Université de Montréal

Identification and characterization of a PKC-like kinase in potato and its role in the activation of the defense response gene *PR-10a*

par
Rajagopal Subramaniam
Département de Biochimie
Faculté des études supérieures

Thèse présentée à la Faculté des études supérieures
en vue de l'obtention du grade de
Philosophiæ Doctor (Ph.D.)
en Biologie Moléculaire

November 1998

Université de Montréal

Faculté des études supérieures

Cette thèse intitulée:

Identification and characterization of a PKC-like kinase in potato and its role in the activation of the defense response gene *PR-10a*

presentée par:

Rajagopal Subramaniam

a été évaluée par un jury composé des personnes suivantes:

Dr. Louise Brisson

Dr. Stephen Michnick

Dr. Daniel Skup

Dr. Normand Brisson

Thèse acceptée le: 30.11.1998

ABSTRACT

The production of PR (pathogenesis-related) proteins is one of the most frequently observed responses in plants to microbial attack. Since the activation of *PR* genes is indicative of the pathogenesis state of the plant, it serves as a useful marker to study the defense response in plants. In potato, a noticeable battery of genes is induced in response to an attack by the pathogen *Phytophthora infestans*, or after treatment with the elicitor arachidonic acid. Among the *PR* genes induced is the *PR-10* gene family.

Our studies of the regulation of the expression of *PR-10a*, a member of the *PR-10* gene family, indicate that the promoter element responsible for the activation of this gene is restricted to a 30 bp region located between -135 and -105 of the transcription start site and called the elicitor response element (ERE). Following elicitor treatment, the factors, named PBF-1 and 2, (*PR-10a* binding factor) bind to this element and the evidence demonstrates a direct proportional relationship between the amount of binding and the expression of the gene. The studies also reveal that phosphorylation event(s) mediate the binding of PBFs to this element.

In the present study, we have obtained pharmacological evidence strongly suggesting that the phosphorylation event(s) is mediated through a protein kinase C-like (PKC) pathway. The treatment of potato tuber discs with specific inhibitors of PKC abolished the elicitor-induced binding of PBF-2 to the ERE. This correlated with a reduction in the accumulation of the PR-10a protein. In contrast, treatment

of the tuber discs with 12-*O*-tetradecanoylphorbol 13-acetate (TPA), an activator of PKC, led to an increase in binding of PBF-2 to the ERE and the corresponding increase in the level of the PR-10a protein, mimicking the effect seen with the elicitor. Biochemical characterization of proteins extracted from the particulate fraction of potato tubers demonstrated that a kinase belonging to the conventional isoforms of PKC is present. This was confirmed by immunoprecipitation with antibodies specific to the conventional isoforms of human PKC and in-gel kinase assays. The ability of the immunoprecipitates to phosphorylate the α-peptide (a PKC specific substrate) in the presence of the coactivators calcium, phosphatidylserine and TPA, strongly suggested that the immunoprecipitated kinase is similar to the kinase characterized biochemically. The similar effects of the various modulators of PKC activity on the elicitor-induced resistance against a compatible race of *Phytophthora infestans* implicate this kinase in the overall defense response in potato.

We further characterized this kinase present in the particulate extracts of the tubers by a series of chromatographic steps. The purification was monitored both by biochemical and immunological methods. The partial sequence of the protein was obtained after a final purification by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The sequence analyses showed a strong similarity with the Hsp70 (heat shock protein 70) family of proteins from a variety of eukaryotes including plants, suggesting that the kinase was contaminated with this comigrating protein. Monoclonal antibodies to the mammalian Hsp70 family indicated that both the Hsp70 protein and the PKC-like kinase have similar mobility in SDS-PAGE.

Finally, we used the two-dimensional electrophoresis method to separate the Hsp70 from the kinase. Results obtained with the broad range ampholytes (pH 3.5-10) indicated that their isoelectric points are very close. However, recent results suggest that it will be feasible to purify the kinase using a combination of narrow ranges of various ampholytes.

Key words: defense response, potato, PR-10a, PBF, phosphorylation, kinase, PKC, Hsp70

Sommaire

La production de protéines PR (reliées à la pathogénèse) chez les plantes est observée fréquemment en réponse à une attaque microbienne. Puisque l'activation de ces gènes *PR* est une signe de pathologie, ils peuvent se montrer de précieux indicateurs lors de l'étude des réponses de défense chez la plante. En réponse au pathogène *Phytophthora infestans* ou à un éliciteur, l'acide arachidonique, un grand nombre de gènes sont activés chez la pomme de terre. Parmi ceux-ci, est la famille de gènes à laquelle appartient *PR-10a*.

Notre étude du contrôle de l'expression de *PR-10a* indique que la partie du promoteur responsable de l'activation du gène se limite à une région de 30 pb située entre les positions –135 et –105, en amont du site de départ de la transcription. Suite à un traitement avec l'éliciteur, deux facteurs que nous désignons PBF-1 et 2 (PR-10a binding factor) se lient à cette région et nos résultats démontrent une fonction directement proportionnelle entre l'ampleur de la liaison et l'expression du gène. Nos études démontrent également qu'un ou plusieurs événement(s) de phosphorylation stimule(nt) la liaison de PBF à la région de 30 pb du promoteur.

Dans la présente étude, nous avons obtenu des preuves pharmacologiques qui suggèrent fortement que le ou les événement(s) de phosphorylation font parti d'un circuit semblable à celui de la PKC (protéine kinase C). La liaison, induite par l'éliciteur, du facteur nucléaire PBF à la région ERE (elicitor-response element) est abolie lors du traitement de disques de tubercules de pommes de terre avec des inhibiteurs spécifiques à la PKC. Ceci coïncide avec une accumulation réduite de

la protéine PR-10a. À l'opposé, la liaison de PBF à l'ERE est stimulée par le traitement des disques de tubercules avec du 12-*O*-tetradecanoylphorbol 13-acetate (TPA), un activateur de la PKC. Ceci est accompagné d'une augmentation de la quantité de protéines PR-10a, reproduisant l'effet obtenu avec l'éliciteur naturel, l'acide arachidonique. Une caractérisation biochimique des protéines extraites de tubercules démontra qu'une kinase appartenant aux isoformes conventionnelles de la PKC est présente chez la pomme de terre. Ceci fut confirmé par immunoprécipitation, à l'aide d'anticorps spécifiques contre les isoformes conventionnelles de la PKC humaine, et par des analyses de phosphorylation *in-gel*. La capacité des protéines immunoprécipitées à phosphoryler le peptide a (un substrat spécifique à la PKC) en présence de coactivateurs tels que le calcium, la phosphatidylsérine, et le TPA suggéra fortement que la kinase immunoprécipitée correspond à celle caractérisée biochimiquement. Les effets semblables des différents régulateurs d'activité de phosphorylation, sur la résistance induite par l'éliciteur contre une race compatible de *Phytophtora infestans*, implique cette kinase dans la réponse de défense générale chez la pomme de terre.

Nous avons caractérisé plus amplement cette kinase, présente dans la fraction membranaire des tubercules, par une série d'étapes chromatographiques. Le progrès de la purification fut suivie par les méthodes biochimiques et immunologiques susmentionnées. La séquence partielle de la kinase fut obtenue suite à une dernière étape de purification par électrophorèse. Cette séquence est fortement identique à celles de protéines de la famille Hsp70 (heat-shock protein 70) de la tomate et d'autres eucaryotes. Des anticorps monoclonaux contre la famille Hsp70 des mammifères furent utilisés pour démontrer que Hsp70, ainsi que notre kinase s'apparentant à PKC, migrent de façon très semblable sur un gel

dénaturant d'acrylamide.

Enfin, nous avons utilisé la méthode d'électrophorèse en deux dimensions pour séparer Hsp70 de notre kinase. Les résultats obtenus à l'aide d'ampholytes créant de larges gradients (pH 3.5-10) indiqua que les points isoélectriques des deux protéines sont très similaires. Cependant, de récents résultats suggèrent qu'il sera possible de séparer la kinase de son contaminant en utilisant une combinaison restreinte d'ampholytes.

TABLE OF CONTENTS

List of Tables

List of Figures

List of abbreviations

AOS	active oxygen species
ATP	adenosine 5'-triphosphate
AA	arachidonic acid
avr	avirulence gene or protein
bp	base pair
Bis	bisindolylmaleimide
cDNA	complementary deoxyribonucleic acid
Cal.C	calphostin C
CDPK	calcium-dependent protein kinase
DAG	1,2-dioleolyl-sn-glycerol
DMSO	dimethylsulfoxide
DTT	dithiothreiotol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylenebis(oxyethylenenitrolo)tetraacetic acid
ELISA	enzyme linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
ERE	elicitor response element
g	gram
GTP	guanosine 5'-triphosphate
hr	hour
HR	hypersensitive reaction
Hsp70	heat shock protein 70
IgG	immunoglobulin G heavy chain
kD	kilodalton
LRR	leucine rich repeat
LZ	leucine zipper

MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
µCi	microcurie
µg	microgram
µL	microlitre
µM	micromolar
mg	milligram
mL	millilitre
mM	millimolar
min	minute
M	molar
NBS	nucleotide binding site
ng	nanogram
p-Sph	palmitoyl D-erythro sphingosine
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
PC	phosphatidylcholine
PCR	polymerase chain reaction
PK	protein kinase
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PR	pathogenesis-related protein
PBF	PR-10a binding factor
PS	phosphatidylserine
R	resistance
SA	salicylic acid
SAR	systemic acquired resistance
SDS	sodium dodecyl sulfate

Sph	D-erythro sphingosine
TBS	Tris-buffered saline
TCA	trichloroacetic acid
TPA	12-*O*-tetradecanoylphorbol 13-acetate
4αTPA	4α-12-*O*-tetradecanoylphorbol 13-acetate
TTBS	TBS-Tween
w/v	weight per volume

PREFACE

This thesis comprises a series of experiments that identify and characterize a signalling pathway involved in the defense response in potato. The thesis has been divided into four Chapters. Chapter 1 introduces the topic of defense response in plants and the various components that embodies the response. Chapter 2 describes the materials and methods that were used to identify the specific components involved in the potato defense response process. Chapter 3 includes results of two manuscripts. The first, described the role of phosphorylation in the defense response process in potato and was entitled, "The activation of the potato *PR-10a* gene requires the phosphorylation of the nuclear factor PBF-1" (Després et al., 1995). The second, elaborated on the work presented in the first publication, and was entitled, "A functional homolog of mammalian protein kinase C participates in the elicitor-induced defense response in potato" (Subramaniam et al., 1997). The rest of Chapter 3 is comprised of detailed analysis of the results presented in the second publication. Chapter 4, discusses the implications of the findings in Chapter 3, and offers perspectives with respect to the overall defense response in plants.

Chapter 1. Introduction

Evolution has imparted plants and animals with many different ways to deal with the elemental scourges of nature. In vertebrates, the task of defending the organism from pathogens has been charged to the immune and the circulatory systems. These specialized systems have proven very effective to mitigate pathogenesis. In the absence of such specialization, plants have evolved distinct mechanisms to resist pathogen attacks.

A dogma of plant pathology is that most plants are resistant to most pathogens. In the face of selection pressures exerted by modern agriculture, it is conceivable that an incompatible plant-pathogen interaction, where the plant resists the infection, will evolve into a compatible one and cause disease. It is therefore essential to learn more about natural resistance mechanisms since, in addition to countering any disease threats, they offer insights into defensive approaches distinct from those that have evolved in animals.

Analyses of incompatible interactions between host and pathogens have revealed that resistance in host cultivars is achieved through a combination of constitutive and induced defense mechanisms (Agrios, 1988). A comprehensive defense response encompasses both of these mechanisms. However, in the majority of cases, pathogen attacks are curtailed by the constitutive mechanism alone. Pathogens are unable to penetrate physical barriers such as the leaf cuticle and lignified walls. Additionally, each plant cell is equipped with chemical defenses that can effectively neutralize ingress of any pathogen. In such instances where the constitutive defense is not sufficient, plants may rely on inducible defense mechanisms. This includes the expression of new genes that lead to

modifications and reinforcements of the cell wall, the production of superoxides and other reactive oxygen species, and the secretion of the antimicrobial phytoalexins. This inducible defense is manifested typically as a localized necrosis of challenged plant cells and referred to as the hypersensitive response (HR), which is believed to restrict pathogen growth (Collinge and Slusarenko, 1987). These localized responses are often followed by the establishment of systemic acquired resistance (SAR), in which tissues distant from the original infection site show an increased resistance to subsequent infection by not only the original pathogen but by many others as well (Gaffney et al., 1993). SAR is associated with the induction of a number of genes, some of which are known or suspected to encode defensive proteins.

Neither HR nor SAR can take place without the perception of the pathogen by the plant cells. This recognition represents the first of three critical stages for an effective defense response, the second stage being the transduction of the recognition signal within the cell and the final stage being the culmination of the two previous stages that results in the mobilization of the defense response genes.

An understanding of the defense response requires a broad knowledge of each of the stages mentioned above. Therefore, an overview of each of the stages of plant defense response is presented beginning with the recognition of the pathogen by the plant. This depends to great extent on the interaction between the various classes of the infecting agent such as viruses, bacteria and fungi. This is elaborated further with pertinent examples in Section 1.1. Section 1.2 deals with the second stage of the defense response process, the transduction of the

recognition signal within the plant cell. These signals include calcium and other ions, lipid molecules, active oxygen species, kinases and phosphatases (Gilroy and Trewavas, 1994). The purpose of these signalling components is to properly integrate, regulate and, more importantly amplify the incoming signals leading eventually to activation of genes necessary to contain the pathogen. Finally, Section 1.3 of the introduction deals with the consequence of the previous two stages, activation of many defense-related genes.

1.1. Recognition of pathogens

The molecular mechanisms of pathogen recognition eventually leading to initiation of plant defense responses can be classified into two categories (De Wit, 1997). In the first category, recognition is said to be governed at the genetic level by the 'gene-for-gene' interaction proposed by Flor in the 1940's (Flor, 1946). This type of interaction is guided by the plant's ability, through its *R* (resistance) genes, to recognize specific molecules produced by a given pathogen. These molecules, referred to as race-specific elicitors, are encoded by the avirulence (*avr*) genes. Consequently, if either the host plant or the pathogen lacks the corresponding *R* or *avr* allele, the pathogen can colonize the host and cause disease provided no other defense mechanisms are activated. In the second category, the initiation of defense response is also based on recognition of elicitor molecules, but in this case the elicitors are not associated with race-specificity. Non race-specific resistance in plants accounts for substantial numbers of plant-pathogen interactions. Examples relevant to both of these categories are reviewed in Section 1.1.3.

1.1.1. Characteristics of *R* genes

In the past few years many *R* genes have been cloned from a variety of plant species as summarized in Table 1. Analyses of the *R* genes, with the exception of the *Pto* and *Hm1* genes, reveal a common structural motif, namely the leucine-rich repeats (LRRs). The LRRs are tandem repeats of 20-30 amino acids, with regularly spaced leucine residues. These motifs have been shown to mediate protein-protein binding in yeast, *Drosophila* and mammalian cells (Kobe and Diesenhofer, 1994). Although the function of LRRs in the *R* genes of plants has not been clearly demonstrated, they appear to be suited to interact with either proteinaceous pathogen elicitors or other proteins of the signalling cascade.

As mentioned earlier, the *Pto* gene from tomato, which confers specific resistance to *Pseudomonas syringae* pv. *tomato* strains expressing the avirulence gene *avrPto*, does not possess the typical LRR repeats. *Pto* encodes a cytoplasmic protein serine/threonine kinase and its conserved kinase domain is essential for the interaction with AvrPto (Martin et al., 1993). This interaction is the only example where physical association between an R and Avr has been demonstrated. Resistance, however, requires the function of another protein, Prf, which interestingly features the LRR repeats (Salmeron et al., 1996). Interaction of Prf with Pto is not specific and therefore its role as an intermediary between other signalling molecules has been postulated.

The three highly related R proteins, N, L6 and RPP5 share amino-terminal sequence similarities with the cytoplasmic domains of the mammalian interleukin-1 (IL-1) and Toll transmembrane receptors (Whitham et al., 1994). Significantly, both IL-1 and the Toll receptor activated pathway control the innate immune

Table 1. Cloned Plant Resistance Genes

Gene	Plant	Pathogen	Structure[a]	References
Hm1	Maize	*Helminthosporium maydis*	Detoxyfying enzyme	1
Pto	Tomato	*Pseudomonas syringae*	PK	2
RPS2	Arabidopsis	*P. syringae*	LZ. NBS. LRR	3
RPM1	Arabidopsis	*P. syringae*	LZ. NBS. LRR	4
Mi	Tomato	*Meloidogyne javanica*	LZ. NBS. LRR	5
I2C-1	Tomato	*Fusarium oxysporum*	NBS. LRR	6
RPP5	Arabidopsis	*Peronospora parasitica*	TIR. NBS. LRR	7
N	Tobacco	*Tobacco mosaic virus*	TIR. NBS. LRR	8
L6	Flax	*Melamspora javanica*	TIR. NBS. LRR	9
Cf-2	Tomato	*Cladosporium fulvum*	LRR. TM	10
Cf-4	Tomato	*C. fulvum*	LRR. TM	11
Cf-9	Tomato	*C. fulvum*	LRR. TM	12
$Hs1^{pro1}$	Sugar beet	*Hetrodera Schachtii*	LRR. TM	13
Xa21	Rice	*Xanthomonas oryzae*	LRR. TM. PK	14

[a] Structural motifs were predicted by analysis of the derived peptide sequence: leucine-rich repeats (LRR); leucine zipper (LZ); nucleotide-binding site (NBS); protein kinase (PK); sequence similar to the cytoplasmic domains of Toll and IL-1R (TIR); transmembrane domain (TM).

1. Johal and Briggs. 1992; **2.** Martin et al.. 1993; **3.** Bent et al.. 1994; **4.** Grant et al.. 1995; **5.** Milligan et al.. 1998; **6.** Ori et al.. 1997; **7.** Parker et al.. 1997; **8.** Whitham et al.. 1994; **9.** Lawrence et al.. 1995; **10.** Dixon et al.. 1996; **11.** Jones et al.. unpublished; **12.** Jones et al.. 1994; **13.** Cai et al.. 1997; **14.** Song et al.. 1995.

response of *Drosophila* adults to pathogen invasion (Heguy et al., 1992) and the activated signalling cascades require a serine/threonine kinase that shares sequence similarities to the tomato Pto kinase (Galindo et al., 1995). This substantial level of evolutionary conservation emphasizes the importance of these intracellular pathways for the survival of organisms.

1.1.2. Characteristics of *avr* genes

Analogous to the animal system, the *avr* gene products or race-specific elicitors of pathogens ascribe antigenicity. These molecules are recognized by a matching receptor in a particular plant genotype, thereby evoking response from the host to defend itself. The cloning of the *avr* genes has revealed a vast diversity of elicitor molecules, as diverse as the pathogen from which they originate. Experiments in tobacco have shown that HR and SAR can be induced by interaction between the host R gene *N* and potentially more than one *avr* gene encoded by its infecting agent, the tobacco mosaic virus (Whitham et al., 1994).

In bacteria, more than 30 *avr* genes have been isolated, mostly from narrow-host-range strains of *Pseudomonas* and *Xanthomonas* (De Wit, 1997). There are no obvious homologies between them and any other known proteins. However, the manifestation of disease symptoms on a susceptible plant, or an avirulence response by a resistant host requires the participation of a cluster of genes named the *hrp* (hypersensitive response and pathogenicity) genes (Pirhonen et al., 1993). The products of *hrp* genes resemble the Type III secretion system of mammalian bacterial pathogens (Cornelis and Wolf-Watz, 1997). The secretion pathway enables bacteria to 'deliver' virulence (disease promoting) proteins directly into the plant. With the exception of the *Pto* and *avrPto* system

described previously, physical interaction between other R and Avr proteins of bacteria have not been documented.

Information regarding the nature of fungal avirulence genes is limited, but insights have come from studies with the tomato pathogen *Cladosporium fulvum*. The *avr4* and *avr9* genes of this fungus encode preproteins that are processed by plant proteases into smaller oligopeptides (Van den Ackerveken et al., 1992; Joosten et al., 1994). These cysteine-rich peptides induce the hypersensitive response in tomato plants carrying the matching *R* genes *Cf-4* and *Cf-9*, respectively. No function has been ascribed to the AVR4 protein, but the AVR9 peptide shows structural homology to cystine-knotted peptides that induce ion-channel blockers, protease inhibitors and growth factors. An *avr* gene, *nip1*, of the barley pathogen *Rhynchosporium secalis* which induces necrosis on barley cultivars, also encodes a similar cysteine-rich race-specific elicitor (Rohe et al., 1995).

Another family of avirulence genes is represented by *avr2-YAMO* from the rice pathogen *Magnaporthe grisea*. This gene encodes a protein with homology to zinc proteases (Chasan, 1994), however, no protease activity has yet been attributed to the protein. A residue predicted to be essential for catalysis is altered in a spontaneous mutant that has reverted to virulence.

1.1.3. Characteristics of non race-specific elicitors

As discussed earlier, the function of many *avr* genes depend on the viability of the *Hrp* loci. One class of *hrp* gene products has been shown to induce defense response independent of the *avr* gene products. The functions of these glycine-

rich harpins are not clear, but mutation in the harpin gene *popA1* from *Pseudomonas solanacearum* renders the bacterium fully pathogenic (Wei et al., 1992).

In another example, the *avrD* locus of *P. syringae* pv. *glycinea* encodes not the race-specific elicitor itself, but a protein required for its production (Keen et al., 1990). The elicitor is a hydroxy lipid required for resistance mediated by the soybean *Rpg4* gene. Other general elicitors include oligogalacturonides, chitin, and fungal glycoproteins. A glycoprotein identified from the cell wall fragments of the fungus *Phytophthora sojae* induces defense response in parsley cells. A 13 amino-acid peptide (Pep-13) has been identified within the glycoprotein as the determinant of defense response in parsley cells (Nürnberger et al., 1994). Any modification of the peptide leads to complete abolishment of binding to its cognate receptor on the plasma membrane of parsley cells resulting in a weakened defense response.

1.1.4. Pathogen recognition in potato *(Solanum tuberosum)*

Defense response in potato against one of its natural pathogens, *Phytophthora infestans*, serves as a useful model system to examine signal transduction processes. *Phytophthora infestans* is the causal agent of potato late blight that results in enormous crop losses. Because of the importance of this crop, processes that lead to disease resistance have been intensively studied. Race-specific resistance in potato has been observed against a variety of pathogens including viruses and bacteria. Recently, the PCR-based approach has been successfully used to identify the *R* locus in *P. infestans* (Leister et al., 1996). Since the exact identity of the *R* or corresponding *avr* genes is not known, a

comparison to other patho-cultivar systems is not yet possible.

Just as the incompatible races of *P. infestans* elicit a defense response in resistant cultivars of potato, mycelial extracts of the pathogen induce a similar defense response in susceptible cultivars. The components in the extracts responsible for induction of the defense response are the two polyunsaturated fatty acids arachidonic acid (AA) and eicosapentaenoic acid (EPA) (Bostock et al., 1981). Since neither the incompatible nor the compatible races of the pathogen differ in their AA or EPA content, they are not the race-specific determinants of the potato-*Phytophthora infestans* interaction. Nevertheless, they constitute a major component of the fungus required for recognition by the potato plant in order to initiate both the hypersensitive (Preisig and Kuc, 1988) and the systemic acquired resistance responses (Doke et al., 1987).

