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Immunological Evaluation of the Levels of Calmodulin and its Binding Proteins in Sr^{2+}-stressed Sugar Beet (*Beta vulgaris* L.) Leaves

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With 3 Figures

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Summary

KIM T. W. & HEINRICH G. 1997. Immunological evaluation of the levels of calmodulin and its binding proteins in Sr^{2+}-stressed sugar beet (*Beta vulgaris* L.) leaves. – Phyton (Horn, Austria) 37 (1): 107–118, 3 figures. – English with German summary.

Calmodulin levels were detected in 7 day-old sugar beet (*Beta vulgaris* L.) leaves. Immunological analysis implied that the apparent molecular weight of calmodulin in root, petiole and leaf is about 17 kDa. The levels of calmodulin in the first leaf grown in 1 mM Ca^{2+} or 1 mM Sr^{2+} for 7 days, respectively, were not significantly different. However, the petiole collected from the seedling grown in the absence of Ca^{2+} and Sr^{2+} showed very much lower calmodulin level. From the in vitro activity test using calmodulin deficient bovine heart cAMP-phosphodiesterase (EC 3.1.4.17) it clearly appeared that the activity was reduced in the presence of higher concentration of Sr^{2+} (>100 μM) when compared to Ca^{2+}. The most remarkable difference between Sr^{2+} and Ca^{2+} in calmodulin binding proteins was observed for Mw 39 kDa protein to which calmodulin could weakly bind in the presence of Sr^{2+}. Difference in only one binding protein could not really explain a metabolic inhibition by Sr^{2+}. Consequently, one could conclude that a calmodulin cascade may not be included in

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metabolic disorder caused by Sr^{2+}. Additionally, results of the immuno-overlay assay suggested that calmodulin does not bind to ribulose bisphosphate carboxylase/oxygenase large and small subunits.

Zusammenfassung

KIM T. W. & HEINRICH G. 1997. Calmodulinspiegel und Calmodulinbindungsproteine in mit Sr^{2+} gestreßten Blättern der Zuckerrübe (*Beta vulgaris* L.). – Phyton (Horn, Austria) 37 (1): 107–118, 3 Abbildungen. – Englisch mit deutscher Zusammenfassung.

In sieben Tagen alten Blättern der Zuckerrübe (*Beta vulgaris* L.) wurden die Calmodulinspiegel bestimmt. Das Molekulargewicht des Calmodulins in Wurzeln, Blattstielen und Blattlamina ist ca. 17 kDa. Die Calmodulinspiegel der Primärblätter von Pflanzen, die 7 Tage in 1mM Ca^{2+} oder 1 mM Sr^{2+} kultiviert wurden, wiesen keine signifikanten Unterschiede auf. Die Blattstiele von Keimpflanzen, die ohne Ca^{2+} oder Sr^{2+} angezogen wurden, hatten viel geringere Calmodulinkonzentrationen als Blattstiele, die in Anwesenheit von Sr^{2+} oder Ca^{2+} gewachsen waren. Bei dem unter Verwendung von calmodulinfreier cAMP-phosphodiesterase (EC 3.1.4.17, aus Rinderherz) durchgeführten in vitro Aktivitätstest zeigte sich die Aktivität bei Zusatz hoher Sr^{2+} –(>100 µM) Konzentrationen verglichen mit der Ca^{2+} Kontrolle gehemmt. Ein 39 kDa-Protein band Calmodulin in Anwesenheit von Sr^{2+} locker, nicht aber bei Zusatz von Ca^{2+}, alle anderen Bindungsproteine zeigten sowohl bei Ca^{2+}- als auch Sr^{2+}-Zusatz übereinstimmendes Bindungsverhalten. Das unterschiedliche Verhalten eines einzigen Bindungsproteins kann die Beeinträchtigung von Stoffwechselprozessen durch Sr^{2+} nicht schlüssig erklären und legt vielmehr nahe, daß eine Calmodulinkaskade dabei keine wesentliche Rolle spielt. Die Resultate des Immunooverlay assays legen außerdem nahe, daß keine Calmodulinbindung an der größeren bzw. kleineren Untereinheit der Ribulosebisphosphat-Carboxylase/Oxygenase erfolgt.

