UNIVERSITY OF PADOVA

DOCTORAL THESIS

DOCTORATE SCHOOL OF CROP SCIENCE

CURRICULUM OF CROP PROTECTION – CYCLE XXII

Department of Land Use and Agro-Forestry Systems – Plant Pathology

Pectin Methylesterase Inhibitors in Plant Defence Against Pathogens

Head of the School: Ch.mo Prof. Andrea Battisti

Supervisor: Dr. Alessandro Raiola

Doctoral Student: Ibrahim Mohammed Kamal Elmaghraby

THESIS SUBMISSION DATE

01st FEBRUARY 2010

Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

01. 02. 2010

Ibrahim Mohammed Kamal Elmaghraby

A copy of the thesis will be available at *http://paduaresearch.cab.unipd.it/*

To who taught me the value of science and education,
To who taught me to stand up for what I believe,
To who always behind me, loving, supporting and believe in me,
To the guidance, knowledge and wisdom,
To the source of inspiration and the mean of sacrifice,
To who I would not be who I am today without them,

To my parents

The source of everything

LIST OF CONTENTS

	Page
CONTENTS	vii
ACKNOWLEDGEMENTS	xi
LIST OF ACRONYMS	xiii
LIST OF FIGURES	xvii
SUMMARY	**1**
RIASSUNTO	**3**
GENERAL INTRODUCTION	**5**
1. The plant cell wall	6
2. Pectic enzymes	7
3. PMEs and plant defences	9
4. Regulation of PME activity	12
5. Pectin methylesterase inhibitors (PMEIs)	14
1. CHAPTER I	
An Increase in Pectin Methylesterification Restricts Bacterial Infection by *Pectobacterium carotovorum* in Arabidopsis	**19**
1.1. Introduction	**21**
1.2. Materials and Methods	**23**
1.2.1. Plant lines and growth conditions	23
1.2.2. Bacterial growth and infection	23
1.2.3. Cell Wall Isolation	24
1.2.4. Bacterial Growth on Different Pectic Substrates	24
1.2.5. RT-PCR analysis	24

1.3. Results **26**

1.3.1. Overexpression of *AtPMEI* genes and *AtPME3* gene knockout reduce susceptibility to *Pectobacterium carotovorum* in Arabidopsis 26

1.3.2. Bacterial growth is impaired by pectin methylesterification 28

1.3.3. Defence genes expression in *AtPMEI* and *Atpme3* plants after *P. carotovorum* infection 30

1.4. Discussion **32**

2. CHAPTER II

Alterations of the *Turnip Vein-Clearing Virus* Spreading in *Arabidopsis thaliana* Expressing Pectin Methylesterase Inhibitors **35**

2.1. Introduction **37**

2.1.1. Plant viruses and their control 37

2.1.2. Plant virus movement 37

2.1.3. Movement of TMV: a model system 38

2.1.4. *Turnip vein-clearing virus* (TVCV): a tobamovirus infecting cruciferous 39