The salient feature of the AA-induced defense response is that it maintains the integrity of an incompatible interaction. My thesis work exploits this system to decipher the underlying signalling cascades that lead to initiation of the defense response in potato. The evidence that already exists suggests that the signalling cascades are numerous and very complex. It is therefore appropriate at this point to describe stage 2 of the defense response process, the components involved in the signalling network.

1.2. Intracellular signals involved in the defense response

Among the earliest events observed after exposure to elicitors are the changes in ion permeability of the plasma membrane of plant cells (Mayer et al., 1988). For example, race-specific elicitors of *C. fulvum* activate a H^+-ATPase in

tomato cells (Xing et al., 1996). A similar observation has also been made in parsley cells exposed to glucan elicitors of *P. megasperma* (Mayer et al., 1988). It has been proposed that the decreased intracellular pH rapidly and directly activates enzymes of the secondary metabolic pathway which mediate incorporation of a caffeic acid derivative into the cell wall (Kneusal et al., 1989). This could result in strengthening of the physical barrier against the pathogen.

1.2.1. Calcium as a signal tranducer

Along with the influx of protons, there is also the influx of calcium into the cell. Modulation of Ca^{2+} levels in the cytoplasm of plant cells has been observed in response to a variety of stimuli that includes hormones, heat or cold shock, light, wind, touch and elicitation (Gilroy and Trewavas, 1994). The scenario that is emerging from studies in animals and in plants is that specificity of response to Ca^{2+} influx is not merely due to its presence but is also governed by its amplitude, frequency and spatial localization (McAinsh and Hethrington, 1998). This has been demonstrated in the growing pollen tubes (Malho and Trewavas, 1996) and at the apex of rhizoids of the marine alga Fucus following osmotic shock (Taylor et al., 1996).

Calcium also plays a major role in plant defense responses. Either omission from the extracellular media or blocking the entrance of calcium into the cell abolishes the activation of defense-related genes (Stab and Ebel, 1987). Recently, the oligopeptide Pep-13 of *P.sojae*, a known inducer of resistance genes in parsley was shown to activate a specific Ca^{2+} channel (Zimmermann et al., 1997). Ca^{2+} antagonists such as $La(NO_3)_3$ and $GdCl_3$ blocked the uptake of calcium into these cells. Similarly, treatments of potato tubers with the calcium ion

channel blockers verapamil or dilitiazem completely abolished the arachidonic-acid-induced defense responses (Miura et al., 1995). However, the exact role calcium plays in the elicitor response process of potato tubers has yet to be characterized.

The number of identified target proteins for Ca^{2+} has grown and several of these are likely to have pleiotropic effects on cell function (McAinsh and Hetherington, 1998), such as, calmodulin, calcium-dependent protein kinases (Harper et al., 1991) and phosphatases (Kauss and Jeblick, 1991), and calcium-stimulated phospholipases (Shorrosh and Dixon, 1991). This indicates that changes in calcium, early or late in the transduction process, are likely to have significant effects on cellular function.

The target protein calmodulin, is one of the major intracellular receptors of calcium. However, its role in plant defense response is ambiguous. Inhibitors of calmodulin have been shown to block elicitor-induced phytoalexins in cells of carrot (Kurosaki and Nishi, 1993) and tobacco (Vogeli et al., 1992). In contrast, these inhibitors have no effect on elicitor induction in soybean cells (Stab and Ebel, 1987). A calcium-dependent protein kinase which contains calmodulin-like domains has been identified that could preclude the need for calmodulin in the signalling cascade (Roberts and Harmon, 1992).

1.2.2. Lipids as second messengers and signal transducers

In animal cells, agonist-induced response activates various phospholipases, including phosphoinositidase C (PIC), and phospholipase A and C. The targets of these phospholipases are the phospholipids present in

membranes whose cleavage generates second messengers. For example, the phospholipid phosphatidylinositol 4,5-biphosphate is hydrolysed by phospholipase C to produce inositol 1,4,5-triphosphate (IP_3) and diacyglycerol (DAG). IP_3 is released in the cytosol where it triggers the release of Ca^{2+} from internal stores. DAG on the other hand, remains in the membrane, where it activates protein kinase C (Nishizuka, 1992).

In plants, the activation of phospholipases leads to the release of lipids such as linolenic acid from the plasma membrane pool (Creelman and Mullet, 1997). In a lipoxygenase-mediated peroxidation of linolenic acid, precursors of jasmonic acid could be derived. Jasmonic acid and its volatile methyl ester have been shown to induce many disease resistance genes in a variety of plant cells (Wasternack and Parthier, 1997). In particular, potato plants treated with either compound results in increased resistance to *P.infestans* (Cohen et al., 1993).

Phospholipase C (PLC)-catalyzed hydrolysis of inositol phospholipids has been shown to play a direct role in the defense response process. Inhibition of this enzyme leads to blockage of phytoalexin production in parsley cells (Renelt et al., 1993). Similarly, inhibition of this enzyme in pea tissues blocks the accumulation of IP_3 and induces local susceptibility to *Mycospaerella pinodes* (Toyoda et al., 1993). In carrot cells, it was shown by direct measurement of IP_3 and DAG that following elicitor treatment there was a very rapid but small (two-fold) increase in these second messengers (Einspahr and Thompson, 1990). Interestingly, in the absence of any elicitors, treatment of the same carrot cells by DAG, an activator of protein kinase C, resulted in the induction of phytoalexins.

1.2.3. Protein kinases as signal transducers

Kinases are one of the major effector molecules of the second messengers described above. Protein kinases have long been recognized to act as amplifiers, propagators and integrators of primary signals (Pawson, 1994). These kinases may phosphorylate several substrates while the substrates themselves may be phosphorylated by several different kinases, reinforcing the idea of cross-talk between various regulatory pathways.

Protein kinases play a central role in plant signal transduction. This is supported by the number of kinases implicated in diverse physiological and stress responses in plants. Examples include, perception of the phytohormone abscisic acid in guard cells (Sheen, 1996), phytochrome mediated gene expression (Neuhaus et al., 1993), the interaction between pollen and stigma (Estruch, et al., 1994) and cold and stress tolerance (Sheen, 1996).

A direct mediation of signalling by protein kinases has been established only in a few systems. In *Arabidopsis thaliana*, perception of ethylene is ascribed to the product of the *ETR1* gene, which resembles members of the two-component signal transduction system in bacteria and contains all the conserved residues required for histidine kinase activity (Swanson et al., 1994). A phosphorylation cascade has been established with the observation that this signal can then relay to another kinase, CTR1, which is related to the Raf-type serine/threonine protein kinases of mammals (Kieber et al., 1993). Although not yet observed in plant cells, the cascade that activates RAF kinase in mammalian cells leads to the activation of a highly conserved mitogen activating protein kinase (MAPK) or the extracellular signal-regulated protein kinase cascades (Kyriakis et al., 1992).

In addition to the abiotic stresses mentioned above, phosphorylation events have long been observed in plant cells as one of the primary events following exposure to pathogens or elicitors. Broad range inhibitors of protein kinases such as staurosporine and K-252a, are known to completely abolish elicitor induction of defense response genes (Felix et al., 1991; Grosskopf et al., 1990). Questions remain: What specific kinases are involved in the elicitation process? What are their substrates and what activities do they modulate? My thesis topic aimed to examine the role of phosphorylation and identify kinase(s) involved in the elicitor/pathogen-induced defense responses in potato tubers. A number of kinases have been implicated in elicitor induction of defense response genes. An overview of these kinases is presented.

1.2.3.1. Pto protein kinase

The disease resistance gene *Pto* is a member of the serine/threonine protein kinase family (Martin et al., 1993). Its specific interaction with the Avr elicitor initiates the defense response. In a yeast two-hybrid assay, Pto also interacts specifically with a second serine/threonine protein kinase, Pti1 (Zhou et al., 1995). In vitro, Pti1 is a substrate for Pto suggesting that Pti1 acts downstream of Pto, thus establishing a kinase cascade. Since *Pto* is present in both resistant and susceptible plants, it likely participates in other cellular activities. This is supported by the fact that Pto has been shown to interact with a family of transcription factors in a yeast two-hybrid system (Zhou et al., 1997). More importantly, expression of *Pto-avrPto* in a heterologous system maintains the integrity of the resistance system implying that the signal transduction machinery may be conserved across the species (Thilmony et al., 1995; Hammondkosak et al., 1998).

1.2.3.2. Calcium-dependent protein kinases

Calcium-dependent protein kinases (CDPK), first identified in soybean are serine/threonine protein kinases and are unique to plant cells (Roberts and Harmon, 1992). As previously described, they possess a calmodulin-like domain that confers the calcium-dependent, calmodulin-independent character to these kinases. The possible physiological functions of the plant CDPKs have been proposed according to their subcellular distribution. CDPKs have been found associated with chromatin (Roberts and Harmon, 1992), plasma membranes (Schaller et al., 1992), and the plant cytoskeleton (Putnam-Evans et al., 1989). The multiple subcellular localizations suggest that this protein family is involved in numerous signal transduction pathways. With respect to defense response, the regulation of the plasma membrane H^+-ATPase, an integral component of defense response in many cells, appears to be under the control of a CDPK (Xing et al., 1996) in tomato. In these cells, CDPK maintains the steady state phosphorylation of the H^+-ATPase. After exposure to race-specific elicitors, ATPase is dephosphorylated resulting in activation of the pump. However, CDPK in conjunction with a PKC-like kinase restores the pump to its normal phosphorylated state.

1.2.3.3. Mitogen-activated protein kinase

Suzuki and Shinshi (1995) initially demonstrated that a MAPK is activated in tobacco cells treated with fungal elicitors and that upstream kinase(s) and Ca^{2+} are likely to be involved in its regulation. Recently, an oligopeptide fragment, Pep 13, derived from the soybean pathogen *Phytophthora sojae*, described previously to elicit a defense reaction in cultured parsley cells, has been shown to induce a MAPK (Ligterink et al., 1997). The activation of this kinase is dependent on the

state of specific ion channels and it appears to be translocated to the nucleus in a manner similar to that is observed in mammalian cells. Once in the nucleus, this MAPK may activate defense-related genes by regulating directly or indirectly regulatory molecules, such as transcription factors responsible for the activation of defense genes.

1.2.3.4. Protein kinase C

PKCs are major regulatory enzymes in animal cells involved in a wide variety of physiological processes including, cell proliferation and differentiation, tumour promotion, membrane receptor function, regulation of ion channels, secretion and gene expression (Nishizuka, 1992). In animals, PKCs are a protein family made up of at least 12 different serine/threonine kinases. Based on biochemical properties and sequence analysis, these isoenzymes have been divided into three groups. The conventional or classical isoforms (cPKCs α, βI, βII, and γ isotypes) meet the original definition of PKCs as Ca^{2+}-activated, phospholipid-dependent enzymes whose affinity for Ca^{2+} is greatly increased by DAG. Members of the second group also referred to as the novel isoforms (nPKCs) exhibit little or no requirement for Ca^{2+}. The last group of PKC isotypes require neither Ca^{2+} nor DAG and belong to the atypical isoforms of PKC. Individual PKC isozymes display subtle biochemical differences and are expressed in a tissue specific and developmentally regulated manner suggesting that they have distinct roles *in vivo*. For example, one of the PKCs appears to regulate the activity of Raf kinase, which in turn regulates a kinase immediately upstream of one of the three MAPK modules described previuosly (Kolch et al., 1993).

Pharmacological evidence suggests that a PKC-like kinase is involved in development of oat tissues (Wolniak and Larsen, 1995) and in phytochrome-mediated regulation of nitrate reductase in maize (Chandok and Sopory, 1996). Pharmacological evidence also indicates a role for this kinase in the plant defense response. Inhibitors of these enzymes block elicitor-induced accumulation of phytoalexins in carrot cells (Kurosaki et al., 1987). As previously described in tomato cells, rephosphorylation of H^+-ATPase is achieved by a CDPK (Xing et al., 1996). However, activators and inhibitors of PKC also suggest that the CDPK is regulated by a PKC-like kinase.

Until recently, biochemical data to support the accumulating pharmacological evidence for PKC in plants was limited. A partial purification by Nanmori *et al.*, (1994) from *Brassica campestris* demonstrated that an enzyme with properties similar to the first group of animal PKCs is present in plant cells. More recently, Chandok and Sopory (1998), reported that an enzyme belonging to the same group of PKCs has been purified to homogeneity from maize. In both instances the activity of the kinase was supported by all three cofactors: calcium, phosphatidylserine and DAG.

1.2.4. Other signal transducers

1.2.4.1. Active oxygen species

One of the earliest events during the hypersensitive response is the production of active oxygen species (AOS) such as, superoxide anion and hydrogen peroxide (Keppler et al., 1989; Bolwell et al., 1995). Interestingly, this was first discovered in potato plant cells infected with an incompatible race of *P. infestans* (Doke et al., 1983). Hydrogen peroxide is presumed to drive the cross-linking of cell wall proteins via dityrosine bridges (Bradley et al., 1992). Consequently, the wall becomes reinforced and more resistant to the pathogens. On the other hand, hydrogen peroxide, in combination with other AOS such as the hydroxyl radical, may be directly anti-microbial.

AOS may function in the generation of bioactive fatty-acid derivatives. Rapid lipid peroxidation is observed in elicited cells and during the HR, but the potential signal activities of the products have not yet been established (Rogers et al., 1988). It is postulated that hydrogen peroxide may act as a substrate for lipoxygenase-mediated production of jasmonic acid precursors from linolenic acid present in the plasma membrane lipid pool (Bradley et al., 1992).

1.2.4.2. Salicylic acid (SA)

SA accumulates to high levels in the region surrounding developing lesions during the HR, and prior to transcriptional activation of many defense related genes such as the pathogenesis-related genes (PR) (Enyedi et al., 1992). A much smaller rise in SA has been observed in non-infected tissues distant from the infection site. A concomitant activation of the PR genes in these distal tissues suggested that SA may be the signal molecule responsible for SAR induction. This

has been supported by constitutive expression of the bacterial salicylate hydroxylase gene (*nahG*) which converts SA into the inactive compound catechol (Gaffney et al., 1993). Transgenic plants expressing NahG fail to exhibit systemic acquired immunity, displayed reduced PR protein accumulation and exhibited severe disease symptoms in infected leaves. Compelling evidence suggests that in tobacco cells, induction of PR genes by SA is mediated by a member of a MAPK family distinct from MAPK previously implicated in stress responses (Zhang and Klessig, 1997). The complexity between the various signalling components is borne out by the observation that SA binds to catalase and inhibits its activity (Chen et al., 1993) resulting in an elevated concentration of hydrogen peroxide. Indeed, transgenic tobacco plants with catalase gene in the antisense display enhanced resistance to tobacco mosaic virus (Chamnongpol, et al., 1996).

1.3. Defense response genes

The culmination of the signal transduction pathway initiated by pathogen ingress is the activation of genes that will successfully thwart the pathogen's progress. The function of many of these genes is directly related to where they are expressed in the plant. In cells directly infected by the pathogen, resistance is associated with HR which leads to the death of infected cells, a process mediated by the activation of specific genes which program the infected cell to the death fate. The end result is that the pathogen infecting these cells also die and the spread of disease is averted.

Cells in the vicinity of the infection site express a distinct suite of defense genes, the functions of which are two fold. In addition to augmenting the existing defensive mechanisms, the proteins encoded by these genes prepare the cells for

pathogen resistance. The genes that contribute to this process encode cell wall proteins (Bradley et al., 1992; Brisson et al., 1994), enzymes of the phenylpropanoid pathway (Dixon and Paiva, 1995), hydrolytic enzymes such as chitinase and glucanase (Kombrink et al., 1988) and pathogenesis-related proteins (Linthorst, 1991). Examples of proteins that contribute both to augment the cellular defense mechanisms and those that actively resist the pathogen are described in the following Sections.

1.3.1. Cell wall proteins

Cell wall modification has been observed soon after pathogen perception (Bradley et al., 1992; Brisson et al., 1994) and is primarily driven by hydrogen peroxide. The architecture of the cell wall may be modified through the production and deposition of hydroxyproline-rich glycoproteins, or reinforced by the deposition of lignin and related wall-bound phenolics. Lignin is a strong and complex polymer formed by the condensation of phenylpropanoid units, and is an integral component of the secondary cell walls of vascular plants (Douglas, 1996). The enzymes in the phenylpropanoid biosynthetic pathway responsible for the synthesis of the phenolic compounds are detected both at infection sites and their peripheries. Other notable products of this biosynthetic pathway include phytoalexins which have direct antimicrobial effect and salicylic acid which as previously described plays a role in systemic disease resistance.

1.3.2. Hydrolytic enzymes

Plants also produce compounds that can degrade cell walls of potential pathogens, thereby reducing the potency of infection. The examples include β-1,3-glucanases and chitinases. Both of these enzymes are encoded by multigene families and are implicated in developmental and physiological processes. For example, members of both enzyme families are expressed in response to ethylene (Boller, 1985). Chitin, the substrate for chitinase, is not generally found in plants, but it is a common constituent of many fungal cell walls. Endochitinase also shows lysozyme activity and its induction may therefore be an effective defense mechanism of plants against invading bacteria (Boller, 1985).

β-1,3-glucanases seem to play a role in pollen formation (Worrall et al., 1992) and seed germination (Vogeli-Lange et al., 1994). In response to fungal attack, the enzymes can directly digest β-1,3-glucans of fungal cell walls (Ebel and Scheel, 1992). The consequence of digestion of fungal and host polysaccharides by both glucanases and chitinases is the release of β-glucans, chitin and chitosan oligosaccharides. These oligisaccharides have proven to be potent signals or endogenous elicitors capable of activating plant defense responses (Ryan, 1994).

1.3.3. Pathogenesis-related proteins

The term PR protein initially referred to numerous extracellular proteins that accumulate in susceptible tobacco leaves following infection with tobacco mosaic virus (Van Loon and Van Kammen, 1970). The proteins accumulate mainly at the peripheries of the HR, but are also detected at lower levels in distant uninfected leaves. A similar pattern has been observed in a wide variety of plant-pathogen interactions. More recently, the definition has been broadened to include intra-

and extracellularly localized proteins that accumulate in intact plant tissue or cultured cells after pathogen attack or elicitor treatment. Their expression also correlates with leaf senescence, exposure to UV, ethylene, cytokinins and salicylic acid (Ebel and Scheel, 1992).

Given the large number of *PR* genes identified from many different plants and their structural and functional heterogeneity, a nomenclature has recently been established to categorize the *PR* genes (Van Loon et al., 1994). The regrouping places the *PR* genes into eleven groups: *PR-1* to *PR-11*. *PR-2* and *PR-3* for example, include families of glucanases and chitinases, respectively. As noted before, both glucanases and chitinases exhibit antifungal and antibacterial properties and play a role in disease resistance. Although other proteins such as PR-1 and PR-4 exhibit some antifungal activity, their function has not been defined (Niderman et al., 1995; Ponstein, et al., 1994). Similarly, functions of the remaining PR proteins have remained obscure. Studies with transgenic plants overexpressing PR proteins suggest that individually, they provide some protection from disease which can be increased through the coordinated expression of several PR proteins (Jach et al., 1995).

1.4. Defense response in potato

Defense reactions in potato as described in Section 1.1.4, have been studied in the context of interaction with the pathogen *Phytophthora infestans* and the elicitor arachidonic acid (Bostock et al., 1981). The basic passive and active defense mechanisms described previously are also observed in this interaction. Potato infected with the incompatible race of the pathogen exhibits the archetypal HR. The factors associated with the HR, such as depolarization of the membrane

(Tomiyama et al., 1983), production of active oxygen species (Doke, 1983), the induction of phytoalexins (Oba et al., 1985), and induction of glucanases and chitinases (Kombrink et al., 1988) are all observed in the potato-*P. infestans* interaction. The expression patterns of many defense-related genes surrounding the infection site and beyond are also similar to other systems. These include the enzymes of the phenylpropanoid biosynthetic pathway (Smith and Rubery, 1981) and the PR proteins. Among the *PR* genes that are induced are members of the *PR-2*, *3*, *5* and *10* groups (Marineau et al., 1987; Beerhues and Kombrink, 1994,; Zhu et al., 1995).

1.4.1. Regulation of *PR-10a* in potato

The expression of PR proteins is intimately associated with the defense response status in plants and understanding the mechanisms that regulate the expression of these genes could yield valuable information about the overall defense response. Our laboratory has focused its research efforts on the pathways leading to the expression of the *PR-10* gene family.

The *PR-10* gene family in potato is comprised of at least three members whose homologs have been identified in many monocot and dicot plant species (Van Loon et al., 1994; Warner et al., 1992). In species where no close homologs are found, a subclass of a distantly related protein referred to as the major latex protein has been identified (Osmark et al., 1998). The two potato *PR-10* genes, *PR-10a* and *PR-10b* were initially isolated by differential screening of a cDNA library from potato tubers induced by the elicitor arachidonic acid (Marineau et al., 1987). A third member has been isolated from potato leaves treated with mycelial homogenate of *P. infestans* (Subramaniam et al., manuscript in preparation).

In potato tubers, the *PR-10a* gene is induced after infection by both compatible and incompatible races of *P. infestans*. However, the incompatible (resistance) interaction results in a more rapid expression and a higher level of accumulation of PR-10a protein (Constabel and Brisson, 1992). The expression of the gene is localized to the vascular tissues of stolons, stems, petioles and leaves of the potato plant and in addition to the site of infection. Under normal circumstances *PR-10a* is developmentally induced in the epidermal layer of the stigma (Constabel and Brisson, 1995). Since the biological function of PR-10 proteins has not been characterized, the significance of these studies remains speculative. Recently, studies have shown that some members of the *PR-10* may possess ribonuclease activity (Moiseyev et al., 1994; Bufe et al., 1996; Swoboda et al., 1996).

Analyses of the of *PR-10a* promoter have localized elements necessary for regulation by arachidonic acid (Matton et al., 1993). The studies have identified a region between -135 and -105 of the promoter necessary for activation by the elicitor known as the elicitor response element (ERE). Furthermore, electrophoretic mobility shift assay (EMSA) analysis revealed nuclear proteins, named PBF-1 and PBF-2, specifically interact with the ERE. There is a direct correlation between the activation of *PR-10a* and increased binding of these factors to the ERE (Després et al., 1995). Furthermore, treatment of nuclear extracts with alkaline phosphatase abolishes this increased binding, suggesting that a phosphorylation step is involved. The treatment of potato tubers with staurosporine, a potent but non-specific inhibitor of protein kinases, reduces the elicitor-induced binding of these factors, while treament with okadaic acid, a phosphatase inhibitor, increases binding to the ERE independent of the elicitor.

This confirms the importance of phosphorylation events in the binding of PBFs to the ERE and the subsequent activation of PR-10a.

1.5. Research objectives

The thesis sets out to address the role of phosphorylation events in the arachidonic acid induction of *PR-10a* and to identify components within this signalling pathway in potato tubers. Initially, pharmacological agents used to modulate the activity of various protein kinases in animal cells were used on tuber discs to determine the effects on binding of the factor PBF-2 to the ERE and the accumulation of PR-10a protein. Results suggested that activation of a PKC-like kinase may be involved in the pathway leading to the activation of *PR-10a*. Biochemical and immunological methods were subsequently used to confirm the role of this kinase in the elicitor-induced defense response in potato.

Finally, in order to characterize this kinase in detail, a purification scheme was designed. Our earlier results suggested that this kinase is present in the membranes of tubers. The efficacy and practicality had to be determined before using the membranes as a source of this kinase. A partial purification of the kinase was achieved and its purity at each purification step was assessed by biochemical and immunological methods. The mechanism of regulation of the kinase and the role it might play in the potato defense system are discussed.

