A study on abscisic acid-responsive protein kinase in stomatal guard cells

Imari O. Mori

A Study on Abscisic Acid-responsive Protein Kinase in Stomatal Guard Cells

孔辺細胞の ABA 応答性プロテインキナーゼに関する研究

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by

Izumi C. Mori

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List of Abbreviations

A23187	(6S-[6α(2S*,3S),8β(R*),9β,11α]-5-(methylamino)-2-[[3,9,11-trimethyl-8-[1-methyl-2-oxo-2-(1*H*-pyrrol-2-yl)ethyl]-1,7-dioxaspiro[5.5]undec-2-yl]methyl]-4-benzoxazolecarboxylic acid
ABA	abscisic acid
ABR kinase	ABA-responsive protein kinase
BA	benzoic acid
BAPTA	1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid
BSA	bovine serum albumin
$[Ca^{2+}]_{cyt}$	cytosolic free calcium ion concentration
CDPK	calmodulin-like domain protein kinase
2,4-D	2,4-dichlrophenoxyacetic acid
EDTA	ethylenediamine-*N,N,N',N'*-tetraacetic acid
EGTA	ethyleneglycol-bis-(ß-aminoethylether)*N,N,N',N'*-tetraacetic acid
ERKs	extracellular signal-regulated protein kinases
FK506	[3S-[3R*[E(1S*,3S*,4S*0)],4S*,5R*,8S*,9E,12R*,14R*,15S*,15R*18S*19S*,26aR*]]-5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-Hexadecahydro-5,19-dihydroxy-3-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylethenyl]-14,16-dimethoxy-4,10,12,18-tetramethyl-8-(2-propenyl)-15,19-epoxy-3H-pyrido[2,1-c][1,4]oxaazacyclotricosine-1,7,20,21(4H,23H)-tetrone
GA_3	gibberellin A_3
GCP	guard cell protoplast
IAA	indoleacetic acid

$I_{K,in}$	inward-rectifying K^+ current
$I_{K,out}$	outward-rectifying K^+ current
IP_3	*myo*-inositol 1,4,5-trisphosphate
IPTG	isopropyl ß-D-thiogalactopyranoside
K-252a	(8R*, 9S*, 11S*)-(-)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H, 8H, 11H-2, 7b,11a-triazadibenzo [a,g]cyclo-octa[c,d,e]-trinden-1-one
K^+_{in} channel	inward-rectifying K^+ channel
K^+_{out} channel	outward-rectifying K^+ channel
KN-62	1-(*N*,*O*-bis[5-isoquinolinesulfonyl]-*N*-methyl-L-tyrosyl)-4-phenylpiperazine
MAP kinase	mitogen activated protein kinases
MBP	myelin basic protein
Mes	2-(*N*-mopholino)ethanesulfonic acid
ML-9	1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine
PAGE	polyacrylamide electrophoresis
PM	plasma membrane
PMSF	phenylmethylsulfonyl fluoride
rlu	relative luminescence unit
SA	salicylic acid
SDS	sodium dodecylsulfate
TCA	Trichloric acid
TMB-8	8-(*N*,*N*-diethylamino)-octyl 3,4,5-trimethoxybenzoate-HCl
Tris	2-amino-2-hydroxymethyl-1,3-propanediol

General Introduction

Stomatal movement is influenced by various environmental and internal signals such as phytohormones, light, temperature, humidity and water potential, and controls transpiration and CO_2 uptake under diverse environmental conditions (Zeiger, 1983; Mansfield et al., 1990). For instance, when plants suffered from drought, abscisic acid (ABA) is accumulated in response to drought, and induces a series of signaling events leading to stomatal closure (Mansfield et al. 1990) to prevent water loss through transpiration. Stomatal closing is driven by a reduction of turgor pressure in guard cells that is caused by the efflux of K^+ and anions and a concomitant conversion of malate to starch (Raschke, 1979; MacRobbie, 1981; Outlaw, 1983). ABA induces rapid activation of non selective ion channels which causes membrane depolarization and allows Ca^{2+} influx from the extracellular space. Increase in cytosolic Ca^{2+} and the production of other signaling intermediates trigger further Ca^{2+} release from intracellular stores. The increases in cytosolic free calcium ion concentration ($[Ca^{2+}]_{cyt}$) in guard cells have been shown to precede ABA-induced stomatal closure (McAinsh et al., 1990; Gilroy et al., 1990; Schroeder and Hagiwara, 1990; Irving et al., 1992; McAinsh et al., 1992), suggesting that Ca^{2+} functions as a second messenger in ABA signal transduction pathway. Gilroy et al. (1990) have reported that cytoplasmic release of Ca^{2+} or *myo*-inositol 1,4,5-trisphosphate (IP_3) from its caged form can initiate stomatal closure in *Commelina* as a result of $[Ca^{2+}]_{cyt}$ elevation. However, effect of the $[Ca^{2+}]_{cyt}$ elevation was variable (MacRobbie, 1989) and ABA-induced $[Ca^{2+}]_{cyt}$ elevation was observed in a minority of cells, even though stomatal closure always occurred in *Commelina* (Gilroy et al., 1991). These observations indicate that ABA-induced stomatal closure occurs in Ca^{2+}-dependent and -independent

pathways. Membrane depolarization, elevated $[Ca^{2+}]_{sip}$, and other signal events activate anion channels, which mediate anion release and long-term membrane depolarization. K^+ efflux through outward-rectifying K^+ channels (K^+_{out} channels) driven by membrane depolarization enhances decrease of turgor pressure resulting in stomatal closure (Ward et al., 1995).

Several factors have been proposed as the modulators of ion transporters in guard cells (for review, see Thiel and Wolf, 1997). Cytosolic Ca^{2+} inhibited an inward-rectifying K^+ current ($I_{K,in}$), and activated a slow-anion channel (Schroeder and Hagiwara, 1989) and a vacuolar K^+ channel (Ward and Schroeder, 1994). The activated GTP-binding protein was reported to inhibit $I_{K,in}$ (Fairley-Grenot and Assmann, 1991; Armstrong and Blatt, 1995). Kelly et al. (1995) reported that the regulation of $I_{K,in}$ by GTP-binding protein was also affected with Ca^{2+} and a rather complicated way. Ca^{2+} and nucleotide changed the voltage dependency of fast anion channel (Hedrich et al., 1990). ATP activated $I_{K,in}$ and this activation was not mediated by phosphorylation (Wu and Assmann, 1995). Actin filaments was reported to have an effect on $I_{K,in}$ in guard cells (Hwang et al., 1997).

Pharmacological studies have suggested that protein phosphorylation is one of the most important regulatory mechanisms of ion transport in guard cells. $I_{K,in}$ was activated by FK506, an inhibitor of protein phosphatase 2B (Luan et al., 1993), and inhibited by either okadaic acid or calycurin A, inhibitors of protein phosphatase 1 and 2A (Li et al., 1994; Thiel and Blatt, 1994). Outward-rectifying K^+ current ($I_{K,out}$) was also affected by the protein phosphatase inhibitors (Li et al., 1994; Thiel and Blatt, 1994). A slow-anion channel which is thought to be important in ABA-signaling was inhibited by K-252a (Schmidt et al., 1995). Grabov et al. (1997) reported

that the conductance of Cl^- in the plasma membrane of tobacco guard cells altered after ABA-treatment, and calycurin mimicked the action of ABA. A slow-vacuolar channel was reported to be modulated by calcineurin (protein phosphatase 2B) (Allen and Sanders, 1995).

There was no direct evidence showing the presence of protein kinases in guard cells. Recently, Kinoshita and Shimazaki (1995) reported Ca^{2+}-dependent and -independent in vivo protein phosphorylation in *Vicia* guard cell extracts. Currently, the identification of protein kinases which transduce the external and phytohormonal signals in guard cells is becoming controversial. In the present study, I focused on the identification of protein kinase which is responsive to ABA in guard cells. I found that a protein kinase was activated when guard cell protoplasts were treated with ABA. The activation of this kinase (named ABA-responsive protein kinase; ABR kinase) was inhibited by protein kinase inhibitors, suggesting the presence of upstream activator kinase(s) on a protein kinase cascade. In addition, the requirement of Ca^{2+} for the activation was indicated. Characterization of ABR kinase will be described in Chapter 1 of this thesis.

The downstream event of activation of ABR kinase leading to stomatal movement is phosphorylation of target protein(s). The aim of studies described in Chapter 2 and 3 is the identification of target protein(s) for ABR kinase, especially proteins related to ion transport. Several approaches have been tried to identify components of protein phosphorylation/dephosphorylation pathways. An *Arabidopsis thaliana* protein phosphatase mutant *abi1-1* was used to analyze role of protein phosphorylation in ion transport after treatment of tobacco guard cells with ABA. $I_{K,in}$ and $I_{K,out}$ were deactivated and activated with ABA, respectively, in wild type cells, but the change in these K^+ currents were suppressed in transgenic guard cells expressing the mutant gene (Armstrong et al. 1995).

It has been reported that a recombinant calmodulin-like domain protein kinase (CDPK) phosphorylated and activated a chloride channel in vacuolar membrane of *Vicia* guard cells when applied during patch-clamp experiments (Pei et al., 1996). I applied an in-gel protein kinase assay method to identify the target protein of ABR kinase. Using recombinant peptides of ion transporters as substrates for the in-gel assay, I found that ABR kinase specifically phosphorylates an inward-rectifying K^+ channel KAT1.

Recently, techniques of electrophysiology and molecular biology have met together, and studies on correlation between structure and function of ion transporters have been markedly stimulated (Hille, 1991). A number of genes encoding ion transporters including channels, carriers and pumps have been isolated, and their primary, secondary and tertiary structures were predicted from the deduced amino acid sequences. Their functions were analyzed by electrophysiological techniques in heterologous expression systems such as *Xenopus* oocytes and vaculovirus-infected insect cells. Several genes encoding members of inward-rectifying K^+ channel (K^+_{in} channel) superfamily have been cloned, expressed in *Xenopus* oocytes and their electrophysiological natures were analyzed by voltage-clamping techniques (for review see, Schroeder et al., 1994). A K^+ channel, KCO1, with four transmembrane domains and tandem calcium-binding EF-hand motifs was cloned from *Arabidopsis* and functional analysis in vaculovirus-infected *Spodoptera* cells showed a depolarization-dependent $I_{K,out}$ with a strong dependency on nanomolar Ca^{2+} (Czempinski et al., 1997). However, animal cells used for heterologous expression have endogenous factors which prevent the electrophysiological analysis interacting with the expressed ion channels.

Another heterologous expression system is budding yeast

Saccharomyces cereviciae cells. The first identification of K^+ channel cDNA from plants was achieved by functional complementation of yeast cells that were defective in K^+ uptake. Two distinct *Arabidopsis thaliana* cDNAs, termed *AKT1* and *KAT1*, were independently cloned (Sentenac et al., 1992; Anderson et al., 1992). Four novel members of CLC family of chloride channel were cloned from *Arabidopsis* and one of these genes functionally substituted for the single yeast CLC protein but none of them elicited chloride currents when expressed in *Xenopus* oocytes (Hechenberger et al., 1996). cDNAs coding ion carriers, a high affinity K^+ transporter (Schachtman and Schroeder, 1994), H^+/Ca^{2+} exchangers (Hirschi et al., 1996), and sulfate transporters (Smith et al., 1995) were isolated from plants by functional complementation of yeast mutants.

Once the genes of ion transporters have isolated, functional analysis will be allowed in heterologous expression in yeast cells. Yeast cells have a big advantage to analyze functions of plant transporters as they have vacuoles like plant cells and plant vacuolar transporters are expected to be expressed in their proper organelle. Recently, the complete nucleotide sequence of whole yeast genome has been established [Nature (1997) **387** supplement]. Now one can find and disrupt transporter genes on yeast genome and can utilize it for functional complementation screening for plant cDNAs encoding transporters. Furthermore, one can disrupt genes which prevent functional analysis of transporters and interactions between ion transporters and protein modulators could be examined in yeast cells. However, electrophysiological analysis in yeast cells is rather difficult because of their small size and difficulty of making protoplasts. Only a couple of laboratories have succeeded to analyze electric current of exogenous ion channels expressed in yeast (Bertl et al., 1995; Zhou et al., 1995). Establishment of electrophysiological analysis in yeast cells starts

from getting large size cells. Production of 4 *n* cells with different mating types is in progress in this laboratory.

The activation of ABR kinase by ABA in guard cell protoplasts required extracellular Ca^{2+} (see Chapter 1). Ca^{2+} is widely accepted as an important second messenger in intracellular signal transduction in eukaryotic cells. In plants, Ca^{2+} was predicted as a modulation factor of ion channels (for review, see Thiel and Wolf, 1997). It is important to analyze the role of Ca^{2+} in yeast cells expressing plant ion channels.

Stimulus-coupled change in $[Ca^{2+}]_{cyt}$ in animal cells have been studied using fluorescence dyes. Plant cells expressing apoaequorin which constitutes a Ca^{2+}-dependent luminescent protein aequorin, with externally added chromophore coelenterazine, were successfully used for investigating the stimulus induced Ca^{2+} transients (Knight et al., 1991). However, studies on Ca^{2+} mobilization in yeast are limited in a few reports. For instance, Iida et al. (1990) reported a $[Ca^{2+}]_{cyt}$ elevation induced by the mating pheromone (α factor) in **a**-mating type cells using a Ca^{2+}-indicator (fura-2). Nakajima-Shimada et al. (1991) transformed yeast cells with apoaequorin and monitored $[Ca^{2+}]_{cyt}$ changes in response to mating pheromone and glucose in glucose-starved yeast cells. Cell cycle control by calcium and calmodulin in yeast has been well documented (Anraku et al., 1991). Stimulus-coupled immediate and transient $[Ca^{2+}]_{cyt}$ elevation has not been reported in yeast except hypoosmotic shock-induced transient observed in apoaequorin-transgenic cells (Batiza et al., 1996). The mechanism of Ca^{2+} mobilizing into the cytosol has remained unclear in yeast cells. In the present study, I found that treatment of the apoaequorin-transgenic yeast with salicylic acid induces $[Ca^{2+}]_{cyt}$ transient. The salicylic acid-induced Ca^{2+} mobilization system in yeast cells was analyzed in Chapter 4.

Chapter 1

Abscisic Acid Activates a 48-kilodalton Protein Kinase in *Vicia* Guard Cell Protoplasts

Summary

A 49- and a 46-kD Ca^{2+}-independent protein kinases and a 53-kD Ca^{2+}-dependent protein kinase were detected in *Vicia faba* guard cell protoplasts (GCPs) by in-gel protein kinase assay using myelin basic protein as substrate. A 48-kD protein kinase which is designated as ABA-responsive protein kinase (ABR kinase) appeared when GCPs were treated with ABA. The activation of ABR kinase was suppressed by a protein kinase inhibitor staurosporine, indicating that a putative activator protein kinase phosphorylates and activates ABR kinase. The treatment of GCPs with BAPTA, a calcium chelator, suppressed the activation of ABR kinase suggesting that an influx of extracellular Ca^{2+} is required for the activation. Staurosporine and K-252a inhibited both the activity of ABR kinase and the stomatal closure induced by ABA-treatment of *Vicia* epidermal peels. These results suggest that ABR kinase and its activator kinase may consist of a protein kinase cascade in signal transduction pathway linking ABA perception to stomatal closure. The mobility of 53-kD Ca^{2+}-dependent protein kinase in SDS-polyacrylamide gel was shifted upon Ca^{2+} binding to the enzyme, indicating the characteristics of CDPK. This kinase is one of candidates for the activator of ABR kinase.

1-1. Introduction

The opening and closing of stomatal apertures are influenced by various environmental factors such as light, humidity and CO_2 level and by internal factors such as plant hormones and $[Ca^{2+}]_{cyt}$ (for review, see Kearns and Assmann, 1993). The contents of K^+ and counter ions in guard cells increase and decrease during stomatal opening and closing, respectively

(for review, see Schroeder and Hedrich, 1989). These ion transports could be mediated by K^+_{in} and K^+_{out} channels (Schroeder, 1988) and slow anion channels (Schmidt et al., 1995). Exogenous ABA causes stomatal closure by depolarization of the plasma membrane and subsequent K^+ efflux across the plasma membrane of guard cells.

The increases of $[Ca^{2+}]_{cyt}$ in guard cells have been shown to precede ABA-induced stomatal closure (McAinsh et al., 1990; Gilroy et al., 1990; Schroeder and Hagiwara, 1990), suggesting that Ca^{2+} functions as a second messenger in the signal transduction pathway. Involvement of second messengers, IP_3 (Blatt et al., 1990; Gilroy et al., 1990; Lee et al., 1996) and diacylglycerol (Lee and Assmann, 1991), in guard cell movement has been also suggested. Regulation of K^+_{in} channels which play a central role in regulating stomatal aperture, by GTP-binding proteins (Fairley-Grenot and Assmann, 1991) was implied in *Vicia faba* guard cells.

Several recent reports suggest that protein phosphorylation/dephosphorylation is the important regulatory mechanism of ion channels and H^+-pump in guard cells. The blue light-induced activation of plasma membrane (PM) H^+-pump of *V. faba* was inhibited by ML9, an inhibitor of myosin light chain kinase (Shimazaki et al., 1992). Protein phosphatase inhibitors were reported to affect the currents of K^+_{in} and K^+_{out} channels (Luan et al., 1993; Thiel and Blatt, 1994; Li et al., 1994). Armstrong et al. (1995) reported that ABI1, an ABA-insensitive gene product which is a putative protein phosphatase 2C regulates K^+_{in} and K^+_{out} channels. The inhibition of slow-type anion channel by K-252a, a serine/threonine-type protein kinase inhibitor was reported (Schmidt et al., 1995). A slow vacuolar channel was reported to be modulated by calcineulin (protein phosphatase 2B) (Allen and Sanders, 1995). In addition, Cousson et al. (1995) showed that stomatal closure by vanadate or a

light/dark transition was canceled with a myosin light chain kinase inhibitor (ML7) or a calmodulin antagonist (*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide-HCl, W-7). The inhibition of vanadate-induced stomatal closure by the protein kinase inhibitor suggests that protein phosphorylation step(s) is involved in stomatal closure in addition to the decrease in H^+-pump activity.

However, there was no direct evidence showing the presence of protein kinases and protein phosphatases in guard cells. Recently, Kinoshita and Shimazaki (1995) reported Ca^{2+}-dependent and -independent in vitro protein phosphorylation in *V. faba* guard cell extract. Currently, the identification of protein kinases which transduce the external and phytohormonal signals in guard cells is becoming controversial.

In the present study, I applied a highly sensitive method, in-gel protein kinase assay, to detect protein kinases in guard cells. I found that the treatment of *V. faba* GCPs with ABA activates a 48-kD protein kinase which phosphorylates preferentially myelin basic protein (MBP). Phosphorylation catalyzed by a putative protein kinase is required for the activation of this kinase, suggesting the presence of protein kinase cascade in signal transduction of ABA-induced stomatal closure.

1-2. Materials and Methods

Plant Material.

Seeds of *Vicia faba* L. c.v. Otafukusanzu (Aisan-shubyo Co., Nagoya) were planted on soil (Hanachan-baiyoudo, Hanagokoro Co., Nagoya) in pots and grown in a growth chamber for 6 to 8-weeks under a 12 h-light/12 h-dark cycle at 20°C. The plants were watered once a day.

Chemicals.

