed that hypoosmotic shock induced a marked transient elevation of $[Ca^{2+}]_{cyt}$ in the tobacco suspension culture cells [18] (see also Fig. 4A). $[Ca^{2+}]_{cyt}$ increased rapidly and peaked 70 s after hypoosmotic shock, decreased quickly for the next 30 s and then gradually returned to the original level. The $[Ca^{2+}]_{cyt}$ elevation was completely suppressed when the extracellular Ca^{2+} was chelated with BAPTA (Fig. 4A, trace-Ca^{2+}). This shows that hypoosmotic shock induces Ca^{2+} influx across the plasma membrane of tobacco cells and results in $[Ca^{2+}]_{cyt}$ elevation. To elucidate the relationship between $[Ca^{2+}]_{cyt}$ elevation and the activation of protein kinases induced by hypoosmotic shock, activities of protein kinases were analyzed with cells hypoosmotically shocked in the presence or absence of the extracellular Ca^{2+} (Fig. 4B,C). Activation of 50-, 75-, and 80-kDa protein kinases occurred after $[Ca^{2+}]_{cyt}$ elevation and the activated states were kept after decrease in $[Ca^{2+}]_{cyt}$ to the original level (Fig. 1). The hypoosmotic shock-induced activation of 50-, 75- and 80-kDa kinases was not observed in the cells shocked in the absence of Ca^{2+} (Fig. 4C). This result shows that hypoosmotic shock alone does not activate these protein kinases, but $[Ca^{2+}]_{cyt}$ elevation is prerequisite for the activation of these protein kinases.

Fig. 5A shows that addition of Ca^{2+} to the cell suspension which had been hypoosmotically shocked in the absence of extracellular Ca^{2+}, induced a rapid and transient $[Ca^{2+}]_{cyt}$ elevation. The cells were killed at the times indicated (a–e) and analyzed their protein kinase activities with the in-gel protein kinase assay (Fig. 5C). Activation of 50-, 75- and 80-kDa protein kinases was observed only after $[Ca^{2+}]_{cyt}$ elevation. Addition of Mg^{2+} neither caused $[Ca^{2+}]_{cyt}$ elevation (Fig. 5B) nor activation of protein kinases (Fig. 5D). These