Chapter 2. Materials and Methods

2.1. Materials

Potato tubers (*Solanum tuberosum*, L. cv Kennebec) were obtained from the Québec Ministry of Agriculture Les Buissons Research Station (Pointe-aux-Outardes, Québec, Canada). They were stored in the dark at 4°C and brought to room temperature 24 hr before use. Arachidonic acid, sphingosine, palmitoyl sphingosine, phosphatidylserine (PS), phosphatidylcholine (PC), 1,2-Dioleolyl-sn-Glycerol (DAG), L-threonine, and carbodiimide were purchased from Sigma. Arachidonic acid, PS, PC, and DAG were dissolved in chloroform and stored at -70°C. Calphostin C and bisindolylmaleimide were purchased from Calbiochem (La Jolla, CA). 12-*O*-Tetradecanoylphorbol 13-acetate (TPA), 4α-12-*O*-Tetradecanoylphorbol 13-acetate (4αTPA), histone H1, Ac-MBP (4-14), and α-peptide {[ser25]PKC(19-31); RFARKGSLRQKNV} were purchased from Gibco BRL (Burlington, Ontario, Canada). TPA and 4αTPA and all the inhibitors were dissolved in 100% DMSO and stored in small aliquots at -20°C. γ-^{32}P-ATP (7000 Ci/mmol) was obtained from ICN (Mississauga, Ont). The anti-PKC antibody (polyclonal) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Hsp70 antibody (monoclonal) was obtained from Stressgen Biotechnology (Victoria, BC). The anti-rabbit and anti-mouse antibodies conjugated to horseradish peroxidase were purchased from BioRad Laboratories (Hercules, CA).

2.2. Experimental methods

2.2.1. Treatments of tuber discs

After a 6-hr aging period, tuber discs were treated with 20 µL of PKC activator and inhibitors in 1% DMSO or with a solution of 1% DMSO as control. After 30 min, the discs were treated with 20 µL of 1 µg/µL emulsion of arachidonic

acid in water.

2.2.2. Immunoblot analyses

Proteins were separated on 14% (for PR-10a), 10% (for PKC and Hsp70) and 12% (for chitinase and glucanase) SDS-polyacrylamide gels, and blotted onto nitrocellulose membranes as previously described (Constabel and Brisson, 1992). The blots were stained with Ponceau S, blocked, and incubated with anti-PR-10a antibodies as described previously (Constabel and Brisson, 1992). Both anti-PKC antibodies and anti-Hsp70 antibodies were used at 100 ng/mL. The antibodies raised against potato chitinase (class I) and 1,3-β-glucanase (class I) were used at a dilution of 1/5000. The blots were then developed with the electrochemiluminescent detection system (Amersham International) according to the manufacturer's instructions. Protein quantification was performed using a protein assay (Bio-Rad) with BSA as a standard.

2.2.3. Preparation of crude nuclear extracts and electrophoretic mobility shift assay

Treatment of potato tubers, extraction of nuclear proteins, and use of probes, and gel conditions for the electrophoretic mobility shift assay (EMSA) were identical to the procedures described previously (Després et al., 1995).

2.2.4. Preparation of the particulate fraction from potato tubers

Potato was taken from cold storage and 60 g of sliced tubers were homogenized by two 45-sec bursts in a blender in 120 mL of buffer A (50 mM Tris-HCl, pH 7.5 containing 300 mM sorbitol, 100 mM $CaCl_2$, 5 µg/mL of leupeptin, 5 µg/mL aprotinin, 100 µM phenylmethylsulfonyl fluoride, and 30 mM β-

mercaptoethanol). The homogenate was filtered through Whatman No.1 MM paper. The filtrate was centrifuged for 10 min at 5000*g*, and the supernatant was centrifuged for an additional 1 hr at 100,000*g* at 15°C. The membrane pellet was gently resuspended in 100 mL of buffer A by 10 strokes in a Dounce homogenizer (Wheaton Scientific, NJ), followed by centrifugation for 1 hr at 100,000*g* at 4°C as before. The washing procedure was repeated three times. In the last two washes, calcium was excluded from the buffer. The pellet was resuspended in 10 mL of ice-cold buffer B (50 mM Tris-HCl, pH 7.5, 5 mM EGTA, 2 mM EDTA, 50 mM NaCl, 0.5% Triton X-100, 5 μg/mL of leupeptin, 5 μg/mL aprotinin,100 μM phenylmethylsulfonyl fluoride, and 30 mM β-mercaptoethanol), incubated with gentle shaking at 4°C for 1 hr followed by centrifugation at 4°C, as before. The supernatant was stored at 4°C and an aliquot (100 μg of protein) was treated with 25 μL of Affigel protein A agarose (Bio-Rad) for 1 hr before it was used in the kinase assay.

2.2.5. PKC assay

PKC activity was quantitated by measuring incorporation of phosphorus-32 into the substrates histone H1 or α-peptide. The reaction mixture (50 μL) contained 25 μL of the particulate fraction (5 μg of protein), 5 mM EGTA, 20 mM $MgCl_2$, 4.95 mM $CaCl_2$, 20 μM γ-^{32}P-ATP (1μCi per assay), 200 μg/mL histone H1 or 10 μM α-peptide, 20 μg/mL PS or PC, 8 μg/mL DAG, and 10 nM TPA or 4αTPA. Phospholipids in chloroform were evaporated to dryness under nitrogen and resuspended in 20 mM Tris-Cl, pH 7.5, with sonication and vortexing. The reaction was started with the addition of the solution of $MgCl_2$, γ-^{32}P-ATP, substrate, and $CaCl_2$ to the incubation mixture containing the particulate fraction, the phospholipid and DAG/TPA. The final concentration of DMSO was 0.002%.

After 10 min at 30°C, the reaction was stopped by the addition of 25 µL of 3x sample buffer (150 mM Tris-HCl, pH 6.8, 6% SDS, 0.3% bromophenol blue, 30% glycerol, 50 mM β-mercaptoethanol). An aliquot (15 µL) of the reaction mixture was separated by 12% SDS-PAGE for histone H1 and 18% SDS-PAGE for α-peptide. The phosphorylated substrates were visualized by autoradiography. For quantifiying the phosphorylated substrates, the gels were stained with Coomassie Brilliant Blue R 250 and gel slices were cut corresponding to the substrate and were subjected to scintillation counting.

The renatured kinase was excised from the gel and eluted overnight in 30 mM Hepes-HCl, pH 7.5, at 4°C. An aliquot of the eluate was used to determine PKC activity by phosphorylation of the α-peptide as described above, except that the reaction volume was 250 µL and $CaCl_2$ was adjusted to 20 µM.

2.2.6. Immunoprecipitation, in-gel kinase assay, and phosphoamino acid analysis

For immunoprecipitation, PKC-specific antibody was bound to the protein G-agarose (Sigma). This was achieved by incubating 500 ng of the antibody to 30 µL of the protein G-agarose overnight at 4°C. The conjugate was washed two times in buffer C (50 mM Tris-HCl, pH 7.5, 2 µg/mL of aprotinin and 2 µg/mL of leupeptin). An aliquot (600 µg of protein) of the particulate extract was then added to the PKC antibody-protein G-agarose conjugate and incubated with gentle agitation for 3 hr at 4°C. The immunoprecipitate was washed three times in buffer D (buffer C containing 5 mM EGTA, 2 mM EDTA, 100 mM NaCl and 0.5% Triton X-100). The pellet was resuspended in sample buffer, and boiled for 5 min, and the sample was electrophoresed for immunoblot analysis and in-gel kinase assay.

The immunoblot analysis of the immunoprecipitates was performed as described above.

The in-gel kinase assay was performed using the method of Kameshita and Fujisawa (1989), with minor modifications. An aliquot (3 µg of protein) of the particulate fraction and the immunoprecipitates were electrophoresed, except that the 10% SDS-PAGE gel was polymerized with 200 µg/mL of histone H1. After renaturation of the proteins in the gel, the gel was washed in Tris buffer (50 mM Tris-HCl, pH 7.5, 5 mM β-mercaptoethanol) for 30 min at room temperature. In some experiments, the gel was treated with 1 µM calphostin C for 2 hr. After several washes with Tris buffer, both the treated and the nontreated gels were incubated in the kinase buffer (Tris buffer containing 10 mM $MgCl_2$, 10 mM $MnCl_2$, 2 mM DTT, 1 µM ATP (50 µCi/mL γ-^{32}P-ATP) for 3 hr at room temperature. The gel was extensively washed with a solution of 5% trichloroacetic acid and 1% pyrophosphate, dried and subjected to autoradiography. The γ-^{32}P-labeled immunoprecipitated proteins from the in-gel kinase assay were excised and subjected to a second SDS-PAGE. After blotting, the Immobilon-P membrane (Millipore) was autoradiographed, and the radiolabeled protein band corresponding to the immunoprecipitate was excised. The proteins were partially hydrolyzed in 200 µL of 6 N HCl at 110°C for 2 hr (Kamps and Sefton, 1989). The supernatant was lyophilized and resuspended in 3 µL of distilled water. The sample and 2 µL of cold phosphoamino acid standards (2 mg/mL each of phosphoserine, phosphothreonine, and phosphotyrosine) were spotted onto 100-µm thin-layer cellulose chromatography plates (Sigma). The three phosphohydroxyamino acids were separated by an ascending solvent containing isobutyric acid:0.5 M NH_4OH {(5:3, v/v); Duclos et al., 1991}. The phosphorylated

amino acids were visualized by autoradiography. The positions of the three phosphoamino acid standards were visualized by spraying the chromatography plates with 0.2% ninhydrin (Sigma) in absolute ethanol followed by heat treatment.

2.2.7. Pathogen infection and ELISA assays

The tuber discs were prepared and treated with PKC activator and inhibitors as described above. Elicitor treatment and inoculation of the treated tubers with *Phytophthora infestans* (race 1, 2, 3, 4) were as described before (Constabel and Brisson, 1992). The tissue was incubated on moist filter paper in Petri dishes and incubated in the dark at 15°C. After the onset of the mycelia growth, the tubers were frozen and stored at -20°C for ELISA analysis. The ELISA analysis was performed as described by Yao et al., (1995) using a *Phytophthora* detection kit (Sigma). The ELISA values, which are proportional to the amount of pathogen in tuber extracts, were determined using a microplate reader (Model MR5000; Dynatech Laboratories Inc., Chantilly ,VN) and expressed as the absorbance at 410 nm.

2.2.8. Purification of a cPKC-like kinase

Chromatographic procedures

All the procedures were performed at room temperature with a fast protein liquid chromatography (FPLC) apparatus of Pharmacia. The elution profiles were monitored by absorbance at 280 nm. Immunoblot analyses with PKC and Hsp70 antibodies were performed on the eluted fractions as described in Section 2.2.2 and the PKC activity assay were performed on the eluted fractions as described in Section 2.2.5. The kinase activity was determined with α-peptide as the

substrate.

Preparation of protein extracts

The protein extracts were prepared as described in Section 2.2.4 with few modifications. Approximately 600 g of tubers were used to prepare the membranes. The tubers were sliced and incubated in the dark for 20 hr. The grinding and the washing buffers did not contain calcium. The proteins were extracted from the membrane pellet with 50 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 0.5 mM EDTA, 1.0% Triton X-100, 5 ug/mL leupeptin, 200 uM phenylmethylsulfonyl fluoride, and 15mM β-mercaptoethanol. All the centrifugation steps were performed at 4°C.

Q-Sepharose Fast Flow

The extract (150 mL) was applied to a Pharmacia Q-Sepharose column (2.0 cm x 10 cm) equilibrated with Buffer E (20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 0.5 mM EDTA, 1 ug/mL leupeptin, 5 mM β-mercaptoethanol) containing 10 mM NaCl. The column was washed with buffer E containing 75 mM NaCl until the absorbance at 280 nm reached the baseline. The bound proteins were eluted batchwise from the column with buffer E containing 350 mM NaCl.

Threonine-Sepharose

The Q-Sepharose fraction which exhibited the PKC activity was diluted with Buffer E to reduce the concentration of NaCl to 100 mM and applied to a threonine-Sepharose column prepared as described by Kikkawa et al., (1986). The column (1.0 cm x 5 cm) was equilibrated with buffer E containing 10 mM NaCl and subsequently washed with buffer E containing 200 mM NaCl until the

absorbance at 280 nm reached the baseline. The bound kinase was eluted from the column with buffer E containing 600 mM NaCl.

Phenyl-Sepharose- 4B

The threonine-Sepharose fraction containing the PKC activity was brought to a final concentration of 2.5 M NaCl and applied to a phenyl-Sepharose 4B (Pharmacia) column (1.0 cm x 2 cm) equilibrated with 2 M NaCl in buffer E. The column was washed with 1 M NaCl in buffer E followed by a wash in 0.5 M NaCl in buffer E containing 0.05 % Triton X-100. The proteins were eluted by a combination of a linear negative gradient of NaCl (0.5 M - 0 M) and a linear positive gradient of Triton X-100 (0.05 - 0.30 %). The flow rate was 1.0 mL/min and 2 mL fractions were collected. The fractions exhibiting PKC-like kinase activity were pooled and precipitated overnight at 4°C with an equal volume of 50 % PEG 4000 (Sigma).

Mono S HR 5/5

The PEG precipitate was centrifuged at 15,000*g* for 20 min at 4°C and the pellet was resuspended in buffer E containing 10 mM NaCl. After a brief centrifugation, the supernatant was applied to a Mono S column (Pharmacia) equilibrated with buffer E containing 10 mM NaCl. The proteins were eluted with a linear gradient of NaCl concentration from 10 mM to 1 M. The flow rate was 1.0 mL/min and 1 mL fractions were collected.

2.2.9. Microsequencing of internal tryptic peptides

The proteins in the active fractions from the Mono S column were precipitated by acetone. The pellets were then dissolved in SDS sample buffer

and separated on a 10% SDS-polyacrylamide gel. After Coomassie blue-staining, the 78 kD band was excised and sent to the Harvard Microchemistry Facility (Cambridge, MA) for amino acid sequence analyses. Briefly, for sequence analyses, the PKC-like kinase was subjected to in-gel digestion with trypsin and resultant peptides were separated by capillary electrophoresis. Two peptides were selected and sequenced by tandem mass spectrometry.

2.2.10. Two-dimensional gel electrophoresis

The proteins in the active fractions from the Phenyl-Sepharose column were precipitated by acetone. The pellets were resuspended in urea-solubilization buffer (9.5 M urea, 2.0% Triton X-100, 5% β-mercaptoethanol, 1.6% Bio-Lyte 5/7 ampholyte and 0.4% Bio-Lyte 3/10 ampholyte (Pharmacia)) and incubated for 1 hr at room temperature. The proteins were separated in the first dimension by isoelectric focusing as described by Marineau, et al., (1987). The isoelectric-focused proteins from the first dimension were separated in the second dimension by 10% SDS-PAGE as described in Section 2.2.2.

Chapter 3. Results

3.1. A Functional homolog of mammalian protein kinase C participates in the elicitor-induced defense response in potato

3.1.1. Elicitor and wound induction of *PR-10a* are mediated through the action of a protein kinase

We had demonstrated that binding of the nuclear factors PBF to the elicitor response element is a consequence of phosphorylation events (Després et al., 1995). Moreover, the studies established a strong correlation between the level of binding of these factors to the ERE and the subsequent activation of the *PR-10a*. Thus, one would predict that protein kinases and/or protein phosphatases are involved in the pathway leading to the activation of this gene and that inhibitors of these enzymes would have an effect on *PR-10a* expression. Figure 1 is an immunoblot showing the effects of an inhibitor of kinases and phosphatases on the elicitor induction of the PR-10a protein. In agreement with our earlier studies, both wounding and the treatment with arachidonic acid leads to the induction of the *PR-10a* gene (Matton and Brisson, 1989) and to the accumulation of the product of this gene (Constabel and Brisson, 1992; and Figure 1, compare lanes 1 (fresh tubers) and 2 (wounded tubers) as well as lanes 2 and 5 (elicited tubers). We also reported that *PR-10c* another member of the *PR-10* gene family sharing 70% identity with PR-10a, is present in fresh tubers (lane 1) and that its pattern of accumulation remains unaffected by wounding or elicitor treatment (Constabel and Brisson, 1992). However, as indicated in Figure 1, treatment of tuber discs with staurosporine, a potent but non-specific inhibitor of protein kinases reduced both the elicitor-induced (compare lanes 5 and 6) as well as the wound-induced (compare lanes 2 and 3) accumulation of PR-10a. In contrast, treatment of tuber discs with okadaic acid, an inhibitor of the serine/threonine phosphatases1 and

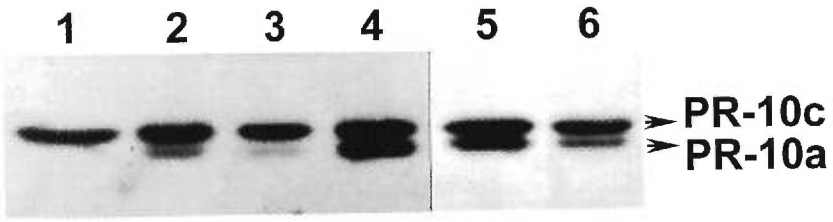


Figure 1

Figure 1. Accumulation of the PR-10a Protein after Treatment of Potato Tubers with Protein Kinase and Protein Phosphatase Inhibitors.

Tuber discs were aged for 6 hr and treated with 1% DMSO (lane 2), 5 nM staurosporine (lane 3), 1 μM okadaic acid (lane 4), arachidonic acid (lane 5), or a combination of 5 nM staurosporine and arachidonic acid (lane 6). The tuber discs were incubated for 48 hr in the dark. Lane 1 represents fresh tuber tissues. Extracted proteins were separated by electrophoresis on 14% SDS-polyacrylamide gels, blotted to nitrocellulose, and analyzed using an anti-PR10a antibody. PR-10a and PR-10c indicate the two proteins detected.

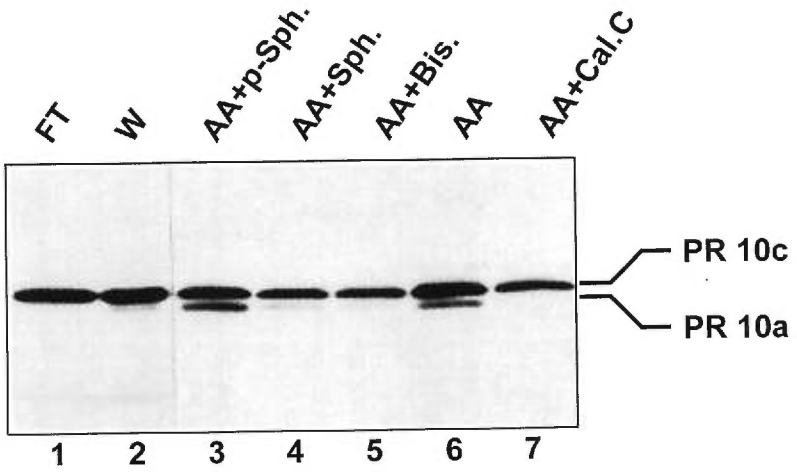
2A, resulted in an increased accumulation of the PR-10a protein to levels comparable with those obtained by treatment with the elicitor arachidonic acid (compare lanes 4 and 5). Accumulation of PR-10c was not affected to the same extent as that of PR-10a. Although it is likely that the expression of many other genes is modified following treatment with staurosporine and okadaic acid, our results suggest, nevertheless, that protein kinases and phosphatases are involved in *PR-10a* gene activation during wounding and elicitation.

3.1.2. Inhibitors of mammalian protein kinase C affect the elicitor-induced expression of *PR-10a*

As demonstrated earlier, staurosporine, a protein kinase inhibitor, has major deleterious effects on the elicitor-induced binding of nuclear factors to an elicitor response domain and on the accumulation of the PR-10a protein (Després et al., 1995). Furthermore, the low concentration of staurosporine (5 nM) required to inhibit PR-10a protein accumulation led us to speculate that a kinase belonging to the PKC family is involved in the activation of *PR-10a*. Based on the diverse properties of PKC isoenzymes, Blobe et al., (1995) proposed a strategy to establish a role for PKC in a biological process. One important element of this strategy is to show that inhibitors of this kinase can block the biological event under study. In view of this, we used specific inhibitors of protein kinase C to observe their effects on the elicitor-induced expression of *PR-10a*.

As seen in the immunoblot in Figure 2A, the application of the elicitor arachidonic acid to the tuber discs induced the accumulation of PR-10a (lane 6), compared to the sliced tuber alone (lane 2). The elicitor-induced accumulation of PR-10a protein was severely curtailed when the tuber discs were pretreated with

A


FT
W
AA+p-Sph.
AA+Sph.
AA+Bis.
AA
AA+Cal.C
PR 10c
PR 10a
1
2
3
4
5
6
7

B

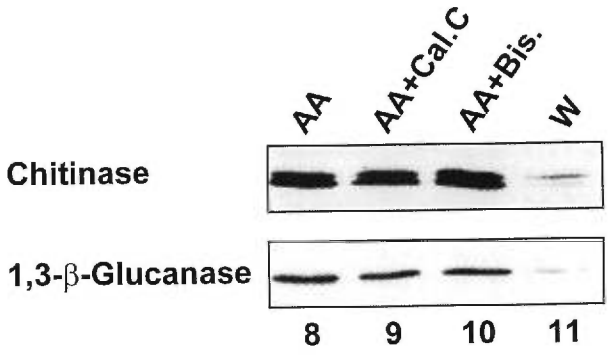

AA
AA+Cal.C
AA+Bis.
W
Chitinase
1,3-β-Glucanase
8
9
10
11

Figure 2

Figure 2. Immunoblots Showing the Effect of Protein Kinase C Inhibitors on the Accumulation of PR-10a, Chitinase, and 1,3-β-Glucanase.

Following a 6-hr aging period, tuber discs were treated with 1% DMSO (lanes 2,6,8 and 11), 2 μM palmitoyl sphingosine (AA + p-Sph; lane 3), 2 μM sphingosine (AA + Sph; lane 4), 50 nM bisindolylmaleimide (AA + Bis; lanes 5 and 10), and 500 nM calphostin C, (AA + Cal.C; lanes 7 and 9). After 30 min, the elicitor, 1 mg/mL arachidonic acid (AA) was applied to the treated tubers (lanes 3 to 10), and incubation was continued for 48 hr in the dark at room temperature. Total proteins were extracted, and 10 μg aliquots from each sample were separated by SDS-PAGE. Lane 1 represent fresh tuber (FT) tissues, lanes 2 and 11 represent wounded tuber (W) tissues not treated with arachidonic acid, and lanes 6 and 8 represent tuber tissues treated with arachidonic acid (AA).

(A) Immunoblot with anti-PR-10a antibodies. PR-10a and PR-10c indicate the two proteins detected. Essentially identical results were obtained in five independent experiments.

(B) Immunoblot with anti-Chitinase, and anti-1,3-β-Glucanase antibodies as indicated. Essentially identical results were obtained in three independent experiments.

the PKC inhibitors sphingosine (Hannun et al., 1991) (Figure 2, lane 4), bisindolylmaleimide (Toullec et al., 1991) (lane 5), or calphostin C (Tamaoki, 1991) (lane 7) before the elicitor arachidonic acid was applied on the discs. The fact that palmitoyl sphingosine, an analog of sphingosine that does not inhibit PKC (Hannun et al., 1991), had no effect on the elicitor-induced accumulation of PR-10a (lane 3) emphasized the specificity of action by sphingosine. Figure 2A also indicates that the level of accumulation of PR-10c, which is constitutively present in fresh tubers (Figure 2A, lane 1) was only moderately affected by these inhibitors. Neverthless, nonspecific effects of the inhibitors were monitored with other elicitor-inducible genes. As Figure 2B shows, both chitinase and 1,3-β-glucanase were induced by elicitor treatment (lane 8) compared with the sliced tuber discs (lane 11). However, pretreatment of the tuber discs with the PKC inhibitor calphostin C (lane 9) or bisindolylmaleimide (lane 10) did not affect the elicitor-inducibility of these genes.

