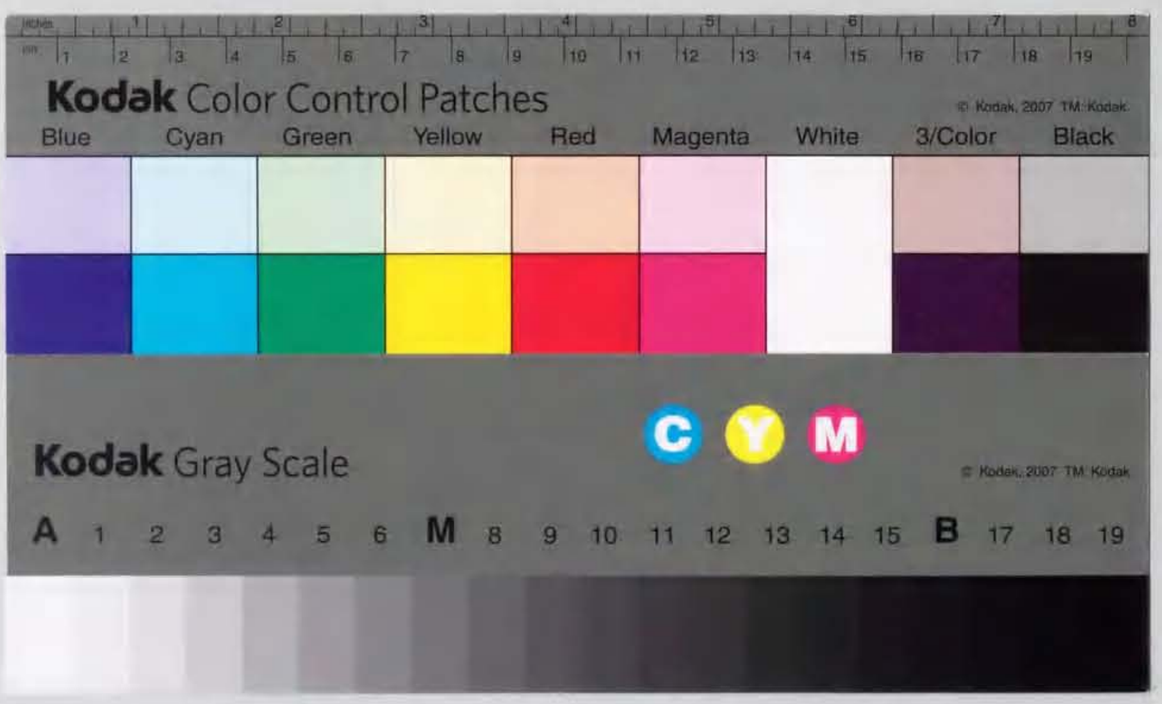
Calcium Signaling in Tobacco Suspension Culture Cells

Koji Takahashi



Calcium Signaling in Tobacco Suspension Culture Cells

タバコ培養細胞におけるカルシウムシグナリング

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the Graduate School of Agricultural Sciences,
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By

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ATA	aurintricarboxylic acid
BAPTA	1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid
$[Ca^{2+}]_{cyt}$	cytosolic free calcium ion concentration
CDPK	Ca^{2+}-dependent protein kinase
CLA	Cripridina luciferin-derived chemiluminescent reagent
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol bis (β-aminoethylether) *N,N'*-tetraacetic acid
ER	endoplasmic reticulum
G-protein	GTP binding protein
Hepes	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
IP_3	inositol 1,4,5-triphosphate
LS	Linsmaier and Skoog
MAPK	mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MBP	myelin basic protein
Mes	4-morpholineethanesulfonic acid
mosmol	milli osmole / kg
NAD	β-nicotinamido adenine dinucleotide
PI	phosphoinositide
PLC	phospholipase C
rlu	relative light unit
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
TCA	trichloroacetic acid

TMB-8	8-(*N,N*-diethylamino)-octyl 3,4,5-trimethoxybenzoate-HCl
Tris	2-amino-2-hydroxymethyl-1,3-propanediol

General Introduction

Many physiological processes in plants have been reported to be under the control of Ca^{2+} (for review see Hepler and Wayne, 1985): for example, the action of phytochrome (Dreyer and Weiseneel, 1979), auxin (Hanson and Trewavas, 1982) and ethylene (Raz and Fluhr, 1992), cell elongation (Brewbaker and Kwack, 1963), protoplasmic streaming (Kamiya, 1981), turgor regulation (Okazaki and Tazawa, 1986), and phototactic and gravitropic movements (Gehring et al., 1990).

Free Ca^{2+} concentration in cytosol ($[Ca^{2+}]_{cyt}$) is usually kept as low as 10^{-7} M (Gilroy et al., 1986; Felle, 1989), which is 10,000-fold lower than that in apoplast, the extracellular milieu. Cellular Ca^{2+} homeostasis is strictly maintained by a balance of various Ca^{2+} transport systems against the Ca^{2+} gradient: extrusion through the plasma membrane and sequestration into the intracellular organelles such as vacuoles, endoplasmic reticulum (ER) and mitochondria. Two Ca^{2+} transport systems in the plasma membrane function for extrusion of Ca^{2+}. Ca^{2+} transporting ATPase (Ca^{2+}-ATPase) pumps Ca^{2+} into extracellular space dependent on ATP hydrolysis and Ca^{2+}/H^+ antiporter extrudes Ca^{2+} by exchange Ca^{2+} for H^+ using H^+ gradient across the plasma membrane which is formed by H^+ transporting ATPase (H^+-ATPase) in the plasma membrane (Kasai and Muto, 1990). For intracellular Ca^{2+} sequestration ATP-dependent Ca^{2+} transport system in the ER (and the vacuole) and Ca^{2+}/H^+ antiport system in the vacuole are operating. It is considered that Ca^{2+}-ATPase in the plasma membrane and the ER are responsible for keeping the basal cytosolic Ca^{2+} because of their high affinity for Ca^{2+}, and the Ca^{2+} transport systems in the vacuole play important roles in intracellular Ca^{2+} sequestration because of high concentration of Ca^{2+} in the vacuole which occupies as much as 90% of the mature plant cell volume.

The general mechanism by which Ca^{2+} modulates a response is through a change in its concentration. Under the low $[Ca^{2+}]_{cyt}$ condition maintained by the transporting systems, stimulus-responsive transport of a relatively small absolute number of Ca^{2+} into the cytosol will cause a large change in $[Ca^{2+}]_{cyt}$. Stimuli-induced increase in $[Ca^{2+}]_{cyt}$ is caused by activation of Ca^{2+} permeable channels which located in the plasma membrane and the membranes of organelles storing intracellular Ca^{2+}. Voltage-dependent Ca^{2+} channels (Ping et al., 1992b; Thuleau et al., 1994) and mechanosensory Ca^{2+} permeable channels (Ding and Pickard, 1993) have been detected in the plasma membrane. These channels must be responsible for influx of Ca^{2+} through the plasma membrane in response to stimuli. It has been known that $[Ca^{2+}]_{cyt}$ is increased via Ca^{2+} influx through the plasma membrane in response to external stimuli such as fungal elicitor (Kauss and Jeblick, 1991) and gibberellin (Gilroy and Jones, 1992).

Evidences indicating the involvement of Ca^{2+} release from the intracellular stores in Ca^{2+} signaling in plants are accumulating. Enhancement of phosphoinositide (PI) turnover has been reported to be caused by various extracellular signals including light (Morse et al., 1987), auxin (Ettlinger and Lehle, 1988) and fungal elicitor (Kamada and Muto, 1994). Legendre et al., (1993) reported that both polygalacturonic acid elicitor and mastoparan promoted a transient increase in inositol 1,4,5-triphosphate (IP_3) content in soybean culture cells, indicating activation of PI-specific phospholipase C (PLC). Activation of PLC by mastoparan has also been reported in carrot cells (Cho et al., 1995). In aminal cells IP_3 is established as major Ca^{2+}-mobilizing ligand. Generation of IP_3 by plasma membrane receptpor-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate by PLC results in Ca^{2+} release through binding of IP_3 to a specific endomembrane Ca^{2+} channel. In plants, the vacuole is considered as the main IP_3-sensitive Ca^{2+} pool (Drøbak,1992) based on in vitro studies which showed IP_3-induced Ca^{2+} release from isolated vacuoles

(Ranjeva et al., 1988) or microsomes (Schumaker and Sze, 1987). In vivo photolysis of caged (photoactivatable)-IP_3 caused an increase in $[Ca^{2+}]_{cyt}$ of guard cell which was followed by stomatal closure (Gilroy et al., 1990). Recently gadolinium-sensitive, voltage-dependent Ca^{2+} channel was detected in the ER of touch-sensitive tendrils of *Bryonia dioica* in which gadolinium completely abolished the response to touch (Klüsener et al., 1995). This suggests Ca^{2+} release from the ER in this plant.

Activation of PLC is mediated by receptor-coupled activation of some types of heterotrimeric GTP binding proteins (G-proteins) in animal cells (Neer, 1995). However, much is not known about the involvement of G-proteins in signal transduction in plant cells. Alpha-subunit of G-protein (G_α) has been detected immunologically in several plants. Heterotrimeric G-protein has not yet been purified and characterized from plants, however, evidence showing its presence and function in plants is accumulating. cDNA encoding G_α (Ma et al., 1990) and G_β (Weiss et al., 1994) have been cloned from *Arabidopsis thaliana*. Activation of G-protein by blue light was reported in the plasma membrane of etiolated pea (Warpeha et al., 1991). Romero and Lam (1993) presented evidence suggesting the involvement of G-protein in early steps of phytochrome-regulated expression of chlorophyll a/b-binding protein gene in soybean cells. Muschietti et al. (1993) repoted that binding of GTPγS to G_α in *Medicago sativa* protoplasts was enhanced by red light irradiation and this enhancement was reversed when far-red light was illuminated immediately. Legendre et al., (1992) presented evidence demonstrating that G-proteins are involved in the elicitation of the defense response of cultured soybean cells.

The main targets of Ca^{2+} signal are Ca^{2+} binding proteins, which must bind it with an affinity at physiological range of $[Ca^{2+}]_{cyt}$. In fact, a number of plant enzymes have been known to be regulated directly or indirectly by micromolar concentrations of Ca^{2+}. Nicotinamide adenine dinucleotide (NAD) kinase is the first plant enzyme shown to be regulated by calmodulin

(Muto and Miyachi, 1977; Anderson and Cormier, 1978). Although this protein has no enzymatic activity, it can activate several enzymes in a calcium-dependent manner. So far, plant enzymes including Ca^{2+}-ATPase (Briskin, 1990; Evans et al., 1991), nuclear nucleoside triphosphatase (Matsumoto, et al., 1984; Chen et al., 1987) and protein kinases (Blowers and Trewavas, 1987) have been reported to be bound and activated by calmodulin. Calcium-dependent protein kinase (CDPK) is the most well-characterized enzyme among enzymes whose activities are modulated by Ca^{2+}. CDPK, first detected by Hetherington and Trewavas (1982) is activated by direct binding of Ca^{2+} without other component such as phospholipids and diacylglycerol which activate protein kinase C in animal cells, and calmodulin (Harmon et al., 1987). The presence of CDPKs in several subcellular locations suggests that this enzymes may be involved in multiple Ca^{2+} signaling pathways (Roberts and Harmon, 1992). The transcripts of calmodulin (TCH1) and CDPK (ATCDPK1 and ATCDPK2) have been reported to be increased by environmental stimuli, such as wind, rain, and drought stress (Braam and Davis, 1990; Urao et al., 1994), suggesting that the Ca^{2+}-modulated proteins are controlled by both posttranslational and transcriptional regulation in plant.

Protein phosphorylation in response to external stimuli is an important biochemical process in eukaryotic cells. It has been reported in plants that the protein phosphorylation is induced by various stimuli, such as light (Fallon and Trewavas, 1994), temperature (Monroy et al., 1993), fungal elicitors (Felix et al., 1991), osmotic shock (Yuasa and Muto, 1996) and phytohormones (Koritsas, 1988). Recently, protein phosphorylation cascades (or protein kinase cascades) have been reported to be involved in intracellular siganl transduction. In particular, the protein kinase cascades including mitogen-activated protein kinases (MAPKs), MAPK kinases (MAPKKs), and MAPKK kinases (MAPKKKs) have been shown to

function in diverse signal transduction pathway in mammalian cells and yeast (Marshall, 1995; Herskowitz, 1995). MAPKs are activated by tyrosine and threonine phosphorylation catalyzed by MAPKKs which are protein kinases with dual specificity for both tyrosine and serine/threonine. MAPKKs are also activated by serine/threonine phosphorylation catalyzed by MAPKKKs. In *Saccharomyces cerevisiae* at least five different MAPK signaling pathways. Among of them the protein kinase C-dependent pathway (BCK1→MKK1/MKK2 → MPK1) was suggested to be triggered by membrane stretch (Kamada et al., 1995). In higher plants it has been demonstrated that a number of genes for protein kinases constituting MAPK cascade (Jonak et al., 1994): e.g. NFT3 (Wilson et al., 1993), NPK2 (Shibata et al., 1995) and NPK1 (Banno et al., 1993) which are structurally related to MAPK, MAPKK, and MAPKKK, respectively have been isolated from *N. tabacum*. The deduced amino acid sequence of NPK1 is closely homologous to that of the protein kinase encoded by BCK1 gene and the catalytic domain of NPK1 specifically activates the signal transduction pathway mediated by BCK1 in yeast, suggesting that a signal transduction pathway mediated by BCK1-related protein kinase is conserved in tobacco cells (Banno et al., 1993). Mizoguchi et al. (1996b) recently detected the interaction between ATMPK4 and AtMEK which are homologs of MAPK and MAPKK in *Arabidopsis thaliana*, respectively, by examining the protein-protein interaction with two-hybrid system. This study and complementation analyses in yeast (Mizoguchi et al., 1996a) strongly suggest the possibility that AtMEK phosphorylates and activates ATMPK4. Such study is effective for determination of relationship between the protein kinase constituting MAPK cascades in plant. Although plant MAP kinases have been shown to be activated by auxin (Mizoguchi et al., 1994) and wounding (Seo et al., 1995), the mechanisms of activation of the kinase cascade have not been elucidated yet.

In my master course study, I have purified a protein cross-reacting with an anti-G_α antibody from a halotolerant green alga *Dunaliella tertiolecta* (Takahashi et al., 1995) to examine the involvement of G-protein in osmotic regulation of this alga. It has been reported in *D. tertiolecta* that hydrolysis of PI is increased upon hypoosmotic shock (Einspahr et al., 1988) and PLC activity is stimulated by GTPγS (Einspahr et al., 1989), suggesting that *Dunaliella* cells have a G-protein-mediated transduction pathway for sensing hypoosmotic signals. Yuasa et al. (1992, 1995) have purified a CDPK from *Dunaliella tertiolecta* and presented evidence for its involvement in hypoosmotic response of *Dunaliella*. Moreover, they showed that hypoosmotic shock induced activation of protein kinases in *Dunaliella* cells (Yuasa and Muto, 1996). Hypoosmotic shock has been reported to be a trigger of MAPK cascade in mammalian cells (Tilly et al., 1996; Schliess et al., 1996) and yeast (Kamada et al., 1995). These reports suggest that $[Ca^{2+}]_{cyt}$ and protein kinases involve in hypoosmotic regulation in widespread organisms. The aim of the present study is to reveal the mechanism of hypoosmotic signal transduction in higher plant, especially in respect to Ca^{2+} signaling.

Chapter I

Preparation of the transgenic tobacco suspension culture cells expressing apoaequorin

SUMMARY

Aequorin is the Ca^{2+}-sensitive photoprotein which participates in the bioluminescence of the hydromedusa *Aequorea victoria* accompanied with a green fluorescent protein. This protein which was microinjected in living cells has been employed for more than 3 decades as a cytoplasmic Ca^{2+} indicator. To elucidate the involvement of cytosolic Ca^{2+} in the signal transduction in plants, I prepared the transgenic tobacco (*Nicotiana tabacum* L.) suspension culture cells expressing apoaequorin. Northern blot analysis showed that the tobacco cells were transformed and expressed apoaequorin. Recombinant aequorin can be reconstituted in situ by incubating of the cells with coelenterazine.