Introduction

It has been suggested that Sr^{2+} could substitute for Ca^{2+} in many cellular processes (ISERMAN 1981, KIM & HEINRICH 1995a, b, QUEEN & al. 1963). Although many plant species can survive in high levels of Sr^{2+} (>mM in nutrient solution), the root or hypocotyl growth is sharply reduced in the presence of Sr^{2+} (BURSTRÖM 1983, JOHNSON & JACKSON 1966, KIM 1994).

It is well-known that calmodulin (CaM), a major intracellular Ca^{2+} receptor, regulates the activity of numerous enzymes and cellular processes (HEPLER & WAYNE 1985, HUANG & al. 1990). It has been known that many enzymes such as cAMP-PDE, adenylate cyclase, protein kinase and Ca^{2+}/Mg^{2+}-ATPase are CaM-dependent (ALLAN & TREWAVAS 1985, CHEN & al. 1987, PALIYAH & POOVAIAH 1985, ROBERTS & HARMON 1992). Interestingly, a Ca^{2+}/nH^{+} antiport system across the tonoplast can also be activated by Sr^{2+} in addition to CaM (KIM & HEINRICH 1994) and Sr^{2+} per se can be incorporated into calcium-oxalate crystals (KIM & HEINRICH 1995a). These data imply that some intracellular processes in the presence of Sr^{2+} could be

linked with a physiological Ca^{2+}/CaM cascade. A change in CaM-dependent cellular processes could then be a direct cause of Sr^{2+}-induced growth inhibition. However, the physiological rationale on the relationship between Sr^{2+} and CaM remains unclear.

In this work, we tried to detect the change in CaM level in seedlings grown in the presence of Sr^{2+} and to measure CaM activity (cAMP–PDE) by comparing the in vitro CaM binding proteins.

Abbreviations: BSA, bovine serum albumin; CaM, calmodulin; cAMP, adenosine 2′:3′-cyclic monophosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis(ß-aminoethyl ether) N,N,N′,N′-tetraacetic acid; FW, fresh weight; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino) propane sulfonic acid; Mw, molecular weight; PDE, phosphate diesterase; PMSF = phenylmethylsulfonylfluoride; PVDF = polyoimylidendifluoride; PVP, polyvinylpyrrolidone; TRIS, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

Materials and Methods

Plant materials

Sugar beet seeds (*Beta vulgaris* L. from Fellmann Samenhandlung, Graz) were sterilised in 1 % sodium hypochlorite for 10 min and then washed several times with sterile deionized water. The sterilized seeds were incubated in sterile deionized water in the dark for 3 days at 22 °C in an aseptic chamber and then transferred to Hoagland's solution (final pH 5.8) with or without 1 mM $Ca(NO_3)_2$ or 1 mM $Sr(NO_3)_2$ as described by Kim & Heinrich 1995 a, b. Control seeds were grown in the presence of 1 mM EGTA without $Ca(NO_3)_2$ and $Sr(NO_3)_2$. The plants were hydrocultured in a controlled growth chamber (Heraphyt HPS 1500, Heraeus Vötsch); 12 h daylight intensity of 600 µmol photon $m^{-2}s^{-1}$ from indecandescent lamps (L58 Osram Lumlux); 25/15 ± 1 °C day/night temperature; 70 % relative humidity for further 7 days. Root apices, primary leaves and petioles of seedlings were divided using aseptic razer blades.The nutritional solutions were refreshed daily. For the in vivo CaM level assay in primary leaves during germination, using CaM free cAMP–PDE (EC 3.1.4.17, No. 709883, Boehringer Mannheim), seeds are germinated in the presence of $CaCl_2$, $SrCl_2$, and EGTA for 4 days.

Preparation of soluble protein for CaM binding protein

Soluble proteins were extracted according to a previous method with some modifications (Dieter & Marmé 1980). The sugar beet leaves were chopped with razor blade in an extraction buffer (1:2, w/v) which consisted of 25 mM MOPS–TRIS, pH 7.5, 5 mM $MgCl_2$, 10 % sucrose, 0.6 % PVP, 5 mM EDTA and 0.2 mM EGTA, 1 mM PMSF and 20 mM β-mercaptoethanol. The leaves were ground with mortar and pestle. After filtration through 8 layers of cheese cloth, the filtrates were pelleted by 1,000 × g centrigugation for 15 min at 2 °C to remove cell debris. The supernatants were recentrifuged at 6,000 × g and were repelleted at 150,000 × g with Beckman rotor 27.1. The supernatants were used as the soluble fraction and were dialysed against the extraction buffer overnight. All precedures were performed by 2 ± 1 °C.