3.1.3. An activator of mammalian PKC regulates expression of *PR-10a*

Other elements of the strategy proposed by Blobe et al., (1995) require that the biological event be mimicked by the addition of PKC activators. Furthermore, this activation should be countered by the PKC inhibitors. Studies with animal cells have established that PKC isoforms are potently activated by diacylglycerol, a product of lipid hydrolysis (for a review, see Nishizuka, 1992). Phorbol esters, such as TPA fully substitute for DAG and are able to activate PKC (Castagna et al., 1982). In contrast to DAGs, phorbol esters are slowly metabolized in the cells, resulting in a protracted stimulation of the PKC enzyme (Blobe et al., 1994). If PKC is involved in the elicitor-mediated response, as implied from the results shown in Figure 2, then application directly on potato tuber discs of phorbol

esters, such as TPA, should lead to the activation of this kinase and result in the activation of *PR-10a*. As the immunoblot in Figure 3 illustrates, treatment of tuber discs with TPA strongly induced the accumulation of the PR-10a protein compared to the wounded tubers (lane W). The inability of 4α-12-*O*-tetradecanoylphorbol 13-acetate (4αTPA), an inactive isomer of TPA to induce accumulation of PR-10a confirmed the specificity of action by TPA.

The PKC inhibitor sphingosine, which inhibited the elicitor-induced accumulation of PR-10a protein (Figure 2, lane 4), is known to be an antagonist of DAGs and phorbol esters (Kahn et al., 1991). Therefore, its ability to counteract the effects of TPA (Figure 3, lane TPA+Sph) satisfies the other criterion by which the action of an activator is overcome by one of its inhibitors.

3.1.4. The binding of nuclear factor PBF-2 is affected by TPA and PKC inhibitors

We have previously demonstrated that after elicitor treatment of potato tubers, increased binding of the nuclear factors PBF-1 and PBF-2 to the ERE of *PR-10a* is preceded by a phosphorylation event (Després et al., 1995). This increase in binding of the factors correlates with the activation of *PR-10a*. Now we have shown that inhibitors of PKC and its activator TPA have significant effects on PR-10a protein accumulation (Figures 2 and 3). To confirm that this kinase is indeed involved in the pathway leading to the activation of *PR-10a*, we determined the ability of PBF-2 to bind to the ERE of *PR-10a* in response to the various modulators of PKC activity.

As the results of the EMSA in Figure 4 indicate, elicitor treatment (lane 3)

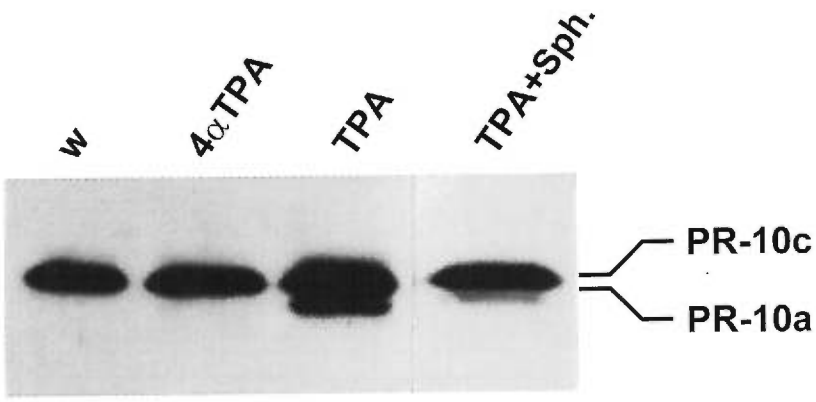


Figure 3

Figure 3. Immunoblot Showing the Effect of TPA on the Accumulation of PR-10a.

Following a 6-hr aging period, tuber discs were treated with 1% DMSO (W), 100 nM of TPA, 100 nM of 4αTPA, and 100 nM of TPA plus 2 μM of sphingosine (TPA+Sph.). After an incubation period of 48 hr in the dark at room temperature, total proteins were extracted, and 10-μg aliquots of each sample were fractionated by SDS-PAGE and immunoblotted with anti-PR-10a antibodies. PR-10a and PR-10c indicate the two proteins detected. Essentially identical results were obtained in five independent experiments.

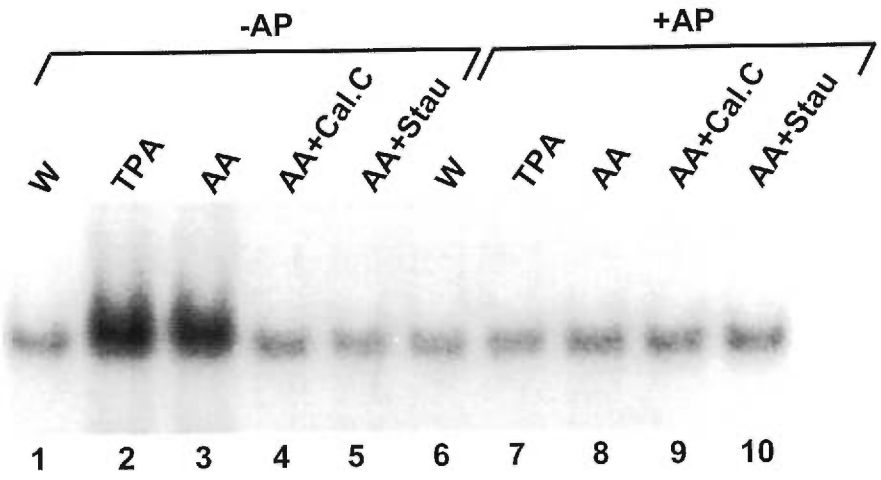

-AP
+AP
W
TPA
AA
AA+Cal.C
AA+Stau
W
TPA
AA
AA+Cal.C
AA+Stau
1
2
3
4
5
6
7
8
9
10

Figure 4

Figure 4. Binding of PBF-2 to a *PR-10a* Promoter Element is Affected by the Activation of a PKC-like Enzyme and by Treatment with Alkaline Phosphatase.

Tuber discs were aged for 6 hr and treated with 1% DMSO (lanes 1, 3, 6, and 8), 100 nM of TPA (lanes 2 and 7), arachidonic acid (AA; lanes 3 and 8), a combination of 500 nM of calphostin C and arachidonic acid (AA+Cal.C; lanes 4 and 9), and a combination of 20 nM of staurosporine and arachidonic acid (AA+Stau; lanes 5 and 10). The treated discs were incubated for 3 hr in the dark. Nuclear proteins were extracted and EMSA were performed with (lanes 6 to 10) or without (lanes 1 to 5) 20 units of alkaline phosphatase (+AP and -AP, respectively). Lanes 1 and 6 represent wounded tuber (W) tissues. The probe is the -135 to -105 DNA fragment that mediates the elicitor-induction of *PR-10a* (ERE) (Després et al., 1995). This is a representative of at least three different experiments.

led to an increase in the binding of the factor to the ERE when compared with the control (lane 1). A similar increase in binding was also seen with the extracts from discs treated with TPA, the PKC activator (Figure 4, lane 2). Pretreatment of the discs with the PKC inhibitor, calphostin C (Figure 4, lane 4) or staurosporine (Figure 4, lane 5) before application of the elicitor resulted in a level of binding similar to that observed with the untreated discs (Figure 4, lane 1). The notion that phosphorylation mediates this increased binding was reinforced by the observation that alkaline phosphatase treatment (Figure 4, lanes 6 to 10) of the nuclear extracts from both the elicitor and the TPA treated tuber discs before the assays led to a decrease in the binding of the factor to the ERE (Figure 4, lanes 7 and 8). Binding of the factor was not greatly affected after alkaline phosphatase treatments of the extracts prepared either from combined treatments of elicitor and calphostin C (lane 9), elicitor and staurosporine (lane 10), or from the control tubers (lane 6). These results confirm our previous findings (Després et al., 1995) that indicated that the capacity to bind to the ERE of *PR-10a* is potentiated by a phosphorylation step affecting the binding activity of the nuclear factors and places the PKC-like kinase directly in the pathway leading to *PR-10a* expression.

3.1.5. A homolog of the conventional isoforms of PKC is present in fresh potato tubers

Proteins isolated from the particulate fraction of fresh tubers were used to demonstrate the activity of PKC in potato. We used this fraction because in animal cells, PKC translocates from the cytosol to the membrane upon activation (Dutil et al., 1994). This translocation can be induced artificially by isolating the membranes at 15°C with the inclusion of calcium in the homogenization buffer, thereby forcing the kinase into the membranes where it can be extracted (Hannun

et al., 1985). The activity of the kinase was measured by the ability of enzymes present in the particulate fraction to phosphorylate the exogenous substrates histone H1 and α-peptide (a substrate specific for PKC), with the addition of appropriate cofactors (Kitano et al., 1986). The assay mixture was subjected to SDS-PAGE, and the extent of phosphorylation of the substrates was determined by autoradiography (Figure 5). For quantification purposes some of the experiments presented in Figure 5 were performed three times and the radiolabeled substrates were excised and counted to determine the extent of phosphorylation.

As indicated in Table 2 and shown in Figure 5, maximum phosphorylation of both histone H1 and α-peptide was attained only when all three cofactors, namely calcium, TPA, and PS, were present in the assay (Figure 5A and 5C, lane 8). There was a five- to sevenfold increase in the phosphorylation level compared with only calcium present in the assay (Table 2, compare lane 8 to lane 2). However, presence of calcium alone in the assay mixture resulted in an 1.5 fold increase in phosphorylation compared with the buffer alone (Table 2, compare lane 2 to lane 1). The specificity of the in vitro phosphorylation assay was demonstrated by the fact that neither 4αTPA nor phosphatidylcholine could substitute TPA and PS, respectively, to support the full activity of the enzyme (Table 2, compare lanes 10 and 11 to lane 8). More critically, however, pretreatment of the particulate fraction with the PKC inhibitor calphostin C mitigated the maximum phosphorylation of both histone H1 and α-peptide that was obtained when all the three cofactors were present in the assay (Table 2, compare lane 9 to lane 8).

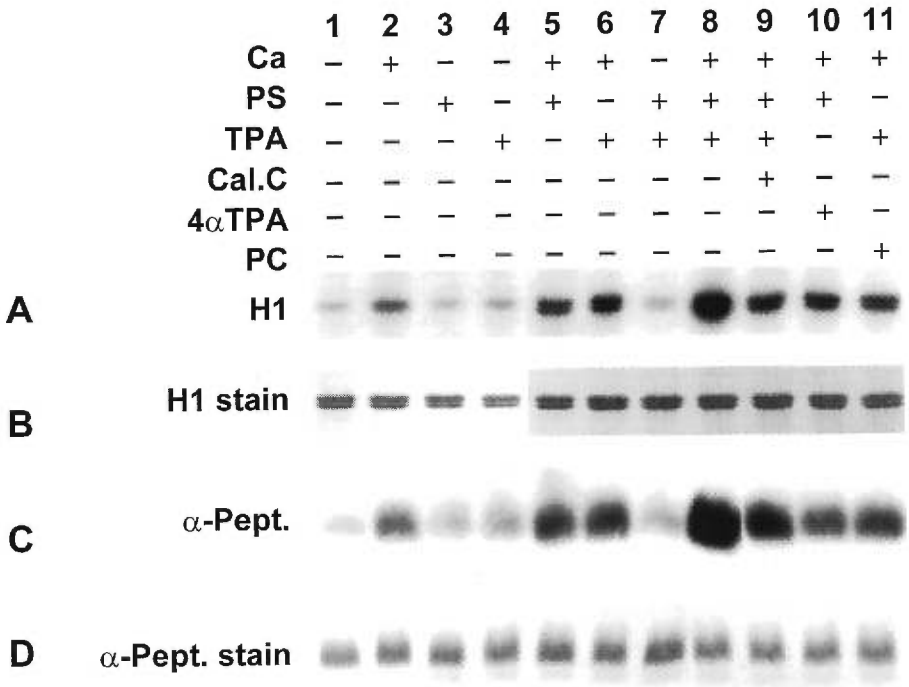

1 2 3 4 5 6 7 8 9 10 11
Ca – + – – + + – + + + +
PS – – + – + – + + + + –
TPA – – – + – + + + + – +
Cal.C – – – – – – – – + – –
4αTPA – – – – – – – – – + –
PC – – – – – – – – – – +
A
H1
B
H1 stain
C
α-Pept.
D
α-Pept. stain

Figure 5

Figure 5. Demonstration of PKC Activity in Protein Extracts of Potato.

PKC activity was measured in the particulate fraction of potato tubers containing the indicated combination of the following compounds: 4.95 mM $CaCl_2$ (Ca), 20 µg/mL PS, 10 nM of TPA, 10 nM 4αTPA, 20 µg/mL PC and 10 µM γ-^{32}P-ATP (1.0 µCi per assay). The assay contained 5 µg of protein. The PKC inhibitor calphostin C (Cal.C) at the concentration of 1 µM was added to an aliquot (100 µg) of the particulate fraction 1 hr prior to the start of the assay. The final concentration of DMSO was 0.002% in all the reaction mixtures. This is a representative of at least four independent experiments. (+) indicates that a compound has been added to the reaction mixture. (-) indicates that a compound has not been added.

(A) PKC activity was measured with histone H1 (200 µg/mL) as substrate. An aliquot (15 µL) of each reaction mixture was separated by SDS-PAGE and visualized by autoradiography.

(B) An aliquot (15 µL) from the reaction mixture in **(A)** was separated as given in **(A)** and visualized by Coomassie blue staining to show relative loading (H1 stain).

(C) PKC activity was measured with the PKC specific substrate α-peptide (10 µM). An aliquot (15 µL) of each reaction mixture was separated by SDS-PAGE and visualized by autoradiography.

(D) An aliquot (15 µL) from the reaction mixture in **(C)** was separated as given in **(C)** and visualized by Coomassie blue staining to show relative loading (α-Pept. stain).

TABLE 1. Specific Activity of Protein Kinase C in the Particulate Extracts of Potato Tubers

		Histone		α-peptide	
Lane[a]	Cofactors	Specific activity[b] (pmol min^{-1} mg^{-1})	Fold increase	Specific activity[b] (pmol min^{-1} mg^{-1})	Fold increase
1	None[c]	164 ± 14.4	1.0	326 ± 39.0	1.0
2	Ca^{+2}	253 ± 13.9	1.5	433 ± 89.0	1.3
5	Ca^{+2} and PS	350 ± 28.0	2.1	977 ± 64.0	3.0
8	Ca^{+2}, PS, and TPA	1630 ± 122.0	9.9	2850 ± 2.0	8.7
9	Ca^{+2}, PS, TPA, and Cal.C	414 ± 87.0	2.5	1180 ± 73.0	3.6
10	Ca^{+2}, PS, and 4αTPA	570 ± 55.0	3.5	1060 ± 190.0	3.2
11	Ca^{+2}, PC, and TPA	414 ± 37.1	2.5	1000 ± 25.0	3.1

[a] Refers to the lanes from Figure 4A and 4C.
[b] Data are expressed as means ± standard deviation (n = 3). An aliquot of each assay was analyzed by SDS-PAGE as described in Figure 5. The substrates were identified by staining the gels with Coomassie blue dye. The phosphorylation of the substrates was quantified by excising gel pieces corresponding to the stained substrate and counting in a scintillation counter.
[c] Represents the activity in the presence of EGTA-EDTA buffer alone.

As shown in Figure 5A and 5C, the presence of calphostin C (lane 9) in the assay reduced the phosphorylation to a level similar to that observed when only calcium and one other coactivator (Ca^{+2} and PS; lane 5) or (Ca^{+2} and TPA; lane 6) were present in the assay. However, the omission of calcium had no apparent effects on the level of phosphorylation of the substrates with either PS (Figure 5A and 5C, lane 3) or TPA (Figure 5A and 5C, lane 4), or even the combined presence of PS and TPA (Figure 5A and 5C, lane 7) in the assay. This suggested that other classes such as the novel and the atypical isoenzymes of PKC may not be present in the particulate extracts of fresh potato tubers. However, a minor contamination by calcium dependent protein kinases might explain the activation obtained by calcium alone (Figure 5A and 5C, lane 2).

3.1.6 Immunological confirmation of PKC isoforms in potato tubers

Analysis of cDNAs of the conventional isoforms of PKC isolated from diverse sources, including rat, mouse, rabbit and human, revealed an overall sequence similarity in two major domains, that is the catalytic domain and the phospholipid/DAG or the phorbol ester binding domain (Dekker and Parker, 1994). On this basis, using affinity-purified polyclonal antibodies raised against PKC, we confirmed the presence of PKC-like enzyme in the same particulate fraction that was used to demonstrate the kinase activity in Figure 5. The antibodies are directed against a short peptide, conserved in the catalytic domain of the conventional isoforms of human PKC (α, βI, βII and γ). Results shown in Figure 6 demonstrate that the antibody recognized a protein of ~78 kD in the particulate fraction of fresh tubers (lane 1). A comigrating band was also revealed in the total cell extract of rat spleen (Figure 6, lane 2). These results are in agreement with previous studies suggesting that PKC α, βI, βII and γ in rat spleen are between

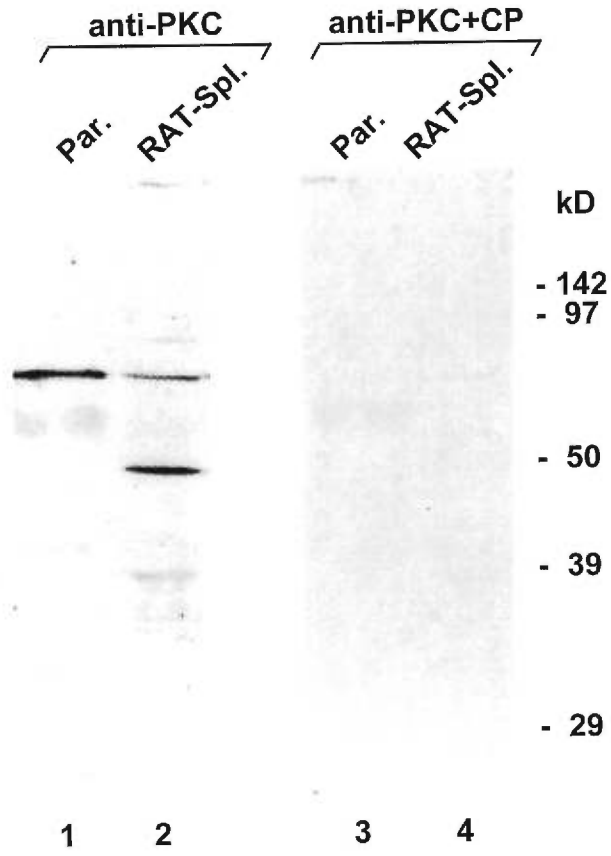

anti-PKC
anti-PKC+CP
Par.
RAT-Spl.
Par.
RAT-Spl.
kD
- 142
- 97
- 50
- 39
- 29
1
2
3
4

Figure 6

Figure 6. Confirmation for the Presence of a PKC Homolog in Potato by Immunoblotting.

Aliquots (15 µg) of total protein extracts of rat spleen (RAT-Spl.; lanes 2 and 4) and proteins from the particulate fraction of potato tubers (Par.; lanes 1 and 3) were fractionated by SDS-PAGE and immunoblotted with anti-PKC antibodies in the absence (anti-PKC) and in the presence (anti-PKC+CP) of 1.75 µM of the immunizing peptide during immunoblot analysis. The PKC antibody was raised and affinity purified against the conserved C4 catalytic domain (residues 528 to 537; PEIIAYQPYG) of the conventional isozymes of human PKC. Numbers at right indicate molecular mass standards in kilodaltons. Essentially identical results were obtained in five independent experiments.

78 - 80 kD (Kosaka et al., 1988; Wetsel et al., 1992). The migration at 50 kD of other immunoreactive species seen in the extracts of rat spleen may be due to the action of a specific protease (Sorimachi et al., 1994), representing the catalytic fragment of the PKC enzyme (Wetsel et al., 1992). The specificity of the antisera is emphasized by the observation that the reaction of the antisera with the protein species in both the extracts of rat spleen and particulate fraction of fresh tubers were succesfully blocked when coincubated with the immunizing peptide in the immunoblot analysis (Figure 6, lanes 3 and 4).

The authenticity of the immunoreactive species was verified by immunoprecipitating the proteins from the particulate fraction of tubers by the PKC-specific antibodies and subjecting them to an in-gel kinase assay. The immunoblot in Figure 7A confirmed that the protein species immunoprecipitated by the antibodies (lane 2, 78-kD band) corresponded to those species observed in the particulate extracts (lane 1). The specificity of the immunoprecipitation was confirmed by the inclusion of the immunizing peptide in the immunoprecipitation reaction (Figure 7A, lane 3). The proteins from the particulate extracts and the immunoprecipitates were subjected to an in-gel kinase assay with histones as substrate. Figure 7B shows that there are at least four proteins present in the particulate extracts that phosphorylate histones (lane 4). These bands most likely correspond to different and/or break down products of kinases. The immunoprecipitates of the particulate extracts, on the other hand, revealed only one strongly labeled band (Figure 7B, lane 5). The specificity of this phosphorylating band was confirmed by the inclusion of the immunizing peptide in the immunoprecipitation reaction (Figure 7B, lane 6). The minor phosphorylating band observed in Figure 7B, lanes 5 and 6, may represent

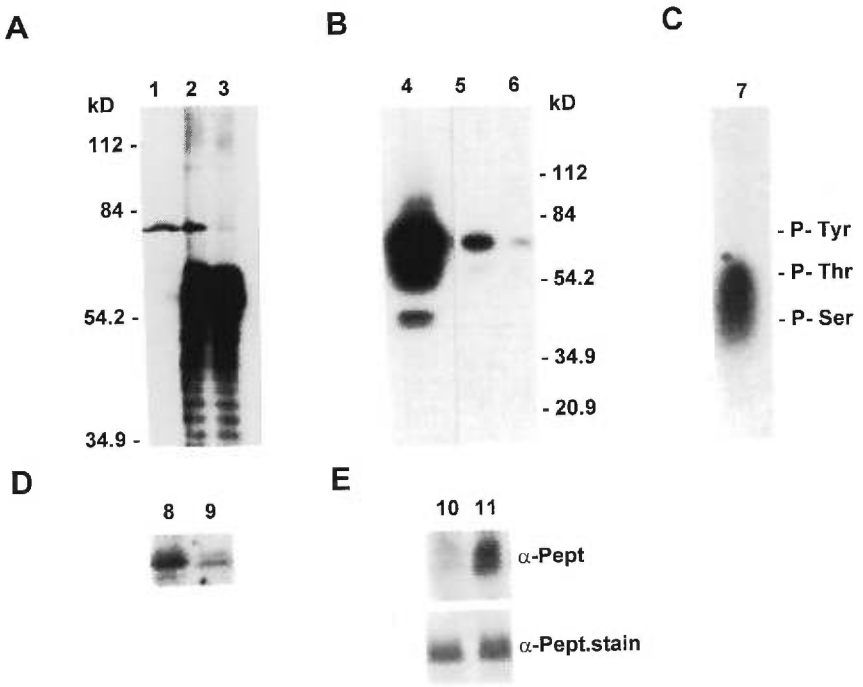

A
B
C
kD
1 2 3
112 -
84 -
54.2 -
34.9 -
4 5 6
kD
- 112
- 84
- 54.2
- 34.9
- 20.9
7
- P- Tyr
- P- Thr
- P- Ser
D
8 9
E
10 11
α-Pept
α-Pept.stain

Figure 7

Figure 7. Analysis of the Immunoprecipitates with PKC-Specific Antibodies.