I-1. INTRODUCTION

Aequorin, a photoprotein which was isolated from the bioluminescent jellyfish *Aequora* (Shimomura et al., 1962) and consists of apoaequorin, a 21-kDa single polypeptide chain and coelenterazine, a luminophore, binds 3 moles of Ca^{2+} per mole of aequorin and then yields blue light (λ_{max} 469 nm), an apoaequorin-coelenteramide complex (discharged aequorin) and CO_2 (Prasher et al., 1985; Fig. 1). Taking advantages of the property, this protein has been employed as an indicator of $[Ca^{2+}]_{cyt}$ in living cells, since its use for the purpose was first proposed in 1963 (Shimomura et al., 1963). Although there are some suitable properties of aequorin for a Ca^{2+} indicator such as ease of signal detection, high sensitivity to Ca^{2+} and lack of toxicity (Blinks et al., 1978), some disadvantages are certainly presented; Loading of aequorin into cytosol requires traumatic procedures, e.g. microinjection, and it is technically difficult that aequorin is introduced into the cytosol of the target cells, especially plant cells possessing the rigid cell wall and huge vacuole. The Ca^{2+}-sensitive fluorescent indicators, such as quin-2, fura-2,

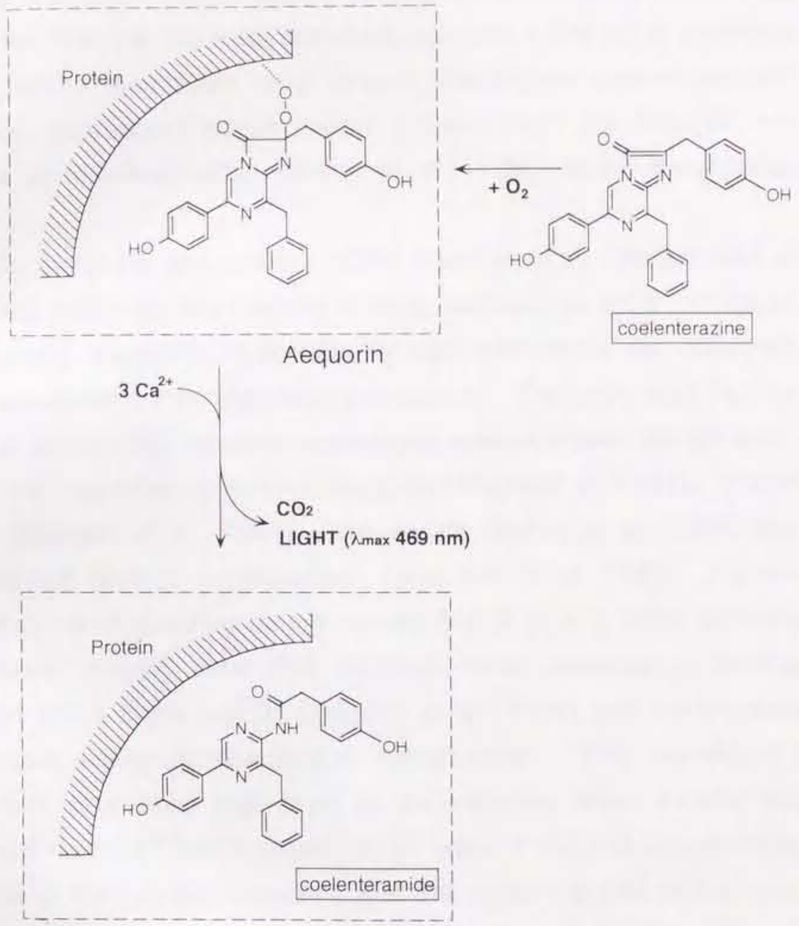

Figure 1. Postulated mechanism of luminescence of aequorin.
Model was drawn based on results obtained by Prasher et al. (1985).

indo-1 and their permeant esters, which are easy to introduce into the animal cells, have come to use as a Ca^{2+} indicator in wide-spread organisms. However, it is limited to use these fluorescent indicators for plant cells: for example, the ester quin-2/AM appears either not to permeate the plant plasma membrane or to already hydrolyzed outside the cell. Alternatively, fluorescent nonpermeant probes might be brought into protoplasts by electroporation (Gilroy et al., 1986) which may cause traumata.

The cloning of the apoaequorin cDNA (Prasher et al., 1985; Inouye et al., 1985) has expanded the method of using aequorin as a Ca^{2+} indicator. The expressing aequorin recombinantly has eliminated the traumatic loading procedures by endogenous production. Trewavas and his co-workers first applied this aequorin-expressing system to plant (Knight et al., 1991b) as early as other organisms; bacteria (Knight et al., 1991a), yeasts (Nakajima-Shimada et al., 1991), slime molds (Saran et al., 1994) and mammalian cells (Button and Browstein, 1993; Sheu et al., 1993). For this purpose, they constructed the binary vector, pMAQ2 (Fig. 2) which contains the cauliflower mosaic virus 35S promoter-fused apoaequorin-coding region from cDNA clone pAEQ1 (Prasher et al., 1985) and transformed tobacco plant using *Agrobacterium tumefaciens*. The transformed tobacco plant expressed high level of apoaequorin which located the soluble fraction over 97% of the total cellular amount and was suggested to concentrate in the cytosol, indicating that this system is one of the most valid method of monitoring $[Ca^{2+}]_{cyt}$. With this transgenic plant the change in $[Ca^{2+}]_{cyt}$ has been observed in response to touch, cold shock, elicitors (Knight et al., 1991b), wind (Knight et al., 1992), wounding (Knight et al., 1993), hydrogen peroxide (Price et al., 1994) and circadian rhythms (Johnson et al., 1995).

To elucidate the involvement of Ca^{2+} in signal transduction pathway, I prepared the transgenic tobacco suspension culture cells expressing

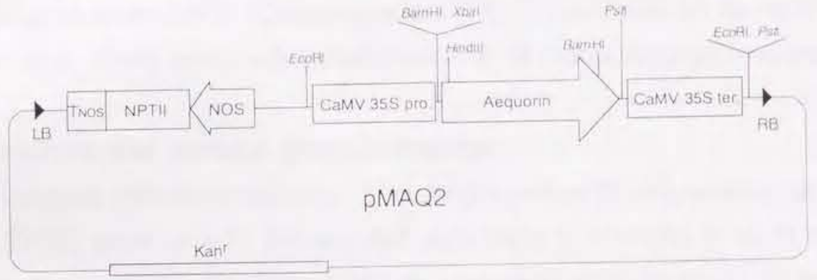

Figure 2. Construction of pMAQ2.
CaMV 35S pro; CaMV 35S promoter, Aequorin; apoaequorin gene, CaMV 35S ter; CaMV 35S terminater, NOS; nopaline synthase promoter, NPTII; neomycin phophotransferase, TNOS; nopalin synthase terminater, LB; left border, RB; right border, Kan^r; kanamycin resistant gene

apoaequorin.

I-2. MATERIALS AND METHODS

Chemicals

The binary vector, pMAQ2 (Fig. 2) was kindly gifted from Prof. A.J. Trewavas, University of Edinburgh, UK. Aurintricarboxylic acid (ATA) and Random primer DNA labeling kit were purchased from Sigma (St. Louis, MO, USA) and Takara Shuzo Co. (Kyoto), respectively. Hybond N^+ nylon membrane filter and [α-^{32}P] d-CTP were obtained from Amersham (Buckinghamshire, UK). Coelenterazine (Fig. 1), synthesized as reported (Isobe et al., 1994) was kindly gifted from Prof. M. Isobe, Nagoya University.

Cell culture and genetic transformation

Tobacco (*Nicotiana tabacum* L. cv bright yellow 2) suspension culture cells (BY-2) were usually propagated according to Nemoto et al. (1988). Briefly, the culture was maintained in Linsmaier and Skoog (LS) liquid medium (Linsmaier and Skoog, 1965) supplemented with 3% sucrose and 0.2 μg/mL 2,4-dichlorophenoxyacetic acid, where the level of KH_2PO_4 and thiamine-HCl was increased to 370 mg/L and 1 mg/L, respectively. Two mL of 7 day-old stationary culture was suspended in 100 mL of fresh medium and incubated at 28℃ with shaking at 130 rpm on a gyratory shaker in darkness.

Transformation of tobacco suspension culture cells was carried out according to An (1985). Briefly, 4 mL of 3-day old, exponentially growing, suspension cell culture was transferred to a 90-mm petri dish and incubated at 28℃ with 100 μL of fresh overnight-culture of *Agrobacterium tumefaciens* LBA4404 containing the binary vector pMAQ2. After 48 h of co-cultivation, tobacco cells were washed and plated on the LS agar

medium containing 500 μg/mL of carbenicillin to prevent growth of *Agrobacterium* and 250 μg/mL of kanamycin to select the kanamycin-resistant transformants. After 3 to 4 weeks selection, the kanamycin-resistant transformants were collected and transferred into LS liquid medium.

Northern blot analysis

Total RNA was extracted from the transgenic tobacco cells and wild-type tobacco cells. Tobacco cells were frozen in liquid nitrogen and homogenizing with a mortar and a pestle. The frozen homogenate was thawed in an extraction buffer containing 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 5 mM EDTA, 2% SDS and 2 mM ATA, and 0.37 M KCl was added to them. After standing on ice for 15 min the homogenate was centrifuged at 8,000 $\times g$ for 5 min at 4℃. The resultant supernatant was kept for 6 h at 4℃ after adding 4 M LiCl and then centrifuged at 15,000 $\times g$ for 20 min at 4℃. The resultant pellet was resuspended in 0.4 mL of distilled water and mixed with 40 μL of 3 M sodium acetate (pH 5.2). After the addition of 450 μL of phenol (saturated with 100 mM Tris-HCl, pH 7.5), the mixture was centrifuged and the aqueous phase was re-extracted twice with phenol and once with chloroform. The total RNA was precipitated with and washed with 70% ethanol. After dried, the total RNA was suspended in 50 μL of TE.

For northern hybridization analysis, total RNA was subjected to electrophoresis on agarose gel, and transferred to Hybond N^+ nylon membrane filter by capillary elution. After prehybridized for 2 h at 42℃ in the prehybridization buffer containing 50% formamide, 6× SSC, 2× Denhardt's reagent (Denhardt, 1966), 0.1% SDS and 0.1 mg/mL salmon sperm DNA, the filter was incubated for 12 h at 42℃ in the prehybridization buffer supplemented with the denatured radiolabeled apoaequorin probe.

The filter was then washed for 20 min at room temperature in 1× SSC, 0.1% SDS and successively washed for 20 min at 65℃ in 0.5× SSC, 0.1% SDS twice. The hybridized bands on the filters were visualized by a Bioimaging Analyzer BAS 2000 (Fuji Photo Film Co., Tokyo).

The apoaequorin probe was prepared by labeling the 0.5-kb HindIII-BamHI fragment of pMAQ2 (Fig. 2) with [α-^{32}P] d-CTP using Random primer DNA labeling kit.

Luminescence measurement

Luminescence emitted from the transgenic cells possessing the reconstituted aequorin was measured with a Chem-Glow photometer (American Instrument Co., Silver Spring, MD, USA) equipped with a pen recorder (Rikadenki Co., Tokyo) and expressed as relative light units (rlu).

I-3. RESULTS

Expression of apoaequorin

Figure 3 shows that the kanamycin-resistant transformants of tobacco cells express apoaequorin.

Aequorin reconstitution and luminescence measurements

The recombinant aequorin was reconstituted in situ by incubating 3-day old transgenic tobacco cells with 1 μM coelenterazine in the culture medium for 24 h in darkness. When the transgenic cells possessing reconstituted aequorin were treated with 10% ethanol containing 1 M $CaCl_2$, the Ca^{2+}-dependent luminescent spike was detected (data not shown), whereas the same treatment did not emit luminescence in the wild-type tobacco cells preincubated with 1 μM coelenterazine or the transgenic cells not preincubated with coelenterazine. This indicates that the luminescent

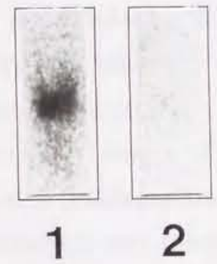

Figure 3. Northern analysis of the transgenic and wild-type tobacco suspension culture cells.
Total RNA was extracted from the transgenic (lane 1) and wild-type (lane 2) tobacco suspension culture cells. The RNA (20 μg) was electrophoresed, blotted and hybridized as described in Materials and Methods.

spike detected was emitted from the reconstituted aequorin but not from coelenterazine or apoaequorin. Figure 4 shows the time course of reconstitution of aequorin. The reconstitution began immediately after the addition of coelenterazine and achieved maximum level at 6 h and the high level was sustained until 24 h.

I-4. DISCUSSION

The advantage of the method using the recombinant aequorin is that $[Ca^{2+}]_{cyt}$ responses of plant cells can be monitored without traumatic procedures such as microinjection within the range of physiological $[Ca^{2+}]_{cyt}$ (Blinks et al., 1978).

The recombinant aequorin can be reconstituted in situ by incubating the transgenic cells with 1 μM coelenterazine as shown in figure 4. I decided to use the transgenic tobacco cells incubated with 1 μM coelenterazine for 6 to 10 h in this study. The amount of reconstituted aequorin was not sufficient to report resting $[Ca^{2+}]_{cyt}$ in these cells (which we expected to be maintained at a relatively low level of $< 10^{-7}$ M), therefore unstimulated cells did not produce any luminescence above background (see Chapter II).

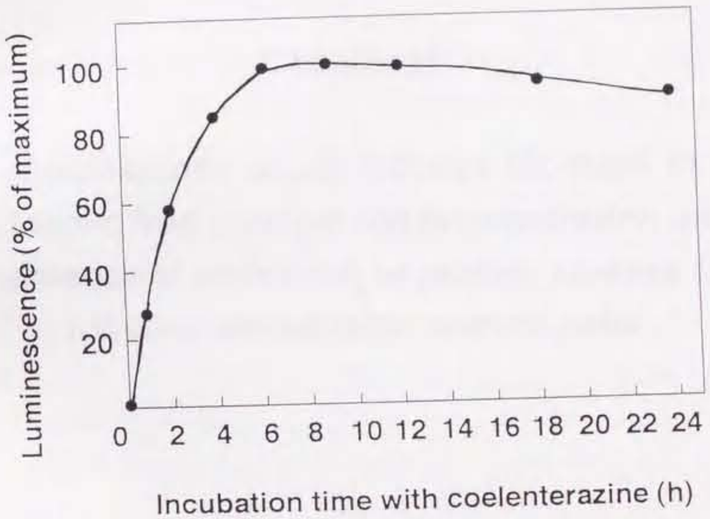

Figure 4. Time course of aequorin reconstitution in the transgenic tobacco suspension culture cells.
Coelenterazine (1 μM) was added to the transgenic tobacco suspension culture at 0 time. After the indicated time, the cell suspension was treated with 1 M $CaCl_2$ and 10% ethanol to discharge the aequorin reconstituted, and resultant luminescence was measured.

Chapter II

Hypoosmotic shock induces increase in cytosolic free calcium ion concentration and subsequent activation of protein kinases in tobacco suspension culture cells

SUMMARY

Hypoosmotic shock induced a transient increase in cytosolic free calcium ion concentration ($[Ca^{2+}]_{cyt}$) and subsequent activation of a 50-, a 75- and a 80-kDa protein kinases in tobacco (*Nicotiana tabacum* L.) suspension culture cells. $[Ca^{2+}]_{cyt}$ measurements were made by genetically transforming these cells to express apoaequorin and by reconstituting the Ca^{2+}-dependent photoprotein, aequorin, in the cytosol by incubation with chemically synthesized coelenterazine. Measurement of Ca^{2+}-dependent luminescence output thus allowed the direct monitoring of $[Ca^{2+}]_{cyt}$ changes. When cells were added to a hypoosmotic medium, a biphasic increase in $[Ca^{2+}]_{cyt}$ was observed; an immediate small elevation (phase 1) was observed first, followed by a rapid large elevation (phase 2). Phase 1 $[Ca^{2+}]_{cyt}$ was stimulated by the vacuolar-type ATPase inhibitor bafilomycin A_1. Phase 2 was inhibited by the protein kinase inhibitor K-252a and required the continued presence of the hypoosmotic stimulus to maintain it. Although Ca^{2+} in the medium was needed to produce phase 2, it was not needed to render the cells competent to the hypoosmotic stimulus. If cells were subject to hypoosmotic shock in Ca^{2+}-depleted medium, increases in luminescence could be induced up to 20 min after the shock by adding Ca^{2+} to the medium. These data suggest that hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation results from the activity of a Ca^{2+} channel in the plasma membrane or associated hypoosmotic sensing components that require Ca^{2+}-independent phosphorylation and a continued stimulus to maintain full activity.

