



Stimulated synthesis of specific proteins by calcium perturbation and heat shock in *Brassica napus*

Fang-Sheng Wu¹, Yuan-Kuey Shieh² and Teng-Yung Feng²

¹Department of Biology, Virginia Commonwealth University, Richmond, VA. 23284-2012, U. S. A.

²Institute of Botany, Academia Sinica, Nankang, Taipei, Taiwan, Republic of China

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Abstract. We investigated the stimulated synthesis of several specific proteins in hypocotyl segments and etiolated seedlings of *Brassica napus* cv. Westar treated with calcium chelator ethyleneglycol-bis (β -aminoethylether)-N, N'-tetraacetic acid (EGTA), or calcium ionophore A23187. EGTA treatment resulted in an increased synthesis of 88, 61, 55, and 45 kD proteins, and A23187 stimulated synthesis of 88, 55, and 45 kD proteins in excised hypocotyl sections. The potassium ionophore valinomycin did not stimulate synthesis of these proteins. Two-dimensional polyacrylamide gel electrophoresis (2-D-PAGE) demonstrated the enhanced synthesis of four additional proteins in A23187-treated hypocotyl segments. Heat shock at 38.5°C stimulated synthesis of 105, 84, 70, and a group of 16-28 kD proteins regardless of whether or not cells were pretreated with EGTA, A23187, or valinomycin. The 2-D PAGE revealed that when A23187 treated cells were subjected to heat shock, synthesis of at least nine proteins was enhanced. The overall protein synthesis in cells treated with EGTA, A23187, or ethylenediaminetetraacetate (EDTA) was more sensitive to inhibition at 38.5°C than at 25°C. A23187 also acts synergistically with EGTA to enhance the inhibition. It can be concluded that specific proteins are induced when plant cells are subjected to calcium perturbation.

Key words: *Brassica napus*; Calcium ion; EDTA; EGTA; Heat shock; Ionophores; Protein synthesis.

Introduction

Calcium plays a key role in numerous plant functions, including cell division, cell growth, phytochrome response, hormone action, tropisms, osmoregulation, fruit ripening, photosynthesis and plant diseases (Poovaiah and Reddy, 1987). The physiological roles of calcium in plant cells have been extensively investigated, but it is not known how calcium acts as a transduction signal for the regulation of gene expression. The identification of specific gene products expressed in response to changes in cellular calcium levels is necessary to study the molecular mechanisms governing calcium regulated gene functions.

Calcium ionophores and chelators are widely used

to manipulate intracellular calcium concentration (Poovaiah and Reddy, 1987). The calcium ionophore A23187 elevates intracellular calcium concentration and has been used extensively to mimic the effects of a variety of physiological stimuli. A23187 has been shown to induce cell movement, cell fusion, transformation, differentiation, hormonal response, and to act directly as a regulator of gene expression in mammalian cells (Resendez *et al.*, 1986). In plant cells, calcium chelator EGTA (ethyleneglycol tetraacetic acid) causes an increased cytoplasmic streaming (Takagi and Nagai, 1983), and a loss of the gravitropism (Miyazaki *et al.*, 1986).

In animal cells, the levels of specific 80 and 100 kD proteins and their corresponding messenger RNAs can be increased several-fold by the calcium ionophore

A23187 (Wu *et al.*, 1981). Another calcium ionophore, ionomycin, and to some extent the calcium chelator EGTA, also cause a similar response (Wu *et al.*, 1981). The localization and characterization of these two proteins, and the isolation, molecular cloning, and DNA sequencing of their genes have been reported (Lee, 1987). It was suggested that a transient increase in cytosolic calcium concentration, or a change in intracellular calcium distribution caused the induction of these genes (Wu *et al.*, 1981; Lee, 1987). Thus, conditions which disturb the delicate balance of calcium systems in animal cells are related to the calcium-dependent regulatory signals which trigger the transcription and translation of specific genes. However, induction of genes or gene products by calcium ionophores or EGTA has not been examined in plant cells.

Induction of heat shock proteins (hsps) in response to elevated temperatures is ubiquitous in eukaryotic cells (Lindquist and Craig, 1988; Kimpel and Key, 1985). Heat shock specifically elevates intracellular calcium, but not the sodium, potassium, and magnesium ions in animal cells (Vidair and Dewey, 1986). The calcium ionophore-inducible promoter sequence in animal cells has several regions sharing homologies with hsp 70 gene and is also inducible by heat shock (Lee, 1987). However, a link between heat shock and calcium perturbation in cells has not been established, nor has its effect on protein synthesis patterns.

We report here the effect of calcium ionophores and chelators on the synthesis of several specific proteins under normal (25°C) and heat shock (38.5°C) conditions in excised hypocotyl sections and newly germinated, etiolated seedlings of *Brassica napus*.

Materials and Methods

Plant Materials and Seed Germination

Seeds of *Brassica napus* cv. Westar were surface-sterilized in 70% ethanol for 30 sec, 30% commercial bleach (Chlorex) for 30 min, followed by rinsing five times in distilled water. Seeds were then placed into 100 ml Erlenmeyer flasks containing 20 ml of 0.5x strength Murashige and Skoog (MS) medium without sucrose at pH 7.0. About 200 seeds/flask were incubated at 25°C in the dark with constant shaking at 120 rpm. Cefotaxime at 500 µg/ml was added into the 0.5x MS during the first 24 h of incubation to minimize bacterial contamination. After 24 h, germinated seeds

were collected and their seed coats were removed with a forceps. Ten germinated seeds were distributed into each well of a 24-well plate containing 1 ml of fresh 0.5x MS and incubated at 25°C in the dark for 24 h without shaking. Under these conditions, seedlings were etiolated and grew approximately 1 cm before being subjected to the described treatments.

Chemical and Heat Shock Treatments

Hypocotyls were excised from the seedlings, cross-sliced into 2-3 mm sections, and placed into a 24-well plate containing 0.4 ml of 0.5x MS in each well. After 1 h or incubation at 120 rpm to stabilize the hypocotyl sections, the medium was removed and replaced with fresh 0.5x MS containing the chemicals for the treatment as indicated below and in the figure legends.

