SAVITA D. VISAL

INHIBITION OF COLORADO POTATO BEETLE (*LEPTINOTARSA DECEMLINEATA* SAY) DIGESTIVE PROTEINASES: THE SEARCH FOR A BROAD-SPECTRUM INHIBITOR

(INHIBITION DES PROTÉASES DIGESTIVES DU DORYPHORE DE LA POMME DE TERRE (*LEPTINOTARSA DECEMLINEATA* SAY): À LA RECHERCHE D'UN INHIBITEUR À SPECTRE LARGE)

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Résumé *(version courte)*

Le but de ce projet consistait à isoler un inhibiteur de protéases (IP) montrant de l'affinité pour l'essentiel des protéases digestives du doryphore de la pomme de terre (*Leptinotarsa decemlineata* Say), un insecte ravageur d'importance considérable à l'échelle mondiale. Après une étude biochimique/électrophorétique du système protéasique digestif de l'insecte, le potentiel de deux nouveaux IPs végétaux - l'un produit de façon constitutive, l'autre en réaction à un stress - a été comparé à celui de l'inhibiteur modèle OCI, un IP exprimé de façon constitutive chez le riz. En accord avec des études antérieures suggérant l'existence chez le doryphore de protéases sensibles et insensibles à OCI, l'insecte utilise deux formes protéasiques majeures - une forme de 28 kDa sensible à l'inhibiteur et une forme insensible de 67 kDa, dont l'importance relative varie selon la diète ingérée. En outre, alors que l'effet d'un IP constitutif de la papaye, la prorégion de la protéase IV, est similaire à celui d'OCI, un inhibiteur induit chez la tomate par un traitement à l'acide γ-linolénique, *TCPI*, s'est montré beaucoup plus efficace, inhibant non seulement la fraction protéasique OCI-sensible mais aussi la fraction insensible. En bref, les résultats obtenus dans le cadre de ce projet suggèrent le potentiel des prorégions protéasiques végétales pour la régulation de protéases impliquées dans certains processus biologiques, et l'intérêt d'inhibiteurs de stress' comme *TCPI* pour le développement de plantes transgéniques résistantes aux herbivores nuisibles.

Savíta Visal, étudiante

Dominique Michaud, directeur de thèse

Résumé *(version longue)*

Les progrès récents dans le domaine des biotechnologies végétales ont permis le développement d'une variété de plantes transgéniques résistantes aux insectes nuisibles. Le développement de plantes transgéniques exprimant des inhibiteurs de protéases (IPs) recombinants, par exemple, constitue une avenue intéressante pour le contrôle des insectes herbivores. En bloquant l'activité des protéases digestives retrouvées dans le tube digestif des insectes, les IPs causeraient un ralentissement de la digestion des protéines alimentaires et une surproduction nocive de protéases digestives, induisant des retards de croissance importants et certaines anomalies de développement. Intégrés à une stratégie de lutte intégrée, les IPs pourraient s'avérer utiles en complément à des approches de contrôle en émergence comme la lutte biologique, la lutte mécanique et la prévention.

En dépit du nombre grandissant d'études faisant état de l'implication des IPs dans la défense des plantes, l'utilité réelle de ces protéines pour le contrôle des insectes reste à démontrer dans bien des cas. L'effet inhibiteur des IPs sur les protéases d'un organisme cible est en général relativement limité, laissant des protéases libres et actives dans le milieu. Ces protéases libres (ou insensibles), qui peuvent permettre une compensation physiologique des fonctions enzymatiques inhibées, peuvent aussi conduire à la dégradation de certains IPs et à une diminution graduelle de leur efficacité. Une compréhension adéquate des interactions survenant entre IPs et protéases digestives des insectes herbivores est donc un pré-requis essentiel à l'optimisation d'une approche de contrôle basée sur l'inactivation des protéases.

D'un point de vue pratique, l'identification d'IPs montrant de l'affinité pour une gamme élargie de protéases apparaît d'intérêt majeur. L'effet de l'oryzacystatine I (OCI) contre le doryphore de la pomme de terre (*Leptinotarsa decemlineata* Say), par exemple, est limité par son spectre d'action relativement étroit contre les protéases de l'insecte. Les protéases insensibles du doryphore, présentes en quantité importante dans son système digestif, lui permettent de

contourner aisément l'effet d'une lignée transgénique de pomme de terre accumulant OCI dans son feuillage et de croître normalement en dépit de l'inhibition effective d'une fraction non négligeable de ses protéases. En réduisant l'activité protéasique digestive à un niveau négligeable, un inhibiteur à spectre d'action plus large pourrait à l'inverse causer une diminution des risques de compensation chez l'insecte et accroître la stabilité relative des diverses protéines de défense parvenues à son tube digestif.

Dans cette optique, le thème central du présent projet de recherche consistait à identifier et à caractériser un IP montrant de l'affinité pour l'essentiel des protéases digestives du doryphore. La poursuite de cet objectif s'est appuyée sur l'hypothèse selon laquelle une plante soumise à un stress a la capacité de produire des IPs à large spectre contre les protéases digestives des organismes phytophages. Après une étude biochimique du système protéasique digestif de l'insecte cible, le potentiel de deux nouveaux IPs végétaux - l'un produit de façon constitutive, l'autre en réaction à un stress - a été comparé à celui de l'inhibiteur modèle OCI, produit de façon constitutive chez le riz. Plus spécifiquement, quatre objectifs principaux ont été poursuivis:

I - confirmer, par l'emploi d'une nouvelle technique électrophorétique et de procédures chromatographiques appropriées, l'existence présumée chez le doryphore de deux formes protéasiques digestives majeures, respectivement sensible et insensible à OCI;

II - déterminer, par des tests de nutrition comparative, dans quelle mesure le spectre d'action d'un IP contre les protéases digestives du doryphore est tributaire de son statut physiologique;

III - déterminer, par des tests protéasiques standards, l'efficacité du propeptide de la protéase IV de la papaye, prorégion régulatrice (inhibitrice) à 'effet IP', à inhiber les protéases digestives du doryphore;

IV - déterminer, après l'avoir isolé de feuilles de tomate traitées à l'acide γ-linolénique, l'efficacité d'un IP 'de stress' à inhiber l'activité des protéases digestives du doryphore.

Les résultats obtenus dans le cadre du projet ont permis d'établir:

I - qu'en accord avec des études antérieures suggérant l'existence chez le doryphore de protéases sensibles et insensibles à OCI, l'insecte à l'étude utilise deux formes protéasiques majeures, l'une d'environ 28 kDa sensible à l'inhibiteur, l'autre de 67 kDa, insensible;

II - que la diète ingérée par le doryphore détermine la nature des formes protéasiques libres (actives) retrouvées dans son tube digestif et le ratio de protéases sensibles/insensibles à OCI. Cette observation suggère que le spectre d'activité d'un IP est fonction non seulement de ses caractéristiques stériques et des réponses compensatoires chez l'insecte cible, mais aussi du statut physiologique de ce dernier;

III - que le propeptide de la PPIV, d'origine végétale, reconnaît une fraction des protéases digestives d'un insecte, en l'occurrence de doryphore de la pomme de la terre. Tel que démontré par des études de complémentation, la fraction reconnue par le propeptide correspond à celle aussi reconnue par OCI. Ces observations, qui suggèrent le 'potentiel IP' des prorégions protéasiques en biocontrôle, suggéraient toutefois le potentiel limité du propeptide de la PPIV pour le contrôle du doryphore;

IV - qu'un IP de 55 kDa, *TCPI (Tomato Cysteine Protease Inhibitor)*, est produit dans des feuilles de tomate soumises à un stress par le biais d'un traitement à l'acide γ-linolénique. Produit par un sentier métabolique restant à élucider, ce nouvel inhibiteur montre de l'affinité pour l'essentiel des protéases du doryphore. Une caractéristique intéressante de *TCPI* est sa capacité de conserver son large spectre d'action contre les protéases induites chez l'insecte en réponse à des conditions diverses, y compris celles induites en présence d'OCI recombinant retrouvé dans du feuillage de pomme de terre transgénique. Ce pouvoir inhibiteur *tampon*, par lequel *TCPI* conserve son large spectre d'activité en dépit de phénomènes compensatoires chez l'insecte, fait de cet inhibiteur un candidat particulièrement prometteur en protection des plantes.

En bref, les travaux effectués dans le cadre du présent projet ont permis (i) d'élucider certains aspects du métabolisme protéasique digestif chez le doryphore de la pomme de terre, (ii) de

démontrer le potentiel des prorégions protéasiques végétales en biotechnologie et (iii) d'isoler un inhibiteur à spectre large montrant du potentiel en phytoprotection. L'efficacité différentielle d'OCI, du propeptide PPIV et de *TCPI* contre les protéases du doryphore est en accord avec l'hypothèse de base du projet, selon laquelle des IPs produits dans les plantes en réponse à un stress montrent un spectre d'action large contre les protéases digestives des organismes phytophages. Des travaux demeurent nécessaires pour démontrer l'efficacité réelle de l'inhibiteur *TCPI* en protection des plantes et pour élucider le sentier métabolique menant à la synthèse de ce nouvel IP chez la tomate.

Sayita Visal, étudiante

Dominique Michaud, directeur de thèse

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ABBREVIATIONS

12-oxo-PDA	12-oxophytodienoic acid
AA	arachidonic acid
ABA	abscisic acid
ALA	α-linolenic acid
AOS	allene oxide synthase
ATP	adenosine triphosphate
BSA	bovine serum albumin
Bt	*Bacillus thuringiensis*
CPB	Colorado potato beetle
CPI	cysteine proteinase (protease) inhibitor
E-64	*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane
g	gravitational acceleration
GLA	γ-linolenic acid
GST	glutathione-*S*-transferase
HOTrE	hydroxy trienoic acid
HPOTrE	hydroperoxy-trienoic acid
HSA	human stefin A
JA	jasmonic acid
kDa	kilo Dalton
LOX	lipoxygenase
LT	leucotriene
M	molar
maspin	mammalian serine proteinase inhibitor
MeJA	methyl jasmonic acid/methyl jasmonate
OCI	oryzacystatin I
OCII	oryzacystatin II
PAGE	polyacrylamide gel electrophoresis
PC	prostacyclin
PG	prostaglandin
PI	proteinase (protease) inhibitor
PI2/pin2	proteinase inhibitor 2
PK	protein kinase

PMC	potato multicystatin
PMSF	phenylmethylsulfonyl fluoride
PPIV	papaya proteinase IV
PUFAs	polyunsaturated fatty acids
rpm	revolutions per minute
SAR	systemic acquired resistance
SDS	sodium dodecyl sulfate
SE	standard error
STI	soybean trypsin inhibitor
TCPI	tomato cysteine proteinase (protease) inhibitor
TMC	tomato multicystatin
Tris	Tris (hydroxymethyl) aminoethane
TX	thromboxane
v/v	volume/volume
w/v	weight/volume
°C	degree Celcius

BRIEF INTRODUCTION

The results presented in this thesis demonstrate for the first time the induction of a novel cysteine proteinase inhibitor (PI), TCPI, in young tomato leaves treated with an unusual fatty acid, γ-linolenic acid (GLA). Previous studies suggested the use of oryzacystatins (OCs: OCI and OCII) for the production of transgenic potato lines (*Solanum tuberosum* L.) resistant to the phytophagous pest, Colorado potato beetle (CPB, *Leptinotarsa decemlineata* Say) (Michaud et al 1995). Given problems of narrow inhibition spectrum of OCs against CPB proteinases, instability of OCII and development of physiological resistance (or compensation) in insects fed on transgenic plants, the present research was aimed at identifying and isolating a cysteine PI either complementary to or more efficient than OCs in inhibiting CPB digestive proteinases.

- The first chapter introduces the problems encountered with the use of pesticides (e.g. the development of resistance in insects) and presents alternate control strategies, in particular the use of pro-regions of protease and plant proteinase inhibitors (PIs), as potentially useful bio-molecules in insect control. Based on the literature related to PIs in insect control, a working hypothesis underlying our research interests is then presented, along with the objectives to attain for achieving our research goal and validating our hypothesis.

- The second chapter, presented in the form of a scientific article, describes an improved *in gel* proteinase detection technique helpful in estimating the molecular weight of cathepsin B- and H-like CPB proteinases, and in discriminating the insect OCI-sensitive and -insensitive digestive proteinases.

- The third chapter briefly introduces the importance of understanding the possible effect of various diets on CPB midgut proteinases. Taking into account these dietary conditions, chapters three and four (presented in form of research articles), assess the potential of a constitutive PI, the pro-region of papaya proteinase IV (PPIV), and of a stress (GLA)-induced PI, in CPB control.

- The fifth chapter summarizes our research observations and suggests cautionary steps to consider when planning to use a particular PI against specific insect midgut proteinases. The thesis concludes with some comments on the putative existence of a novel, yet to be elucidated, hypothetical GLA-induced defense pathway in plants.

- Finally, Annexes I and II give some information on additional (review) papers to be published as a result of our studies, and on meeting communications presented during the course of the project.

CHAPTER I

PLANT-INSECT INTERACTIONS AND INSECT RESISTANCE: LITERATURE REVIEW

1.1. GENERAL INTRODUCTION: THE CASE OF COLORADO POTATO BEETLE

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) is the most destructive phytophagous insect pest of potato in eastern US and Canada (Ferro 1985, Hare 1990). If uncontrolled, the population growth rate could easily increase up to 40-fold per generation, with an over-wintering survival rate exceeding 60% (Harcourt 1971). This growth of the insect population results in complete defoliation of the crop, and decrease in potato yields by up to 85% (Roush and Tingey 1994). In order to prevent population from getting out of control, insecticides are heavily employed (Roush and Tingey 1992). The heavy use of insecticides poses a selection pressure on CPB which results in the selection of insecticide resistant strains and adversely affects their natural predators (Roush and Tingey, 1994), thus resulting in an ecological imbalance of the natural fauna.

The major obstacle in controlling CPB is its rapid adaptation and evolution against old as well as newly formulated pesticides (Table 1.1, Georgiou 1986). These toxic chemicals are responsible for eliciting biological responses in the insect population, which can lead either to extinction or to the evolution of adaptive responses (Grafius 1995). The latter gives rise to a resistant population, which ultimately leads to both increased frequency of pesticide applications and increased dosage in the field (Roush et al 1990, Huang et al 994). Insect resistance is a facilitated co-evolution process, where the host plant acquires one or a series of new chemical defenses, either a) endogenous, biosynthesized by the plant itself (e.g. by activation of a defense cascade and by production of secondary metabolites like alkaloids or deterrents) or b) exogenous, by spraying of chemicals (Kessmann et al 1994). Correspondingly, the insect evolves counter-defenses to these chemicals, i) by modifying its feeding behavior where the exposure to the lethal dose of toxic compound is avoided (Sparks et al 1989); ii) by finding an

alternate way to survive normal lethal doses (e.g. by reducing the cuticular penetration or by modifying the receptors by altering the target sites for the toxic compound), and/or iii) by increasing the metabolic activation or detoxification by gene amplification (Devonshire and Field 1991).

The continuous presence of insecticides thus acts as a selection pressure on insect populations, leading to evolution and selection of resistant strains (Metcalf 1989). Although proved to be effective as a mean to control CPB, the use of multiple insecticides at the same time or during the season (Tabashnik 1989) has led with time to public concern over pesticide residues in foods and in the environment, now making their use less favorable. Resistance to whole classes of insecticides in target insects has also increased problems in controlling agriculturally important pests, which has led to large investments for the development of new, safe, and cost effective insecticides. The cost associated with management practices and chemical control of insects approaches nearly $10 billion per year (Oerke EC, 1994), which includes 75 million dollars for the CPB alone.

Table 1.1. Evolution of resistance to insecticides in CPB

Insecticide	Yr. Introduced	Yr. of Resistance	No. of Yr. of Control
DDT	1945	1952	7
Dieldrin	1954	1957	3
Ensulfan*	1991	1991	<1
Endrin	1957	1960	3
Esfenvalerate*	1991	1991	<1
Azinphos methyl	1959	1964	5
Monocrotophos	1973	1973	<1
Phorate	1973	1974	1
Carbaryl	1959	1963	4
Carbofuran	1974	1976	2
Oxamyl	1978	1978	>1
Fenvalerate	1979	1981	2
Permethrin	1979	1981	2

Data from Forgash (1981) and Metcalf (1989); (*) data from Huang et al (1994)

1.2. ALTERNATE STRATEGIES FOR INSECT CONTROL

As an alternative to the present methods of insect control involving externally applied, unspecific, hazardous or potentially hazardous organo-chemicals or pesticides, exploiting biological sources is the most favorable strategy. This is accomplished:

1) by deploying predatory or parasitic insects, nematodes, fungi or micro-organisms against the target insects. However, there are certain limitations that must be overcome when biological organisms are to be used as biocontrol agents (Ferro 1994), including: (a) the inability to control target pests at high density population (Hill, 1987), (b) foraging activity of the biocontrol organism that must be in synchrony with behavior and development of the target pest, (c) limited availability of biocontrol agents due to the lack of exploration and limited knowledge of their biology and (d) the fact that inundative releases of natural enemies is questionable due to the heavy costs associated with rearing. The success of biological control is further dependent on the quality of the natural enemies, and in the long run has been predicted to lead to biological control failures and to a negative image of natural enemy producers (van Lenteren et al 1997). Nevertheless, various organisms have been successfully deployed for the biocontrol of CPB and a better understanding of the biology and ecology of CPB and its natural enemies, along with the improvement of cultural practices and management, may contribute to an effective control of CPB (Ferro 1994).

2) by using bio-molecules. With the advent of molecular techniques, genetic engineering of insect resistance into crops is a promising way to reduce or control insect damage. This can be achieved by expressing (a) **biocidal molecules,** which eliminate the pest itself, like the δ-endotoxins of *Bacillus thuringiensis* (Goldberg and Marlait 1977, Koziel et al 1993), vegetative insecticidal proteins (Estruch et al 1996), lectins (Czapla and Lang, 1990, Murdock et al 1990, Chrispeels and Raikhel 1991, Harper et al 1995, Hilder et al 1995), anti-insect toxins (Rochat et al 1979, Chejanovsky et al 1995, Nakagawa et al 1997) and recombinant baculoviruses

expressing insect-specific neurotoxins [referred to as viral insecticides] (Maeda, 1995), or (b) **anti-nutritive/anti-metabolic proteins,** which interfere with the target insect metabolism or development and lead to the control of insects at the population level. Examples of antinutritive/antimetabolic proteins are PIs (Gatehouse and Boulter 1983, Gatehouse et al 1991, Ryan, 1990), proteinase pro-regions (Taylor and Lee 1997), ribosome-inactivating proteins (Gatehouse et al 1990, 1992), α-amylase inhibitors (Ishimoto and Kitamura 1989, Huesing et al 1991, Pueyo et al 1995, Piergiovanni et al 1991), polyphenol oxidases (Felton et al 1993), and cholesterol oxidases (Purcell et al 1993, Cho et al 1995, Greenplate et al 1995).

1.2.1. Limitations of insecticidal molecules

The most well-known and commonly used insecticidal bio-molecules are the δ-endotoxins from the soil bacterium *Bacillus thuringiensis*. In the bacterial cell, δ-endotoxins are deposited in intracellular spores, in the form of a paracrystalline body. The use of δ-endotoxins as a biopesticide dates back to 1960, when it was commercialized and marked as 'Thuricide'. Amongst the strains of *B. thuringiensis* isolated, the toxin from *B. thuringiensis* var. *tenebrionis,* was found to be toxic to the CPB (Krieg et al 1983, Gasser and Fraley 1989, Boulter et al 1990, Brunke 1991). The δ–endotoxin sprayed on the plants in the form of a pro-endotoxin, when ingested, is broken down to an active toxin by the insect gut proteinases. The endotoxins act either by paralyzing the midgut via disruption of the midgut epithelial cells (Wolfersberger and Spaeth 1987, Schwartz 1991), by blocking the amino acid uptake (Giordana et al 1993, Reuveni and Dunn 1991) or by binding to specific receptors of the brush-border membrane vesicles (Martinez-Ramirez and Real, 1996), thereby blocking the ATP-dependent proton pump and the flow of amino acids through the epithelial membrane (Leibeg, 1995). In short, the δ-endotoxin is recognized by insect midgut epithelial cell receptors like many other chemical pesticides. This exerts a very high selection pressure on the target insect

population and may lead to the selection of resistant strains, which has led to a major concern of insects developing resistance against these endotoxins (a) ***by mutations***, where they become insensitive to the endotoxins by modifying the membrane proteins or receptors (Van Rie et al 1989, Bravo et al 1992, Tabashnik 1994); (b) ***by protease-mediated resistance*** (Oppert et al 1994, Forcada et al 1996, Michaud 1997), where Bt-resistant strains show an altered profile of midgut proteinases which alter the integrity of the δ-endotoxin, and/or (c) ***by precipitation of the pro-toxin*** by toxin-precipitating proteins in the insect midgut (Milne et al 1995). The toxin has been suggested to be inefficient in controlling insect pests in the long term (Brattsen 1991), and several insects including CPB have been shown to actually develop resistance to this biopesticide (McGaughey and Whalon 1992, Whalon et al 1993). Similarly, CPB larvae fed on transgenic potato plants expressing the CryIIIA gene were recently shown to develop resistance to the toxin in a stage-specific manner (Wierenga et al 1996).

As an alternative to the use of biocidal proteins, the use of anti-nutritive or anti-digestive molecules has been suggested (Duffey and Stout 1996). By simply altering growth and development of the target insect, these proteins would cause low selection pressure on the target populations, thereby avoiding or at least delaying the appearance of resistant strains. Amongst the various anti-nutritive molecules known until now, most emphasis is put on proteinaceous PIs. These proteins exist naturally in plants as regulators of both endogenous proteinases and 'foreign' proteases of pests and pathogens; they can also be exploited to engineer plants for resistance to specific target pest or pathogens.

1.3. PI-BASED CONTROL OF INSECTS

1.3.1. Introduction

PIs block the activity of proteolytic enzymes, usually in a competitive manner (Barrett 1986). Since long, PIs are known to be anti-nutritive molecules for a variety of organisms, including humans. Several previous studies in animals in the past have revealed their effects on growth

and development, essentially (i) by inhibiting digestive proteinases, which results in poor digestion of dietary proteins and in reduction in the uptake of free essential amino acids (Alumot and Nitsan 1961, Pearce et al 1983); and (ii) by causing the hyperproduction of proteinases in response to the inhibition of digestive proteinases, which leads to loss of appetite and growth reduction (Broadway and Duffey 1986, Smith and Gibbs 1987, Hill et al 1990).

Insects, like humans and other living organisms, use one or a combination of serine, cysteine and (or) aspartic proteinases as major digestive proteinases (Wolfson and Murdock 1990). Digestive proteinases hydrolyze dietary proteins, thereby releasing essential amino acids, which are assimilated and further utilized for growth and development. In insects, digestive proteinases are abundant in the midgut, being secreted extracellularly in the endoperitrophic space (Terra 1990). In plants, proteolysis also plays a major role in maintaining the intracellular levels of proteins, reallocating organic nitrogen, reducing the toxic effects of abnormal proteins and regulating the levels of rate-limiting enzymes. Four types of proteinases (or endo-proteolytic enzymes) have been identified, namely the serine, cysteine, aspartic and metallo-proteinases, their name being based on the amino acid (or metallic ion) active in the reaction center.

PIs, the inhibitors of proteolytic enzymes, are categorized according to the class of proteinase they inhibit (Table 1.2). They are found in abundance in most living cells and usually are highly stable and specific for a particular class of proteinases (Ryan and Walker-Simons 1981, Ryan 1990). In plants, PIs vary from ~4 to ~90 k-Da in size (Richardson et al 1979, Hojima et al 1980, Abe et al 1987a, Walsh and Strickland 1993). Under normal conditions, they occur constitutively in storage organs like seeds (Abe et al 1987a, Brzin et al 1990, Hines at al 1991, Terada et al 1994), tubers (Hoff et al 1972, Rodis and Hoff 1984), reproductive organs (Ausloos et al 1994, Atkinson et al 1993, Lim et al 1996), cereal embryos and endosperms (Boisen and Djurtaft 1982), and vegetative cells (Akers and Hoff 1980), where they are involved in the regulation of endogenous proteinases. When induced as defense molecules

following stress, they are thought to inhibit foreign proteinases of pests and pathogens, exerting an anti-nutritional effect and resulting in pest or pathogen control. Interestingly, PIs show little activity (if any) against most endogenous plant proteinases (Michaud et al 1994, and previous laboratory observations, unpublished), making them good candidates for plant genetic engineering.

Table 1.2. Families of plant PIs

CLASS/FAMILIES	PROTEINASES INHIBITED
Serine PIs	
Soybean trypsin inhibitor (Kunitz family)	Trypsin, Elastase and
Bowman-Birk Family	Chymotrypsin
Barley trypsin inhibitor family	
Potato inhibitor I family	
Potato inhibitor II family	
Squash inhibitor family	
Serpin family	
Cysteine PIs	
Cystatin super family	Papain, Cathepsins B, H, L
Potato cysteine proteinase inhibitor	
Aspartic PIs	Cathepsin D, Pepsin
Metallo-PIs	Carboxypeptidases A and B
Bifunctional PIs	
Ragi I-2/maize trypsin inhibitor family	Trypsin (and α-amylase)

1.3.2. Mode of action of PIs

In most cases, PIs interact with their target proteinases by combining their reactive (inhibitory) site with the enzyme (proteinase) in a substrate-like manner, thereby serving as a pseudo-substrate (Laskowski and Kato 1980). The exact mode of action of PIs *in vivo* is not yet understood. By definition, an anti-digestive molecule detrimentally limits the rate of enzymatic conversion of ingested food, while an anti-nutritive molecule detrimentally limits utilization of food by altering its physical availability and (or) its chemical identity (Duffey and Stout, 1996). For example, for *Spodoptera exigua* and *Heliothis zea* fed potato leaves-expressing PI-2 and soybean trypsin inhibitor (STI) respectively, a massive hyperproduction of PI-2- and STI-

sensitive proteinases is observed (Broadway and Duffey 1986). Essential free amino acids are thus diverted towards the production of digestive proteinases in a compensatory response, thereby making them unavailable for the production of other proteins and resulting in growth retardation. In parallel, the hyperproduction of digestive proteinases has been proposed to be responsible for the depletion of sulfur-containing amino acids (Broadway and Duffey 1986). Thus, PIs would act as **anti-nutritive** molecules by altering the physical availability of food, while PI-proteinase interactions would limit the assimilation of by-products, posing an **anti-digestive effect** and resulting in retardation of insect growth, development and alteration of reproductive functions (Broadway and Duffey 1986, Hilder et al 1987, Johnson et al 1989, Atkinson et al 1995, Urwin et al 1995, 1997).