Cellulase YC and Pectolyase Y23 were purchased from Seishin Pharmaceutical Co. (Tokyo, Japan). [γ-^{32}P]ATP (111 TBq/mmol) were from ICN (Cost Mesa, CA). (8R*, 9S*, 11S*)-(-)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H, 8H, 11H-2, 7b,11a-triazadibenzo [a,g]cyclo-octa[c,d,e]-trinden-1-one (K-252a) and staurosporine were products of Kyowa Hakko Kogyo (Tokyo, Japan). 1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine (ML-9) and 1-(*N*,*O*-bis[5-isoquinolinesulfonyl]-*N*-methyl-L-tyrosyl)-4-phenylpiperazine (KN-62) were from Seikagaku Kogyo (Tokyo, Japan). Prestained SDS-PAGE standard proteins were from BioRad laboratories (Hercules, CA). ABA [a mixture of (+)- and (-)-form], 2,4-D, kinetin, IAA and GA_3 were from Wako Pure Chemical Industries (Osaka, Japan). Myelin basic protein, histone type III, and dephosphorylated casein were from Sigma. Cycloheximide was purchased from Nacalai Tesque (Kyoto, Japan). Anti-phosphotyrosine monoclonal antibody (PY-20) and Protein A-Sepharose CL4B were from Transduction Laboratories (Lexington, KY) and Pharmacia, respectively.

Isolation of Guard Cell and Mesophyll Cell Protoplasts.

GCPs were prepared essentially according to Gotow et al. (1984). Abaxial epidermal strips were peeled from fully expanded leaves of 6 to 8-week old plants and incubated for 20 min at 30°C with 4 %(w/v) Cellulase YC and 0.4 %(w/v) Pectolyase Y23 in 0.25 M mannitol, 10 mM Mes-KOH (pH 5.5), 1 mM $CaCl_2$ and 1 %(w/v) bovine serum albumin (BSA). The epidermal peels were recovered on nylon net (opening, 80 µm), washed with a medium containing 0.35 M mannitol, 10 mM Mes-KOH (pH 5.5), and 1 mM $CaCl_2$ (suspension medium), and further incubated with 4 %(w/v)

Cellulase YC and 0.4 % (w/v) Pectolyase Y23 in the suspension medium containing 1 %(w/v) BSA for 1 h at 30°C. The released protoplasts were isolated by passing successively through nylon nets (openings, 80 and 30 μm). The resulting protoplasts were washed twice with the suspension medium by centrifugation at 800*g* for 5 min.

The mesophyll protoplasts were isolated by a similar procedure. Leaves of which lower epidermis were removed were treated with the enzymes in the suspension medium containing 1 %(w/v) BSA for 1 h at 30°C. The released protoplasts were isolated by passing through nylon net (opening, 80 μm), collected and washed with the above medium by centrifugation at 800*g* for 5 min.

The guard cell and mesophyll cell protoplasts were suspended in the suspension medium and kept on ice at least 1 h before use.

In-gel Protein Kinase Assay.

This was performed essentially according to the method of Kameshita and Fujisawa (1989) as described by Yuasa and Muto (1995, 1996). Samples (5 to 50 μg protein) precipitated by TCA were washed twice with 80 % acetone, dried in vacuo and dissolved in Laemmli's sample buffer containing 20 mM b-glycerophosphate, 100 μM Na_3VO_4, 1 mM NaF, 5 mM ε-amino-*n*-capronic acid, 1 mM benzamidine, 1 mM benzamide, and 1 mM PMSF. The artificial substrate protein (0.2 mg mL^{-1}) for protein kinases was mixed with SDS-PAGE gel (10 % polyacrylamide) before polymerization. Samples were electrophoresed with mini-gels (85 x 60 x 1 mm) at 30 mA gel^{-1} until the 40-kD marker protein run out from the end of the gel, otherwise indicated. SDS in gels was removed by washing with 20 % (v/v) isopropanol in 20 mM Tris-HCl (pH 8). Proteins separated in gels were denatured completely by the treatment with 6 M guanidine-HCl,

then renatured by washing the gels with 20 mM Tris-HCl (pH 8) containing 1 mM 2-mercaptoethanol and 0.03 % (w/v) Tween 20. The gels were equilibrated with 25 mL of the reaction mixture containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 4 mM 2-mercaptoethanol, and 1 mM EGTA (unless indicated), and incubated in 10 mL of the reaction mixture supplemented with 25 μM [γ-^{32}P]ATP (740 kBq). Phosphorylation was carried out for 1 h at 30°C and terminated by removing the reaction mixture and adding 30 mL of 5 % (w/v) TCA-1 % (w/v) $Na_4P_2P_7 \cdot 10H_2O$ (TCA-PPi). Unreacted ^{32}P was removed by successive washing with TCA-PPi. The washed gels were dried on filter papers and radioactivities were visualized by an imaging analyzer (BAS 2000, Fuji Film Co., Tokyo).

Immunoprecipitation by Anti-phosphotyrosine Monoclonal Antibody.

This procedure was modified from Yuasa and Muto (1996). GCPs-extracts in Laemmli's sample buffer (50 μL) were diluted to 1 mL with the washing buffer containing 50 mM Tris-HCl (pH 8.8), 1 mM EDTA, and 1 % (w/v) Triton X-100 and centrifuged at 15,000*g* for 10 min at 4°C. The resulting supernatants were transferred into new tubes and supplemented with anti-phosphotyrosine antibody (PY-20, final concentration, 2 μg protein mL^{-1}) and Protein A Sepharose CL4B [final concentration, 0.9 %(v/v)] equilibrated with washing buffer. The mixtures were incubated on a rotator (3-4 rpm) for 3 h at 4°C. Sepharose beads were washed with washing buffer 4 times. The washed Sepharose beads were suspended in Laemmli's sample buffer and boiled for 1 min, then centrifuged at 15,000*g* for 10 min at 4°C. The supernatants were used for the in-gel protein kinase assay.

Measurement of Stomatal Aperture.

Abaxial epidermal strips (2 x 5 mm) were floated on 50 μL of an incubation mixture containing 50 mM KCl, 10 mM Mes-KOH (pH 5.7), and 100 μM $CaCl_2$ on a slide glass and incubated under a fluorescent lamp at 100 μmol m^{-2} s^{-1} for 45 min. A piece of epidermal strip with fully opened stomata was set on the stage of a microscope. The incubation mixture was replaced by the fresh incubation mixture containing protein kinase inhibitors, and the epidermal strip was incubated for 10 min. One μM ABA was applied to the epidermal strip, and stomatal apertures were measured after 10 min. The images of stomata were recorded on a video tape through a CCD camera (C-2400 77H, Hamamatsu Photonics, Hamamatsu, Japan) and width of the apertures was analyzed by a graphic analysis software, NIH image 1.58 (provided by Dr. Wayne Rasband, NIH).

Viability of protoplasts.

The viability of GCPs were examined essentially according to Ono et al. (1995). Just before the killing of GCPs with TCA, 10 μL aliquots were withdrawn and diluted 10-fold with the suspension medium containing 0.35 M mannitol, 10 mM Mes-KOH (pH 5.5), and 1 mM $CaCl_2$. Two μL of 0.5 % (w/v) fluorescein diacetate in acetone was added to this. After incubation for 20 min at room temperature, the cells were washed twice with the medium indicated above. Viable cells stained were counted under a fluorescence microscope.

1-3. Results

Protein Kinases in GCPs and Mesophyll Cell Protoplasts.

The protein kinases in GCPs and mesophyll cell protoplasts were analyzed by the in-gel protein kinase assay using MBP as the substrate (Fig.

1-1). Extracts from ABA-treated or -untreated protoplasts were electrophoresed in the presence or absence of Ca^{2+}, and the protein kinase activities were assayed also in the presence or absence of Ca^{2+}. When assayed in the absence of Ca^{2+} (left panel), a 46-kD and a 49-kD band with protein kinase activities were detected in GCPs extracts (lanes 1 to 4). A 48-kD band appeared in ABA-treated GCPs (lanes 1 and 2). This 48-kD protein kinase was designated as ABR kinase (ABA-responsive protein kinase).

In this experiment, the extracts were electrophoresed in a large gel (130 x 110 x 1 mm) and the condition for separating these three protein kinase bands was not optimized well. The mobility of the 48-kD ABR kinase on SDS-PAGE changed from experiment to experiment. For instance, the ABR kinase band was close to the 49-kD protein kinase band (see Fig. 1-4). On the other hand, the ABR kinase band was close to the 46-kD protein kinase band in figure 1-5. In some cases, the ABR kinase band was hardly distinguished from the 46- or 49-kD protein kinase band. When electrophoresed at a low current and for a short running distance, the 46- and 49-kD protein kinase bands positioned nearby, and the 48-kD ABR kinase band fused with these bands. To resolve these bands on SDS-PAGE, samples were electrophoresed at a rather high current and until 40-kD marker has been overrun as described in Materials and Methods. This enhanced the strength of the band between the 46- and 49-kD protein kinase bands in ABA-treated samples, even though it failed to separate these bands.

In addition to the 46-, 48- and 49-kD protein kinases, a smear faint band was detected at 40 to 45 kD. Activities of the 40 to 45-, 46-, and 49-kD kinases, were much lower in mesophyll cell protoplasts compared with GCPs, even though a double amount of sample proteins were used (lanes 5 to 8). In addition, the activation of the 48-kD protein kinase by ABA was

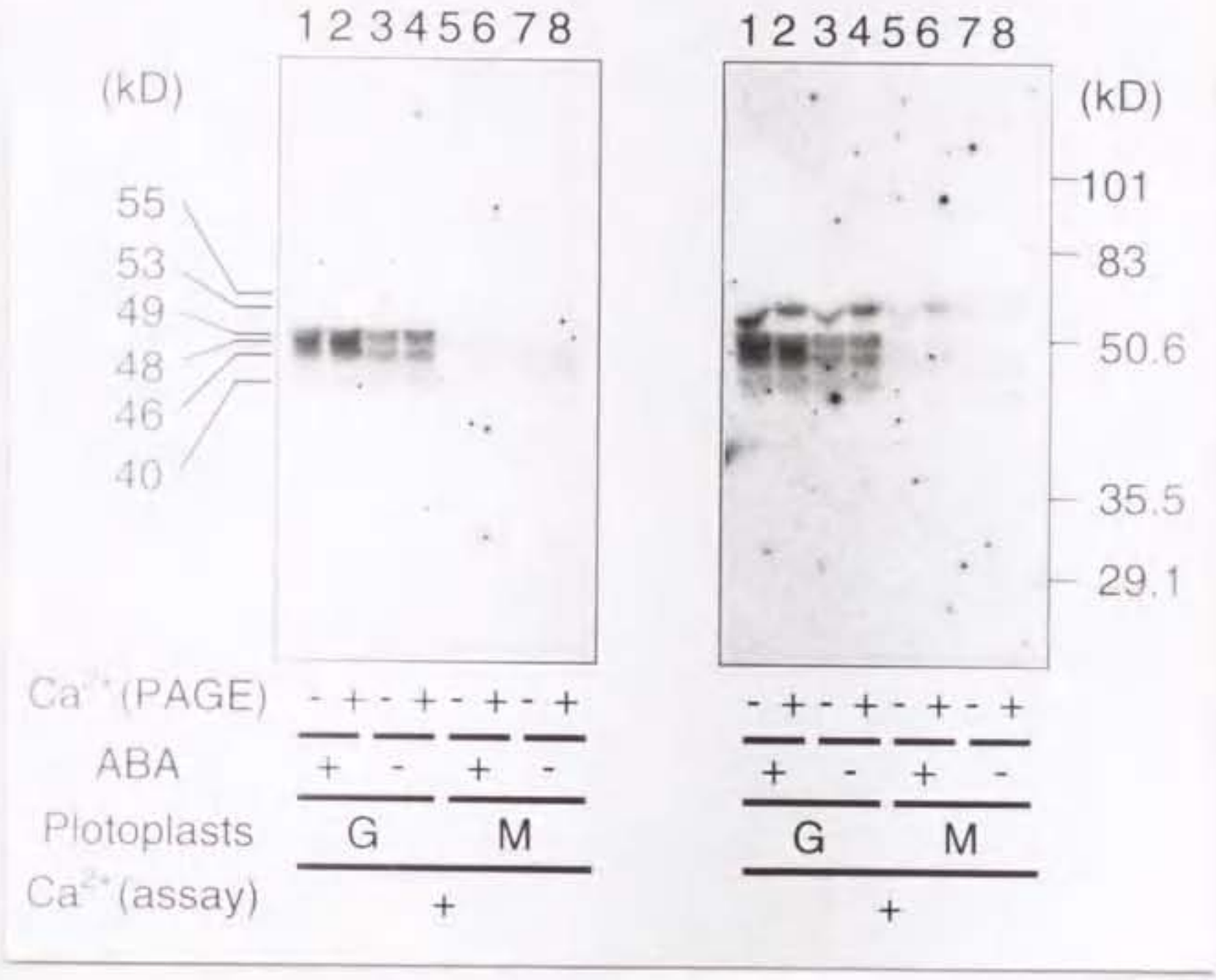

Figure 1-1. Protein kinase activities of GCPs and mesophyll cell protoplasts in the in-gel protein kinase assay using MBP gel. The protoplasts from guard cells (G; 25 μg protein) and mesophyll cells (M; 50 μg protein) were treated with 1 μM ABA (ABA,+) or 0.002 % (w/w) ethanol (solvent control; ABA, -) for 5 min at 20 ℃. Samples were dissolved in SDS-PAGE sample buffer containing 1 mM EGTA [Ca^{2+}(PAGE), -] or 1 mM Ca^{2+} [Ca^{2+}(PAGE), +]. Assay was carried out as described in "Materials and Methods" in the presence of 1 mM EGTA [Ca^{2+}(assay), -] or 1 mM Ca^{2+} [Ca^{2+}(assay), +]. Molecular masses of protein kinases and standard proteins are indicated on the left and right side of the gels, respectively.

not observed in mesophyll cells.

In the presence of Ca^{2+} during protein kinase reaction (right panel), activity bands with molecular masses of 46, 49, 53 kD were detected when GCPs were electrophoresed without Ca^{2+} (lane 3). A 48-kD band was detected in ABA-treated GCPs (lanes 1 and 2) as same as observed in left panel. The mobility of the 53-kD band was shifted to 55 kD in the presence of Ca^{2+} during electrophoresis (lanes 2 and 4). This band was not detected in the absence of Ca^{2+} in the assay mixture (left panel), indicating that this band may show the activity of CDPK, as this protein kinase may bind Ca^{2+} directly and require only Ca^{2+} for its activation. The low molecular mass faint band was detected in GCPs also in right panel. Protein kinase activities in mesophyll cell protoplasts were very low except a significant activity of the 53-kD protein kinases which was forecasted as CDPK (lanes 5 to 8).

Characterization of ABR Kinase and 46- and 49-kD Protein Kinases.

Substrate specificity --- The extracts of both GCPs and mesophyll cell protoplasts scarcely phosphorylated histone and casein (data not shown). The 46- and 49-kD protein kinases detected in MBP-gel were designated as MBP kinases because of their substrate specificity. No band was observed in the gel which contained no substrate protein, irrespective of whether protoplasts were treated with ABA or not.

Effects of protein kinase inhibitors and related compounds on the activities --- After electrophoresis and renaturation, each of lanes was divided by cutting the gel and subjected to protein kinase assay in the presence of protein kinase inhibitors. Among protein kinase inhibitors tested, only staurosporine and K-252a inhibited the protein kinase activities (Fig. 1-2a). Figure 1-2b shows dose-dependencies of staurosporine and K-252a on the inhibition of protein kinases. Staurosporine inhibited both the

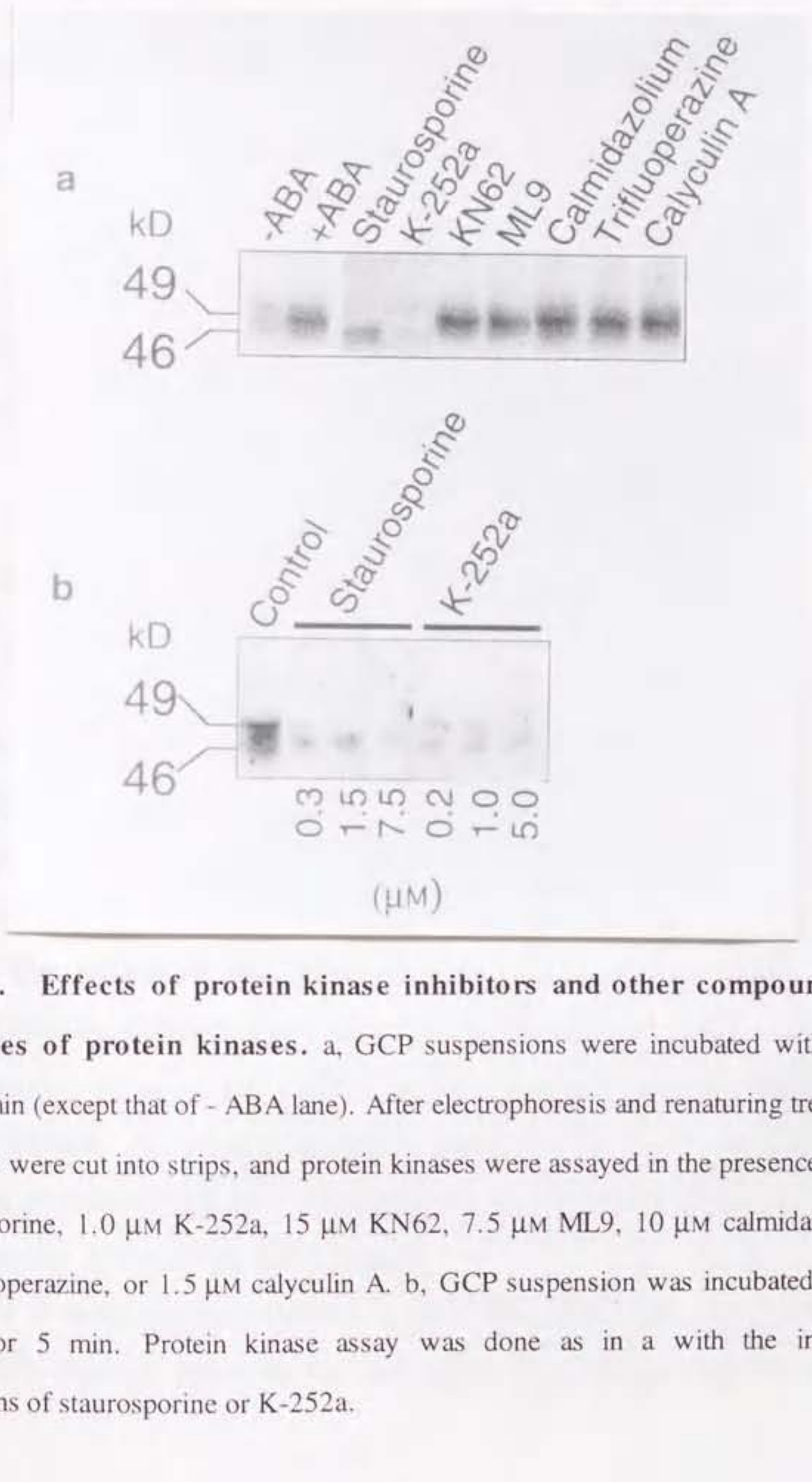

Figure 1-2. Effects of protein kinase inhibitors and other compounds on the activities of protein kinases. a, GCP suspensions were incubated with 1 μM ABA for 5 min (except that of - ABA lane). After electrophoresis and renaturing treatment, each of lanes were cut into strips, and protein kinases were assayed in the presence of 1.5 μM staurosporine, 1.0 μM K-252a, 15 μM KN62, 7.5 μM ML9, 10 μM calmidazolium, 10 μM trifluoperazine, or 1.5 μM calyculin A. b, GCP suspension was incubated with 1 μM ABA for 5 min. Protein kinase assay was done as in a with the indicated concentrations of staurosporine or K-252a.

activities of the 49-kD MBP kinase and the ABR kinase at 1.5 μM, while 1 μM K-252a inhibited the ABR kinase but not the 49-kD MBP kinase. The threshold concentration of staurosporine for the inhibition of the ABR kinase and the 49-kD MBP kinase was 0.3 to 1.5 μM. The inhibitory effect of staurosporine on the 46-kD MBP kinase was much weaker. The activities of the 49-kD MBP kinase and the ABR kinase were completely inhibited by 5 μM K-252a, while that of the 46-kD MBP kinase was slightly inhibited. The threshold concentration of K-252a for the inhibition of the ABR kinase and the 49-kD MBP kinase was 1 to 5 μM. Calmidazolium and trifluoperazine, calmodulin antagonists, and calyculin A, an inhibitor of protein phosphatase 1 and 2A, had no effect on the activities of these protein kinases.