Changes in protein kinase activities were measured by an in-gel protein kinase assay method, with parallel monitoring of $[Ca^{2+}]_{cyt}$. Depletion of extracellular Ca^{2+} suppressed both $[Ca^{2+}]_{cyt}$ elevation and the activation of protein kinases in response to hypoosmotic shock. This indicates that $[Ca^{2+}]_{cyt}$ elevation is prerequisite for hypoosmotic shock-

induced activation of protein kinases. Pharmacological analyses indicated that the shock-activated protein kinases were activated by phosphorylation, suggesting the activities of these protein kinases are regulated by putative protein kinases. These results suggest that hypoosmotic signal is transduced to protein kinase cascades which are triggered by $[Ca^{2+}]_{cyt}$ elevation.

II-1. INTRODUCTION

The maintenance and regulation of turgor pressure is essential for many aspects of plant growth, development and movements. Although turgor pressure can be regulated via the synthesis and degradation of osmotic protectants, rapid changes usually occur by modifications of the flux of inorganic ions across the plasma membrane (Schroeder and Hedrich, 1989).

A great deal of research effort has been directed at investigating the responses of higher plants to hyperosmotic signals (Greenway and Munns, 1980), because hyperosmotic stress during salination or drought for example, is agriculturally important. In contrast, plant responses to hypoosmotic signals have rarely been studied even though they are directly relevant to understanding both stomatal and leaf movements. Hypoosmotic shock induces increases in both cell volume and turgor pressure, even in walled cells, and the magnitude of the water influx is, in part, dependent on the osmotic potential of the cell. After hypoosmotic shock plant cells can usually recover both their cell volume and their original osmotic potential, a phenomenon termed "hypoosmotic regulation" (Okazaki and Tazawa, 1990). To accomplish this, plant cells must have mechanisms both for sensing hypoosmolarity and for transducing this information to affect responses leading to hypoosmotic regulation; the challenge is to identify these components.

Hypoosmotic regulation, which occurs after hypoosmotic shock, involves a reduction in turgor pressure. Substantive evidence in the literature suggests that $[Ca^{2+}]_{cyt}$ may regulate reductions in turgor pressure. Leaf closure in *Mimosa pudica*, which is caused by a decrease in turgor pressure on one side of the pulvinus, can be inhibited by EDTA and La^{3+} (Campbell and Thompson, 1977). Dark-induced stomatal closure in *Commelina communis* is also mediated by a decrease in turgor pressure and can be accelerated by external Ca^{2+} and inhibited by EGTA (Schwartz, 1985). Increases in $[Ca^{2+}]_{cyt}$ in guard cells of *Vicia faba* and *C. communis* precede ABA-induced stomatal closure (McAinsh et al., 1990; Irving et al., 1992). Photolysis of loaded caged Ca^{2+} or caged IP_3 causes stomatal closure in *C. communis* (Gilroy et al., 1990) again indicating the involvement of $[Ca^{2+}]_{cyt}$ and internal IP_3-sensitive Ca^{2+} stores in closure. These reports suggest that $[Ca^{2+}]_{cyt}$ plays a significant role in controlling turgor decrease. In algal cells, $[Ca^{2+}]_{cyt}$ has been postulated to play a significant role in hypoosmotic responses. Okazaki and Tazawa (1990) reported that hypoosmotic shock induces a transient $[Ca^{2+}]_{cyt}$ elevation and Ca^{2+} acts in turgor regulation in the euryhaline characean alga *Lamprothamnium*.

The above information suggests that hypoosmotic signals in plant cells might be transduced via $[Ca^{2+}]_{cyt}$. Ca^{2+} functions as a primary second messenger in signal transduction (Muto, 1992; Bush, 1995) and has been shown to modify a number of physiological processes (Hepler and Wayne, 1985; Nagai, 1993; Trewavas and Knight, 1994). To understand the involvement and functions of Ca^{2+} in hypoosmotic signaling it is necessary to continuously monitor the change in $[Ca^{2+}]_{cyt}$. It has been developed a novel method for this purpose that uses plants genetically transformed to express apoaequorin which on incubation with coelenterazine reconstitutes the Ca^{2+}-dependent luminescent protein aequorin. Because of the simplicity and ease of this method, I used it to examine the possible transduction of hypoosmotic signaling. However hypoosmotic shock is

technically difficult to administer to whole plants, so I decided to transform tobacco (*Nicotiana tabacum* BY-2) suspension culture cells to express apoaequorin and reconstitute aequorin by incubation with chemically-synthesized coelenterazine. This approach has the added benefit that biochemical inhibitors can be used more effectively and in the physiological range.

Protein phosphorylation in response to external stimuli is an important biochemical process in eukaryotic cells. In plants, it has been reported that phytohormones (Koritsas, 1988) and external stimuli, such as light (Fallon and Trewavas, 1994), cold shock (Monroy et al., 1993), fungal elicitor (Felix et al., 1991; Suzuki and Shinshi, 1995) and wounding (Usami et al., 1995; Seo et al., 1995) induce the phosphorylation of specific proteins and activate protein kinases. Transient elevation of $[Ca^{2+}]_{cyt}$ has been reported to be caused by various external stimuli. Therefore the above stimuli were supposed to activate protein kinases via calcium signal. It was 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 (Yuasa and Muto, 1992). Involvement of a CDPK which has been purified and characterized (Yuasa et al., 1995) in the above process was suggested and several other protein kinases seemed likely to participate in the mechanism of hypoosmotic signaling in *Dunaliella*. (Yuasa and Muto, 1996). In order to elucidate the relationship of Ca^{2+} and protein kinases in hypoosmotic signal transduction pathway in tobacco cells, I further 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.

II-2. MATERIALS AND METHODS

Chemicals

K-252a, calyculin A and ML-9 were purchased from Seikagaku Kogyo Co. (Tokyo). Bafilomycin A_1, verapamil, diltiazem, bromophenacyl bromide, neomycin sulfate, genistein and ruthenium red were purchased from Wako Pure Chemical Industries (Osaka). Dephosphorylated casein, histone type III-S (histone H1), myelin basic protein (MBP), calf intestinal alkaline phosphatase, trifluoperazine, nifedipine, KN-62 and M-9 were from Sigma (St. Louis, MO, USA). [γ-^{32}P]ATP (148 TBq/mmol) was obtained from ICN (Cost Mesa, CA, USA). Calmidazolium and thapsigargin were obtained from Boehringer Mannheim (Mannheim, Germany). Anti-phosphotyrosine antibody (PY-20) was purchased from Transduction Laboratories (Lexington, KY, USA). Geldanamycin was a gift from Dr. Yamaki, Institute of Applied Microbiology, University of Tokyo. Prestained SDS-PAGE standards and protein A-Sepharose CL-4B were products of Bio-Rad (Hercules, CA, USA) and Pharmacia (Uppsala, Sweden), respectively. Coelenterazine was a generous gift from Prof. Isobe, and dissolved in ethanol at 1 mM and stored at -30℃.

Plant cell culture

The transgenic tobacco (*Nicotiana tabacum* L. cv bright yellow 2) suspension culture cells expressing apoaequorin prepared as described in Chapter I, were used.

Hypoosmotic shock and luminescence measurements

Aequorin was reconstituted by incubating 3-day old transgenic BY-2 cells with 1 μM coelenterazine in the culture medium in darkness for 8 h. The cells were washed with and resuspended in fresh medium, and used after 30 min resting incubation. The cells were subjected to hypoosmotic

shock, by diluting 200 μL of the cell suspension with 400 μL of the culture medium depleted of sucrose in a glass tube for luminescence measurement. Luminescence emitted from the transgenic cells was measured and expressed as rlu as described in Chapter I. The osmotic pressure of solutions was measured with an Advanced Osmometer, model 3D3 (Advanced Instruments, Norwood, MA, USA).

Calibration of $[Ca^{2+}]_{cyt}$

After each experiment all remaining aequorin were discharged with 1 M $CaCl_2$ and 10% ethanol, and the resultant luminescence was measured to estimate the amount of remaining aequorin. $[Ca^{2+}]_{cyt}$ was calibrated according to Knight et al. (1996) using the calibration equation:

$$pCa = 0.332588(-\log k) + 5.5593$$

where k is a rate constant equal to luminescence counts per second divided by total counts.

Measurement of free calcium ion concentration

The concentration of free calcium ion in solutions was measured with a Ca^{2+} ion-selective electrode (model IS 561-Ca^{2+}, Philips, Eindhoven, The Netherlands)

Determination of cell viability

Cell viability was determined according to Ono et al. (1995) as the percentage of cells that accumulated fluorescein and as the percentage of plasmolyzed cells. Briefly, 5-mL aliquots of the suspension of cells were withdrawn after various treatment. One hundred microliters of 0.5% solution (w/v) of fluorescein diacetate in acetone was added to 5-mL aliquots of the cell suspension and cells were incubated for 20 min at 28℃. The cells were washed twice with fresh medium and cells that accumulated fluorescein were counted using fluorescence microscopy. For monitoring

plasmolysis, 5-mL aliquots of the cell suspension were centrifuged (500×*g* for 1 min) and harvested cells were resuspended in 2 mL of 1 M sorbitol in the culture medium and incubated for 20 min at 28℃ and the plasmolyzed cells counted under a light microscope.

Protein kinase assay

The activities of protein kinases were assayed by an in-gel protein kinase assay as reported previously (Yuasa and Muto, 1996). The cells were subjected to hypoosmotic shock and killed by adding one-forth volumes of chilled 50% (w/v) trichloroacetic acid (TCA) at appropriate time and kept for 30 min on ice. After centrifugation at 15,000×*g* for 15 min at 4℃, the resulting pellets were resuspended in 80% acetone by sonication. The suspensions were centrifuged at 15,000×*g* for 15 min at 4℃. This procedure was repeated twice. The pellets were solubilized in the SDS-PAGE sample buffer and applied on 10% polyacrylamide gel (Laemmli, 1970) which was polymerized in the presence of 0.2 mg/mL of substrate proteins (MBP, histone H1 or casein) and 0.1% SDS. After SDS-PAGE, the gels were washed successively with 20 mM Tris-HCl (pH 8.0) containing 20% isopropanol, and then with 20 mM Tris-HCl (pH 8.0) containing 5 mM 2-mercaptoethanol (washing buffer). Denaturation of proteins were completed by shaking the gels in the washing buffer containing 6 M guanidine-HCl. In order to renature protein kinases, the gels were gently shaken in the washing buffer containing 0.05% Tween 20 at 4℃ for 15 to 20 h. After equilibrating with 20 mM Hepes-KOH (pH 7.6) containing 10 mM $MgCl_2$, 1 mM EGTA and 1 mM dithiothreitol (reaction buffer) at room temperature for 30 min, the gels were incubated in the reaction buffer containing 25 μM [γ-^{32}P]ATP (final 37 kBq/mL) at 25℃ for 1 h. The reaction was terminated by transferring the gels into 5% (w/v) TCA containing 1% (w/v) $Na_4P_2O_7$-$10H_2O$. The gels were successively washed

with the same solution to remove the unreacted ATP. After drying, radioactive bands on the gels were visualized by a Bioimaging Analyzer BAS 2000 (Fuji Photo Film Co., Tokyo).

Phosphatase treatment

The cells treated as in the previous section were solubilized in 100 mM Tris-HCl (pH 8.8) containing 0.5% SDS instead of the SDS-PAGE sample buffer and the solution was supplemented with 4 volumes of 1% Triton X-100. The phosphatase treatment was started by adding 0.5 U/mL calf instinal alkaline phosphatase and then terminated by adding 20 mM 2-glycerophosphate, 20 mM NaF and 0.1 mM Na_3VO_4 after 10 min. Proteins were recovered by precipitation with 80% acetone and analyzed with an in-gel protein kinase assay.

Immunoprecipitation

Samples solubilized in the sample buffer for SDS-PAGE were incubated for 3 min at 65℃ and centrifuged at 15,000×*g* for 5 min. The resultant supernatants were diluted in 20 volumes of 50 mM Tris-HCl (pH 8.0), 1 mM EDTA and 1% Triton X-100 (washing buffer) and mixed for 15 min at 4℃. After centrifugation at 15,000×*g* for 5 min at 4℃, 970 μL of the supernatants were transferred to new tubes. To these tubes 2 μL of anti-phosphotyrosine antibody (PY-20) was added and mixed for 30 min at 4℃, and then 30 μL of 20 % (v/v) protein A-Sepharose CL-4B equilibrated in the washing buffer was added. After mixing for 1.5 h, Sepharose CL-4B beads were precipitated by centrifugation at 15,000×*g* for 1 min at 4℃ and the supernatants were discarded by aspiration. The beads were washed twice by the washing buffer and then incubated with the sample buffer at 95℃ for 3 min. The solubilized proteins from the beads were subjected to an in-gel protein kinase assay.

II-3. RESULTS

Hypoosmotic shock transiently increases $[Ca^{2+}]_{cyt}$

Transgenic tobacco suspension culture cells expressing apoaequorin were incubated with 1 μM coelenterazine for 8 h to reconstitute aequorin. When the transgenic tobacco cell suspension in the 200 mosmol medium was diluted with 2 volumes of the culture medium depleted of sucrose (90 mosmol), a dramatic increase in the $[Ca^{2+}]_{cyt}$-dependent luminescence was observed (Fig. 5, trace A). An immediate small increase in $[Ca^{2+}]_{cyt}$ was followed by a rapid large increase starting 35 s after dilution. Luminescence intensity peaked at 70 s and rapidly decreased for the next 30 s and then gradually returned to the original level after 4 to 5 min. The peaked $[Ca^{2+}]_{cyt}$ was estimated to be 321.4 $\pm$ 1.2 nM (n=10) using the calibration equation described in Materials and Methods. Dilution with the ordinary medium (containing 3% sucrose, 200 mosmol, trace B) or with a medium containing 120 mM mannitol (320 mosmol, data not shown) did not cause the increase in $[Ca^{2+}]_{cyt}$. When a cell suspension preincubated in culture medium in which mannitol replaced sucrose and with a final osmolarity of 200 mosmol was diluted with 2 volumes of culture medium depleted in sucrose (90 mosmol), a similar luminescence profile to figure 5A was observed (data not shown). On the other hand, dilution of the same cell suspension with 200 mosmol mannitol solution did not induce a $[Ca^{2+}]_{cyt}$ elevation and the trace was similar to figure 5B (data not shown). Taken together these results indicate that the $[Ca^{2+}]_{cyt}$ elevation observed in figure 5A was caused by a "hypoosmotic shock" induced by reducing the extracellular osmolarity from 200 to 125 mosmol. This response was not because of the lack of sucrose as a nutrient and was specific for hypoosmotic shock, because it could not be mimicked by a hyperosmotic shock.

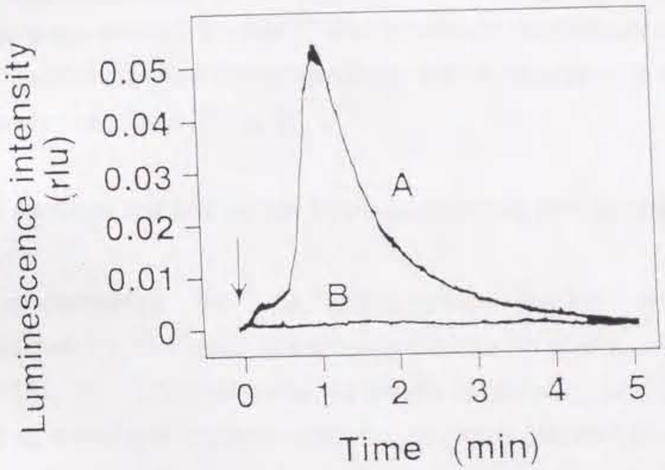

Figure 5. Effect of hypoosmotic shock on the $[Ca^{2+}]_{cyt}$ of cultured tobacco cells. Three-day old transgenic tobacco suspension culture cells were incubated with 1 μM coelenterazine to reconstitute aequorin. At the arrow the suspension was diluted (A) with 2 volumes of the medium depleted of sucrose (90 mosmol) (B) with the normal medium (200 mosmol) and luminescence recorded. These experiments were repeated ten times and the traces represented have been chosen to best represent the average result.