1.4. THE RESEARCH PROBLEM

Unlike toxins, PIs do not require 'receptor' molecules to act on *in vivo,* but rather directly act on digestive proteinases by forming an enzyme/inhibitor (E/I) complex. This functional characteristic of PIs, in contrast with the antibiotic effect of Bt δ-endotoxins, makes them interesting molecules to genetically engineer plants resistant to insects (Broadway and Duffey 1986, Hilder et al 1987, Johnson et al 1989, Ryan CA 1990, Hilder et al 1993, Atkinson et al 1995, Urwin et al 1995, 1997, Gatehouse et al 1993). As noted earlier, CPB is the most destructive phytophagous insect pest of potato in eastern US and Canada (Ferro 1985, Hare 1990), and its effective biocontrol by other means than the use of Bt toxins has not been achieved. Transgenic potato plants expressing the cryIIIA gene have shown to induce resistance in CPB larvae in a stage-specific manner (Wierenga et al 1996), which further stresses the necessity to explore alternate bio-molecules to achieve effective biocontrol of CPB over a long period. PIs constitutively expressed in plants would act directly on the insect digestive proteinases, which may limit the hydrolyses of dietary proteins, and thereby controlling the availability of essential amino acids required for the normal growth and development of the pest. Recently, a fungal non-protein inhibitor of cysteine proteinases, E-64, when administered at

'physiological concentrations' has proven effective in controlling CPB at the population level without causing direct mortality (Wolfson and Murdock 1995, Bolter and Latoszek-Green 1997).

CPB commonly uses cysteine proteinases for dietary protein digestion (House 1965). *In vitro,* the insect digestive proteolytic activity is susceptible to inhibition by cysteine PIs (CPI), being significantly decreased (~80%) in the presence of E-64 (Wolfson and Murdock 1987). Based on *in vitro* studies with various class-specific substrates and inhibitors, CPB digestive cysteine proteinases were further classified into two major sub-classes, namely cathepsin B- and cathepsin H-like proteinases (Thie and Houseman 1990; refer to Fig.1.1.). *In vitro* studies with oryzacystatins I and II (OCI and OCII) (Abe et al 1987) and the human cysteine PI, stefin A (HSA), showed that CPIs of the cystatin superfamily could inhibit approximately 40% of the CPB cysteine digestive proteinase activity at pH 6.0, while 80% inhibition was observed with E-64 (Michaud et al 1993). Based on *in vitro* tests with leupeptin, OCI and OCII were then suggested to recognize the insect cathepsin H-like but not the cathepsin B-like proteinase fraction (Michaud et al 1993), making these two cystatins poor inhibitors of the insect proteinases. Accordingly, results with CPB larvae fed on transgenic potato plants expressing active OCI showed the absence of adverse effects of OCI on the insect (Cloutier et al 1998). Similarly, *Spodoptera exigua* larvae, when fed on Pin2-expressing transgenic tobacco plants, were not affected (Jongsma et al 1995). Instead, *in vitro* studies with their digestive proteinases demonstrated the expression of a new Pin2-insensitive proteinase (Jongsma et al 1995). These observations while showing the limited effect of 'narrow-spectrum' PIs expressed in transgenic plants were also demonstrating the ability of herbivorous insects to overcome the effects of dietary PIs, even those expressed as recombinant proteins in transgenic plants.

Different mechanisms by which the insect may become resistant to PIs were suggested. Insects, when fed on transgenic plants over-expressing a single PI physiologically adapt by expressing insensitive proteinases which probably compensate for the loss of PI-sensitive proteinase

activity (Jongsma et al 1995). The degradation of PIs expressed in plants by the insensitive proteinases has also been proposed (Michaud 1997). Therefore, for a given single PI gene product in transgenic plants, problems such as compensation by PI-insensitive proteinase(s) associated or not with degradation of the inhibitor thus become a major concern in efficiently controlling insect pests by digestive proteinase inhibition.

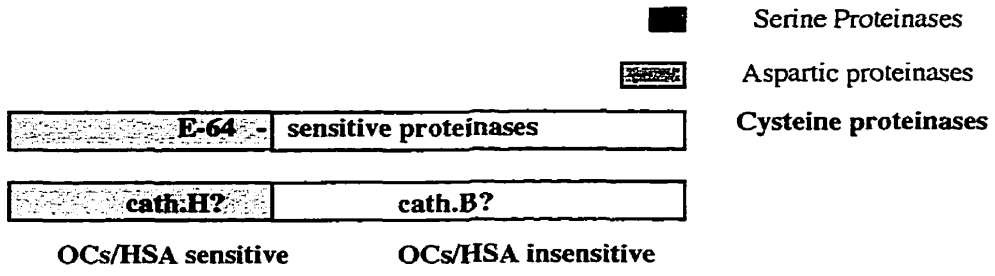

Fig.1.1. Colorado potato beetle digestive proteinases *(adapted from Thie and Houseman 1990).*

In this context, the main goal of the study, which focuses on the biocontrol of CPB, was ***"to identify proteinaceous PI(s) of plant origin, effective in inhibiting CPB digestive proteinases. These PIs would either complement the effect of OCI, OCII and HSA by recognizing the insect cathepsin B-like proteinase fraction, or exhibit a broad inhibitory spectrum against CPB digestive proteinases, as compared to the above mentioned cystatins".*** Two strategies were considered to achieve these goals.

1.4.1. Ist strategy: Assessing the potential of proteinase pro-regions as inhibitors of insect pest proteinases

The regulation of proteinases is an integral component of biochemical processes essential for growth, development and defense of any organism. In cells, most proteinases are synthesized as a pre-pro-enzyme consisting of a typical signal peptide followed by a pro-peptide and a mature enzyme (Baker et al 1993; Fig. 1.2 below). The pro-peptide (or pro-region) plays an

important role in controlling the activity of the enzyme (Chang et al 1994). Recently, elucidation of the crystal structure of human pro-cathepsin B has enabled the understanding of the three dimensional structure of pro-proteinases (i.e. the pro-region complexed to the mature enzyme), showing how the pro-region interacts with its cognate proteinase and blocks access to the active site (Turk et al 1996). Several functions have been proposed for the pro-regions: (i) they aid in proper folding of the mature enzyme both *in vitro* and *in vivo*, (ii) they mediate the secretion of mature proteinases (Shinde et al 1993, Chang et al 1994), (iii) they inhibit the activity of the mature proteinase until they reach their final location in the cell (Sansegunido et al 1982, Fusek et al 1991, Baker et al 1992a, Baker et al 1992b), and (iv) they may help anchoring the proteinase to the membrane and maintaining it in an inactive state until it is released by the cell (Tatsumi et al 1991).

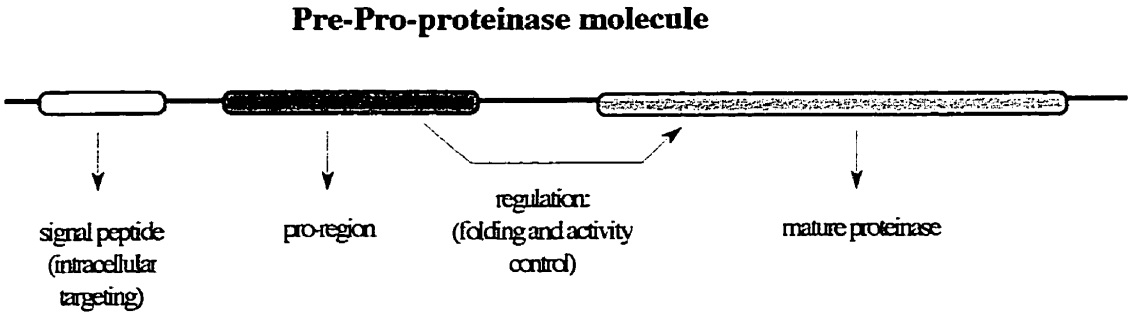

Fig.1.2. Regulation of a mature proteinase by its pro-region I *(adapted from Baker et al 1993).*

Pro-peptides of serine- and metallo-proteinases from *Aspergillus fumigatus* have been shown to bind specifically and inhibit their respective mature enzymes (Markaryan et al 1996). Similarly, the pro-region of the midgut serine proteinase of the insect *Manduca sexta* was shown to inhibit its cognate proteinase (Taylor and Lee 1997). These observations led to the definition of a new approach in pest biocontrol, wherein pest midgut proteinases, in the form of pro-proteinases, could be exploited to isolate pro-regions with PI-activity. These pro-regions could then be further utilized in genetically engineering plants as insect specific digestive proteinase inhibitors (Taylor and Lee 1997). In this study, the pro-region of a cysteine proteinase, papaya proteinase

IV (PPIV) (Taylor et al 1995), was assessed for its potential to inactivate the digestive cysteine proteinases of CPB.

1.4.2. IInd strategy: Assessing the potential of stress-induced PIs in plants

1.4.2.1. The plant defense system

Plants are sedentary organisms exposed to both biotic and abiotic stresses. They have a built-in machinery to combat these stresses, which acts as signals leading to the activation of defense pathway(s), to the simultaneous suppression of some genes, accompanied to either the activation or overexpression of genes required for the synthesis of defense molecules (Bowles 1990). For example, synthesis of phytoalexins, phenolics (Maher et al 1994, Bate et al 1994), defense-related proteins like hydroxyproline-rich glycoproteins or glycine rich-proteins (Cassab and Varner 1988, Brisson et al 1994), hydrolytic enzymes like chitinases and ß-1,3 glucanases and PIs capable of inhibiting insect (or pathogen) proteinases are known to occur in plants exposed to both biotic and abiotic stresses (Dixon et al 1994). The chemical compounds (or the signaling molecules) released following invasion of the tissues by parasites or following predator attack, are transported locally by diffusion through intercellular and extracellular fluids. The physiological responses to these signaling molecules, which occur in plant tissues away from the place of induction (Fig. 1.3), provide a long term (weeks to months) protection against a broad range of unrelated pathogens or herbivores (Hildebrand et al 1987, 1989).

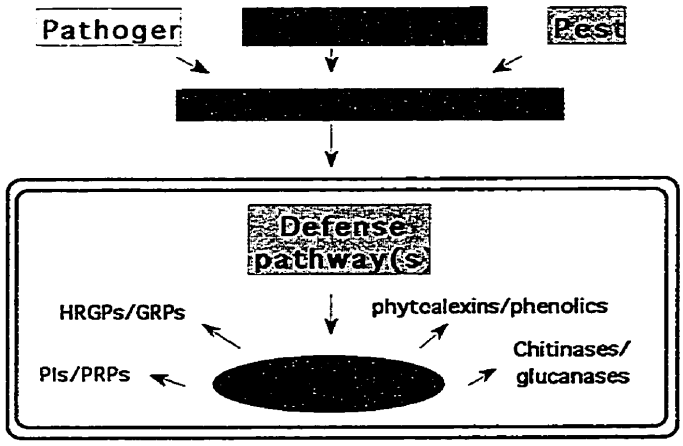

Fig.1.3. Defense-related signaling in plants. A defense signal is generated in response to local and systemic signals. Local signals elicit response at the site of injury, while systemic signals are translocated through the vascular system (adapted from Ryals et al 1996).

In plants, PIs are expressed either constitutively (Botella et al 1996) or in response to various stress signals including ethylene (Boller 1982, Brederode et al 1991, Ecker and Davis 1987), systemin (Pearce et al 1991), oxidative burst (Mehdy 1994, Sutherland 1991), electric potentials (Malone 1993, Wildon et al 1992), oligosaccharides (Ryan 1994, Ryan and Farmer 1991), growth regulators (Péna-Cortés et al 1989), wounding (Green and Ryan 1972, Botella 1996), pathogen or pest invasion (Johnson 1989, Pautot et al 1991, Mueller et al 1993, El-Shamel et al 1996, Zhao et al 1996), and jasmonic acid and its derivatives or precursors (Staswick 1992, Farmer and Ryan 1992, Schweiser et al 1993, Cohen et al 1993, Bolter 1993, Farmer 1994, Wasternack and Parthier, 1997 *and references therein*).

1.4.2.2. Fatty acids as (stress) signals in plant defense

The exact inducers or signaling molecules which co-ordinate the complex changes involved in the induction of resistance responses in plants are not completely understood. The primary stress signals, which may be due to mechanical damage, pathogenesis, herbivory, pest attack or endogenous molecules act primarily on the cell membrane either by changing the membrane electric potential and causing an oxidative burst, by releasing cell wall components, or by activating membrane receptors. In most cases these stress signals have been shown to increase

the levels of endogenous jasmonic acid (JA), a product of octadecanoid signaling pathway that induces the expression of defense genes (Farmer and Ryan 1992, Sembdner and Parthier 1993, Schaller and Ryan 1995, Blechert et al 1995). Airborne JA or methyl jasmonate (MeJA) activates the expression of defense genes, leading to the expression of PIs, similar to the response towards wounding or oligosaccharide fragments from plants or pathogens (Farmer and Ryan 1990, 1992a, 1992b). A model describing the production of defense genes in plants involves lipase as a key enzyme responsible for the activation of both systemic and local signals (Pearce et al 1991, Farmer and Ryan 1992a), and 'JA' as a key molecule of the signal transduction system that regulates inducible defensive genes (Farmer and Ryan, 1990, 1992b; Fig. 1.4). Apart from JA and its precursors, a variety of different compounds and stimuli have been reported to increase the endogenous levels of JA/MeJA (Mueller et al 1993, Creelman et al 1992, Falkenstein et al 1991, Doares et al 1995, Creelman and Mullet 1995, Péna-Cortés et al 1995, Conconi et al 1996, Staswick 1992, Bostock et al 1981, Choi and Bostock 1994, Ricker and Bostock 1994, Choi et al 1994). The exact events along the JA-signaling pathway inducing developmental, structural and defense genes in plants is not yet well understood. Questions about perception of JA signals, their transduction pathways or the mechanisms responsible for promoter activation of the responsive genes remain unanswered.

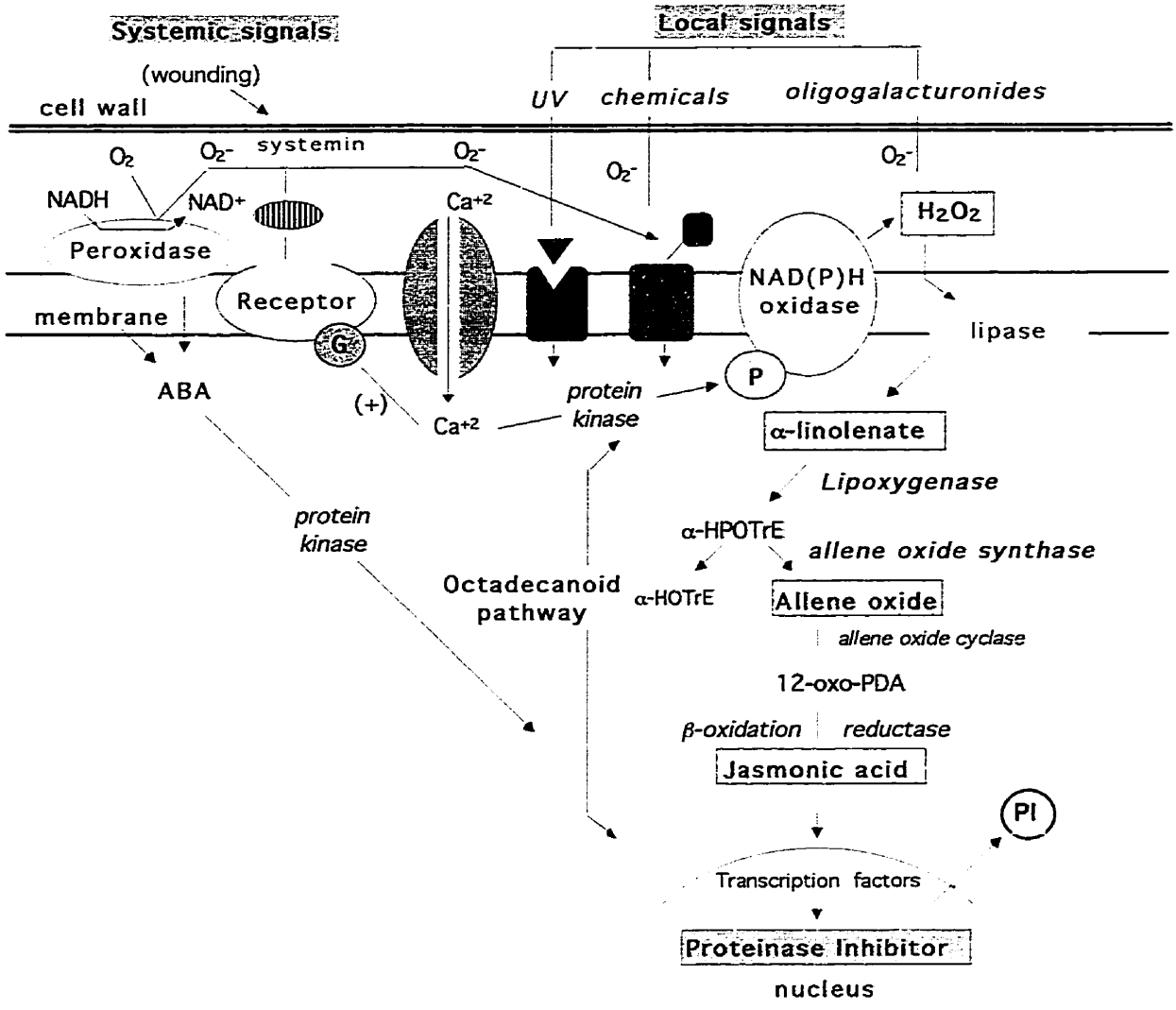

Fig.1.4. The octadecanoid signaling pathway leading to the expression of defense genes (adapted from Farmer and Ryan 1992).

In animals, a parallel system involves prostaglandins, leukotrienes, prostacyclins and thromboxanes (structurally similar to JA), in inflammatory and pain responses. These molecules originate from the arachidonic acid (AA) pathway (Bergey et al 1996). Figure 1.5 compares stress-activated fatty acid signaling pathways in plants and animals.

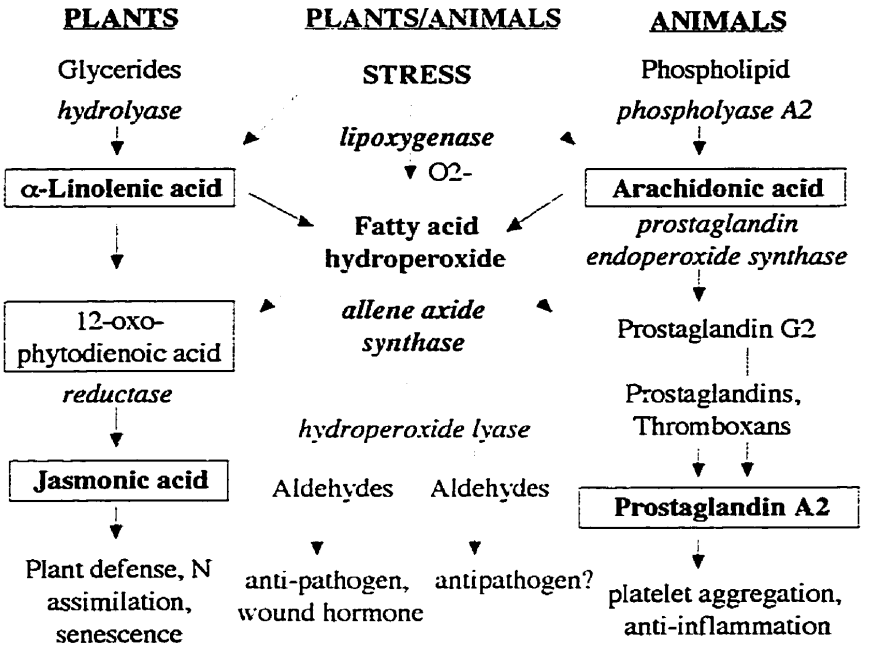

Fig.1.5. Stress-activated pathways common to plants and animals (Gardener 1995). The common steps appear in red.

In plants, free fatty acids or cell membrane phospholipids would act as second messengers in the cell signaling cascade via the activation of protein kinase(s) (PK) (Farmer et al 1989, Asaoka et al 1992, Farmer 1994), leading to the defense/HR response. Lipoxygenase was shown to oxygenate the membrane lipids, thereby producing corresponding hydroperoxy polyunsaturated fatty acids (PUFAs) (Regdel et al 1994), which are enhancers of PK activity. PK, through a series of metabolic intermediates, is responsible for the activation of various genes. The cellular responses to PK activation are rapid cell proliferation, cell differentiation (Asaoka et al 1992) and induction of PIs (Farmer et al 1989). Although in plants the most studied signaling pathway is the JA-pathway (or octadecanoid pathway), alternate pathways also exist. For example:

1) In tomato, MeJA, but not wounding, induces papain inhibitory activity through a 87-kDa protein analogous to potato multicystatin (Bolter 1993), indicative of differential signaling pathways involved in PI induction.

2) A high endogenous concentration of JA in a transgenic potato plant constitutively over-expressing allene oxide synthase does not show a corresponding increase in the expression of

pin2 genes (Harms et al 1995). Instead, these plants respond to stress by further increasing the concentration of JA and activating the corresponding stress-inducible genes (Harms et al 1995). This study therefore reveals that JA is not the only molecule leading to the induction of defense-related genes in plants.

3) In soybean, where cysteine proteinase inhibitors (CPIs) are represented by a multigene family, differential expression of CPIs during development or in response to wounding and methyl jasmonate has been reported (Botella et al 1996). In addition to the constitutively expressed CPI, wounding and methyl jasmonate differentially induce two CPIs with distinct affinities towards papain, a model cysteine proteinase (Botella et al 1996). In another study, two wound-induced soybean CPIs were shown to exhibit substantially greater inhibitory activities than their constitutive homologue against the gut proteinases of third instar larvae of western corn rootworm and CPB (Zhao et al 1996). In both cases, physiological status of the tissue or the whole plant coupled with the type of stress signal may determine the expression of specific defense genes. These reports were also indicating that stress-induced PI(s), unlike their constitutive homologues, would exhibit high affinity towards exogenous (pest) proteinases.

Noteworthy, LOX, a key enzyme in the induction of defense pathways in both plants and animals (Kühn et al. 1990, Kato et al. 1992), dioxygenates PUFAs with varying reaction specificities, thus producing corresponding hydroperoxides (Kühn et al 1990). LOX purified from the tomato fruit pericarp shows decreasing activities with the following PUFAs: α-linolenic acid (ALA) > γ-linolenic acid (GLA) > arachidonic acid (AA) (Regdel et al 1994). Although all the resulting hydroperoxy- intermediates have been shown to induce defense genes (Choi et al 1994, Ricker and Bostock 1994, Blechert et al 1995), and although the biosynthesis of AA in algae via a GLA-intermediate as in animals has been documented (Nichols and Appleby 1969), the occurrence of GLA and AA is rare in plants. Both fatty acids have a restricted distribution in the plant kingdom and are thus classified as 'unusual' fatty acids (van

de Loo et al 1993). In humans, both GLA and AA are catalyzed by cyclooxygenases or LOX to form prostaglandins (PG), thromboxanes (TX) or leukotrienes (LT), which are either anti-inflammatory (e.g. PGE1, TXA1, LTC3 and 15-hydroxy-dihomo GLA) or inflammatory (e.g. PGE2, TXA2, LTC4, LTD4, LTE4, LTB4) molecules.

Recently, GLA was shown to elevate, *in vitro,* the expression of maspin- a mammalian serine proteinase inhibitor, in human cancer cells. In plants, these unusual fatty acids have been suggested to undergo α–oxidation or double dioxygenation, forming reactive hydroperoxy intermediate molecules which might be involved in plant defense reactions (van de Loo et al 1993, Vick 1993). Considering (**i**) that various fatty acids like AA induce the accumulation of defense-related proteins in plants via JA-independent pathways (Fidantsef and Bostock 1998), (**ii**) that GLA is present in plants and metabolized by LOX (Rigdel et al 1994), (**iii**) that GLA does not activate the ALA mediated (or JA-) pathway (Farmer and Ryan 1992) and (**iv**) that stress-induced PIs in plants seem to exhibit higher inhibitory activity against pest digestive proteases than their constitutive homologues (Zhao et al 1996), we postulate the following working hypothesis:

1.5. WORKING HYPOTHESIS AND RESEARCH OBJECTIVES

"PLANTS UNDER STRESS CONDITIONS HAVE EVOLVED THE CAPACITY TO PRODUCE PIS WITH BROAD SPECTRUM ACTIVITY AGAINST TARGET (DIGESTIVE) PROTEINASE OF PHYTOPHAGOUS ORGANISMS. STRESSING PLANTS WITH GLA, AN ANALOG OF ALA (PRECURSOR OF JA), MAY LEAD TO THE ACCUMULATION OF BROAD SPECTRUM PI(S) OTHER THAN THE PIS CURRENTLY KNOWN."

1.5.1. AIM AND OBJECTIVES OF THE RESEARCH PROJECT

Our primary aim of research is to identify a cysteine PI of plant origin, more efficient or complementary to the model inhibitors OCI, OCII and HSA. In brief, we aim at identifying a

broad-spectrum inhibitor recognizing both cathepsin B-like and H-like CPB digestive proteinases, **or** to identify an inhibitor specific to cathepsin B-like CPB proteinase(s), insensitive to the action of both OCs and HSA. To achieve our research goals and to verify our working hypothesis, the following objectives are pursued:

1.5.1.1 OBJECTIVE 1

" To discriminate, *in gel*, the OCI-, OCII- and HSA-sensitive and -insensitive digestive proteinases of CPB by utilizing a modified version of the existing gelatin/PAGE technique".