Immunoprecipitation of proteins from the particulate fraction of the potato tubers were performed as described in Section 2.2.2. The nonimmunoprecipitated and the immunoprecipitated samples were fractionated by SDS-PAGE.

(A) Immunoblot analysis using the PKC-specific antibodies. An aliquot (30 µg of protein) of the particulate fraction (lane 1) and the immunoprecipitates with the PKC-specific antibodies in the absence (lane 2) and in the presence (lane 3) of the immunizing peptide (5 µM) in the immunoprecipitation reaction were separated by SDS-PAGE and immunoblotted with PKC-specific antibodies. Intense bands at 54 kD represent IgG. Numbers at left indicate molecular mass standards in kilodaltons.

(B) Autoradiography of the in-gel kinase assay. An aliquot (3 µg of protein) from the particulate fraction (lane 4) and the immunoprecipitates in the absence (lane 5) and in the presence of the immunizing peptide (lane 6) was subjected to an in-gel kinase assay as described in Section 2.2.6. This is a representative of at least five independent experiments. Numbers at right indicate molecular mass standards in kilodaltons.

(C) Phosphoamino acid analysis of the radiolabeled immunoprecipitate in **(B)**. Autophosphorylated protein kinase (Figure **6B**, lane 5) was separated from histones by SDS-PAGE. The ^{32}P-labeled immunoprecipitate was hydrolyzed with HCl and subjected to one-dimensional thin-layer chromatography. The positions of phosphoserine, phosphothreonine, and phosphotyrosine standards are as

indicated.

(D) Autoradiography of an in-gel kinase assay following calphostin C treatment. After the immunoprecipitated proteins in the gel were renatured, the gels were treated (lane 9) or not with 1 μM calphostin C (lane 8). The in-gel kinase assay was performend as described in Section 2.2.6. This is representative of three independent experiments.

(E) PS and TPA increase phosphorylation of the α-peptide. The kinase immunoprecipitated by the PKC-specific antibodies was eluted and PKC activity was measured with the PKC-specific substrate, α-peptide (10 μM). Lane 10 is a control reaction with calcium alone and lane 11 represents the effect of calcium plus PS and TPA. The reaction mixture was separated by SDS-PAGE and the labelled α-peptide was visualized by autoradiography (α-pept). Relative loading was monitored by Coomassie blue staining (α-pept.stain). This is representative of three independent experiments.

another kinase associated with the dominant species recognized by the PKC antibody because it was not detected in the immunoblots. It might, however, also be a degradative product of the kinase(s).

The bands revealed in the in-gel kinase assay (Figure 7B) are the result of combined effect of phosphorylation of the histone substrate and autophosphorylation of the protein kinase. To verify autophosphorylation, the dominant band observed after immunoprecipitation (Figure 7B, lane 5) was isolated from the gel and subjected to a second SDS-PAGE. Autoradiography indicated the presence of a unique radiolabeled 78 kD protein (data not shown) identical to the one seen in the immunoblot analysis. Figure 7C shows the result of the phosphoamino acid analysis of this protein, which indicated that autophosphorylation occurs on serine and threonine residues and confirmed that the immunoprecipitated kinase belongs to the serine/threonine family of kinases.

The criteria that were used to determine that the kinase characterized biochemically as shown in Figure 5 belonged to the conventional isoforms of PKC were also used with the kinase immunoprecipitated by the PKC-specific antibodies. Figure 7D shows that treatment with calphostin C led to a marked decrease in the activity of the kinase in the gel (compare lane 9 to lane 8). However, unlike the kinase characterized in Figure 5, we were unable to augment the activity of the kinase in the gel by the addition of calcium, PS, and TPA, presumably because of the inability of the lipid cofactors to enter into the gel matrix. Therefore, we eluted the renatured kinase from the gel and observed the effects of cofactors on the ability of the kinase to phosphorylate α-peptide in vitro. As shown in Figure 7E, addition of the cofactors PS and TPA led to a two- to

threefold increase in the phosphorylation of this PKC-specific substrate (α-Pept.; compare lane 11 to lane 10). These results strongly suggest that the kinase detected by the PKC-specific antibodies corresponds to the kinase characterized biochemically in Figure 5 because both enzymes share properties common to the conventional isoforms of PKC.

It is known that phorbol esters, because of their metabolic stability, prolong the activation of PKC. This results in the eventual down-regulation (depletion) of PKC, a characteristic associated with this enzyme (Huang et al., 1989). The immunoblot in Figure 8 shows that the level of the 78-kD protein kinase was noticeably increased after a 48-hr exposure of the potato tubers to TPA (lanes TPA_{500}, at 48 hr), compared with the control discs (lanes W, at 48 hr). However, an additional 24-hour exposure to TPA led to a significant decrease in the level of the protein (compare lanes W, at 72 hr and lanes TPA_{500}, at 72 hr). This suggested that prolonging the activation by TPA of this kinase in potato also led to its eventual down regulation, characteristically similar to what is observed with PKC in the animal cells.

Figure 8 also shows a comparable effect of prolonged exposure to TPA on the PR-10a protein accumulation. This effect, however, was not observed with other PR proteins, like the chitinase and the 1,3-β-glucanase. This strong correlation between TPA treatment and the effect on PR-10a accumulation underscores the specificity of the signaling pathway leading to *PR-10a* activation. Overall, the behaviors of this 78-kD protein kinase immunologically and in combination with the biochemical data reinforce our proposition that a PKC-like kinase is present in potato tubers and that it participates in the pathway leading

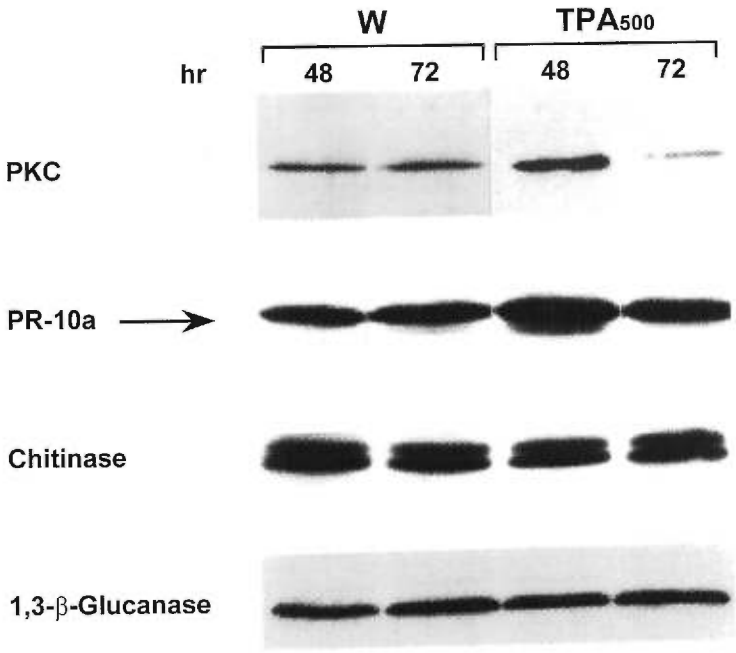


Figure 8

Figure 8. Immunoblot Showing the Effects of Chronic Exposure to TPA on the PKC-like Kinase, PR-10a, Chitinase, and 1,3-β-Glucanase.

After a 6-hr aging period, potato discs were treated with 1% DMSO (W) or 500 nM TPA (TPA_{500}) and incubated in the dark at room temperature. Proteins were extracted from the treated tuber discs after 48 and 72 hr, respectively. Aliquots (10 µg of protein) from each sample were fractionated using SDS-PAGE and immunoblotted with antibodies as indicated. The arrow indicates PR-10a. Essentially identical results were obtained in three independent experiments.

to *PR-10a* activation.

3.1.7. Acquired resistance to *P. infestans* after elicitor treatment is mediated through PKC

Treatment of potato tuber discs with the elicitor arachidonic acid completely prevents fungal growth when infected with the compatible races of *P. infestans* (Bostock et al., 1981). It is likely that the resistance to the fungus involves a plethora of genes induced by the elicitor. We have already demonstrated that one of the induced genes, *PR-10a*, was markedly affected by modulators of PKC activity (Figures 2 and 3). We were interested to see whether the resistance to the pathogen induced by the elicitor was similarly affected by the same modulators of PKC function. Therefore, we inoculated the tuber discs with zoospores of a compatible race of *P. infestans* and assessed the infection by an ELISA designed for the quantification of *Phytophthora* species.

Figure 9 shows that, as expected, the application of the elicitor on the tuber discs prevented growth of the pathogen (compare bars W and AA). However, pretreatment of the discs with the PKC inhibitors calphostin C or bisindolylmaleimide before the application of arachidonic acid abolished the effect of the elicitor and restored the growth of the fungus to a level similar to that of the untreated discs (bar W). On the other hand, the PKC activator, TPA, mimicked the action of the elicitor and completely prevented the infection by *P. infestans*. At the same concentrations, neither calphostin C nor TPA interfered with the normal growth of the fungus as confirmed by the ability of the fungus to grow on rye agar plates impregnated with either TPA or calphostin C (data not shown). These results suggest that the PKC homolog present in potato tubers plays an important

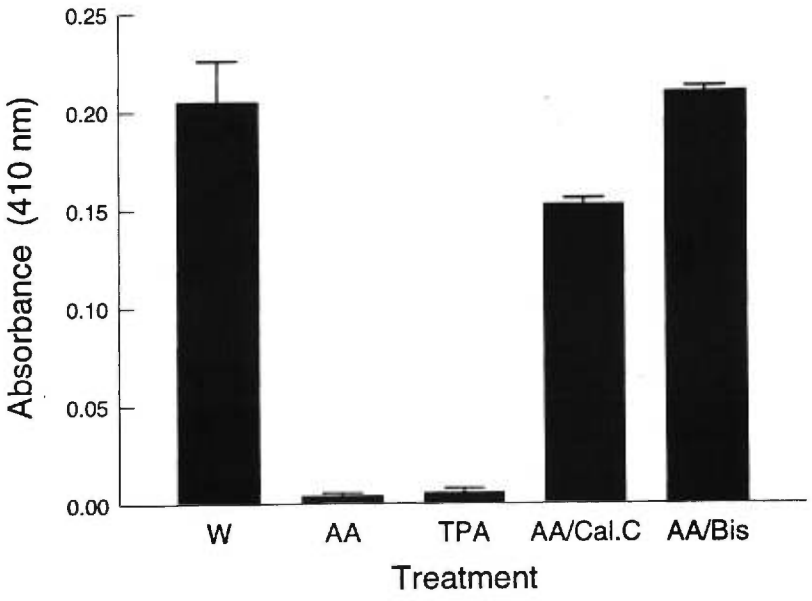

0.25
0.20
0.15
0.10
0.05
0.00
Absorbance (410 nm)
W
AA
TPA
AA/Cal.C
AA/Bis
Treatment

Figure 9

Figure 9. Acquired Resistance to *P. infestans* is Mediated through a PKC Homolog in Potato.

The potato discs were aged for 6 hr and treated with 1% DMSO (W), arachidonic acid plus 1% DMSO (AA), 100 nM TPA, a combination of 500 nM calphostin C and arachidonic acid (AA/Cal.C), and combination of 50 nM bisindolylmaleimide and arachidonic acid (AA/Bis). The discs were treated with the compounds for 30 min before the application of the elicitor arachidonic acid. After the elicitor was absorbed, the potato discs were inoculated with 3000 zoospores of *P. infestans* (race 1,2,3,4) and incubated for 6 days at 15°C in the dark. Quantification of the fungal mycelial growth on the inoculated discs was done by ELISA using a *Phytophthora* detection kit (Sigma). The data are from two independent experiments and standard error range is indicated above each bar.

role in the defense response signaling pathway. The results also validate the usefulness of *PR-10a* as a marker for the defense response.

3.2. Partial purification and characterization of a PKC-like kinase from the membranes of potato tubers

3.2.1. Partial purification of a PKC-like kinase by column chromatography

The activation of cPKCs in mammalian cells is demonstrated by their ability to translocate to membranes. Similarly, antibodies specific to the human cPKCs detected a 78 kD protein in the membrane extracts of potato tubers that had been wounded (Figure 10, W). It is also evident that this protein species is not present in the membrane extracts of fresh tuber (Figure 10, FT). In Section 3.1, we established that activation of *PR-10a* by the elicitor occurs via the PKC signalling pathway. Interestingly, the results in Figure 10 suggest that the kinase in the tuber is activated during the wounding period, before the elicitor treatment. This is in agreement with our earlier results which showed a moderate accumulation of the PR-10a protein after wounding (Figure 1, lane 2). In view of this evidence and for convenience, we used proteins prepared from the membranes of tubers wounded for 20 hours for further characterization of this kinase. Using the Triton X-100 extracted proteins, a partial purification of the kinase was achieved using four chromatographic steps.

Table 3 describes the purification achieved with this strategy. The strong binding of the kinase to the Q-Sepharose, the first chromatographic step, resulted in a seven-fold purification of the kinase. Threonine-Sepharose affinity chromatography is used routinely in PKC purification, because it allows for the separation of different PKC isoforms. The interaction between the various isoforms of PKC and threonine is not known. The PKC-like kinase of the potato tubers also binds strongly to this affinity column. The 200 mM NaCl wash eliminated proteins

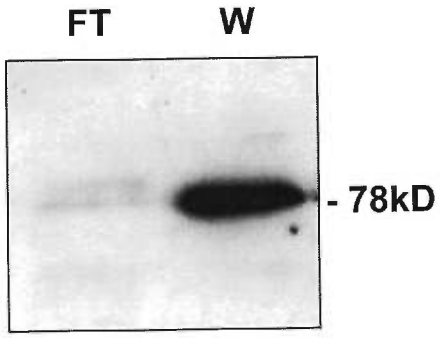


Figure 10

Figure 10. The PKC-like Kinase Translocates to Membranes of Tubers after Wounding.

The tuber discs were cut and incubated for 20 hr in the dark. The proteins were extracted from the membranes of fresh tubesr (FT) and from tubers that were wounded (W) as described in Section 2.2.8. Aliquots (25 µg of protein) were separated by SDS-PAGE and immunoblotted with anti-PKC antibodies as described in Section 2.2.2. A 78 kD protein was detected as indicated.

Table 3. Purification of the PKC-like Kinase from the Membranes of Potato Tubers

Fraction	Total Protein (mg)	Total Activity (pmol min-1)	Specific Activity[a] (pmol min-1 mg-1)	Yield (%)	Purification (fold)
Membrane[b]	160	172114	10.75	100	1
Q-Sepharose	9.96	75000	75.3	44	7
Thr-Sepharose[c]	2.94	37150	126.4	21.6	12
Phe-Sepharose[d]	0.61	24375	399.59	14	37
Mono S	0.005	1548	3096.0	0.9	288

[a] Represents activity in the presence of all the cofactors minus EGTA-EDTA buffer control.

[b] Protein extracted from membranes of 600g of potato tubers.

[c] Thr-Sepharose, threonine-Sepharose affinity column.

[d] Phe-Sepharose, phenyl-Sepharose-4B

bound to the Sepharose backbone non-specifically. The kinase eluted at a NaCl concentration between 400 mM and 600 mM. This elution profile is typical of the cPKC isozyme family in animals. However, we did observe a large variation in the level of purification (up to 150-fold) achieved with this column. This depended mostly on the age of the tuber and the time lapsed between cutting and the isolation of the membranes from the tubers.

Phenyl-Sepharose chromatography has been used particularly for the purification of PKC species, because the matrix prevents binding of many other kinases, including CDPKs (Macintosh et al., 1996). The fractions that exhibited cPKC activity were precipitated by polyethylene glycol. An in-gel kinase assay with the PKC-specific substrate α-peptide was also performed to assess the number of kinases present in the PEG precipitate. We detected only a single phosphorylated band suggesting that only a single species of kinase is present after the Phenyl-Sepharose column fractionation (data not shown). Subsequently, the kinase precipitated by PEG was subjected to Mono S column fractionation. The kinase eluted at a NaCl concentration between 150 mM and 200 mM. Although the fractionation through this column resulted in a 10-fold purification over the previous step, we did incur substantial losses of the kinase.

3.2.2. Characterization of the PKC-like kinase

As indicated in Table 4, the kinase eluted in fraction 7 from the Mono S column possessed all the biochemical features of the conventional isoforms of the animal PKC family. The maximum phosphorylation of α-peptide was obtained only when all the cofactors, namely, Ca^{2+}, phosphatidylserine, and TPA were present in the assay. The kinase was equally responsive in the presence of DAG, a

Table 4. Biochemical Characterization of PKC-like Kinase Eluted from the Mono S[a] Column

Cofactors	Activity[c] (pmol min^{-1})	Fold Increase
None[b]	11.5 ± 2.8	1
Ca^{2+}	51.5 ± 6.6	4.5
Ca^{2+} and PS	73.0 ± 7.2	6.3
Ca^{2+}, PS, and TPA	110.6 ± 6.8	9.6
Ca^{2+}, PS, TPA, and Cal.C[d]	34.2 ± 3.9	3.0
Ca^{2+}, PC, and TPA	63.2 ± 4.9	5.4
Ca^{2+}, PS and DAG[e]	92.3 ± 7.6	8.1

[a] The kinase assay was performed on fraction 7 eluted from the Mono S column.

[b] Represents the activity in the presence of EGTA-EDTA buffer alone.

[c] Data are expressed as means ± SE (n =3).

[d] Cal.C, Calphostin C.

[e] DAG, 1,2-Dioleoyl-sn-Glycerol.

product of phospholipid hydrolysis and a well known activator of PKC *in vivo*. Calphostin C, a selective inhibitor of PKC, substantially abrogated the activity of the kinase. Altogether, the biochemical data suggested that a kinase with properties similar to the mammalian cPKC has been purified from the membranes of potato tubers.

It is known that kinases within the same family exhibit subtle differences in their substrate specificities. As documented in Table 5, α-peptide is the most efficient substrate for the kinase purified from the potato tuber cell membranes. The least efficient substrate is the peptide derived from the myelin basic protein, Ac-MBP (4-14). This peptide has been shown to be an efficient phosphate acceptor for the cPKC in animals. Similarly, the kinase purified from *Brassica* also phosphorylates this substrate very efficiently (Nanmori et al., 1994). The differences in the plant material and the tissues used to purify the kinase may account for the differences in the substrate preferences of the potato and the *Brassica* kinase.

We confirmed our biochemical results with the immunological data. As indicated in Figures 11A and 11B (lane 6), the antibodies raised against the catalytic regions of the human cPKC isoforms, recognized a protein of ~78 kD in each of the purification steps. There was a strong correlation between the kinase activity and the presence of the 78 kD PKC-like protein. The silver stain of proteins eluted from fraction 7 of the Mono S column indicated that one of the band (Figure 11B, arrow, lane 5) corresponds to the protein species detected by the PKC-specific antibodies (Figure 11B, lane 6).

Table 5. Substrate Specificity of PKC-like Kinase Eluted from the Mono S[a] column

Substrate	Activity[b] (pmol min^{-1})	Percent (%)
α-Peptide	100.6 ± 5.8	100
Histone H1	60.8 ± 4.9	60
Histone HIIIS	28.8 ± 4.2	28.8
MBP[c]	52.9 ± 6.2	53
Ac-MBP (4-14)	1.28 ± 0.05	1

[a] The kinase assay was performed on fraction 7 of the Mono S column elution

[b] Data are expressed as means ± SE (n=3). It represents activity in the presence of Ca^{2+}, PS and TPA.

[c] MBP, myelin basic protein.

[d] Ac-MBP (4-14), synthetic peptide derived from MBP and acetylated at the N-terminal.

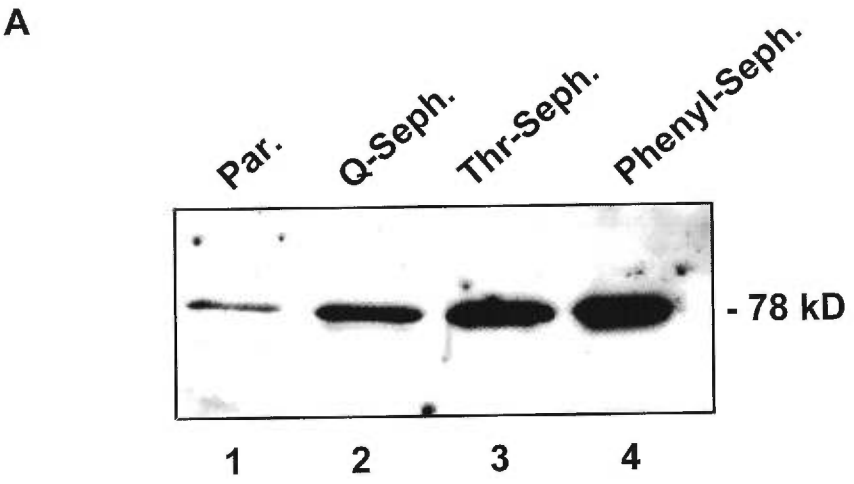

A
Par.
Q-Seph.
Thr-Seph.
Phenyl-Seph.
- 78 kD
1
2
3
4
B
Mono S
Silver St.
anti-PKC
175 -
83 -
63 -
5
6

Figure 11

Figure 11. Confirmation of the Purification of a Potato PKC-like Kinase by Immunoblotting.

Aliquots (25 µg of protein) from the membrane extracts (Par.; lane 1), eluent of the Q-Sepharose column (Q.Seph.; lane 2), eluent of the threonine-Sepharose column (Thr.Seph.; lane 3), eluent of the phenyl-Sepharose column (Phen.Seph.; lane 4), and fraction 7 of the Mono S column (lanes 5 and 6) were separated by SDS-PAGE.

A. Immunoblot with anti-PKC antibodies. A 78 kD protein is detected as indicated.

B. Proteins eluted in fraction 7 from the Mono S column were silver stained (Silver St.; lane 5) by the method of Oakley (1980) and immunoblotted with anti-PKC antibodies (anti-PKC; lane 6). Numbers at left indicate molecular mass standards in kilodaltons. The arrow indicates the position of the 78 kD protein.

3.2.3. Characterization of the 78 kD protein isolated from the Mono S fraction

The 78 kD protein identified in Figure 11B (arrow) was excised and digested with trypsin in-gel. Following capillary electrophoresis, two peptides were selected and sequenced by mass spectrometry. The partial amino acid sequence of both the peptides revealed strong identity with the Hsp70 proteins from a wide variety of species including human and tomato (Figure 12). This result suggested that the 78 kD protein kinase comigrates with another protein belonging to the Hsp70 protein family. Monoclonal antibodies raised against the human Hsp70 confirmed that proteins identified by silver staining (Figure 11B, lane 5), in addition to being recognized by the PKC-specific antibodies (Figure 13, anti-PKC), also crossreact with the anti-Hsp70 antibodies (Figure 13, anti-Hsp70). Similar results were obtained with the proteins eluted from all the earlier purification steps (data not shown).