2.2. Materials and Methods **41**

2.2.1. Virus purification 41

2.2.2. Virus infection of Arabidopsis plants 41

2.2.3. TVCV CP assay 42

2.2.4. Expression and purification of TVCV MP 42

2.2.4.1. cDNA preparation of TVCV MP 42

2.2.4.2. Construction of the expression vector 42

2.2.4.3. Expression of 6xHis-tagged TVCV MP 43

2.2.4.4. Purification of 6xHis-tagged TVCV MP 44

2.2.4.5. Western blot analysis 44

2.2.5. Study of TVCV MP-PME interaction 45

2.2.5.1. Intercellular washing fluid (IWF) isolation 45

2.2.5.2. Detection of PME activity 45
2.2.5.3. MP-PME binding by affinity chromatography 46
2.2.5.4. Immunodetection of MP immobilized to the Sepharose matrix 46
2.2.5.5. Isoelectrofocusing and gel detection of PME 47
2.3. Results **48**
2.3.1. Pectin methylesterase and virus movement 48
2.3.2. Infection of Arabidopsis by TVCV 48
2.3.3. Viral coat protein detection 50
2.3.4. Systemic accumulation of TVCV in rosette leaves of wild-type and overexpressing AtPMEI-1 and AtPMEI-2 Arabidopsis plants 51
2.3.5. Cloning and expression of TVCV encoded movement protein (TVCV MP) 54
2.3.6. Purification and detection of 6xHis-tagged TVCV MP 57
2.3.7. Isolation of Cell Wall-Associated Arabidopsis PMEs and examination of the binding to TVCV MP 59
2.3.7.1. Intercellular Washing Fluid (IWF) Isolation 59
2.3.7.2. Binding of the TVCV MP to PME 60
2.3.7.3. IEF gel detection of interacting PME 61
2.4. Discussion **62**

CONCLUSIONS **65**

REFERENCES **69**

ACKNOWLEDGEMENTS

First, I would like to express my deepest gratitude and appreciation to my family for their continuous encouragement, support, love and care.

My sincere thanks and gratefulness to Dr. Alessandro RAIOLA who supervised this work for his overall guidance, precious advices and help in each phase of this thesis.

I would like to express my deep thankful and appreciation to the staff members of doctorate school, for their help, support, and giving me the opportunity to carry out this work. Sincerely thanks to Prof. Andrea BATTISTI, director of the school for his generous support and encouragement.

Special thanks to all staff members of the "Plant Pathology Group" at Padova University for their professional help and coordination, in particular; Prof. Francesco FAVARON, Dr. Luca SELLA, Ms. Carla CASTIGLIONI, Ms. Silvana ODORIZZI

My thanks also awarded to Ms. Laura NICOLETTI and secretary members of plant pathology and TeSAF Department for their following up and deep concern.

Acknowledgements are also extended to the collaborative group at Rome University "La Sapienza" for their help and sharing their knowledge and experiences to brought this work to completion, In particular Prof. Felice CERVONE, Prof. Daniela BELLINCAMPI, Prof. Giulia De LORENZO and Dr. Vincenzo LIONETTI.

Grateful thanks to Italian Ministry of Foreign Affairs, Cooperation office, for supporting scholarship and facilitating my presence in Italy.

Furthermore, I would like to thank the staff members of CLOA, Agricultural Research Center, Giza, Egypt for their help, support and encouragement with special thanks to Prof. Dr. T. ABD ELMOITY, Prof. Dr. B. ALI, Prof. Dr. E. HASSAN, Prof. Dr. A. RAGAB, Prof. Dr. A. Tolba, Prof Dr. M. Abdelmoniem, Eng. A. RABIE and Eng. M. MOSTAFA, also Grateful thanks to Dr. Mona HEFNI for her continuous encouragement.

My cordial thanks, appreciation and gratefulness to Mohammed HEWIDY and Maha ELSAYED for everything.

Finally, my deepest thanks to all my friends and colleagues at Padova University for their help, friendship and unforgettable moments we shared together.

Ibrahim M. K. ELMAGHRABY

Italy, Padova, January 2010

Scientific Acknowledgements:

- Dr. Vincenzo Lionetti, Rome University “La Sapienza” for selecting homozygous Atpme3 lines and cell wall characterization of mutant.
- Prof. Ulrich Melcher, Oklahoma State University for kindly supplying pTVCV50 plasmid.

Financial Source:

Research was supported by a grant from University of Padova (Progetto di Ateneo CPDA075725).