A number of proteinase activity bands exhibiting a large range of 'molecular weights' are detected when a crude protein extract from CPB midguts is fractionated by the standard gelatin/PAGE approach, in part as a result of gelatin-protease interactions during electrophoresis (Michaud et al 1995, Michaud 1998). In contrast, with class-specific inhibitors, only 2 families of cysteine proteinases, namely cathepsin B-like and H-like proteinases are observed in *in vitro* assays. To avoid this discrepancy between *in vitro* data and in gel activity patterns, and to permit in gel discrimination of OCI-sensitive and -insensitive proteinases in crude complex extracts, a two-step gelatin/PAGE will be developed. The proteins will be first fractionated by standard reducing SDS/PAGE (thereby reducing the protein-protein interactions, both by the presence of SDS and β-mercapto-ethanol, and by the absence of gelatin in the resolving gel). After electro-transfer of these fractionated proteins in a gelatin-containing polyacrylamide gel, two major cysteine protease activity bands should be observed, as is evident with *in vitro* assays. Thus by using this two-step approach, one should be able to visualize two major cysteine proteinases as two distinct activity bands, which would be completely inhibited by E-64, a CPI inhibiting both cathepsin B and H of CPB larvae (Michaud et al 1993). After two-step gelatin/PAGE, if the gels are incubated with class-specific inhibitors during proteolysis (for example with OCI recognizing CPB cathepsin H-like proteinase), proteins 'sensitive' and 'insensitive' to this inhibitor should be discriminated. This would enable to visualize *in gel* the efficiency of candidate PIs for pest control, and confirm the occurrence of OCI-sensitive and -insensitive cysteine proteases in CPB. Since the proteins are first fractionated under reducing SDS/PAGE with no interference by gelatin, the estimation of approximate molecular weights of these proteinases should also be possible.

1.5.1.2 OBJECTIVE 2

" To investigate whether an alteration in diet leads to an alteration in the insect digestive proteinases, and to determine whether or not this possible change leads to a change in the ratio of OCI-sensitive to -insensitive proteinases".

Previous studies in our laboratory and elsewhere have demonstrated that a change in diet brings about 'qualitative' and 'quantitative' changes in the E-64- and OCI-sensitive insect midgut proteinases. Genetic engineering of plants, tissue culture or stresses occasionally cause unexpected changes in the plant characteristics (Anonymous, 1992), which may indirectly represent stress conditions for insects feeding on these transgenic plants. This stress can be reflected as a change in the insect digestive proteinase pool, as was recently reported by our group (Overney et al 1997). Here we want to assess if such changes in the insect midgut proteinases occur in CPB larvae in response to various stress conditions, including starvation, and feeding on non-host plants or on PI-expressing transgenic potato. Such experiments are important since they allow (in part), to foresee 'unexpected' physiological resistance (or compensation) in insects, probably due to change in the basic nature of the plants genetically engineered with PI gene(s).

1.5.1.3 OBJECTIVE 3

"To determine the efficiency and stability of the pro-region of papaya proteinase IV (glycyl endopeptidase {EC 3.4.22.25}) in the inhibition of CPB digestive proteinases from insects reared on various diets".

As mentioned in the introduction (1.4.1), proteinases and PIs are expressed in all living organisms to regulate protein turnover and maintain metabolic harmony in any living cell. Hence, in plants there are PIs which are constitutively expressed and are basically involved in regulating the level of proteolysis by the action of proteinases. By analogy, one of our objective was to assess the potential of constitutive PIs in inhibiting CPB midgut proteinases. Pro-regions of proteinases which control the activity of immature proteinases in cells will be used as a 'constitutive PI' model, along with the different diet-related protease complements (variants) induced in 'objective 2'. Considering that constitutive inhibitors could be less efficient than their stress-induced homologues (Zhao et al 1996), the inhibitory spectrum of the pro-peptide may not be wider than that of the model (constitutive) PI, OCI.

1.5.1.4 OBJECTIVE 4

"To identify and isolate a novel stress-induced broad-spectrum cysteine PI from a non-host plant, and to characterize its inhibitory spectrum against both OC-sensitive and -insensitive CPB digestive proteinases fed on various diets."

The importance of fatty acids in the induction of defense responses in plants is well documented (see introduction, 1.4.2.2). Stress, whether biotic or abiotic, primarily disturbs the cell membrane integrity by releasing free fatty acids or cell membrane phospholipids, which act as second messengers in signaling pathways and lead to the over-expression of several defense-related molecules. The octadecanoid signaling pathway, which involves ALA as a primary fatty acid, is the best known signaling pathway in plants, being involved in various defense responses including the expression of PIs (Farmer and Ryan 1992). The same authors reported the inefficiency of GLA to activate either the JA-pathway or the expression of PIs (Farmer and Ryan 1992). In contrast, while studies carried out in humans demonstrate the importance of GLA (or other eicosanoids) in diverse pathologies, notably in inducing the expression of serine PIs in cancerous cells (Jiang et al 1997), the defense-inducing role of this fatty acid has not been studied in plants. Taking tomato as a non-host model, this part of the thesis will address the possible 'stress-PI'-inducing effect of GLA in plants.

CHAPTER II

Visual Discrimination of Protease Inhibitor-'Sensitive' and 'Insensitive' Proteinases Using an Electroblotting Gelatin/PAGE System

(General Résumé)

This chapter is presented in the form of a scientific article dealing with the first objective of the thesis, i.e. " To discriminate in gel, OCI-, OCII- and HSA-sensitive and -insensitive digestive proteinases of CPB by modifying the existing gelatin/PAGE technique." A pure commercial cysteine proteinase, bromelain was used as a model to validate the new technique. Bromelain showed a differential behavior when analyzed by traditional and two-step substrate (gelatin) SDS/PAGE under mildly-denaturing (non-reducing) or denaturing (reducing) conditions. Even under reduced conditions, which completely denatures most proteins, multiple bands of bromelain were detected following traditional gelatin-SDS/PAGE, while a single activity band of 33 kDa (as observed after standard SDS/PAGE) was evident following the two-step gelatin/PAGE under non-reducing conditions.

The usefulness of this technique in discriminating class(es)/sub-class(es) of proteinases, as well as in determining their molecular weights from crude protein extracts, was assessed with CPB digestive proteinases as a model system. The presence of two major sub-classes of cysteine proteinases in the CPB midgut, namely cathepsins B-like (OC-insensitive) and H-like (OC-sensitive) proteinases, was first demonstrated by affinity gel chromatography of the crude midgut extract by utilizing cathepsin B- (Divicell-ε-aminocaproic acid-Phe-Phe-CH_3) and cathepsin H- (GST-OCI) affinity columns. Accordingly, the improved two-step gelatin/PAGE technique enabled the detection of two bands of molecular weights ~28 kDa, and ~67 kDa, respectively sensitive (cathepsin H) and insensitive (cathepsin B) to OCs and HSA. This new technique simplified the complex proteinase patterns usually observed *in gel* and eliminated the discrepancy observed between *in vitro* data and 'traditional' in gel inhibition studies.

In the context of the thesis, this study allowed the identification of the OCI-insensitive cysteine proteinase of CPB as a ~67-kDa protein, presumably the insect cathepsin B-like proteinase (Michaud et al 1998). This OCI-insensitive proteinase is the 'target molecule' for achieving broad-spectrum inhibition of CPB digestive cysteine-type proteinase activity.

Visual Discrimination of Protease Inhibitor-'Sensitive' and 'Insensitive' Proteinases Using an Electroblotting Gelatin/PAGE System

Savita D. Visal, Binh Nguyen-Quoc, Serge Yelle, and Dominique Michaud[1]

Centre de Recherche en Horticulture, Département de phytologie, FSAA, Université Laval, Québec, Canada G1K 7P4

[1] To whom reprint requests should be addressed. Fax: (418) 656-7871. E-mail: Dominique.Michaud@crh.ulaval.ca.

[2] Abbreviations used: GST, glutathione *S*-transferase; HSA, human stefin A; OCI, oryzacystatin I; OCII, oryzacystatin II; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino) butane.

Savita Visal, Binh Nguyen-Quoc, Serge Yelle, and Dominique Michaud[1] (1998):

*Analytical Biochemistry (submitted)

2.1. Abstract

An electroblotting gelatin-PAGE system was devised for *in gel* detection and analysis of proteinases from complex biological extracts. The proteins are first separated by reducing or non-reducing SDS-PAGE, and then electrotransferred into a 0.75 mm-thick polyacrylamide slab gel containing 0.1% (w/v) porcine gelatin. The active proteinase bands are then developed in the accompanying gel in the presence or absence of non-protein or proteinaceous proteinase inhibitors, allowing determination of proteinase classes and visual discrimination of inhibitor-'sensitive' and 'insensitive' proteinases in complex biological extracts. Where possible, the use of reducing conditions during SDS-PAGE also eliminates ambiguity when estimating the molecular weight of proteinases. In comparison with the standard gelatin/PAGE technique, that involves copolymerization of gelatin with acrylamide in the resolving gel, this technique simplifies proteinase patterns by preventing interference of gelatin during migration, and by avoiding overestimation of the number of proteinases present in the extracts. Bromelain (EC 3.4.22.32), bovine trypsin (EC 3.4.21.4) and the digestive cysteine proteinases of the insect pest, Colorado potato beetle (*Leptinotarsa decemlineata* Say) were used as models to assess the usefulness of this approach in detecting proteinases and in studying their interactions with specific proteinaceous protease inhibitors.

2.2. Introduction

The detection of proteinases in gelatin-containing polyacrylamide gels, first described by Heussen and Dowdle (1980), has been widely used to study proteinases from various sources (Lantz and Ciborowski 1994, North 1994, Michaud and Asselin 1995, Michaud 1998). In most cases, the gelatin substrate is copolymerized with acrylamide in the resolving gel. After electrophoretic migration, the proteins are renatured by equilibrating the gel in an aqueous Triton X-100™ solution, the proteinases are allowed to digest gelatin in an appropriate activation buffer, and finally visualized as clear bands against a blue background after staining with

Coomassie Brilliant Blue. This technique is highly sensitive and allows the detection of proteinases present in crude extracts at very low levels (Michaud et al 1993, Kleiner and Stetler-Stevenson 1994, Gordon and Lilly 1995). It has notably been used to rapidly determine the molecular weight of several proteinases (e.g. North et al 1988, Harrington and Russell 1994), to discriminate proteinase classes by the addition of class-specific proteinase inhibitors either directly in the crude extract (Michaud and Asselin 1995, Michaud et al 1993) or after migration in the activation buffer (Gordon and Lilly 1995), and to study the characteristics of proteinase/inhibitor complexes (Michaud 1998, Michaud et al 1996a, Michaud et al 1996b, Nawata et al 1995, Nawata et al 1995).

Despite its well recognized usefulness in the study of various proteinase-related biological processes, the gelatin/PAGE technique shows some limitations (Michaud and Asselin 1995, Hummel et al 1996). Inclusion of gelatin in polyacrylamide gels was notably shown to reduce the migration rate of proteins by 15 to 20%, making questionable the reliability of this approach to determine molecular weights (Hummel et al 1996). Apart from this general effect on protein migration, gelatin was shown to bind differentially to purified proteinases and proteinases from complex extracts during the electrophoretic migration (Michaud et al 1993, Hummel et al 1996), causing background trails in the gel and possible overestimation of the number of proteinase forms. Extracellular cysteine proteinases of the protozoan parasite *Trypanosoma brucei*, for instance, were resolved as two major bands after standard (reducing) SDS-PAGE, but multiple bands were detected after mildly-denaturing (non-reducing) gelatin/SDS-PAGE (Huet et al 1992). This apparent microheterogeneity of proteinases observed in gels, that possibly reflects a certain structural variability of proteinase species in complex extracts, may make it difficult to establish correlations between gel proteinase patterns and data obtained by *in vitro* assays or SDS-PAGE, and to take advantage of the high sensitivity of PAGE systems when studying specific proteinase/inhibitor interactions.

As a complement to standard gelatin/SDS-PAGE, this study describes a simple two-step, electroblotting gelatin/SDS-PAGE system enabling both the detection of proteinases without interference by gelatin, and the visual discrimination of proteinases 'sensitive' and 'insensitive' to specific proteinase inhibitors showing potential in biotechnology. The proteins are first resolved by SDS-PAGE under reducing or non-reducing conditions, and then electrotransferred into a 0.75 mm-thick polyacrylamide slab gel containing 0.1% (w/v) porcine gelatin. Following transfer, the proteinases are visualized after appropriate proteolysis and staining steps, with or without inhibitors in the assay buffer. Bromelain, bovine trypsin and midgut proteinases of the insect pest, Colorado potato beetle (CPB; *Leptinotarsa decemlineata* Say) were taken as model enzymes to demonstrate the usefulness of the approach.

2.3. Materials and methods

2.3.1. Proteinases

Bromelain (from pineapple stem, EC 3.4.22.32) and trypsin (type III, from bovine pancreas; EC 3.4.21.4) were purchased from Sigma (St. Louis, MO), and dissolved in 100 mM citrate phosphate, pH 6.0, at a concentration of 5 mg/ml. Insect digestive proteinases were extracted from 3rd-instar CPB larvae, essentially as described by Overney et al. (1997). Briefly, the insect material was ground to a fine powder in liquid nitrogen, and the total soluble proteins were extracted in a 100 mM citrate phosphate buffer, pH 6.0 (2 ml buffer per g of insect material). After incubation on ice for 30 min., the mixture was centrifuged at 13,000 *g* for 30 min. at 4°C, and the supernatant was stored at -20°C until use. Before protease analyses, the protein content in the extracts was adjusted to 5 mg/ml with extraction buffer. Protein concentrations were estimated according to Bradford (1976), with bovine serum albumin as a protein standard.

2.3.2. Proteinase inhibitors

Trans-epoxysuccinyl-L-leucylamido(4-guanidino) butane (E-64) was from Sigma (St. Louis, MO). Oryzacystatin I (OCI), oryzacystatin II (OCII) and human stefin A were expressed in *Escherichia coli* using the glutathione *S*-transferase (GST) gene fusion system (Smith and Johnson 1988) and purified with reduced glutathione-embedded beads as described previously (Michaud et al 1994, Michaud et al 1996). The GST/cystatin fusions, as active against cysteine proteinases as the natural cystatins (Michaud et al 1994, Michaud et al 1996), were used for *in gel* inhibition assays.

2.3.3. *Purification of oryzacystatin I-sensitive and -insensitive digestive cysteine proteinases of CPB*

OC I-'sensitive' and 'insensitive' digestive cysteine proteinases in CPB were purified by differential affinity chromatography, using an Affigel-GST/OCI gel for the OCI-sensitive protease, and a Divicell-ε-aminocaproic acid-Phe-Phe-CH_3 gel for the OCI-insensitive enzyme. The OCI-affinity column was prepared by incubating a preparation of 30 mg GST/OCI fusion protein in 50 mM Tris-HCl (Michaud et al 1994) with 1 ml of Affigel 15 preparation (BioRad, Richmond CA), according to the supplier's instructions. The second column was prepared by coupling H-Phe-Phe-CH_3 x HCl to Divicell-AcaOH using water soluble carbodiimide (K. Peters, unpubl.). The gels (500 µl) were packed in a 2 ml-column, and incubated with 1 ml (5 mg protein) of the CPB crude midgut extract for one hour on ice. The columns were then washed with several volumes of 100 mM citrate phosphate, pH 6.0, to remove unbound proteins, and the bound proteinases were eluted with a 100 mM citrate phosphate buffer, pH 6.0, containing 50% (v/v) isopropanol, 1 M NaCl, and 1% (v/v) β–mercaptoethanol. The resulting preparations were desalted and concentrated with Microcentricon-10 microfilters (Amicon Inc., Beverly CA), and analyzed by reducing SDS-PAGE.

2.3.4. SDS-PAGE

SDS-PAGE was carried out in 0.1% (w/v) SDS/10% (w/v) acrylamide/0.4% (w/v) *bis*-acrylamide slab gels (Laemmli 1970), using the Mini-Protean II™ electrophoretic unit (Bio-Rad, Richmond, CA). After migration, the gels were stained with Coomassie Blue [0.3% (w/v) Coomassie Blue R-250 (Bio-Rad) in 10% (v/v) methanol/5% (v/v) acetic acid].

2.3.5. Gelatin/SDS-PAGE.

Standard gelatin/SDS-PAGE was carried out in 0.1% (w/v) gelatin/0.1% (w/v) SDS/10% (w/v) acrylamide/0.4% (w/v) *bis*-acrylamide slab gels under either reducing or non-reducing conditions (Heussen and Dowdle 1980, Michaud et al 1993). Samples containing 1 µg of bromelain or 10 µg of insect midgut proteins were diluted twice in electrophoresis sample buffer [62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 2% (w/v) sucrose, 0.001% (w/v) Bromophenol Blue, in the presence or absence of 0.7 M ß-mercaptoethanol], and subjected to electrophoresis at 4°C using the Bio-Rad Mini-Protean II™ unit. After migration for 90 min. at 120 V, the gel was transferred to a 2.5% (v/v) Triton X-100 solution for 30 min. at room temperature, and then placed in an activation buffer [100 mM citrate phosphate, pH 6.0, 0.1% (v/v) Triton X-100, 5 mM L-cysteine] for 30 min. at 37°C, in the presence or absence of class-specific proteinase inhibitors. Staining with Coomassie Blue was performed as described previously. For inhibition studies, E-64 was added to the activation buffer at 100 µM concentration. The recombinant GST-cystatins were used in excess, at a concentration of 2 mg/ml.

2.3.6. Two-step gelatin/SDS-PAGE

Protein extracts were first resolved by SDS-PAGE in a 0.75 mm-thick 10% (w/v) acrylamide/0.4% (w/v) *bis*-acrylamide slab gel. After migration, the proteins were electrotransferred into a 0.75 mm-thick 10% (w/v) acrylamide/0.4% (w/v) *bis*-acrylamide slab gel containing 0.1% (w/v) gelatin, using the Bio-Rad mini-transfer unit (Bio-Rad). Electrotransfer was carried out for various periods at 4°C in a 25 mM Tris buffer, pH 8.3, containing 192 mM glycine, under a constant voltage of 60 V. After transfer, the proteins were

renatured with Triton X-100, and placed in the activation buffer containing or not proteinase inhibitors (see above). Proteolysis was performed for 30 min. at 37°C, and the proteinases were visualized by staining the gel with Coomassie Blue. Semiquantitative analysis of gelatinase activity in gels was carried out by densitometry of the clear bands, with a Microtek Scanmaker II scanner (Microtek Lab., Torrence, CA) and the image analysis software NIH Image 1.51 (National Institutes of Health, Bethesda, MD).

2.4. Results and discussion

2.4.1. Behavior of bromelain in gelatin/PAGE systems

Migration behavior of the cysteine proteinase bromelain in gelatin/SDS-PAGE systems was assessed with regard to the presence of gelatin in the resolving gel, the reduction status of the electrophoresis sample buffer, and the temperature of the proteinase sample preparation before migration (Fig. 2.1). Despite the presence of a single 33-kDa band on Coomassie Blue-stained gels following SDS-PAGE (not shown), multiple bands were detected following gelatin/SDS-PAGE under both non-reducing and reducing conditions (lanes 1 and 2). In contrast, fewer bands were detected following two-step gelatin/SDS/PAGE under mild denaturing (non-reducing) conditions while a 33-kDa single band was observed under reducing conditions (lanes 3-6), clearly showing the effect of gelatin on the migration of bromelain in standard gelatin/SDS-PAGE systems. The migration of bromelain was not only affected by gelatin, but also by the reduction status of the sample (lanes 3 and 5), and by the temperature of the complete mixture before electrophoresis (lanes 3-9). While mild denaturation of bromelain by SDS in non-reducing conditions was favored by increasing the temperature from 4 to 37°C (lanes 3 and 4), an effective denaturation was observed at 4°C in reducing conditions (lane 5). Noteworthy, stronger denaturing conditions (reduction + heating at 37°C) resulted in a visible loss of bromelain activity (lane 6). A similar temperature-dependent loss of activity was also noted for trypsin, but for higher temperatures (lanes 7 and 9), indicating the need to calibrate the denaturing conditions for each particular enzyme or enzymatic system to be analyzed.

Despite this apparent multiplicity of factors interfering with proteinase migration and subsequent detection, it clearly appears that the gelatin substrate may cause overestimation of 'proteinase species' detected on gels, probably owing to chemical interactions taking place during migration. By preventing direct interference by gelatin, the two-step approach described here provides a simple mean 1) to minimize microheterogeneity in extracts for which complex patterns are obtained, and 2) to facilitate the estimation of proteinase molecular weights. The electrotransfer step is rapid, simple, and reproducible in optimal conditions (20 to 30 min. transfer for a 0.75 mm-thick slab gel), allowing semi-quantitative estimation of proteinase activities (Table 1).

2.4.2. Digestive cysteine proteinases of the Colorado potato beetle

Usefulness of the two-step gelatin/PAGE approach in studying the proteinases from complex extracts was assessed with the digestive proteinases of CPB as a model system. Thie and Houseman (1990) previously showed that the bulk of digestive proteinase activity in this insect was explained by cysteine-type proteinases. Based on their affinity for specific substrates and inhibitors, these proteinases were classified into two groups, cathepsin H-like and cathepsin B-like proteinases (Thie and Houseman 1990), respectively 'sensitive' and 'insensitive' to the action of the rice cysteine-type inhibitors, OCI and OCII (Michaud et al 1993). In agreement with these conclusions, we isolated two proteinases from the insect extract showing differential affinity for GST/OCI- and ε-aminocaproic acid-Phe-Phe-CH_3- affinity gels (Fig. 2.2). When OCI was used as an affinity ligand, a ~28-kDa protein was partially purified, while a ~67-kDa protein was purified with the *N*-blocked -Phe-Phe- ligand, known to inhibit the endopeptidase cathepsin B, but not the aminoendopeptidase cathepsin H (Brömme et al 1989).

In contrast with this simple mechanistic discrimination of the insect digestive cysteine proteinases, complex proteinase patterns composed of several bands were detected after gelatin/SDS-PAGE, either under non-reducing (Michaud et al 1995) or reducing conditions (S. Visal et al., unpubl.). Probably due to interactions between gelatin and the insect midgut

proteinases during migration, this interference by the gelatin substrate exemplifies the difficulty to correlate gel activity patterns from gelatin-containing gels with quantitative data obtained *in vitro*. In contrast, by using the electroblotting gelatin/PAGE system, the insect proteinases were resolved as two main bands under reducing conditions (Fig. 2.3A, lane 1). Assuming that the proteins in the extract were completely denatured during SDS-PAGE, the molecular weight of these two proteinases could be estimated at ~28 and ~67 kDa, respectively. In accordance with chromatographic data (Fig. 2.2), the 67-kDa proteinase was insensitive to inhibition by either OCI and OCII, while the 28-kDa protein was sensitive to both inhibitors (Fig. 2.3A and 2.3B). Interestingly, human stefin A also had a differential inhibitory effect (Fig. 2.3B), despite its reported affinity for the insect cathepsin B-like enzyme (Michaud et al 1996). This differential effect of the human inhibitor may reflect the very low affinity between this inhibitor and the insect proteinase (Michaud et al 1996), thereby suggesting that the effective *in gel* inhibition of proteinases by proteinaceous proteinase inhibitors depends on the affinity between this inhibitor and its target enzymes. In summary, the two-step electroblotting gelatin-PAGE system appears useful not only in proteinase detection and class determination, but also in discriminating proteinases (strongly) sensitive and insensitive (or weakly-sensitive) to specific proteinaceous inhibitors.

2.4.3. Concluding remarks

Standard gelatin/SDS-PAGE, that allows rapid detection of proteinases in complex biological samples, has proved useful in the analysis of several proteinase-related processes. Although it is increasingly evident that the patterns observed not only reflect the nature of proteinases present but also the interactions between these enzymes and various extrinsic factors like the gelatin substrate itself, the presence of reducing agents and the temperature, this approach appears particularly useful when studying fine variations of proteolytic systems during specific physiological or developmental processes (Heussen and Dowdle 1980, Gordon and Lilly 1995, North et al 1988, Michaud et al 1996a, Michaud et al 1996b, Overney et al 1997, Michaud et al 1995, North 1988). Despite the apparent variety of factors interfering with proteinases during

migration, the proteolytic patterns observed are generally highly reproducible for specific protease complements, making even possible the development of gelatinase standards useful for calibration purposes (Makowski and Ramsby 1996). As a complement to this commonly-used approach, this study described a simple two-step method for *in gel* detection and analysis of proteinases (gelatinases). By preventing interference of gelatin during migration and thus minimizing the number of proteinases detected, this technique may prove useful 1) in eliminating ambiguity in the estimation of molecular weights, 2) in obtaining simpler proteolytic patterns useful to compare data from *in vitro* and *in gel* assays, and 3) in allowing to identify protease inhibitors effective against particular proteinase systems. Two-step approaches involving the electrotransfer of proteins onto nitrocellulose membranes after SDS-PAGE (Moos 1991), the diffusion of proteinases in gelatin-containing gel overlays (Hummel et al 1996), or the simple diffusion of gelatin in the gel after migration (Garcia-Carreno et al 1993) were previously proposed, but we found it difficult to ensure reproducibility of either the protein transfer and the inhibition reactions using these approaches. With the electroblotting gelatin/PAGE technique, both transfer yield and inhibition rates are reproducible, making this approach useful for semi-quantitative analysis of proteinase and proteinase inhibitor activities.

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TABLE 2.1. Bromelain activity detected on gel after two-step gelatin/PAGE. After SDS-PAGE, bromelain was electrotransferred into a 0.75 mm-thick gelatin-containing polyacrylamide gel for different time periods. [a] As compared to the maximal activity (arbitrarily fixed at 1.00; after 20 min.). Data are the mean of four values ± SE.