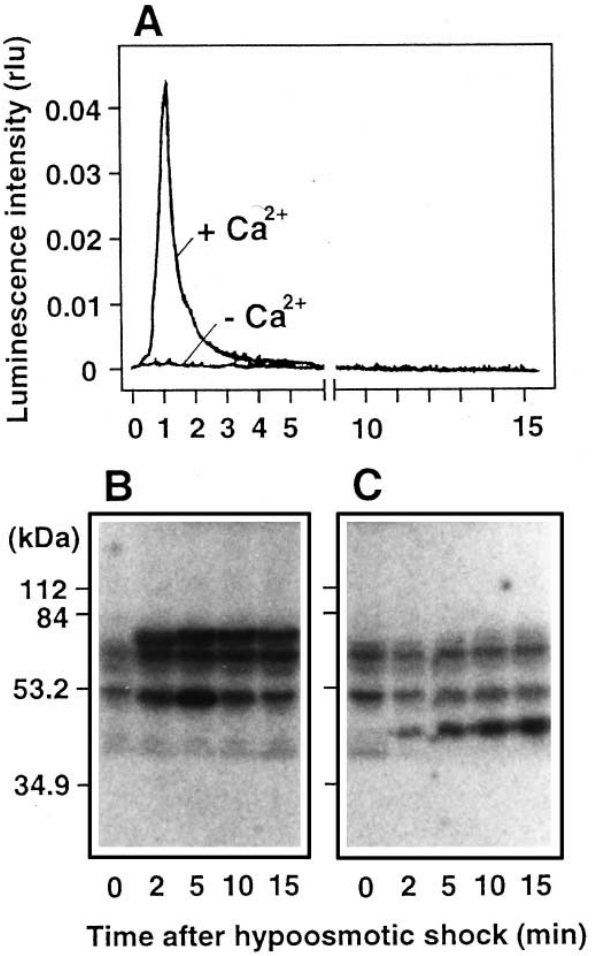

Fig. 4. The effect of extracellular Ca^{2+} on $[Ca^{2+}]_{cyt}$ elevation and on the activation of protein kinases in response to hypoosmotic shock. The tobacco suspension culture cells expressing apoaequorin were incubated with 1 μM coelenterazine to constitute aequorin. The cells were washed with and incubated in a fresh medium (normal medium) or a fresh medium containing 5 mM BAPTA (Ca^{2+}-depleted medium) for 30 min. The cell suspensions in the normal medium and the Ca^{2+}-depleted medium were subjected to hypoosmotic shock. A: Luminescence in the normal ($+Ca^{2+}$) and Ca^{2+}-depleted ($-Ca^{2+}$) medium. Changes in activities of protein kinases in the normal (B) and the Ca^{2+}-depleted medium (C).

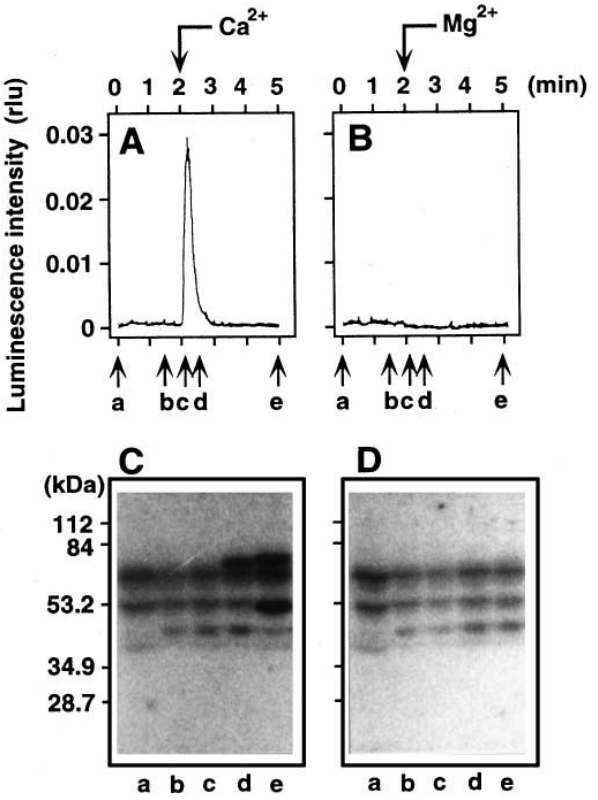

Fig. 5. The relationship between $[Ca^{2+}]_{cyt}$ elevation and activation of protein kinases. The tobacco cells expressing apoaequorin were incubated with 1 μM coelenterazine and were washed with and incubated in the fresh medium containing 5 mM BAPTA for 30 min. The cells were subjected to hypoosmotic shock as indicated by (a). Two min after hypoosmotic shock, 10 mM $CaCl_2$ (A) or 10 mM $MgCl_2$ (B) was added to the cell suspension. Changes in activities of protein kinases were analyzed 0, 1.5, 2.1, 2.5 and 5 min after the shock (indicated by a, b, c, d and e, respectively). C: Activities in the cells which received $CaCl_2$. D: Activities in the cells which received $MgCl_2$.

results coincide with the above observation that $[Ca^{2+}]_{cyt}$ elevation is prerequisite for activation of 50-, 75- and 80-kDa kinases.

Hypoosmotic shock in the absence of Ca^{2+} activated a 45-kDa protein kinase (Fig. 4C and 5C,D) which was deactivated when $[Ca^{2+}]_{cyt}$ increased (Fig. 5C). This protein kinase might be activated by hypoosmotic shock but strongly down-regulated by a Ca^{2+}-dependent protein phosphatase.

3.5. Presence of Ca^{2+}-dependent protein kinase

When protein kinase reaction was performed in the presence of Ca^{2+}, a 56-kDa protein kinase was detected (Fig. 6, lane 1). This protein kinase band shifted to 50 kDa when the sample was electrophoresed in the presence of Ca^{2+} (lane 2), indicating that the kinase directly bound Ca^{2+}. This protein kinase was not detected in the absence of Ca^{2+} in the reaction mixture. These are typical characteristics of CDPK [19].