Preparation of soluble protein for in vivo CaM level test

CaM was partially purified according to ALLAN & TREWAVAS 1985. But following the dialysis of partially purified CaM, the dialysates were centrifuged at 150,000 × g and then desalted by gel filtration on a Sephadex G-25 column and freed from the inhibitor by passage through a CM-cellulose column eluted with 10 mM HEPES (pH 7.5), 50 mM KCl and 500 μM $CaCl_2$ for $CaCl_2$ or EGTA treated seedlings or 500 μM $SrCl_2$ for $SrCl_2$-treated seedlings respectively.

In vivo CaM level assay

CaM level in desalted 150,000 g supernatants extracted from leaves were calculated by measuring the activity of cAMP-PDE as described by COCUCCI 1984 and SCHIEFER 1986 with some modifications. When required, 500 μM $SrCl_2$ was substituted for 500 μM $CaCl_2$ in the incubation medium.

cAMP–PDE activity

In vitro cAMP–PDE activities in various concentration of $CaCl_2$ or $SrCl_2$, respectively, were also performed in reaction medium A (final volume 1.0 ml) which consisted of 50 mM TRIS (pH 7.5), 3 mM $MgCl_2$, 0.1 mM cAMP, 1 μg spinach CaM (Sigma-5779), 1 mg albumin, 2 units adenosine deaminase, 1 unit alkaline phosphatase and the amount of bovine heart PDE to give 3.0 μmol min^{-1}. The assay was performed at 30 °C by monitoring the decrease in optical density at 260 nm in a Hitachi U-2000 spectrophotometer. For the in vivo CaM level assay, CaM was omitted.

Protein contents were measured using BSA as a standard (BRADFORD 1976).

Western–blotting and immuno–overlay assay

Soluble proteins from root apex and primary leaf were preincubated in a medium (100 μl) which consisted of 50 mM HEPES (pH 7.0) and 5 mM $MgCl_2$ with 2 mM ATP-TRIS at 30 °C for 1 min. The reaction was stopped by the addition of 100 μl SDS-PAGE sample buffer.

After SDS-PAGE following LAEMMLI'S method 1970, the gels were washed three times in 50 mM TRIS-HCl (pH 7.4). The gels were silver-stained (SWITZER & al. 1979) or electrotransferred on PVDF (Bio-Rad) equilibrated in a transfer buffer containing 0.1 mM $CaCl_2$ or $SrCl_2$ when indicated. The electrotransfer was carried out at 4 °C, 250 mV for 90 min according to sandwich double diffusion method (TOWBIN & al. 1979). After the electrotransfer, the PVDF (Bio-Rad) membranes were soaked in a fixing solution (25 % isopropanol and 10 % acetic acid), vacuum-packed and then fixed on a rotary shaker (25 rpm) for 10 min. The membranes were washed three times in TBS (0.9 % NaCl, 10 mM TRIS–HCl, pH 7.4) for 10 min and then saturated in 3 % BSA in TBS for 2 hr, shaking gently. For the CaM overlay-assay, the membranes were finally washed with 0.1 mM $CaCl_2$ or $SrCl_2$ in TBS for 10 min and incubated in 0.1% BSA, 1 mM $CaCl_2$ or $SrCl_2$ in TBS with 100 ng spinach CaM ml^{-1} (Sigma P-5779) overnight at room temperature. After being washed in TBS for 10 min, the membranes were washed twice in 0.05% Nonidet P 40 in TBS for 10 min, shaking. The membranes were washed in TBS for 10 min and were incubated with spinach CaM antibody (Sigma C-6784) diluted in 1 % BSA in TBS for 2 hr (1:1000, w/v), following the washing steps above. After incubation with akaline phosphatase conju-

gated rabbit anti-goat IgG (Sigma A-2168) diluted in 3 % BSA in TBS (1:1500) for 90 min, the washing steps were performed again. The phosphatase activity was measured following manufacturer's standard method (Bio-Rad). The reaction was stopped through adding 20 mM EDTA. The membranes were dried and photographed.