Activation of Protein Kinases in GCPs.

Effect of ABA --- Figure 1-3 shows a time course of the activation of the ABR kinase in GCPs by ABA. The ABR kinase was activated slightly within 2 min, when GCPs were incubated with 1 μM ABA. The activity was increased gradually up to 10 min during incubation. The activated state of the enzyme was sustained at least for 30 min (data not shown). The activation of the ABR kinase was dependent on the concentration of ABA (Fig. 1-4). The activation was detected with 1 μM and seemed not to be saturated within the concentration range used. The 46-kD and 49-kD MBP kinases seemed to be activated slightly at high concentrations of ABA (5 to 25 μM). Incubation of extracts of GCPs with ABA (up to 25 μM) had no effect of the protein kinase activities (data not shown), indicating that ABA does not directly activate the ABR kinase.

Effects of other phytohormones --- Kinetin, IAA, GA_3 and 2,4-D (up to 10 μM each) had no effect on the activation of protein kinases in GCPs

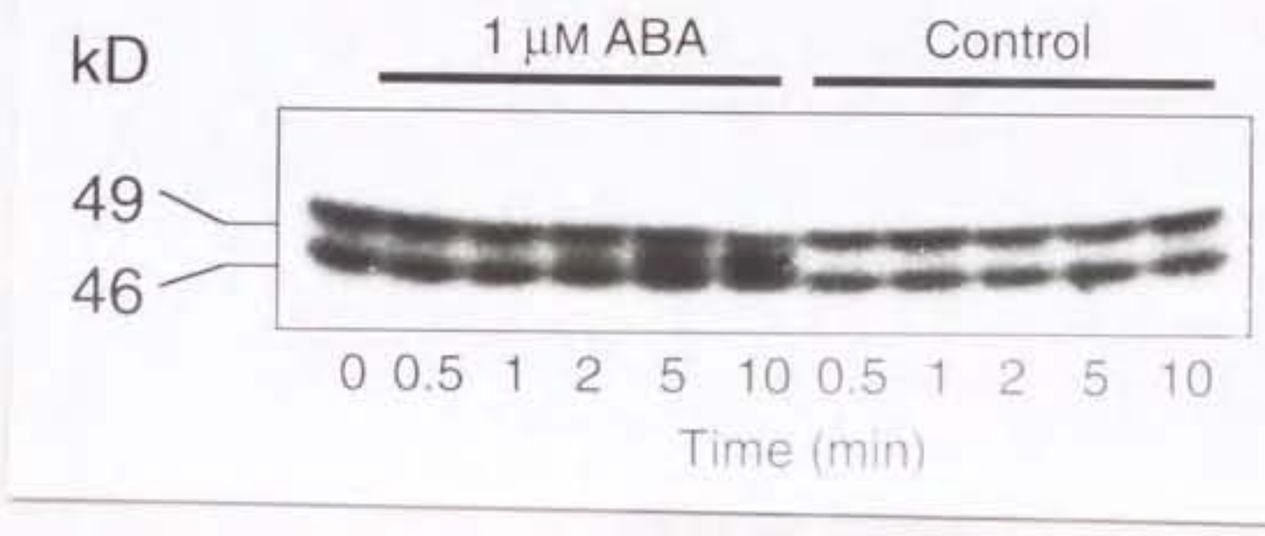

Figure 1-3. Time course of activation of the ABR kinase by ABA. GCPs were incubated with 1 μM ABA for the indicated times. Protein kinase assay was done as described in "Materials and Methods".

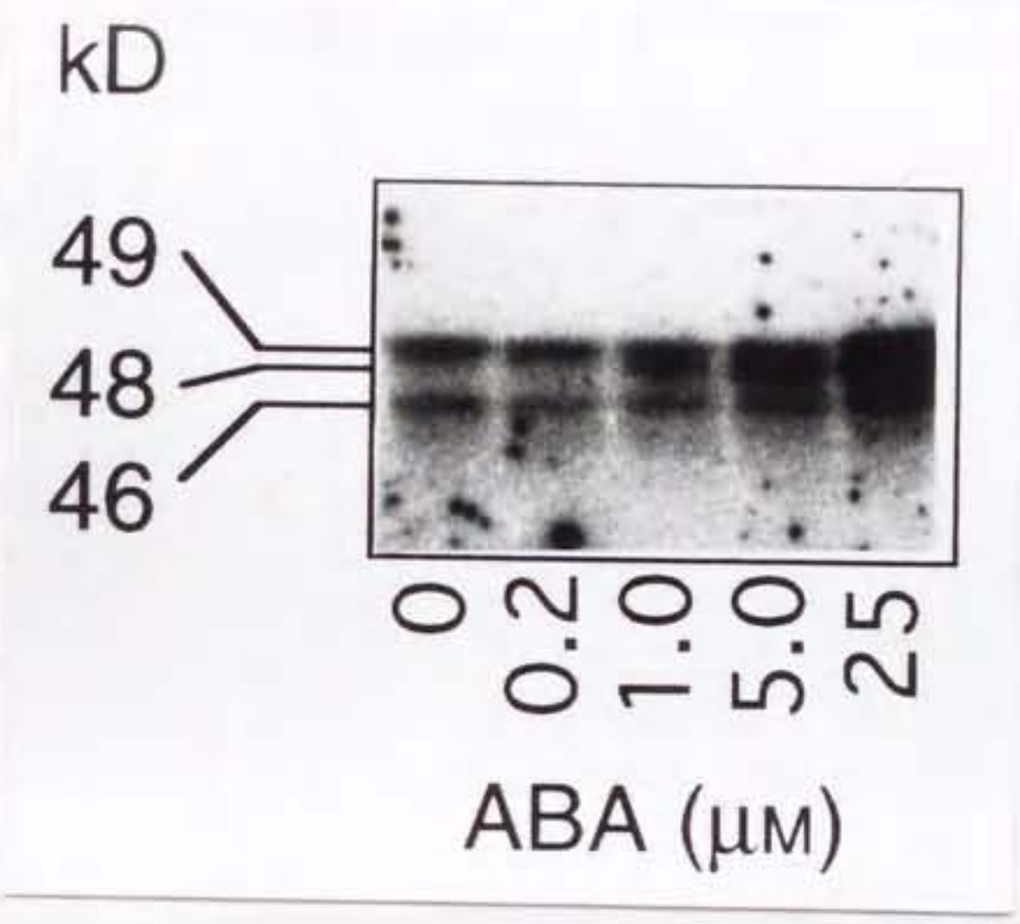

Figure 1-4. Effect of ABA concentration on the activation of the ABR kinase. GCPs were incubated with the indicated concentrations of ABA for 5 min. Protein kinase assay was done as in figure 3.

(data not shown).

Effects of protein kinase and protein phosphatase inhibitors --- Figure 1-5 shows the effects of protein kinase inhibitors and calyculin A on the activation of the ABR kinase by ABA. GCPs were treated with an appropriate inhibitor for 1 min prior to the application of ABA and the activity of the ABR kinase was assayed after 5 min. The activation of the ABR kinase was apparently suppressed by 1.5 μM staurosporine but not by KN62 and ML9. The activation of the ABR kinase seemed not to be inhibited by K-252a in this experiment, though it was slightly inhibited by K-252a in some of the repeated experiments. These results suggest that phosphorylation process(es) catalyzed by protein kinases which is sensitive to staurosporine (and K-252a), is involved in the activation of the ABR kinase. Calmidazolium and calyculin A had no effect on the activation. The activation of the 46- and 49-kD MBP kinases was inhibited a little with staurosporine or K-252a.

Effects of compounds related to regulation of $[Ca^{2+}]_{cyt}$ and cycloheximide --- GCPs were preincubated for 1 min with chemicals which might affect $[Ca^{2+}]_{cyt}$ and treated with 1 μM ABA. The Ca^{2+} chelator BAPTA suppressed the activation of the ABR kinase (Fig. 1-6). However, verapamil, La^{3+} and TMB-8 had no effect on the ABA-induced activation (Fig. 1-6). Nifedipine (10 μM), 1 mM Gd^{3+}, 1 mM Cd^{3+} and 1.5 μM A23187 had also no effect on the ABA-induced ABR kinase activation (data not shown).

Cycloheximide had no effect (Fig. 1-6), suggesting that protein synthesis de novo is not involved in the activation of the ABR kinase.

Viability of GCPs was examined 20 min after the treatment with the chemicals. As much as 99 % of GCPs were intact as judged by fluorescein diacetate staining.

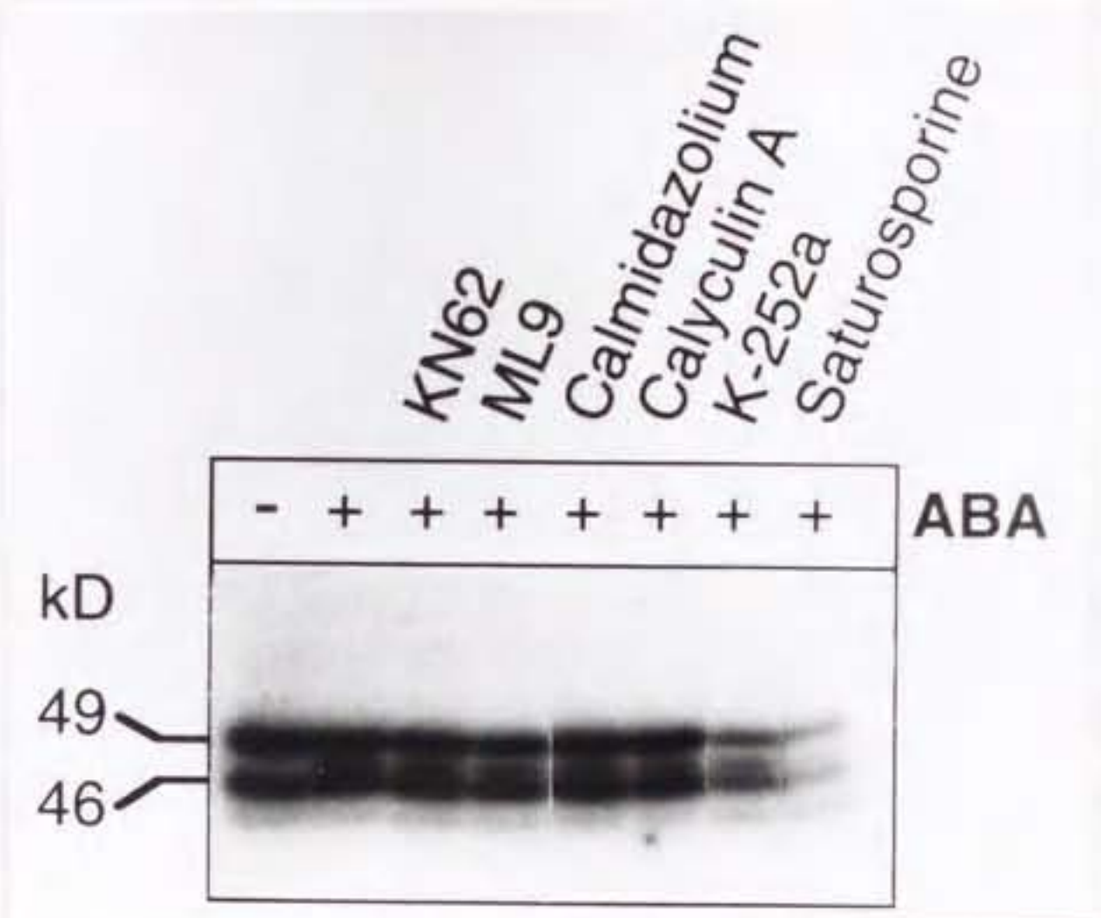

Figure 1-5. Effects of inhibitors of protein kinase or protein phosphatase on the activation of the ABR kinase. GCP suspension was incubated for 5 min in the absence (-) or the presence of 1 μM ABA (+). Inhibitors (15 μM KN62, 7.5 μM ML9, 10 μM calmidazolium, 1.5 μM calyculin A, 2.0 μM K-252a, 1.5 μM staurosporine) were added to GCP suspension 1 min before the addition of ABA.

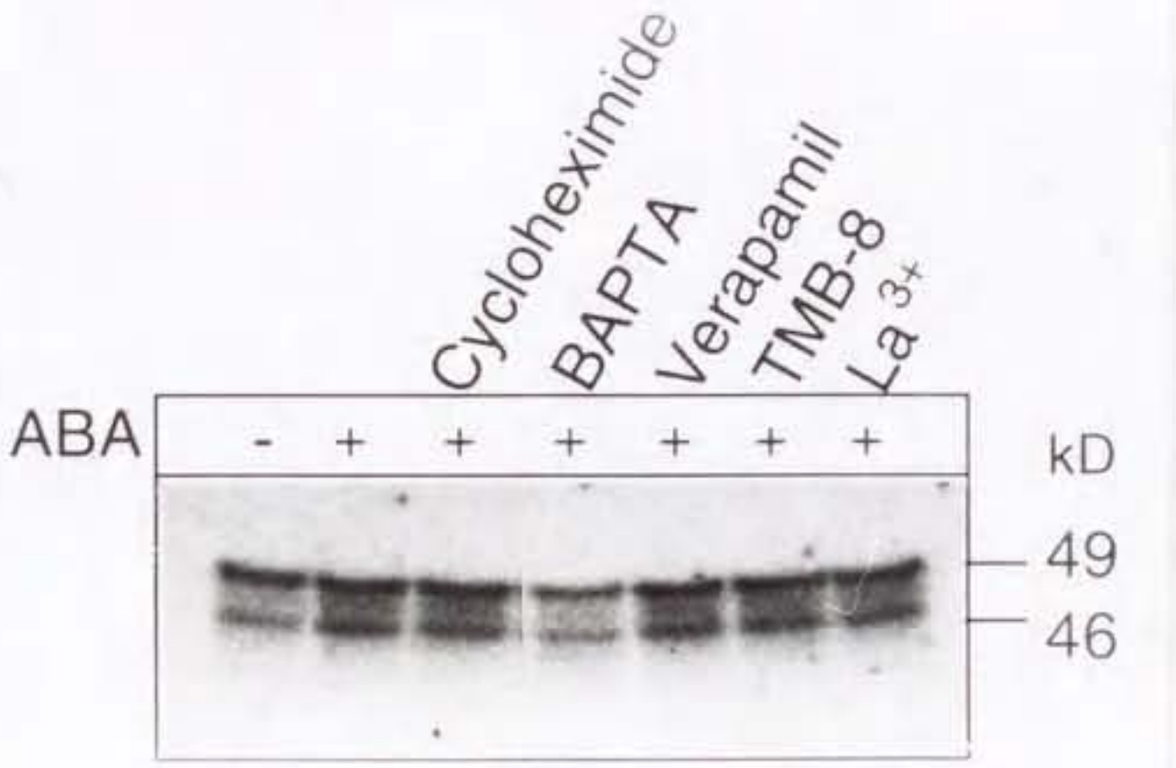

Figure 1-6. Effects of the chemicals which are related to cytoplasmic calcium concentration or transcription on the activation of the ABR kinase.

GCP suspensions were incubated for 5 min in the absence (-) or the presence of 1 μM ABA (+). Inhibitors (10 μM cycloheximide, 3 mM BAPTA, 30 μM verapamil, 200 μM TMB-8, 3 mM La^{3+}) were added to GCP suspensions 1 min before the addition of ABA.

Immunoprecipitation of Protein Kinases with an Anti-phosphotyrosine Antibody.

Extract of GCPs was immunoprecipitated with a monoclonal anti-phosphotyrosine antibody (PY-20) and the immunoprecipitated protein kinase was analyzed by the in-gel assay. The activity bands of the 46-kD and 49-kD MBP kinases were detected but the ABR kinase was not (Fig. 1-7). This indicates that the 49-kD and 46-kD MBP kinases may be phosphorylated at their tyrosine residue(s), but the ABR kinase may not be.

Effects of Staurosporine and K-252a on ABA-induced Stomatal Closure.

Epidermal peels were used to examine the effects of protein kinase inhibitors on the stomatal closure (Table 1-1). Stomata fully opened after incubating the epidermal peels under white light for 45 min. The stomatal aperture size was reduced by 20 % during 10-min incubation of epidermal peels with 1 μM ABA. The ABA-induced stomatal closure was completely inhibited by 2 μM staurosporine or 2 μM K-252a. These inhibitors did not apparently affect the stomata which were not treated with ABA.

1-4. Discussion

The treatment of *V. faba* GCPs with ABA activated a 48-kD protein kinase (ABR kinase). The activation was dose-dependent and specific for ABA. The activation was inhibited by a protein kinase inhibitor staurosporine indicating that the ABR kinase is phosphorylated by a putative activator protein kinase which might be activated directly or indirectly by ABA, though the activity band of the activator kinase might not be detected in the assay used here. A possibility of the ABR kinase activation by autophosphorylation may be eliminated, since no band was detected in

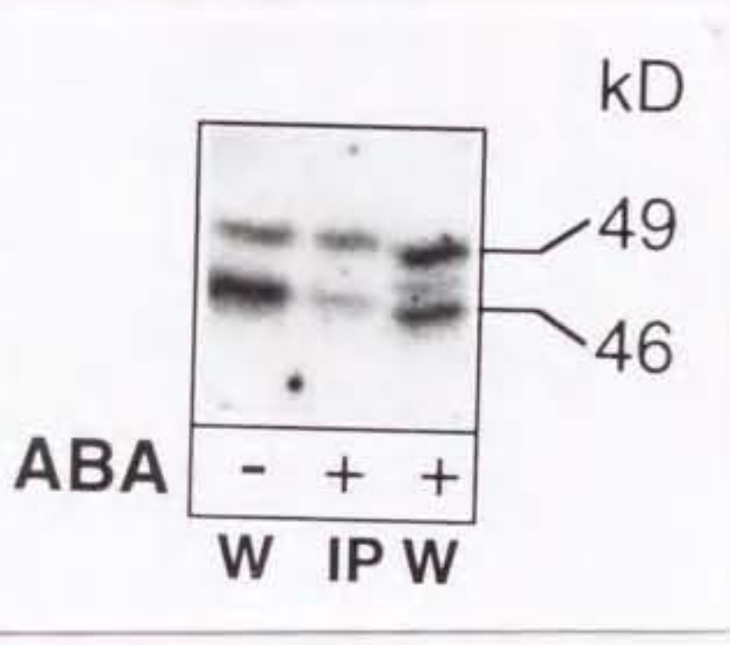

Figure 1-7. Immunoprecipitation of the MBP kinases by anti-phosphotyrosine monoclonal antibody. GCPs were incubated in the absence (-) or presence (+) of 1 μM ABA for 5 min. Immunoprecipitation was performed as described in "Materials and Methods". IP, immunoprecipitate; W, whole cell extracts.

Table 1. Effects of staurosporine and K-252a on the ABA-induced stomatal closure.

Treatment	Initial width (μm)	Width at 10 min (μm)	% of initial
No ABA	15.6±2.4 (n=6)	15.3±2.3	98.1
1 μM ABA	16.2±2.3 (n=10)	11.3±1.6	75.4
+1 μM staurosporine	13.4±3.6 (n=12)	13.8±3.2	103
+1 μM K-252a	16.1+1.5 (n=12)	16.3±1.5	101

ABA-treated GCPs in the in-gel assay using a no substrate protein-gel. The ABR kinase and its activator kinase may be components of a protein kinase cascade in signal transduction pathway linking ABA perception to stomatal closure.