For whole etiolated seedlings, the 0.5x MS medium in each well was removed and replaced with 0.5 ml of the same medium containing the other chemicals as follows: ethylenediaminetetraacetate (EDTA), ethyleneglycol-bis (β -aminoethylether)-N, N'-tetraacetic acid (EGTA), A23187, ionomycin, glucose, manitol, glutamine, 2-mercaptoethanol, and valinomycin. Except for the ionophores which were prepared as 10 mM stock solutions in 95% ethyl alcohol (with mild sonication to disperse the ionophores), all other chemical solutions were prepared as 1 M, pH 7.0 stocks. The treatments involved replacing one-half volume of the culture medium (0.5x MS) in each well with the same medium but with the addition of above chemicals at 2X the indicated final concentrations. The durations and concentrations for each treatment are indicated in the figure legends. Heat-shock treatment was performed in a water bath preset at 38.5°C for 1.5 h.

In vivo Labeling, [³⁵S]-methionine Incorporation, and Protein Separation by Electrophoresis

For *in vivo* labeling, 20 µl of [³⁵S]-methionine (1000 Ci/mmol; Amersham Corp., Chicago) prepared in 0.5x MS was added to each well to reach a final concentration of 40 µCi per ml of the culture medium. After 2-5 h of incubation at the indicated temperature, the medium was removed, and the samples were washed five times with 1 ml of pre-chilled (4°C) 0.5x MS with the vacuum suction. The samples were then collected into test tubes, frozen with liquid nitrogen, and stored at -70°C for no more than two weeks prior to processing for electrophoresis.

The samples were ground into fine powders in liquid nitrogen with a mortar and pestle and resuspended in an extraction buffer containing 0.3 M Tris (pH 7.8), 5% 2-mercaptoethanol, and 2% sodium lauryl sulfate (SDS). They were then centrifuged in an Eppendorf microfuge at full speed for 3 min, and the supernatants were quantified using a Biorad protein analysis kit. For measuring the incorporation of [³⁵S]-methionine into protein, 5 μ l of the supernatant was placed on a Whatman fiberglass filter disc (GF/A) and precipitated with 10% trichloroacetic acid (TCA) at 4°C. The filter paper was then washed three times with 5% TCA, dried, and its radioactivity determined with a Beckman LS7800 liquid scintillation counter.

For one-dimensional (1-D) SDS polyacrylamide gel electrophoresis (PAGE), the extraction buffer containing the protein samples was adjusted to contain the same amount of protein, and the SDS content was adjusted to 7% with a 25% stock solution. Twenty μ g of proteins from each sample were analyzed, and the electrophoresis was carried at 70 volts for 14 h in either 12.5% or 8–15% gradient polyacrylamide gel as indicated in the figure legends. After electrophoresis, the gels were stained with 0.3% Coomassie blue R-250, destained in several changes of a solution containing 40% methanol and 10% acetic acid, dried, and exposed to Kodak XAR film for 4–7 d. Two-dimensional (2-D) electrophoresis was carried out as described previously (Wu *et al.*, 1981). After electrophoresis, the gels were processed as described above, except that they were treated with an "EN³Hance" solution (NEN) for 30 min before they were dried for autoradiography.

Results

EGTA and A23187 Stimulated Synthesis of Several Proteins in Excised Hypocotyl Sections

Treating the excised hypocotyl sections of *Brassica napus* with EGTA resulted in an increased synthesis of four proteins with mol wt of 88, 61, 55, and 45 kD, respectively (Fig. 1). Increases of the 55 and 45 kD proteins were greater than those of 88 and 61 kD proteins, but required EGTA higher than 2.5 mM. The presence of 50 μ M A23187 during the 10 mM EGTA treatment increased these four proteins, especially the 55 kD protein. The incorporation of [³⁵S]-methionine into proteins was not notably affected by EGTA at 10 mM, but was completely inhibited by EDTA at 5 mM (Fig. 1).

A23187 at 25 μ M slightly stimulated the synthesis of 88, 55 and 45 kD proteins, but not the 61 kD protein (Fig. 1). A23187 at 50 and 100 μ M did not result in further increases. In fact, the 55 kD protein was less distinguishable because the protein pattern in this area was changed. Treatment with another calcium ionophore, ionomycin, also resulted in protein synthesis patterns similar to those of A23187 treated cells (data not shown). Treating the cells with the potassium ionophore, valinomycin, did not stimulate synthesis of these proteins, but significantly increased the amount of a 42 kD protein. In addition, synthesis of a 70 kD protein was inhibited by valinomycin treatment. High concentration (800 μ g/ml) of glutamine did not change the protein synthesis pattern in treated cells, but 0.5% of 2-mercaptoethanol completely inhibited protein synthesis.

Effect of Heat Shock on the Protein Synthesis Patterns in Hypocotyl Cells Treated with EGTA and A23187

Heat shock at 38.5°C stimulated the synthesis of 105, 84 and 70 kD high mol wt, and a group of 16–28 kD low mol wt proteins (Fig. 2). The presence of 70 kD protein was especially prominent. These heat shock proteins were induced regardless of treatment with EGTA, A23187, valinomycin, glutamine, and 2-mercaptoethanol.

Heat shock greatly reduced the overall protein synthesis in cells pretreated with 5 mM of EGTA, as revealed by the incorporation of [³⁵S]-methionine into proteins separated by the SDS-gel electrophoresis (Fig. 2, lane g5). EGTA at 10 mM further reduced the protein synthesis. Our results on the incorporation of [³⁵S]-methionine into acid-insoluble proteins on the basis of cpm per μ g protein indicated that at 15 mM of EGTA, the incorporation rates for cells without and with heat shock treatment were 25% and 5% of the control's (without EGTA), respectively. Clearly, protein synthesis in EGTA treated cells was more sensitive to heat shock treatment than that in untreated, control cells. Another ion chelator EDTA completely inhibited all protein synthesis of the cells at 5 mM under the heat shock condition.