Results and Discussion

CaM level in seedlings

CaM molecular weights (≅ 17 kDa) from different sources are similar to that of standard spinach CaM. When sugar beet seeds were germinated in a solution without Ca^{2+} or Sr^{2+}, the reduction of CaM level in petioles is observed by immunoblotting (Fig. 1). Minor difference in CaM levels occurs in growing first leaf. In the presence of Sr^{2+}, CaM levels in the first leaves of 7 day-old seedlings are somewhat lower (72 %) than those grown in the presence of Ca^{2+} but higher than those in untreated control seedlings on the basis of cAMP-PDE activity (Table 1). However, the leaves of seedlings grown in Sr^{2+} do show a shrieveling and thereafter a severe chlorosis (data not shown). CaM amount in cytoplasm and plasmalemma may be closely linked with extracellular Ca^{2+} (and Sr^{2+} also) level (NEGRINI & al. 1995). If one follows DIETER'S suggestion 1984 that the regulation of Ca^{2+}-CaM mediated processes seems to be due to variations in the free, cytoplasmic Ca^{2+} concentration rather than to changes in the cellular content of CaM, the only slightly lower levels (72 %) of CaM observed in leaves grown in Sr^{2+} compared to Ca^{2+} cannot link Sr^{2+}-toxicity to changes in CaM level. CaM synthesis seems to be preserved even under Ca^{2+}-deficiency, in a first leaf grown in the presence of Sr^{2+} in the nutritional solution without Ca.

Table 1

Effect of Sr^{2+} and Ca^{2+} on calmodulin levels in *Beta vulgaris*. The soluble proteins of the 150000 g supernatant of the first leaves of 7 day-old seedlings were desalted and eluted through a CM-cellulose column to eliminate CaM-inhibitor protein. Reactions were started by adding 100 µg soluble protein to raction medium A (final volume 1.0 ml) and 500 µM $CaCl_2$ or $SrCl_2$. The assay was performed at 30 °C by monitoring the decrease in optical density at 260 nm.

Treatments	Added divalent in assay	CaM level ng mg^{-1} Protein	µg FW^{-1}
H_2O	$CaCl_2$	19.4 (59 %)[a]	6.48
	$SrCl_2$	16.2 (50 %)	–
$CaCl_2$	$CaCl_2$	32.7 (100 %)	12.42
	$SrCl_2$	27.8 (85 %)	–
$SrCl_2$	$CaCl_2$	23.5 (72 %)	12.93
	$SrCl_2$	21.6 (66 %)	–

[a] The percentages to activity in the presence of $CaCl_2$.

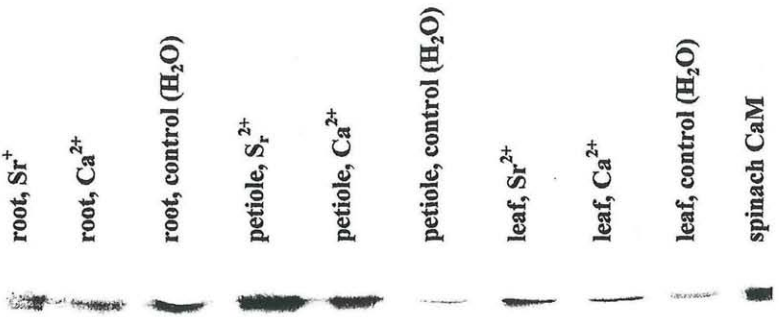


Fig. 1. Western blotting analysis of soluble proteins from *Beta vulgaris*. Seedlings were grown in nutrient solutions with 5 mM of Sr^{2+} or Ca^{2+}. After SDS-PAGE and electrophoretic transferation to a PVDF membrane in the presence of 0.1 mM $CaCl_2$ the soluble proteins from roots, petioles, and leaves were immunoblotted using anti-CAM and made visible with alkaline phosphatase conjugated rabbit anti-goat IgG.

Namely, since Ca^{2+} are not mobile even under Ca^{2+} deficient conditions (LONERAGAN & SNOWBALL 1969), Sr^{2+} in newly growing first leaves may at least partly substitute for the role of Ca^{2+}. We would rather postulate that the inhibitory action of Sr^{2+} may be attributed to an inefficient 'on' and 'off' rate of the Ca^{2+}-CaM-dependent enzymes. Thus, we have tried to test in vitro cAMP-PDE activity in the presence of Ca^{2+} or Sr^{2+}.