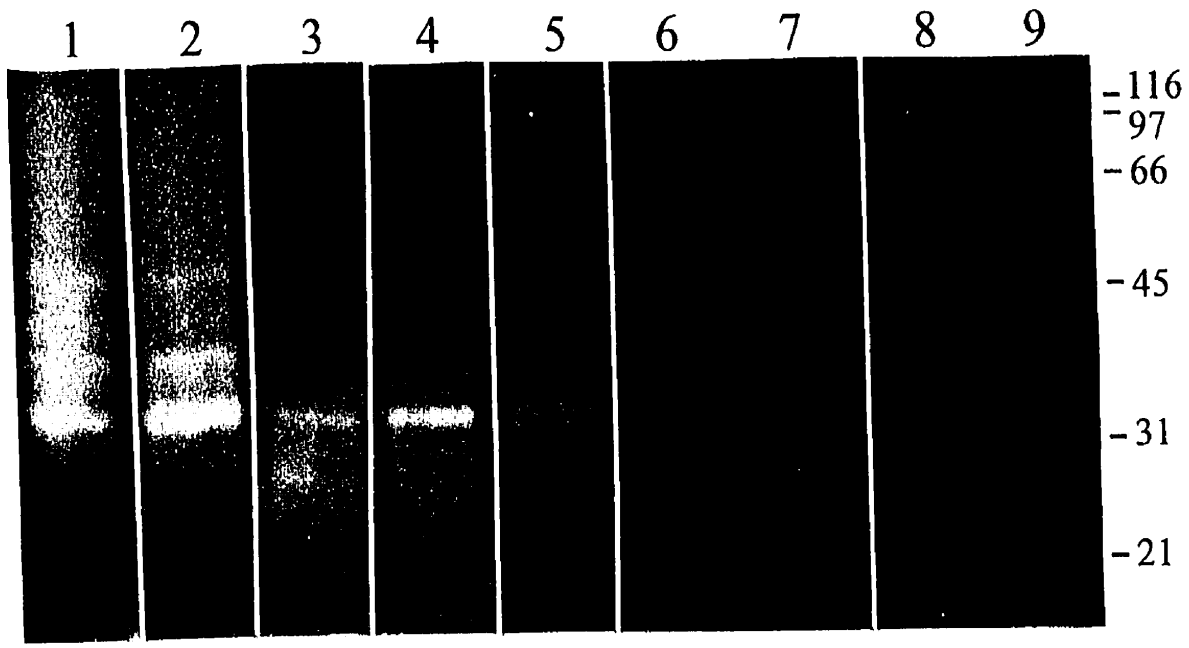
Transfer time (min.)	Relative activity[a]
10	0.44 ± 0.10
20	1.00 ± 0.05
30	0.80 ± 0.07
45	0.48 ± 0.10
60	0.06 ± 0.04

Figure legends

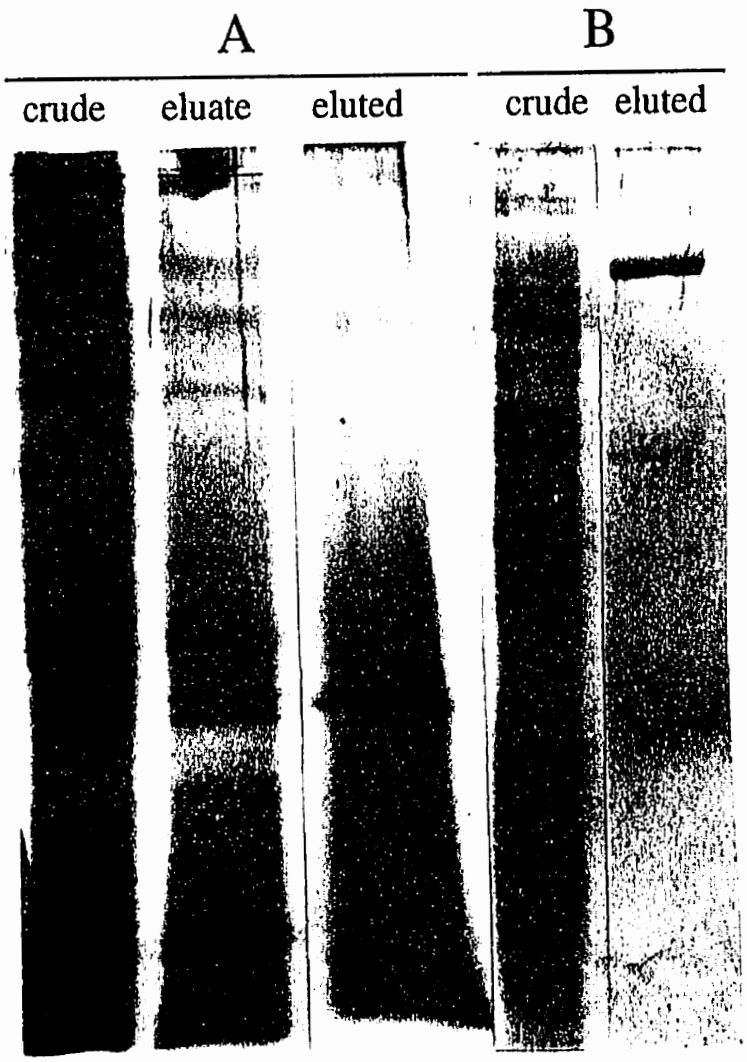
FIG. 2.1. Migration behavior of bromelain (lanes 1-6 and 8) and trypsin (lanes 7 and 9) in gelatin/SDS-PAGE systems. The enzymes were prepared and resolved under various conditions, and detected after appropriate renaturation, proteolysis and staining. Lanes 1 and 2: standard gelatin/PAGE under non-reducing (lane 1) or reducing (lane 2) conditions. Lanes 3-9: two-step gelatin/PAGE under non-reducing (lanes 3 and 4) or reducing (lanes 5-9) conditions. Before migration, the samples were incubated either at 4°C (lanes 1-3, and 5), 37°C (lanes 4, 6, and 7), or 65°C (lanes 8 and 9). Values on the right represent the molecular weight of commercial protein markers (kDa).

FIG.2.2. Partial purification of Colorado potato beetle OCI-sensitive (A) and -insensitive (B) digestive proteinases. The insect enzymes were isolated with either a GST/OCI gel or a Divicell-ε-aminocaproic acid-Phe-Phe-CH_3 gel, subjected to SDS-PAGE, and stained with silver nitrate (OCI-sensitive proteinase) or Coomassie Brilliant Blue (OCI-insensitive proteinase). Molecular weights were estimated to 28-kDa and 67-kDa for the OCI-sensitive and -insensitive forms, respectively, using commercial protein markers.

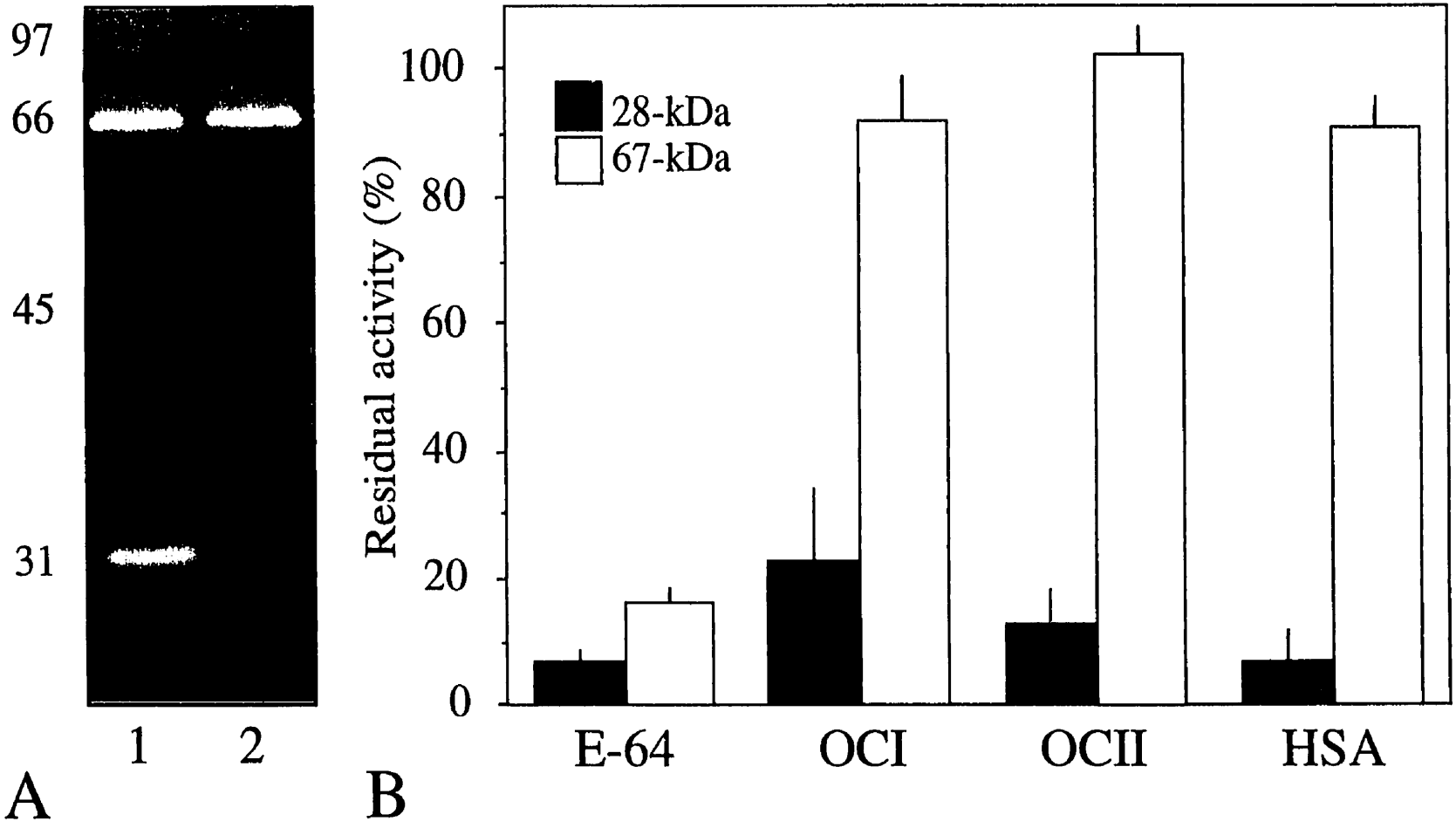
FIG.2.3. Detection of Colorado potato beetle digestive proteinases following two-step gelatin/SDS-PAGE. The insect proteinases were denatured at 4°C in reducing conditions, resolved and detected after appropriate renaturation, proteolysis at pH 6.0 and staining. A. *In gel* detection of the insect proteinases, in the absence (lane 1) or presence (lane 2) of OCI. B. Inhibitory effect of E-64, OCI, OCII, and HSA on the activity of the insect proteinases. Activity values were estimated by scanning and image analysis of the clear (lysis) zones. Data represent residual relative activities as compared to the activity of a non-inhibited control (100% activity). The inhibitors were added in the activation buffer during the proteolysis step. Values on the left represent the molecular weight of protein markers (kDa).



Visal et al., Figure 1



Visal et al., Figure 2



Visal et al., Figure 3

CHAPTER III

The pro-region of papaya proteinase IV inhibits Colorado potato beetle digestive cysteine proteinases

(General Résumé)

This chapter is presented in the form of a scientific article aimed at identifying a novel PI for the inhibition of CPB digestive proteinases. More precisely, it deals with the second and third objectives of the thesis which are:

- " to investigate whether or not an alteration in the diet leads to an alteration of the insect digestive proteinases, and to determine whether this change leads to a change in the ratio of OCI-sensitive to -insensitive proteinases", and

- "to determine the efficiency, stability and potency of the pro-region of papaya proteinase IV (glycyl endopeptidase {EC 3.4.22.25}) in the inhibition of CPB digestive proteinases from insects reared on various diets"

- ***Diet-related plasticity in CPB***

Previous studies in mammals (Johnson et al 1977, Schick et al 1984, Giorgi et al 1985, Lhoste et al 1993) and insects (Gooding 1973, Briegel and Lea 1975, Noriega et al 1994) demonstrate that both the quantity and the quality of proteins in the diet influence the availability of specific proteinases for their digestion. Insects, fed with high levels of PI in their diets, either included in artificial diets (Broadway and Villani 1995, Broadway et a 1995, 1996, 1997) or over-expressed in transgenic plants (Jongsma et al 1995, Wu et al 1996), elevate or induce proteinases insensitive to the PI present, that may compensate for the inhibited proteolytic functions. Previous studies in our laboratory has demonstrated that changes in the diet bring about 'qualitative' and 'quantitative' changes in CPB midgut E-64-sensitive proteinases (Overney et al 1997), suggesting that the nature of the insect digestive proteinases is in part determined by the type of diet consumed. Further, it is known that genetic engineering, tissue

culture and stress conditions occasionally cause unexpected changes in the plant characteristics (Anonymous 1992), and eventually, an 'indirect stress' to the insects.

As noted earlier, the aim of the present thesis is to isolate PIs with a broad inhibition spectrum against CPB digestive proteinases or PIs complementary to OCs (or HSA) against these proteinases. Given the evident plasticity of the beetle digestive proteolytic system, any study aiming to assess the potential of specific PIs against the insect proteinases must take into account this variability.

Specifically, the aim of this chapter was 1) to understand the nature and abundance of basic digestive proteinases in CPB, and possible micro-heterogeneous changes in the proteolytic enzymes, with respect to the diet; and 2) to determine whether a change in diet cause changes in the CPB digestive proteinases, and if so, to what degree the cystatins would inhibit these proteinases. Three different diet (stress) conditions were employed: transgenic potato expressing active OCI, tomato- a non-host plant, and starvation conditions. In this chapter, presented in the form of a scientific article, such diet-related studies have been carried out in order to predict the eventual development of PI- resistance in CPB.

- ***Protease pro-regions***

Pro-regions of proteinases play an important role in down-regulating the activity of their cognate proteinase, and therefore may demonstrate its potential as 'PI-molecules' in insect control (Taylor and Lee 1997). In this study, inhibition of CPB midgut proteinases from insects fed on potato leaves, tomato leaves or OCI-expressing transgenic potato leaves, or from starved insects reared under similar growth conditions, indicated the ability of the PPIV pro-peptide to recognize a fraction of the insect digestive proteinases, presumably the cathepsin H-like proteinase also inhibited by OCs and HSA. The inhibitory potential of the pro-peptide was strongly enhanced by maintaining its structural and functional integrity, under either lower

temperature conditions or by preventing its hydrolysis by blocking the insect non-target proteinase, cathepsin D, with pepstatin A.

Overall, our observations have led to the following findings: (1) the inhibitory spectrum of a PI is dependent not only on the basic affinities between this inhibitor and its target proteinases, and on the insect ability to produce corresponding insensitive proteinases, but is also influenced by its physiological status, especially when it is exposed to different stress conditions; (2) although only a fraction of CPB digestive proteinase activity is recognized by the PPIV pro-peptide, plant proteinase pro-regions could show potential as PI molecules in pest control; and (3) PIs could be useful in maintaining the integrity of 'companion' defense proteins susceptible to degradation by the action of pest or pathogen insensitive proteinases.

Footnote: The current procedure for estimating *in vitro* proteolysis by CPB larval proteinases, either starved or reared on potato, tomato or transgenic potato plants expressing OCI, was slightly modified for the present analysis. It was observed that if equal concentrations of proteins 'in terms of equal proteinase activities' were taken for *in vitro* assays, starved insects showed five times and four times more cysteine proteinase activities than the insects fed on potato and tomato plants, respectively. This is therefore a major criterion to be considered when carrying *in vitro* inhibition assays, especially when dealing with diet-related studies.

The Pro-region of Papaya Proteinase IV Inhibits Colorado Potato Beetle Digestive Cysteine Proteinases

Savita D. Visal[a], Mark A.J. Taylor[b], Dominique Michaud[a,*]

[a] *Centre de Recherche en Horticulture, FSAA, Université Laval, Québec G1K 7P4, Canada*

[b] *Institute of Food Research, Reading Laboratory, Early Gate, Reading RG6 6BZ, England*

Keywords: Insect digestive proteases, Cysteine proteinase, Papaya proteinase IV pro-region, Proteinase inhibitor, Colorado potato beetle, *Leptinotarsa decemlineata* Say

Abbreviations: CPB, Colorado potato beetle; OCI, oryzacystatin I; OCII, oryzacystatin II; PPIV, papaya proteinase IV.

*Author for correspondence Fax (418) 656-7871; E-mail: Dominique.Michaud@crh.ulaval.ca.

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3.1. Abstract

Three distinct digestive protease complements were induced in larvae of the herbivorous pest, Colorado potato beetle (CPB; *Leptinotarsa decemlineata* Say), and used as a model system to assess the ability of the pro-region of papaya proteinase IV (PPIV) to act as an inhibitor of insect digestive cysteine proteinases. As shown by gelatin/PAGE and complementary inhibition assays, a recombinant form of the pro-region produced in *Escherichia coli* inhibited a fraction of the insect proteases also inhibited by the well-characterized inhibitor of cysteine proteinases, oryzacystatin I (OCI). In contrast with OCI, the inhibitory potential of the pro-region was affected by an increase of the temperature, suggesting a certain alteration of its structural integrity by the insect non-target proteases. This apparent susceptibility to proteolysis was confirmed by SDS-PAGE, after challenging the pro-region with the different insect extracts. As seen on gel, selective inhibition of the insect aspartate proteinase, cathepsin D, with the inhibitor pepstatin A preserved the activity of the pro-region against cysteine proteinases by preventing its hydrolysis. Taken together, these observations suggest the potential of plant protease pro-regions as regulators of cysteine proteinases in biotechnological systems, and show the ability of protease inhibitors to preserve the integrity of 'companion' defense-related proteins from the action of insensitive proteases in target pests.

3.2. Introduction

The importance of extracellular proteases in plant/pest and plant/pathogen interactions is well-recognized (Ryan 1990), and control strategies based on their inhibition with selective inhibitors have been proposed as a way to control herbivorous insects (Hilder et al 1993), parasitic nematodes (Urwin et al 1995), and microbial pathogens (Lorito et al 1994, Dunaevskii et al 1994). Although the exact metabolic functions altered by plant protease inhibitors remain to be elucidated in most cases, the importance of proteolytic enzymes in target pests and pathogens appears obvious. The repressive effects of protease inhibitors on insect growth or fecundity, for

instance, have been described for several species (Broadway et al 1986, Murdock et al 1988, Burgess et al 1991, Chen et al 1992, Michaud et al 1995), and the implication of microbial extracellular proteases in plant pathogenic processes has been suggested in several instances (Hislop et al 1982, Bashan et al 1986, Ball et al 1991, Dow et al 1990, Dow et al 1993). Based on these data, the use of protease inhibitor-expressing transgenic plants has been proposed as a mean of protecting crops from their natural enemies, and several plants of economic importance have been genetically engineered with inhibitor-encoding cDNA sequences during the last ten years (Urwin et al 1995, Hilder et al 1987, Johnson et al 1989, Masoud et al 1993, Hosoyama et al 1994, Leplé et al 1995, Michaud and Vrain 1998).

Despite these important advances, the general usefulness of recombinant protease inhibitors in plant protection still remains equivocal. The inhibitory range of protease inhibitors is usually limited to proteinases in one of several mechanistic classes, leaving free proteases in the surrounding medium after inhibition. Possibly due to co-evolution processes, the inhibitory spectrum of plant protease inhibitors against herbivorous pest proteinases is even more limited, being restricted in several cases to the subclass level (Michaud et al 1993a, Jongsma et al 1995, Bolter and Jongsma 1995, Broadway 1995, Broadway 1996). The occurrence of insensitive proteases in target pests, that may allow physiological compensation of inhibited proteolytic functions (Jongsma and Bolter 1997), may also challenge the structural integrity of certain inhibitors (Michaud 1997). The ability of cysteine-type protease inhibitors to retain their structural integrity in the presence of insect insensitive proteases, for instance, was assessed with human stefin A and two inhibitors from rice, oryzacystatin I (OCI) and oryzacystatin II (OCII) as model inhibitors (Michaud et al 1995, Michaud et al 1996c). While OCI remained stable in the presence of insect insensitive proteases, OCII and the human inhibitor were subjected to extensive hydrolysis, gradually leading to a complete loss of their inhibitory activity.

Understanding the dynamic interactions between plant protease inhibitors and the digestive proteases of herbivorous pests clearly appears important to correctly assess the actual usefulness of extracellular protease inhibition in plant protection. From a practical point of view, the development and the identification of alternative or complementary inhibitors is also important, to achieve broad-spectrum inhibition of pest protease complements, and thus to minimize the occurrence of compensatory or degradation processes in target pests (Michaud 1997, Jongsma et al 1996). Several strategies are currently considered for the improvement of protease inhibitor-based control approaches, including: (i) the improvement of inhibitor binding characteristics by site-directed mutagenesis (Urwin et al 1995), (ii) the isolation of effective inhibitor variants by phage display (Jongsma et al 1996, Koiwa et al 1998), (iii) the isolation of novel, stress-induced inhibitors from plant tissues (Zhao et al 1996, Visal et al 1996), (iv) the use of insect protease inhibitors exhibiting high affinity for insect digestive proteinases (Thomas et al 1995), and (v) the use of insect regulatory pro-peptides specific to their cognate proteinase (Taylor and Lee 1997). In this study we assessed the ability of a plant protease pro-region, the pro-region of papaya proteinase IV (PPIV) (Taylor et al 1995), to act as an inhibitor of herbivorous pest digestive cysteine proteases and to remain stable in the presence of non-target, insensitive proteases. Diet-related variants of the well-characterized digestive protease system of the insect pest, Colorado potato beetle (CPB; *Leptinotarsa decemlineata* Say) were used as an insect model for the inhibition assays.

3.3. Materials and methods

3.3.1. Reagents

Azocaesin, bovine serum albumin (BSA), L-cysteine, gelatin (porcine type A), pepstatin A, trichloroacetic acid and Triton X-100™ were purchased from Sigma (St. Louis, MO). Electrophoretic reagents were from Bio-Rad (Richmond, CA). All other reagents were of the highest purity commercially available.

3.3.2. Inhibitors

Pepstatin was dissolved in methanol to a 1 mM final concentration. The pro-region of PPIV was expressed as a soluble polypeptide in *Escherichia coli* and purified by chromatography on a 1 ml Hitrap Q column (Pharmacia, Uppsala), as described previously (Taylor et al 1995). Recombinant OCI was produced in *E. coli* JM109 using the glutathione *S*-transferase gene fusion system (Smith and Johnson 1988), with the plasmid pGEX3X-OCI as the expression vector (Michaud et al 1994). Purity of the recombinant inhibitors was controlled by 15% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). Inhibitor concentration in the resulting preparations was adjusted to 1.5 mg/ml with 50 mM Tris, pH 8.0. Protein concentrations were determined according to Bradford (1976), with BSA as a protein standard.

3.3.3. Insect digestive proteases

Third-instar CPB larvae reared on greenhouse-grown potato plants (cv. Kennebec) were placed at 22°C in 100 ml aerated plastic arenas containing water-saturated cotton sticks and a moist filter paper, under a 16 h/8 h (L:D) photoperiod provided by cool white fluorescent lights. The fifth leaves of either 'control' potato (cv. Kennebec) or OCI-expressing transgenic potato (cv. Kennebec) (Michaud and Vrain 1998) were supplied to the insects at 24 h intervals over a three-day period. In parallel, third instars were starved for three days under the same growth conditions. Each experiment was repeated three times, with 10 larvae in each arena. The insect digestive soluble proteases were then extracted as described previously (Overney et al 1997), in a 100 mM citrate phosphate buffer, pH 6.0, containing 10% (w/v) ethylene glycol. After centrifuging the mixture at 4°C for 30 min. at 17000 *g*, the supernatant was passed through a Sephadex G-25 column, and used as a source of proteases for subsequent analyses. Protein content in the extracts was determined as described above.

3.3.4. Gel protease assay

The insect digestive proteinases were visualized by mildly-denaturing gelatin/SDS-PAGE (Michaud et al 1993b). The protein extracts were fractionated into 0.1% (w/v) SDS-10% (w/v) polyacrylamide slab gels containing 0.1% (w/v) gelatin. Samples containing 0.25 unit of protease activity (see below) were first subjected to electrophoresis at 4°C using a Bio-Rad Mini-Protean II™ unit. After migration at 200 V, the gel was transferred to a 2.5% (v/v) aqueous solution of Triton X-100 for 30 min. at room temperature to allow enzyme renaturation, and then placed in an assay (proteolysis) buffer (100 mM citrate phosphate, pH 6.0, 0.1% (v/v) Triton X-100, 5 mM L-cysteine) for 3 h at 37°C. Proteolysis was stopped by transferring the gel into a staining solution [0.1% (w/v) Coomassie Brilliant Blue in 25% (v/v) isopropanol/10% (v/v) acetic acid], and the proteases (gelatinases) were visualized as clear bands against a dark background.

3.3.5. Protease inhibition assays

Total protease activity in the insect extracts was determined according to a previously described procedure (Michaud et al 1993). An appropriate amount of insect extract (corresponding to 0.25 unit of protease activity) was mixed with 7.5 µg of inhibitor solution, and the total volume of the mixture was adjusted to 50 µl with assay buffer (see above). After a 30 min. incubation at 37°C, 50 µl of 2% (w/v, 1mg/100µl) azocaesin dissolved in assay buffer was added to the enzyme/inhibitor solution, and the complete mixture was incubated for 3 h at 37°C. After proteolysis, 300 µl of 10% (w/v) trichloroacetic acid was added to the mixture, and the residual azocaesin was removed by centrifugation for 5 min. at 13000 *g*. The supernatant (350 µl) was added to 300 µl of 1N NaOH, and the absorbance (*A*) was measured at 440 nm using a Spectronic 1000 Plus spectrophotometer (Milton Roy, Rochester, NY). The A_{440} of blanks, which consisted of complete mixtures incubated for 0 h, was subtracted from each value. One unit of activity was defined as the amount of insect extract needed to cause an absorbance change of 1.0 in a 1 cm cuvette, under the conditions of the assay. All measures were done in triplicate.

3.3.6. Stability of the PPIV pro-region

Susceptibility of the recombinant pro-region to the action of CPB digestive proteases was assessed by incubating the inhibitor with the different insect extracts. Briefly, an amount of insect extract containing 0.125 unit of protease activity was incubated for 60 min. at 37°C with 5 µl of the pro-region preparation (7.5 µg protein). After incubation, the reaction was stopped by adding an equal volume of SDS-PAGE sample buffer 2X (Laemmli 1970) to the enzyme/inhibitor solution, and then placing the complete mixture in a boiling water bath for 3 min. The residual pro-region was visualized as a 13-kDa band following 15% (w/v) SDS-PAGE (Laemmli 1970). The protective effect of pepstatin A on the integrity of the propeptide was visualized by adding 1 µl of 1 mM pepstatin in methanol to the insect proteases/pro-region mixture, before the incubation at 37°C.

3.4. Results and discussion

3.4.1. The ratio of OCI-sensitive to insensitive cysteine proteinase activity in CPB larvae is drastically increased during starvation

In CPB, most of the digestive protease activity is explained by multiple proteinase forms present throughout development of the insect (Michaud et al 1995) and presumably belonging to the cathepsin B, cathepsin H, cathepsin D, and chymotrypsin protease families (Thie and Houseman 1990, Novillo et al 1997). This apparent complexity of the insect digestive protease complement, which possibly reflects a certain physiological advantage for the insect of using different proteinase forms with various specificities, also helps it to adapt its digestive protease metabolism to the presence in the diet of compounds interfering with dietary protein hydrolysis. The nature of the CPB digestive protease system is strongly influenced, notably, by the type of diet ingested (Overney et al 1997) and by the presence of protease inhibitors in host plant tissues (Bolter and Jongsma 1995). Such compensatory responses of the insect digestive proteolytic metabolism, while providing a good example of the complexity of dietary protein digestion in

herbivorous pests, also suggest the usefulness of this system as a tool for studying the complex and dynamic interactions between pest digestive proteases and plant protease inhibitors. In this study, 'new' proteinase complements were induced in the insect midgut by providing third instars previously reared on control (untransformed) potato plants with OCI-expressing transgenic potato foliage, or by submitting the larvae to starvation for a three-day period (Fig. 3.1). In accordance with the occurrence of OCI-sensitive digestive proteinases in CPB larvae (Michaud et al 1993), the activity of a major proteinase form detected in gel was lower in the extracts prepared from insects supplied with OCI-expressing potato (Fig. 3.1, arrow 1), demonstrating the effective inhibitory potency of recombinant OCI expressed *in planta*. Assuming that the initial basic level of OCI-sensitive proteinase activity was similar in the midgut of control and OCI-fed insects, this decrease in activity noted after electrophoresis in the presence of SDS was also suggesting the high stability of the OCI/CPB target proteinase complex. Unlike complexes formed by weak cystatin-proteinase interactions, which are completely dissociated in the presence of SDS, strong complexes remain (at least partly) stable during the mildly-denaturing gelatin/SDS-PAGE process (Michaud et al 1996a, Michaud et al 1996b).