Protein kinase cascades composed of extracellular signal-regulated protein kinases (ERKs) and mitogen activated protein kinases (MAP kinases) in eucaryotic cells have been shown to serve as key components in signal transduction pathways (for review see, Pelech and Sanghera, 1992). MAP kinases are phosphorylated at both tyrosine and threonine residues, and are consequently activated. With this respect, the ABR kinase may not be a MAP kinase since it was not immunoprecipitated by an anti-phosphotyrosine antibody. Recently, MAP kinase homologues were cloned from plants. A cDNA encoding a 44-kD MAP kinase has been cloned for the first time from alfalfa in plant and expressed in *Escherichia coli* (Duerr et al., 1993). Mizoguchi et al., (1994) have cloned two cDNAs encoding MAP kinases from *Arabidopsis thaliana* and expressed them in *E. coli*. The expressed proteins were phosphorylated by *Xenopus* MAP kinase kinase and activated. They also found that a 46-kD protein kinase which phosphorylated MBP was activated rapidly and transiently in auxin-starved tobacco BY-2 cells when treated with 2,4-D, and that protein kinase activities which phosphorylated one of the recombinant MAP kinase also increased rapidly by auxin treatment.

The 46-kD and 49-kD MBP kinases were immunoprecipitated with an anti-phosphotyrosine antibody. These MBP kinases possessed phosphotyrosine residue, they possibly belong to MAP kinase family. Their activities were high in GCPs, and slightly activated when high concentrations of ABA were applied to GCPs. However, the MBP kinases may not be the activator kinase for the ABR kinase, because ABA-

dependency for the activation of the MBP kinases is not consistent with that of the ABR kinase.

The 53-kD Ca^{2+}-dependent band which showed a comparable activity to those of the MBP kinases, is one of candidates for the activator of the ABR kinase. ABA-induced rapid increase in $[Ca^{2+}]_{cyt}$ of guard cell has been reported (McAinsh et al., 1990). The increase in $[Ca^{2+}]_{cyt}$ may cause the activation of the 53-kD calcium-dependent protein kinase located in the cytoplasm. Kinoshita and Shimazaki (1995) showed Ca^{2+}-dependent phosphorylation of several proteins in *Vicia* guard cell extracts. The 53-kD calcium-dependent protein kinase may respond to this phosphorylation. However, the effect of ABA on $^{45}Ca^{2+}$ fluxes into *Commelina* guard cells was variable (MacRobbie, 1989), and ABA-induced $[Ca^{2+}]_{cyt}$ elevation was observed in a minority of cells, even though stomatal closure always occurred in *Commelina* guard cells (Gilroy et al., 1991). These observations indicate that ABA-induced stomatal closure occurs in Ca^{2+}-dependent and -independent pathways. Our results showed that calcium channel inhibitors had no effect on the activation of the ABR kinase. Nevertheless, the addition of BAPTA in the suspension medium of GCPs suppressed the activation of the ABR kinase (Fig. 1-6), indicating that the activation of the ABR kinase required the external Ca^{2+} (Fig. 1-6) and that Ca^{2+} influx across the plasma membrane of GCPs may occur through a channel or transporter insensitive to the channel inhibitors used.

The activation of the ABR kinase started 2 min after ABA-treatment followed by a gradual increase up to 10 min, and the activated state was kept for at least 30 min. This suggests that the ABR kinase can continuously phosphorylate its target proteins during ABA-treatment after activation. The ABR kinase might phosphorylate directly or indirectly proteins related to ion transport in guard cells and regulate their activities.

The ion channels which are thought to be regulated by phosphorylation are the candidates for the target protein of the ABR kinase. A slow-anion channel which depolarized the plasma membrane of *Vicia* guard cell was suggested to induce the activation of K^+_{out} channel (Ward et al. 1995). Schmidt et al. (1995) reported that a slow anion channel was regulated by the phosphorylation level. This channel was strongly activated by the elevation of cytosolic ATP and *vice versa* abolished by removal of cytosolic ATP or the protein kinase inhibitors. Down-regulation of slow anion channels was inhibited by okadaic acid, a protein phosphatase inhibitor, indicating that the phosphorylation of the slow-anion channels leads to the stomatal closure. K^+ channels in the plasma membrane of guard cells are thought to be key components in stomatal movements. It was reported that okadaic acid inhibited K^+_{in} channels in guard cells (Li et al., 1994). This inhibition is consistent with our working hypothesis *i.e.* phosphorylation of the K^+_{in} channels by the ABR kinase would cause the inhibition of K^+ influx. Li et al. (1994) reported that okadaic acid stimulated an K^+_{out} channel. On the other hand, Thiel and Blatt (1994) reported that okadaic acid inhibited both the K^+_{in} and K^+_{out} channels. Slow-vacuolar channels which transport K^+ and Ca^{2+} may play a role in stomatal closing (Ward et al., 1995). Allen and Sanders (1995) reported that the slow-vacuolar channel was bimodally regulated by calcineulin. Low concentrations of calcineulin activated the slow vacuolar channel and high concentrations of calcineulin inhibited it.

ABA-induced stomatal closure in epidermal peels was inhibited by K-252a and staurosporine, indicating the involvement of protein kinase(s) in this process. These inhibitors also inhibited the activity of the ABR kinase. However, I can not conclude that ABA-induced activation of the ABR kinase is directly coupled with the ABA-induced stomatal closure, as the

both inhibitors have broad specificities against serine/threonine type kinases. Schmidt et al. (1995) reported that the ABA-induced stomatal closures in *Vicia* leaves was inhibited by K-252a or H7, and enhanced by okadaic acid, though the target protein kinases for these inhibitors were not identified.

Chapter 2

Phosphorylation of an Inward-rectifying Potassium Channel KAT1 by ABA-responsive Protein Kinase in *Vicia* Guard Cells

Summary

A 48-kD protein kinase was detected in *Vicia faba* guard cell protoplasts by an in-gel protein kinase assay using a recombinant peptide (KAT1C) of the carboxyl terminus of a voltage-dependent K^+_{in} channel cloned from *Arabidopsis thaliana*, KAT1. This protein kinase (KAT1C kinase) was detected when guard cell protoplasts were pretreated with ABA, but not detected by pretreatment with IAA, 2,4-D, kinetin nor GA_3. The activation of KAT1C kinase was dependent on time and concentration of ABA. The kinase activity was sensitive to staurosporine and K-252a, protein kinase inhibitors and insensitive to Ca^{2+}. These results indicate that KAT1C kinase phosphorylated the voltage-dependent K^+_{in} channel in response to ABA stimulation of stomatal guard cells. KAT1C kinase phosphorylated myelin basic protein, but not histone and casein. No activity of KAT1C kinase was detected in mesophyll cell protoplasts. These characteristics of KAT1C kinase was consistent with those of ABR kinase described in Chapter 1.

2-1. Introduction

Stomatal aperture is influenced by various environmental and internal signals such as phytohormones, light, temperature, humidity and water potential, and controls transpiration and CO_2 uptake under diverse environmental conditions (Zeiger, 1983; Mansfield et al., 1990). ABA is accumulated in response to drought, and induces a series of signaling events leading to stomatal closure (Mansfield et al. 1990). Stomatal closing is driven by a reduction of turgor pressure in guard cells that is caused by the efflux of K^+ and anions and a concomitant conversion of malate to starch

(Raschke, 1979; MacRobbie, 1981; Outlaw, 1983). ABA induces the rapid activation of non selective ion channels which causes membrane depolarization and allows Ca^{2+} influx from the extracellular space. The increase in cytosolic Ca^{2+} and the production of other signaling intermediates trigger further Ca^{2+} release from intracellular stores. Membrane depolarization, elevated cytosolic Ca^{2+}, and other signal events activate anion channels, which mediate anion release and long-term membrane depolarization. K^+ efflux through K^+_{out} channels driven by membrane depolarization enhance decrease of turgor (Ward et al., 1995). Most studies on the responses of guard cells to ABA tend to ABA-induced stomatal closure, but ABA inhibits stomatal opening, too (De Silva et al., 1985). Thus, similar ABA-induced events may occur in the inhibition of opening such as membrane depolarization and suppresion of K^+ and Cl^- uptake. For instance, it was reported that blue light-induced activation of PM H^+-pump was antagonized by ABA (Shimazaki et al., 1986).

Several factors have been proposed as the modulators of ion transporters in guard cells (Thiel and Wolf, 1997). Cytosolic Ca^{2+} inhibited $I_{K,in}$, and activated a slow-anion channel (Schroeder and Hagiwara, 1989) and a vacuolar K^+ channel (Ward and Schroeder, 1994). The activated GTP-binding protein was reported to inhibit $I_{K,in}$ (Fairley-Grenot and Assmann, 1991; Armstrong and Blatt, 1995). Kelly et al. (1995) reported that the regulation of $I_{K,in}$ by GTP-binding protein was also affected with Ca^{2+} and a rather complicated way. Ca^{2+} and nucleotide changed the voltage dependency of fast-anion channel (Hedrich et al., 1990). ATP activated $I_{K,in}$ and this activation was not mediated by phosphorylation (Wu and Assmann, 1995). Actin filaments was reported to have an effect on $I_{K,in}$ in guard cells (Hwang et al., 1997).

Protein phosphorylation is one of important regulatory mechanisms of

ion transport in guard cells. $I_{K,in}$ was activated by FK506, an inhibitor of protein phosphatase 2B (Luan et al., 1993), and inhibited by either okadaic acid or calycurin A, inhibitors of protein phosphatase 1 and 2A (Li et al., 1994; Thiel and Blatt, 1994). $I_{K,out}$ was also affected by the protein phosphatase inhibitors (Li et al., 1994; Thiel and Blatt, 1994). $I_{K,in}$ and $I_{K,out}$ were deactivated and activated, respectively, by the treatment of guard cells with ABA, but the change in these K^+ currents were suppressed in transgenic tobacco guard cells expressing *Arabidopsis thaliana abi1-1*, a mutant of protein phosphatase, (Armstrong et al. 1995). Slow-anion channel which is thought to be important in ABA-signaling was inhibited with K-252a (Schmidt et al., 1995). Grabov et al. (1997) reported that the conductance of Cl^- in the plasma membrane of tobacco guard cells altered after ABA-treatment, and calycurin mimicked the action of ABA. A slow-vacuolar channel was reported to be modulated by calcineurin (protein phosphatase 2B) (Allen and Sanders, 1995). However, protein kinases which phosphorylate the ion transporters in guard cells have not been identified. It has been reported that a recombinant CDPK phosphorylated and activated a chloride channel in vacuolar membrane of *Vicia* guard cells when applied during patch-clamp experiments (Pei et al., 1996). However, *in vivo* protein kinase in guard cells has not been identified. I identified a 48-kDa protein kinase as ABR kinase in *Vicia* guard cells by an in-gel protein kinase assay using myelin basic protein as a substrate (Chapter 1; Mori and Muto, 1997), but *in vivo* substrate was not identified and the role of this kinase in ABA-signaling remains unknown. Li and Assmann (1996) identified a similar protein kinase (AAPK) which was increased its autophosphorylation level by ABA-stimulus of *Vicia* guard cell protoplasts.

Several genes which encode ion transporters and express in guard cells have been reported. *KAT1* encoding a K^+_{in} channel (Anderson et al., 1992;

Schachtman et al., 1992; Ichida et al., 1997) expressed primarily in *A. thaliana* guard cells (Nakamura et al., 1995). *KST1*, a homolog of *KAT1* cloned from potato (Müller-Röber et al., 1995), was also expressed in guard cells. Expression of a PM H^+-pump (VHA1) cloned from a guard cell-cDNA library was not limited only in guard cells, but in mesophyll cells (Nakajima et al., 1995).

The recombinant products of genes described above would be a specific substrate for a specific protein kinase and be useful to identify the protein kinases which phosphorylate these peptides. In the present study, I examined the phosphorylation of the recombinant carboxyl terminal peptide of KAT1 by the ABR kinase in *V. faba* guard cell protoplasts to evaluate the role of the ABR kinase in stomatal movement in response to ABA-stimulus.

2-2. Materials and Methods

Plant material and isolation of GCPs and MCPs

V. faba L. were grown and GCPs and MCPs were isolated as described in Chapter 1.

Chemicals

TALON™ metal affinity resin and amylose resin were purchased from Clontech (Palo Alto, CA) and New England Biolabs (Beverly, MA), respectively. Lysozyme was from Wako Pure Chemical Industries (Osaka, Japan). TaKaRa Ex *Taq* polymerase was a product of Takara shuzo (Osaka, Japan). The other chemicals used were described in Chapter 1.

Construction and purification of recombinant KAT1 peptides.

A histidine-tagged 345-amino acid peptide of carboxyl terminus of KAT1 (KAT1C-H) was constructed as follows. I first introduced *Nde* I site at 997 bases downstream from the first ATG codon and *Bam*H I site at 26 bases downstream from the termination codon of *KAT1* cDNA by PCR using 23 mer and 21 mer oligonucleotides, TACATATGTTATCACACA-TTTGC and GGATCCATAAATGAAGTCGAG. PCR was performed in 50-μL volume in a thermal cycler (TaKaRa Thermal Cycler MP, Takara shuzo, Osaka, Japan). Twenty ng of DNA template was used in Ex Taq buffer with 1 unit of TaKaRa Ex *Taq* polymerase. Amplification was performed in 25 cycles as follows: 30 sec at 94°C, 30 sec at 55°C, 2 min at 72°C, and additional 10 min at 72°C at the end of cycles. The resultant DNA fragment which was ligated in pT7Blue T-vector (Novagen, Madison, WI) was sequenced to ensure no mutation occurred in its nucleotide sequence during PCR, digested with *Nde* I and *Bam*H I, and ligated into *Nde* I site and *Bam*H I site on pET-16b vector (Novagen, Madison, WI).

E. coli (BL21) harboring the recombinant plasmids was grown under ampicillin selection in LB medium. Overnight cultures grown at 37°C was diluted 100-fold and grown until OD_{600} reached up to 0.5 at 30°C. After adding 0.5 mM isopropyl ß-D-thiogalactopyranoside (IPTG), growth was continued for an additional 2.5 h. All subsequent purification steps were carried out at room temperature. A 400-mL aliquots of culture was centrifuged for 10 min at 5,000 *g*, and the pellet was resuspended in 20 mL of a lysis buffer containing 20 mM Tris-HCl (pH 8.0), 0.1 M NaCl, and 8 M urea. The resuspended cells were disrupted for 30 sec 8 times at volume 10 with a dip-type sonicator (Handy sonic model UR-20P, Tomy seiko, Tokyo). Broken cells were centrifuged at 400,000 *g* for 20 min. The resultant supernatant was adsorbed on TALON™ metal affinity resin (bed volume, ~4 mL). The column was washed with 5-column volume of a washing

buffer containing 20 mM Tris-HCl (pH 8.0), 8 M urea, 0.1 M NaCl, and 10 mM imidazole. KAT1C-H peptide was eluted with an elution buffer containing 20 mM Tris-HCl (pH 8.0), 8 M urea, 0.1 M NaCl, and 0.1 M imidazole. Finally, the resultant eluate was dialyzed against 190 mM Tris-HCl (pH 8.9) containing 8 M urea. This peptide should consist of MGHHHHHHHHHHSSGHIGGRH at amino-terminus and 345 amino acids from methionine at 333 downstream of the first methionine to carboxyl end of KAT1 at carboxyl terminus.

A peptide, 10-H, the product of the empty vector (pET-16b) was purified as in KAT1C-H. The amino acid sequence of this peptide should be MGHHHHHHHHHHSSGHIGGRHMLEDPAANKARKEAELAAATAEQ.

The 356 amino acids of carboxyl terminus from arginine 322 to carboxyl end of KAT1 was fused with maltose-binding protein at the carboxyl terminal (KAT1C-M). A *KAT1* cDNA which contains some silent mutations and has a unique *Xba* I site at 961 base from the first ATG codon (Uozumi et al., 1995) was subcloned into *Hin*d III site and *Not* I site on a modified pYES2 as described. This *KAT1* cDNA was digested with *Xba* I and *Pst* I, and the released DNA fragment coding a carboxyl terminal peptide of KAT1 was ligated into *Xba* I and *Pst* I sites on pMAL-c2 (New England Biolabs, Beverly, MA).

E. coli (BL21) harboring the recombinant plasmid was grown, treated with IPTG, and harvested. All subsequent purification steps were done at 4°C or on ice. The cells were resuspended in 20 mL of an extraction buffer containing 20 mM Tris-HCl (pH 8.0), 30 mM NaCl, 10 mM EDTA, 0.2 mM PMSF and 20 $\mu g\ mL^{-1}$ lysozyme, and incubated for 1 h. After adding 0.6% Triton X-100 and 150 mM NaCl, the cell suspension was incubated for 10 min, followed by sonication for 30 sec 6 times at volume 10. The disrupted cells were centrifuged at 100,000 *g* for 30 min. The resultant supernatant

was adsorbed on an amylose resin column (bed volume, 2 mL). The column was washed with 5-column volume of a column buffer containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA and 1 mM 2-mercaptoethanol, and eluted with the column buffer supplemented with 10 mM maltose. The eluate was dialyzed against 190 mM Tris-HCl (pH 8.9). KAT1C-M peptide should consist of a carboxyl-terminal peptide of KAT1 (356 amino acids from arginine at 322 downstream of the first methionine to the carboxyl end of KAT1) at carboxyl terminal and maltose-binding protein at amino-terminal.

As shown in Fig. 2-1 KAT1C-H and 10-H were highly purified but KAT1C-M was partially. These three recombinant peptides were used as the artificial substrates for the in-gel protein kinase assay.

In-gel protein kinase assay.

The in-gel protein kinase assay was performed according to the method described in Chapter 1 with a slight modification. Guard cell extracts were prepared as described previously (Mori and Muto, 1997). KAT1C-H (0.5 mg mL^{-1}), KAT1C-M (1.0 mg mL^{-1}), and 10-H (0.3 mg mL^{-1}) were immobilized in 10% polyacrylamide gels. After electrophoresis, washing out SDS from the gels and renaturing protein kinases, phosphorylation reaction was performed in a reaction mixture which contained 20 mM Tris-HCl (pH 7.5), 4 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 1 mM EGTA (unless otherwise indicated), and 25 μM [γ-^{32}P] ATP (740 kBq). The gels were dried after removing the unreacted ATP and radioactive bands were visualized by autoradiography using X-ray film (Fuji medical X-ray film RX, Fuji film, Ashigara, Japan).