As seen in Fig. 2, the cAMP-PDE activity is very much dependent upon the concentration of Ca^{2+} and Sr^{2+}. One should note that the CaM level could be further lower estimated by the addition of Sr^{2+} to cAMP–PDE assay media as was seen in Table 1. This result is clearly derived from the fact that Sr^{2+} over 100 μM range activates less effectively than Ca^{2+} (Fig. 2). It is as yet unclear why cAMP-PDE activity is slightly reduced at high concentration of Sr^{2+}. It could be attributed to a conformational change induced by a nonspecific Sr^{2+}-binding to this enzyme since Sr^{2+} has larger ion radius (1.12 Å) than Ca^{2+} (0.99 Å). Even heavy metals such as Pb^{2+} and Cd^{2+} have an ability to bind to CaM through which cAMP-PDE is activated within a limited range from 10 to 100 μM (BEHRA 1993). However, although such heavy metals can bind to CaM (CHAO & al. 1984), their complexes with CaM cannot effectively stimulate CaM-dependent enzyme activity. Some comparison experiments to investigate the substituting effects of Sr^{2+} for Ca^{2+} were carried out in the mM range (DREIER & al. 1992, MITSUI & al. 1984). Although the role of cAMP as a second messenger in higher plants is still debated (ASSMANN 1995), Sr^{2+} in high concentration (>100 μM) could be less effective than Ca^{2+} in the intracellular processes related to cAMP and CaM such as in signal induction for growth and development processes. Thus, plants can survive in the presence of Sr^{2+} but

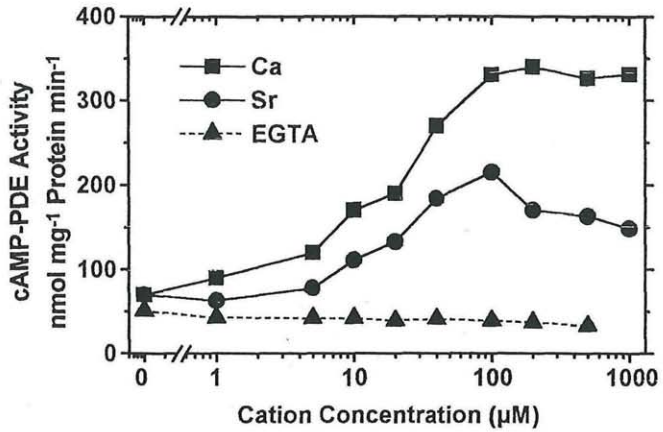


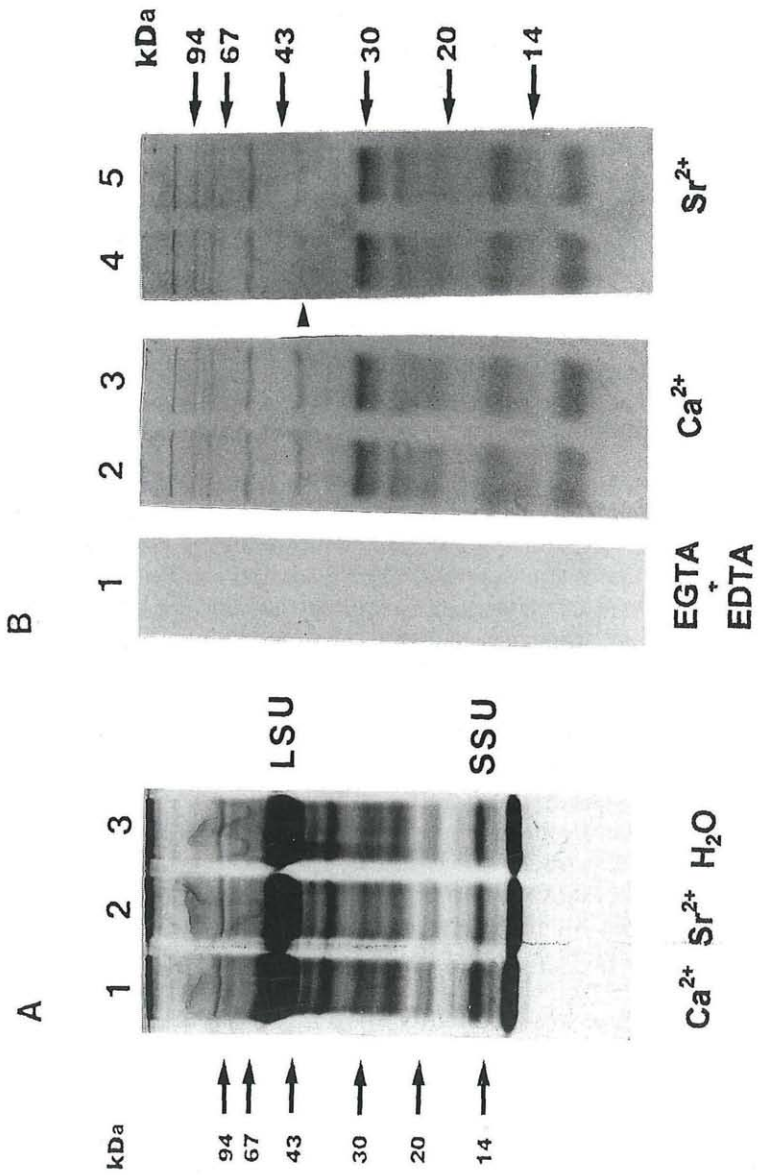
Fig. 2. Changes in in vitro cAMP–PDE activities in the presence of Sr^{2+}, Ca^{2+}, or 2mM EGTA. The reactions were carried out in reaction medium A (final volume 1.0 ml). The assay was performed at 30 °C by monitoring the decrease in optical density at 260 nm.