The increased synthesis of the 88, 61, 55, and 45 kD proteins with EGTA, and 88 and 55 kD proteins with A23187 treatments at 25°C (Fig. 1) were not observed in 1-D PAGE when cells were subsequently subjected to the heat shock treatment (Fig. 2). The synthesis of a 69

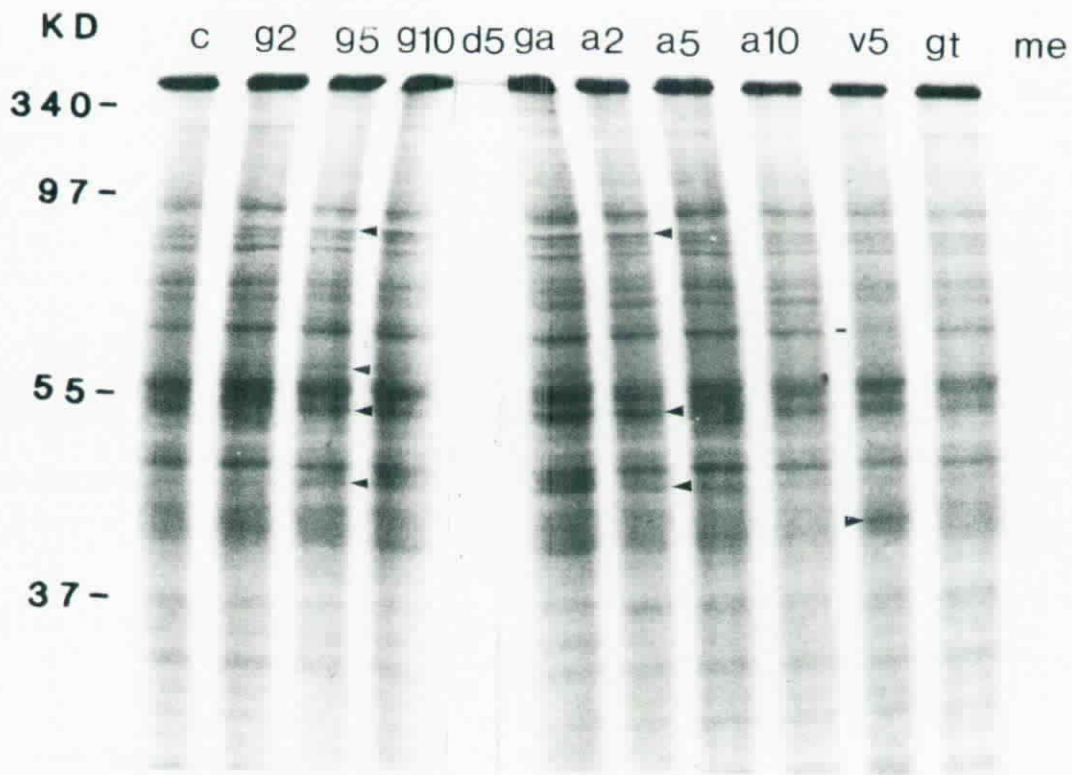


Fig. 1. (A): Protein synthesis patterns in excised hypocotyl sections of *B. napus* after various treatments at 25°C. Excised hypocotyl sections were collected from two-day old etiolated seedlings and were incubated in 0.5x MS medium for 5 h and then for 15 h under the conditions indicated. c: control; g2, g5, and g10: EGTA at 2.5, 5, and 10 mM, respectively; d5: 5mM EDTA; ga: 5 mM EGTA for 2 h followed by the addition of 50 μ M A23187; a2, a5, and a10: A23187 at 25, 50, and 100 μ M, respectively; v5: 50 μ M valinomycin; gt: 800 μ g/ml glutamine; me: 0.5% of 2-mercaptoethanol. After the treatment, they were labeled with [35 S]-methionine for 2.5 h, and analyzed by SDS-PAGE. The numbers at left represent mol wt of marker proteins in kilo-Dalton (kD). The " \blacktriangleleft " between lanes g5 and g10 indicate EGTA-stimulated proteins with mol wt of 88, 61, 55, and 45 kD from top to bottom, respectively. The " \blacktriangleleft " between lanes a2 and a5 represent A23187-stimulated proteins with mol wt of 88, 55, and 45 kD from top to bottom, respectively. The " \blacktriangleright " in lane v5 indicates a valinomycin-induced 42 kD protein, and the "-" indicates a 70 kD protein inhibited by valinomycin.

kD protein was slightly stimulated by 2.5 mM EGTA, but diminished by 5 and 10 mM EGTA, probably as a result of the overall protein synthesis inhibition.

Co-treatment of 5 mM EGTA and 50 μ M A23187, followed by heat shock, completely inhibited protein synthesis, except for the 105, 84, and 70 kD heat shock proteins which were still present in trace amounts (Fig. 2). Treatment of 10 mM EGTA together with 50 μ M A23187 at 38.5°C completely inhibited all protein synthesis (data not shown). A23187 treatment followed by heat shock slightly increased the synthesis of 85, 69, and 63 kD proteins, but they were present only in small amounts. At 50 μ M of A23187, synthesis of a 45 kD protein was also increased, but at 100 μ M, the overall

protein synthesis was inhibited, including the 45 kD protein.

At 50 μ M, valinomycin greatly inhibited overall protein synthesis and did not stimulate the synthesis of any specific proteins under the heat shock condition. As at 25°C, 800 μ g/ml of glutamine did not change the protein synthesis patterns, but 0.5% of 2-mercaptoethanol completely inhibited protein synthesis, although there were still trace amounts of the 105, 84, and 70 kD high mol wt heat shock proteins present in the cells. In all cases of protein synthesis inhibition, the low mol wt heat shock proteins appeared to be inhibited to a greater extent than high mol wt proteins, and the heat shock-induced proteins are more resistant than normal