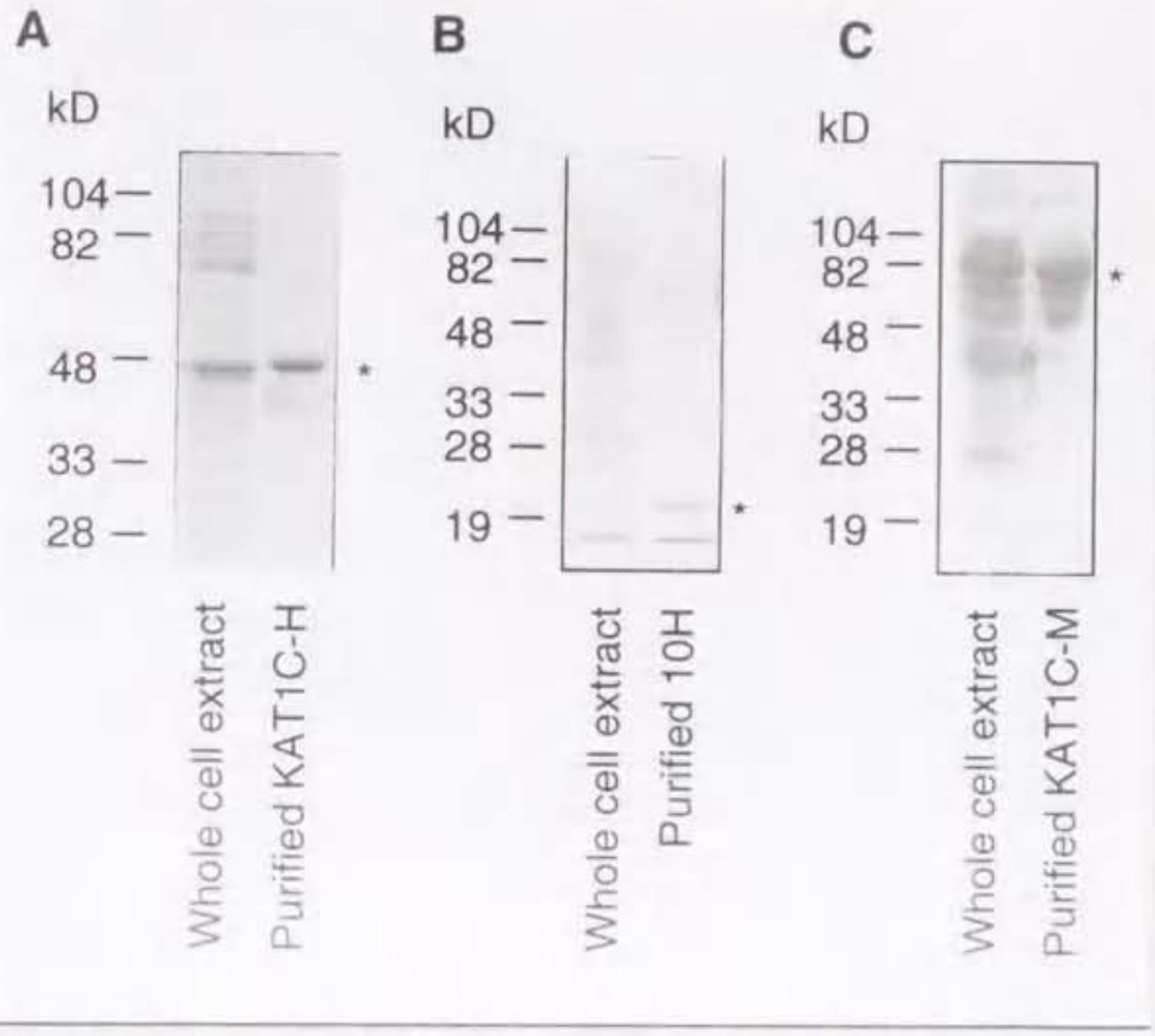

Fig. 2-1 Purification of KAT1C-H, KAT1C-M and 10H peptides from *E. coli* cells. The peptides were purified as described in "Materials and Methods". The purified peptides and the whole cell extracts were electrophoresed in SDS-polyacrylamide gels and stained with coomassie brilliant blue R-250. The molecular masses of standard proteins were indicated left of gels. Asterisks indicate the purified peptides. A, The whole cell extract from *E. coli* cells carrying pKAT1C-H and the purified peptides were electrophoresed in 10 % gel. B, The whole cell extract from *E. coli* cells carrying pKAT1C-M and the partially purified peptides were electrophoresed in 12.5 % gel. C, The whole cell extract from *E. coli* cells carrying pET-16b and the purified peptides were electrophoresed in 12.5 % gel.

Determination of protein concentration.

Protein concentration was determined by the methods of Bradford (1976) using BSA as a standard.

2-3. Results

Phosphorylation of KAT1 carboxyl terminal peptide by guard cell protoplast extracts.

Figure 2-2 shows in-gel protein kinase assays of guard cell protoplast (GCP) extracts and mesophyll cell protoplast (MCP) extracts in gels containing KAT1C-H, KAT1C-M, 10-H, or myelin basic protein. The treatment of GCPs with ABA activated a 48-kDa protein kinase (ABR kinase) which phosphorylated myelin basic protein (Fig. 2-2D) as in Chapter 1. Note that resolution of 49-, 48-, and 46-kDa protein kinase was not good, because the improved electrophoresis condition adopted in Chapter 1 was not employed. The ABR kinase activity was observed in GCP extracts, but not in MCP extracts. Only a single band indicating protein kinase activity was detected in ABA-treated GCP extracts using KAT1C-H as a substrate (Fig. 2-2A). A protein kinase in ABA-treated GCP extracts also phosphorylated the partially purified KAT1C-M (Fig. 2-2B). Both activities were detected at 48 kDa. No band was observed when the 10-H peptide was used as a substrate (Fig. 2-2C). There are two serine residue in the amino terminus of KAT1C-H which originated from pET-16b sequence. These serine residues were not phosphorylated by the 48-kDa protein kinase, because 10-H was not phosphorylated though it also had the same serine residues (see Materials and Methods). MCP extracts did not phosphorylate these substrates. Histone type III and dephosphorylated casein were not phosphorylated by GCP and MCP extracts (data not shown). These results

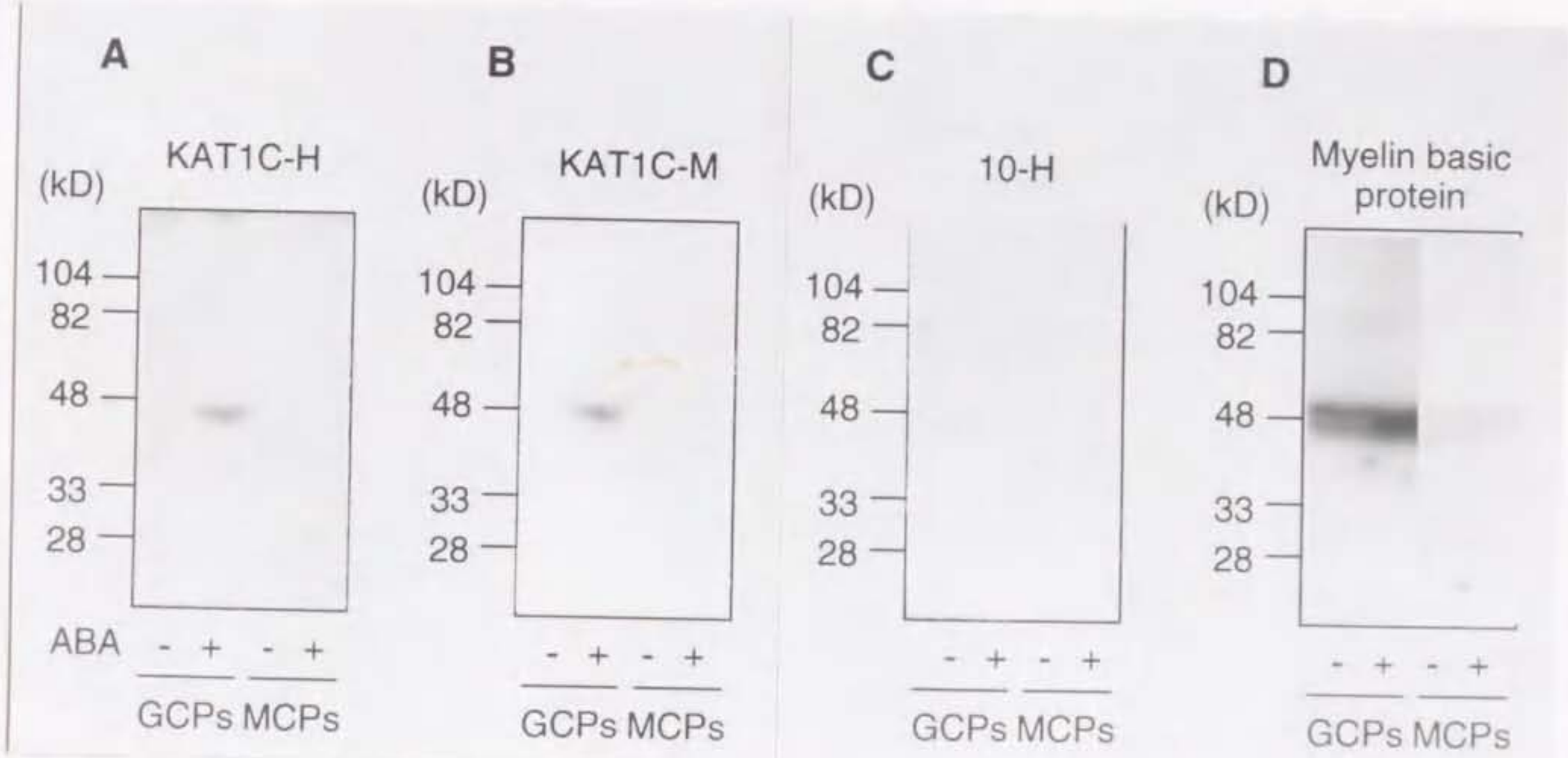

Fig. 2-2 Protein kinase activity in GCPs and MCPs from *Vicia faba*. GCPs and MCPs were incubated with 1 μM ABA (+) or 0.1% EtOH (solvent control, -) at 20℃ for 10 min. Each sample contained 10 μg protein (GCPs) or 20 μg protein (MCPs). KAT1C-H (A), KAT1C-M (B), 10H (C) and myelin basic protein (D) were used as the artificial substrates. The protein kinase assay was done as described in

indicate that the protein kinase has substrate specificity to the carboxyl terminus of KAT1 and myelin basic protein. This protein kinase was named as KAT1C kinase in the present study. Further studies were conducted with KAT1C-H as the substrate.

Activation of KAT1C kinase.

KAT1C kinase was activated by the treatment of GCPs with 10 μM ABA for 10 min, but neither by auxins (IAA, 2,4-D), cytokinin (kinetin), nor gibberellin (GA_3) at 10 μM (Fig. 2-3A). The radioactive band indicating the KAT1C kinase activity was detected with 0.2 μM ABA, and was further enhanced with the increasing concentrations of ABA (Fig. 2-3B), but it seemed not to be maximized up to 25 μM. Figure 2-3C shows the activation time course of the KAT1C kinase. When 1 μM ABA was applied to GCPs, a weak band was observed at 0.5 min and gradually enhanced up to 10 min. The activity remained at 30 min, even though reduced.

The phytohormone specificity in the activation of the KAT1C kinase was quite similar to the ABR kinase. The activation of KAT1C kinase occurred at slightly lower concentration of ABA (0.2 μM for the KAT1C kinase and 1.0 μM for the ABR kinase), and quicker (0.5 min for the KAT1C kinase and 2 min for the ABR kinase) compared with the ABR kinase assayed in the gel containing myelin basic protein.

Effects of Ca^{2+} on the activity and mobility in SDS-PAGE of KAT1C kinase.

The presence of Ca^{2+} during electrophoresis had no effect on the mobility of KAT1C kinase. The presence of Ca^{2+} in the protein kinase assay mixture caused no additional band and no activation of KAT1C kinase (Fig. 2-4). These results indicate that CDPK which has been shown to exist in

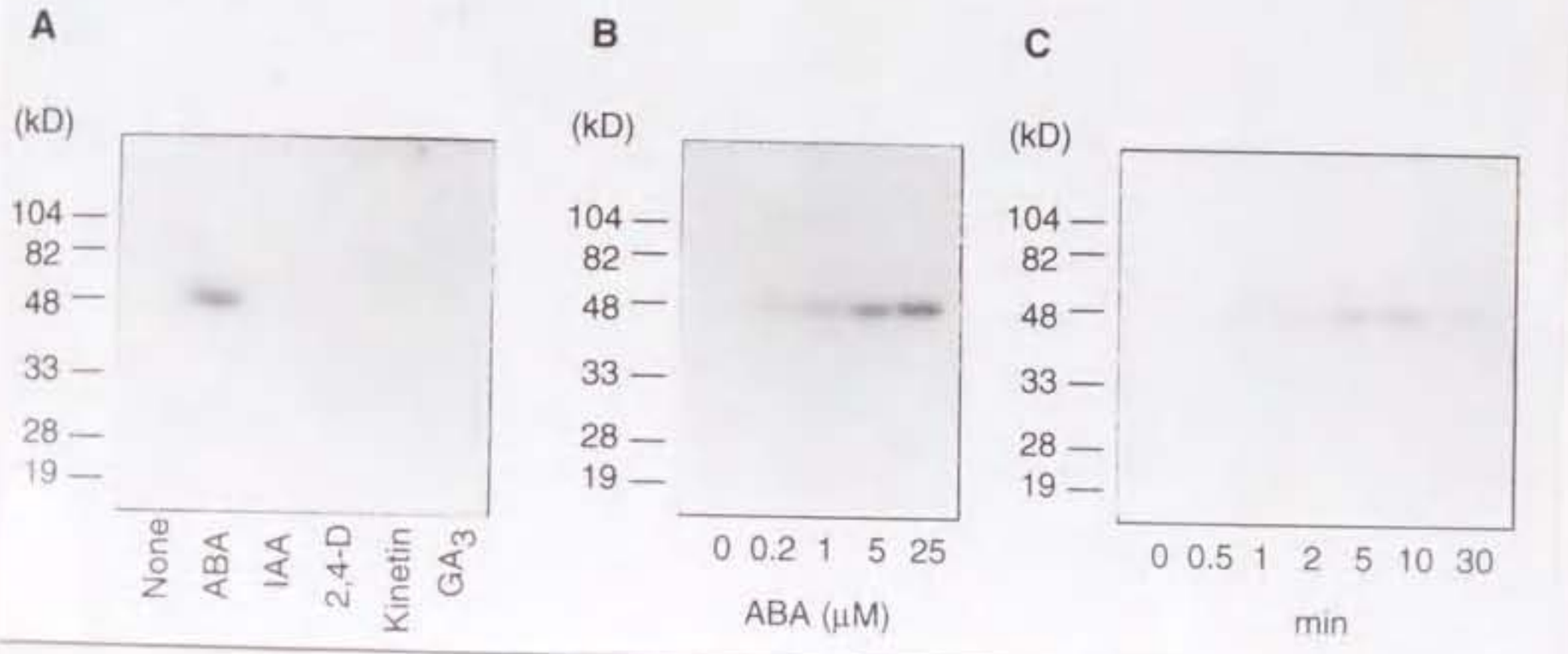

Fig. 2-3 Effect of phytohormones on the activation of KAT1C kinase.

A, GCPs were treated with 10 μM each phytohormone for 10 min. B, GCPs were incubated with the indicated concentrations of ABA for 10 min. C, GCPs were incubated with 1 μM ABA for the indicated periods. The protein kinase assay was done as in Figure 2. KAT1C-H was used as the substrate.

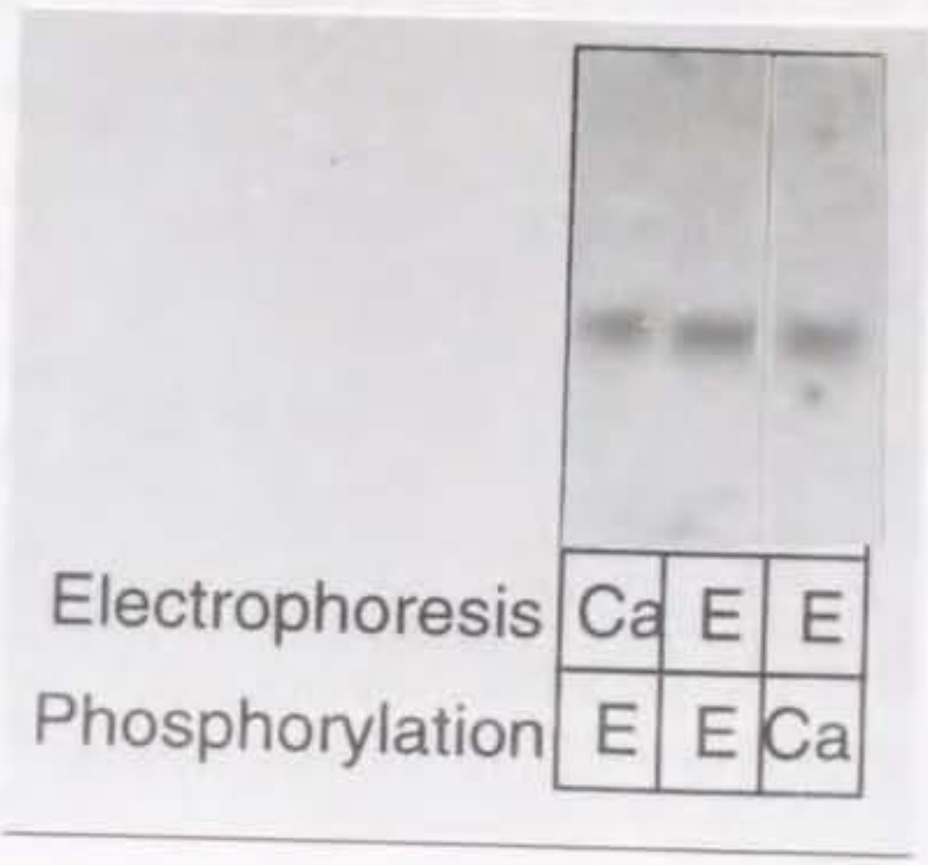

Fig. 2-4 Effects of Ca^{2+} on the protein kinase in GCP extracts. GCPs were treated with 1 μM ABA for 10 min. SDS-PAGE was carried out in the presence of 0.1 mM $CaCl_2$ (Electrophoresis, Ca) or 1 mM EGTA (Electrophoresis, E). The phosphorylation reaction was carried out with 1 mM EGTA (Phosphorylation, E) or 0.1 mM $CaCl_2$ (Phosphorylation, Ca). KAT1C-H was used as the substrate.

GCP extracts and to phosphorylate myelin basic protein did not phosphorylate KAT1C-H, and that KAT1C kinase was not Ca^{2+}-dependent.

Effects of several protein kinase inhibitors on the activity of the KAT1C kinase.

Phosphorylation reactions were performed in the presence of inhibitors after cutting the lanes separately. Among the protein kinase inhibitors tested, staurosporine and K-252a inhibited KAT1C kinase activity (Fig. 2-5A). KN-62 or ML-9 did not inhibit it at all. Figure 2-5B shows effect of concentrations of staurosporine and K-252a on the activity of KAT1C kinase. No band appeared in the presence of 1.5 μM staurosporine (Fig. 2-5A), and a weak band appeared at 0.75 μM (Fig. 2-5B). Five μM K-252a completely inhibited the activity of KAT1C kinase and the activity was detected at 1.0 μM (FIG. 2-5B). The concentrations of staurosporine and K-252a which completely inhibited the KAT1C kinase activity were similar to those for ABR kinase activity.

2-4. Discussion

KAT1 is a voltage-dependent and K^+_{in} channel, showing a remarkable homology to members of the animal outward-rectifying K^+ channel superfamily (for review, see Schroeder et al., 1994). It has six transmembrane hydrophobic domains S1-S6 which can be aligned with the core hydrophobic membrane-spanning domains S1-S6 of the animal outward-rectifying K^+ channel superfamily genes. It has a short amino terminus (~70 amino acids) facing to the cytosol and a long carboxyl terminus (~380 amino acids) facing to the cytosol.