Increasing the magnitude of the hypoosmotic shock, produced a correspondingly increased $[Ca^{2+}]_{cyt}$ response (Fig. 6). The first phase of the response, the small rapid elevation, became more clearly pronounced with increasing hypoosmotic shock and was clearly detectable at the 96.7 and 126.9 mosmol treatments (Fig. 6, indicated by arrows). These results indicate that there are two phases in $[Ca^{2+}]_{cyt}$ elevation in response to hypoosmotic shock in tobacco suspension culture cells. These two phases, the immediate small elevation and subsequent large major $[Ca^{2+}]_{cyt}$ elevations, were termed "phase 1" and "phase 2" respectively. A second correlation with increased hypoosmolarity was a decrease in the lag time before the start of phase 2 (Fig. 6).

Effect of various inhibitors on hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation

To characterize the Ca^{2+}-transporting system activated by hypoosmotic shock, the effect of various inhibitors on $[Ca^{2+}]_{cyt}$ elevation was examined (Fig. 7). To emphasize the peaks of phases 1 and 2, cells were subjected to a stronger hypoosmotic shock (from 200 to 103 mosmol) in these experiments. The inhibitors of voltage-dependent Ca^{2+} channels, verapamil, nifedipine and diltiazem (5 μM each) did not affect $[Ca^{2+}]_{cyt}$ elevation. Bromophenacyl bromide (10 μM) and neomycin (100 μM), respective inhibitors of PI metabolism and synthesis of IP_3, and thapsigargin (10 μM), an inhibitor of Ca^{2+}-ATPase on the ER, also had no effect. Calmodulin inhibitors calmidazolium and trifluoperazine (100 μM each) had no effect. Among the various inhibitors and chemical compounds I tested, only K-252a and bafilomycin A_1 affected the hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation. The protein kinase inhibitor K-252a inhibited phase 2 (Fig. 8A) and the vacuolar-type H^+-ATPase inhibitor bafilomycin A_1 (Yoshimori et al., 1991) enhanced phase 1 (Fig. 8B). When the cells were treated with 1 μM K-252a 1 min before hypoosmotic shock, phase 2 was

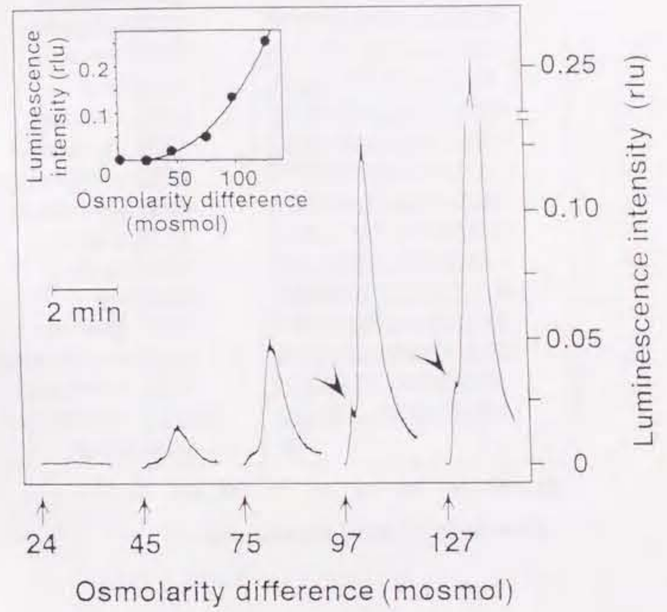

Figure 6. Effect of osmolarity difference on hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation.
The suspension of tobacco cells containing reconstituted aequorin was diluted with 2 volumes of various concentration of mannitol solution (175, 150, 90, 50 and 0 mM) as indicated by the arrows. The inset shows the peak values of the luminescence intensity. The values on the abscissa shows the osmolarity difference (24, 45, 75, 97, 127 mosmol) before and after the dilution. The arrowheads indicate the peaks of phase 1 $[Ca^{2+}]_{cyt}$ elevation. These experiments were repeated five times and the traces represented have been chosen to best represent the average result.

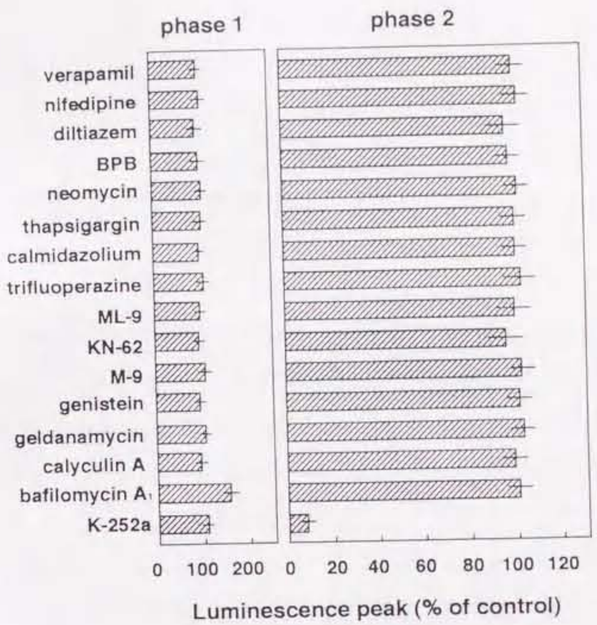

Figure 7. Effect of various inhibitors on hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation. The tobacco cells containing reconstituted aequorin were pretreated with verapamil, nifedipine, diltiazem (5 μM each), bromophenacyl bromide (BPB) (10 μM), neomycin (100 μM), thapsigargin (10 μM), calmidazolium, trifluoperazine (100 μM each), ML-9, KN-62, M-9, genistein, geldanamycin (10 μM each), calyculin A (5 μM) for 10 min and bafilomycin A_1 (500 nM) for 60 min and K-252a (1 μM) for 1 min, and then subjected to hypoosmotic shock and luminescence was recorded. The phase 1 (left) and phase 2 (right) luminescence peaks were plotted as % of control. Bars are standard errors (n=4).

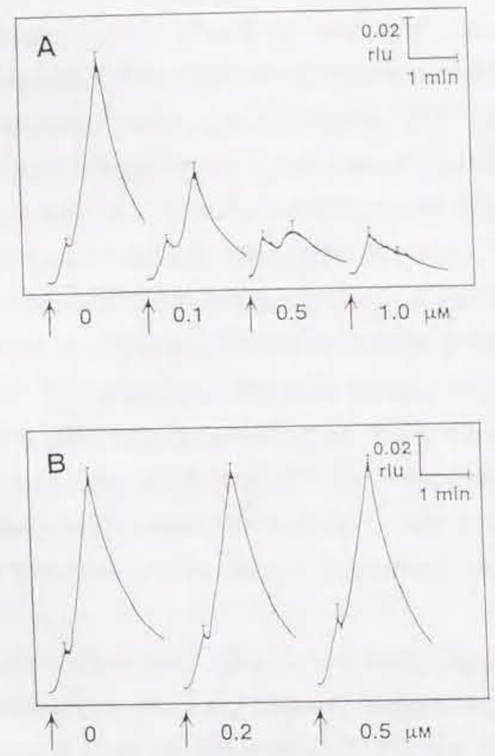

Figure 8. Effect of K-252a and bafilomycin A_1 on hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation.
The suspension of tobacco cells containing reconstituted aequorin were pretreated for 1 min with K-252a at indicated concentration (A) or for 1 h with bafilomycin A_1 at indicated concentration (B). Then, the cells were subjected to hypoosmotic shock by diluting with 2 volumes of the 50 mM mannitol solution as indicated by the arrows. The osmolarity difference between the before and after the shock is 97 mosmol. As a solvent control, 0.05% DMSO was treated to cells. These experiments were repeated four times and the traces represented have been chosen to best represent the average result. Vertical lines at the time of 20 s and 70 s represent ±SE.

completely inhibited, although no effect of K-252a on phase 1 was observed (Fig. 8A). The inhibitory effect of K-252a on phase 2 was also concentration dependent. This demonstrates that phase 2 is sensitive to K-252a and suggests that phase 2 depends upon one or more phosphorylation events. The other protein kinase inhibitors tested, ML-9, KN-62, M-9, genistein and geldanamycin (10 μM each) and a phosphoprotein phosphatase inhibitor, calyculin A (5 μM), however, did not affect the $[Ca^{2+}]_{cyt}$ elevation. In cells pretreated with 0.5 μM bafilomycin A_1 for 60 min, phase 1 was markedly stimulated but phase 2 was not affected (Fig. 8B). The effect of bafilomycin A_1 on phase 1 was also concentration dependent. These results show that the sensitivity to inhibitors of phase 1 and 2 is different. The viability of the cells treated with K-252a (1 μM) or bafilomycin A_1 (0.5 μM) was determined as the percentage of cells that accumulated fluorescein (97.2 and 97.1%, respectively) and as the percentage of plasmolyzed cells (100% each). Both examinations showed that the treatment of cells with K-252a or bafilomycin A_1 did not affect cell viability.

Either $LaCl_3$ (3 mM) or $GdCl_3$ (3 mM) markedly suppressed both phase 1 and 2 $[Ca^{2+}]_{cyt}$ elevations (data not shown), suggesting that these $[Ca^{2+}]_{cyt}$ elevations may result from an influx of Ca^{2+} into the cytosol through the plasma membrane. However these two channel blockers may have potential side effects on $[Ca^{2+}]_{cyt}$, because in long-term incubations they seemed to induce cell aggregation. Also ruthenium red (20 μM), a putative inhibitor of mitochondrial and ER Ca^{2+} channels, seemed to cause some quenching of aequorin luminescence and consequently I have not further examined its effects on hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation.

Effect of extracellular Ca^{2+} concentration on hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation

To ascertain the involvement of extracellular Ca^{2+} on the hypoosmotic $[Ca^{2+}]_{cyt}$ response I altered the extracellular Ca^{2+} concentration and examined its effect on the hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation. The ordinary culture medium that I used contained 3 mM Ca^{2+}. The transgenic cells containing reconstituted aequorin were washed with and transferred to culture medium depleted of $CaCl_2$ (contaminating free Ca^{2+} concentration, 0.025 mM) and incubated for 30 min. To this suspension 3 mM $CaCl_2$, or 5 mM EGTA or 10 mM mannitol were added. After 1 min the cells were hypoosmotically shocked and the $[Ca^{2+}]_{cyt}$ elevation was monitored (Fig. 9). The biphasic $[Ca^{2+}]_{cyt}$ elevation was observed in the presence of 3.0 mM Ca^{2+} (trace A) and this was greatly reduced at 0.025 mM Ca^{2+} (trace B). No $[Ca^{2+}]_{cyt}$ elevation was detected in cells treated with 5 mM EGTA (trace C). These results suggest that the $[Ca^{2+}]_{cyt}$ elevation mediated by hypoosmotic shock may result from an influx of Ca^{2+} into the cytosol through the plasma membrane.

To examine further the effect of external Ca^{2+} on the $[Ca^{2+}]_{cyt}$ elevation, the cell suspension was subjected to hypoosmotic shock in a Ca^{2+}-depleted medium (free Ca^{2+}, 0.025 mM), and then 3 mM $CaCl_2$ was added back to the cell suspension after the onset of the osmotic shock. An immediate and sharp luminescence emission was observed by the addition of Ca^{2+} to the hypoosmotically shocked cell suspension (Fig. 10, trace A). The luminescence emission was observed 1 min after the shock, was highest at 2 to 3 min, and observed as late as 20 min. After the addition of Ca^{2+}, $[Ca^{2+}]_{cyt}$ increased without a lag period and peaked after only a few seconds before rapidly returning to basal levels after 30 to 40 s. When cells depleted of Ca^{2+} were then treated with 3 mM Ca^{2+} in the absence of any previous hypoosmotic shock only slight changes in luminescence were observed (Fig. 10, trace B). These results would suggest that the Ca^{2+} channels responsible for hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation is activated dependent on time and can maintain their competence to allow

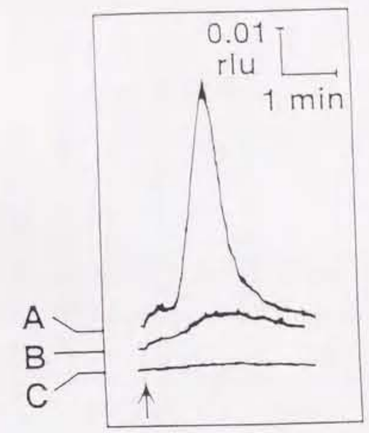

Figure 9. Effect of extracellular Ca^{2+} concentration on hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation.
The tobacco cells containing reconstituted aequorin were suspended in a medium depleted of $CaCl_2$. Either 3 mM $CaCl_2$ (A), 10 mM mannitol (B) or 5 mM EGTA (C) was then added to this cell suspension. After 1 further min (arrow) the cell suspension was diluted with 2 volumes of each of the media depleted of sucrose to provide hypoosmotic shock. Free Ca^{2+} concentration in each medium was 3.02 mM (A), 0.025 mM (B) and <0.001 mM (C). These experiments were repeated five times and the traces represented have been chosen to best represent the average result.

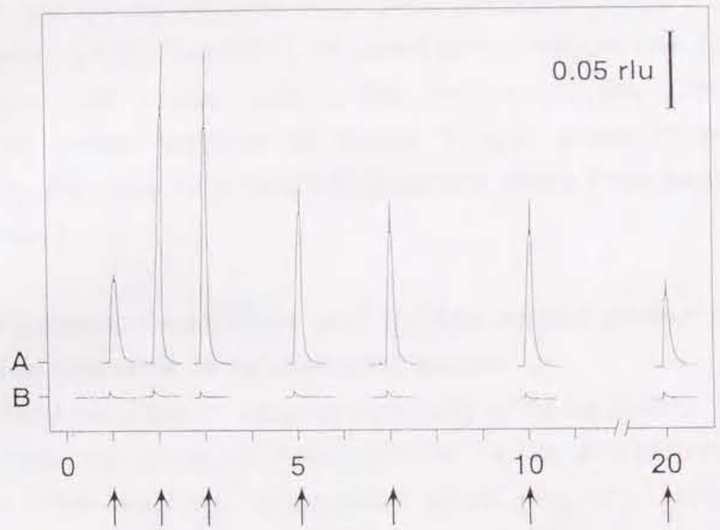

Figure 10. Effect of delay in adding externally-added Ca^{2+} on $[Ca^{2+}]_{cyt}$ elevation in cells subjected to hypoosmotic shock.
Tobacco cells containing reconstituted aequorin were transferred to the Ca^{2+}-depleted medium for 60 min. (A), The cell suspension was subjected to hypoosmotic shock by adding 2 volumes of the Ca^{2+} and sucrose-depleted medium, (B), isoosmotic treatment with the Ca^{2+}-depleted medium. Then, 3 mM $CaCl_2$ was added to the cell suspension at the indicated time after the shock. These experiments were repeated five times and the traces represented have been chosen to best represent the average result.

Ca^{2+} influx for at least 20 min, if Ca^{2+} is initially depleted from the medium. When this experiment was repeated with cells pre-treated with K-252a, the $[Ca^{2+}]_{cyt}$ elevation observed was markedly suppressed (Fig. 11C). This K-252a-suppression indicated the presence of characteristics of the phase 2 $[Ca^{2+}]_{cyt}$ elevation. In the cells pretreated with bafilomycin A_1 both in the presence and the absence of K-252a, the $[Ca^{2+}]_{cyt}$ elevation was enhanced (Fig. 11, E and G), therefore showing characteristics of phase 1 as well. These results imply that the $[Ca^{2+}]_{cyt}$ elevation shown in figure 10A, resulting from external Ca^{2+} added back to the medium of cells undergoing hypoosmotic shock, consists of phase 1 and phase 2 merged. Furthermore this observation would suggest that phase 2 can be induced without phase 1.