LIST OF ACRONYMS

AcPMEI	*Actinidia chinensis* - Pectin methylesterase inhibitor
AtPMEI(s)	*Arabidopsis thaliana* - Pectin methylesterase inhibitor(s)
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	Base pair
°C	Celsius degree
CaMV	*Cauliflower mosaic virus*
CaPMEI1	*Capsicum annuum* - Pectin methylesterase inhibitor
cDNA	Complementary deoxyribonucleic acid
CFU	Colony Forming Unit
CNBr	Cyanogen bromide
CP	Coat protein
CWDE(s)	Cell wall degrading enzyme(s)
DM	Degree of methylesterification
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DPI	Day post-infection
E81	Lime pectin with 81% degree of methylesterification
E. coli	*Escherichia coli*
ER	Endoplasmic reticulum
g	Gram
h	Hour
HGA	Homogalacturonan
His	Histidine
IEF-PAGE	Isoelectric focusing - Polyacrylamide gel electrophoresis
IgG	Immunoglobulin G
IPTG	Isopropyl-β-D-thiogalactoside
IWF(s)	Intercellular Washing Fluid(s)

kDa	Kilo Dalton
KO	knockout
LB	Luria-Bertani
M	Molar
MeOH	Methanol
µg	microgram
mg	Milligram
min	Minute
µl	Microliter
ml	Milliliter
mM	Millimolar
MM	Minimal medium
mm^2	Millimeter square
MP	Movement protein
NBT	4-nitro blue tetrazolium chloride
Ni-NTA	Nickel-nitrilotriacetic acid
nm	Nanometer
nt	Nucleotide
OD	Optical density
OG(s)	Oligogalacturonide(s)
ORF(s)	Open reading frame(s)
P60	Lime pectin with 60% degree of methylesterification
PAA	Polyacrylamide gel
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Plasmodesmata
PEG	Polyethylene glycol
PEL(s)	Pectate lyase(s)

PG(s)	Polygalacturonase(s)
PGA	Polygalacturonic acid
p*I*	Isoelectric point
PME(s)	Pectin methylesterase(s)
PMEI(s)	Pectin methylesterase inhibitor(s)
PNL(s)	Pectin lyase(s)
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Round per minute
RT-PCR	Reverse transcription-polymerase chain reaction
sec	Second
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
ssRNA	Single stranded ribonucleic acid
subsp.	Subpecies
TAE	Tris-Acetate-Ethylenediaminetetraacetic acid
TBS	Tris-buffered saline / alkaline phosphate buffer
TMV	*Tobacco mosaic virus*
TVCV	*Turnip vein-clearing virus*
v/v	Volume per volume
WT	Wild-type
w/v	Weight per volume

LIST OF FIGURES

	Page
General Introduction	
Figure 1. Pectinolytic enzymes.	**9**
Figure 2. De-methylesterification of pectins by pectin methylesterases (PME).	**12**
Figure 3. Calcium-pectin-crosslink as egg box model structure.	**13**
Figure 4. Molecular surface of the PME-PMEI complex.	**16**
Chapter I	
Figure 1.1. Resistance of *AtPMEI-1* and *AtPMEI-2* plants to *P. carotovorum* infection.	**27**
Figure 1.2. Resistance of *Atpme3* to *P. carotovorum*.	**28**
Figure 1.3. Bacterial growth on liquid synthetic media containing PGA or two series of model pectins (E81 and P60).	**29**
Figure 1.4. Reduction of bacterial growth on cell walls isolated from transformed *AtPMEI* plants and *Atpme3* mutant after 12 h of culture.	**30**
Figure 1.5. Gene expression in response to *P. carotovorum* infection.	**31**
Chapter II	
Figure 2.1. Tobamovirus phylogenetic analysis.	**40**
Figure 2.2. pQE30 vector with multiple cloning site.	**43**
Figure 2.3. Purified TVCV.	**49**
Figure 2.4. Symptoms of TVCV infection in Arabidopsis plants.	**49**
Figure 2.5. SDS-PAGE analysis of total proteins isolated from Arabidopsis wild-type rosette leaves.	**50**
Figure 2.6. Infection kinetics following the TVCV CP accumulation in uninoculated Arabidopsis rosette leaves (systemic infection) in wild-type and AtPMEI plants.	**52**