the whole growth is greatly inhibited. It should be pointed out that intracellular mM levels of Ca^{2+} or Sr^{2+} are no physiological concentrations and are generally toxic. For example, CaM–mediated soluble starch synthase could be activated in the presence of 5 mM Ca^{2+} but not by the same concentration of Sr^{2+} (DREIER & al. 1992). The result presented here indicates that cAMP-PDE can be stimulated by Sr^{2+} within a range of 1 to 100 µM (to about 70 % of the activity in the presence of Ca^{2+}) but the stimulation is reduced at higher concentrations than 100 µM (to less than 50 % of the activity in the presence of Ca^{2+}).

SASAKI & HIDAKA 1982 have hypothesized that a role of CaM can be included in the control of DNA synthesis and cell mitosis. BURSTRÖM 1983 indicated that Sr^{2+} has no effect on cell division. Taking into account the fact that CaM is localized in the mitotic apparatus (VANTARD & al. 1985), Sr^{2+} could not fatally hamper cell division when associated with CaM at least at levels within the µM range. Furthermore, CaM binding to soluble proteins seems not to be inhibited by Sr^{2+} (see following section) and in our environment, there is no possibility for Sr^{2+} to occur at high level (>mM).

CaM binding proteins in leaf soluble proteins

RubisCO large (55 kDa) and small (14 kDa) subunit are major proteins in sugar beet leaf (Fig. 3, pannel A). Appearent protein band patterns do not vary with the different ion treatments. The pannel B of figure 3 also shows CaM-binding proteins among the leaf soluble proteins. CaM-binding seems to be inhibited by the addition of 1 mM EGTA and 1 mM EDTA



to the CaM-overlay medium (lane 1). A similar result was recently reported by LING & ASSMANN 1992 who had washed and incubated in the presence of 1 mM EDTA. Interestingly, a 39 kDa peptide is clearly detectable when the PVDF blots are washed and incubated in the presence of $CaCl_2$. Although the soluble proteins from Ca^{2+}-grown seedling are preincubated in the presence of $CaCl_2$ before subjection on SDS-PAGE, the electrotransfer and CaM-overlay assay in the presence of Sr^{2+} has an inhibitory effect on the CaM binding to 39 kDa protein (lane 5). This differential binding of CaM may be due to a change in the affinity of Sr^{2+}-CaM complexes or may be associated with a distruption of CaM-binding domains to CaM by Sr^{2+}.

Some proteins which were not visualized in the previous observation by LING & ASSMANN 1992 were detected through the preincubation with spinach CaM. They detected only 3 bands on overlay assay using ^{125}I–labeled CaM. Our result presented shows 12 polypeptides to which CaM is to bind. The reason for this difference remains to be explained in future experiments. Additionally, it is suggested that CaM does not bind to ribulose bisphosphate carboxylase/oxygenase large (Mw 55 kDa) and small subunits (Mw 14 kDa) since broad banding patterns do not occur.

The information on the identities and functions of CaM binding proteins is very scarce in plant cells. O'NEIL & DEGRADO 1990 reported that a conserved structure, a basic amphiphilic α-helix, could be identified as a conserved binding domain in several animal CaM-regulated enzymes. The conserved structure is characterized as the amphiphilic nature of the α-helix and the presence of tryptophan residues which may be necessary for high binding affinity to CaM (O'NEIL & DEGRADO 1990, STRYNANAKA & JAMES 1989). Unfortunately, no information is available to provide an explanation for the different binding affinity induced by the difference in ionic strength between Ca^{2+} and Sr^{2+} in plant CaM binding proteins.