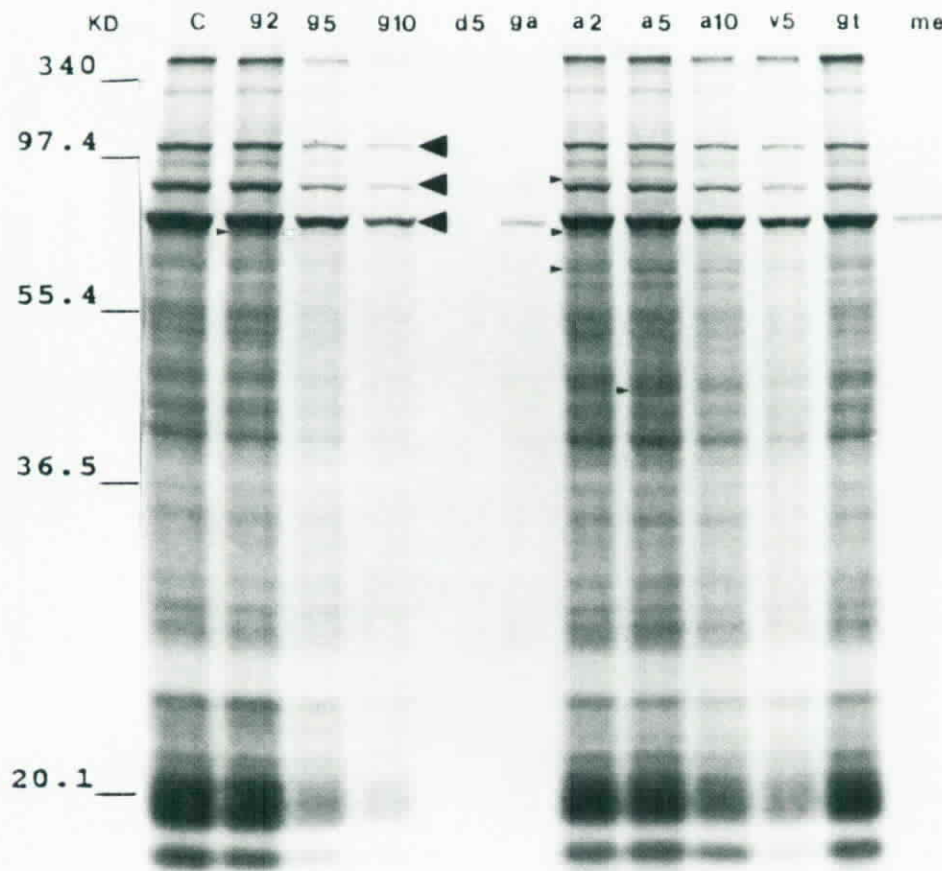


Fig. 2. Protein synthesis patterns in excised hypocotyl sections of *B. napus* after various treatments at 38.5°C. Excised hypocotyl sections were collected from two-day old etiolated seedlings and were incubated in 0.5x MS medium for 5 h followed by the following treatments for 15 h at 25°C before they were subjected to heat shock (38.5°C) for 1.5 h. They were then labeled with [³⁵S]-methionine for 2.5 h at 38.5°C, and harvested for SDS-PAGE in an 8–15% acrylamide gel. c: control; g2, g5, and g10: EGTA at 2.5, 5, and 10 mM, respectively; d5: 5 mM EDTA; ga: 5 mM EGTA for 2 h followed by the addition of 50 μM A23187; a2, a5, and a10: A23187 at 25, 50 and 100 μM, respectively; v5: 50 μM valinomycin; gt: 800 μg/ml glutamine; me: 0.5% of 2-mercaptoethanol. The numbers at left represent mol wt of marker proteins in kD. The large "◄" pointing toward lane g10 indicate the 105, 84, and 70 kD major high mol wt heat shock proteins. The small "►" indicate the follows: the 69 kD protein in lane g2; the 85, 69, and 63 kD proteins in lane a2; and the 45 kD protein in lane a5.

cellular proteins to the inhibition.

Effect of EGTA and A23187 Treatment on the Protein Synthesis in Etiolated Seedlings at 25°C and 38.5°C

When newly germinated, etiolated seedlings were treated with 5 mM EGTA at 25°C, the protein synthesis pattern was similar to that of untreated, control cells (Fig. 3A). EDTA at 5 mM failed to inhibit protein synthesis, but it slightly elevated the level of a 70 kD proteins. A23187 treatment slightly increased the 45 and 41 kD proteins. However, since the protein bands in these areas were not distinct (due to the presence of many

proteins with similar mol wts), and since they seemed to be also present in valinomycin treated cells, their identities were unclear. In general, the observed changes in protein synthesis patterns of hypocotyl sections treated with EGTA and A23187 were not evident in whole seedlings with the same treatment.

Heat shock at 38.5°C of the etiolated seedlings resulted in the synthesis of hsp's similar to those observed in cells of excised hypocotyl sections (Fig. 3B). These included the 105, 84, and 70 kD high mol wt, and the 12–28 kD low mol wt proteins. In contrast to cells treated at 25°C, 5 and 10 mM of EGTA or EDTA at