Effect of osmotic restoration and K-252a added during $[Ca^{2+}]_{cyt}$ elevation in response to hypoosmotic shock

To look at the effect of restoring osmolarity of the medium on $[Ca^{2+}]_{cyt}$ the extracellular osmolarity was returned to the original level (200 mosmol) at various times after the hypoosmotic shock (Fig. 12). When the hypoosmotic condition was canceled 30 s after the shock, i.e. at the time when the phase 1 elevation had already peaked, the peak height of the phase 2 was lowered and $[Ca^{2+}]_{cyt}$ rapidly returned to the original resting level (Fig. 12B). When 60 s were allowed to elapse before cancellation of the shock, no change in the peak height of phase 2 was seen; however the decrease in $[Ca^{2+}]_{cyt}$ was more rapid (Fig. 12C) than that of controls (Fig. 12A). These results indicate that osmotic restoration in some way either suppresses the activation of the Ca^{2+} channel involved in phase 2 or stimulates Ca^{2+} pumping activity. If K-252a was added after 30 s, instead of osmotic restoration being carried out, the peak luminescence was lowered but the rate of decline of the luminescence intensity was similar to the controls (Fig. 12D). The addition of K-252a, 60 s after the shock, had

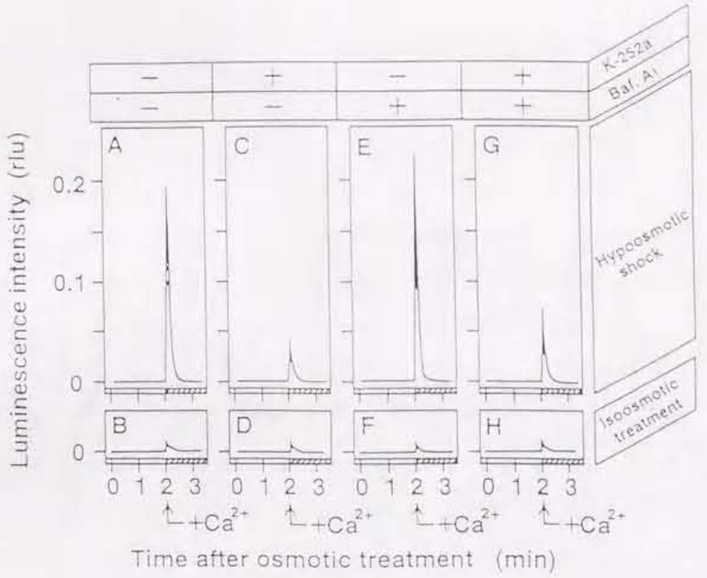

Figure 11. Effect of K-252a and bafilomycin A_1 on $[Ca^{2+}]_{cyt}$ elevation induced by adding external Ca^{2+} in cells which had been subjected to hypoosmotic shock in the absence of external Ca^{2+}.
Tobacco cells containing reconstituted aequorin were transferred to the Ca^{2+}-depleted medium for 60 min. The cell suspension was treated with 1 µM K-252a (panel C, D, G and H) or DMSO as a solvent control (panel A, B, E and F) for 1 min, or with 0.5 µM bafilomycin A_1 (Baf. A_1, panel E, F, G and H) or DMSO (panel A, B, C and D) for 60 min. Then, the cell suspension was subjected to hypoosmotic shock by adding 2 volumes of the Ca^{2+} and sucrose-depleted medium (panel A, C, E and G), or isoosmotic treatment with the Ca^{2+}-depleted medium (panel B, D, F and H). These experiments were repeated five times and the traces represented have been chosen to best represent the average result.

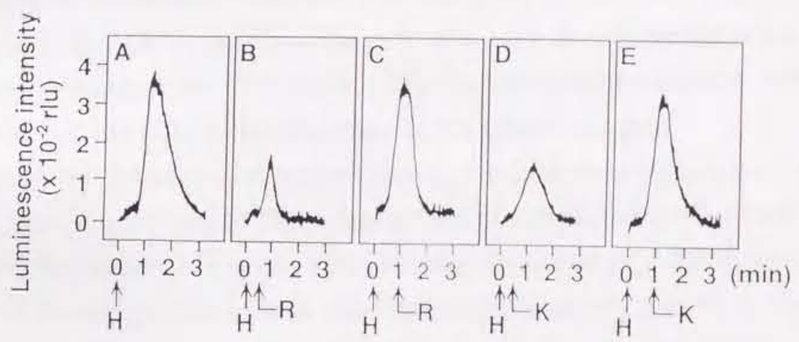

Figure 12. Effect of osmotic restoration and K-252a added during $[Ca^{2+}]_{cyt}$ elevation induced by hypoosmotic shock.
The tobacco cells containing reconstituted aequorin were subjected to hypoosmotic shock by adding 2 volumes of the sucrose-depleted medium (A) and then the hypoosmotic condition was canceled by adding 0.275 volumes of a medium containing 400 mM mannitol 30 s (B) or 60 s (C) after the shock, or 1 μM K-252a was added 30 s (D) or 60 s (E) after the shock. H, R and K indicated the time of hypoosmotic shock, osmotic restoration and addition of K-252a, respectively. These experiments were repeated five times and the traces represented have been chosen to best represent the average result.

no effect on the elevation and also did not change the rate of decline (Fig. 12E). These results indicate that K-252a added after the shock can suppress the Ca^{2+} channel activity responsible for phase 2 but has little effect on Ca^{2+} pumping activity.

Activation of protein kinases in response to hypoosmotic shock

Protein kinase activity was detected with in-gel protein kinase assays using MBP, histone H1 and casein as protein substrates. In unstimulated cells, several bands with molecular masses of 38, 50, 65 and 75 kDa were observed (Fig. 13B, D and F). These bands were also detected in the gel containing no substrate (Fig. 13H). The bands detected without protein substrate indicate autophosphorylation of the protein kinases.

When the tobacco cell suspension in the 200 mosmol medium was diluted with 2 volumes of the culture medium depleted of sucrose (90 mosmol), activation of protein kinases was observed in a MBP-gel (Fig. 13A). A 50-kDa protein kinase was activated transiently 2 to 10 min after the shock with a maximum at 5 min. A 75-kDa protein kinase was activated 2 to 15 min after the shock and remained to be activated till 30 min. Activation of a 80-kDa protein kinase was observed 2 to 15 min after the shock. These changes in activities of protein kinases were not observed in the cells treated with the ordinary medium (200 mosmol; Fig. 13B) and also in the cells treated with 200 mM mannitol solution (200 mosmol; data not shown). Therefore the activation of these protein kinases observed was caused by hypoosmotic shock induced by reducing the extracellular osmolarity but not by the lack of sucrose as a nutrient. The similar changes in activities of protein kinases were detected in a histone-gel but the activities were lower than in MBP-gel (Fig. 13C). Activation of these protein kinases were scarcely detected in casein- and no-substrate-gel, except that a 80-kDa faint band appered 2 to 15 min after hypoosmotic shock in both gels (Fig. 13E and G). The activation of this kinase indicates

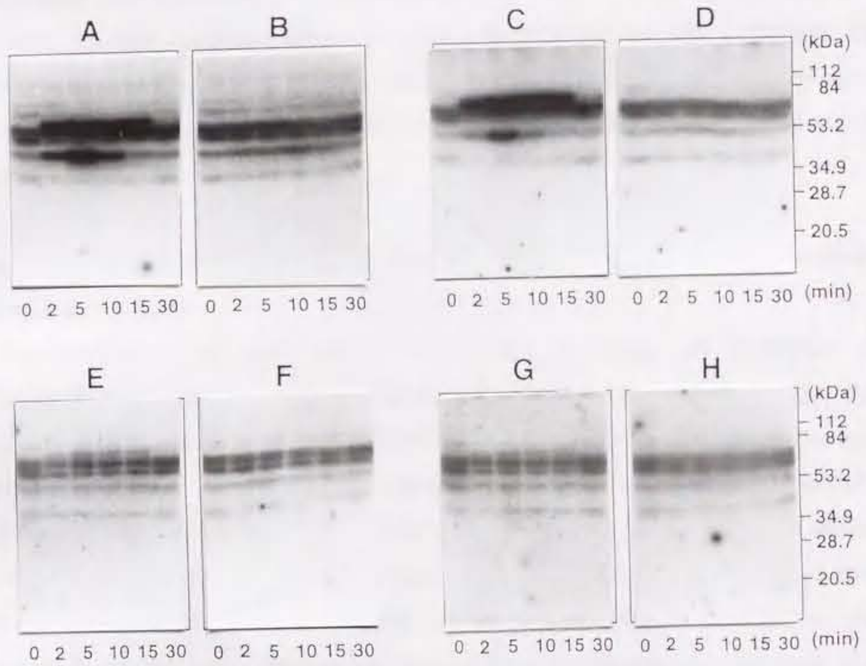

Figure 13. Effect of hypoosmotic shock on the protein kinase activities in tobacco suspension culture cells.
Tobacco suspension culture cells were killed by 10% (w/v) trichloroacetic acid at indicated times after addition of hypoosmotic medium (A, C, E and G) or isoosmotic medium (B, D, F and H). The cell extracts were subjected to SDS-PAGE gels containing MBP (A and B), histone H1 (C and D), casein (E and F) or nothing (G and H) as substrate proteins 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). Molecular masses of standard proteins are indicated at the right of gel.

that its autophosphorylation activity was induced in response to hypoosmotic shock. The pronounced activation of 80-kDa protein kinase in MBP- and histone-gel suggests that the activity for external substrates of this kinase was increased by autophosphorylation. The activities of the 50- and 75-kDa protein kinases are seemed not to be activated by autophosphorylation. Since the protein kinases detected on the gels prefer MBP and histone to casein as substrates, MBP-gels were used thereafter.

The 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 the 50-, 75- and 80-kDa protein kinases (Fig. 14A). When preincubated for 5 min with calyculin A, a potent inhibitor of protein phosphatase 1 and 2A, the 50-kDa protein kinase which was activated 5 min after the shock sustained in its activated state till 15 min (Fig. 14B). The activities of the 75- and 80-kDa protein kinases in the calyculin A treated cells were much higher than the respective controls at 0 time, increased after the shock and kept their activated state till 30 min (Fig. 14B). These results indicate that the protein kinases are up-regulated by protein phosphorylation in response to hypoosmotic shock and suggests 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 (Fig. 15), indicating that the hypoosmotic shock-activated protein kinases were activated by phosphorylation.

Tyrosine phosphorylation of a 50-kDa protein kinase

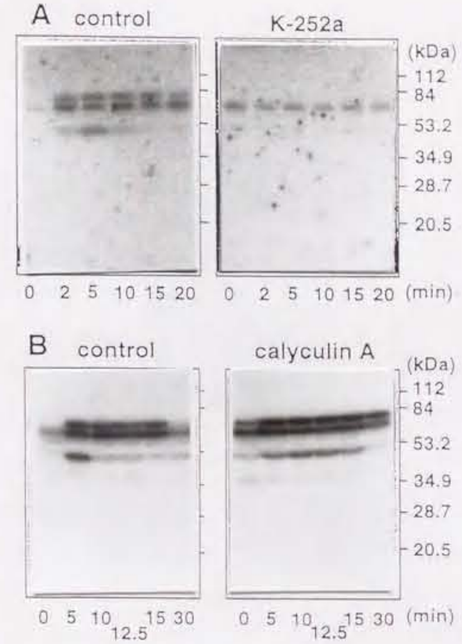

Figure 14. Effects of K-252a and calyculin A on the activation of protein kinases in the tobacco cells in response to hypoosmotic shock.
Tobacco suspension culture cells were preincubated for 5 min with 1 μM K-252a (A) or 0.5 μM calyculin A (B), and subjected to hypoosmotic shock. Controls were treated with 0.01% DMSO. The cells were killed at indicated time after the shock and analyzed by in-gel protein kinase assays using MBP-gels.

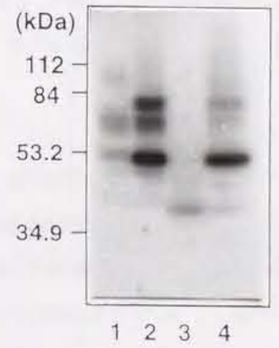

Figure 15. Deactivation of protein kinases by treatment with alkaline phosphatase. The tobacco cells were subjected to hypoosmotic shock and the cells were killed after 3 min. The extracts of the cells subjected to hypoosmotic shock were treated with alkaline phosphatase (0.5 U/mL, lane 3) or boiled alkaline phosphatase (lane 4) for 10 min as described in Materials and Methods. The protein kinases were assayed in an MBP-gel. Lane 1 and 2 represent the protein kinase activity in the extracts of the cells subjected to isoosmotic treatment and hypoosmotic shock, respectively.

Immunoprecipitates with an anti-phosphotyrosine monoclonal antibody were analyzed with an in-gel protein kinase assay (Fig. 16A). 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 figure 16B, 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.

An increase in $[Ca^{2+}]_{cyt}$ precedes activation of protein kinases

I described that hypoosmotic shock induced a marked transient elevation of $[Ca^{2+}]_{cyt}$ in the tobacco suspension culture cells (Fig. 5). 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 while monitoring $[Ca^{2+}]_{cyt}$. As shown in figure 5, $[Ca^{2+}]_{cyt}$ increased transiently after hypoosmotic shock only in the presence of extracellular Ca^{2+} (Fig. 17A). The activation of the 50-, 75-, and 80-kDa protein kinases occurred after the $[Ca^{2+}]_{cyt}$ elevation and the activated states were kept after decrease in $[Ca^{2+}]_{cyt}$ to the original level (Fig. 17B). The hypoosmotic shock-induced activation of the 50-, 75- and 80-kDa kinases was not observed in the cells shocked in the absence of Ca^{2+} (Fig. 17C). These results show that hypoosmotic shock alone does not activate these protein kinases, but $[Ca^{2+}]_{cyt}$ elevation is prerequisite for the activation of these protein kinases.

Figure 18A shows that the addition of Ca^{2+} to the cell suspension which had been hypoosmotically shocked in the absence of extracellular Ca^{2+},

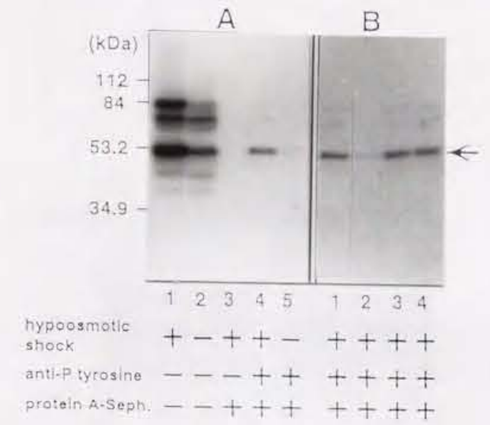

Figure 16. Activities of protein kinases in immunoprecipitates from cell extracts treated with anti-phosphotyrosine antibody.
The tobacco cells were subjected to hypoosmotic treatment and killed after 3 min. The cell extracts were solubilized in Laemmli's sample buffer, and immunoprecipitated with an anti-phosphotyrosine 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. In panel B, the antibody was treated in the absence (lane 1), or presence of 10 μM phosphotyrosine (lane 2), phosphothreonine (lane 3) or phosphoserine (lane 4). MBP-gels were used. The arrow indicates the 50-kDa protein kinases.

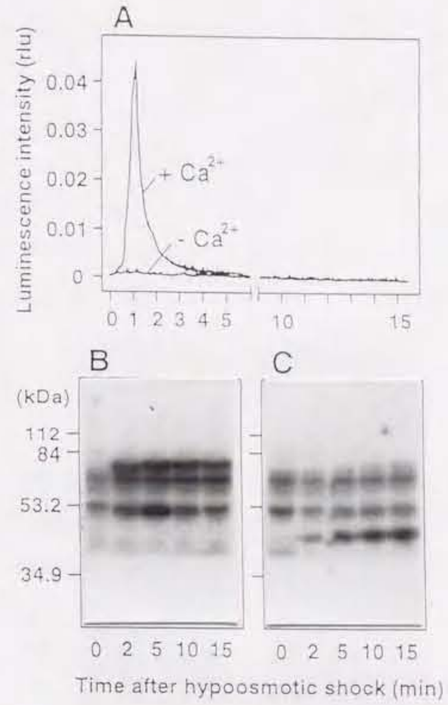

Figure 17. Effect of extracellular Ca^{2+} on $[Ca^{2+}]_{cyt}$ elevation and on the activation of protein kinases in response to hypoosmotic shock.
The tobacco suspension culture cells expressing apoaequorin were incubated with 1 μM coelenterazine to constitute aequorin. The cells were washed with and 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) were assayed in MBP-gels.