To investigate the possibility that the PKC antibodies might cross react with the Hsp70 proteins, we immunoprecipitated the total proteins from the membrane extract with the anti-PKC antibodies and subjected them to immunoblotting with PKC and Hsp70 antibodies, respectively. As indicated in Figure 14A, a 78 kD protein was immunoprecipitated and detected by the anti-PKC antibodies (lane 2, arrow). This protein comigrated with the 78 kD band detected in the membrane extracts (lane 1). The Hsp70 antibodies also recognized a 78 kD protein in the membrane extract (Figure 14B, lane 3, arrow), but they failed to crossreact with any proteins immunoprecipitated by the PKC antibodies (Figure 14B, lane 4). These results clearly indicated that the two proteins are distinct and that the anti-PKC antibodies do not react with the Hsp70 protein.

Tomato Hsp70	FEELNNDLFR
Peptide 1	FEELNNDLFR
	**
Human Hsp70	FEELCSDLFR

Tomato Hsp70	NQVALNPINTVFDAK
	*
Peptide 2	NQVAMNPINTVFDAK
	* *
Human Hsp70	NQVALNPQNTVFDAK

Figure 12

Figure 12. Alignment of Two Internal Peptide Sequences of the potato PKC-like Kinase with the Sequences of the Human and Tomato Hsp70 Protein.

The amino acid sequences of human Hsp70 (Milner and Campbell, 1990) and tomato Hsp70 (Lin et al., 1991) proteins were deduced from the respective cDNA sequences. Only the regions in the two respective proteins that align with the two sequenced peptides are shown. Asterisks represent the amino acid residues which do not match with the peptide sequences.

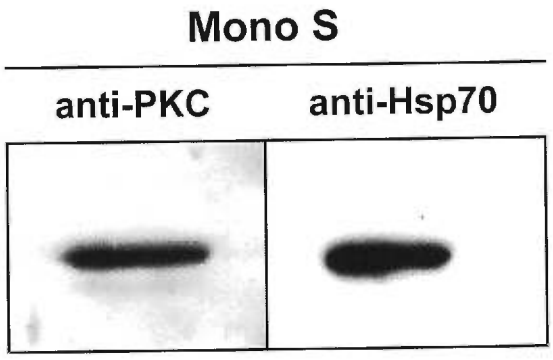


Figure 13

Figure 13. The PKC-like Protein Eluted in Fraction 7 from the Mono S Column Comigrates with a Hsp70 Protein.

Aliquots (25 µg of protein) were separated by SDS-PAGE and immunoblotted with anti-PKC antibodies (anti-PKC) and anti-Hsp70 antibodies (anti-Hsp70) as indicated.

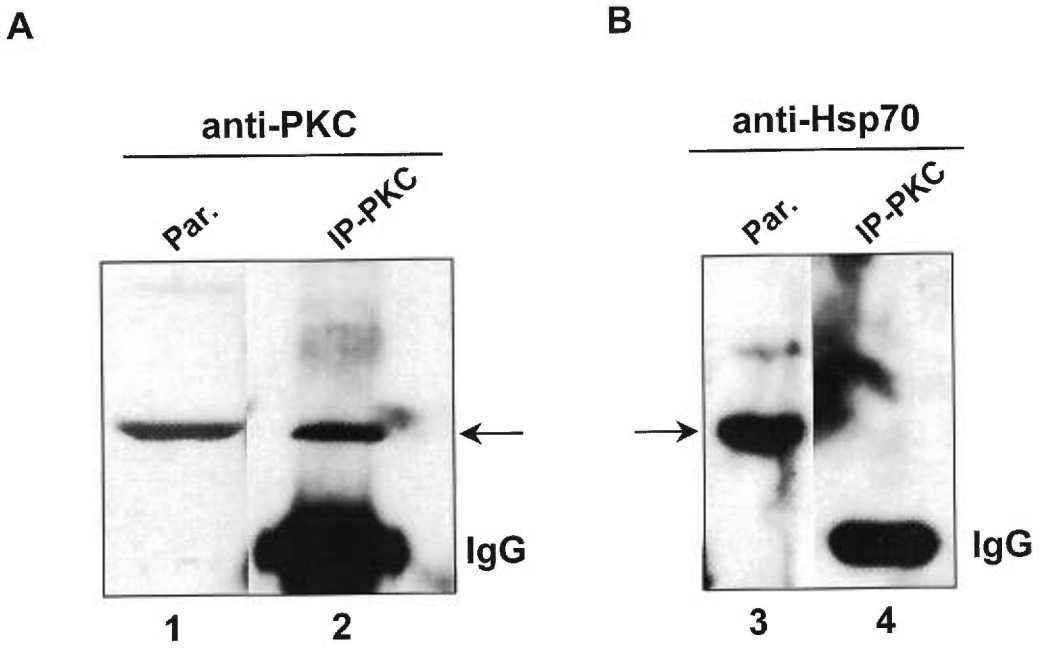


Figure 14

Figure 14. The Hsp70 Protein does not Crossreact with the Anti-PKC Antibodies.

Immunoprecipitation of proteins from the particulate fraction of the potato tubers was performed as described in Section 2.2.6. Aliquots (50 µg of protein) of the particulate fraction (lanes 1 and 3) and the immunoprecipitated samples (lanes 2 and 4) were fractionated by SDS-PAGE.

A. Immunoblot analysis using the anti-PKC antibodies. The arrow indicates the 78 kD protein PKC-like protein.

B. Immunoblot analysis using the anti-Hsp70 antibodies. The arrow indicates the 78 kD Hsp70 protein.

IgG in the immunoprecipitates (lanes 2 and 4) are as indicated.

3.2.4. Separation of the PKC-like kinase and Hsp70 proteins

In order to separate this kinase from the Hsp70 proteins, we modified some of the steps in the purification scheme described earlier. This included re-chromatographing the eluates from the Q-Sepharose and the threonine-Sepharose through a Mono Q column with different gradients of NaCl concentration. We were able to reduce the level of the Hsp70 contamination, but significant amounts still coeluted with the kinase (data not shown). Numerous other techniques that have been used to purify Hsp70 proteins, including gelatin affinity chromatography (Nandan et al., 1994) and hydroxyapatite chromatography (Welch and Feramisco, 1985), also did not successfully separate the two proteins (data not shown).

3.2.5. Two-dimensional electrophoresis analyses of the PKC-like kinase and the Hsp70 protein

Two-dimensional electrophoretic analysis as described by O'Farrell (1977) separates the proteins by isoelectric point (pI) in the first dimension and by molecular weight in the second dimension. The proteins eluted from the Phenyl-Sepharose column was precipitated by acetone and solubulized in a buffer containing broad range ampholytes (pH 3.5-10) and subjected to two-dimensional SDS-PAGE. Under these conditions, as indicated by the immunoblot in Figure 15B, the PKC antibodies crossreacted with a protein (arrow 1) corresponding to the protein indicated in the silver stained gel (Figure 15A, arrow). The faster migrating protein detected by the PKC antibodies (arrow 1a) in Figure 15B may represent a breakdown product of the larger protein.

To ascertain the relationship between the kinase and the Hsp70 protein in

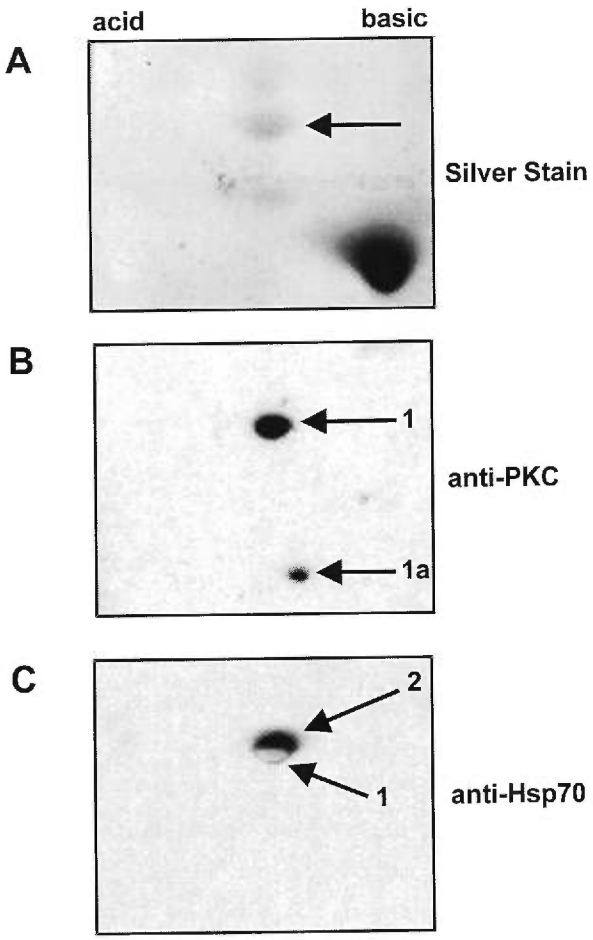


Figure 15

Figure 15. Separation of the PKC-like kinase and Hsp70 Protein by Two-Dimensional Electrophoresis.

Two-dimensional electrophoresis as described by O'Farrell (1977) were performed with the proteins eluted from the Phenyl-Sepharose column fractionation. An aliquot (100 µg of protein) was separated in the first dimension isoelectric focusing gel, and subsequently separated in the second dimension by 10% SDS-PAGE.

A. Silver stain of the proteins separated by 2D-electrophoresis. The acidic and the basic ends of the isoelecric focusing gel are indicated. Only the area where the PKC-like kinase and Hsp70 proteins migrate are shown. The protein spot of ~78 kD corresponding to both the proteins is indicated by an arrow.

B. Immunoblot analysis by the anti-PKC antibodies of the proteins separated by 2D-electrophoresis. A protein of ~78 kD detected by the antibodies is indicated (arrow 1). Another protein of ~65 kD detected by the antibodies is also indicated (arrow 1a). The exposure of the X-ray film was 10 minutes.

C. Immunoblot analysis by the anti-Hsp70 antibodies was performed on the same nitrocellulose paper that had been previously immunoblotted with anti-PKC antibodies described in **B**. The exposure of the X-ray film was 1 minute. The protein detected by the anti-Hsp70 antibodies is indicated (arrow 2). A clear zone that coincides with the PKC is also indicated (arrow 1).

this two-dimensional gel, the immunoblot in Figure 15B was reprobed with the Hsp70 antibodies. As revealed by the short exposure of the film in Figure 15C, the protein detected by the Hsp70 antibodies (arrow 2) appears slightly above a clear zone (arrow 1) which coincides with the signal obtained with the PKC antibodies in Figure 15B. These results indicate that the kinase and the Hsp70 protein have a very similar isoelectric point.

In the experiments described above, the protein samples were solubilized in broad-range ampholytes. However, when the proteins were solubilized in narrrow-range ampholytes (pH 5-8), the subsequent immunoblot only revealed a signal with the Hsp70 antibodies (data not shown). This suggested that the pI values of the PKC-like kinase is between 3.5 and 5 and was retarded from entering into the first-dimension isoelectric focusing gel. The results also suggested that the pI value of Hsp70 is higher than 5. Therfore, these experiments indicate that a combination of various narrow ampholytes between pH 3 and 6 may effectively separate the kinase from the Hsp70 proteins.

Chapter 4. Discussion

Many lines of evidence have indicated a role for protein kinases as mediators of the elicitor response (Dixon et al., 1994; Després et al., 1995; Suzuki and Shinshi 1995; Xing et al., 1996; Ligterink et al., 1997). Inhibitors of protein kinases have been used successfully to block this response, in particular the induction of defense genes (Raz and Fluhr, 1993). Similarly, our earlier work suggested that phosphorylation events may play a role in the activation of the defense response in potato tubers (Després et al., 1995). The objective of this thesis was to assess this role and identify components within this important signalling event. Chapter 3 follows the strategy outlined by Blobe et al., (1995) to determine if PKC plays a role in a biological process such as the elicitor-induced defense response in potato. After it was established that PKC is a central player in the elicitation process, we initiated steps to isolate this specific component. In the second section of Chapter 3, the procedures followed to isolate the PKC-like kinase from potato tuber membranes are described. The many issues that arose from each facet of these studies are the subject of the following discussion.

4.1. PKC mediates *PR-10a* expression

In potato, *PR-10a* is one among the many defense-related genes that are activated in response to infection or elicitor treatment (Matton and Brisson, 1989). Since its expression in potato tuber is indicative of the pathogenesis state, it serves as a useful marker of the response to pathogens (Constabel and Brisson, 1992). Studies on the regulation of *PR-10a* identified the promoter elements necessary for the elicitor to activate the gene (Després et al., 1995). A strong correlation was established between the binding of the PBF nuclear factors to their cognate element, the ERE. In the same study, staurosporine, a broad range kinase inhibitor was used to confirm the relationship between the binding of PBF

to the ERE and the expression of *PR-10a*. Even at concentrations lower than 10 nM, the kinase inhibitor disrupted elicitor-induced binding of PBF to the ERE and dramatically reduced the accumulation of PR-10a protein. These results alluded to the possibility that a PKC-like kinase might be the target of the inhibitor.

Following these initial observations, a specific kinase pathway involved in the defense response of potato tubers has now been identified. As detailed in Section 3.1, specific pharmacological agents with different molecular structures and modes of action against PKC were initially used to demonstrate a role for this kinase in the elicitor-induced accumulation of PR-10a. For example, both calphostin C and sphingosine inhibit PKC activity by interacting with different regions of its regulatory domain (Tamaoki, 1991; Hannun et al., 1991). On the other hand, the inhibitor bisindolylmaleimide interacts specifically with the catalytic domain of the enzyme (Toullec et al., 1991). PKC activators such as the phorbol ester, TPA, have been used extensively to study the cellular functions of PKC (Castagna et al., 1982). Thus, events stimulated by TPA or abolished by long-term phorbol ester treatment (i.e. down regulation of PKC) are attributed to PKC (Huang et al., 1989). Both of these effects are documented in this thesis with respect to the accumulation of PR-10a.

Two issues arise regarding the use of pharmacological agents. The first is the non-specific effects of these agents. This was addressed by observing the effect of these agents on other defense-related genes such as the β-glucanases and chitinases. No deleterious effects caused by these pharmacological agents on the accumulation of these proteins were observed. Moreover, the fact that these agents have significant effects on the binding of PBF to the ERE further

suggested that a PKC-like kinase is involved specifically in the pathway leading to the expression of *PR-10a*. The second issue relates to the efficacy of these agents. There are many reports that suggest that inhibitors like sphingosine mediate some of their effects independently of PKC (Zhang et al ., 1990). Furthermore, phorbol ester receptors other than PKC have been described (Areces et al., 1994). For these reasons, additional evidences were sought to support the involvement of PKC in the defense response process.

4.2. Characterization of a PKC-like kinase from the membranes of potato tubers

In animals, PKC is activated in a two-step process. In the initial step, calcium and PS recruit inactive PKC from the cytosol to the membrane where it remains inactive, but has an increased affinity for substrates. This inactive PKC then interacts with DAG at the membrane. DAG serves to increase the affinity of PKC for calcium and PS so that physiological levels are now able to activate PKC. It was later observed that the actvity of the kinase could be sustained for longer periods in the presence of TPA (Castagna et al., 1982). These unique features enabled us to biochemically assay the kinase activity in membrane peparations from potato tubers. We determined the activity of the kinase by its ability to transfer phosphate to substrates such as histone H1 and α-peptide. The maximum kinase actvity of the kinase is obtained only in the presence of all the three cofactors Ca^{2+}, PS, and TPA. Of the three groups of the PKC family, only the group of conventional isoforms is dependent on all three cofactors to be maximally activated. In addition, it was demonstrated that the activity of the kinase could be drastically reduced in the presence of calphostin C. Thus, the ability of this inhibitor to affect PKC-like activity *in vitro* and its proposed role *in vivo* as a PKC

inhibitor address the efficacy of this particular pharmacological agent.

The catalytic domains of the PKC family are highly conserved (Dekker et al., 1995). Several regions of near complete identity within the kinase domain can be distinguished between PKC family members. The antibodies directed to one such subdomain enabled us to confirm the presence of a cPKC. The antibodies detected a protein species of ~78 kD in the membrane extracts of tubers, consistent with what is known in mammalian cells, where the molecular weights of cPKC members range between 75 and 80 kD. The proteins that crossreacted with these antibodies exhibited kinase activity in-gel kinase assays. Furthermore, the immunoprecipitated kinase was only able to phosphorylate the α-peptide when assayed in the presence of Ca^{2+}, PS, and TPA.

Employing the strategy proposed by Blobe et al. (1995), evidence was collected to confirm that a PKC-like kinase plays a role in the elicitation process of potato tubers. The process renders the tubers resistant to infection by its natural pathogen *P. infestans*. ELISA assays to detect *Phytophthora* proteins provide the opportunity to test the role of PKC in the defense response and validate the use of *PR-10a* as a marker for the pathogenesis-state of potato. The ELISA assays unambiguously indicated that pharmacological agents that perturb the activity of PKC have a direct impact on the infection process reflected in the level of PR-10a protein accumulation. For example, calphostin C, which inhibits PKC-like kinase activity in vitro, completely blocked the arachidonic acid-induced resistance and rendered the tubers susceptible to infection.

In order to firmly establish the involvement of a specific PKC isozyme in a

biological process, certain molecular features had to be characterized. A PCR based approach was considered to isolate the kinase. The drawback to this approach is to establish the link between the isolated kinase and the biological process under study. Another approach attempted was to screen cDNA expression libraries with the PKC-specific antibodies. One clone possessed many motifs common to the cPKC family, including the phorbol ester binding and calcium binding sites. Unfortunately, the clone exhibited no kinase activity when expressed either in bacteria, yeast or insect cells. For these reasons, protocols were developed to purify the kinase from potato tubers. As described in Section 3.2, a partial purification of the kinase has been achieved. The discussion that follows highlights the common features and the differences between this kinase and other PKC kinases that have been characterized including those from *Brassica* and *Zea mays*. The discussion also explores alternate strategies to purify this kinase to homogeneity.

4.3. Characterization of a partially purified PKC-like kinase from potato tuber membranes

One of the hallmarks of PKC enzymes is that they translocate to membranes upon activation. As described in Section 3.1, this was achieved artificially by supplementing the membrane extraction buffers with calcium. It was subsequently shown that sliced or wounded tubers also stimulated the translocation of this kinase to the membranes. Interestingly, the kinase translocated in response to wounding required much less calcium for its activation. This phenomenon is strikingly similar to that observed in animal cells where translocated kinase displays higher affinity to calcium. This suggests that the PKC-like kinase is also activated during wounding. However, this activation does

not lead to a resistance response. It should be reiterated at this point that only treatment of wounded tubers with arachidonic acid induces resistance. The question that arises is, what is the role of arachidonic acid with respect to PKC in inducing the resistance response in potato tubers? This issue is addressed in Section 4.5.

In animals, purification of PKC has been achieved by many different methods. Typically, initial chromatography steps through Q-Sepharose, DEAE-Sepharose or DE-52 cellulose are used to eliminate many non-specific proteins. A major yield in the purification is achieved by affinity to various ligands like threonine, serine, and lysine. A high level of purification of PKC from *Caenorhabditis elegans* (Sassa and Miwa, 1992) and from *Brassica* and *Zea mays* (Nanmori et al, 1994; Chandok and Sopory, 1998) has also been obtained with a phosphatidylserine affinity column. Finally, the various PKC isozymes are separated by hydroxyapatite chromatography.

Chromatography purification of the PKC-like kinase from potato employed many of the steps outlined above. Interestingly, unlike the two other PKC-like kinases purified from plants (Nanmori et al, 1994; Chandok and Sopory, 1998), the PKC-like kinase from potato was not retained on a PS-affinity column. It has been noted that cPKC βI isozyme from bovine brain does not bind to the phosphatidylserine ligand very efficiently (Allen and Katz, 1991). However, the kinase from potato did bind effectively to a threonine-affinity column and as mentioned in Chapter 3 resulted in a purification as high as 150-fold. The reasons for this are unclear but could reflect the age, and therefore, the physiology of tubers. The kinase proved to be very hydrophobic and adhered very tightly to a

Phenyl-Sepharose column even in the absense of salt or detergent. Elution was only achieved by the addition of detergent. Finally, it had been observed during optimisation of various chromatographic steps that the kinase could bind to both cation and anion exchangers. This amphitrophic behaviour provided another method to purify this kinase. Following hydrophobic chromatography, the kinase was further purified using a Mono S column.

The kinase that eluted from the Mono S column exhibited many properties associated with the cPKC family. These included maximal activation in the presence of cofactors Ca^{2+}, PS, and TPA. Interestingly, DAG, the natural counterpart of TPA was equally effective in activating this purified kinase. As described before, DAG is a product of hydrolysis of plasma membrane-associated phosphoinositides by phospholipase C. Evidence from animal cells indicate that the lifespan of the DAG molecule produced by hydrolysis of phosphoinositides is in the order of minutes (Nishizuka, 1995). However, the level of DAG increases with a relatively slow onset, and persists for several hours. It is hypothesized that for a long-term cellular response by PKC, a sustained elevation of DAG levels is required and this is achieved mainly by hydrolysis of phosphatidylcholine by phospholipase D (PLD) (Nishizuka, 1995). Interestingly, both PC-specific PLD and phosphoinositol-specific PLC are present in plants and their genes have been cloned (Hirayama et al., 1995; Shi et al., 1995; Yamamoto et al., 1995). PLC is activated in soybean cells exposed to polygalacturonic acid elicitor, suggesting that phosphoinositides may participate as second messengers in the elicitor-induction of the defense-related response in these cells (Legendre et al., 1993; Chandra et al., 1996). Recently, Young et al., (1996) observed that PLD accumulates around the pathogen ingress site during a resistance response in

rice.

Preliminary experiments conducted in our laboratory suggest that indeed hydrolysis of PC by PLD increases following elicitor treatment in potato tubers. The DAG level also increases by three-fold (Yao, K., personal communication). Altogether, these observations suggest that in potato a similar two-tier system for the production of DAG may exist. The hypothesis is that at the first level, wounding tubers could activate a PI-specific PLC and the resulting IP_3 molecules would release calcium from the internal storages and translocate PKC to the membrane. Once translocated, DAG molecules would activate PKC. As demonstrated in Figure 10, translocation of PKC was observed soon after the tubers were wounded. Therefore, a detection of PLC activity in these wounded tubers would lend credance to the hypothesis. At the second level, treatment with elicitor would trigger the activation of PLD to produce sustained elevated levels of DAG for long-term activation of PKC. It is tempting to speculate that the activation of PKC by the two-tier system outlined above may also be the driving force behind the activation of the *PR-10a* gene. The mRNA levels of *PR-10a* have been shown to accumulate only transiently during the wounding period. In contrast, the mRNA level continues to accumulate and is maintained long after the tubers are exposed to the elicitor (Matton and Brisson, 1989), reflecting the sustained PKC activity induced by the elicitor arachidonic acid.

The cPKC-like kinase from potato exhibits differences when compared to the cPKC family member identified in *Brassica* (Nanmori et al., 1994). For example, the peptide Ac-MBP (4-14), which was shown to be an excellent substrate of the *Brassica* and other cPKCs, was as a poor substrate of the potato

kinase. The reason for this difference is not known. It is clear that kinases have evolved to respond to different extracellular stimuli and phosphorylate a unique set of targets in the cell. It therefore follows that substrate specificities of kinases are crucial determinants of their fidelity and efficacy during signalling events. Explanation of subtle differences that may exist between the two cPKC family members will likely require the knowledge of their molecular structure. To this end, we obtained partial sequences of the putative PKC from potato tuber membranes. The amino acid analyses of the two peptides revealed that the kinase was contaminated by a protein belonging to the Hsp70 family.