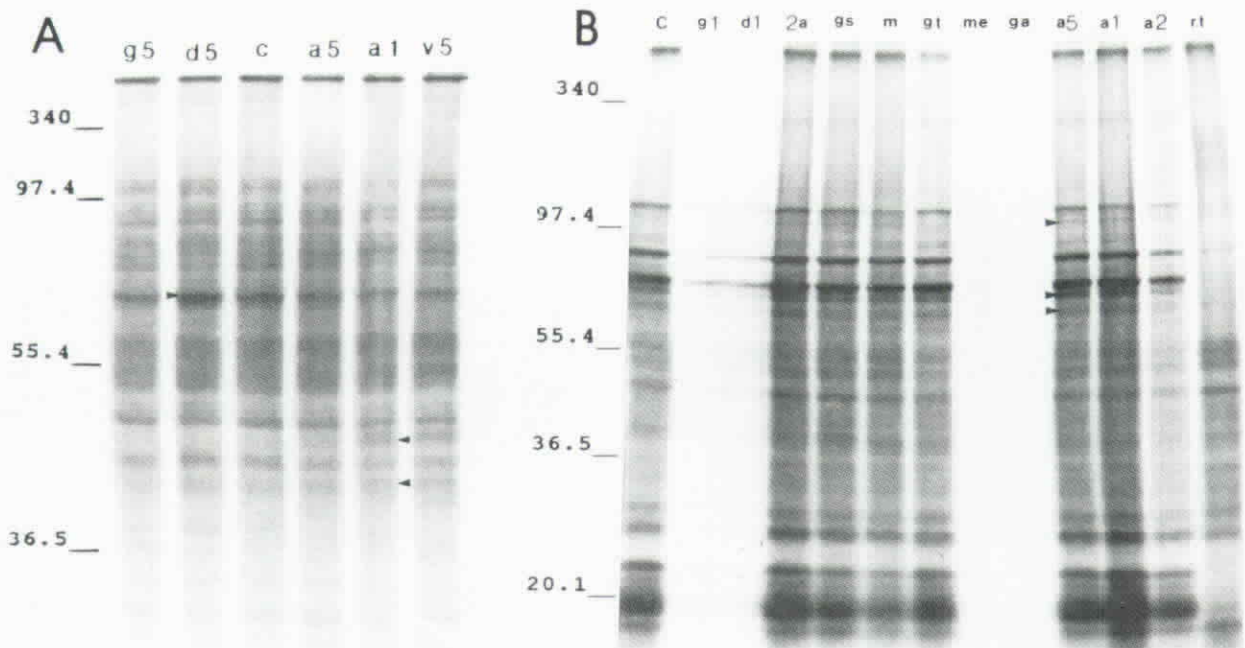


Fig. 3. Protein synthesis patterns in etiolated seedlings of *B. napus* after various treatments at 25°C (A) and 38.5°C (B). Two-day old etiolated seedlings grown in 0.5x MS medium were subjected to the following treatments for 15 h at 25°C unless as otherwise indicated. The seedlings were then either continuously incubated at 25°C for 1.5 h (A), or were subjected to 38.5°C for 1.5 h (B). They were then labeled with [³⁵S] methionine for 2.5 h, harvested, and processed for 1-D PAGE in 12.5% (A) and 8–15% gradient (B) gels as described in Materials and Methods. (A): g5: 5 mM EGTA; d5: 5 mM EDTA; a5 and a1: 50 and 100 μ M A23187, respectively; v5: 50 μ M valinomycin. The "►" indicates a 70 kD protein in lane d5 and the "◄" indicates a 45 and a 41 kD proteins in lane a1. (B): c: control; g1: 10 mM EGTA; d1: 10 mM EDTA; 2a: 2 h of treatment with 50 μ M of A23187; gs: 0.3 M of glucose; m: 0.3 M of manitol; gt: 800 μ g/ml of glutamine; me: 0.5% of 2-mercaptoethanol; ga: 5 mM of EGTA for 2 h followed by 50 μ M of A23187 for 15 h; a5, a1, and a2: A23187 at 50, 100 and 200 μ M, respectively; rt: room temperature (25°C) untreated control. The "►" in lane a5 indicate 98, 69, and 63 kD proteins from top to bottom, respectively. The numbers at left represent mol wt of marker proteins in kD.

38.5°C resulted in an almost complete inhibition of protein synthesis, indicating that the ion chelators rendered the seedlings much more susceptible to heat stress.

A23187 slightly stimulated the synthesis of a 98 kD protein following treatment at 38.5°C. There was no significant difference in the proteins synthesis patterns between 2 h and 15 h of pre-treatment, indicating that proteins synthesis was a continuous process and was performed at low levels. The 98 kD protein was also slightly increased in cells from etiolated seedlings treated with 0.3 M glucose or manitol, suggesting that the osmotic changes may in part be related to the stimulation of this protein. The 85 kD protein, stimulated by A23187 treatment of excised hypocotyl sections, was not observed in etiolated seedlings. The 69 and 63

kD proteins were slightly stimulated in seedling treated with 100 μ M of A23187; however, untreated seedlings or those treated with glucose, manitol, and glutamine also have high levels of proteins in these mol wt ranges. A23187 at 100 μ M appeared to transiently increase the levels of low mol wt heat shock proteins. Higher concentration of A23187 (200 μ M) resulted in an overall inhibition of protein synthesis in treated cells including those proteins that were stimulated at 100 μ M.

Again, glutamine treatment did not affect the protein synthesis, but 2-mercaptoethanol completely inhibited it. No specific protein was induced under either of these treatments.