Fig. 3. Separation of leaf soluble proteins (9 µg/lane) from *Beta vulgaris* on 12 % SDS–PAGE. A: Proteins were isolated from leaves of seedlings grown in the dark for 3 days and then in Hoagland's nutrient solution containing 1 mM $Ca(NO_3)_2$ (lane 1), 1 mM $Sr(NO_3)_2$ (lane 2) or H_2O (lane 3) for 4 days further. Proteins were preincubated in the presence of 2 mM ATP-TRIS and 5 ng CaM per ml with 2 mM $CaCl_2$ (lane 1) or $SrCl_2$ (lane 2), or without divalent ions (lane 3) respectively, for 1 min. B: CaM-binding proteins. After electrophoretic transfer to a PVDF membrane without $CaCl_2$ or $SrCl_2$, the PVDF membrane was incubated with spinach CaM (5 µg/ml) in TBS containing 100 µM Sr^{2+} or Ca^{2+}. Then, the CaM-binding soluble proteins were immunodetected using alkaline phosphatase conjugated rabbit anti-goat IgA: 1 mM EGTA and 1 mM EDTA (lane 1); in 100 µM $CaCl_2$ (lane 2 and 3); electrotransfer in 100 µM. $SrCl_2$ (lane 4 and 5). Lanes 2 and 4 show the soluble proteins isolated from roots grown in the presence of $Ca(NO)_2$ whereas lanes 3 and 5 represent the ones grown in the presence of $SrCl_2$. LSU; RuBisCO large subunit (55 kDa). SSU; RuBisCO small subunit (14 kDa).

In conclusion, we would like to suggest that Sr^{2+} also has a high affinity to CaM and does not prevent the CaM-Sr^{2+} complexes to bind to the proteins. However, the Sr^{2+}-binding CaM is relatively less active than Ca^{2+}-binding ones at least on the basis of cAMP-PDE activity. Thus the growth-inhibitory effects of Sr^{2+} may be due to unspecific binding to many different target proteins regulated by Ca^{2+} and CaM by which the enzyme per se could be conformationally changed resulting in reduction of activity. The nature of one polypeptide (Mw 39 kDa) remains to be elucidated.

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References

ALLAN E. & TREWAVAS A. 1985. Quantitative changes in calmodulin and NAD kinase during early cell development in the root apex of *Pisum sativum* L. – Planta, 165: 493–501.

ASSMANN S. M. 1995. Cyclic AMP as a second messenger in higher plants. – Plant Physiol. 108: 885–889.

BEHRA R. 1993. In vitro effects of cadmium, zinc and lead on calmodulin-dependent actions in *Oncorhynchus mykiss*, Mytilus sp., and *Chlamydomonas reinhardtii*. – Arch. Environ. Contam. 24: 21–27.

BRADFORD M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. – Anal. Biochem. 72: 248–254.

BURSTRÖM H. G. 1983. Calcium, strontium and root growth. – Z. Pflanzenphysiol. 109: 91–93.

CHAO S.-H., SUZUKI Y., ZYSK J. R. & CHEUNG W. Y. 1984. Activation of calmodulin by various cations as a function of ionic radius. – Mol. Pharmacol. 26: 75–82.

CHEN Y.-R., DATTA N. & ROUX S. J. 1987. Purification and partial characterization of a calmodulin stimulated nucleotide triphosphatase from pea nuclei. – J. Biol. Chem. 262: 10689–10694.

COCUCCI M. 1984. Increase in calmodulin level in the early phases of radish seed (*Raphanus sativus*) germination. – Plant Cell Physiol. 7: 215–221.

DIETER P. 1984. Calmodulin and calmodulin-mediated processes in plants. – Plant Cell Environ. 7: 371–380.

— & MARMÉ D. 1980. Calmodulin activation of plant microsomal Ca^{2+} uptake. – Proc. Natl. Acad. Sci. USA. 77: 7311–7314.

DREIER W., PREUSSER E. & GRÜNDEL M. 1992. The regulation of the activity of soluble starch synthase in spinach leaves by a calcium-calmodulin dependent protein kinase. – Biochem. Physiol. Pflanzen. 188: 81–96.

HEPLER P. K. & WAYNE R. O. 1985. Calcium and plant development. – Ann. Rev. Plant Physiol. 36: 397–439.