Figure 2.7. Electrophoretic protein pattern obtained from directly TVCV-inoculated leaves (local infection) 6 DPI. **53**
Figure 2.8. Accumulation of TVCV CP determined after seven days post-infection. **54**
Figure 2.9. Amino acids alignment between TMV MP and TVCV MP. **55**
Figure 2.10. TVCVMP-pQE30 expression vector used to transform *E. coli.* **56**
Figure 2.11. Agarose gel analysis of TVCVMP-pQE30 construct. **57**
Figure 2.12. Expression and purification of His-tagged TVCV MP (30 kDa). **58**
Figure 2.13. Western blot analysis for 6xHis tagged TVCV MP. **59**
Figure 2.14. Gel diffusion assay showing the PME activity. **60**
Figure 2.15. Immunodetection of MP immobilized to the Sepharose matrix using an anti- polyhistidine antibody. **60**
Figure 2.16. Thin layer IEF separation in the pH range 3-10 of PME before and after affinity chromatography. **61**

SUMMARY

The plant cell wall is a physical barrier against pathogens and its modification is often associated to changes in plant defence responses. Pectin is responsible for mechanical properties of the wall such as porosity, adhesion and rigidity and is the substrate of pectin-degrading enzymes such as polygalacturonases and pectin methylesterases (PMEs). The action of these enzymes is prerequisite for subsequent cell wall degradation by other polysaccharide degrading enzymes. Pectin is secreted into the wall in a highly methylesterified form and subsequently de-esterified *in muro* by PME. The level and pattern of pectin methylesterification play a role in constitutive resistance to fungal and bacterial pathogens influencing the susceptibility of the wall to microbial pectin-degrading enzymes. PME activity is finely regulated by the presence of multiple enzyme isoforms and by the action of pectin-methylesterase inhibitors (PMEI). PMEI over-expression has been used to stably increase pectin methylesterification and decrease PME activity in Arabidopsis plants. Transformed plants, so far resulted more resistant to the necrotrophic fungus *Botrytis cinerea*, have been also found less susceptible to the soft rot pathogen *Pectobacterium carotovorum*. Also a knockout of a selected PME isoform has been found less susceptible to bacterial infection.

On the other hand, plant PME has been shown to interact with viral movement protein (MP) of some viruses and in the case of *Tobacco mosaic virus* (TMV) this interaction has been shown to be important for cell-to-cell and systemic spread of the virus through the plant. However, the role of the PME-MP interaction to virus movement has not been elucidated. Because PME is finely regulated *in planta* by several factors, including proteinaceous inhibitors, Arabidopsis plants over-expressing PMEIs have been analysed for

the *Turnip vein clearing virus* (TVCV) propagation in comparison with untransformed plants and obtained data reveal an alteration of the virus spreading in Arabidopsis plants overexpressing a PME inhibitor. In addition, a plant PME interacting with TVCV MP has been detected.

RIASSUNTO

La parete cellulare costituisce una barriera fisica contro i patogeni e le sue modificazioni sono spesso associate a cambiamenti nelle risposte di difesa delle piante. La pectina è responsabile delle proprietà meccaniche della parete come porosità, adesione e rigidità ed è il substrato di enzimi degradativi come poligalatturonasi e pectine metilesterasi. L'azione di questi enzimi è il prerequisito per la degradazione della parete da parte di altri enzimi. Le pectine sono sintetizzate come polimeri altamente metilesterificati e secrete nello spazio apoplastico dove sono de-esterificate dalla pectina metilesterasi. Evidenze sperimentali indicano che il livello e pattern di metilazione delle pectine svolge un ruolo nella resistenza costitutiva della pianta a patogeni batterici e fungini. La regolazione dell'attività della PME è determinata sia dalla differente espressione di isoforme diverse che ad opera di inibitori proteici dell'enzima (PMEI). La sovraespressione di PMEI è stata impiegata per ottenere un incremento della metilesterificazione delle pectine in piante di Arabidopsis. La riduzione dell'attività metilesterasica totale, associata all'incremento del grado di metilesterificazione delle pectine rispetto alle piante di tipo selvatico, ha comportato nelle linee transgeniche per gli inibitori una significativa riduzione della suscettibilità a due agenti di marciume, *Pectobacterium carotovorum* e *Botrytis cinerea*. E' risultata anche significativamente diminuita la suscettibilità al betterio *P. carotovorum* in un mutante "knockout" per un'isoforma di PME.