Analysis of Proteins Synthesized in A23187 Treated

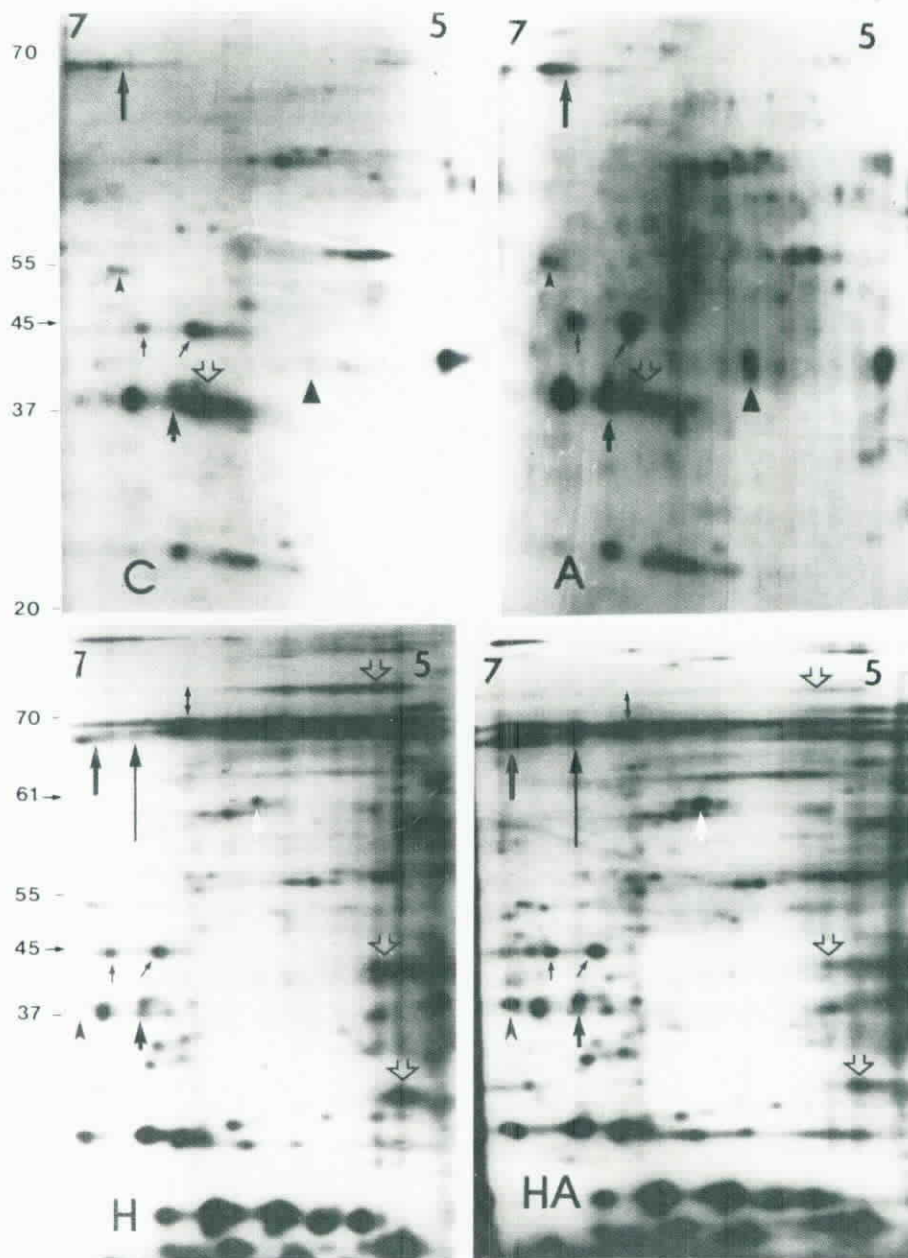


Fig. 4. Two-dimensional polyacrylamide gel electrophoresis analysis (2-D PAGE) of protein synthesis in excised hypocotyl sections of *B. napus* after A23187 treatment at 25°C (C, A) and 38.5°C (H, HA). Excised hypocotyl sections were collected from two-day old etiolated seedlings and were incubated in 0.5x MS for 5 h, and then for 15 h under the indicated conditions. C: control, untreated cells at 25°C; A: cells treated with 100 μ M of A23187 at 25°C; H: control, untreated cells at 38.5°C; HA: cells treated with 100 μ M A23187 at 38.5°C. After the treatment, the cells were labelled with [³⁵S]-methionine for 2.5 h and analyzed by 2-D PAGE as described in Materials and Methods. The numbers at left represent mol wt marker proteins in kilo-Dalton (kD). The numbers at upper left and right corners indicate the positions of pI values on the gels. Different styles of arrows or arrowheads are used to indicate different proteins that are stimulated (pointing upward), inhibited (pointing downward), or unchanged (pointing toward both directions) in A23187-treated cells. The details of the changes are described in the text.

Cells at 25°C by 2-D PAGE

The 88 kD protein that was stimulated by the A23187 treatment of hypocotyl cells at 25°C, as shown in 1-D PAGE (Fig. 1), was resolved by the 2-D PAGE into a polypeptide with an isoelectric point (pI) of 5.1 (data not shown). In addition, a 69 kD polypeptide with pI of 6.7 as indicated by a bold, long arrow was induced by A23187 (Fig. 4A). This polypeptide was not present in the control cells (Fig. 4C).

As shown in Fig. 4A, A23187-stimulated 55 kD protein (indicated by a small arrowhead) with pI of 6.7 was present at low level in the untreated cells. Two distinct spots with pI of 6.6 and 6.3 (small arrows), respectively, both with mol wt of 45 kD, were stimulated in A23187-treated cells. The pI 6.3 spot was present in a larger amount than the pI 6.6 spot in either the treated or untreated cells.

Two additional proteins with mol wt of 40 and 38 kD, and pI of 5.7 (solid triangle) and 6.5 (short, bold arrow), respectively, were present in greater amounts upon A23187 treatment (Fig. 4A). The increase of these two proteins was not observed in 1-D PAGE, perhaps due to the presence of other proteins in similar mol wt ranges. However, their increases were clearly distinguishable by the 2-D PAGE. In all, at least seven proteins were present in greater amounts in A23187-treated cells than in the untreated cells at 25°C.

In general, more [³⁵S]-methionine labeled proteins were observed in A23187 treated cells when similar amounts of proteins were loaded into the gels. However, as indicated by empty arrowheads in Fig. 4 C and A, a group of proteins with mol wt of 38 kD and pI of 6.0-6.4 were present in reduced amounts in A23187 treated cells. These proteins appeared to be greatly inhibited when the cells were subjected to heat shock (Fig. 4 C and H).

Analysis of Proteins Synthesized in A23187-Treated Cells at 38.5°C by 2-D PAGE

As demonstrated in Fig. 4 H and HA, the A23187-stimulated synthesis of 88, 55, and 45 kD proteins from excised hypocotyl cells treated at 38.5°C, although not observed in 1-D PAGE (Fig. 2) were observed in 2-D PAGE with apparent pIs similar to those observed at 25°C (Fig. 4 C and A). The 69-71 kD mol wt areas had a number of proteins with a wide range of pIs which were not well separated. The distributions of 69-71 kD proteins in isoelectric point gels indicated that the A23187

-treated cells produced more proteins in high pI areas; whereas, the untreated cells produced more proteins in low pI areas. Two 69 kD proteins, one with pI of 6.7 (bold, long arrow) and one with pI of 6.5 (thin, long arrow), showed enhanced levels in A23187-treated cells.

A23187-stimulated synthesis of a 61 kD protein (white arrow, pI 6.0) at 38.5°C as shown in the 1-D PAGE (Fig. 2), was observed with the 2-D PAGE. This protein was also present, at low level, in both the treated and untreated cells at 25°C. In addition, two 38 kD proteins, one with a pI of 6.8 (arrowhead) and the other with a pI of 6.5 (short, bold arrow), were induced. The enhanced synthesis of pI 6.5 protein was also observed in cells treated at 25°C (Fig. 4 C and 4A), but the pI 6.8 protein was only observed in cells treated at 38.5°C.

In all, the 2-D PAGE demonstrated the enhanced synthesis of many proteins, at least nine of which are clearly distinguishable in A23187-treated hypocotyl cells at 38.5°C. However, not all proteins were present in greater amounts: the synthesis of three proteins (large, empty arrows) with pI of 5.2, 5.3, and 5.4 and mol wt of 33, 43, and 84 kD, respectively, were prominently reduced when the A23187 treated cells were subjected to heat shock. The 84 kD protein appeared to be one of the three major groups of high mol wt hsp. The low mol wt proteins induced by heat shock were not significantly affected by the presence of A23187, although there were some minor differences between them (data not shown).