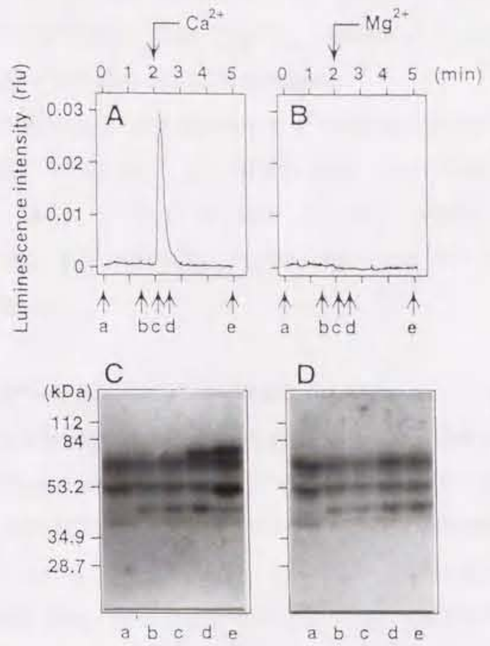

Figure 18. Relationship between $[Ca^{2+}]_{cyt}$ elevation and activation of protein kinases. The tobacco cells expressing apoaequorin were incubated with 1 μM coelenterazine and were washed with and incubated in the fresh medium containing 5 mM BAPTA for 30 min. The cells were subjected to hypoosmotic shock at 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 in MBP-gels 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 received $CaCl_2$. D, activities in the cells received $MgCl_2$.

induced a rapid and transient $[Ca^{2+}]_{cyt}$ elevation as observed in figure 10. The cells were killed at the indicated times (a-e) and analyzed their protein kinase activities with the in-gel protein kinase assay (Fig. 18C). Activation of the 50-, 75- and 80-kDa protein kinases was observed only after $[Ca^{2+}]_{cyt}$ elevation. The addition of Mg^{2+} caused neither $[Ca^{2+}]_{cyt}$ elevation (Fig. 18B) nor the activation of protein kinases (Fig. 18D). These results coincide with the above observation that $[Ca^{2+}]_{cyt}$ elevation is prerequisite for the activation of the 50-, 75- and 80-kDa kinases.

Hypoosmotic shock in the absence of Ca^{2+} activated a 45-kDa protein kinase (Figs. 17C, 18C and D) which was deactivated when $[Ca^{2+}]_{cyt}$ increased (Fig. 18C). This protein kinase might be activated by hypoosmotic shock but strongly down-regulated by a Ca^{2+}-dependent protein phosphatase.

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. 19, 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 (Roberts and Harmon, 1992).

II-4. DISCUSSION

In the present study genetically transformed *N. tabacum* suspension cells in culture were used to investigate the effect of hypoosmotic shock on $[Ca^{2+}]_{cyt}$. Hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation consists of two phases (Fig. 5A), an immediate small $[Ca^{2+}]_{cyt}$ elevation (phase 1) followed by a rapid and more prolonged elevation (phase 2). Hypoosmotic shock

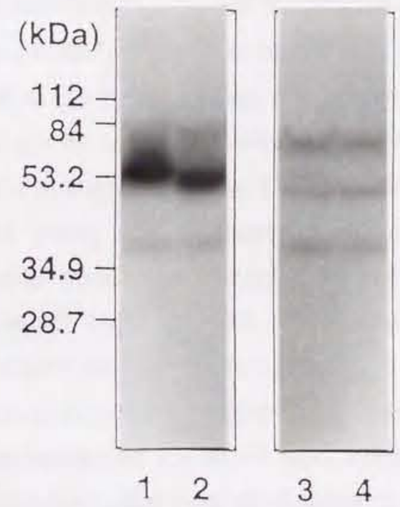

Figure 19. Detection of Ca^{2+}-dependent protein kinase in an MBP-gel.
The tobacco cell extracts were solubilized in Laemmli's sample buffer and to the aliquot was added 2 mM EGTA (lanes 1 and 3) or 2 mM $CaCl_2$ (lanes 2 and 4). The assay mixture contained 0.5 mM $CaCl_2$ (lanes 1 and 2) or 0.5 mM EGTA (lanes 3 and 4).

causes water influx due to the osmotic potential of plant cells, and as a result increases turgor pressure thus causing extension of the plasma membrane. In a brackish water charaphyte *Lamprothamnium succinctum*, increases in turgor pressure were shown to be caused by hypoosmotic shock (Okazaki et al., 1984). Microinjection of aequorin into the cytosol of *L. succinctum* cells was used to demonstrate that hypoosmotic shock induced a transient $[Ca^{2+}]_{cyt}$ elevation (Okazaki et al., 1987) and this elevation was shown to be a prerequisite for turgor regulation of the cells (Okazaki and Tazawa, 1990). However, the change in turgor pressure caused by hypoosmotic shock was not measured in tobacco suspension cells because it is technically difficult to do. Hypoosmotic shock may then result in an increase in the turgor pressure of tobacco suspension culture cells and thus could act as a mechanical stress. Plant cells are known to be very sensitive to mechanical stress (Trewavas and Knight, 1994). It has been reported using the transgenic tobacco plants expressing aequorin that instantaneous touch- and wind-induced transient $[Ca^{2+}]_{cyt}$ elevations (Knight et al., 1991b, 1992). Touch and wind stimuli cause tension and compression in plant cells because of bending of the tissue. However the kinetics of hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation are different from those described for touch and wind (Knight et al., 1991b, 1992) where the increases in luminescence were observed without obvious lag periods.

The phase 2 $[Ca^{2+}]_{cyt}$ elevation occurred some 30 s after imposition of stress and required the continued presence of hypoosmotic stress. This biphasic $[Ca^{2+}]_{cyt}$ elevation observed in the tobacco cells shows features similar to Ca^{2+}-induced Ca^{2+} release in animal cells in which intracellular Ca^{2+} pools are mobilized. However the results obtained in the present study suggest that elevated $[Ca^{2+}]_{cyt}$ originated instead from extracellular sources. The magnitudes of both the phase 1 and 2 elevations were definitely dependent on the extracellular Ca^{2+} concentration. This again is

in contrast to the situation with touch- and wind-induced $[Ca^{2+}]_{cyt}$ elevation in whole tobacco seedlings, epidermal strips and protoplasts (Knight et al., 1992; Haley et al., 1995) and *Bryonia dioica* (Klüsener et al., 1995) where the Ca^{2+} source is thought to be predominantly intracellular. This contrast provides an argument for a separate mechanism for hypoosmotic sensing/signaling. However in mosses extracellular Ca^{2+} is thought to be involved in the touch-induced $[Ca^{2+}]_{cyt}$ response (Haley et al., 1995) so the situation is not yet clear. With regard to the Ca^{2+}-induced Ca^{2+} release it is unlikely that phase 1 stimulates the appearance of phase 2, because the two phases occurred independently and phase 1 was not a prerequisite for phase 2 (Fig. 11).

K-252a inhibited $[Ca^{2+}]_{cyt}$ elevation in phase 2 but not in phase 1 (Fig. 8A), suggesting that a protein phosphorylation step is involved in the control of phase 2. The implication of this result is that hypoosmotically induced $[Ca^{2+}]_{cyt}$ elevation involves the activation of either a Ca^{2+} channel(s) which requires phosphorylation for activity or a Ca^{2+} pump(s) which is inactivated by phosphorylation or associated hypoosmotic sensing component(s).

Bafilomycin A_1, a vacuolar-type H^+-ATPase inhibitor is thought to negate the H^+ gradient between the vacuole and the cytosol. Consequently it suppresses Ca^{2+} sequestration into the vacuole which is facilitated by a Ca^{2+}/H^+ antiporter(s) on the vacuolar membrane. Thus the presumed effect of bafilomycin A_1 is a reduction of storage Ca^{2+} in the vacuole. If hypoosmotic shock induces intracellular Ca^{2+} release, the cells treated with bafilomycin A_1 might then show a reduced response in $[Ca^{2+}]_{cyt}$ elevation. However, the inhibitor stimulated phase 1 $[Ca^{2+}]_{cyt}$ elevation (Fig. 8B). This indicates that the effect does not result from the presumed mechanism, and therefore the stimulatory effect of bafilomycin A_1 on phase 1 $[Ca^{2+}]_{cyt}$ remains unknown.

When Ca^{2+} was added to the cell suspension subjected to hypoosmotic shock in the low-Ca^{2+} medium, the K-252a-sensitive (phase 2) and -

insensitive (phase 1) $[Ca^{2+}]_{cyt}$ elevation were observed immediately without a lag period (Fig. 11). This suggests that the Ca^{2+} channel(s)/pump(s) or associated hypoosmotic sensing component(s) involved in phase 2 are controlled through protein phosphorylation without Ca^{2+} elevation and that the phase 1 $[Ca^{2+}]_{cyt}$ elevation is not required for the activation of phase 2.

None of the inhibitors of voltage-dependent Ca^{2+} channels had any effect on the hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation, suggesting that these types of Ca^{2+} channels are not a component of this system. Mechanical stimuli probably induce physical changes in the plasma membrane and it has been suggested that these signals are perceived by mechanosensory Ca^{2+} sensitive channels (Pickard and Ding, 1993). Although the sources of $[Ca^{2+}]_{cyt}$ elevated by wind and touch are likely to be intracellular (Knight et al., 1992; Haley et al., 1995; Klüsener et al., 1995), the data presented here strongly suggest that hypoosmotic shock induced Ca^{2+} influx through the plasma membrane. Mechanosensory Ca^{2+} permeable channels on the plasma membrane have been reported in tobacco protoplasts (Falke et al., 1988), guard cells of *Vicia faba* (Cosgrove and Hedrich, 1991) and epidermal cells of sweet red onion (Ding and Pickard, 1993). The Ca^{2+} channels involved in phases 1 and 2 may then be of this type, with the possibility of Ca^{2+}-independent phosphorylation-sensitive channel(s) in phase 2. It is also possible that the associated hypoosmotic sensing system is regulated by protein phosphorylation.

In-gel assays of protein kinase indicated that hypoosmotic shock induced activation of the 50-, 75- and 80-kDa protein kinases in tobacco suspension culture cells (Fig. 13). Pharmacological analyses with K-252a and calyculin A suggest that the activation of these protein kinases was accompanied by phosphorylation. The 50-kDa protein kinase was tyrosine phosphorylated but its deactivation was inhibited by calyculin A, an inhibitor of protein phosphatase 1 and 2A (Ishihara et al., 1989). 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 (Brewster et al., 1993; Galcheva-Gargova et al., 1994). MPK1 (a MAPK) was activated by hypoosmotic shock in budding yeast (Kamada et al., 1995). These reports and our present results suggest that protein kinases accompanying with 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 (Suzuki and Shinshi, 1995; Usami et al., 1995) and wounding to activate a MAPK (Seo et al., 1995). The fungal elicitor-induced activation of protein kinase(s) was suggested to require $[Ca^{2+}]_{cyt}$ elevation by the pharmacological study (Suzuki and Shinshi, 1995). Knight et al. (1991b) 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 the activation of the 50-, 75- and 80-kDa protein kinases in response to hypoosmotic shock and both the activation of protein kinases and the $[Ca^{2+}]_{cyt}$ elevation did not occur in the absence of extracellular Ca^{2+} (Fig. 17). This indicates that the $[Ca^{2+}]_{cyt}$ elevation is essential for the activation of protein kinases. The activation of protein kinases was inhibited by K-252a (Fig. 14) which inhibited phase 2 $[Ca^{2+}]_{cyt}$ elevation but not phase 1 elevation (Fig. 8). It seems likely to indicate that protein kinases are activated by phase 2 $[Ca^{2+}]_{cyt}$ elevation but not by phase1 elevation. If the activation of protein kinases is dependent on phase 1 elevation, K-252a must inhibit the activation step itself. Other inhibition steps of K-252a might be phase 2

$[Ca^{2+}]_{cyt}$ elevation or both phase 2 elevation and the activation of protein kinases after phase 2 elevation. Because the protein kinases did not require Ca^{2+} for their activities (Fig. 13), the elevated Ca^{2+} may activate their regurator(s). The CDPK which was activated by direct binding of Ca^{2+} without other components, was detected in tobacco cells by in-gel assay (Fig.19). This is supposed to be the first candidate for the activator protein kinase of the hypoosmotic shock responsive protein kinases, since the hypoosmotically increased cytosolic Ca^{2+} may directly or indirectly activate putative upstream protein kinases. Calmodulin-dependent protein kinase is also a candidate for the activator protein kinase though its activity was not detected by the in-gel assay.

In this study, I showed that 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 the 50-, 75- and 80-kDa protein kinases are components of a same protein kinase cascade or they consist independent cascades.

Although $[Ca^{2+}]_{cyt}$ increased by hypoosmotic shock is essential for driving the protein kinase cascades, it may play an alternative role in hypoosmotic regulation. In *Characeae*, elevated $[Ca^{2+}]_{cyt}$ has been reported to result in decreases in cell volume (Pierce and Politis, 1990) and in turgor pressure (Okazaki et al., 1987; Okazaki and Tazawa, 1990) by activating outward-rectifying ion channels. This is also the case for guard cell closure (Gilroy et al., 1991). The implication is that the hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation which I have observed in tobacco subsequently induces hypoosmotic regulation leading to a reduction in cell volume and turgor pressure. However, in wheat leaf protoplasts $[Ca^{2+}]_{cyt}$ elevation can cause increases in cell volume presumably resulting from increased turgor (Shacklock et al., 1992). Specific cell types therefore may interpret the same basic $[Ca^{2+}]_{cyt}$ signal in entirely different ways. The implication is that differentiation and development provide specific aspects

to the interpretation of signals; that $[Ca^{2+}]_{cyt}$ alone cannot specify these responses and some form of signal discrimination must occur. The hypoosmotic shock-triggered protein kinase cascades may help explain some aspects of the specificity of the hypoosmotic shock-signaling pathway.

Chapter III

Mastoparan induces increase in cytosolic free calcium ion concentration and subsequent activation of protein kinases in tobacco suspension culture cells

SUMMARY

Mastoparan, a wasp venom peptide induced a transient elevation of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in tobacco suspension culture cells which were transformed to express aequorin, a Ca^{2+}-sensitive photoprotein, whereas Mas-17, an inactive analogue of mastoparan induced no $[Ca^{2+}]_{cyt}$ elevation. The mastoparan-induced $[Ca^{2+}]_{cyt}$ elevation was inhibited by 8-(*N,N*-diethylamino)-octyl 3,4,5-trimethoxybenzoate-HCl and neomycin but not by depletion of extracellular Ca^{2+}, suggesting that the elevation was resulted from inositol 1,4,5-triphosphate-induced Ca^{2+} release from the intracellular stores caused by stimulation of phosphoinositide turnover coupled with the activation of trimeric GTP-binding protein. Activation of a 50-, a 75- and a 80-kDa protein kinases after the mastoparan-induced $[Ca^{2+}]_{cyt}$ elevation was shown by an in-gel protein kinase assay. This activation was inhibited by neomycin, suggesting that the $[Ca^{2+}]_{cyt}$ elevation is necessary for the mastoparan-induced activation of protein kinase. Hydrogen peroxide also induced a transient $[Ca^{2+}]_{cyt}$ elevation in the tobacco cells. However, mastoparan-induced oxidative burst which has been reported in soybean cells (Legendre et al., 1992) was not observed in the tobacco cells when treated with mastoparan.