HUANG Y., CHEN C. & KAO C. H. 1990. Senescence of rice leaves XXIV. Involvement of calcium and calmodulin in the regulation of senescence. – Plant Cell Physiol. 31: 1015–1020.

ISERMAN K. 1981. Uptake of stable strontium by plants and effects on plant growth. In: SKORYNA S. C. (ed.), Handbook of stable strontium, pp. 65–86. Plenum Press, New York, London.

JOHNSON R. E. & JACKSON W. A. 1966. Effect of calcium and strontium on the preferential formation of an adenosine triphosphatase in wheat roots. – Nature 210: 869–870.

KIM T. W. 1994. Biochemical and immunological studies on strontium action in higher plants: Uptake, localisation and effect of strontium on different metabolic reactions related to calcium and calmodulin. – Ph.D. Thesis, University of Graz, Graz.

— & HEINRICH G. 1994. Calmodulin stimulation of Sr^{2+}/nH^{+} antiport in vacuoles of red beet (*Beta vulgaris* L.) storage tissue. – Biol. Plant. (Abstract No. 1525) 36: S–381.

— & — 1995a. Use of a laser microprobe mass analyzer for detection of strontium incorporation in oxalate-crystals of *Beta vulgaris* leaf. – J. Plant Physiol. 146: 217–221.

— & — 1995b. Effects of Sr^{2+}, Ca^{2+}, and spermine on thylakoid protein and chlorophyll degradation during dark incubation of sugar beet leaf discs. – Photosynthetica 31: 315–319.

LAEMMLI U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. – Nature 227: 680–685.

LING V. & ASSMANN S. M. 1992. Cellular distribution of calmodulin and calmodulin-binding proteins in *Vicia faba* L. – Plant Physiol. 100: 970–978.

LONERAGAN J. F. & SNOWBALL K. 1969. Calcium requirements of plants. – Aust. J. Agric. Res. 20: 465–478.

MITSUI T., CHRISTELLER J. T., HARA-NISHIMURA I. & AKAZAWA T. 1984. Possible roles of calcium and calmodulin in the biosynthesis and secretion of α-amylase in rice seed scutellar epithelium. – Plant Physiol. 75: 21–25.

NEGRINI N., RIVETTA A. & COCCUCI M. 1995. Calmodulin levels in radish (*Raphanus sativus* L.) seeds germinating at low calcium availability induced by EGTA treatments. – Plant Cell Environ. 18: 159–167.

O'NEIL K. T. & DEGRADO W. F. 1990. How calmodulin binds its targets: sequence independent recognition of amphiphilic (-helices. – Trends Biochem. Sci. 15: 59–64.

PALIYAH G. & POOVAIAH B. W. 1985. Calcium and calmodulin-promoted phosphorylation of membrane proteins during senescence in apples. – Plant Cell Physiol. 26: 977–986.

QUEEN W. H., FLEMMING H. W. & O'KELLEY J. C. 1963. Effects on *Zea mays* seedlings of a strontium replacement for calcium in nutrient media. – Plant Physiol. 38: 410–413.

ROBERTS D. M. & HARMON A. C. 1992. Calcium-modulated proteins: Targets of intracellular calcium signals in higher plants. – Ann. Rev. Plant Physiol. Plant Mol. Biol. 43: 375–414.

SASAKI Y. & HIDAKA H. 1982. Calmodulin and cell proliferation. – Biochem. Biophys. Res. Commun. 104: 451–456.

SCHIEFER S. 1986. Calmodulin. In: BERGMEYER U. H. & GRASSL M. (Eds), Methods of enzymatic analysis, 3rd Edition, Vol. IX, pp. 317–331. VCH Verlagsgesellschaft, Weinheim.

STRYNANAKA N. C. & JAMES M. N. G. 1989. Crystal structures of the helix-loop-helix calcium-binding proteins. – Annu. Rev. Biochem. 58: 951–998.

SWITZER III R. C., MERRIL C. R. & SHIFRIN S. 1979. A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. – Anal. Biochem. 98: 231–237.

TOWBIN H., STAEHELIN T. & GORDEN J. 1979. Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheet: procedure and some application. – Proc. Natl. Acad. Sci. USA 76: 4350–4354.

VANTARD M., LAMBERT A.-M., DE MEY J., PIQUOT P. & VAN ELIK J. 1985. Characterization and immunocytochemical distribution of calmodulin in higher plant endosperm cells: Localization in the mitotic apparatus. – J. Cell Biol. 101: 488–499.