Discussion

Calcium plays a vital role in many key metabolic activities and is one of the most essential of those major ions which control the life processes of plant cells. Plant cells possess delicate mechanisms to balance cellular calcium level, and an enormous amount of information is available on the role of calcium in the regulation of physiological activities in plant systems (Poovaiah and Reddy, 1987). However, very little is known about the action of calcium ions on gene regulation. Our results demonstrate that calcium perturbations caused by treating the cells with EGTA and A23187 result in enhanced synthesis of several specific proteins in excised hypocotyl cells.

EGTA is a chelator of free calcium ions in the medium as well as in the cell wall, where a large

amount of calcium ions are located (Demarty *et al.*, 1984). Therefore, the treatment of EGTA is likely to deplete extracellular calcium. However, since EGTA releases protons upon chelating calcium, which may promote calcium efflux through the calcium/proton antiport system, it may lead to a loss of intracellular calcium to the extracellular calcium milieu. EGTA treatment can result in changes in gravitropism, root and root-hair growth, and many other physiological activities (Poovaiah and Reddy, 1987). However, no study has demonstrated that EGTA acts to regulate gene expression. Our finding of EGTA-stimulated proteins suggests that such a possibility may exist; however, transcriptional analysis of the effect of EGTA on gene expression is necessary before a conclusion can be made.

EDTA chelates divalent cations, especially for magnesium and calcium ions, and it is permeable to cell membranes. It is known to be capable of removing calmodulin from its target enzymes (Klee *et al.*, 1980). Since the cytosols have high levels of magnesium ions, but low levels of calcium (Poovaiah and Reddy, 1987), the inhibitory effect of 5 mM EDTA on protein synthesis reported here is likely to be the result of calcium, rather than magnesium depletion.

Calcium ionophore A23187 elevates intracellular calcium level of the cells since it can bind to calcium ions and move freely across cellular membranes (Poovaiah and Reddy, 1987). It forms $(A23187)^-_2Ca^{2+}$ complex and its movement is not influenced by the membrane potential (Cambell, 1983). The 0.5x MS medium contains 1.5 mM of Ca^{2+} , which is many orders of magnitude higher than the normal intracellular calcium concentration of $0.1 \mu M - 1 \mu M$ (Bush and Jones, 1990; Clarks *et al.*, 1988). Treating the cells with A23187 can result in changes in a number of physiological processes such as blockage of cytoplasmic streaming (Doree and Picard, 1980) and auxin-induced cell elongation (Reddy *et al.*, 1988). The enhanced synthesis of the specific proteins stimulated by A23187 may therefore reflect a cellular response to the perturbation of intracellular calcium balances.

In animal cells, calcium ionophores represent a new class of initiation blockers that can effectively and selectively suppress the calcium-dependent component of protein synthesis, possibly through the mobilization of sequestered intracellular calcium (Brostrom *et al.*, 1989a). A23187 is capable of selectively inhibiting the

translation of muscle-specific mRNAs. This inhibitory effect can also be caused by EGTA, but it is more pronounced with A23187. When both are present, they become extremely effective in the inhibition of protein synthesis in animal cells (Endo and Nadal-Ginard, 1987). Our results using excised hypocotyl sections of *Brassica* indicate that high concentrations of either A23187 or EGTA inhibit protein synthesis, and that together, they act synergistically to enhance the inhibition. These similarities between plant and animal cells suggest that protein synthesis in eukaryotic cells is sensitive to intracellular calcium perturbation.

Thermal stress can result in a destruction of calcium-dependent translational initiation of mRNA for normal cellular proteins in animal cells (Brostrom *et al.*, 1986b) and a selective destabilization of specific mRNA in plant cells (Belanger *et al.*, 1986). Thermal stress can also rapidly raise the cytosolic free calcium level (Stevenson *et al.*, 1986). However, it is not known whether the inhibition of normal cellular protein synthesis and the induction of heat shock proteins by thermal stress are related to the elevation of calcium level. In animal cells, thermal stress can nullify the inhibitory effect of A23187 and EGTA on amino acid incorporation (Brostrom *et al.*, 1986b); however, our results with *Brassica* cells demonstrate that heat shock enhances the inhibitory effect of EGTA and A23187 on protein synthesis. The reason is not known, but one may speculate that in plant cells, the cell membrane becomes leaky and more permeable to EGTA under heat shock condition. Once inside the cells, EGTA may chelate cytosolic calcium, and either acts directly or plays a contributory role in the inhibition of protein synthesis. It is unlikely that sodium, potassium, and magnesium ions are involved since heat shock does not increase intracellular concentrations of these ions (Vidair and Dewey, 1986). The synergistic effect of EGTA and A23187 on protein synthesis inhibition under heat shock condition suggests that the cytosolic calcium ions which can be released from organelle reservoirs by A23187 treatment (Moore and Akerman, 1984) may have been lost to the chelation of EGTA, resulting in a depletion of cytosolic calcium at high temperature.

Eukaryotic cells respond to a variety of stimuli as diverse as heat shock, heavy metals, and calcium ionophores by synthesizing different groups of polypeptides. The expression of genes for these polypeptides can be induced independent of one another but calcium

ionophores can also induce the expression of some heat shock protein genes (Welch *et al.*, 1983). Our results demonstrate that under normal temperature, both EGTA and A23187 stimulate synthesis of 88, 55, and 45 kD proteins. However, only EGTA stimulates synthesis of a 61 kD protein. The potassium ionophore, valinomycin, which is known to inhibit oxidative phosphorylation (Montal *et al.*, 1970), failed to stimulate synthesis of these proteins, suggesting that the stimulation is not related to the reduction of ATP level or potassium ions. This observation is somewhat analogous to the report in animal cells that the A23187-induced proteins are not affected by other ionophores (Wu *et al.*, 1981). The mol wt of the proteins stimulated by calcium ionophores between plant and animal cells are clearly different, and our analysis of protein synthesis by 2-D PAGE revealed that A23187 treatment results in enhanced levels of several additional specific proteins undetectable with the 1-D PAGE. Apparently, more proteins are induced in plant cells than in animal cells upon A23187 treatment.