III-1. INTRODUCTION

Mastoparan, a tetradecapeptide toxin which was isolated from wasp venom has been known to activate heterotrimeric G-proteins by catalyzing GTP/GDP exchange and the mechanism of action of G-protein-coupled receptors in animal cells, and has been used to manipulate cellular signaling pathways involving in G-proteins (Ross and Higashigima, 1994). G-proteins serve to transduce and amplify the signals which are initially

perceived by integral plasma membrane receptor proteins. The existence of G-proteins in plant cells is well documented and mastoparan has been shown to induce physiological responses and modify functions of various enzymes in plants; The peptide induces oxidative burst in soybean suspension culture cells (Legendre et al., 1992) and deflagellation in *Chlamydomonas reinhardtii* (Quarmby and Hartzell, 1994), promotes a transient increase in IP_3 content in *C. reinhardtii* and soybean culture cells (Yueh and Crain, 1993; Legendre et al., 1993), activates PLC in carrot cells (Cho et al., 1995), phospholipase A in zucchini hypocotyl (Scherer, 1992) and soybean culture cells (Chandra et al., 1996), and phospholipase D in *C. eugametos* and carnation petals (Munnik et al., 1995).

Mastoparan causes an increase in intracellular Ca^{2+} in many types of animal cells (Perianin and Snyderman, 1989; Komatsu et al., 1993). In plants, it has been reported that mastoparan induces accumulation of extracellular ^{45}Ca in *C. reinhardtii* and mastoparan microinjected also induces transient increase in $[Ca^{2+}]_{cyt}$ in staminal hair of *Setcreasea purpurea* (Tucker and Boss, 1996). Calcium ion functions as a primary second messenger in signal transduction of plant cells (Muto, 1992; Bush, 1995) and has been shown to modify a number of physiological processes (Hepler and Wayne, 1985; Nagai, 1993; Trewavas and Knight, 1994). Transient increase in $[Ca^{2+}]_{cyt}$ has been reported to be caused by external stimuli such as cold shock, fungal elicitor, wounding and H_2O_2 (Knight et al., 1991b, 1993; Price et al., 1994). In particular, I have shown in Chapter II that $[Ca^{2+}]_{cyt}$ elevation induced by hypoosmotic shock triggers activation of protein kinase cascades in tobacco suspension culture cells.

To elucidate the mastoparan-triggered signal transduction via Ca^{2+} signaling, I analyzed the change in $[Ca^{2+}]_{cyt}$ and protein kinase activities using the transgenic tobacco suspension culture cells expressing aequorin. In this study, I demonstrated that mastoparan induces $[Ca^{2+}]_{cyt}$ elevation resulted from Ca^{2+} release from intracellular stores which might be caused

by stimulation of PI turnover, and subsequently activates protein kinases in the transgenic tobacco suspension culture cells.

III-2. MATERIALS AND METHODS

Materials

Mastoparan, MBP, bafilomycin A_1, K-252a, catalase and superoxide dismutase were purchased from Sigma (St. Louis, MO, USA). Mas-17 was purchased from Peninsula Laboratories (Belmont, CA, USA). 2-Methyl-6-phenyl-3,7-dihydroimidazo [1,2-a] pyrazin-3-on, a Cripridina luciferin-derived chemiluminescent reagent (CLA) and tiron were purchased from Tokyo Kasei Kogyo (Tokyo). *N,N'*-dimethylthiourea was from Nacalai tesque (Kyoto). Neomycin sulfate and 8-(*N,N*-diethylamino)-octyl 3,4,5-trimethoxybenzoate-HCl (TMB-8) were from Wako Pure Chemical Industries (Osaka) and Aldrich Chem. (Milwaukee, WI, USA), respectively. [γ-^{32}P]ATP (148 Tbq/mmol) was obtained from ICN (Cost Mesa, CA, USA). Prestained SDS-PAGE standards were products of Pharmacia (Uppsala, Sweden). Cellulase Onozuka RS and Pectolyase Y-23 were from Yakult Co. (Tokyo) and Seishin Pharmaceutical Co. (Tokyo), respectively. Coelenterazine was a geneous gift from Prof. Isobe.

Cell culture

The transgenic tobacco (*Nicotiana tabacum* L. cv bright yellow 2) suspension culture cells expressing apoaequorin prepared as described in Chapter I were used.

Preparation of protoplasts

The tobacco cell protoplasts were prepared as described previously (Ping et al., 1992a). Briefly, 3-day old transgenic tobacco cells, expressing apoaequorin were treated with 1% (w/v) Cellulase Onozuka RS and 0.1%

(w/v) Pectolyase Y-23 in 500 mM mannitol and 10 mM Mes-KOH (pH 5.5) for 1 h at 28℃. The protoplast suspension was passed through nylon mesh (opening, 80 μm) and the filtrate containing protoplasts was centrifuged at 1,000 $\times g$ for 5 min. The precipitated protoplasts were suspended in medium A (530 mosmol) containing 100 mM KCl, 1 mM $CaCl_2$, 300 mM mannitol and 10 mM Mes-KOH (pH 5.5) and centrifuged as above. This procedure was repeated twice.

Measurement of $[Ca^{2+}]_{cyt}$

Aequorin was reconstituted in 3-day old transgenic tobacco cells as described in Chapter I. The cells were washed with and resuspended in fresh medium and used after a 30-min resting incubation. For reconstitution of aequorin in protoplasts incubation the time with coelenterazine was 3 h and medium A was used for suspension. Luminescence emission from the transgenic cells was measured as described in Chapter I and expressed as rlu. $[Ca^{2+}]_{cyt}$ was calibrated according to Knight et al. (1996) as described in Chapter II.

In-gel protein kinase assay

This was done as described in Chapter II using MBP-gels.

Measurement of active oxygen

Cells were used without reconstitution of aequorin. CLA (10 μM) was added to the cell suspension and O_2^--dependent chemiluminescence was monitored with a Chem-Glow photometer.

Phosphatase treatment

This was done as described in Chapter II.

III-3. RESULTS

Mastoparan induces a transient $[Ca^{2+}]_{cyt}$ elevation

When the tobacco cells containing aequorin were treated with 10 μM mastoparan, a transient increase in luminescence was observed (Fig. 20A). Luminescence intensity raised immediately after the mastoparan treatment and returned to the original level within 1 min. The $[Ca^{2+}]_{cyt}$ at the peak of luminescence was estimated to be 453 ± 14 nM (n=5) using the calibration equation described in Chapter II. The $[Ca^{2+}]_{cyt}$ elevation was dependent on the concentration of mastoparan and a detectable response was observed with 2 μM mastoparan (Fig. 20C). No $[Ca^{2+}]_{cyt}$ elevation was induced by treating the cells with an inactive synthetic analogue, Mas-17 at 10 μM (Fig. 20B).

Mastoparan-induced $[Ca^{2+}]_{cyt}$ elevation occurs without production of hydrogen peroxide

Legendre et al. (1992) reported that an oligogalacturonide elicitor induced a rapid oxidative burst in cultured soybean cells and mastoparan mimicked the elicitor. Hydrogen peroxide was shown to induce a transient $[Ca^{2+}]_{cyt}$ elevation in tobacco seedlings (Price et al., 1994). Thus I examined the effect of H_2O_2 on $[Ca^{2+}]_{cyt}$. A transient $[Ca^{2+}]_{cyt}$ elevation was observed in tobacco suspension culture cells when treated with H_2O_2 (Fig. 21). Aequorin luminescence raised immediately after the H_2O_2 treatment and returned to the original level within 1 min as observed in the mastoparan treatment. The $[Ca^{2+}]_{cyt}$ elevation was dependent on the concentration of H_2O_2. These observations suggest that mastoparan may induce H_2O_2 production and subsequently H_2O_2 may induce the $[Ca^{2+}]_{cyt}$ elevation. To examine this possibility, I attempted to detect production of active oxygen species in response to mastoparan in the tobacco cells. Treatment of the tobacco cells with mastoparan did not produce

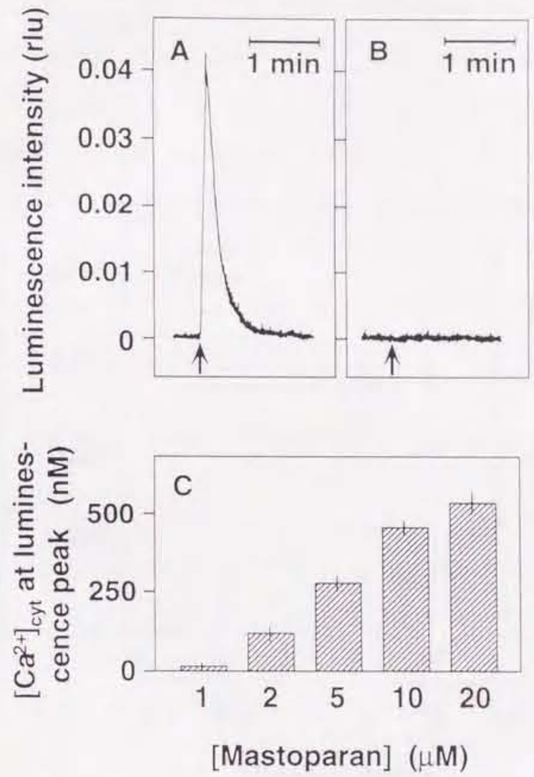

Figure 20. Effect of mastoparan on $[Ca^{2+}]_{cyt}$ of tobacco suspension culture cells. Three-day old tobacco suspension culture cells containing aequorin were treated with 10 μM mastoparan (A) or with 10 μM Mas-17 (B) at the time indicated with arrows and luminescence was recorded. Similar results were obtained in five independent experiments. The $[Ca^{2+}]_{cyt}$ at the luminescence peak induced by various concentration of mastoparan was estimated using calibration equation and plotted (C). Bars are standard errors (n=5).

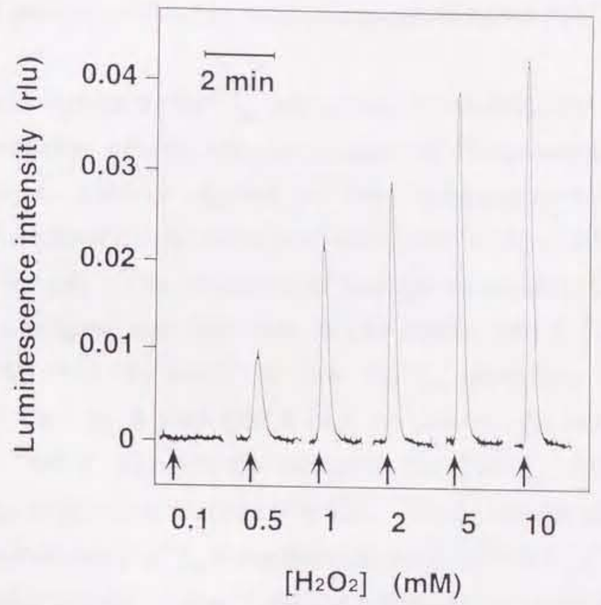

Figure 21. Effect of H_2O_2 on $[Ca^{2+}]_{cyt}$ of tobacco suspension culture cells.
The tobacco cells containing aequorin were treated with the indicated concentrations of H_2O_2 at the indicated time with arrows and luminescence was recorded.

luminescence of CLA, an O_2^- specific luminescence probe (Nakano et al., 1986), indicating that mastoparan did not induce O_2^- burst in these cells. Effect of active oxygen scavengers on the mastoparan-induced $[Ca^{2+}]_{cyt}$ elevation was next examined. Pretreatment of tobacco cells with catalase (125 μg/mL), superoxide dismutase (330 U/mL), an O_2^- scavenger tiron (5 mM), an ·OH scavenger *N,N'*-dimethylthiourea (5 mM), had no effect on the mastoparan-induced $[Ca^{2+}]_{cyt}$ elevation. These results indicate that H_2O_2, O_2^- and ·OH are not involved in the mastoparan-induced $[Ca^{2+}]_{cyt}$ elevation.

Mastoparan-induced $[Ca^{2+}]_{cyt}$ elevation is caused by Ca^{2+} release from intracellular stores via stimulation of PI turnover

To identify calcium source of the mastoparan-induced $[Ca^{2+}]_{cyt}$ elevation, the tobacco cells were pretreated with various inhibitors of Ca^{2+} transport (Fig. 22). The inhibitors of voltage-dependent Ca^{2+} channels, verapamil, nifedipine and diltiazem (5 μM each), and a Ca^{2+} antagonist $LaCl_3$ (1 mM) had no effect on the $[Ca^{2+}]_{cyt}$ elevation. Depletion of extracellular Ca^{2+} by 5 mM EGTA had no effect. An intracellular Ca^{2+} antagonist, TMB-8 significantly reduced the $[Ca^{2+}]_{cyt}$ elevation in its concentration dependent manner (Fig 23). These results suggest that the mastoparan-induced $[Ca^{2+}]_{cyt}$ elevation may be caused by Ca^{2+} release from the intracellular stores. I examined the effect of neomycin which binds PI and inhibits PI turnover, on the mastoparan-induced $[Ca^{2+}]_{cyt}$ elevation. Figure 24 shows that neomycin inhibited the $[Ca^{2+}]_{cyt}$ elevation in a dose-dependent manner. This result supports an assumption that mastoparan induces Ca^{2+} release mediated by IP_3. However, there is another possible inhibitory effect of neomycin on the function of mastoparan. Cho et al. (1995) reported that neomycin binds to cell wall and inhibits uptake of mastoparan in carrot culture cells. Hence, I examined the effect of mastoparan on $[Ca^{2+}]_{cyt}$ in protoplasts prepared from the tobacco cells.

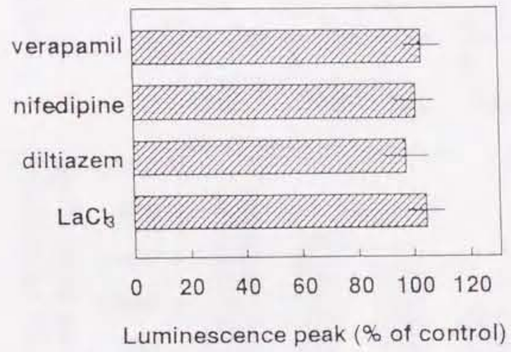

Figure 22. Effect of various inhibitors on the mastoparan-induced $[Ca^{2+}]_{cyt}$ elevation. The tobacco cells containing aequorin were pretreated with verapamil, nifedipine, diltiazem (5 μM each) and $LaCl_3$ (1 mM) for 10 min and then treated with 10 μM mastoparan and luminescence was recorded. The luminescence peak was plotted as % of control. Bars are standard errors (n=4).

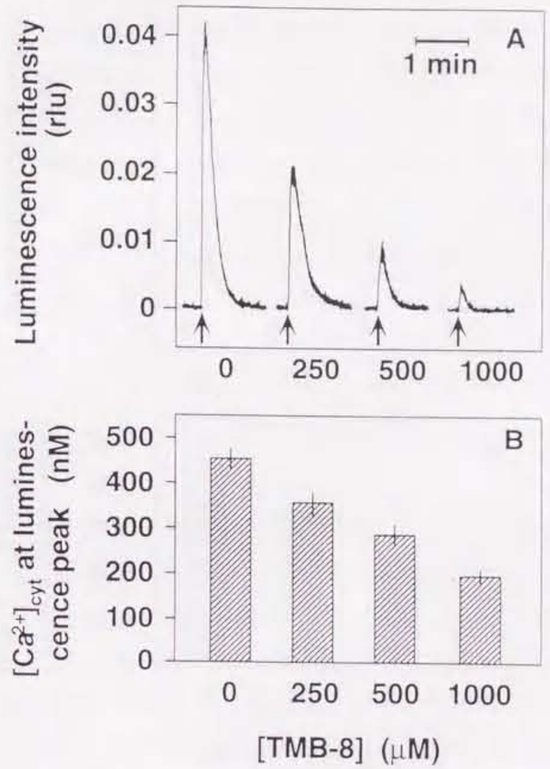

Figure 23. Effect of TMB-8 on the mastoparan-induced $[Ca^{2+}]_{cyt}$ elevation.
The tobacco cells containing aequorin were pretreated with the indicated concentrations of TMB-8 for 20 min. At the arrows the cells were treated with 10 μM mastoparan and luminescence was recorded (A). Similar results were obtained in five independent experiments. The $[Ca^{2+}]_{cyt}$ at the luminescence peak induced by various concentration of mastoparan was estimated using calibration equation and plotted (B). Bars are standard errors (n=5).