4. Discussion

The present study has indicated that hypoosmotic shock induced activation of 50-, 75- and 80-kDa protein kinases in tobacco suspension culture cells (Fig. 1). The activation of these protein kinases was accompanied by phosphorylation and deactivation. The 50-kDa protein kinase was tyrosine phosphorylated but its deactivation was inhibited by calyculin A, an inhibitor of protein phosphatase 1 and 2A [20]. The putative protein phosphatase which deactivates the 50-kDa protein kinase might not be the target for calyculin A. The target might be the protein phosphatase which deactivates the upstream protein kinase of the 50-kDa protein kinase. The 75- and 80-kDa protein kinases might be dephosphorylated by protein phosphatase 1- or 2A-type phosphatases.

Tyrosine phosphorylation in response to hypoosmotic shock in budding yeast and animal cells has been reported [21,22]. A mitogen-activated protein kinase MPK1 was activated by hypoosmotic shock in budding yeast [23]. These reports and our present results suggest that protein kinases accompanying tyrosine phosphorylation in protein kinase cascade play significant roles in hypoosmotic signal transduction pathway in these organisms. In tobacco cells, fungal elicitor and cutting have been reported to induce transient activation and tyrosine phosphorylation of protein kinases [5,6] and wounding to activate a mitogen-activated protein kinase [7]. The fungal elicitor-induced activation of protein kinase(s) was suggested to require $[Ca^{2+}]_{cyt}$ elevation by the pharmacological study [5]. Knight et al. [8] reported an elicitor-induced transient increase in $[Ca^{2+}]_{cyt}$ in tobacco plants genetically transformed aequorin.

The present study showed that the $[Ca^{2+}]_{cyt}$ elevation precedes activation of 50-, 75- and 80-kDa protein kinases in response to hypoosmotic shock and strongly suggested that the $[Ca^{2+}]_{cyt}$ elevation is indispensable for hypoosmotic activation of these protein kinases. The elevated Ca^{2+} may activate a regulatory component(s) for the protein kinases, but not directly these protein kinases, because these protein kinases themselves do not require Ca^{2+} for their activities (Fig. 1). The CDPK which is activated by direct binding of Ca^{2+} without other components was detected in tobacco cells by an in-gel assay (Fig. 6). This is supposed to be the first candidate for the activator protein kinase of the hypoosmotic shock responsive protein kinases directly or indirectly via putative upstream protein kinases. Calmodulin-dependent pro-

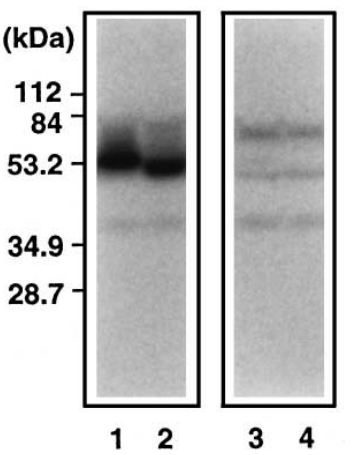

Fig. 6. Detection of Ca^{2+}-dependent protein kinase. The tobacco cell extracts were solubilized in Laemmli's sample buffer and to the aliquot was added 2 mM EGTA (lanes 1 and 3) or 2 mM $CaCl_2$ (lanes 2 and 4). The assay mixture contained 0.5 mM $CaCl_2$ (lanes 1 and 2) or 0.5 mM EGTA (lanes 3 and 4).

tein kinase is also a candidate for the activator protein kinase although its activity was not detected by the in-gel assay.

In this report, we have shown that the hypoosmotic signal is transduced to protein kinase cascades which are triggered by $[Ca^{2+}]_{cyt}$ elevation in tobacco suspension culture cells; however, it is not known at present whether 50-, 75- and 80-kDa protein kinases are components of the same protein kinase cascade or whether they consist of independent cascades.

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