In accordance with the recently described diet-related plasticity of the CPB digestive protease system (Overney et al 1997), the proteinase pattern observed for starved insects markedly differed from those observed for control potato and OCI-potato-fed insects (Fig. 3.1). As compared with control larvae, the starved insects overexpressed the OCI-sensitive proteinase, while not expressing most of the insensitive species. The presence of a proteinase form absent from the extracts of both potato- and OCI/potato-fed insects (Fig. 3.1, arrow 2), and from those of eggplant- or tomato-fed insects (not shown) was also detected, suggesting the existence of a starvation-specific digestive proteinase in CPB larvae. Although this observation indicated the possible occurrence of starvation-specific proteolytic events in CPB larvae, the exact role of this particular protease remains to be elucidated. Nevertheless, regardless of its physiological significance, this starvation-induced protease complement, mainly composed of OCI-sensitive

cysteine proteinase activity, provided an additional argument supporting the usefulness of CPB digestive proteases as a model system to study the relative importance of inhibitor-sensitive and insensitive digestive proteases in herbivorous pests, and to assess the potential of novel cysteine proteinase inhibitors in plant protection.

3.4.2. The pro-region of PPIV inhibits the insect OCI-sensitive digestive proteinase

In this study, the ability of the PPIV pro-region to inhibit insect digestive cysteine proteinases was assessed by measuring its inhibitory activity against the different protease complements described in Fig. 3.1. In cells, proteinase pro-regions are thought both to assist folding of the mature enzyme (Ikemura et al 1987), and to prevent uncontrolled proteolysis by acting as inhibitors of their cognate enzyme (Baker et al 1993). After expressing the PPIV propeptide in *E. coli*, Taylor et al. (1995) showed that it could inhibit not only PPIV but also other papaya proteinases, including papain. As shown here by standard inhibition assays, the plant pro-region also shows affinity for a fraction of the CPB digestive cysteine proteinases (Fig. 3.2), suggesting that it could actually represent a general inhibitor of papain-like cysteine proteinases. In accordance with the distinct ratios of OCI-sensitive to insensitive proteinase activity in control, OCI/potato-fed and starved insects, the inhibitory spectrum of both OCI and PPIV pro-region against the insect proteinases drastically varied depending on the enzyme complement assessed. Roughly, the inhibitory spectrum of OCI ranged from ~25-40% of total protease activity for control and OCI-fed insects to more than 75% for the starved insects. Similar inhibitory spectra were noted for the PPIV pro-region when the assays were done at 25°C, although these spectra were narrower for inhibition assays carried out at 37°C. Interestingly, no complementary inhibition was noted when the pro-region and OCI were used in combination, strongly suggesting that the proteinase recognized by the first inhibitor corresponded to the OCI-sensitive cysteine proteinase visualized in gel (Fig. 3.1, arrow 1), presumably a cathepsin H-like enzyme (Michaud et al 1993). Taken together, these observations provide evidence that the inhibitory spectrum of proteinase inhibitors against insect digestive proteinases is determined

not only by the basic affinity between these inhibitors and their target enzymes, and by the ability of insects to produce insensitive proteases following ingestion of dietary inhibitors (Jongsma et al 1995, Bolter and Jongsma 1995, Broadway 1995, 1996), but may also be strongly influenced by the physiological status of the target organism submitted to stress conditions such as starvation.

Our results also point out the possible usefulness of plant cysteine proteinase pro-regions as a new tool for the inhibition of cysteine proteinases in biological systems. The narrow activity spectrum of the PPIV propeptide against CPB digestive cysteine proteinases, although showing the limited potential of this inhibitor in CPB control, does not exclude interesting effects on alternative protease systems. Complex dissociation studies involving OCI and OCII as model inhibitors clearly revealed the differential effects of these closely-related plant inhibitors on the extracellular cysteine proteinases of herbivorous pests (Michaud et al 1996a, 1996b), strongly suggesting the importance to assess the effect of several inhibitors when planning the control of a particular pest. As a 'general' inhibitor of cysteine proteinases, the pro-region of PPIV (and possibly those of other plant cysteine proteinases) could provide an interesting complement to the currently used cysteine-type inhibitors, extending the diversity of inhibitors available for the regulation of cysteine proteinases in various systems. Pest extracellular protease complements composed of a limited number of OCI-sensitive cysteine proteinase species, notably, could represent interesting target enzyme systems for plant cysteine proteinase pro-regions expressed as recombinant proteins in transgenic plants.

3.4.3. Pepstatin preserves the inhibitory potency of the PPIV pro-region against the insect OCI-sensitive proteinase

Besides its potential in the regulation of cysteine proteinases, the PPIV pro-region could prove useful as a model protein substrate for studying protease-mediated resistance in insects, and for assessing the usefulness of protease inhibitors in the protection of potentially useful, but unstable defense-related proteins (Michaud 1997). In contrast with OCI, the inhibitory

efficiency of the pro-region was affected by increasing the temperature from 25°C to 37°C (see Fig. 3.2), suggesting a certain alteration of its structural integrity by the insect non-target proteases (Michaud et al 1995, Michaud et al 1996). This apparent susceptibility to proteolysis was confirmed by SDS-PAGE, after incubating the pro-region at 37°C with the different larval extracts (Fig. 3.3). Interestingly, the temperature-dependent efficiency and the hydrolysis of the propeptide were noted even with the extracts prepared from starved insects. Considering the negligible importance of OCI-insensitive cysteine proteinases in the corresponding extract (see Figs. 3.1 and 3.2), this observation was suggesting that the extensive hydrolysis of the pro-region was due to the presence of a non-cysteine protease in the surrounding medium, presumably the insect cathepsin D-like aspartate proteinase, which is not visualized following gelatin/SDS-PAGE (Michaud et al 1995) but easily detected by standard assays under mild conditions (Michaud et al 1995, Thie and Houseman 1990).

In agreement with this hypothesis, structural and functional integrity of the PPIV pro-region was preserved by the addition of pepstatin A, a specific inhibitor of aspartate proteinases, to the enzyme/inhibitor mixture (Fig. 3.4). By using a combination of *in vitro* and diet-based assays, Orr et al. (1994) previously suggested that the repressive effect of cysteine proteinase inhibitors against herbivorous insects not only depends on their affinity for target proteinases, but also on their capacity to remain stable in the insect midgut environment. Inclusion of different cystatins in the diet of Southern corn rootworm larvae, for instance, resulted in quite different effects on the growth of the insect, although all inhibitors caused inactivation of the insect digestive proteases *in vitro*. Interestingly, the simultaneous inclusion of a complementary, non-cysteine protease inhibitor restored both the activity of the less effective cystatins in the insect gut and their antinutritive effect *in vivo* (Orr et al 1994). While suggesting the importance of digestive cathepsin D-like activity in CPB larvae, the pro-region-stabilizing effect of pepstatin noted here provides an additional evidence suggesting the usefulness of protease inhibitors in protecting 'companion' defense-related proteins susceptible to the action of free proteases in the surrounding medium, and in avoiding protease-mediated resistance in pests (Michaud 1997).

3.5. Concluding remarks

This study assessed the potential of a plant cysteine proteinase pro-region, the pro-region of PPIV, as a tool for the inhibition of cysteine (papain-like) proteinases in biological systems. After developing an insect model system useful in studying the dynamic interactions taking place between plant protease inhibitors and the digestive cysteine proteinases of herbivorous pests, we showed that the PPIV pro-region could inhibit not only the cysteine proteinases of papaya, but also those found in the digestive tract of insect pests. Although important questions remain regarding their actual efficiency and stability *in vivo*, plant protease pro-regions could thus prove an interesting complement to the various inhibitors currently used for regulating proteinase activity in complex biological systems. The use of plant pro-regions in combination with highly specific pro-regions like those of insect midgut proteinases (Taylor and Lee 1997), notably, could prove particularly useful in designing molecular control strategies tailored for the inhibition of specific insect target systems. Studies are currently underway to further characterize the stabilizing effect of protease inhibitors on the PPIV propeptide, and to assess the general potential of protease pro-regions in plant biotechnology.

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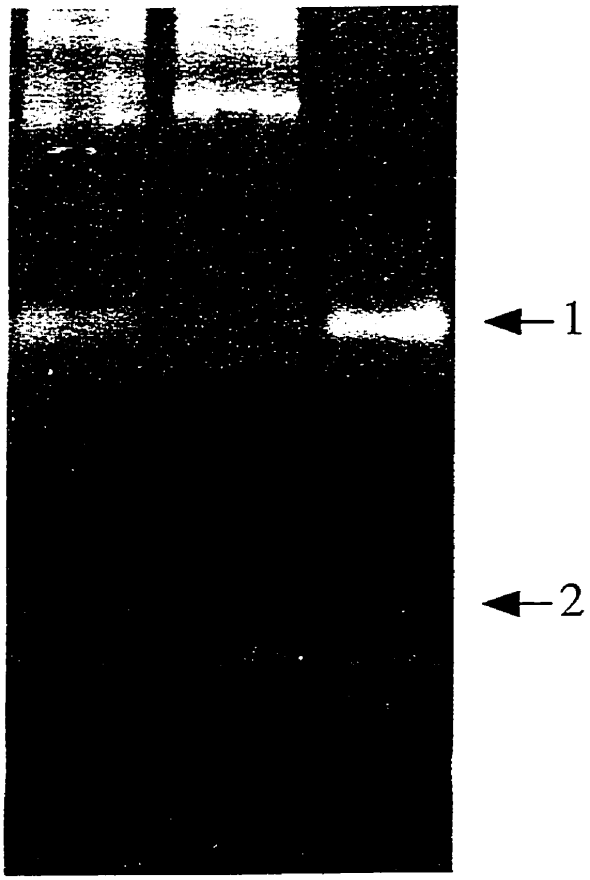
Figure legends

Fig. 3.1. Detection of CPB digestive proteinase forms after mildly-denaturing gelatin/SDS-PAGE. The arrows show the OCI-sensitive proteinase (arrow 1) and a starvation-specific proteinase (arrow 2).

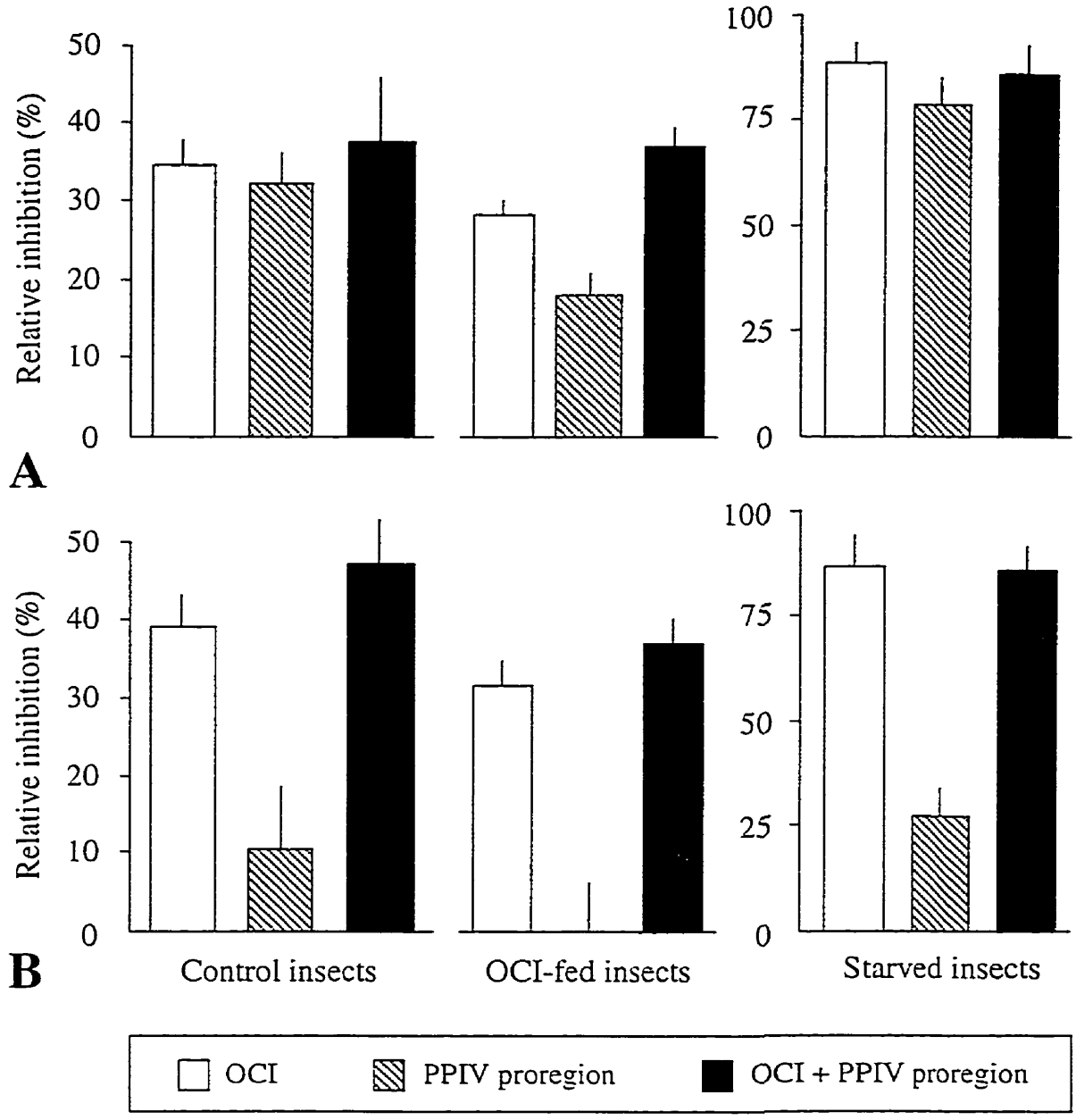
Fig. 3.2. Inhibition of CPB digestive protease activity by OCI and PPIV pro-region, alone or in combination. The assays were carried out at 25°C (A) or 37°C (B) as described in Materials and Methods. Results are expressed as relative inhibitions (%), as compared to controls for which no inhibitor was added. Each bar represents the mean of three values ± SE.

Fig. 3.3. Response of recombinant PPIV pro-region to the action of CPB digestive proteases from potato-fed (control), OCI/potato-fed, or starved insects. After incubation for 60 min. at 37°C, the protein mixture was subjected to 15% (w/v) SDS-PAGE and the pro-region was visualized as a 13-kDa band following Coomassie Brilliant Blue staining. 7.5 µg of pro-region ('time 0' control equivalent) was loaded in each well. I, insect extract; 0', i + pro-region before incubation; 60', i + pro-region after incubation.

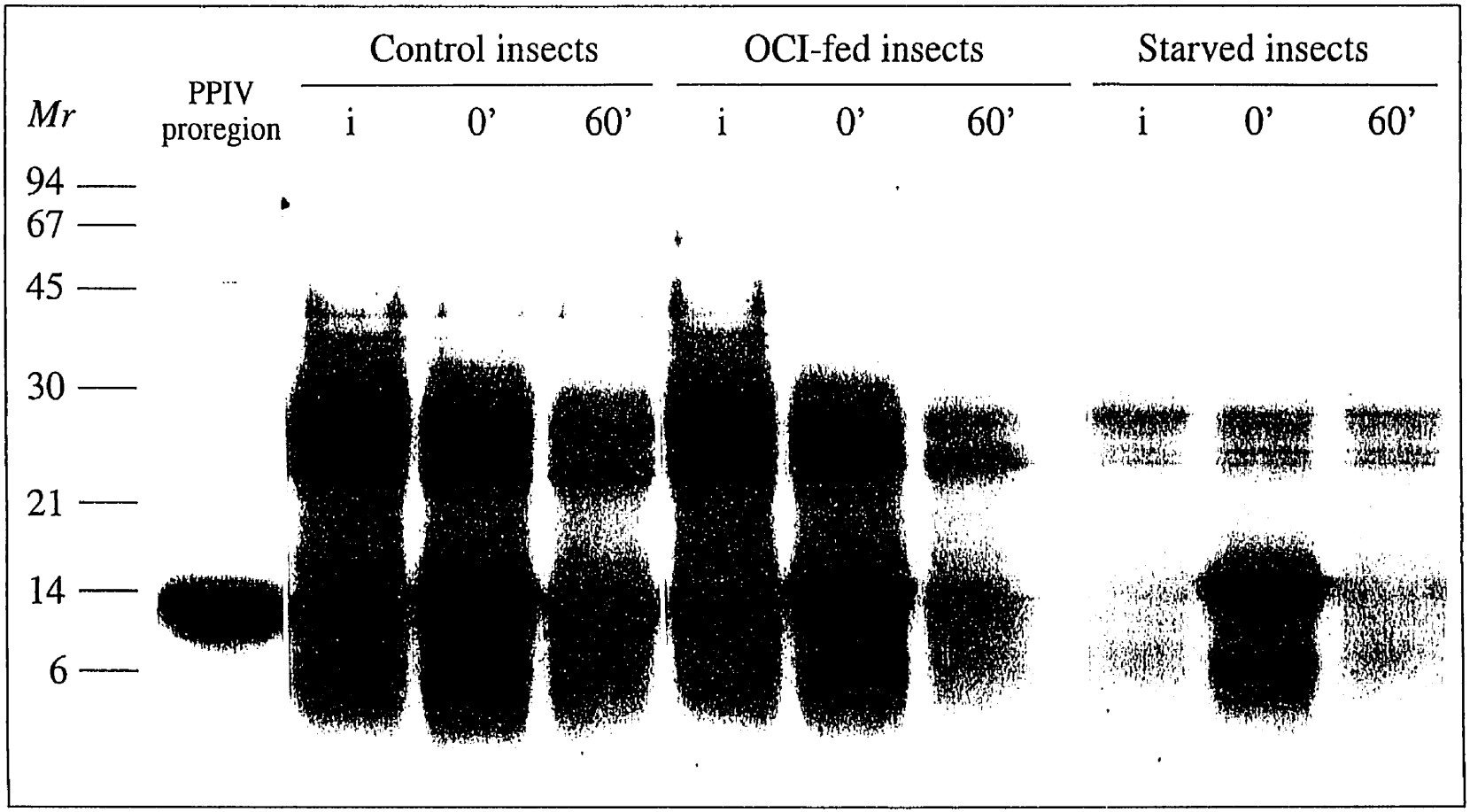
Fig. 3.4. Stabilization of the PPIV pro-region by pepstatin A. (A) Susceptibility of the recombinant pro-region to the action of potato-fed insects' digestive proteases, in the presence (+p) or absence (-p) of pepstatin A. (B) Inhibition of CPB digestive protease activity at 37°C by the PPIV pro-region and pepstatin A, alone or in combination. Inhibition assays and stability studies were carried out as described in Figs. 3.2 and 3.3; insect extract; p; pepstatin A.



Visal et al., Fig. 1

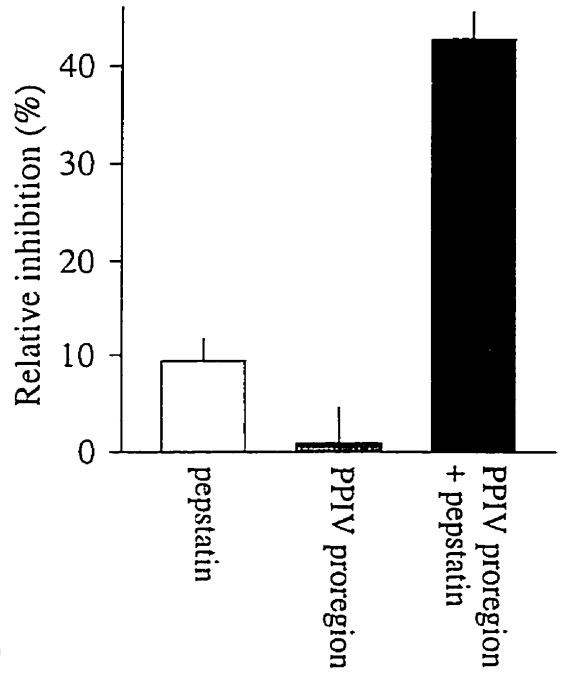


Visal et al., Fig. 2



Visal et al., Figure 3

A



B

Visal et al., Fig. 4

CHAPTER IV

γ-Linolenic Acid Induces the Accumulation of a Broad-Spectrum Inhibitor of Insect Digestive Cysteine Proteinases in Tomato

(General Résumé)

The literature review in chapter I led to the definition of a working hypothesis stating that "plants under stress conditions have evolved the capacity to produce PIs with broad spectrum inhibitory activity against the target (digestive) proteinases of phytophagous organisms. Therefore stressing plants with GLA, an analog of ALA, a precursor of jasmonic acid, may lead to the accumulation of broad-spectrum PIs, other than the PIs currently known". In this article we validate this hypothesis, and thus attain our fourth research objective, which was "to identify and isolate a novel stress-induced broad-spectrum cysteine PI from a non-host plant, and to characterize its inhibitory spectrum against both OC-sensitive and -insensitive digestive cysteine proteinases of CPB larvae fed on various diets."

A 55 kDa PI (TCPI) was induced in young tomato leaves in response to GLA treatment. Unlike the PPIV propeptide, TCPI was shown to possess a broad spectrum of inhibition against CPB midgut proteinases, which was unchanged or slightly affected by inclusion of OCI in the reaction mixtures, or in the diet. This study demonstrates the potential of TCPI as a broad-spectrum inhibitor of cysteine proteases which, if exploited in plant genetic engineering, could represent an efficient 'biochemical weapon' for protecting plants from insect damage. It also suggests the existence of an alternate fatty acid signaling pathway in plants, with as yet unknown intermediary products.

CHAPTER IV

γ-Linolenic Acid Induces The Accumulation Of A Broad-Spectrum Inhibitor Of Insect Digestive Cysteine Proteinases In Tomato

Savita Visal, Conrad Cloutier, Serge Yelle, Claude Willemot
and Dominique Michaud *

Centre de Recherche en Horticulture, Département de phytologie, FSAA, Université Laval, Québec, Canada G1K 7P4

Abbreviations used: GLA, γ-linolenic acid; TCPI, tomato cysteine proteinase inhibitor; OCI, oryzacystatin I; OCII, oryzacystatin II; HSA, human stefin A

*Author for correspondence. Fax: (418) 656-7871; E-mail: dominique.michaud@crh.ulaval.ca.

Visal S, Cloutier C, Yelle S, Willemot C and D Michaud (1998) (submitted to *Plant Physiology)

4.1. Abstract

Several strategies are currently being developed for the improvement of protease inhibitor-based pest control approaches, including the search for effective, broad-spectrum inhibitors. In this study we assessed the potential of γ-linolenic acid (GLA), a plant unusual polyunsaturated fatty acid, for inducing the accumulation of effective cysteine PIs in tomato (*Lycopersicon esculentum* Mill.). Using papain as an affinity ligand, a 55-kDa single polypeptide absent from untreated tomato leaves, TCPI (tomato cysteine proteinase inhibitor), was isolated from young leaves harvested 24 h after spraying with 40 μM GLA. As shown by immunoblotting, this inhibitor was immunologically distinct from the 88-kDa tomato multicystatin induced in tomato via the α-linolenate/jasmonate signaling pathway. In contrast with the other proteinaceous cysteine PIs tested (e.g. oryzacystatin I, oryzacystatin II and human stefin A), TCPI exhibited broad-spectrum affinity for digestive cysteine proteinases of the herbivorous insect, Colorado potato beetle (*Leptinotarsa decemlineata* Say). This broad inhibitory spectrum of TCPI was noted not only for cysteine proteinases of larvae fed 'normal' potato plants, but also for those of diet-related protease variants and those of larvae physiologically adapted to recombinant oryzacystatin I expressed in potato. Taken together, these observations show the potential of TCPI for controlling herbivorous pests relying on cysteine proteinases for dietary protein hydrolysis, and suggest the usefulness of GLA to direct the accumulation of novel defense-related molecules in plants.

4.2. Introduction

The importance of proteinase inhibitors (PIs) in plant defense is now well-recognized (Ryan 1990, Koiwa et al 1998), but recently it became evident that important limitations are associated with the use of recombinant PIs in plant protection (see Jongsma and Bolter 1997, for a review). In the last ten years, several plants of economic importance have been genetically engineered with inhibitor-encoding cDNA sequences (Schuler 1998, Michaud and Vrain 1998),

but convincing protective effects were reported only for a limited number of pests (Hilder et al 1987, Johnson et al 1989, Leplé et al 1995, Urwin et al 1995, 1998). The occurrence of both natural and recombinant PIs in plant tissues is now known to induce *de novo* synthesis of proteases either 'sensitive' or 'insensitive' to these inhibitors in the midgut of several herbivorous pests, as a way to compensate for inhibited proteolytic functions (Jongsma et al 1995, Bolter and Jongsma 1995, Bown et al 1997, Cloutier et al 1998). The appearance of such compensatory responses, along with the degradation of certain PIs by insensitive proteases (Michaud et al 1995, Michaud et al 1996a, Giri et al 1998, Girard et al 1998a), the poor binding affinity between plant PIs and certain pest proteinases (Michaud et al. 1996b, 1996c; Girard et al. 1998b), and the general occurrence of complex patterns of digestive proteases in herbivorous pests (Terra and Ferreira 1996) would help them eluding the antinutritive effects of plant PIs, thereby limiting the effectiveness of these defense-related proteins and the usefulness of recombinant PIs in pest control.