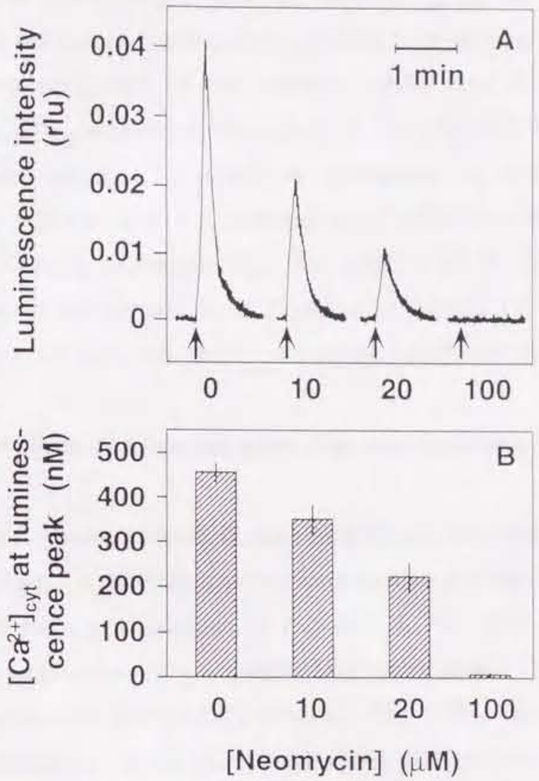

Figure 24. Inhibition of the mastoparan-induced $[Ca^{2+}]_{cyt}$ elevation by neomycin. The tobacco cells containing aequorin were pretreated with neomycin sulfate at the indicated concentration for 1 min. At the arrows the cells were treated with 10 μM mastoparan and luminescence was recorded (A). Similar results were obtained in five independent experiments. The $[Ca^{2+}]_{cyt}$ at the luminescence peak induced by various concentration of mastoparan was estimated using calibration equation and plotted (B). Bars are standard errors (n=5).

Mastoparan induced $[Ca^{2+}]_{cyt}$ elevation in the protoplasts (Fig. 25A) and this was inhibited by preincubating the protoplasts with neomycin (Fig. 25B and C). Mas-17 did not induce $[Ca^{2+}]_{cyt}$ elevation (Fig. 25D). These results suggest that the effect of neomycin on the $[Ca^{2+}]_{cyt}$ elevation is not only due to blocking the uptake of mastoparan but also to inhibiting PI turnover.

As I have observed in the tobacco cells that hypoosmotic shock induced the $[Ca^{2+}]_{cyt}$ elevation consisting of two phases; first an immediate small elevation (phase 1) which is activated by bafilomycin A_1 and insensitive to K-252a, and the second large elevation (phase 2) which is inhibited by K-252a (Chapter II), the effects of these inhibitors were examined. Both bafilomycin A_1 (0.5 μM) and K-252a (1 μM) had no effect on the mastoparan-induced $[Ca^{2+}]_{cyt}$ elevation (data not shown).

Activation of protein kinases after the mastoparan-induced $[Ca^{2+}]_{cyt}$ elevation

Figure 26A shows protein kinase activities in the tobacco cells treated with mastoparan. A 50-kDa protein kinase was transiently activated after the treatment with a maximum at 5 min. A 75- and a 80-kDa protein kinases were also transiently activated by mastoparan. No such activation of these kinases was induced by Mas-17 (Fig. 26B), which did not induce the $[Ca^{2+}]_{cyt}$ elevation. A 78-kDa protein kinase was detected in the gel but activated by neither mastoparan nor Mas-17. When extracts of mastoparan-treated tobacco cells were treated with alkaline phosphatase, the activities of these protein kinases were completely inactivated (data not shown). This indicates that the mastoparan-activated protein kinases were activated by phosphorylation and the 78-kDa protein kinase was somehow activated also by phosphorylation. Neomycin (100 μM) which completely suppressed mastoparan-induced $[Ca^{2+}]_{cyt}$ elevation, inhibited the mastoparan-induced activation of protein kinases (Fig. 26C), suggesting that the $[Ca^{2+}]_{cyt}$ elevation was requisite for the activation of

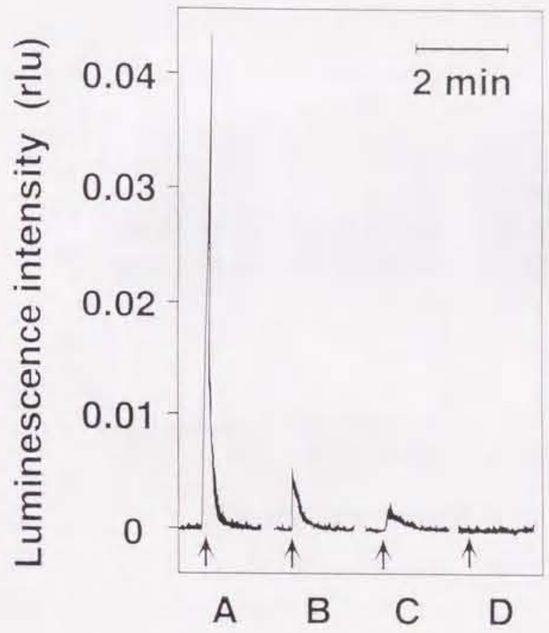

Figure 25. Effect of mastoparan on $[Ca^{2+}]_{cyt}$ of tobacco cell protoplasts.
The protoplasts prepared from 3-day old transgenic tobacco cells were incubated with 1 μM coelenterazine for 3 h. At the arrows, 20 μL of 50 μM mastoparan (final concentration: 5 μM; A, B and C) or 50 μM Mas-17 (final concentration: 5 μM; D) was added to the protoplast suspension (180 μL), and the luminescence was recorded. The protoplasts in B and C were pretreated with 100 and 200 μM neomycin, respectively. Mastoparan and Mas-17 were added gently to prevent mechanical stimulus. Similar results were obtained in five independent experiments.

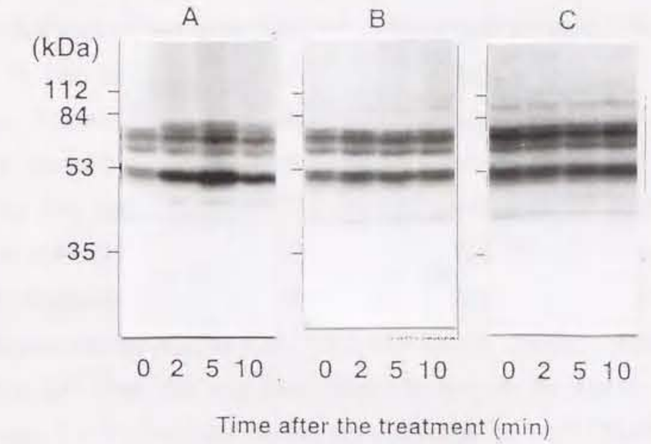

Figure 26. Mastoparan-induced activation of protein kinases in the tobacco suspension culture cells and its inhibition by neomycin.
The tobacco cells were killed by 10% trichloroacetic acid at indicated times after the treatment with 10 μM mastoparan (A) or 10 μM Mas-17 (B). The cells were preincubated for 1 min with 100 μM neomycin sulfate and treated with 10 μM mastoparan, and killed at indicated times (C). The cell extracts were subjected to SDS-PAGE followed by in-gel protein kinase assays. Molecular masses of standard proteins are indicated at the right of gels.

protein kinases. The activity of 78-kDa protein kinase had no effect of neomycin.

III-4. DISCUSSION

The present study indicated that mastoparan induced a transient $[Ca^{2+}]_{cyt}$ elevation in the tobacco cells, and which was prevented by TMB-8 and neomycin but not by depletion of extracellular Ca^{2+} and by compounds which inhibit influx of extracellular Ca^{2+}. Mastoparan also induced $[Ca^{2+}]_{cyt}$ elevation in the tobacco cell protoplasts, and which was inhibited by neomycin. These results suggest that the mastoparan-induced $[Ca^{2+}]_{cyt}$ elevation is caused by trimeric G-protein-coupled stimulation of PI turnover, followed by IP_3-dependent Ca^{2+} release from the intracellular stores. Stimulation of PLC and increase in IP_3 level by mastoparan treatment were reported in soybean (Legendre et al., 1993), carrot cells (Cho et al., 1995) and *Chlamydomonas reinhardtii* (Yueh and Crain, 1993). The vacuole as well as the ER and the mitochondrion is known to serve as a major compartment for the sequestration and storage of Ca^{2+} (Muto, 1992). It was reported that IP_3 induces Ca^{2+} release from vacuolar membrane vesicles (Schumaker and Sze, 1987) and whole vacuoles (Allen and Sanders, 1994). TMB-8 was reported to block IP_3-induced Ca^{2+} release from vacuolar membrane vesicles of oat roots (Schumaker and Sze, 1987).

I have reported the activation of protein kinases with molecular masses of 50, 75 and 80 kDa in response to hypoosmotic shock in tobacco cells (Chapter II). The mastoparan-activated protein kinases have also molecular masses of 50, 75 and 80 kDa. Both the osmo-responsive and the mastoparan-responsive protein kinases were activated by phosphorylation, and the preceding $[Ca^{2+}]_{cyt}$ elevation was necessary for the activation of both types of protein kinases. However, the source of Ca^{2+} was different; hypoosmotic shock is extracellular and mastoparan

intracellular. The molecular masses of respective counterparts are not distinguishable but it is not known whether they are identical molecules or not. It has been found that hypo- and hyper-osmotic shocks activate protein kinases with the same molecular mass but with different substrate specificities and different activation time courses in a halotolerant green alga *Dunaliella tertiolecta* (Yuasa and Muto, 1996).

The mastoparan-induced $[Ca^{2+}]_{cyt}$ elevation was insensitive to K-252a which inhibited the phase 2 of hypoosmotic induced $[Ca^{2+}]_{cyt}$ elevation and to bafilomycin A_1 which activated the phase 1. This indicates that K-252a-sensitive protein phosphorylation is not involved in the process of mastoparan-induced intracellular Ca^{2+} release.

Mastoparan has been reported to induce oxidative burst in soybean culture cells (Legendre et al., 1992). On the other hand, oxidative burst was not observed in the tobacco cells after mastoparan treatment, however, H_2O_2 induced a transient $[Ca^{2+}]_{cyt}$ elevation in the tobacco cells (Fig. 21). This indicates that the soybean and the tobacco cells differently respond to mastoparan. In the tobacco cells, mastoparan and H_2O_2 may induce the $[Ca^{2+}]_{cyt}$ elevation via independent signal transduction pathways.

General Discussion

The present study demonstrated that both the hypoosmotic shock and mastoparan induced transient $[Ca^{2+}]_{cyt}$ elevation in tobacco suspension culture cells. The former signal induced an influx of extracellular Ca^{2+} and the later a release of intracellular storage Ca^{2+}. Following to the $[Ca^{2+}]_{cyt}$ elevation, protein kinases with molecular masses of 50, 75 and 80 kDa were activated in both cases. Hypothetical schemes for the $[Ca^{2+}]_{cyt}$ elevation and the subsequent activation of protein kinases are depicted in figure 27.

Hypoosmotic shock causes water influx and results in increase in turgor pressure of the cells and subsequently causes extension of the plasma membrane. Phase 1 responsible Ca^{2+} channel which gates immediately after the shock may be a mechanosensory channel which is directly activated by extension of the plasma membrane. Ca^{2+} channel gating in phase 2 which is fully activated after a 35-s lag time may be activated by direct phosphorylation of the channel protein or by phosphorylation of associated sensing component(s). This phosphorylation process does not require $[Ca^{2+}]_{cyt}$ elevation and the activated state is maintained until $[Ca^{2+}]_{cyt}$ elevation occurs. This suggests that phosphorylated form of the phase 2 responsible Ca^{2+} channel or the associated sensing component(s) may be dephosphorylated and inactivated by a Ca^{2+}-dependent phosphatase(s). Increased Ca^{2+} may activate CDPK (or calmodulin dependent protein kinase) and the activated CDPK may activate putative upstream protein kinases of the 50-, 75-, and 80-kDa protein kinases. Regarding the 50-kDa protein kinase, the putative upstream protein kinase is a protein tyrosine kinase.

Mastoparan binds to and activates G_{α}, and the activated G_{α} activates PLC located in the plasma membrane. PLC hydrolyzes phosphatidylinositol-4,5-bisphosphate to IP_3 and diacylglyerol. IP_3 binds to

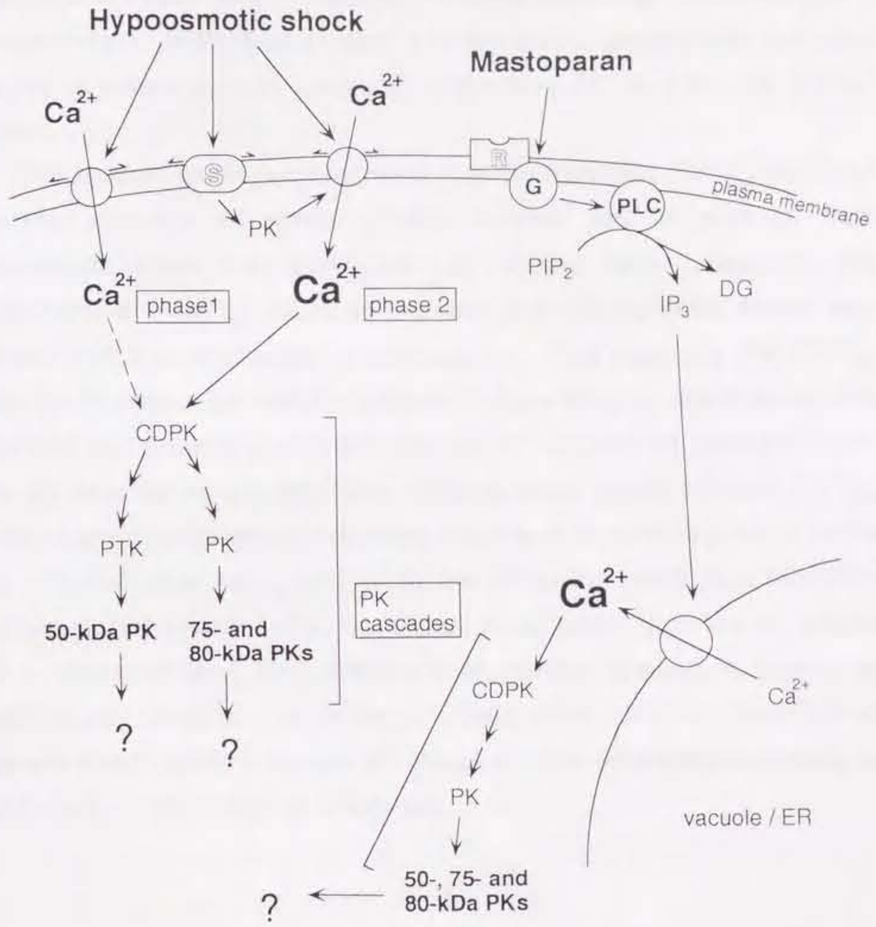

Figure 27. Hypothetical schemes of signal transduction pathways in tobacco cells upon hypoosmotic shock and mastoparan treatment.
S, putative hypoosmotic sensor; PK, protein kinase; PTK, protein tyrosine kinase; R, receptor; PIP2, phosphatidylinositol 4,5-bisphosphate; DG, diacylgrycerol.

a putative IP_3 receptor on the membrane of intracellular Ca^{2+} storage organelles and induces Ca^{2+} release. The increased Ca^{2+} activates CDPK (or calmodulin dependent protein kinase) and subsequently activates putative upstream protein kinase(s) of the 50-, 75- and 80-kDa protein kinases.

The present study demonstrated that the transient $[Ca^{2+}]_{cyt}$ elevation and the activation of similar protein kinases are induced by both hypoosmotic shock and mastoparan in tobacco cells. However, the mechanism of $[Ca^{2+}]_{cyt}$ elevation induced by hypoosmotic shock was different from that of induced by mastoparan. This suggests that $[Ca^{2+}]_{cyt}$ within single cells is geometrically different depending on which signal was perceived and accordingly the activities of Ca^{2+}-dependent protein kinases in single cells varies geometrically. Hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation and protein kinase activation might lead to osmoregulation of the cells. On the other hand, what might be led by the mastoparan-induced changes is not known. Diverse stimuli have been reported to induce $[Ca^{2+}]_{cyt}$ elevation and also activation of protein kinases in plants as described in General Introduction. Cells must provide complicated networks which discriminate specific stimuli and transduce them properly to lead the proper physiological responses.

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