Our results showed that heat shock induces the synthesis of 105, 84, 70, and 16-28 kD proteins in *B. napus*. The 105 and 84 kD proteins are somewhat larger than those reported previously (Baszczynski, 1988), but the 70 and 16-28 kD proteins are in similar range. The overall incorporation of labels into these proteins is sensitive to the presence of EGTA and A23187, and the high mol wt hsp appear to be more resistant to the inhibition than those of low mol wt hsp and normal cellular proteins, indicating that calcium stress at high temperature may selectively inhibit synthesis of certain proteins. The 2-D PAGE demonstrated the enhanced synthesis of a number of proteins in A23187 treated cells under normal and heat shock conditions. This result indicates that the effect of A23187 treatment on protein synthesis is influenced by temperature. It remains to be established whether the intracellular calcium levels in A23187 treated cells are different between 25°C and 38°C, and whether such differences, if any, play a role in the regulation of protein synthesis. A major hindrance to studies of this kind is caused by the fact that the cytosolic calcium level is difficult to measure, particularly in plant cells, due to the presence of the cell wall and vacuoles, both of which are rich in calcium ion.

It is interesting that the calcium ionophore-stimulated proteins from excised hypocotyl sections

differed from those of young seedlings, except for a 45 kD protein which is present in both tissues. Studies indicate that the translocation of calcium ions is upward in the seedling of cruciferous plants (Bittner and Bushchmann, 1983). Since only the roots and lower parts of the hypocotyls were immersed in the treatment solution, perhaps the treatment of seedling resulted in a calcium redistribution within different parts of the seedling. The protein synthesis patterns may therefore be a reflection of the perturbation in either the calcium redistribution of different tissues or in the intracellular calcium level of certain specific tissues. The excised hypocotyl sections, on the other hand, are unlikely to form different calcium gradients after the treatment because they were fully submerged in the culture medium. Although one may argue that the use of protoplasts should completely eliminate the calcium gradient problem, the removal of the cell wall, the presence of an osmotic stabilizing agent, and the use of single cells make it more difficult to extrapolate the results with whole plant systems.

The A23187-inducible 94 and 78 kD proteins are ubiquitous in animal cells. They have been identified as the glucose-regulatory proteins (grp) and are also inducible by EGTA, glucose starvation, insulin treatment, and conditions that cause underglycosylation of proteins. Both proteins are located in the endoplasmic reticulum and are presumably related to the processing of abnormal membrane proteins (Lee, 1987). It is yet to be established whether the 88, 55, and 45 kD proteins induced by A23187 in *Brassica* are also present in other species of plants, and if they are also inducible by other stresses. Our study indicates that heat shock neither facilitates, nor prevents A23187 from stimulating the synthesis of these proteins. However, heat shock does appear to nullify to some extent the stimulated synthesis of specific proteins by EGTA and A23187, and *vice versa*. This result suggests that the synthesis of these proteins and hsp is controlled by separate mechanisms, and that they are both sensitive to a common factor, perhaps the balance of intracellular calcium ions.

The sulfhydryl-reducing agent 2-mercaptoethanol is capable of inhibiting protein glycosylation and activating the promoters of the A23187 inducible grp 78 and grp 94 genes in animal cells at concentrations of 0.2-0.5% (Kim and Lee, 1987). However, we observed that it completely blocked all the protein synthesis in

Brassica hypocotyl cells. Further studies are needed to determine whether plant cells are more sensitive to 2-mercaptoethanol than animal cells and whether the synthesis of A23187-stimulated proteins is related to the underglycosylation process in plant cells.

It can be concluded that specific proteins can be induced when eukaryotic cells are subjected to calcium perturbation caused by EGTA and A23187 treatments. The physiological roles of these proteins are unclear. Although EGTA reduces, while A23187 increases the intracellular calcium level, they both are capable of causing the mobilization of intracellular calcium stores. Thus, the stimulated synthesis may be related to the calcium mobilization process. A recent study also indicates that pretreatment of plant cells with calcium ionophores, calcium chelators, or heat shock results in changes of protein phosphorylation which is known to modulate plant gene expression (Wu *et al.*, 1992). It is therefore possible that calcium perturbation in plant cells may cause changes in gene expression at both transcriptional and translational levels. The identification and characterization of these proteins and their corresponding genes would further our knowledge on calcium as a second messenger in regulating gene expressions of plant cells. In fact, when 20-mers of the 5' and 3' regions of the *grp 78* gene (Lin and Lee, 1984) were used as primers, the polymerase chain reactions using *Nicotiana plumbagifolia* DNA as a template consistently produced a 1.8 kD fragment which is partially homologous to the *grp 78* gene (data not shown). Work is in progress to compare the sequence homologies between plant and animal genes that are responsive to calcium perturbation.

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鈣失調與熱休克對油菜 (*Brassica napus*) 蛋白質合成之影響

吳方盛¹ 薛淵貴² 馮騰永²

¹美國維吉尼亞州立大學生物系

²中央研究院植物研究所

我們已經證明以鈣離子鉗制劑 EGTA 或鈣離子運送劑 A23187 處理油菜的細胞，可激發產生一些特殊蛋白質的合成。以 EGTA 處理，會增加分子量 88, 61, 55 及 45 kD 蛋白質的合成；以 A23187 處理下胚軸片段，則會增加分子量 88, 55 及 45 kD 蛋白質的合成；而鉀離子運送劑 valinomycin，並不會刺激上述蛋白質的合成。以雙向電泳分析 (2-D PAGE) 結果發現經 A23187 處理過的下胚軸組織，又新增 4 種蛋白質的合成。不論細胞是否經 EGTA, A23187, 或 valinomycin 處理過，若以 38.5°C 熱處理，皆會刺激 105, 84, 70 及 16~28 kD 蛋白質的合成。雙向電泳分析顯示：A23187 處理過之細胞受到熱休克時，至少會促進 9 種蛋白質的合成。細胞蛋白質的總量也會因 EGTA, A23187, EDTA 的處理而降低；且在 38.5°C 下比在 25°C 下降低更多。當 A23187 與 EGTA 同時施用時則有協同抑制之現象。我們的結論是：當植物細胞受到鈣離子失調與熱休克時，某些特殊蛋白質就會被誘導或抑制。