The carboxyl terminus of KAT1 was phosphorylated with GCP extracts

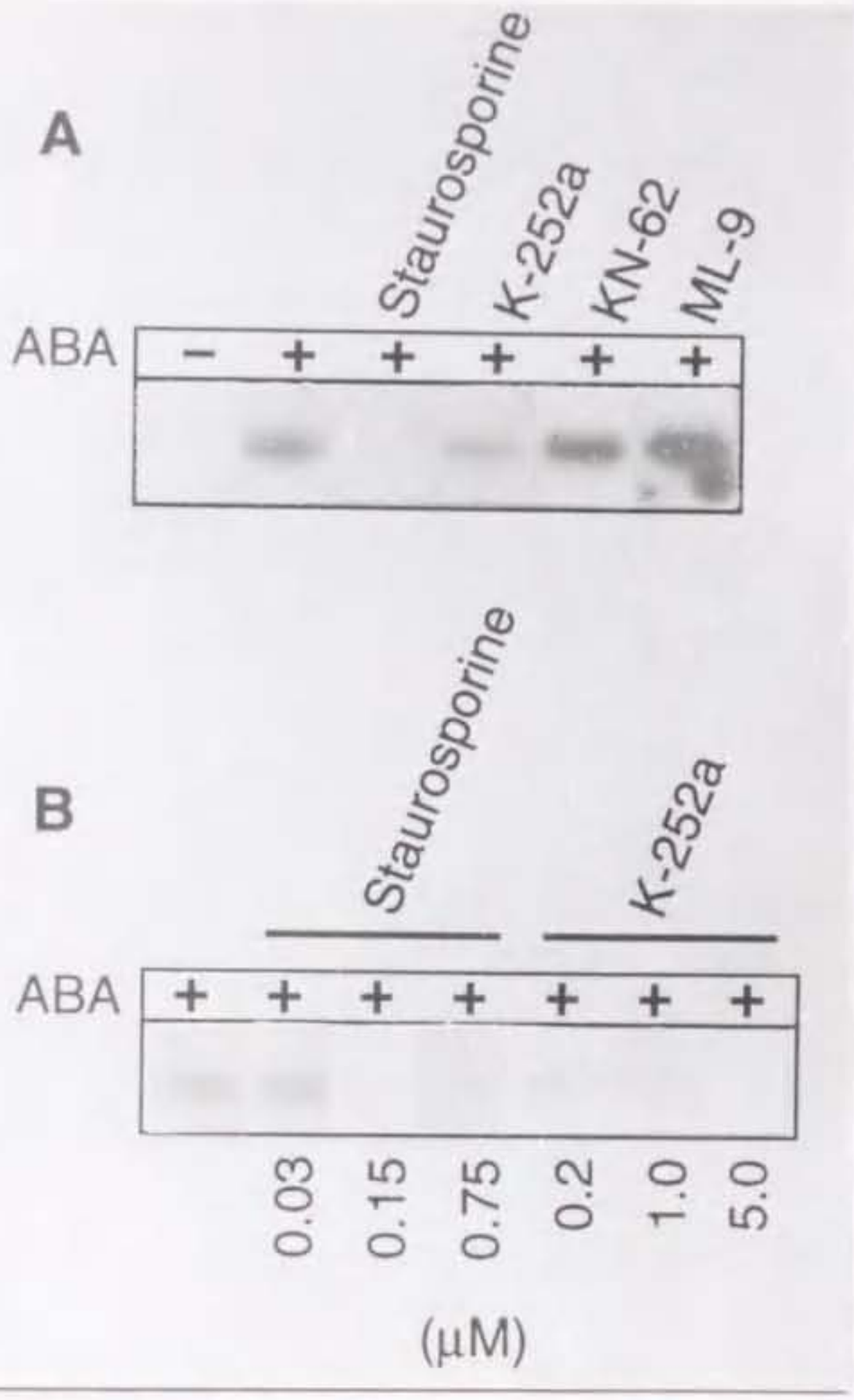

Fig. 2-5 Effects of protein kinase inhibitors on the activity of KAT1C kinase. A, GCPs were incubated without (-) or with 1 μM ABA (+) for 10 min. After electrophoresis and renaturing treatment, each lanes were cut into strips and protein kinase was assayed in the presence of 1.5 μM staurosporine, 1.0 μM K-252a, 15 μM KN-62, or 7.5 μM ML-9. B, GCPs were incubated with 1 μM ABA for 10 min. The protein kinase assay was done as in A, with the indicated concentration of staurosporine or K-252a. KAT1C-H was used as the substrate.

of *V. faba*. The protein kinase which phosphorylated KAT1 had an apparent molecular mass of 48 kD, was named as KAT1C kinase based on its substrate specificity. KAT1C kinase has similar characteristics to ABR kinase (see Chapter 1), i.e. 48-kD molecular mass, ABA-specificity among phytohormones in *in vivo* activation and protein kinase inhibitor-specificity of the activity. This strongly suggests that KAT1C kinase is identical to the ABR kinase. Compared with the ABR kinase, the KAT1C kinase was activated quicker and by lower concentration of ABA. This may be due to that KAT1C kinase prefer KAT1 peptide to myelin basic protein as substrate.

The sensitivity to the inhibitors suggests that KAT1C kinase may be a serine/threonine-kinase. There are 32 serine and 16 threonine residues in the carboxyl terminus of KAT1 used in this study. KAT1 and KST1 which is another K^+_{in} channel expressing in the guard cells have several conserved motifs containing serine and/or threonine residues. These motifs may be conserved in the K^+_{in} channels of guard cells in higher plants and may serve as a phosphorylation site to regulate the channel activities. In the further study, the phosphorylated amino acid residue(s) should be identified.

A putative cyclic nucleotide binding domain and an interaction site with microtubule-associated protein are suggested in the primary structure of the carboxyl terminal peptide of KAT1 (Schroeder et al., 1994; Marten and Hoshi, 1997). The carboxyl terminus of KAT1 seems to be important in the regulation of channel activity. Marten and Hoshi (1997) reported that the carboxyl terminus involves in the voltage-sensing and the stability of the channel activity interacting with microtubule. In the *Shaker*-related transient voltage-dependent K^+ channels, a function of the amino terminus has been authorized to be the fast-inactivation of the channel referred as "N-type inhibition" or "ball and chain model" (Hoshi et al., 1990; Zagotta et

al., 1990). Another model which is referred as "C-type inhibition" for the inhibition mechanism of *Shaker*-type K^+ channels showing slow inactivation (Hoshi et al., 1991) was also proposed. Recently, it was reported that the phosphorylation of the distal carboxyl terminus modulated the voltage-dependent activation of the *Snab*-related slow voltage-dependent K^+ channel, Kv2.1 (Murakoshi et al., 1997). This indicates that the phosphorylation of the carboxyl terminus can altar the activity of voltage-dependent K^+ channels. Since the amino terminus of *KAT1*-related plant K^+_{in} channel is too short to function as the ball of the ball and chain model (Schroeder et al., 1994), the carboxyl terminus may function as a modulator of the channel activity as Kv2.1.

Decrease in $I_{K,in}$ by phosphorylation of the K^+_{in} channel in *Vicia* guard cells was suggested from the observations that calycurin A and okadaic acid, potent inhibitors of protein phosphatase 1 and 2A, inhibited $I_{K,in}$(Li et al., 1994; Thiel and Blatt, 1994). In contrast, FK506, a specific inhibitor of protein phosphatase 2B, activated $I_{K,in}$ (Luan et al., 1993). These studies implicate alternative regulatory pathways involving phosphorylation/dephosphorylation of inward K^+ channels. The KAT1C kinase may phosphorylate K^+_{in} channel and regulate the $I_{K,in}$ in cooperation with protein phosphatase 1 and/or 2A. This model is favorable to explain the mechanism of inhibition of light-induced stomatal opening by ABA. In the further study, the inhibition of KAT1 by the KAT1C kinase should be examined. However, this model can not explain the ABA-induced stomatal closure. KAT1C kinase would phosphorylate channels other than K^+_{in} channel such as slow-anion channel and K^+_{out} channel. Slow-anion channel in the plasma membrane is thought to play a key role in ABA-induced long-term depolarization in guard cells. The depolarization evoked by the activation of the slow-anion channel may trigger the efflux of K^+. The slow-anion

channel was inhibited with K-252a, an inhibitor of serine/threonine-kinase, suggesting that this channel was activated by phosphorylation (Schmidt et al. 1995). The ABR kinase would be responsible to the phosphorylation of slow-anion channel. Li et al. (1994) reported that calycurin A and okadaic acid activated the $I_{K,out}$ examined in a whole cell configuration of patch-clamp experiments. The K^+_{out} channel would be also phosphorylated and activated by the ABR kinase.

Use of recombinant peptides for protein kinase assay was limited in a few reports. A recombinant MAP kinase (Gomez and Cohen, 1991) and MAP kinase kinase (Dent et al., 1994) have been utilized to analyze the MAP kinase cascades. In the present study, I successfully identified and characterized a protein kinase which phosphorylated KAT1C in the in-gel protein kinase assay. The results in this study showed that the in-gel protein kinase assay using recombinant peptides is a powerful method for identifying the protein kinases which phosphorylate their target proteins.

Chapter 3

Does ABA-responsive Protein Kinase in *Vicia* Guard Cells Phosphorylate a Plasma Membrane H^+-ATPase VHA1?

Summary

Phosphorylation of a H^+-ATPase of *Vicia* guard cell by guard cell protoplast (GCP) extracts was examined by in-gel protein kinase assay. Recombinant peptide of VHA1, F2 (cytosolic loop between 4th and 5th hydrophobic domains) and HF35 (carboxyl terminus) were not phosphorylated by GCP extracts in the presence and absence of Ca^{2+}. This result suggests that neither ABR kinase nor CDPK in guard cells phosphorylated the cytosolic loop of VHA1 where a characteristic phosphorylation site of P-type ATPase locates, and the carboxyl terminus where the autoinhibitory domain of P-type ATPase locates.

3-1. Introduction

Stomatal movements is coupled with ion transports, and ion fluxes through ion channels are driven by membrane potential. Plasma membrane (PM) H^+-pumps primarily regulate membrane potential of PM and play a key role in ion transport in guard cells and in regulation of stomatal aperture. The activity of PM H^+-pumps is regulated in response to environmental fluctuations to control ion fluxes in guard cells, e.g. blue light activates PM H^+-pumps and induces subsequent stomatal opening (Shimazaki et al., 1986). The modulation mechanisms of H^+-pumps in guard cells have been studied vigorously by Shimazaki and colleagues. They showed that PM H^+-pumps were regulated via phosphorylation by myosin light chain kinase-like protein kinase based on pharmacological experiments (Shimazaki et al., 1992). However, the protein kinase which involved in the modulation of the PM H^+-pumps is not identified.

PM H^+-pumps possess commonly 10 membrane-spanning regions that

anchor them in PM (for review, see Michelet and Boutay, 1995). Amino and carboxyl termini of the protein are on the cytosolic side of the membrane. The aspartate residue phoshporylated during the catalitic cycle and four ATP-binding sites are on the loop between the 4th and the 5th membrane-spanning region. An autoinhibitory domain is present at the carboxyl terminus. Many cDNAs coding PM H^+-pump have been isolated from plants. Two PM H^+-pump genes (*VHA1* and *VHA2*) from cDNA library originated from isolated *Vicia* guard cell protoplasts (GCPs) were cloned by Nakajima et al. (1995). The complete nucleotide sequence of *VHA1* predicted that VHA1 possesses 8 membrane-spanning , a loop region existing between the 4th and the 5th hydrophobic regions and an autoinhibitory domain at the carboxyl terminus. The autoinhibitory domain which regulates the activity of H^+-pump is predicted to be phosphorylated by protein kinase and modulate the activity. Schaller and Sussman (1988) reported that CDPK phosphorylate H^+-ATPase in oat roots.

In the present study, I focused on the identification of a protein kinase which phosphorylates the H^+-pump (VHA1) in *Vicia* guard cells using the in-gel protein kinase assay.

3-2. Materials and Methods

Plant material and isolation of GCPs.

V. faba L. was grown and GCPs were isolated as described in Chapter 1.

Construction of recombinant peptides.

The plasmid of recombinant peptides containing the fragments of VHA1 was constructed by Dr. Nobuyoshi Nakajima, National Institute for

Environmental Studies, Tukuba 305. T7 gene 10 fusion proteins of VHA1 (HF35, from Lys305 to Val653; F2, from Trp853 to Thr956) were constructed as follows. VHA1 cDNA fragment was amplified by PCR using primers AAGCTTTCTCAGCAAGGTGC and GAATTCTGACACTGCA-TAGATCG for HF35, and AAGCTTTGGTCAAATCTAGA and GAAT-TCTGTGTTGCCTTTGTAC for F2. The resultant DNA fragments which was ligated in pT7Blue T-vector (Novagen, Madison, WI) were sequenced to ensure no mutation occurred in its nucleotide sequence during PCR, digested with *Hin*d III and *Eco*R I, and ligated into *Hin*d III and *Eco*R I sites on pGEMEX-1 vector (Promega, Madison, WI). As a negative control, the transcript of antisence VHA1, BF, was also constructed using the native restriction enzyme site in *VHA1*, *Hin*d III at 1064 and *Eco*R I at 998.

Purification of recombinant peptides

BF, HF35 and F2 were purified as follows. The expressed peptides were accumulated in inclusion bodies in *E. coli* cells. *E. coli* (JM109) harboring the recombinant plasmids was grown under ampicillin selection in LB medium. Overnight cultures grown at 37°C was diluted 100-fold and grown until OD_{600} reached up to 0.5 at 30°C. After adding 0.5 mM IPTG, growth was continued for an additional 2.5 h. All subsequent purification steps were carried out at 4°C, otherwise indicated. A 100-mL aliquot of culture was centrifuged for 10 min at 5,000 *g*, and the pellet was resuspended in 5 mL of a lysis buffer containing 50 mM Tris -HCl (pH 8), 1 mM EDTA, 100mM NaCl, 100 μM PMSF and 20 μg/mL lyzozyme. The resuspended cells were stirred for 20 min and supplemented with 1.3 mg/mL deoxycholic acid and incubated at 37°C for 15 min. After the suspension became viscous, DNase I (20 μg/mL) were added, and then the suspension was incubated for 30 min on ice and centrifuged at 12,000 *g* for

15 min at 4°C. The resultant pellet was suspended in 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM EDTA, 100 mM NaCl and 0.5% (w/v) Triton X-100 and incubated for 5 min at room temperature, and then recentrifuged at 12,000 *g* for 15 min at 4°C. Finally, the resultant pellet was dried *in vacuo* and dissolved in 1% SDS. The purified inclusion bodies were used as the substrate for the in-gel protein kinase assay.

In-gel protein kinase assay.

The in-gel protein kinase assay of ABA-treated protoplasts was performed as described in Chapter 2 using 10% polyacrylamide gels in which HF35 (1.0 mg mL^{-1}), F2 (1.0 mg mL^{-1}), and BF (1.0 mg mL^{-1}) were immobilized

3-3. Results

In-gel protein kinase assay

F2 peptide corresponds with the cytosolic loop between 4th and 5th membrane-spanning region in VHA1. HF35 peptide corresponds with the carboxyl terminus of VHA1 including the autoinhibitory domain. No band was observed in the gel containing F2 or HF35 peptides, when assayed in the presence of Ca^{2+} (Fig. 3-1) and in the absence of Ca^{2+} (data not shown). BF peptide which is a transcript of an antisense *VHA1*, used as a negative control, and not phosphorylated with the GCP extracts, too (data not shown). These results suggest that CDPK and ABR kinase in *Vicia* guard cells did not phosphorylate the center loop nor carboxyl terminus of VHA1.

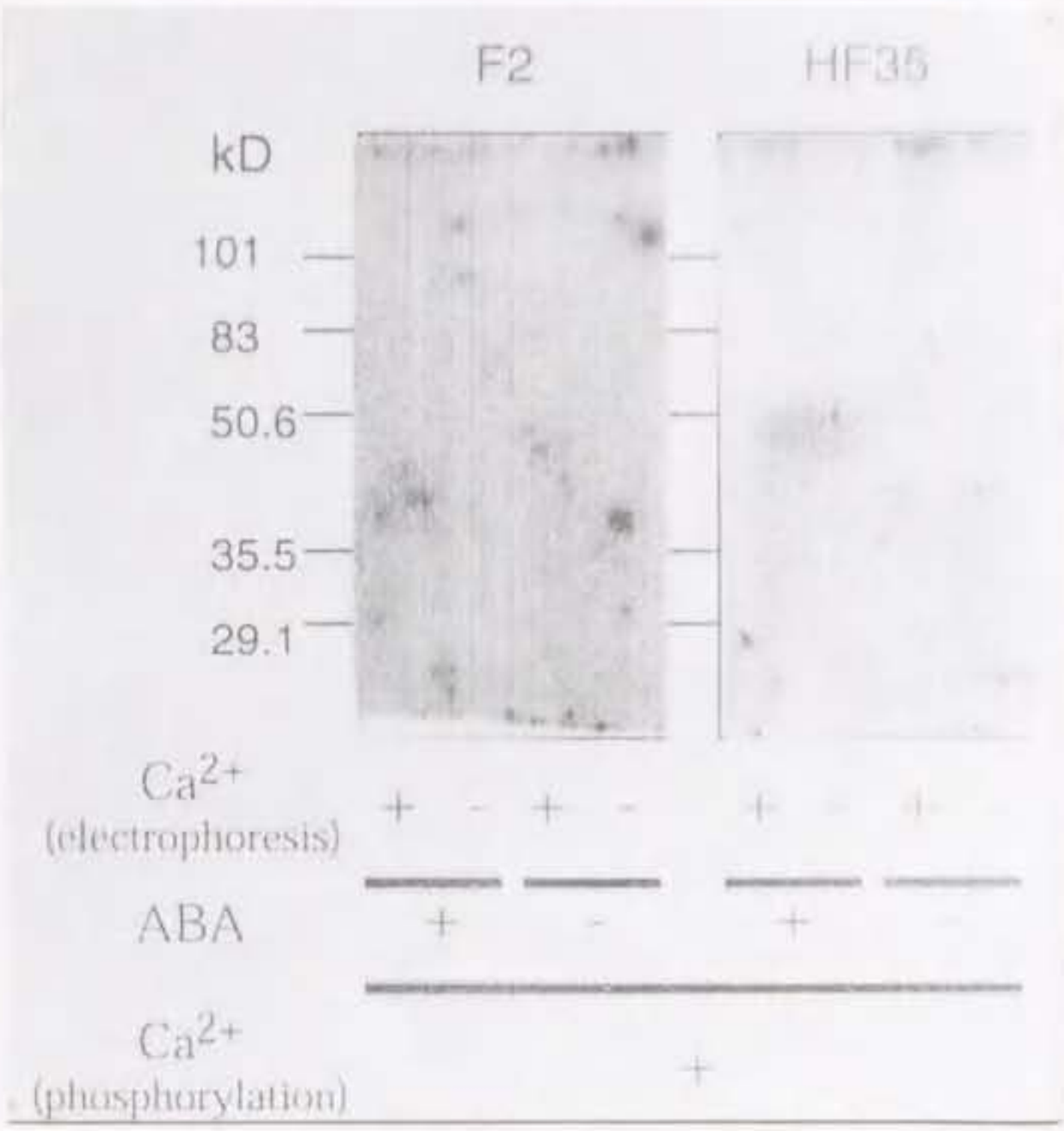

Fig. 3-1 Phosphorylation of the recombinant peptides of VHA1 with GCPs extracts. GCPs were incubated with 1 μM ABA (+) or 0.1% EtOH (solvent control, -) at 20℃ for 10 min. Each sample contained 10 μg protein (GCPs) or 20 μg protein (MCPs). Samples were electrophoresed in the presence (+) or the absence (-) of Ca^{2+}. F2 and HF35 were used as the substrates. The protein kinase assay was done as described in "Materials and Methods" in the presence of 100μM Ca^{2+}.

3-4. Discussion

Schaller and Sussman (1988) reported that PM H^+-ATPase was phosphorylated in a Ca^{2+}-dependent manner in oat root. VHA1 was not phosphorylated by guard cell extract, though the 53-kD CDPK in *Vicia* guard cells phosphorylated myelin basic protein (Chapter 1). A member of cDNA homologue encoding CDPK isoforms has been isolated from *Arabidopsis* (Harper et al., 1991), suggesting the presence of CDPKs with different substrate specificities. No CDPK in guard cells may have specificity to an isoform of PM H^+-ATPase in guard cells, VHA1. The fact that the recombinant VHA1 peptides were not phosphorylated with the ABR kinase nor CDPK, supporting that ABR kinase is highly specific to KAT1C.

Chapter IV

Salicylic Acid Induces a Cytosolic Ca^{2+} Elevation in Yeast.

Summary

Cytosolic free calcium ion concentration ($[Ca^{2+}]_{cyt}$) after a salicylic acid (SA)-stimulus was monitored in *Saccharomyces cerevisiae* cells expressing apoaequorin in which a Ca^{2+}-sensitive luminescent protein, aequorin, is constituted by after incubating cells with coelenterazine. SA induced a transient $[Ca^{2+}]_{cyt}$ elevation which was dependent on the concentration of SA and pH of the SA solution. The SA-induced $[Ca^{2+}]_{cyt}$ elevation was not reduced in Ca^{2+}-deficient medium, suggesting that Ca^{2+} was mobilized from an intracellular Ca^{2+} store(s). Benzoic acid, butyric acid and sorbic acid did not induced a $[Ca^{2+}]_{cyt}$ elevation.