4.4. Role of chaperones in signal transduction

Plant Hsp70, like its counterpart in humans, is encoded by a multigene family (Schöffl et al., 1998). It remains unknown to what degreé the proteins encoded by these gene families are functionally distinct. However, differential regulation of specific *Hsp70* genes supports the hypothesis that diversity within the family is not merely due to gene redundancy (Boston et al., 1996). Plant Hsp70s are expressed in a variety of tissues under normal and stressful conditions. In maize, Hsp70 increases the translocation efficiency of precursor proteins into the microsomal membranes (Miernyk et al., 1992). In pea, Hsp70 aids in the uncoating of clathrin-coated vesicles (Kirsch and Beevers, 1993). The consensus from numerous studies in plants and other eukaryotes is that Hsp70s are specialized proteins that bind nascent polpeptides and assist them in attaining a functional conformation. Furthermore, studies suggest that Hsp70 chaperone proteins recognize the exposed hydrophobic surfaces of nonnative species and through noncovalent interactions stabilize them against irreversible multimeric

aggregation.

As described in Section 3.2, the PKC-like kinase from potato demonstrated strong affinity to Phenyl-Sepharose matrix, suggesting that this protein is hydrophobic. Given the tendency of Hsp70 protein to interact preferentially with hydrophobic proteins, combined with the observation that the kinase always coeluted with the Hsp70 protein, we propose that the two proteins may interact with each other. This hypothesis is not without precedent. For example, in lymphocytes, PKC has been found to aggregate with Hsp70 and a cytoskeleton protein, spectrin (Di et al., 1997). However, preliminary immunoprecipitation experiments performed with the partially purified PKC-like kinase, demonstrated that the kinase and the Hsp70 protein from potato tubers do not interact. It is possible that relevant cofactors such as ATP, which affects Hsp70-substrate interactions or some accessory proteins were not present in the immunoprecipitation reactions.

In a number of systems where an interaction between Hsp70 and other proteins have been documented, the Hsp70 proteins act as accessory proteins of various signalling molecules. BAG-1, a multifunctional protein, has been shown to interact with signalling molecules such as the anti-apoptotic protein Bcl-2, the protein kinase Raf-1, and tyrosine kinase receptors of growth factors such as hepatocyte growth factor and platelet-derived growth factor (Takayama et al., 1997). In this complex signalling network, Hsp70 interacts with BAG-1 and facilitates its interaction with other signalling molecules. A similar role for Hsp70 has been described in the interaction between RAP46, a nuclear receptor-associated protein, and the transcription factor c-Jun (Zeiner et al., 1997). If such

an interaction takes place between Hsp70 and the PKC-like kinase in potato, it could maintain the kinase in a state of readiness favouring interaction with other signalling molecules.

Alternatively, interaction with Hsp molecules may shield the kinase from cellular proteases. This function of Hsp has been demonstrated in the Hsp90-chaperone system. In this system, which includes the Hsp70, is involved in trafficking the signalling molecules Src and Raf. Disruption of the Hsp-90 heterocomplex with inhibitors of Hsp90 function, markedly decreases the half-life of Raf protein kinase, suggesting that Hsp90 protects Raf kinase from protease action (Schulte et al., 1995).

4.5. Role of free fatty acids in signal transduction

In Section 4.3, a question was raised about the relevance of the elicitor arachidonic acid in the defense response in potato with respect to the activation of PKC. This section will address the role of free fatty acids in the overall context of cellular signalling. An emerging body of evidence from animal systems suggest that free unsaturated fatty acids such as arachidonic acid play a pivotal role in many cellular functions. This is supported by the fact that AA can interact with many cellular proteins including phospholipases, G-proteins, ion channels, GTPase activating proteins, and protein kinases (Khan et al., 1995). This suggests that there are mechanisms to control and regulate the release of AA in response to cellular stimulation. Intracellular AA is rapidly metabolized or reincorporated as fatty acyl chains of glycerolipids. Therefore, the majority of the free AA mass released in cells in response to cellular activators results from the activation of one or more phospholipases such as phospholipase A_2, and mono-

and diacylglycerol lipases (Khan et al., 1995).

Similarly, studies on the metabolic fate of AA in potato tubers indicate that most of the free AA applied exogenously is incorporated primarily into the potato acyl lipids, phosphatidylcholine and phoaphatidylethanolamine, with approximately 2% being continually recovered in the free fatty acid form (Preisig and Kuc, 1988). Interestingly, the fate of exogenously applied esterified AA is the same as that of free AA. Given that the hypersensitive response is induced faster by free AA than by its esterified form and the fact that matabolites of AA do not induce this response, Preisig and Kuc, (1988) argued that the free acid plays a central role in triggering the hypersensitive response in potato tubers.

In animals, the interactions between free fatty acids and PKC have been characterized *in vitro* and *in vivo* (Blobe et al., 1995). *In vitro*, PKC is activated by *cis*-unsaturated fatty acids such as AA and oleic acid. Activation by these fatty acids is independent of PS, but the roles of calcium and diacylyglcerol remain obscure. In plants, Van der Hoeven et al., (1996) recently reported that *cis*-unsaturated fatty acids including arachidonic acid activate a protein kinase in oat root plasma membranes. This activation is mitigated by calphostin C, suggesting that a PKC-like kinase may be affected by arachidonic acid, similar to what is observed in the animal cells. Preliminary experiments with kinase purified from potato tuber membranes indicate that AA can increase kinase activity in a PS independent manner. Furthermore, the addition of AA augments the activity induced by all the PKC cofactors (data not shown). These results support the hypothesis that PKC may be regulated by direct interactions with arachidonic acid.

4.6. Perspectives

It is premature at this point to assess the importance of the PKC-like kinase identified in potato in the overall defense response in plants. The molecular cloning of this kinase will help establish its relationship with the PKC family. By examining regulatory and catalytic domains, one can then attempt to elucidate the mechanisms that control its activity. For example, one of its activator *in vivo* is diacylglycerol. Demonstration of binding of this lipid molecule to the kinase would establish a link between the kinase and one of its regulators. Moreover, since the regulation of DAG levels *in vivo* is controlled by the hydrolysis of membrane lipids by phospholipases, this would confirm their role in the signal transduction cascade.

A direct role of the PKC-like kinase in the pathway leading to the expression of *PR-10a* could be examined by creating a kinase-defective mutant and monitoring the expression of *PR-10a*. This could be achieved by transiently co-expressing the kinase-defective mutant and the promoter of *PR-10a* fused to a reporter gene in a potato protoplast system. The dominant negative effect of the kinase-defective mutant would be expected to interfere with the signalling cascade and subsequently, block the expression of the reporter gene. Alternatively, the PKC-like kinase could be expressed stably in potato plants, both in the sense and the anti-sense orientation. Assuming no growth defects occur due to the transgenic expression of the kinase, the ability of these transgenic plants to resist pathogens could be tested.

Among the kinase families, MAPK has been shown to play important role in the defense response pathways (Ligterink et al., 1997; Suzuki and Shinshi,

1995). Like in other eukaryotes, plant MAPK pathways may integrate a variety of upstream signals including those arising from PKC (Kolch et al., 1993). Therefore, by monitoring the MAPK activity in a potato protoplast system transfected with PKC clones (wild-type and mutants) as outlined before, a link between the MAPK and the PKC-like kinase could be established.

The ultimate challenge in cellular signal transduction is to ensure that the various signalling components are regulated properly and that these components are effectively coordinated within the signalling pathway. In animal cells, this responsibility is harbored by the growing number of scaffold and anchoring proteins (Mochley-Rosen and Gordon, 1998). As described in section 4.4, Hsp proteins could also fit this role. By using the PKC-like kinase as a bait in a yeast two-hybrid system, components interacting with the kinase, like the Hsp proteins, could be identified. This would build upon the PKC-based signal transduction pathway involved in the overall disease resistance mechanisms in potato.

Bibliography

Agrios, G.N. (1988). Plant pathology. Academic Press inc. San Diego.

Allen, B.G., and Katz, S. (1991). Isolation and characterization of the calcium- and phospholipid-dependent protein kinase (protein kinase C) subtypes from bovine brain. Biochemistry **30,** 4334-4343.

Arecas, L.B., Kazanietz, M.G., and Blumberg, P.M. (1994). Close similarity of baculovirus-expressed n-chimerin and protein kinase Cα as phorbol ester receptors. J. Biol. Chem. **269,** 19553-19558.

Beerhues, L., and Kombrink, E. (1994). Primary structure and expression of mRNAs encoding basic chitinase and 1,3-beta-glucanase in potato. Plant Mol. Biol. **24,** 353-367.

Bent, A.F., Kunkel, B.N., Dahlbeck, D., Brown, K.L., Schmidt, R., Giraudat, J., Leung, J., and Staskawicz, B.J. (1994). RPS2 of Arabidopsis thaliana: a leucine-rich repeat class of plant disease resistance genes. Science **265,** 1856-1860.

Blobe, G.C., Obeid, L.M., and Hannun, Y.A. (1994). Regulation of protein kinase C and role in cancer biology. Cancer Metastasis Rev. **13,** 411-431.

Blobe, G.C., Khan, W.A., and Hannun, Y.A. (1995). Protein kinase C: cellular target of the second messenger arachidonic acid? Prostaglandins Leukotrienes Essent. Faty Acids **52,** 129-135.

Boller, T. (1985) Induction of hydrolases as a defense reaction against pathogens. In Cellular and Molecular Biology of Plant Stress, J.L. Key and T. Kosuge, eds (A.R. Liss New York, NY) pp. 247-262.

Bolwell, G.P., Butt, V.S., Davies, D.R., and Zimmerlin, A. (1995). The origin of the oxidative burst in plants. Free Radical Res. **23,** 517-532.

Bostock, R.M., Kuc, J.A., and Laine, R.A. (1981). Eicosapentaenoic and arachidonic acids from *Phytophthora infestans* elicit fungitoxic sesquiterpenes in

the potato. Science **212,** 67-69.

Boston, R.S., Viitanen, P.V., and Vierling, E. (1996). Molecular chaperones and protein folding in plants. Plant Mol. Biol. **32,** 191-222.

Bradley, D.J., Kjellbom, P., and Lamb, C.J. (1992). Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. Cell **70,** 21-30.

Brisson, L.F., Tenhaken, R., and Lamb, C.J. (1994). Function of oxidative cross-linking of cell wall structural proteins in plant disease resistance. Plant Cell **6,** 1703-1712.

Bufe, A., Spangfort, M.D., Kahlert, H., Schlaak, M., and Becker, W.M. (1996). The major birch pollen allergen, Bet v 1, shows ribonuclease activity. Planta **199,** 413-415.

Cai, D., Kleine, M., Kifle, S., Harloff, H.J., Sandal, N.N., Marcker, K.A., Klein-Lankhorst, R.M., Salentijn, E.M.J., Lange, W., Stiekema, W.J., Wyss, U., Grundler, F.M.W., and Jung, C. (1997). Positional cloning of a gene for nematode resistance in sugar beet. Science **275,** 832-834.

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. (1982). Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumour-promoting phorbolesters. J. Biol. Chem. **257,** 7847-7851.

Chamnongpol, S., Willekens, H., Langenartels, C., Van Montagu, M., Inze, D., and Van Camp, W. (1996). Transgenic tobacco with a reduced catalase activity develops necrotic lesions and induces pathogenesis-related expression under high light. Plant J. **10,** 491-503.

Chandok, M.R., and Sopory, S.K. (1996). Phosphorylation/dephosphorylation steps are key events in the phytochrome-mediated enhancement of nitrate reductase mRNA levels and enzyme activity in maize. Mol. Gen. Genet. **251,** 599-608.

Chandok, M.R., and Sopory, S.K. (1998). ZmcPKC70, a protein kinase C-type enzyme from maize. J. Biol. Chem. **273,** 19235-19242.

Chandra, S., Heinstein, P.F., and Low, P.S. (1996). Activation of phospholipase A by plant defense elicitors. Plant Physiol. **110**, 979-986.

Chasan, R. (1994). Plant-pathogen encounters in Edinburgh. Plant Cell **6,** 1332-1341.

Chen, Z., Ricigliano, J.W. and Klessig, D.F. (1993). Purification and characterization of a soluble salicylic acid-binding protein from tobacco. Proc. Natl. Acad. Sci. USA **90,** 9533-9537.

Cohen, Y., Gisi, U., and Niderman, T. (1993). Local and systemic protection against *Phytophthora infestans* induced in potato and tomato plants by jasmonic acid and jasmonic methyl ester. Phytopathology **83,** 1054-1062.

Collinge, D.B and Slusarenko, A.J. (1987). Plant gene expression in response to pathogens. Plant. Mol. Bio. **9,** 389-410.

Constabel, C.P., and Brisson, N. (1992). The defense-related *STH-2* gene product of potato shows race-specific accumulation after inoculation with low concentrations of *Phytophthora infestans* zoospores. Planta **188,** 289-295.

Constabel, C.P., and Brisson, N. (1995). Stigma- and vascular-specific expression of the *PR-10a* gene of potato - a novel pattern of expression of a pathogenesis-related gene. Mol. Plant-Microbe Interact. **8,** 104-113.

Cornelis, G.R., and Wolf-Watz, H. (1997). The *Yersinia* Yop virulon: a bacterial system for subverting eukaryotic cells. Mol. Microbiol. **23,** 861-867.

Creelman, R.A., and Mullet, J.E. (1997). Biosynthesis and actions of jasmonates in plants. Annu. Rev. Plant Physiol. **48,** 355-381.

Dekker, L.V., and Parker, P.J. (1994). Protein kinase C-A question of specificity. Trends Biochem. Sci. **19,** 73-77.

Dekker, L.V., Palmer, R.H., and Parker, P.J. (1995). The protein kinase C and protein kinase C related gene families. Curr. Opin. Struct. Biol. **5,** 396-402.

Després, C., Subramaniam, R. Matton, D.P., and Brisson, N. (1995). The activation of the potato *PR-10a* gene requires the phosphorylation of the nuclear factor PBF-1. Plant Cell **7,** 589-598.

De Wit Pierre, J.G.M. (1997). Pathogen avirulence and plant resistance: a key role for recognition. Trends in Plant Sci. **2,** 452-458.

Di, Y-P., Repasky, E.A., and Subjeck. (1997). Distribution of hsp70, protein kinase C, and spectrin is alterd in lymphocytes during a fever-like hyperthermia exposure. J. Cell. Physiol. **172,** 44-54.

Dixon, R.A., Harrison, M.J., and Lamb, C.J. (1994). Early events in the activation of plant defense responses. Annu. Rev. Phytopathol. **32,** 479-501.

Dixon, R.A., and Paiva, N.L. (1995). Stress-induced phenylpropanoid metabolism. Plant Cell **7,** 1085-1097.

Dixon, M.S., Jones, D.A., Keddie, J.S., Thomas, C.M., Harrison, K., and Jones, J.D.J. (1996). The tomato Cf-2 disease resistance locus comprises two functional genes encoding leucine-rich repeat proteins. Cell **84,** 451-459.

Doke, N. (1983) Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans* and to the hyphal wall components. Physiol. Plant Pathol. **23,** 345-357.

Doke, N., Ramirez, A.V., and Tomiyama, K. (1987). Systemic induction of resistance in potato against Phytophthora infestans by local treatment with hyphal wall components of the fungus. J. Phytopathol. **119,** 232-239.

Douglas, C.J. (1996). Phenylpropanoid metabolism and lignin biosynthesis: from weeds to trees. Trend. Plant. Sci. **1,** 171-178.

Duclos, B., Marcandier, S., and Cozzone, A.J. (1991). Chemical properties and separation of phosphoamino acids by thin-layer chromatography and/or electrophoresis. Methods Enzymol. **201**, 10-21.

Dutil, E.M., Keranen, L.M., De-Paoli-Roach, A.A., and Newton, A.C. (1994). In vivo regulation of protein kinase C by trans-phosphorylation followed by autophosphorylation. J. Biol. Chem. **269,** 29359-29362.

Ebel, J., and Scheel, D. (1992). Elicitor recognition and signal transduction. In Genes Involved in Plant Defense, T. Boller and F. Meins, jr., eds (Vienna/New York: Springer-Verlag), pp. 183-205.

Einspahr, K.J., and Thompson, jr., G.A. (1990). Transmembrane signaling via phosphatidylinositol 4,5-biphosphate hydrolysis in plants. Plant Physiol. **93,** 361-366.

Enyedi, A.J., Yalpani N., Silverman, P., and Raskin, I. (1992). Localisation, conjugation and function of salicylic acid in tobacco during the hypersensitive reaction to tobacco mosaic virus. Proc. Natl. Acad. Sci. **89,** 2480-2484.

Estruch, J.J., Kadwell, S., Merlin, E., and Crossland, L. (1994). Cloning and characterization of a maize pollen-specific calcium-dependent calmodulin-independent protein kinase. Proc. Natl. Acad. Sci. **91,** 8837-8841.

Felix G., Grosskopf, D.G., Regenass, M., and Boller, T. (1991). Rapid changes of protein phosphorylation are involved in transduction of the elicitor signal in plant cells. Proc. Natl. Acad. Sci. **88,** 8831-8834.

Flor, H.H. (1946). Genetics of pathogenicity in *Melampsora lini.* J. Agric. Res. **73,** 335-357.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H., and Ryals, J. (1993). Requirement of salicylic acid for the induction of systemic acquired resistance. Science **261,** 754-756.

Galindo, R.L., Edwards, D.N., Gillespie, S.K.H., and Wasserman, S.A. (1995).

Interaction of the pelle kinase with the membrane-associated protein tube is required for transduction of the dorsoventral signal in *Drosophila* embryos. Development **121,** 2209-2218.

Gilroy, S., and Trewavas, T. (1994). A decade of plant signals. Bioessays **16,** 677-682.

Grant, M.R., Godiard, L., Straube, E., Ashfield, T., Lewald, J., Sattler, A., Innes, R.W., and Dangl, J.L. (1995). Structure of the Arabidopsis RPM1 gene enabling dual specificity disease resistance. Science **269,** 843-846 .

Grosskopf, D.G., Felix, G., and Boller, T. (1990). K-252a inhibits the response of tomato cells to fungal elicitors in vivo and their microsomal protein kinase in vitro. FEBS Lett. **275,** 177-180.

Hammondkosack, K.E., Tang, S.J., Harrison, K., and Jones, J.D.J. (1998). The tomato *CF-9* disease resistance gene functions in tobacco and potato confer responsiveness to the fungal avirulence gene product AVR9. Plant Cell **10,** 1251-1266.

Hannun, Y.A., Loomis, C.R., and Bell, R.M. (1985). Activation of protein kinase C by Triton X-100 mixed micelles containing diacylglycerol and phosphatidylserine. J. Biol. Chem. **260,** 10039-10043.

Hannun, Y.H., Merrill, A.H., jr., and Bell, R.M. (1991). Use of sphingosine as inhibitor of protein kinase C. Methods Enzymol. **201,** 316-328.

Harper, J.J., Sussman, M.R., Schaller, G.E., Putnam-Evans, C., Charbonneau, H., and Harmom, A.C. (1991). A calcium-dependent protein kinase with a regulatory domain similar to calmodulin. Science **252,** 951-954.

Heguy, A., Baldari, C.T., , Macchia, G., Telford, J.L., and Melli, M. (1992). Amino acids conserved in interleukin-1 receptors (IL-1Rs) and the *Drosophila* Toll protein are essential for IL-1R signal transduction. J. Biol. Chem. **267,** 2605-2609.

Hirayama, T., Ohto, C., Mizoguchi, T., and Shinozaki, K. (1995). A gene

encoding a phosphatidylinositol-specific phospholipase C is induced by dehydration and salt stress in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA **92**, 3903-3907.

Huang, F.L., Yoshida, Y., Cunha-Melo, J.R., Beavan, M.A., and Huang, K.P. (1989). Differential down regulation of protein kinase C isozymes. J. Biol. Chem. **264,** 4238-4243.

Hug, S., and Sarre, T.F. (1993). Protein kinase C isoenzymes: divergence in signal transduction? Biochem. J. **291**, 329-343.

Jach, G., Gornhardt, B., Mundy, J., Logemann, J., Pinsdorf, E., Leah, R., Schell, J., and Maas, C. (1995). Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. Plant J. **8,** 97-109.

Johal, G.S., and Briggs, S.P. (1992). Reductase encoded by the *Hm1* disease resistance gene in maize. Science **258,** 985-987.

Jonak, C., Kiegerl, S., Ligterink, W., Barker, P.J., Huskisson, N.S., and Hirt, H. (1996). Stress signalling in plants: a MAP kinase pathway is activated by cold and drought. Proc. Natl. Acad. Sci. **93,** 11274-11279.

Jones, D.A., Thomas, C.M., Hammond-Kosack, K.E., Balint-Kurti, P.J., and Jones, J.D. (1994). Isolation of the tomato *Cf-9* gene for resistance to Cladosporium fulvum by transposon tagging. Science **266,** 789-793.

Joosten, M.H.A.J., Cozijnsen, A.J., and De Wit, P.J.G.M. (1994). Host resistance to a fungal tomato pathogen lost by a single base-pair change in an avirulence gene. Nature **367,** 384-386.

Kameshita, I., and Fujisawa, H. (1989). A sensitive method for detection of calmodulin-dependent protein kinase II activity in sodium dodecyl sulfate-polyacrylamide gel. Anal. Biochem. **183**, 139-143.

Kamps, M.P., and Sefton, B.M. (1989). Acid and base hydrolysis of

phosphoproteins bound to Immobilon facilitates analysis of phosphoamino acids in gel-fractionated proteins. Anal. Biochem. **176**, 22-27.

Kauss, H., and Jeblick, W. (1991). Induced Ca^{2+} uptake and callose synthesis in suspension-cultured cells of *Catharanthus roseus* are decreased by the protein phosphatase inhibitor okadaic acid. Physiol. Plant. **81,** 309-312.

Keen, N. T., Tamaki, S., Kobayashi, D., Gerhold, D., Stayton, M., Shen, H., Gold, S., Lorang, J., Thordal-Christensen, H., Dahlbeck, D., and Staskawicz, B. (1990). Bacteria expressing avirulence gene *D* produce a specific elicitor of the soybean hypersensitive reaction. Mol. Plant-Microbe Interact. **3,** 112-121.

Keppler, L.D., Baker, C.J., and Atkinson, N.M. (1989). Active oxygen production during a bacteria induced hypersensitive reaction in tobacco suspension cells. Phytopathology **79,** 974-978.

Khan, W.A., Mascarella, S.W., Lewin, A.H. Wyrick, C.D., Carroll, F.I., and Hannun, Y.A. (1991). Use of D-erythro-sphingosine as a pharmacological inhibitor of protein kinase C in human paltelets. Biochem. J. **278,** 387-392.

Khan, W.A., Blobe, G.C., and Hannun, Y.A. (1995). Arachidonic acid and free fatty acids as second messengers and the role of protein kinase C. Cell. Signall. **7,** 171-184.

Kieber, J.J., Rothenberg, M., Roman G., Feldman, K.A., and Ecker J.R. (1993). *CTR1*, a negative regulator of the ethylene response pathway in *Arabidopsis*, encodes a member of the Raf family of protein kinases. Cell **72,** 427-441.

Kikkawa, U., Masayoshi, G., Koumoto, J., and Nishizuka, Y. (1986). Rapid purification of protein kinase C by high performance liquid chromatography. Biochem. Biophys. Res. Comm. **135,** 636-643.

Kirsch, T., and Beevers, L. (1993). Uncoating of a clathrin-coated vesicles by uncoating ATPase from developing peas. Plant Physiol. **103,** 205-212.