At this point, the development and/or the identification of inhibitors more efficient than the currently available PIs appears crucial to achieve broad-spectrum inhibition of pest protease complements, and to minimize the occurrence of compensatory or degradative processes in target pests (Jongsma et al 1996, Michaud 1997). Several strategies are currently being developed for the improvement of protease inhibitor-based control approaches, including: 1) the improvement of inhibitor binding characteristics -e.g. with reduced K_i for target pest enzymes- by site-directed mutagenesis (Urwin et al 1995), 2) the construction of bifunctional fusion inhibitors (Urwin et al 1998), 3) the isolation of effective inhibitor variants by molecular phage display (Jongsma et al 1996, Koiwa et al 1998), 4) the use of insect protease inhibitors exhibiting high affinity for insect digestive proteinases (Thomas et al 1995), 5) the use of protease regulatory pro-peptides specific to their cognate proteinase (Taylor and Lee 1997) or active against 'foreign', exogenous pest proteases (Visal et al 1998a), and 6) the isolation of novel, stress-induced inhibitors from plants exhibiting broad-spectrum activity against herbivorous pest digestive proteinases (Zhao et al 1996).

In this study we assessed the potential of γ-linolenic acid (GLA), a polyunsaturated fatty acid structurally homologous to α-linolenic acid (ALA), in inducing the synthesis of novel PI(s) in tomato. In a previous study, Farmer and Ryan (1992) clearly showed that GLA does not induce synthesis of the well-characterized serine-type inhibitors, PI-I and PI-II in tomato leaves while ALA, a precursor of jasmonic acid (JA), mediates their rapid accumulation in the plant. This observation, while confirming the specificity of ALA as a precursor of JA along the octadecanoid signaling pathway (Vick and Zimmermann 1984), does not exclude a parallel PI gene-inducing effect for GLA, as noted in animal cells (Jiang et al 1997). In plants, various unsaturated fatty acids -including linoleic acid, eicosapentaenoic acid and arachidonic acid- are potent inducers of systemic resistance to pathogens (Cohen et al 1993). Arachidonic acid (AA), produced naturally by the late blight fungus *Phytophtora infestans*, activates defense-related genes in potato via an ALA/JA-independent inducing pathway (Choi et al 1994, Fidantsef and Bostock 1998). Using papain as an affinity ligand for cysteine PIs and diet-related variants of the well-characterized digestive protease system of the insect pest, Colorado potato beetle (CPB; *Leptinotarsa decemlineata* Say) as a model (Overney et al 1997, Visal et al 1998a), GLA is shown here to induce in tomato leaves the accumulation of a 55-kDa inhibitor with broad-spectrum affinity the CPB digestive cysteine proteinases.

4.3. Materials and methods

4.3.1. Reagents

Azocaesin, bovine serum albumin (BSA), *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64), papain, Triton X-100, phenylmethylsulfonyl fluoride (PMSF), and γ-linolenic acid were purchased from Sigma (St. Louis, MO). Electrophoretic reagents and Bio Affigel-15 were from Bio-Rad (Richmond, CA). Oryzacystatin I (OCI), oryzacystatin II (OCII) and human stefin A were expressed as glutathione *S*-transferase fusion proteins in *Escherichia coli* using the plasmids pGEX-3X/OCI, pGEX-3X/OCII and pGEX-2T/HSA as expression vectors

(Michaud et al 1994, Michaud et al 1996). All other reagents were of the highest purity commercially available.

4.3.2. Plant material

Eight weeks-old tomato (*Lycopersicon esculentum* var. Vendor) seedlings, 'control' (untransformed) potato (*Solanum tuberosum* var. Kennebec) plants and oryzacystatin I-expressing potato (var. Kennebec, clone K52: Benchekroun et al 1995) plants, were grown under greenhouse conditions under a 16h:8h D/L photoperiod. For GLA treatment, the plants were sprayed with 40 μM GLA in 0.125% (v/v) Triton X-100. The leaves were harvested 24 h after induction, and stored at -80°C until use. Before papain affinity chromatography, the frozen leaves were homogenized in buffer A (50 mM Tris-HCl, pH 7.5, 10% (w/v) ethylene glycol, 100 mM PMSF). The homogenate was centrifuged at 17,000 rpm for 30 min. at 4°C. Proteins in the supernatant were concentrated at 4°C by ammonium sulfate precipitation (80% saturation), and dialyzed overnight at 4°C against buffer B (50 mM Tris-HCl, pH 7.5, 10% (w/v) ethylene glycol, 0.1% (v/v) β-mercaptoethanol). The resulting protein preparation was finally passed through a Sephadex G-25 column, and the protein content was estimated according to Bradford (1976), with BSA as a protein standard. This protein extract was used for isolation of papain inhibitor by papain affinity chromatography.

4.3.3. Insect material

Third-instar CPB larvae reared on greenhouse-grown potato plants (cv. Kennebec) were placed at 22°C in 100 ml aerated plastic arenas containing water-saturated cotton sticks and a moist filter paper, under a 16 h/8 h (L:D) photoperiod provided by cool white fluorescent lights. The fifth leaves of either 'control' potato (cv. Kennebec), OCI-expressing transgenic potato (cv. Kennebec) (Benchekroun et al 1995), tomato or eggplant were supplied to the insects at 24 h intervals over a three-day period. In parallel, third instars were starved for three days under the same growth conditions. Each experiment was repeated three times, with 10 larvae in each arena. The insect digestive soluble proteases were then extracted as described previously

(Overney et al 1997) in buffer C (100 mM citrate phosphate buffer, pH 6.0, 10% (w/v) ethylene glycol). After centrifuging the mixture at 4°C for 30 min. at 17000 rpm, the supernatant was passed through a Sephadex G-25 column (to remove low molecular-weight compounds), and used as a source of insect digestive proteases for subsequent analyses. Protein content in the extracts was determined according to Bradford (1976), with BSA as a standard.

4.3.4. Papain affinity chromatography

Cysteine proteinase inhibitor(s) in the plant extracts were purified by affinity chromatography, using a papain-BioAffigel-15 affinity matrix. The affinity matrix was prepared by incubating 30 mg of papain in deionized formamide with 1 ml of Affigel-15 (BioRad, Richmond CA), according to the supplier's instructions. After incubating the gel (1 ml) with the plant soluble protein extract (100 µg protein) for 1 h at 4°C with gentle agitation, the affinity column was packed into a 2 ml-column, and washed with 20 volumes of buffer B to remove unbound proteins. The proteins bound to the column were eluted with buffer D (1 M NH_4OH in water, containing 10% (w/v) ethylene glycol and 0.1% (v/v) ß-mercaptoethanol), plunged immediately in liquid nitrogen, and concentrated in a cold-trap automatic Speedvac (Model A5160, Savant Instruments, Inc., Farmingdale NY).

4.3.5. SDS-PAGE and immunoblotting

Proteins were subjected to 10% (w/v) SDS-PAGE (Laemmli 1970) using a Bio-Rad Mini Protein II electrophoresis unit. The gels were either stained with Coomassie Brilliant Blue R-250 or transferred onto an Hybond C membrane (Pharmacia) using the Bio-Rad mini-transfer unit. Immunoblotting was performed according to Towbin et al (1979), using a polyclonal antibody directed against potato multicystatin as primary antibody (Bolter 1993), and an alkaline phosphatase-labelled goat anti-rabbit secondary antibody (Gibco-BRL, Burlington, ON). Antibody/antigen complexes were visualized by chromogenic development, with 5-bromo-4-chloro-3-indolylphosphate and Nitro blue tetrazolium as a color indicator (Gibco-BRL).

4.3.6. Protease inhibition assays

Total protease activity in the insect extracts was determined according to a previously described procedure (Michaud et al 1993). An appropriate amount of insect extract (corresponding to 0.25 unit of protease activity) was mixed with 5 µl of inhibitor solution (~10 µg protein for OCI, OCII, human stefin A and TCPI; 10 µM for E-64), and the total volume of the mixture was adjusted to 50 µl with assay buffer (see above). After a 30 min. incubation at 37°C, 50 µl of 2% (w/v) azocaesin dissolved in assay buffer was added to the enzyme/inhibitor solution, and the complete mixture was incubated for 4 h at 37°C. After proteolysis, 300 µl of 10% (w/v) trichloroacetic acid was added to the mixture, and residual azocaesin was removed by centrifugation for 5 min. at 13000 *g*. The supernatant (350 µl) was added to 300 µl of 1N NaOH, and the absorbance (*A*) was measured at 440 nm using a Spectronic 1000 Plus spectrophotometer (Milton Roy, Rochester, NY). The A_{440} of blanks, which consisted of complete mixtures incubated for 0 h, was subtracted from each value. One unit of activity was defined as the amount of insect extract needed to cause an absorbance change of 1.0 in a 1-cm cuvette, under the conditions of the assay. All measurements were carried out in triplicate.

4.4. Results

4.4.1. GLA induces the accumulation of a novel papain inhibitor in young tomato leaves

A 55-kDa polypeptide showing affinity for papain, presumably an inhibitor of papain-like cysteine proteinases, was synthesized *de novo* in young tomato leaves sprayed with GLA (Fig. 1). This inhibitor, thereafter referred to as TCPI (tomato cysteine proteinase inhibitor), was not found at detectable levels either in non-treated (control) or in ALA-treated leaves (not shown), suggesting that it is produced via an ALA/JA-independent induction pathway. To confirm that TCPI was not a 55-kDa proteolytic fragment of tomato multicystatin (TMC), an 88-kDa cysteine PI immunologically related to potato multicystatin (PMC) (Bolter 1993), and to determine whether or not TMC (and PMC) synthesis could be induced in GLA-treated leaves, immunoblot analysis was carried out using an anti-PMC polyclonal antibody and a purified preparation of

PMC (Fig. 2). Whereas TMC is easily recognized by the PMC antibody in extracts prepared from methyl jasmonate-treated tomato leaves (Bolter 1993), no cross-reaction was noted with TCPI, indicating that TCPI and TMC are distinct inhibitors produced in the plant via apparently independent pathways. In agreement with a previous study showing that GLA does not induce accumulation of PIs activated by the ALA/JA signaling pathway (Farmer and Ryan 1992), TMC was not induced in tomato leaves treated with GLA (Fig. 2). Noteworthy, a weak signal detected in control (untreated) potato leaf extracts with the PMC antibody, the same size as PMC, was absent in GLA-treated potato leaves (Fig. 2), further suggesting that multicystatins and TCPI are expressed via distinct pathways.

4.4.2. TCPI is a broad-spectrum inhibitor of CPB digestive cysteine proteinases

The ability of TCPI to inhibit herbivorous pest digestive cysteine proteinases was assessed using quantitative inhibition assays and diet-related variants of the well-characterized CPB digestive proteolytic system as a working model (Table 1). For dietary protein hydrolysis, CPB larvae use a complex and plastic protease complement strongly influenced by the diet ingested and altered by the presence of PIs in plant tissues (Overney et al 1997, Bolter and Jongsma 1995, Visal et al 1998a). In most cases, the insect protease complement includes two major cysteine proteinases - or cysteine proteinase fractions: i) a cathepsin H-like fraction easily inhibited by the well-characterized cystatin, OCI; and ii) a cathepsin B-like fraction insensitive to this inhibitor (Thie and Houseman 1990, Michaud et al 1993). While the nonprotein inhibitor of papain-like proteinases, E-64 recognizes both fractions (i.e. about 85% of total azocaesinase activity at pH 6.0 in potato-fed larvae), currently available proteinaceous inhibitors like OCI, OCII and HSA primarily inhibit the insect cathepsin H-like fraction and show no (or weak) affinity for the cathepsin B-like fraction (Michaud et al 1993, 1995, 1996). In contrast with these narrow-spectrum inhibitions, TCPI was shown here to inhibit the insect digestive cysteine proteinases almost as efficiently as E-64, regardless of the diet provided (Table 1). In agreement with recent studies showing variations in the relative importance of cysteine and/or OCI-sensitive proteinases in CPB (Overney et al 1997, Visal et al 1998a), the inhibitory effects of

OCI, OCII and HSA drastically varied with the diet as compared to the E-64 effect, while most of the E-64-sensitive activity was also suppressed by TCPI in all extracts analyzed. Taken together, these observations suggest that TCPI effectively recognized most cysteine proteinases in the midgut of CPB larvae, and that it could act as a broad-spectrum inhibitor of the insect digestive cysteine proteinases *in vivo*.

4.4.3. TCPI retains its broad-spectrum inhibitory potency against cysteine proteinases from larvae adapted to recombinant OCI

An additional evidence showing the effectiveness of TCPI in inhibiting CPB cysteine proteinases was provided by feeding the larvae with OCI-expressing transgenic potato foliage (Fig. 3). Several recent studies showed the remarkable ability of insects to rapidly adapt their digestive protease complement to the presence of either natural or recombinant PIs in the diet, by producing insensitive proteinases compensating for inhibited proteolytic functions (for a review, see Jongsma and Bolter 1997). CPB larvae, in particular, were shown to express cysteine proteinases insensitive to PMC in response to the presence of high PMC levels in the host plant treated with methyl jasmonate (Bolter and Jongsma 1995). In a similar way, the larvae were shown here to produce cysteine proteinases sensitive to E-64 but insensitive to recombinant OCI expressed transgenic potato (Fig. 3). While specific protease activity in insects provided for 5 days with OCI-expressing potato was comparable to the activity found in control insects, susceptibility of their cysteine proteinase(s) to OCI differed markedly. In accordance with previous studies reporting partial but significant inhibitions of CPB cysteine proteinases by OCI (Michaud et al 1993, 1995), this PI inhibited protease activity by about 30% in the extracts prepared from control larvae, while this inhibition rate drastically decreased in larvae fed the transgenic material (Fig. 3). Noteworthy, this adaptation of larvae to recombinant OCI resulted in a visible loss of inhibitory potential for two structural homologues, OCII and HSA, but this steric-based 'cross-compensation' was low with TCPI, which retained most of its inhibitory potency against the E-64-sensitive proteinases of larvae fed OCI-transgenic foliage.

4.5. Discussion

A quick review of the recent literature suggests the existence of several distinct stress-induced metabolic pathways in plants. Unsaturated fatty acids, in particular, were shown to induce the accumulation of various defense-related molecules in plant tissues, either via the well-characterized octadecanoid, ALA/JA-based signaling pathway (Farmer and Ryan 1992) or via ALA-independent pathways (Choi et al 1994, Fidantsef and Bostock 1998). In this study we assessed the potential of GLA, a structural analog of ALA, in inducing the expression of cysteine PIs in tomato. In accordance with a previous paper reporting the inefficiency of GLA to induce PI genes naturally induced by ALA or JA (Farmer and Ryan 1992), TMC, rapidly induced in tomato leaves treated with methyl jasmonate (Bolter 1993), was not induced by GLA. Instead, a novel inhibitor of cysteine proteinases, TCPI, was expressed in leaves, apparently via a distinct pathway.

At this point, the induction pathway leading to TCPI accumulation remains entirely unknown. GLA is an 'unusual' fatty acid in plants, present only in a few specific families possessing a Δ^6-desaturase functional in desaturating linoleic acid [C18:2 $\Delta^{9,12}$] to GLA [C18:3 $\Delta^{6,9,12}$] (Gunstone 1992). In animals, GLA may be either elongated to C20:3, and then desaturated to AA [C20:4] (Gill and Valivety 1997), or peroxidized by LOX and lead to the synthesis of prostaglandins or leukotrienes via eicosanoid intermediates (Anderson 1989, Hildebrand and Grayburn 1991). Although AA from pathogenic fungi is a potent elicitor of defense responses in plants (Bostock 1981), conversion of GLA to AA in plant cells seems unlikely. Desaturation of GLA to stearidonic acid (18:4) was shown to occur in transgenic tobacco expressing a Δ^6-desaturase gene from *Synechocystis* (Reddy and Thomas 1996) or from *Borago officinalis* (Sayanova et al 1997), but no AA could be detected. Alternatively, GLA could act directly as a stress signal and activate defense-related genes, or, indirectly, be transformed via a metabolic pathway similar to the well-characterized octadecanoid pathway (see Fig. 4). In this way, GLA

would be transformed by a tomato leaf LOX to give GLA hydroperoxides (e.g. 9-HPOT-γ) that could be metabolized by allene oxide synthase to yield cyclopentenones structurally homologous to 12-oxo-PDA, and finally be transformed into JA analogs with gene inducing activity. The latter hypothesis, although essentially speculative, appears plausible considering that: i) LOX isoforms including at least one stress-induced form are present in tomato leaves (Heitz et al 1997), ii) a LOX isoform purified from tomato fruit not only metabolizes ALA, but also acts on LA, AA and GLA to form corresponding hydroperoxides (Regdel et al 1994), and iii) 9-HPOT-γ, a GLA hydroperoxide, is metabolized to cyclopentenones by a plant (flax) allene oxide synthase (Grechkin 1994).

Although the inducing effect of GLA remains to be elucidated, and although the general occurrence of this fatty acid in plants appears unlikely, GLA may prove particularly useful as a potent inducer of systemic resistance to pests in plants (Cohen et al 1993), and to direct the accumulation of previously uncharacterized defense-related molecules potentially useful in plant protection. In this study we isolated TCPI, an inhibitor of papain showing broad-spectrum affinity for the digestive cysteine proteinases of CPB larvae. Unlike the other proteinaceous cysteine PIs assessed until now for CPB control (Michaud et al 1993, 1995, 1996, Visal et al 1998a), TCPI shows affinity for most cysteine proteinases used by the insect to hydrolyze dietary proteins, regardless of the diet ingested and the protease complement found in the midgut environment. This observation, together with the apparent ability of TCPI to inhibit (at least partially) the OCI-/OCII-/HSA- 'insensitive' cysteine proteinase(s) induced in the larval midgut following recombinant OCI ingestion, suggests the potential of TCPI for inhibiting not only the digestive cysteine proteinases found in 'normal', potato-fed larvae, but also those accumulated *de novo* in response to various diet-related stresses (Bolter and Jongsma 1995, Overney et al 1997, Visal et al 1998a).

In this context, transforming potato with a cDNA sequence encoding TCPI could represent an attractive way to effectively alter protein digestive functions in the insect midgut, and to protect the plant from predation. Feeding assays carried out recently with the non-protein inhibitor, E-64, showed that both growth and reproductive functions of the insect were affected by large-spectrum inhibition of its midgut cysteine proteinases (Wolfson and Murdock 1995, Latoszek-Green and Bolter 1997), despite the occurrence of prote(in)ases from other mechanistic classes in the insect midgut (Thie and Houseman 1990, Novillo et al 1997). Recombinant TCPI expressed in potato, by allowing an E-64-like inhibitory effect, could lead to similar adverse effects on the insect and thus prove useful as a complement to the various approaches currently being developed for CPB control. Further studies are currently underway to assess the actual potential of TCPI in pest control, and to understand better the gene-inducing effect of GLA in tomato.

Acknowledgments. This work was supported by an operating grant from the Natural Science and Engineering Research Council of Canada to D.M.

Table 4.1. Inhibitory effect of E-64, TCPI, OCI, OCII and HSA on the digestive proteases of CPB larvae provided with different diets. Data are presented as relative inhibition rates, as compared to control extracts to which no inhibitor was added (100%). Specific activity values were 0.25 unit for potato-fed, tomato-fed, eggplant-fed and starved insects, respectively. Each datum represents the mean of 3 values ± se.

Diet	Relative inhibition (%)				
	E-64	TCPI	OCI	OCII	HSA
Potato	85 ± 4	81 ± 3	23 ± 2	30 ± 3	49 ± 3
Tomato	89 ± 2	81 ± 4	42 ± 3	47 ± 5	50 ± 5
Eggplant	58 ± 5	59 ± 6	34 ± 4	31 ± 4	29 ± 3
None (starvation)	83 ± 4	76 ± 3	61 ± 3	69 ± 2	62 ± 3

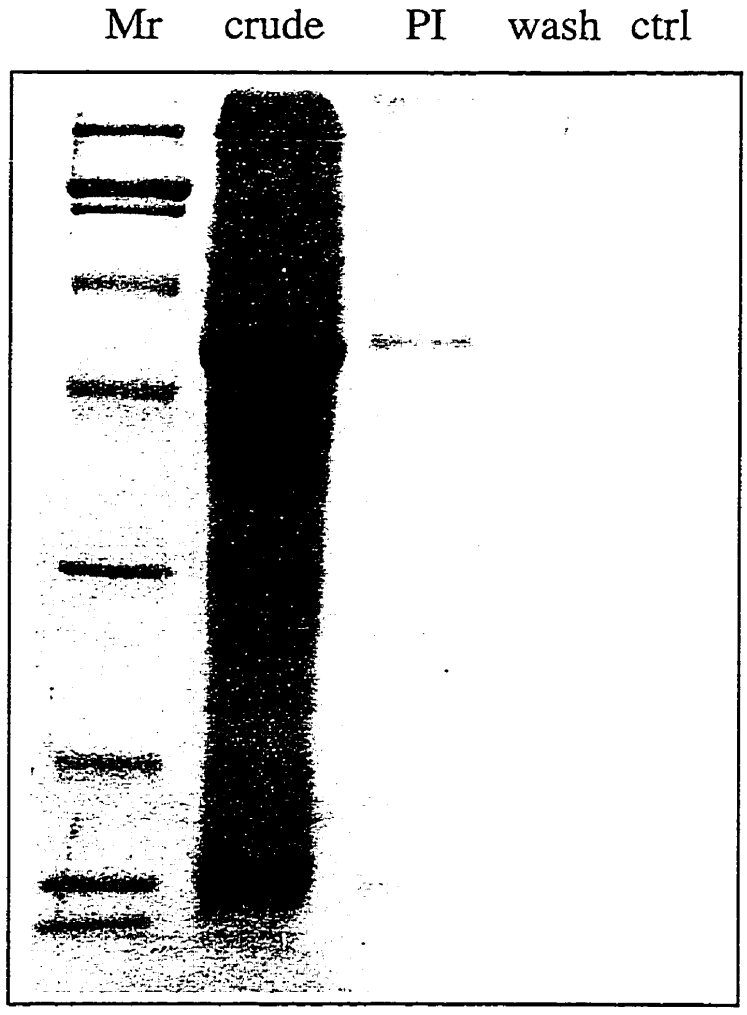
Figure legends

Figure 4.1. Purification of cysteine PI(s) by papain affinity chromatography. Mr, MW markers (200, 116, 97, 67, 45, 30, 21, 14 and 6 kDa); crude, crude extract from GLA-treated tomato leaves; PI, cysteine PI eluted from the papain column; wash, washing solution; ctrl, eluate from control (non-treated) tomato leaves.

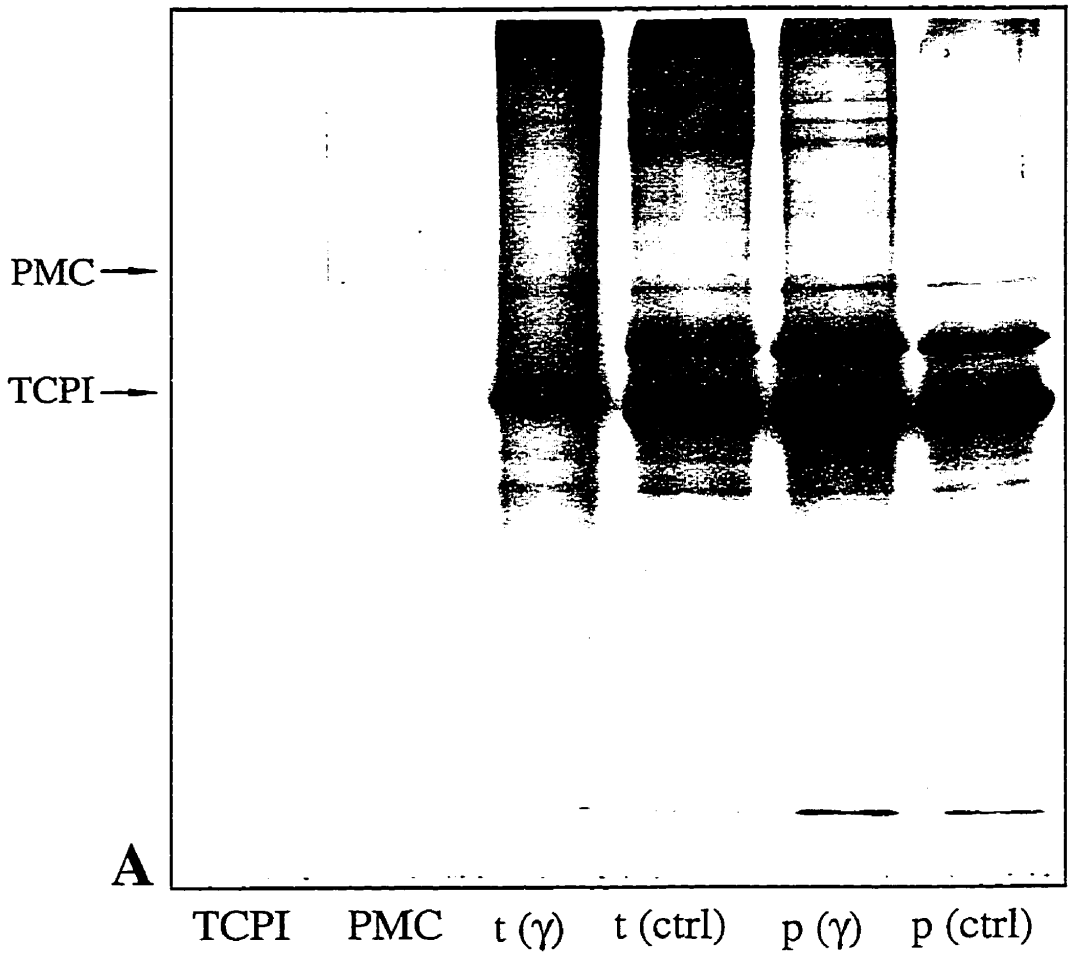
Figure 4.2. Immunoblot analysis of TCPI, tomato multicystatin (TMC), and potato multicystatin (PMC) using an anti-PMC polyclonal antibody. t (γ), crude extract from GLA-treated tomato leaves; t (ctrl), crude extract from non-treated tomato leaves; p (γ), crude extract from GLA-treated potato leaves; p (ctrl), crude extract from non-treated potato leaves. **(A):** Coomassie Blue-stained soluble proteins prefractioned by SDS/PAGE, **(B):** Immunodetection of PMC and immunologically-related proteins using an anti-PMC polyclonal antibody.