4-1. Introduction

The experiments in Chapter 1 demonstrated that ABA activates a 48-kD protein kinase (ABR kianse) in guard cells which is predicted to involve in ABA-signaling. Specific phosphorylation of a K^+_{in} channel by ABR kinase was shown in Chapter 2. In the further study, changes of K^+_{in} channel activity after phoshorylation by ABR kinase should be examined. A pharmacological experiment in Chater 1 indicated that a $[Ca^{2+}]_{cyt}$ elevation occurred prior to the activation of the ABR kinase. Ca^{2+} has been reported to have various effect on the activity of ion transporters in plant (for review, see Thiel and Wolf, 1997). The aim of study in this chapter is to establish the experimental system in which Ca^{2+}-related functions of plant ion transporters can be analyzed.

Stimulus-coupled change in $[Ca^{2+}]_{cyt}$ in animal cells have been studied using fluorescence dyes. Plant cells expressing apoaequorin which constitutes a Ca^{2+}-dependent luminescent protein, aequorin, with the

chromophore coelenterazine, were successfully used for investigating the stimulus induced Ca^{2+} transients (Knight et al., 1991). However, studies on Ca^{2+} mobilization in yeast are limited in a few reports. For instance, Iida et al. (1990) reported a $[Ca^{2+}]_{cyt}$ elevation induced by the mating pheromone (α factor) in **a**-mating type cells using a Ca^{2+}-indicator (fura-2). Nakajima-Shimada et al. (1991) transformed yeast cells with apoaequorin and monitored $[Ca^{2+}]_{cyt}$ changes in response to mating pheromone and glucose in glucose-starved yeast cells. An immediate and transient $[Ca^{2+}]_{cyt}$ elevation in response to a hypoosmotic shock has been reported in apoaequorin transgenic yeast cells (Batiza et al., 1996). However, the Ca^{2+} mobilizing mechanism into cytosol in yeast cells remained unclear in yeast cells. Thus, I examined that Ca^{2+} mobilization in yeast cells using apoaequorin-expressing yeast cells.

Nonsteroidal anti-inflammatory agents, such as salicylic acid (SA) or acetyl SA (aspirin), have been reported to induce cell cycle arrest and/or apoptosis in cancer cells in animal (Elder et al., 1996; Shiff et al., 1996) and heat shock response in human cells (Jurivich et al., 1992 and 1995). In plant cells, SA functions as an intercellular and/or an intracellular signal relating to the systemic acquired resistance, a disease response induced by pathogen infection (Klessig and Malamy, 1994). In yeast, it has been reported that SA decreased the viability of the cell (Zetterberg, 1979; Romano and Suzzi, 1985). Recently, Giardina and Lis (1995) reported that sodium salicylate stimulates the binding of a heat shock transcription factor to the promoter region of a heat shock gene and represses the expression of a HSP gene. However, the upstream in the signal transduction pathway involving in SA-signaling has not been elucidated well in any organisms.

In the present study, I focused on a $[Ca^{2+}]_{cyt}$ change in response to SA treatment in yeast cells.

4-2. Materials and Methods

Culture of cells and constitution of aequorin.

Yeast cells [*S. cerevisiae* H208-3B carrying pGAPAQ1 (Nakajima-shimada, 1991)] were grown in a synthetic medium (SD medium) minus tryptophan at 25°C up to 5 x 10^6 cells/mL. The cells were collected by centrifugation at 700 x *g* for 10 min, washed twice, and resuspended at 1 x 10^8 cells/mL in a glucose-deficient SD (SD -glucose) medium to suppress the growth of the cells. The resuspended cells were incubated with 20 μM coelenterazine at 25°C for 3 h with shaking (130 rpm) to constitute aequorin (Nakajima-shimada, 1991). Cells were washed twice with SD -glucose medium to remove the remaining coelenterazine and resuspended in SD -glucose medium at 2.5 x 10^7 cells/mL. This cell-suspension was used for the monitoring of aequorin luminescence within 2 h.

Chemicals.

Coelenterazine, 2-(*p*-hydroxylbenzoyl)-6-(hydroxyphenyl)-3,7-dihydroimidazo[1,2-**a**]pyrazin-3-one), were chemically synthesized as reported (Isobe et al., 1994). SA was dissolved in 400 mM Mes-KOH (pH 4.0, unless otherwise indicated) containing 20% ethanol and added to the cell suspension at 1/20 volume (10 μL). The final concentration of SA was 5 mM, unless otherwise indicated. The other chemicals were highest grade commercially available.

Monitoring of aequorin luminescence.

Monitoring of aequorin luminescence was done essentially as reported (Takahashi et al., 1997). The coelenterazine-loaded cell-suspension (190

μL) was transferred to a glass tube settled in a luminometer (Chem-Glow photometer, American Instrument Co., Silver Spring, MD). Aequorin luminescence induced by the addition of SA or other compounds was recorded with a pen recorder (Rikadenki Co. Tokyo), and was expressed as relative luminescence unit (rlu).

4-3. Results

SA-induced $[Ca^{2+}]_{cyt}$ elevation

The aequorin luminescence indicating $[Ca^{2+}]_{cyt}$ elevation was observed following the addition of SA to the aequorin-containing yeast cells (Fig. 4-1). When treated with 2 mM SA, a gradual luminescence increase was observed after a lag period (Fig. 4-1A, left middle trace). The higher concentrations of SA induced the stronger luminescence with a shorter lag period. When treated with 5 mM SA , luminescence initiated at 5 s and peaked at 30 s and returned quickly to the original level within 5 min (Fig. 4-1A, right bottom trace). No increase in luminescence was observed below 1 mM SA. The peak-height of aequorin luminescence was dependent on the concentration of SA (Fig. 4-1B). The peak-time also shifted depending on SA concentration.

The SA-induced $[Ca^{2+}]_{cyt}$ elevation was dependent on the pH of SA solution (Fig. 4-2). The lower pH was much effective within pH range examined (pH 4 - 5.5). No aequorin luminescence was observed when the buffer without SA was applied to the cell suspension at any pH value.

Effects of the other organic acids on $[Ca^{2+}]_{cyt}$

Several organic acids were subjected to the aequorin-containing cells. SA induced a marked $[Ca^{2+}]_{cyt}$ elevation with a lag period as observed above.

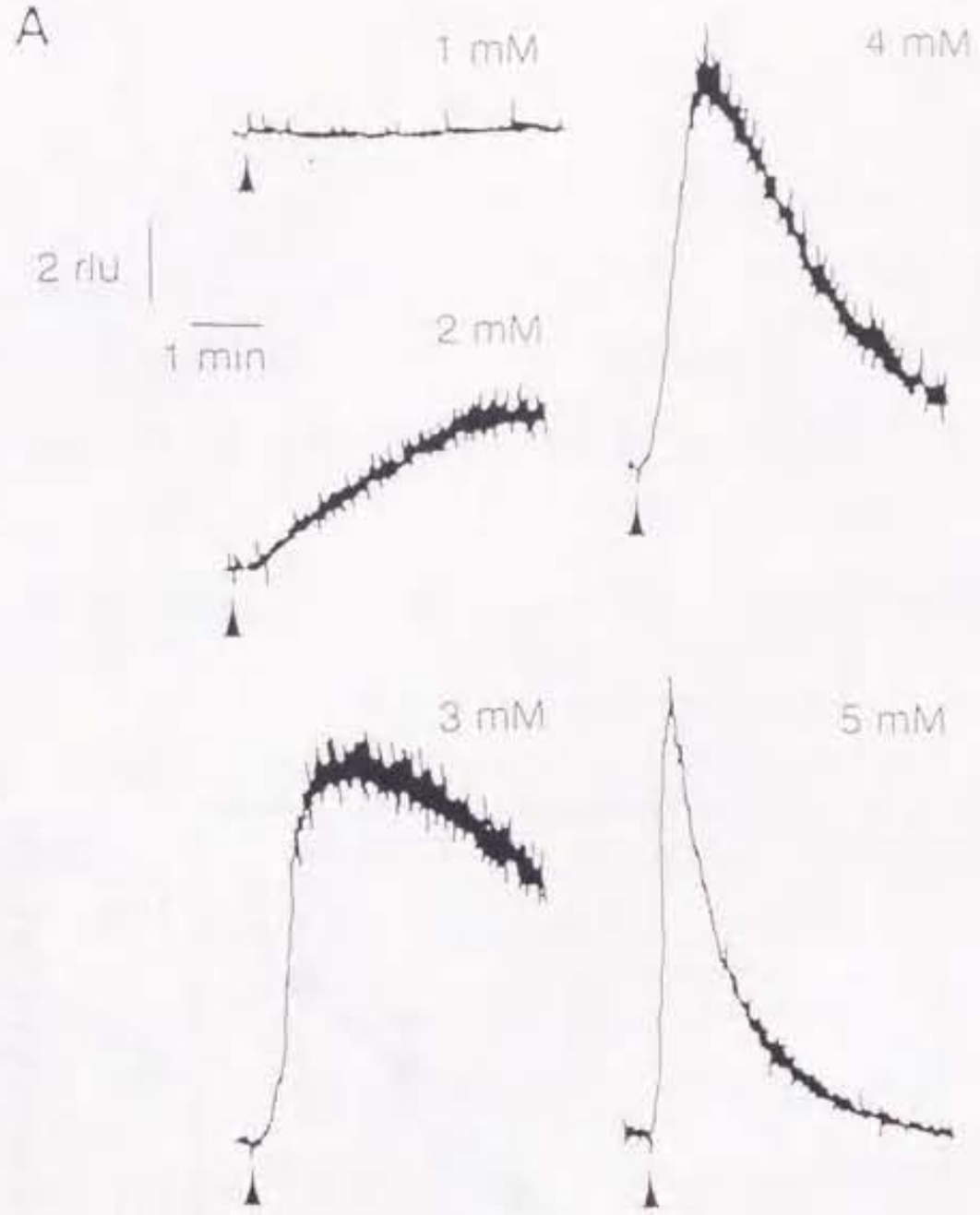

Fig. 4-1. Effect of various concentration of SA on the $[Ca^{2+}]_{cyt}$ in yeast cells. SA was added to the coelenterazine-loaded cells as described in "Materials and Methods". A, Various concentration of SA (0-5 mM) was added to the cell suspension where indicated by the arrowheads. B, The peak height of the luminescence was plotted as a function of SA concentration.

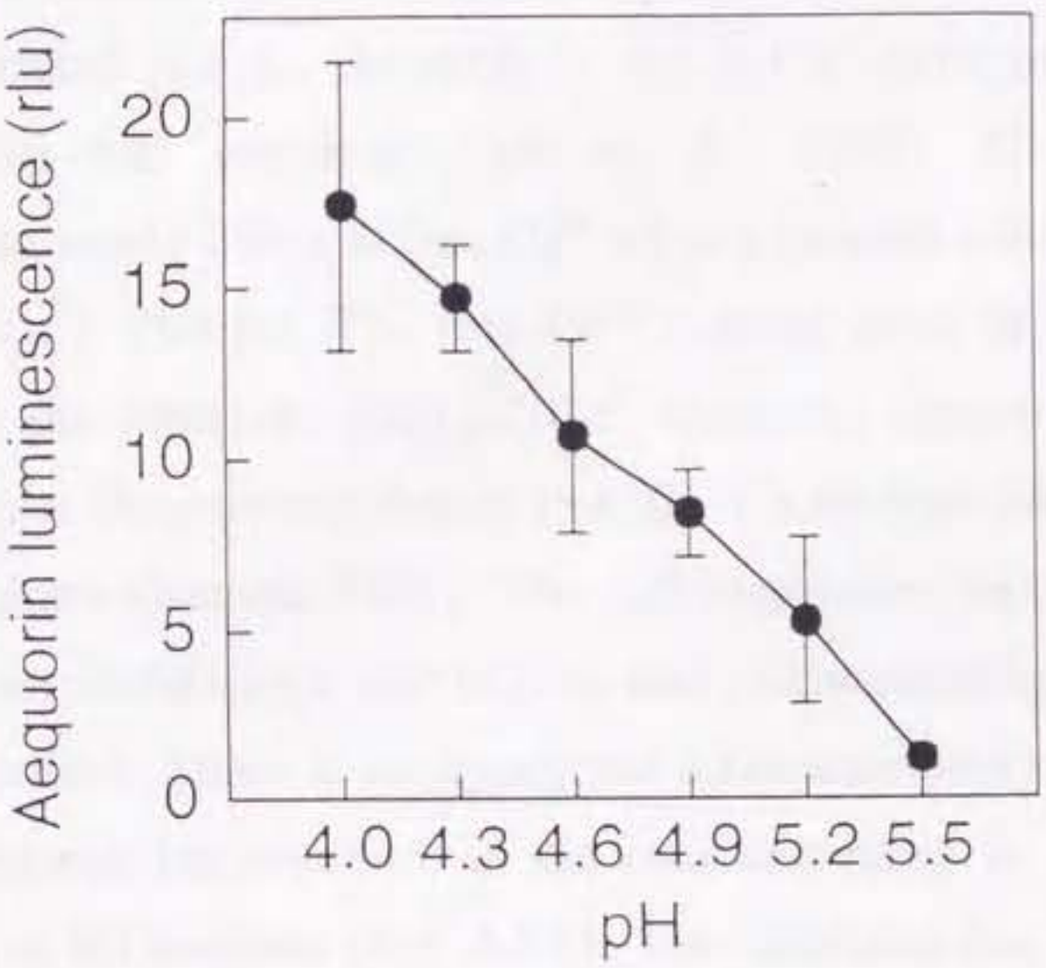

Fig. 4-2. Effect of pH on the SA-induced $[Ca^{2+}]_{cyt}$ elevation. SA dissolved in Mes-KOH with indicated pH was added to the cell suspension. The peak height of the aequorin luminescence after SA addition was plotted as a function of pH of SA solution.

Sorbic acid, butyric acid, or benzoic acid (BA) induced an immediate, small and transient $[Ca^{2+}]_{cyt}$ elevation (Fig. 4-3). In spite of structural similarity between SA and BA, BA did not induce the marked $[Ca^{2+}]_{cyt}$ elevation, suggesting that SA is highly specific in the induction of $[Ca^{2+}]_{cyt}$ elevation.

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

To examine whether influx of the extracellular Ca^{2+} is required for the SA-induced $[Ca^{2+}]_{cyt}$ elevation or not, a Ca^{2+}-deficient synthetic (SD -Ca) medium was prepared (Iida et al., 1990). SD medium contained approximately 250 μ M free Ca^{2+} when estimated with an Ca^{2+}-electrode (IS 561- Ca^{2+}, Philips). The free Ca^{2+} concentration of SD -Ca medium was below the detection limit of Ca^{2+}-electrode (below 10 μ M). This data coincides the previous data in that SD -Ca medium contained 0.24 μ M Ca^{2+} (Nakajima-shimada, 1991). The cell suspension was washed with SD -Ca medium containing 1 mM EGTA, and resuspended in the standard or SD -Ca medium. There is no significant difference between the profiles of the SA-induced luminescence in the cells suspended in SD -Ca medium and those in SD medium (Fig. 4-4A). This indicates that Ca^{2+} was not derived from the extracellular Ca^{2+} in the medium.

When cells were incubated for 90 min in SD -Ca medium, the SA-induced luminescence decreased by 60 % (Fig. 4-4B, center trace). The intensity of the luminescence was not recovered to that in the high Ca^{2+} medium, even when 680 μ M $CaCl_2$ (comparable to the total Ca^{2+} in SD medium) was supplemented to SD -Ca medium prior to the addition of SA (Fig. 4-4B, right trace). This result indicates that the intracellular Ca^{2+}-store was starved during the incubation in SD -Ca medium and was not refilled within a short period (1.5 min) by the supplement of $CaCl_2$.

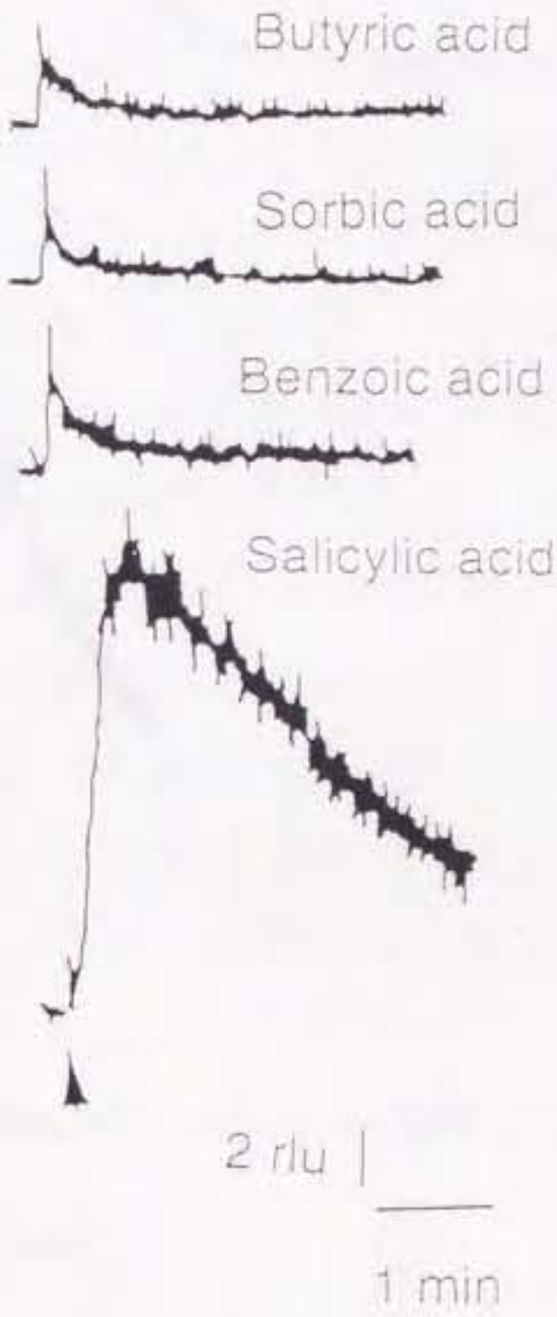

Fig. 4-3. Effect of several organic acids on the $[Ca^{2+}]_{cyt}$ in yeast cells.

Butyric acid, sorbic acid, BA and SA were added to the cell suspension at 5 mM where indicated by the arrowhead. All of the organic acid was dissolved in 0.4 M Mes-KOH (pH 4.0)/20 % ethanol.