Kitano, T., Go, M., Kikkawa, U., and Nishizuka, Y. (1986). Assay and purification

of protein kinase C. Methods Enzymol. **124,** 349-352.

Kneusal, R.E., Matern, U., and Nicolay, K. (1989). Formation of *trans*-caffeoyl CoA from *trans*-4-coumaroyl CoA by Zn^{++}-dependent enzymes and its activation by an elicitor-indueced pH shift. Arch. Biochem. Biophys. **269,** 455-462.

Kobe, B., and Deisenhofer, J. (1994). The leucine-rich repeat: a versatile binding motif. Trends Biochem. Sci. **19,** 415-421.

Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenseller, G., Marne, D., and Rappo, U.R. (1993). Protein kinase Cα activates RAF-1 by direct phosphorylation. Nature **364**, 249-252.

Kombrink, E., Schroeder, M., and Hahlbrock, K. (1988). Several pathogenesis-related proteins in potato are 1,3-β-glucanases and chitinases. Proc. Natl. Acad. Sci. USA **85,** 782-786.

Kosaka, Y., Ogita, K., Ase, K., Nomura, H., Kikkawa, U., and Nishizuka, Y. (1988). The heterogeneity of protein kinase C in various rat tissues. Biochem. Biophys. Res. Comm. **151,** 973-981.

Kurosaki, F., Tsurusawa, Y., and Nishi, A. (1987). Breakdown of phosphatidylinositol during the elicitation of of phyoalexin production in cultured carrot cells. Plant Physiol. **85,** 601-604.

Kurosaki, F., and Nishi, A. (1993). Stimulation of calcium influx and calcium cascade by cyclic AMP in cultured carrot cells. Arch. Biochem. Biophys. **302,** 144-151.

Kyriakis, J.M., App, H., Zhang, X.F., Banerjee, P., Brautigan, D.L., Rapp, U.R., and Avruch, J. (1992). Raf-1 activates MAP kinase-kinase. Nature **358,** 417-421.

Lawrence, G.J., Finnegan, E.J., Ayliffe, M.A., and Ellis, J.G. (1995). The *L6* gene for flax rust resistance is related to the Arabidopsis bacterial resistance gene *RPS2* and the tobacco viral resistance gene *N*. Plant Cell **7,** 1195-1206.

Legendre, L., Heinstein, P.F., Crain, R.C., and Low, P.S. (1993). Phospholipase activation during elicitation of the oxidative burst in cultured plant cells. J. Biol. Chem. **268**, 24559-24563.

Leister, D., Ballvora, A., Salamini, F., and Gebhardt, C. (1996). A PCR-based approach for isolating pathogen resistance genes from potato with potential for wide application in plants. Nature Genetics **14,** 421-428.

Ligterink,W., Kroj, T., Nieden, U.Z., Hirt, H., and Scheel, D. (1997). Receptor-mediated activation of a MAP kinase in pathogen defense of plants. Science **276,** 2054-2057.

Lin, T-Y. Duck, N.B., Winter, J., and Folk, W.R. (1991). Sequences of two hsc70 cDNAs from *lycopersicon esculentum*. Plant Mol. Biol. **16,** 475-478.

Linthorst, H.J.M. (1991). Pathogenesis-related proteins of plants. Crit. Rev. Plant Sci. **10,** 123-150.

Lopez, I., Burns, D.J., and Lambeth, D.J. (1995). Regulation of phospholipase D by protein kinase C in human neutrophils. J. Biol. Chem. **270,** 19465-19472.

McAinsh, M.R., and Hetherington, A.M. (1998). Encoding specificity in Ca^{2+} signalling systems. Trends Plant Sci. **3,** 32-36.

MacIntosh, G.C., Ulloa, R.M., Raíces, M., and Téllez-Iñón, M.T. (1996). Changes in calcium-dependent protein kinase activity during in vitro tuberization in potato. Plant. Physiol. **112,** 1541-1550.

Malho, R., and Trewavas, A.J., (1996). Localized apical increase of cytoplasmic free calcium control pollen tube orientation. Plant Cell **8,** 1935-1949.

Marineau, C., Matton, D.P., and Brisson, N. (1987). Differential accumulation of potato mRNAs during the hypersensitive response induced by arachidonic acid elicitor. Plant Mol. Biol. **9,** 335-342.

Martin, G.B., Brommonschenkel, S.H., Chunwongse, J., Frary, A., Ganal,

M.W., Spivey, R., Wu, T., Earle, E.D., and Tanksley, S.D. (1993). Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science **262,** 1432-1436.

Matton, D.P., and Brisson, N. (1989). Cloning, expression, and sequence conservation of pathogenesis-related gene transcripts of potato. Mol. Plant-Microbe Interact. **2,** 325-331.

Matton, D.P., Prescott, G., Bertrand, C., Camirand, A., and Brisson, N. (1993). Identification of *cis*-acting elements involved in the regulation of the pathogenesis-related gene STH-2 in potato. Plant Mol. Biol. **22,** 279-291.

Mayer, M.G., and Ziegler, E. (1988). An elicitor from *Phytophthora megasperma* f.sp.*glycinea* influences the membrane potential of soybean cotyledonary cells. Physiol. Mol. Plant Pathol. **33,** 397-407.

Miernyk, J.A., Duck, N.B., Shatters, R.G., Jr., and Folk, W.R. (1992). The 70-kilodalton heat shock cognate can act as a molecular chaperone during the membrane translocation of a plant secretory protein precursor. Plant Cell **4,** 821-829.

Milner, C,M., and Campbell, R.D. (1990). Structure and expression of the three MHC-linked *HSP70* genes. Immunogenetics **32,** 242-251.

Milligan, S,B., Bodeau, J., Yaghoobi, J., Kaloshian, I., Zabel, P., and Williamson, V.M. (1998). The root knot nematode resistance gene *M1* from tomato is a member of the leucine zipper, nucleotide binding, leucine-rich repeat family of genes. Plant Cell **10,** 1307-1319.

Mindrinos, M., Katagiri, F., Yu, G.L., and Ausubel, F.M. (1994). The A. thaliana disease resistance gene *RPS2* encodes a protein containing a nucleotide-binding site and leucine-rich repeats. Cell **78,** 1089-1099.

Miura,Y., Yoshioka, H., and Doke, N. (1995). An autophotographic determination of the active oxygen generation in potato tuber discs during hypersensitive response to fungal infection or elicitor. Plant Sci. **105,** 45-52.

Mochley-Rosen, D., and Gordon, A.S. (1998). Anchoring proteins for protein kinase C: a means for isozyme selectivity. FASEB. J. **12,** 35-42.

Moiseyev, G.P., Beintema, J.J., Fedoreyeva, L.I., and Yakovlev, G.I. (1994). High sequence similarity between a ribonuclease from ginseng calluses and fungus-elicited proteins from parsley indicates that intracellular pathogenesis-related proteins are ribonucleases. Planta **193,** 470-472.

Nandan, D., Daubenberger, C., Mpimbaza, G., and Pearson, T.W. (1994). A rapid, single-step purification for immunogenic members of the hsp 70 family: validation and application. J. Imm. Methods **176,** 255-263.

Nanmori, T., Taguchi, W., Kinugasa, M., Oji, Y., Sahara, S., Fukami, Y., and Kikkawa, U. (1994). Purification and characterization of protein kinase C from a higher plant, *Brassica campestris* L. Biochem. Biophys. Res. Commun. **203,** 311-318.

Neuhaus, G., Bowler, R., Kern, R., and Chua, N.-H. (1993). Calcium/calmodulin-dependent and -independent phytochrome signal transduction pathways. Cell **73,** 937-952.

Niderman, T., Genetet, I., Bruyere, T., Gees, R., Stintzi, A., Legrand, M., Fritig, B., and Mosinger, E. (1995). Pathogenesis-related PR-1 proteins are antifungal. Isolation and characterization of three 14-kilodalton proteins of tomato and of a basic PR-1 of tobacco with inhibitory activity against *Phytophthora infestans*. Plant Physiol. **108,** 17-27.

Nishizuka, Y. (1992). Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science **258,** 607-614.

Nishizuka, Y. (1995). Protein kinase C and lipid signaling for sustained cellular responses. FASEB J. **9**, 484-496.

Nürnberger, T., Nennstiel, D., Jabs, T., Sacks, W.R., Hahlbrock, K., and Scheel, D. (1994). High affinity binding of a fungal oligopeptide elicitor to parsley plasma membranes. Cell **78,** 449-460.

Oakley, B.R., Kirsch, D.R., and Morris, N.R. (1980). A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal. Biochem. **105,** 361-363.

Oba, K., Kondo, K., Doke, N., and Uritani, I. (1985). Induction of 2-hydroxy-3-methylglutaryl CoA reductase in potato tubers after slicing, fungal infection or chemical treatment, and some properties of the enzyme. Plant Cell Physiol. **26,** 873-880.

O'Farrell, P.Z., Goodman, H.M., and O'Farrell. (1977). High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell **12,** 1133-1142.

Ori, N., Eshed, Y., Paran, I., Presting, G., Aviv, D., Tanksley, S., Zamir, D., and Fluhr, R. (1997). The I2C family from the wilt disease resistance locus I2 belongs to the nucleotide binding, leucine-rich repeat superfamily of plant resistance genes. Plant Cell **9,** 521-532.

Osmark, P., Boyle, B., and Brisson, N. (1998). Sequential and structural homology between intracellular pathogenesis-related proteins and a group of latex proteins. Plant Mol. Biol. (In Press).

Parker, J.E., Coleman, M.J., Szabo, V., Frost, L.N., Schmidt, R., van der Biezen, E.A., Moores, T., Dean, C., Daniels, M.J., and Jones, J.D. (1997). The Arabidopsis downy mildew resistance gene RPP5 shares similarity to the toll and interleukin-1 receptors with N and L6. Plant Cell **9,** 879-894.

Pawson, T. (1994). Introduction: protein kinases. FASEB J. **8,** 1112-1113.

Pirhonen, M., Flego, D., Heikinheimo, R., and Palva, E.T. (1993). A small diffusable signal is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora*. EMBO J. **12,** 2467-2476.

Ponstein, A.S., Bres-Vloemans, S.A., Sela-Buurlage, M.B., van den Elzen, P.J., Melchers, L.S., and Cornelissen, B.J. (1994). A novel pathogen- and wound-inducible tobacco (Nicotiana tabacum) protein with antifungal activity. Plant

Physiol. **104,** 109-118.

Pratt, W.B. (1997). The role of the hsp90-based chaperone system in signal transduction by nuclear receptors and receptors signaling via MAP kinase. Annu. Rev. Pharmacol. Toxicol. **37,** 297-326.

Preisig, C.L., and Kuc, J.A. (1988). Metabolism by potato tuber of arachidonic acid, an elicitor of hypersensitive response. Physiol. Mol. Plant Pathol. **32,** 77-88.

Putnam-Evans, C., Harmon A.C., Palevitz, B.A., Fechheimer, M., and Cormier, M.J. (1989). Calcium-dependent protein kinase is localized with F-actin in plant cells. Cell Motil. Cytoskeleton **12,** 12-22.

Raz, V., Fluhr, R. (1993). Ethylene signal is transduced via protein phosphorylation events in plants. Plant Cell **5,** 523-530.

Renelt, A., Colling, E., Hahlbrock, K., Nürnberger, T., and Parker, J.E. (1993). Studies on elicitor recognition and signal transduction in plant defense. J. Exp. Bot. **44,** 257-268.

Roberts, D.M., and Harmon, A.C. (1992). Calcium modulated proteins: targets of intracellular calcium signals in higher plants. Annu. Rev. Plant Physiol. Mol. Biol. **43,** 375-414.

Rogers, K.M., Albert, F., and Anderson, A.J. (1988). Lipid peroxidation is a consequence of elicitor activity. Plant Physiol. **86,** 547-553.

Rohe, M., Gierlich, A., Hermann, H., Hahn, M., Schmidt, B., Rosahl, S., and Knogge, W. (1995). The race-specific elicitor, NIP1, from the barley pathogen *Rhynchosporium secalis*, determines avirulence on host plants of the *Rrs1* resistance genotype. EMBO J. **14,** 4168-4177.

Ryan, C.A. (1994). Oligosaccharide signals: from plant defense to parasite offense. Proc. Natl. Acad. Sci. **91,** 1-2.

Salmeron, J.M., Oldroyd, G.E., Rommens, C.M., Scofield, S.R., Kim, H.S.,

Lavelle, D.T., Dahlbeck, D., and Staskawicz, B.J. (1996). Tomato *Prf* is a member of the leucine-rich repeat class of plant disease resistance genes and lies embedded within the *Pto* kinase gene cluster. Cell **86,** 123-133.

Sassa, T., and Miwa, J. (1992). Purification and characterization of protein kinase C from the nematode *Caenorhabditis elegans*. Biochem. J. **282,** 219-223.

Schaller G.E., Harmon, A.C., and Sussman, M.R. (1992). Characterization of a calcium- and lipid-dependent protein kinase associated with the plasma membrane of oat. Biochemistry **31,** 1721-1727.

Schöffl, F., Prändl, R., and Reindl, A. (1998). Regulation of heat shock response. Plant Physiol. **117,** 1135-1141.

Schulte, T.W., Blagosklonny, M.V., Ingui, C., and Neckers, L. (1995). Disruption of Raf-1-hsp90 molecular complex results in destablization of Raf-1 and loss of Raf-1-Ras association. J. Biol. Chem. **270,** 24585-24588.

Sheen, J. (1996). Ca^{2+}-dependent protein kinases and stress signal transduction in plants. Science **274,** 1900-1902.

Shi, J., Gonzales, R. A., and Bhattacharyya, M.K. (1995). Characterization of a plasma membrane-associated phosphoinositide-specific phospholipase C from soybean. Plant J. **8**, 381-390.

Shorrosh J.I., and Dixon, R.A. (1991). Molecular cloning of a putative plant endomembrane protein resembling vertebrate protein disulfideisomerase and a phosphatidylinositol-specific phospholipase C. Proc. Natl. Acad. Sci. **88,** 10941-10945.

Smith, B.G., and Rubery, P.H. (!981). The effects of infection by *Phytophthora infestans* on the control of phenylpropanoid metabolism in wounded potato tissue. Planta **151,** 535-540.

Song, W.Y., Wang, G.L., Chen, L.L., Kim, H.S., Pi, L.Y., Holsten, T., Gardner, J., Wang, B., Zhai, W.X., and Zhu, L.H. (1995). A receptor kinase-like protein

encoded by the rice disease resistance gene, *Xa21*. Science **270,** 1804-1806.

Sorimachi, H., Saido, T.C., and Suzuki, K. (1994). Discovery of tissue-specific calpains. FEBS Lett. **343**, 1-5.

Stab, M.R., and Ebel, J. (1987). Effects of Ca^{2+} on phytoalexin induced by fungal elicitor in soybean cells. Arch. Biochem. Biophys. **257,** 416-423.

Subramaniam, R., Després, C., and Brisson, N. (1997). A functional homolog of mammalian protein kinase C participates in the elicitor-induced defense response in potato. Plant Cell **9,** 653-664.

Suzuki, K., and Shinshi, H. (1995). Transient activation and tyrosine phosphorylation of a protein kinase in tobacco cells treated with fungal elicitor. Plant Cell **7,** 639-647.

Swanson, R.V., Alex, L.A., and Simon, M.I. (1994). Histidine and aspartate phosphorylation: two component systems and the limits of homology. Trends Biochem. Sci. **19,** 485-490.

Swoboda, I., Hoffmannsommergruber, K., Oriordain, G., Scheiner, O., Heberlebors, E., and Vicente, O. (1996). BET v 1 proteins, the major birch pollen allergens and members of a family of conserved pathogenesis-related proteins, show ribonuclease activity in vitro. Physiologia Plantarum. **96,** 433-438.

Takayama, S., Bimston, D.A., Matsuzawa, S-I., Freeman, B.C., Aime-Sempe, C., Xie, Z., Morimoto, R.I., and Reed, J.C. (1997). BAG-1 modulates the chaperone activity of hsp70/hsc70. EMBO J. **16,** 4887-4896.

Tamaoki, T. (1991). Use and specificity of staurosporine, UCN-01, and calphostin C as protein kinase inhibitors. Methods Enzymol. **201**, 340-347.

Taylor, A.R., Manison, N.F.H., Fernandez, C., Wood, J., and Brownlee, C. (1996). Spatial organization of calcium signalling involved in cell volume control in the Fucus rhizoid. Plant Cell **8,** 2015-2031.

Thilmony, R.L., Che, Z., Bressan, R.A., and Martin, G.B. (1995). Expression of the tomato *Pto* gene in tobacco enhances resistance to *Pseudomonas syringae pv. tabaci* expressing *avrPto*. Plant Cell **7,** 1529-1536.

Tomiyama, K., Okamoto, H., and Katoh, H. (1983). Effect of infection by *Phytophthora infestans* on the membrane potential of potato cells. Physiol. Plant Pathol. **22,** 233-243.

Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F., Duhamel, L., Charon, D., and Kirilovsky, J. (1991). The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. J. Biol.Chem. **266**, 15771-15781.

Toyada, K., Shiraisi, T., Yamada, T., Ichinose, Y., and Oku, H. (1993). Rapid changes in phosphoinositide metabolism in pea in response to fungal signals. Plant Cell Physiol. **34,** 729-735.

Van den Ackerveken, G.F.J.M., Van Kan, J.A.L., and De Wit, P.J.G.M. (1992). Molecular analysis of the avirulence gene *avr9* of the fungal tomato pathogen *Cladosporium fulvum* fully supports the gene-for-gene hypothesis. Plant J. **2,** 359-366.

Van der Hoeven, P.C.J., Siderius, M., Korthout, H.A.A.J., Drabkin, A.V., and De Boer, A.H. (1996). A calcium and free fatty acid-modulated protein kinase as putative effector of the fusicoccin 14-3-3 receptor. Plant Physiol. **111**, 857-865.

Van Loon, L.C., and Van Kamman, A. (1970). Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var. "Samsun" and "Samsun NN" II. Changes in protein constitution after infection with tobacco mosaic virus. Virology **40,** 199-211.

Van Loon, L.C., Pierpoint, W.S., Boller, T., and Conejero, V. (1994). Recommendations for naming plant pathogenesis-related proteins. Plant Mol. Biol. Reptr. **12,** 245-264.

Vogeli-Lange, R., Frundt, C., Hart, C.M., Nagy, F., and Meins, jr., f. (1994).

Evidence for role of β-1,3-glucanase in dicot seed germination. Plant J. **5,** 273-278.

Vogeli, U., Vogeli-Lange, R., and Chappell, J. (1992). Inhibition of phytoalexin biosynthesis in elicitor-treated tobacco cell suspension cultures by calcium/calmodulin antagonists. Plant Physiol. **100,** 1369-1376.

Warner, S.A.J., Scott, R., and Draper, J. (1992). Characterisation of a wound-induced transcript from the monocot aspargus that shares similarity with a class of intracellular pathogenesis-related (PR) proteins. Plant Mol. Biol. **19,** 555-561.

Wasternack, C., and Parthier, B. (1997). Jasmonate-signalled plant gene expression. Trends in Plant Sci. **2,** 302-307.

Wei, Z.-M., Laby, R.J., Zumoff, C.H., Bauer, D.W., He, S.Y., Collmer, A., and Beer, S.V. (1992). Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. Science **257,** 85-88.

Welch, W.J., and Feramisco, J.R. (1982). Purification of the major mammalian heat-shock proteins. J. Biol. Chem. **257,** 14949-14953.

Wetsel, W,C., Khan, W.A., Merchenthaler, I., River, H., Halpren, A.E., Phung, H.M, Negro-vilar, A., and Hannun Y.A. (1992). Tissue and cellular distribution of the extended family of protein kinase C isoenzymes. J. Cell. Biol. **117,** 121-133.

Whitham, S., Dinesh-Kumar, S.P., Choi, D., Hehl, R., Corr, C., and Baker, B. (1994). The product of the tobacco mosaic virus resistance gene N: similarity to toll and the interleukin-1 receptor [published erratum appears in Cell 1995 May 5;81(3):466]. Cell **78,** 1101-1115.

Wolniak, S.M., and Larsen, P.M. (1995). The timing of protein kinase activation events in the cascade that regulates mitotic progression in *Tradescantia* stamen hair cells. Plant Cell **7**, 431-445.

Worrall, D., Hird, D.L., Hodge, R., Paul,W., Draper, J., and Scott, R. (1992). Premature dissolution of the microsporocyte callose wall causes male sterility in

transgenic tobacco. Plant Cell **4,** 759-771.

Xing, T., Higgins, V.J. and Blumwald, E. (1996). Regulation of plant defense response to fungal pathogens: Two types of protein kinases in the reversible phosphorylation of the host plasma membrane H^+-ATPase. Plant Cell **8,** 555-564.

Yamamoto, Y.T., Conkling, M.A., Sussex, I.M., and Irish, V.F. (1995). An *Arabidopsis* cDNA related to animal phosphoinositide-specific phospholipase genes. Plant Physiol. **107**, 1029-1030.

Yao, K., De Luca, V., and Brisson, N. (1995). Creation of a metabolic sink for tryptophan alters the phenylpropanoid pathway and the susceptibility of potato to *Phytophthora infestans*. Plant Cell **7,** 1787-1799.

Young, S.A., Wang, X., and Leach, J.E. (1996). Changes in the plasma membrane distribution of rice phospholipase D during resistant interactions with *Xanthomonas oryzae* pv *oryzae*. Plant Cell **8**, 1079-1090.

Zeiner, M., Gebauer, M., and Gehring, U. (1997). Mammalian protein RAP46: an interaction partner and modulator of 70 kDa heat shock proteins. EMBO. J. **16,** 5483-5490.

Zhang, H., Buckley, N.E., Gibson, K., and Spiegel, S. (1990). Sphingosine stimulates cellular proliferation via a protein kinase C-independent pathway. J. Biol. Chem. **265,** 76-81.

Zhang, S., and Klessig, D.F. (1997). Salicylic acid activates a 48-kD MAP kinase in tobacco. Plant Cell **9,** 809-824.

Zhou, J., Loh, Y.T., Bressan, R.A., and Martin, G.B. (1995). The tomato gene *Pti1* encodes a serine/threonine kinase that is phosphorylated by Pto and is involved in the hypersensitive response. Cell **83,** 925-935.

Zhou, J., Tang, X., and Martin, G.B. (1997). The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a *cis*-element of pathogenesis-related genes. EMBO J. **16,** 3207-3218.

Zhu, B.L., Chen, T.H.H., and Li, P.H. (1995). Expression of three osmotin-like protein genes in response to osmotic stress and fungal infection in potato. Plant Mol. Biol. **28,** 17-26.

Zimmermann, S., Nürnberger, T., Frachisse, J.M., Wirtz, W., Guern, J., Hedrich, R., and Scheel, D. (1997). Receptor-mediated activation of a plant Ca^{2+}-permeable ion channel involved in pathogen defense. Proc. Natl. Acad. Sci. **94,** 2751-2755.

Acknowledgements

This thesis, personally, started as a journey into unchartered territory. Soon, it became the territory with many signs and posts. Navigation required diligence. My supervisor, Normand Brisson proved to be the right helmsman for this journey and for that Iam very grateful. Much of this journey was not without intemperance and co-conspirators. In this regard, Iam indebted to my fellow graduate students within the lab and from other labs. In particular, I like to thank Charles Deprés, with whom I had many discussions and fruitful collaboration. I also like to thank Alex Joyeux for his translation skills.