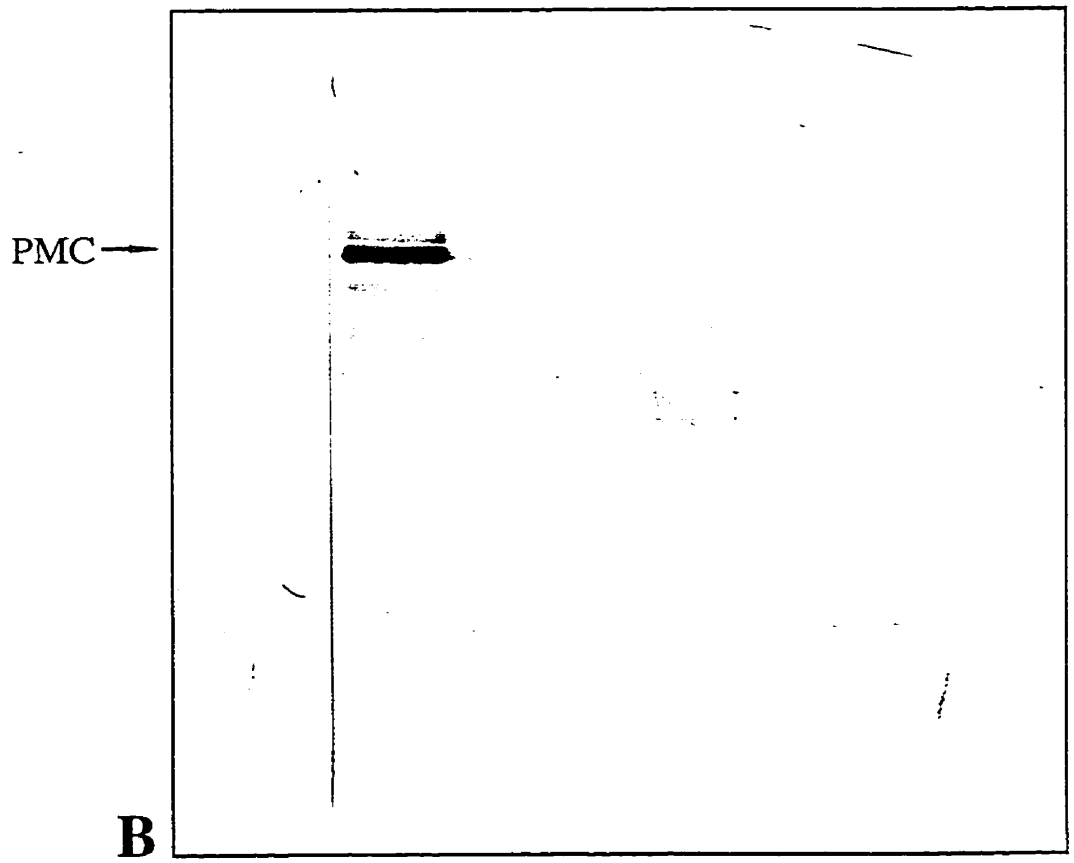
Figure 4.3. Inhibitory effect of E-64, TCPI, OCI, OCII and HSA against digestive proteases from CPB provided with OCI-expressing transgenic foliage. Insects were provided with either 'normal' (control insects) or OCI-expressing (OCI-fed insects) potato foliage for 5 days. Each datum is the mean of three values ± SE. Specific protease activity in the extracts was 0.25 unit for control and OCI-fed insects, respectively.

Figure 4.4. Synthesis of TCPI in GLA-treated tomato leaves via a hypothetical pathway similar to the jasmonic acid/octadecanoid pathway. AOS, allene oxide synthase; LOX, lipoxygenase; PI-I, proteinase inhibitor I; PI-II, proteinase inhibitor II; TMC, tomato multicystatin; 13-HPOT, 13-hydroperoxy-9(*Z*),11(*E*)15(*Z*)-octadecatrienoic acid; 9-HPOT, 9-hydroperoxy-6(*Z*),10(*E*)12(*Z*)-octadecatrienoic acid.

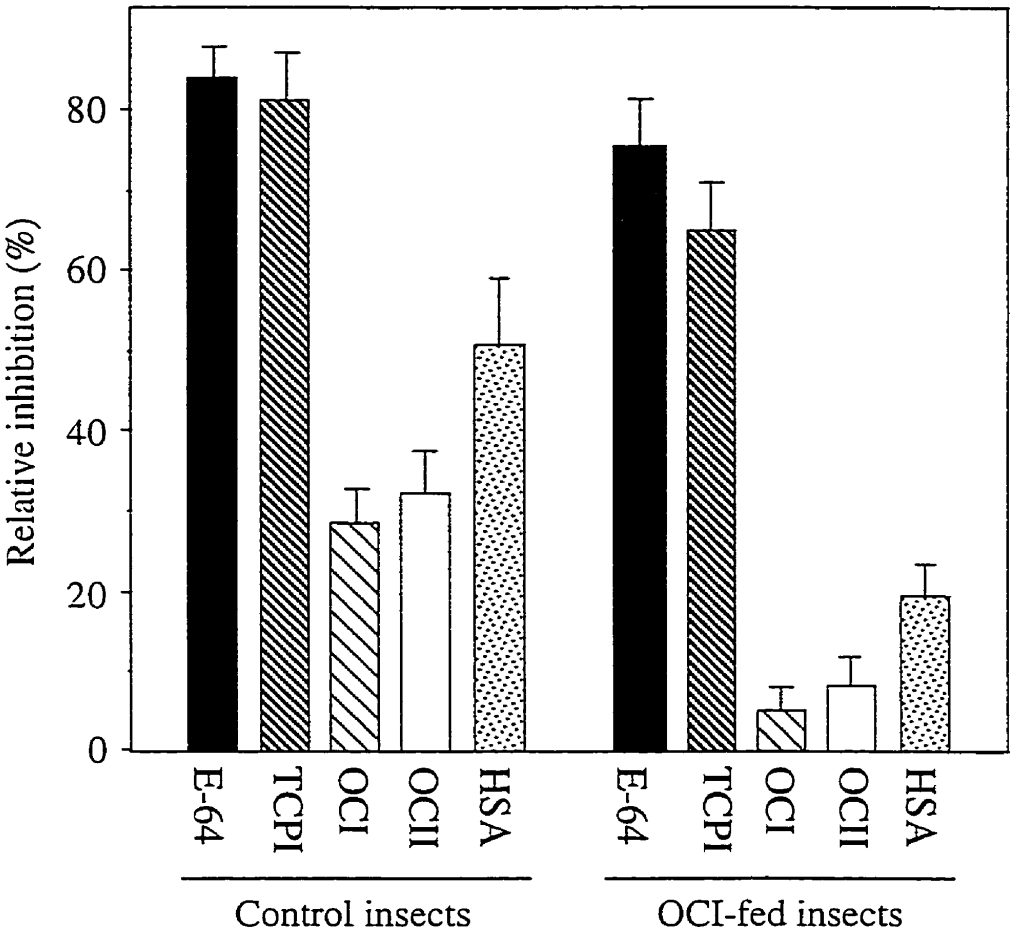


Visal et al., Figure 1

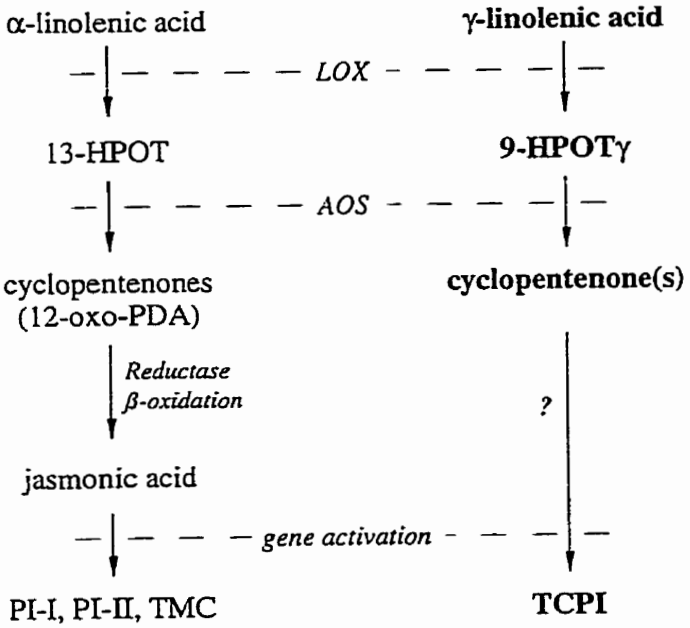




Visal et al., Figure 2



Visal et al., Figure 3



Visal et al., Figure 4

CONCLUSION

5.1. SUMMARY

Until now, approximately forty different genes have been incorporated in plants for insect resistance (reviewed by Schuler et al, 1998), including resistance genes from micro-organisms like those of cholesterol oxidase from streptomycetes and various toxins from *Bacillus* strains; and from higher plants like those of proteinase inhibitors, amylase inhibitors, chitinases and lectins, and from animals, in particular insects, like those of PIs and chitinases.

Taking into account the potential of PIs for pest control, and the known information on CPB, much of the research related to induction of defense genes in plants is currently carried out in view of identifying broad-spectrum PIs of plant origin, specific toward its major digestive cysteine proteinases. Much of the literature concentrates on fatty acids, in particular ALA, in the signaling cascade leading to the over-production or expression of various defense genes, including those coding for PIs. As discussed in the first chapter, recent studies have demonstrated the existence of JA-independent fatty acid signaling in plants. This literature review led us to build a working hypothesis stating that 'plants under stress conditions have evolved the capacity to produce proteinase inhibitors with broad-spectrum inhibitory activity against the target (digestive) proteinases of phytophagous organisms'. To achieve our goals and to verify our hypothesis, research was directed with four objectives:

5.1.1 Objective 1

"To discriminate, *in gel,* OCI-, OCII- and HSA-sensitive and -insensitive proteinases in CPB, by modifying the existing gelatin/PAGE technique" *(See **Chapter II**)*

As demonstrated by *in vitro* inhibition assays with E-64, a non-protein cysteine PI of fungal origin, ~80% of the CPB total digestive proteinase activity measured at pH 6.0 is of the cysteine-type. In addition, *in vitro* studies with specific inhibitors and substrates demonstrated that the CPB cysteine proteinases comprise two distinct sub-classes, namely the cathepsin B- and

H-like proteinases (Thie and Houseman, 1990), insensitive and sensitive to oryzacystatins I and II, respectively (Michaud et al 1993).

To validate these findings, substrate affinity chromatography was carried out to identify the molecular weight of the corresponding proteinases. By taking advantage of specificities of Divicell-ε-aminicaproic acid-Phe-Phe-CH_3 and OCI towards the cathepsin B- (K. Peters, personal communications), and cathepsin H-like proteinases (Michaud et al 1993), the CPB cysteine proteinases were fractionated into 2 forms of molecular weights of ~67 kDa and ~28 kDa, respectively. In contrast with these observations, complex proteinase patterns composed of several bands were detected after gelatin/PAGE, either under reducing or non-reducing conditions (Michaud et al 1995). This apparent complexity was also found true with bromelain, a 33-kDa model cysteine proteinase.

In substrate gelatin/PAGE systems, migration of the proteinases is affected by the strong affinity between gelatin and the hydrolytic enzymes, leading to the formation of various gelatin/proteinase complexes visualized in the form of many bands (Michaud et al 1995, Michaud 1998). To simplify these complex patterns and to make possible the determination of proteinase molecular weights, a two-step gelatin/PAGE was developed. Two bands of molecular weights ~67 kDa and ~28 kDa were detected with this two-step gelatin/PAGE technique. In addition, *in gel* inhibition assays with OCs and HSA allowed us to demonstrate that the 28 kDa band probably belongs to the OCI-sensitive, cathepsin H-family, while the 67 kDa proteinase probably belongs to the cathepsin B-family, insensitive to OCI.

By preventing the interference of gelatin during migration, and thereby minimizing false estimations about the number of 'true' protease sub-families *in gel*, this technique has helped in simplifying and eliminating ambiguity of results obtained with *in vitro* and in gel assays regarding CPB proteinase activities. In brief, CPB larvae use several proteases including two cysteine proteinases, an OCI-insensitive cathepsin B-like proteinase with a molecular weight of ~67 kDa, and an OCI-sensitive cathepsin H-like proteinase with a molecular weight of ~28

kDa., The OCI-insensitive 67 kDa proteinase is therefore 'the target molecule' to be inhibited by PI for an efficient biocontrol of CPB.

5.1.2. Objective 2

"To investigate whether or not an alteration in the diet leads to an alteration of the insect digestive proteinases, and further determine whether or not this possible change leads to a change in the ratio of OCI-sensitive to -insensitive proteinases". *(See **Chapter III**)*

Diet is known to influence the digestive physiological status of insects, including their proteolytic metabolism (Overney et al 1997). Studies with *Spodoptera exigua* fed on PI2-expressing transgenic plants have shown the expression of PI2-insensitive proteinases in the insect midgut, thereby demonstrating compensation for the PI2-sensitive proteinases (Jongsma et al 1995). In this case the diet, containing a high concentration of PI2, probably posed a diet stress which affected the physiological status of the insect, then reflected by a change in the midgut proteinases. In CPB, previous studies have shown 'qualitative' diet-related variations in the proteinase activities, either as a change in the activities of some forms, or as the appearance or disappearance of specific proteinase forms, along with quantitative changes in the ratio of E-64 sensitive to insensitive proteinases (Overney et al 1997, Visal et al 1997). To better understand the insect response to a change in diet in terms of digestive proteinases, CPB larvae were fed on different diets including potato, tomato -a non-host plant, and OCI-expressing transgenic potato, or starved for three days under similar growth conditions. Since gelatin/PAGE clearly reveals the microheterogenity in proteinase forms (Michaud 1998), this technique was utilized to visualize diet-related variation of the CPB midgut proteinases*. The results included in Chapter 3 show the over-expression of a starvation-specific proteinase (see fig. 3.1, chapter 3), thus demonstrating that a diet stress brings about changes in the digestive proteinase pool.

The main aim of this thesis was to find cysteine PIs with a broad inhibition spectrum against the digestive proteinases of the CPB, submitted to normal or dietary 'stress' conditions. As indicated above, the diet determines the physiological status of the insect, reflected in terms of change or over-expression of certain midgut proteinases. It is thus important to assess the potential of PIs not only with 'normal' proteinase complements, but also on 'proteinase variants' induced in stressed insects. In this thesis, the different proteinase complements induced in CPB larvae were used as a tool to assess the inhibition potential of two 'novel' PIs, namely 1) the pro-region of papaya proteinase IV, the 'constitutive' N-terminal regulatory sequence of the pre-mature proteinase *(see objective 3),* and 2) a cysteine PI induced by the 'stress' fatty acid GLA *(see objective 4).*

5.1.3. Objective 3

"To determine the efficiency and stability of the pro-region of papaya proteinase IV [glycyl endopeptidase (EC 3.4.22.25)] in the inhibition of CPB digestive proteinases reared on various diets." *(see **Chapter III**).*

The pro-regions of proteinases play an important role in down-regulating the activities of their cognate proteinases. In this perspective, their mode of action in inhibiting proteinases can thus be compared to PIs. Studies with the pro-region of trypsin digestive proteinase from the insect *Manduca sexta* have demonstrated the potential of insect proteinase pro-regions for insect control (Taylor and Lee 1997). The aim of this study was to determine the potential of a plant constitutive inhibitor, the pro-region of PPIV, in CPB control. This pro-region, in addition to PPIV, also inhibits papain and other papaya proteinases (Taylor et al 1995). Its possible specificity towards animal sub-families of cysteine proteinases was assessed here by *in vitro* inhibition assays with the digestive proteinases of CPB fed either on potato, tomato and OCI-expressing plants, or starved for three days under similar conditions. In summary, our results demonstrated that the pro-peptide recognizes a fraction of the insect proteinases, presumably the cathepsin H-like fraction which is also recognized by OCs and HSA. However our studies have shown the susceptibility of the pro-peptide to hydrolysis by the non-target insect midgut proteinases, which could be prevented by either decreasing the temperature of the assay or by

adding pepstatin, an aspartic PI known to block the activity of cathepsin D. The structural and functional integrity of the PPIV pro-region should thus be preserved to maintain its inhibitory effect towards CPB proteinases, clearly suggesting that the repressive effects of PIs depend on both their affinity towards the target proteinases and their stability in the midgut environment. The relatively narrow inhibition spectrum of the pro-peptide and its susceptibility to the non-target gut proteinases make it a poor inhibitor for CPB control. Nevertheless these studies suggest the general potential of plant protease pro-regions in 'pest biocontrol' biotechnology.

5.1.4. Objective 4

"To identify and isolate a novel stress-induced cysteine PI from a non-host plant, and to characterize its inhibitory spectrum against both OC-sensitive and -insensitive digestive proteinases of CPB larvae reared on various diets. " *(see **Chapter IV**)*

There are a few reports on the induction of cysteine PIs in response to JA or its precursor molecules (Farmer and Ryan 1992, Bolter 1993, Bolter and Jongsma 1995, Botella 1996). ALA is a well-known key precursor of the octadecanoid fatty acid signaling pathway in plants, known to induce, notably, the synthesis of multicystatins in both tomato and potato (Farmer and Ryan 1992, Bolter 1993). In contrast, almost nothing is known about the involvement of GLA, an isomer of ALA, as a signaling molecule in plants. Farmer and Ryan (1992) have demonstrated the inefficiency of GLA in inducing Pin1, Pin2 and PMC genes in plants. However, in view of previous findings showing the production of hydroperoxy-GLA by tomato fruit lipoxygenase (Regdel et al 1994), the production of stable epoxides by the action of flax allene oxide synthase on hydroperoxy-GLA (Greshkin 1995), and the induction of defense responses by various PUFAs in plants (Gandhi and Weete 1991, Bostock et al 1981, 1992, Bloch et al 1984, Ricker and Bostock 1994, Fidantsef and Bostock 1998), GLA was used here as an 'inducer' of 'novel' (unknown) PIs in young tomato plants, a non-host plant for CPB.

Young tomato leaves were treated with 40 μM GLA, and a CPI of 55 kDa (TCPI) was induced and purified by papain affinity chromatography. Its inhibitory potency was tested with CPB digestive proteinases reared on various diets or under starvation conditions. *In vitro* inhibition

assays demonstrated that TCPI inhibited ~90% of the CPB E-64-sensitive proteinases, whereas in combination with the OCs and HSA, it showed no complementary proteinase inhibition. In addition, the inhibition spectrum of TCPI against the proteinases of CPB reared on various diets remained essentially unchanged (or poorly affected) by their modified proteinase system, suggesting that TCPI is a broad-spectrum inhibitor of CPB proteinases regardless of the diet, and that it probably recognizes both cathepsin B-like and cathepsin H-like CPB proteinases. Interestingly, immunodetection studies with an anti-potato multicystatin (PMC) antibody failed to demonstrate the induction or over-expression of PMC in GLA-treated potato plants. On the other hand, a basal level of PMC observed in the untreated plants disappeared after GLA treatment, suggesting the existence of a JA-independent (and competitive) defense signaling pathway induced by GLA, interfering with the ALA (JA)-signaling pathway.

This part of the research confirms our working hypothesis stating that "plants *(tomato)* under stress conditions have evolved the capacity to produce PIs with broad-spectrum inhibitory activity against target (digestive) proteinases of phytophagous organisms *(CPB)* ".

In addition, the research presented in this thesis has led to the important finding of the existence of an alternate fatty acid signaling pathway in plants, induced by GLA and producing unknown intermediates that lead to the expression of defense genes, including the gene of a novel inhibitor, TCPI.

{* Although the two-step gelatin/PAGE technique may help in defining the 'basic nature' of insect proteinases, gelatin/PAGE remains interesting for revealing the microheterogenity amongst proteinases. Hence this technique, rather than two-step gelatin/PAGE has been utilized in studying the fine variations characterizing the insect protease complements induced in response to diet *(please refer to subsequent objectives)*}.

5.2. FUTURE PERSPECTIVES

The potential of PIs in pest control depends primarily on the insect proteinase/plant PI interactions (Jongsma et al 1996, Michaud 1997). Studies with insects fed on artificial diets as well as on transgenic plants expressing recombinant PIs have shown repressive effects of PIs on growth and fecundity of certain insects. Recently, however, many studies have also reported that insects may develop physiological resistance (compensation) toward these PIs. The insect midgut proteinase system is complex, with several protease families and sub-families. The effectiveness of PIs therefore depends on their inhibition spectrum, their ratio with gut proteinases, the spectrum of gut proteinases and the quality of the diet. Our studies, carried out with these parameters in mind, led us to conclude that, overall, four main 'points' should be considered to improve the general usefulness of PIs in pest control:

1) understanding diet-related digestive proteinase plasticity in insects,

2) understanding insect proteinase/PI interactions,

3) improving the inhibition spectrum and stability of known PIs, and

4) finding novel PIs via novel 'stress' induction pathways.

5.2.1. Understanding diet-related digestive proteinase plasticity in insects

In CPB larvae, only ~40% of the E-64-sensitive proteinases, represented by a cathepsin H-like proteinase are inhibited by OCs and HSA. Studies have also shown that the diet has a tremendous influence on the insect midgut proteinases (Overney et al 1997, Visal et al 1997). For example, under stress conditions such as starvation, feeding on a non-host plant or on a PI-expressing transgenic plant, differential expression of insect proteinases is evident (chapter III, fig.3.1). Studies with the PPIV pro-region demonstrated their potential in insect control (chapter III), but also demonstrated the importance of non-target insect gut proteinases, when assessing the inhibitory effect of PIs. Finally, studying the nature of protease sub-families in insects, for example cathepsin B- and H-like cysteine proteinases in CPB, would be of great help in

identifying (novel) PIs or in 'fabricating' effective inhibitors tailored for the inhibition of specific target proteinases.

5.2.2. Understanding insect proteinase-PI interactions

Although the over-expression of PIs in transgenic plants was shown to reduce the growth of some insects (Hilder et al 1987, Johnson et al 1989), studies in recent years demonstrated the inefficiency of several PIs to alter insect growth and development, mainly due the adaptability of the insect repeatedly exposed to PIs (Broadway and Villani, 1995, Broadway 1996, 1997). Resistance or adaptability to PIs may develop either by the expression of non-target proteinases which inactivate/degrade the inhibitor (Michaud et al 1995, Ishimito and Chrispeels, 1996, Michaud et al 1996, Michaud 1997), by the presence or the production of proteinases insensitive to these PIs (Jongsma et al 1995, Michaud 1995, Broadway 1995), by enhanced activity or over-expression of the digestive enzymes, or by the modification of a set of related digestive hydrolase activities (Konarev 1996). In parallel, plants have also co-evolved various mechanisms to overcome the destructive effects of insect proteinases, for instance by increasing the complexity of their protease inhibitory set, by increasing their inhibitory activity and heterogeneity, or by producing 'special' inhibitors like the bifunctional inhibitors (e.g. protease/α-amylase inhibitors) (Konarev, 1996). The following scheme (fig.5.1) represents a paradigm for further studies aimed at elucidating the complex and various insect proteinase/plant PI interactions and the expected plant-insect responses.

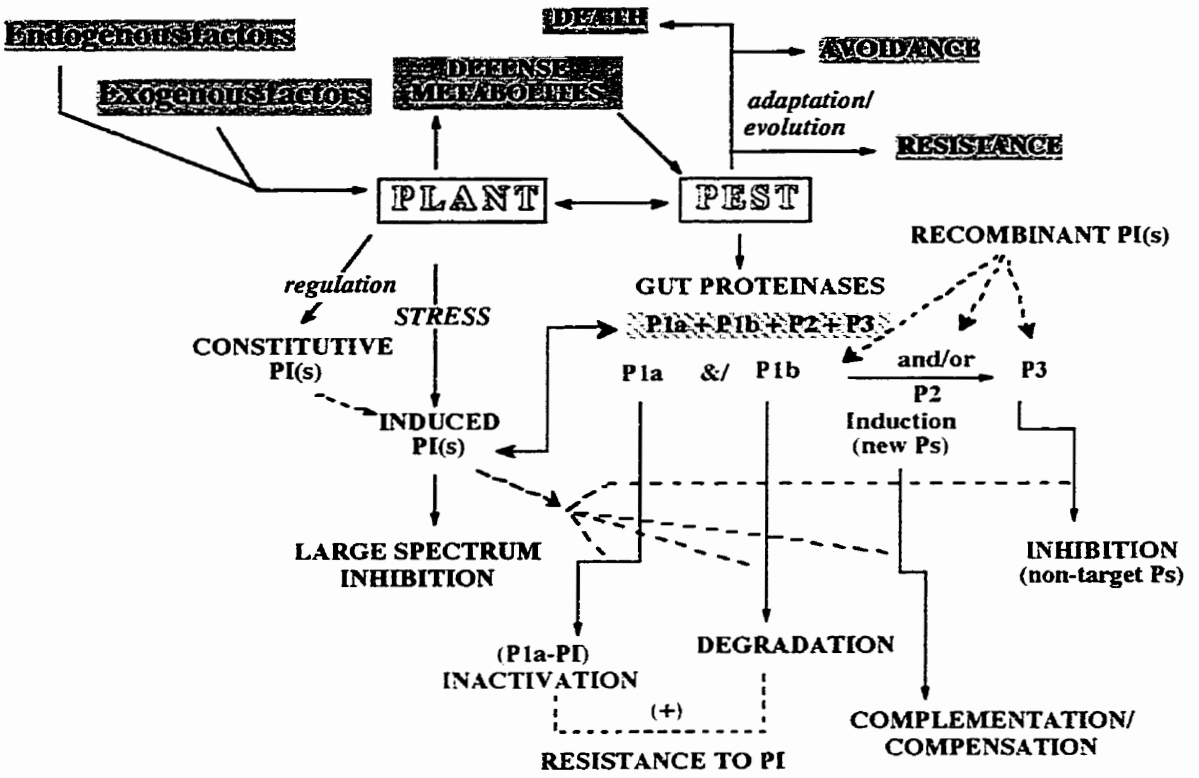


Fig. 5.1. Schematic representation of pest proteinase-plant PI interactions, and expected responses in plants and pests. **PI** -plant proteinase inhibitor, **P1a, P1b, P2, P3** pest proteinase classes or sub-classes, differentially inhibited by plant PIs.

5.2.3. Improving the inhibition spectrum and stability of known PIs

Several observations in the present thesis revealed the importance of studying the multiple interactions between PIs and the whole set of digestive proteinases. Such studies probably will help us in predicting resistance problems due to the plasticity of the insect proteinase complements, which results from an 'indigenous defense phenomenon' under natural feeding conditions, or from a 'thirst for survival' generally represented by a rapid adaptation of the digestive proteinase complements. It is therefore necessary to primarily and carefully scrutinize the structures of both target proteinases and their putative inhibitors, and their interactions at the molecular level including those implicating non-target proteases. Such studies will yield important information about the structural characteristics of proteinases and their cognate PIs. They will help in screening novel PIs with a broad inhibition spectra, in choosing more than one

PI, in engineering the existing PIs by site-directed mutagenesis to favor their activity or stability, and in isolating effective inhibitor variants of specific proteinases by phage display. In any case, the effect of non-target proteinases against the modified PI should also be considered. Once basic information is available on the structure of the insect proteinases and plant PIs, the structure of PIs could be efficiently manipulated by protein engineering techniques. Finally these PIs, either alone or in combination, could be expressed in transgenic plants to achieve a more efficient biocontrol of insect pests.