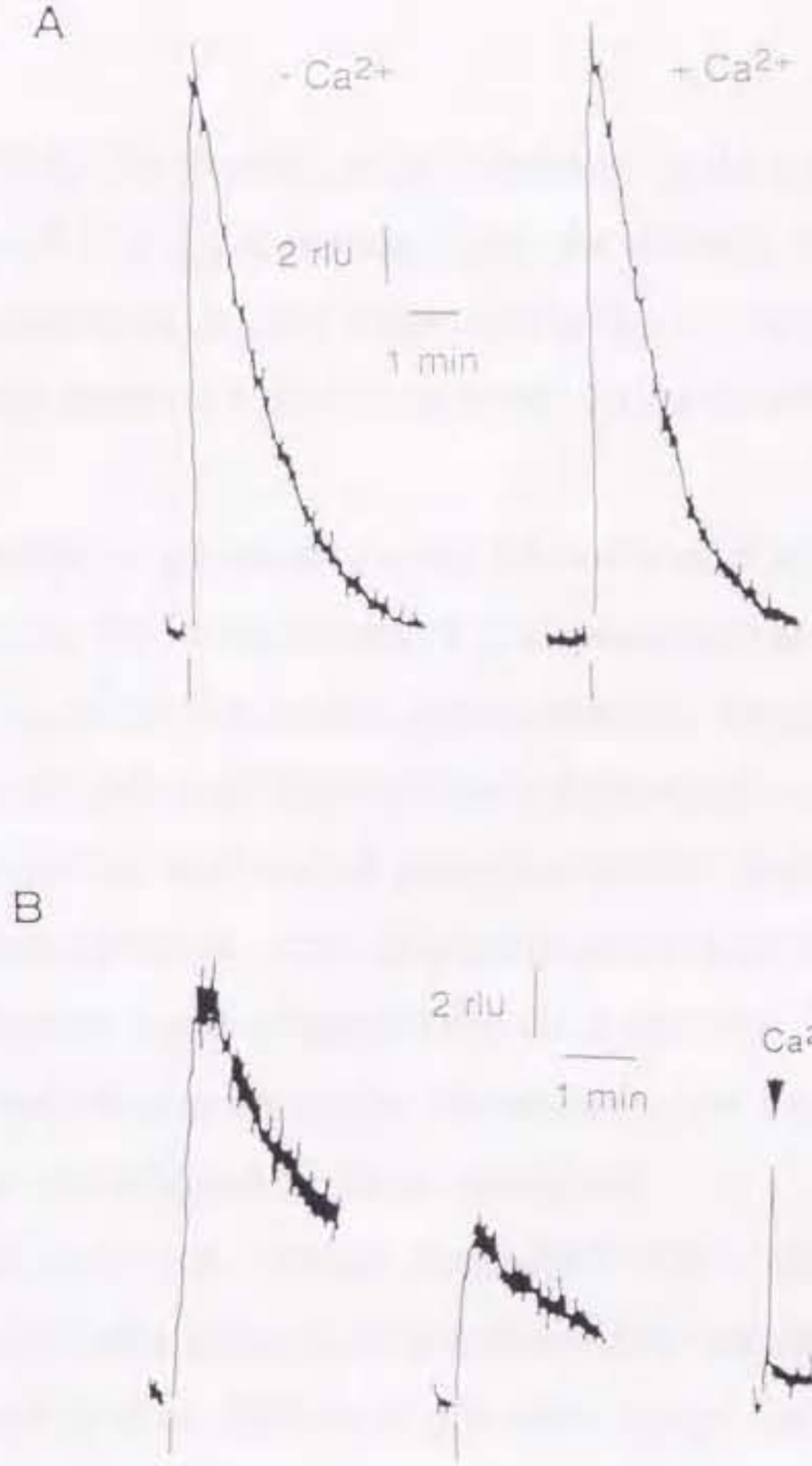

Fig. 4-4. Effect of Ca^{2+} depletion in the medium on the SA-induced $[Ca^{2+}]_{cyt}$ elevation. A, Yeast cells were resuspended in SD medium (+ Ca^{2+}) or SD - Ca medium (- Ca^{2+}). SA was added (indicated by vertical bars) within 3 min after resuspension. SA solution was added where indicated by the vertical bars. B, Yeast cells were resuspended in SD medium or SD -Ca medium and incubated for 90 min. 680 μM $CaCl_2$ was added to the cells suspended in SD -Ca medium (arrowheads), and then SA was added (vertical bars).

Inclusion of BAPTA (5 mM), a Ca^{2+} chelator, in the medium did not reduce the SA-induced $[Ca^{2+}]_{cyt}$ elevation (data not shown). These results suggest that Ca^{2+} required for the SA-induced $[Ca^{2+}]_{cyt}$ elevation is mobilized from an intracellular store(s) rather than from the extracellular medium.

Effects of various compounds on the SA-induced $[Ca^{2+}]_{cyt}$ elevation

To examine the involvement of phosphoinositide response in the SA-induced $[Ca^{2+}]_{cyt}$ elevation, either bromophenoxy bromide (a phospholipase C inhibitor, 100 μM), LiCl (an inhibitor of phosphoinositide metabolism, 10 mM), neomycin (an inhibitor of phosphoinositide metabolism, 500 μM) or 3,4,5-trimethoxybenzoic acid 8-[diethylamino])octyl ester [TMB-8, a blocker of inositol 1,4,5-trisphosphate (IP_3) receptor, 200 μM] were added to the cell suspension prior to the SA-addition, but the SA-induced $[Ca^{2+}]_{cyt}$ elevation was not affected by these chemicals.

The inhibitors of voltage-dependent Ca^{2+} channels of animals, verapamil, nifedipine and diltiazem had no effect on the SA-induced $[Ca^{2+}]_{cyt}$ elevation up to 20 μ M. Effects of the wide range Ca^{2+} permeable channel blockers, La^{3+} and Gd^{3+}, were not able to evaluate, since they coprecipitated with phosphate in the medium, made the medium cloudy, and caused light scattering.

4-4. Discussion

The signal transduction pathway following SA-stimulus has been unclear in any organism. In plants, SA-signal transduction pathway has been extensively studied (Klessig and Malamy, 1994), and the initial event in SA recognition has been proposed to be the direct inhibition of catalase and the subsequent generation of H_2O_2 (Chen et al., 1993). However, this model is

questioned in recent reports (Wobbe and Klessig, 1996). Requirement of extracellular Ca^{2+} in SA-induced defense gene accumulation was reported in tobacco leaves (Raz and Fluhr, 1992) and carrot suspension culture (Schneider-Müller et al., 1994), suggesting that Ca^{2+} functions as a second messenger of SA-signaling in these plants. In the yeast cells, the involvement of Ca^{2+} in SA-signaling has not been reported. In the present study, I successfully monitored the $[Ca^{2+}]_{cyt}$ elevation after SA-treatment in yeast cells by means of apoaequorin-expressing system.

The SA-induced $[Ca^{2+}]_{cyt}$ elevation in yeast cells was dose- and pH-dependent. The pH-dependence may be explained by pH-dependent membrane permeability of SA (Zetterberg, 1979). As p*K*a value of SA is 2.98, SA dissociates at neutral pH, but approximately 10 % of SA is protonated and discharged at pH 4.0. The discharged SA (SAH) might diffuse across the plasma membrane, stimulate a Ca^{2+} influx machinery(ies), and cause the $[Ca^{2+}]_{cyt}$ elevation. In the present study, it is suggested that the $[Ca^{2+}]_{cyt}$ elevation is not induced by the simple acidification of the cytosol which was caused by the diffusion of SAH into the cytosol. Because both SA and BA (p*K*a=4.19) can permeate into the cytosol by simple diffusion and acidify the cytosol (Henriques, 1997).

Ca^{2+} depletion in the medium did not affect the SA-induced $[Ca^{2+}]_{cyt}$ elevation in a short period, suggesting that Ca^{2+} was not originated from the extracellular medium nor the cell-wall bound Ca^{2+}, but might be released from an intracellular store(s). If Ca^{2+} was from the extracellular medium or the cell-wall bound Ca^{2+}, the $[Ca^{2+}]_{cyt}$ elevation would be reduced in a Ca^{2+}-deficient medium. A prolonged incubation of cells in the Ca^{2+}-deficient medium caused a decrease in the SA-induced $[Ca^{2+}]_{cyt}$ elevation and this decrease was not recovered by the supplement of Ca^{2+} to the medium. The intracellular Ca^{2+} store might be depleted during the long term incubation in

Ca^{2+}-deficient medium and result in the reduction of the $[Ca^{2+}]_{cyt}$ elevation. Refilling of Ca^{2+} in the intracellular store might be a relatively slow process, as the $[Ca^{2+}]_{cyt}$ elevation was not fully recovered within 1.5 min after Ca^{2+} supplement. If Ca^{2+} came from the medium or the cell wall-bound Ca^{2+}, the reduced $[Ca^{2+}]_{cyt}$ would be recovered by the Ca^{2+} supplement.

The IP_3-induced Ca^{2+} mobilization is a well-documented mechanism of Ca^{2+} release from the intracellular stores in animal cells. The involvement of this mechanism may be excluded from the SA-induced $[Ca^{2+}]_{cyt}$ elevation, because the chemicals which affect phosphoinositide response in animal cells had no effect. Since homologous gene of animal authentic IP_3-receptors or phospholipase C is not found in the yeast genome, inositol metabolism followed by Ca^{2+}-release from an intracellular pool(s) may not exist in yeast cells.

Several voltage-dependent Ca^{2+} channel blockers which is commonly used in animal cells had no effect on the SA-induced $[Ca^{2+}]_{cyt}$ elevation. It is not known whether these channel blockers are effective on yeast Ca^{2+} channels or not. Recently, a homologue of mammalian L-type voltage-dependent Ca^{2+} channels, *CCH1*, was cloned from yeast (Paidhungat and Garrett, 1997). Mammalian L-type voltage-dependent Ca^{2+} channels are sensitive to the blockers used in the present study. If this type of Ca^{2+} channel was involved in the SA-induced $[Ca^{2+}]_{cyt}$ elevation, it might be inhibited by the Ca^{2+} channel blockers. It is still unknown what type of Ca^{2+}-permeable channel was involved in the SA-induced $[Ca^{2+}]_{cyt}$ elevation.

A H^+/Ca^{2+} antiporter (*VCX1*; Cunningham and Fink, 1996) and a Ca^{2+}-pump (*PMC1*; Cunningham and Fink, 1994) located in the tonoplast, and a Ca^{2+}-pump (*PMR1*) located in the Golgi apparatus (Antebi and Fink, 1992). Intracellular localization of these Ca^{2+} transporters suggests that Ca^{2+} is stored in the vacuole and the Golgi apparatus in yeast cells. However, the

SA-induced $[Ca^{2+}]_{cyt}$ elevation was not inhibited by the preincubation with bafiromycin A (100 nM), a V-type ATPase inhibitor (data not shown).

A gene which is responsible to pheromone (α-factor)-induced Ca^{2+} uptake (*MID1*) was isolated by Iida et al. (1994). The gene product was located in the plasma membrane, thus Ca^{2+} was mobilized extracellularly through the plasma membrane in response to pheromone stimulus. I monitored the SA-induced $[Ca^{2+}]_{cyt}$ elevation in *mid1* mutant cells expressing apoaequorin, but no difference was observed as compared with wild type cells (data not shown). This result suggests that Mid1 does not involve in the SA-induced $[Ca^{2+}]_{cyt}$ elevation.

When hypotonic shock was applied to yeast cells, a biphasic $[Ca^{2+}]_{cyt}$ elevation occurred (Batiza, 1996). Since only the second phase of $[Ca^{2+}]_{cyt}$ elevation was canceled by BAPTA, Ca^{2+} for the second phase elevation might be originated from the medium, and Ca^{2+} for the first phase from the intracellular store. The first phase Ca^{2+} was considered to come through a stretch-activated channel, because it was blocked by gadolinium. However, the reported effects of gadolinium should be carefully evaluated, since it caused light scattering when it was added to the culture medium as indicated in the present study.

The detailed mechanism of SA-induced $[Ca^{2+}]_{cyt}$ elevation remained unclear, but it is different from that of the pheromone- or the hypotonic shock-induced $[Ca^{2+}]_{cyt}$ elevation. It is evident that Ca^{2+} is mobilized via different mechanism in response to different stimulus in yeast cell.

General Discussion

ABR kinase and ABA-signaling in guard cells

The present study demonstrated that the treatment of guard cell protoplast of *Vicia faba* L. with ABA activated a 48-kDa protein kinase (ABR kinase) using the in-gel protein kinase assay. At first, ABR kinase was discovered as a protein kinase which phosphorylates myelin basic protein. The activation of ABR kinase was highly specific to ABA among phytohormones, and was sensitive to staurosporine and K-252a, serine/threonine-kinase inhibitors. These protein kinase inhibitors inhibited not only the activation of ABR kinase, but also the ABA-induced stomatal closure. The results suggest that ABR kinase is a component of ABA-signal transduction pathway leading to stomatal movement, and its activation requires a putative protein kinase(s).

Ca^{2+} signaling ------- Pretreatment of GCPs with BAPTA revealed that $[Ca^{2+}]_{cyt}$ elevation was required prior to the activation of ABR kinase. Pretreatment with TMB-8 revealed that IP_3-induced Ca^{2+} release from intracellular store(s) is not involved in ABA signal transduction pathway leading to the activation of the ABR kinase. The involvement of IP_3-induced Ca^{2+} release in the ABA-induced stomatal closure has been shown in several reports (McAinsh et al., 1990; Gilroy et al., 1990; Irving et al., 1992; Lee et al., 1996). TMB-8 was reported to be a blocker of IP_3-induced Ca^{2+} release in plants (Schumaker and Sze, 1987). These experimental results suggest ABA induces $[Ca^{2+}]_{cyt}$ elevation inguard cells in two ways, one from the extracellular space which was inhibited with the Ca^{2+} chelator, and another from the intracellular store(s) which was inhibited with the inhibitors of phosphoinositide turn over. The results in Chapter 1 suggest that ABR kinase was activated after the Ca^{2+} influx from the extracellular space, but

not the release from the intracellular stores. Though the involvement of Ca^{2+} in ABA-response in guard cells seems to be established, Gilroy et al. (1991) suggested the existence of both Ca^{2+}-dependent and -independent pathways in guard cells. The relationship between ABA-induced $[Ca^{2+}]_{cyt}$ elevation and the activation of ABR kinase in guard cells should be examined in the next step.

Protein kinase cascades -------- The present study suggested that the involvement of a protein kinase cascade including the ABR kinase in ABA-signaling. The MAP kinase cascade is the most established protein kinase cascade which transduces the extracellular stimulus (for review, see Pelech and Sanghera, 1992). This cascade consists of three kinases, MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. Both threonine and tyrosine residues in MAP kinase is phosphorylated by MAP kinase kinase. A 48-kDa protein kinase was activated by treatment of tobacco cells with salicylic acid and identified as a MAP kinase (Zhang and Klessig, 1997). Generally, MAP kinases were known to detected in the in-gel protein kinase assay using myelin basic protein as the substrate (Mizoguchi et al., 1994). ABR kinase has a 48-kDa molecular mass and phosphorylates myelin basic protein, but it was not recognized by an anti-phosphotyrosine antibody, thus ABR kinase is a different type of kinase from MAP kinase. The finding of the ABR kinase provided the first step for discovery of novel protein kinase cascade. To identify the upstream-activator kinase, purification of this protein kinase is essential. The purification of protein kinase from guard cell protoplasts seems difficult. Application of an affinity chromatography, using the carboxyl terminus peptide of KAT1 and co-precipitation with antibody raised against this peptide would be an effective strategy for purification of ABR kinase. The isolation of cDNA encoding this kinase by two-hybrid cloning with KAT1 gene is an alternative strategy. Cloning of

cDNA or purification of this protein kinase may reveal the protein kinase cascade which transduces ABA-signal transduction in guard cells.

$[Ca^{2+}]_{cyt}$ elevation in yeast and analysis of ion transporters using yeast

Analysis of ion transporters in yeast expression system has several advantages compared with those in *Xenopus* oocytes. For instance, 1) As isolation of cDNAs encoding ion transporters by complementation and electrophysiological analysis of ion transporters can carried out in the same system, only gene products which functionaly complemented can be analyzed by electrophysiological techniques. 2) Background currents caused from endogenous gene product can be eliminated by disrupting the gene, and the activity of transformed gene product can be analyzed clealy. 3) Interactions between ion transporters and the modulators can be analyzed without prevention with the endogenous factors, after disrupting their genes.

A number of ion transporters are affected with Ca^{2+} directly or indirectly in plants (for review, see Thiel and Wolf, 1997). To examine the effect of Ca^{2+} on the activity of ion transporter in yeast system, the mobilization of Ca^{2+} in yeast cell should be determined. The present study demonstrated that SA induces a transient and large $[Ca^{2+}]_{cyt}$ elevation in yeast cell. Some Ca^{2+}-sensitive ion transporter expressed in yeast may be affected with SA-treatment of the yeast cells. Molecular interactions between ion transporters and Ca^{2+}, and Ca^{2+}-dependent modulators for ion transporters and Ca^{2+}, can be examined in yeast by applying the SA-induced $[Ca^{2+}]_{cyt}$ elevation.

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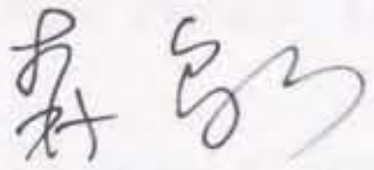

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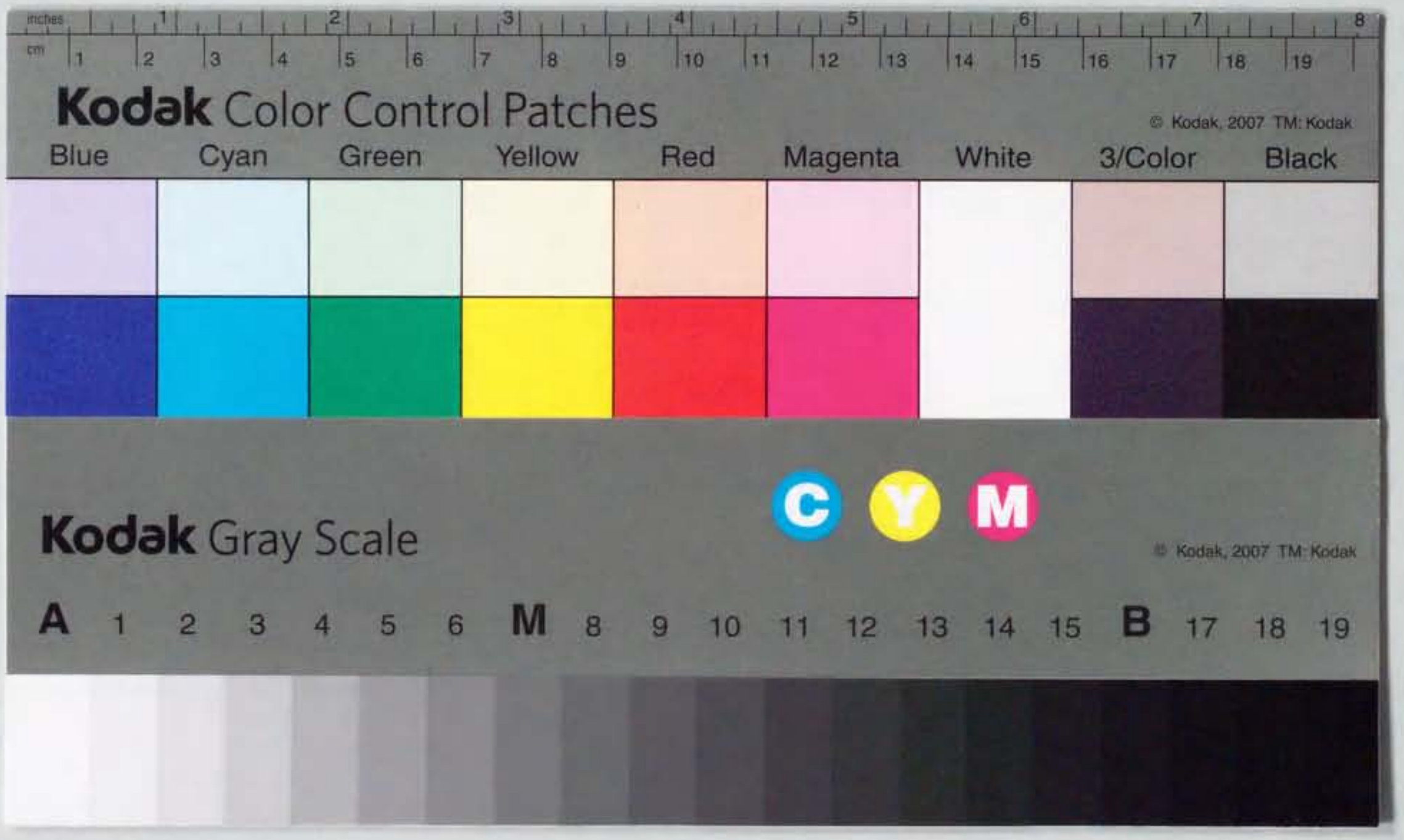
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