5.2.4. Finding novel PIs via novel induction pathways

Most living organisms respond to different stimuli and are able to induce genes via specific signaling cascades, many of which are still unknown. Tapping these signaling pathways may lead to the elucidation of new cascades, to the identification of their intermediate molecules, their effect on gene expression, the number of genes expressed/suppressed and the end products synthesized (e.g. enzymes or metabolites), and to the understanding of these pathways in plant protection.

The jasmonic acid (JA) pathway is one of the best understood defense-related signaling pathways in plants (review Wasternack and Parthier, 1997, Mueller, 1997), where ALA is the key precursor of the octadecanoid-signaling pathway leading to the biosynthesis of JA. The role of other PUFAs, for instance AA and eicosapentaenoic acid (EPA), in defense responses have been studied (Bostock et al 1981, 1992). In any case, LOX is an important key enzyme responsible for the hydro-peroxidation of these fatty acid molecules in plants. Recently, Fidantsef and Bostock (1998) observed a differential expression of Pin2 and P4 (pathogenesis-related protein 4) in potato tubers infected with *Phytophthora infestans* or treated with MJ/JA and AA. P4 was not induced by the MJ/JA treatment nor was Pin2 induced by AA or fungal infection. These results thus demonstrated the differential effects of fatty acids, namely MJ/JA (derivatives of ALA) and AA, as inducers of signal-response pathways (Fidanstef and Bostock 1998; Fig. 5.2)

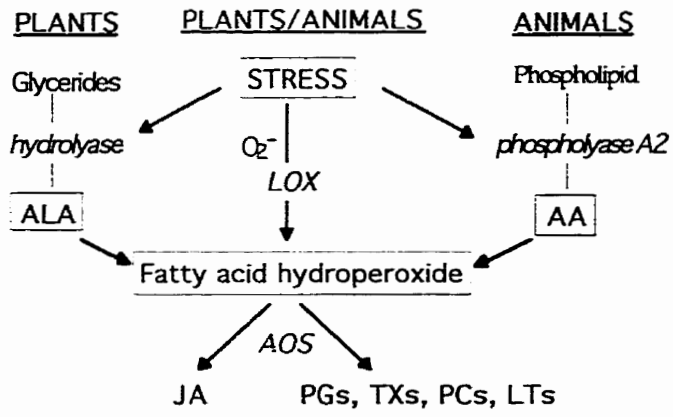


Fig. 5.2. Fatty acid signaling in plant and animal systems (the common steps are shown in red).

GLA, an isomer of ALA, triggers the over-expression of maspin (mammalian serine proteinase inhibitor) in human cancerous cell cultures, but its involvement in plant defense was not documented. In Chapter IV, we described the induction, isolation and functional characterization of a 55 kDa CPI in young tomato leaves in response to GLA treatment. This is the first report on the induction of PI in response to GLA in plants.

The signaling pathway induced by GLA, however, remains to be elucidated. The octadecanoid (ALA) pathway in plants has been compared to an analogous fatty acid pathway in animals, known to trigger the synthesis of PGs, LTs, PCs and TXs, which are involved in inflammatory responses in animals (Blechert et al 1995). Another parallel pathway, the GLA pathway is also known in mammals, where the reduction of hydroperoxy-GLA forms ($PGs_{(\gamma)}$, $LTs_{(\gamma)}$, $PCs_{(\gamma)}$ and $TXs_{(\gamma)}$), is responsible for the anti-inflammatory responses (Pullman-Mooar et al, 1990). It is not yet clear how GLA induced the expression of the TCPI gene in tomato, but the existence of a similar pathway involving LOX and AOS as intermediary enzymes in the signaling cascade in plants cannot be ruled out (see Fig. 4.4)

In addition, while the biochemical signaling cascade leading to the expression of TCPI following GLA treatment remains to be elucidated, another question remains to be answered: Is

GLA produced in 'non-GLA' producing plants? Although restricted to only few families in the plant kingdom, GLA has been reported to accumulate in soybean leaves exposed to high temperatures (Rennie and Tanner 1991), suggesting that plants, which otherwise do not accumulate GLA, do possess the ability to produce this fatty acid when exposed to extreme stress conditions. If so, one could speculate that plants may possess a mechanism for efficiently metabolizing this fatty acid, as supported by the greater affinity of tomato fruit lipoxygenase for GLA than for AA to form hydroperoxy-GLA, 9-HOPT(γ) (Regdel 1994). Further, like 9-HOPT(α), a hydroperoxy product of ALA, 9-HOPT(γ) has been demonstrated to be an effective cyclopentone substrate for the flax allene oxide synthase (AOS) (Greshkin 1994). Taken together, these data enable us to postulate occurrence of similar kind of metabolic cascade in GLA-treated tomato leaves. Although this pathway remains to be demonstrated, an attempt could be made to hypothetically identify the probable steps involved in tomato leaf GLA-signaling. Figure 6.3, essentially speculative, might be useful to unravel the probable steps involved in putative GLA-signaling pathway in tomato leaves.

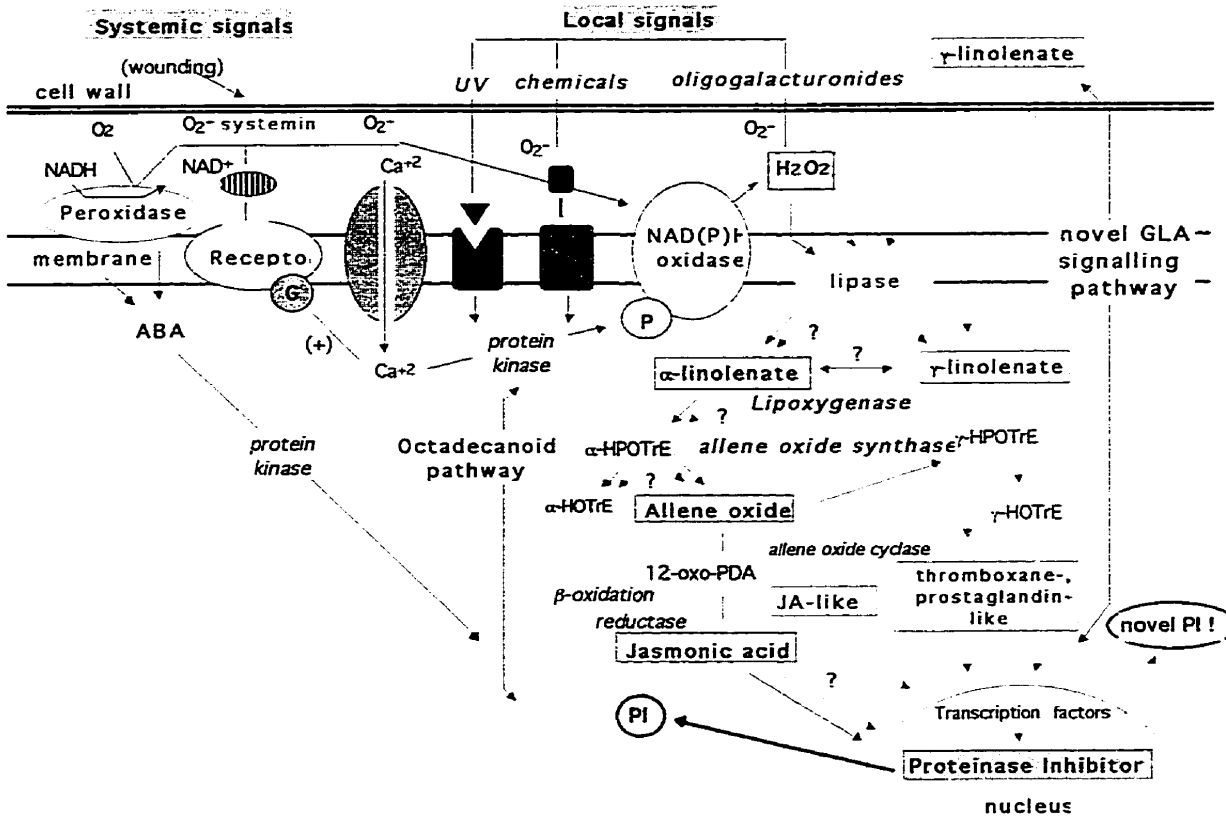


Fig.5.3. A hypothetical alternate fatty acid signaling pathway in tomato leaves, responsible for the induction of TCPI in response to γ-linolenic acid treatment (the hypothetical pathway is shown in red).

5.3. CONCLUDING REMARKS

In brief, the recent literature in the field of insect PI-based biocontrol (including the content of this thesis), stresses out the importance of evaluating the complexity, the plasticity and the specificity of pest proteinases (both at the class and sub-class levels), as well as their differential interactions with proteinaceous PIs. Along with the various approaches currently considered for improving PI-based control, understanding and elucidating the GLA-induced pathway appears a possible logical step for designing control measures against pests and pathogens, notably by identifying novel defense-related proteins. Finally, it is clear that simultaneous multi-dimensional studies must be carried when planning PI-based control of insects. Such studies may help in understanding and putting together, piece-by-piece, the important parameters responsible for plant resistance and insect compensatory responses in plant-insect systems.

ANNEX I - REVIEW ARTICLES

A.1.1.

INHIBITION OF PROTEIN DIGESTION IN HERBIVOROUS INSECTS: THE CHOICE OF EFFECTIVE INHIBITION STRATEGIES

Michaud D & S Visal

A quick survey of the recent literature clearly shows the remarkable ability of herbivorous insects to rapidly adapt their digestive proteolytic system to the diet ingested. In particular, the presence of both natural and recombinant protease inhibitors (PIs) in plant tissues was shown to induce synthesis *de novo* of either 'sensitive' and/or 'insensitive' proteases in the midgut of several insects, as a way to compensate for inhibited proteolytic functions. The appearance of such compensatory responses, together with the degradation of certain protein PIs by insensitive proteases and the general occurrence of a various set of digestive proteases in herbivorous insects would help them eluding the antinutritive effects of plant PIs, thereby limiting the effectiveness of these plant defense-related proteins and the usefulness of recombinant PIs in pest control. After presenting a general paradigm integrating the multiple interactions taking place in plant/insect systems between plant PIs and their target proteases, a general strategy will be proposed (i) to assess the actual effect of specific PIs in the inhibition of insect digestive proteases, and (ii) to identify inhibitors or inhibitor combinations potentially efficient in the control of specific pests. Distinct protease complements induced in Colorado potato beetle (*Leptinotarsa decemlineata* Say) larvae by various diet treatments will be used as a model, along with PIs exhibiting different affinity spectra for the beetle digestive proteases, including the nonprotein inhibitors E-64 and pepstatin A, and the protein inhibitors oryzacystatin I, oryzacystatin II, human stefin A, tomato cathepsin D inhibitor, a 55-kDa cysteine PI from tomato, and the pro-region of papaya proteinase IV.

(Invited Review: Arch. Insect Biochem. Physiol., 1998)

A.1.2.

'PLANT PI/PEST PROTEASE BIOCHEMICAL INTERACTIONS'- A WORKING MODEL

Visal S and D Michaud

(NO ABSTRACT)

(In 'Recombinant protease inhibitors in plants', Michaud D (Eds.), Landes RG/Academic Press).

ANNEX II : MEETING COMMUNICATIONS

A.2.1.

INSECT PEST CONTROL: SEARCH FOR PUTATIVE PROTEINASE INHIBITORS AGAINST COLORADO POTATO BEETLE

Visal S, Overney S, Nguyen-Quoc B, Michaud D and S Yelle

Bioengineering economically important plants with proteinase inhibitors (PIs) is a promising method for the control of pests. The major digestive proteinases (80%) of Colorado potato beetle (CPB) are of cysteine type, *viz.* cathepsin B and H. We showed that 60% of the cysteine proteinases are inhibited by oryzacystatins (OCs) and suggested the specificity of OCs towards CPB cathepsin H. In plants, there is an apparent absence of direct interference of OCs on the endogenous protease pool. Using the "glutathione-S-transferase" fusion protein system, we have produced stefin A, a human cathepsin B inhibitor. *In vitro* assays with stefin A showed no increase in inhibition as compared with OCs. Therefore, to inhibit the remaining insect proteinases for an efficient control of CPB development, a search for other PIs has been carried out. These inhibitors should be complementary to OCs. We have purified class-specific proteinases, using various affinity matrices. At first, the OC-affinity column allowed us to discriminate which kind of the insect proteinases are bound to this family of cysteine PIs. We assume that these proteinases are inhibited by OCs. Secondly, the use of leupeptin-affinity column allowed us to purify both serine and cysteine proteinases which are not inhibited by OCs. These are used as ligands to isolate corresponding inhibitors from various plants.

(Oral presentation 37th Annual Meeting of the Canadian Federation of Biological Sciences, Montréal, Québec, Canada, June 16-18, 1994, Abstract no. 201)

A.2.2.

IDENTIFICATION AND PURIFICATION OF A TOMATO CYSTEINE PROTEINASE INHIBITOR: POTENTIAL USE FOR CONTROL OF COLORADO POTATO BEETLE

Visal S, Overney S and S Yelle

Many defense responses in plants, like the activation of secondary metabolic pathway and the production of defense-related proteins are induced by signaling molecules in response to pathogen and insect attack. Plants, when treated with methyl jasmonate or its precursors are reported to induce the synthesis of defense proteins, among which are the proteinase inhibitor (PI) proteins. Bioengineering plants with PIs is a promising method to control insect pests. Colorado potato beetle (CPB) is one of the most economically important pests of potato. Its effective control has not yet been achieved. Previous studies have shown that 80% of the digestive proteins are of the cysteine type. A protein of ≈ 55 kDa from gamma-linolenic acid treated tomato leaves shows cysteine proteinase inhibitory activity, characteristic to the cystatins. *In vitro* inhibition of larval proteases with tomato cystatins was compared with already available oryzacystatins (OCI and OCII) and human stefin A. *In vitro* inhibitory assays with individual PIs showed that the tomato cystatin inhibits 70% of proteinase activity, which is 4 times more efficient than OCI and 2 times more efficient than OCII and stefin A. Also, when individual PIs- OCI, OCII and stefin A were combined with tomato cystatin, no considerable additive increase in the inhibition of proteinases was seen as compared to the inhibition with the tomato cystatin alone. Further characterization and application of this tomato cystatin in pest control will be discussed.

(Presented in the form of a poster at The XIIIth International Plant Protection Congress, The Hague, The Netherlands, July 2-7, 1995)

A.2.3.

IDENTIFICATION OF A γ-LINOLENIC ACID-INDUCED TOMATO LEAF CYSTATIN-LIKE PROTEIN WITH POTENTIAL FOR BIOCONTROL OF THE HERBIVORY PEST COLORADO POTATO BEETLE

Visal S, Michaud D and S Yelle

Plants treated with jasmonate or its precursor molecules have been shown to induce defense-related proteins in plants, notably serine and cysteine proteinase inhibitors (PIs). In this study, a ~55-kDa protein showing like cysteine PIs (cystatins) papain-inhibitory activity *in vitro* was isolated from γ-linolenic acid-treated tomato leaves by papain-affinity chromatography. The ability of this cystatin-like protein to inhibit *in vitro* digestive proteinases of the potato pest, Colorado potato beetle (CPB), was then compared with those of the cystatins oryzacystatin I (OCI), oryzacystatin II (OCII), and human stefin A (HSA), which are known to inhibit 30 to 40% of the total CPB digestive proteinase at pH 6.0. The tomato cystatin has proved to be the most efficient inhibitor, inactivating ~70% of the total activity. When combined with the tomato inhibitor, OCI, OCII and HSA did not show any complementation effect, suggesting that the tomato cystatin inactivates cysteine proteinases either sensitive and insensitive to the three other cystatins. Until now, efficient control of the CPB has not been achieved using the well-known rice cystatin OCI as a biopesticide. The inhibitor isolated from tomato leaf, with its larger inhibition spectrum against CPB digestive proteinases, may represent a good alternative to rice cystatins for developing CPB-resistant transgenic potato plants.

(Oral presentation at the Annual Meeting of the American Society of Plant Physiologists, San Antonio, TX, July 26-31, 1996, pp. 87).

A.2.4.

DIFFERENTIAL SUSCEPTIBILITY OF ORYZACYSTATIN I AND ORYZACYSTATIN II TO PROTEOLYTIC CLEAVAGE

Michaud D, Cantin L, Visal S and TC Vrain

The relative susceptibilities of oryzacystatin I (OCI) and oryzacystatin II (OCII) to proteolytic cleavage were assessed by peptide mapping using trypsin, chymotrypsin, and vacuolar proteases from strawberry leaves as test enzymes. Although the potential cleavage sites for trypsin (basic residues) and chymotrypsin (aromatic residues) are conserved in OCI and OCII, the proteolytic patterns observed in SDS polyacrylamide gels after either tryptic or chymotryptic cleavage differed. In parallel, the cystatin-insensitive proteases from strawberry leaf vacuoles caused a sequential hydrolysis of both cystatins, but OCII seemed more sensitive than OCI to the action of the plant proteases. Interestingly, the proteolytic intermediates produced after cleavage of the native inhibitors by either trypsin and chymotrypsin were active. While the differential proteolysis of OCI and OCII suggests a distinct steric accessibility of their cleavage sites, the ability of these inhibitors to remain active during the first steps of their hydrolysis by serine proteinases indicates the high conformational stability of their active (inhibitory) site. Considering the roles generally attributed to cystatins and other proteinase inhibitors in plant defense, this latter observation could also indicate a certain adaptation of OCI and OCII to 'non-target' proteases of pests and pathogens, which use in many cases cystatin-insensitive proteinases of the trypsin and the chymotrypsin families for protein hydrolysis. Work supported by a grant from the BC Research Council to TCV.

(Presented in the form of a poster at the Annual Meeting of the American Society of Plant Physiologists, San Antonio, TX, July 26-31, 1996).

A.2.5.

PHYSIOLOGICAL 'CROSS-RESISTANCE' TO CYSTEINE PROTEINASE INHIBITORS IN COLORADO POTATO BEETLES FEEDING ON ORYZACYSTATIN I-EXPRESSING POTATO PLANTS

Visal S, Yelle S & D Michaud

It has been recently proposed that the efficiency of plant proteinase inhibitors to interfere with the development of herbivorous insects is partly determined by their inhibitory spectrum against the pest proteinases, and by the ability of the insects to overcome their inhibitory effects by synthesizing new, insensitive proteinases. In this study, the ability of different cystatins to inhibit digestive proteases of the Colorado potato beetle (CPB; *Leptinotarsa decemlineata* Say) was assessed after providing the insect with transgenic potato plants expressing oryzacystatin I (OCI), a rice inhibitor showing affinity for a fraction of the insect proteinases. Interestingly, most of the cysteine proteinases newly-synthesized in the insect digestive tract following OCI ingestion were insensitive not only to this inhibitor but also to oryzacystatin II and human stefin A, two cystatins showing affinity for the same proteinases in the CPB midgut. Similarly, the effect of a tomato cystatin-like protein showing large-spectrum activity against CPB digestive proteinases (Visal et al., 1996, Plant Physiol. 111s: 40) was also significantly affected after feeding the insect with the OCI-expressing plants. Taken together, these observations suggest the occurrence of physiological 'cross-resistance' to cystatins in CPB adapted to OCI. This kind of cross-resistance to proteinase inhibitors in insects should be taken into account when considering their integrated use for insect control. This work was supported by a grant from the Conseil de Recherches en Pêches et Agroalimentaire du Québec.

(Presented in the form of a poster at the Quadrennial Joint Annual Meeting of the American Society of Plant Physiologists and the Canadian Society of Plant Physiologists, Vancouver, BC, August 2-6, 1997, P1114, pp. 220).

A.2.6.

THE PRO-REGION OF PAPAYA PROTEINASE IV INHIBITS INSECT DIGESTIVE CYSTEINE PROTEINASES

Visal S, Taylor MAJ and D Michaud

Three distinct digestive protease complements were induced in larvae of the herbivorous pest, Colorado potato beetle (CPB; *Leptinotarsa decemlineata* Say), and used as a model system to assess the ability of the pro-region of papaya proteinase IV (pro-PPIV) to act as an inhibitor of insect digestive cysteine proteinases. As shown by gelatin/PAGE and complementary inhibition assays, a recombinant form of pro-PPIV produced in *Escherichia coli* inhibited a fraction of the insect proteases also inhibited by the well-characterized inhibitor of cysteine proteinases, oryzacystatin I (OCI). In contrast with OCI, the inhibitory potency of pro-PPIV was affected by increasing the temperature of the assay, suggesting a certain alteration of its structural integrity by the insect non-target proteases. This apparent susceptibility of the pro-region to proteolysis was confirmed by SDS-PAGE, after incubating the inhibitor with the different insect extracts. As seen on gel, selective inhibition of the insect aspartate proteinase, cathepsin D, with the aspartate-type inhibitor, pepstatin A, preserved the activity of pro-PPIV against cysteine proteinases by preventing its hydrolysis. Taken together, these observations suggest the potential of plant protease pro-regions as regulators of cysteine proteinases in biotechnological systems, and show the ability of protease inhibitors to protect the integrity of 'companion' defense-related proteins from the action of insensitive proteases in target pests.

(Presented in the form of a poster at The Annual Meeting of the American Society of Plant Physiologists, Madison, WI, June 27-July 1, 1998).

A.2.7.

PLANT PROTEINASE INHIBITORS AND INTERACTION WITH INSECT GUT PROTEINASES

Visal S

NO ABSTRACT (Invited Talk)

(Invited talk at the minisymposium 'Plant insect interactions' Annual Meeting of the American Society of Plant Physiologists, Madison, WI, June 27-July 1, 1998)

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IMAGE EVALUATION
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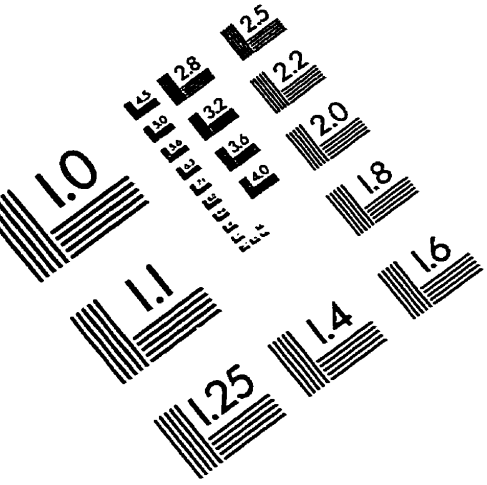

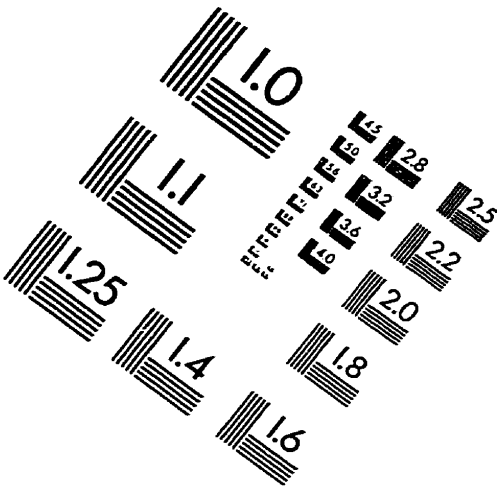

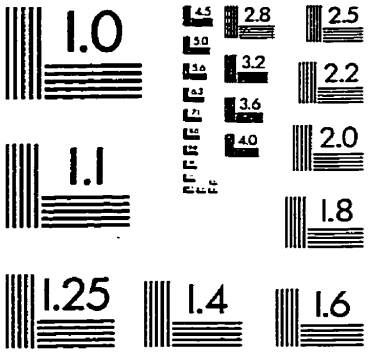

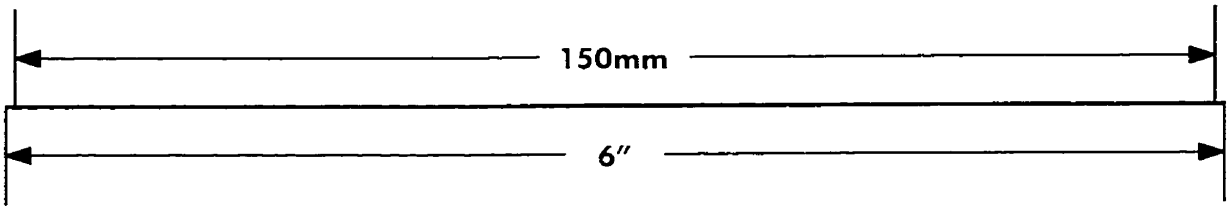

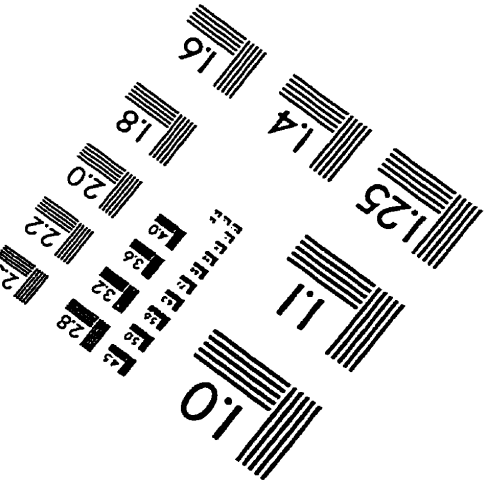

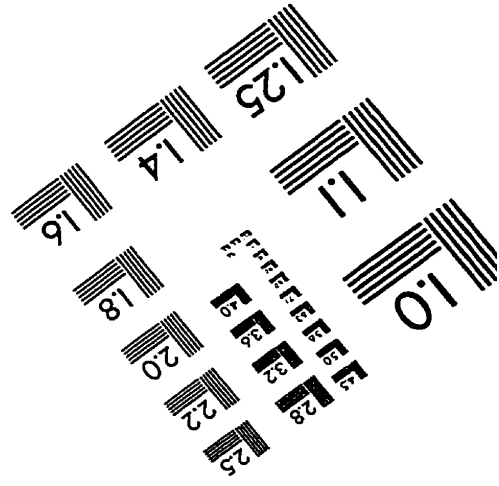

APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989