Studi recenti hanno evidenziato che la PME di pianta riveste un ruolo attivo nel movimento cellula-cellula e sistemico del TMV interagendo con la proteina di movimento codificata dal virus. Tuttavia il ruolo della interazione PME-MP riguardo il movimento non è stato fino ad ora chiarito. Con lo scopo di studiare le possibili interferenze della PMEI durante il processo di infezione virale, le piante di Arabidopsis sovraesprimenti inibitori di pectina metilesterasi sono state analizzate per la resistenza a un tobamovirus delle crocifere (TVCV) e i risultati ottenuti hanno evidenziato una alterazione nella diffusione sistemica

del virus in piante di Arabidopsis sovraesprimenti un inibitore di PME rispetto alle piante di tipo selvatico.

GENERAL INTRODUCTION

Plant cell walls represent the first cell layer encountered by the pathogens. At the simplest level, the walls provide a physical barrier between pathogens and the internal contents of plant cells. The majority of fungi and bacteria need to breakdown this barrier to gain access to plant tissues and, once inside, to release nutrients to sustain their growth and to complete the invasion process. An increased understanding of the interactions between pathogens and their host plants, also at the cell wall level, could be used to improve disease resistance.

1. The plant cell wall

Plant cell walls are highly sophisticated structures mainly composed of complex mixture of polysaccharides (i.e. cellulose, hemicellulose and pectin), enzymes and structural proteins. In addition, may also contain other non-carbohydrate components, such as lignin or cutin. Hemicelluloses and pectins are synthesized within Golgi vesicles whereas cellulose, one of the major cell wall polysaccharides, is created at plasma membrane and arranged in microfibrils and often referred to the cell wall skeleton (Fry, 2004).

Plant cell walls give cells their structure, strength, rigidity and protect against abiotic and biotic factors including osmotic, drought stress and pathogens. The primary cell wall is deposited during cell growth, and needs to be mechanically stable and enough extensible to permit cell expansion, it is composed of cellulose microfibrils embedded in a highly hydrated polysaccharide matrix which is derived from two major classes of polysaccharides, pectins and hemicelluloses and proteins that interact in various ways, forming macromolecular structures that have some degrees of randomness but many shared features (Pelloux *et al*., 2007, Wolf *et al*., 2009). While secondary cell walls are deposited after the cessation of cell growth and confer mechanical stability upon cell types such as xylem elements and sclerenchyma cells.

Pectins, probably the most complex macromolecules in nature, are considered one of the main structural components of the middle lamellae and primary plant cell

walls in dicotyledonous plants and non-graminaceous (non-grass) monocots, where they comprise 30–35% of cell wall dry weight, while lower levels (2–10%) are found in grass plants (Mohnen, 2008). Pectins are highly complicated polysaccharides that might contain as many as 17 different monosaccharides as pectic constituents, including galacturonic acid (Ridley *et al*., 2001; Vincken *et al*., 2003). Moreover, five pectinaceous polysaccharides can be distinguished; homogalacturonan (HGA), rhamnogalacturonan I (RG-I), rhamnogalacturonan II (RG-II), xylogalacturonan (XGA) and apiogalacturonan (APG) which differ in both the structure of the macromolecule backbone and the presence and diversity of side chains. RG-I is the only pectic not built on a HGA backbone, but rather on a polymer of galacturonic acid and rhamnose disaccharide subunits (Wolf *et al*., 2009).

The most abundant pectic polysaccharides is HGA, constituting 65% of total pectin, it is composed of a linear homopolymer of α-1,4-linked galacturonic acid, which can be either methylesterified at the C-6 carboxyl (Mort *et al.*, 1993) and/or carry acetyl groups at O-2 and/or O-3 (Rombouts and Thibault, 1986; Ishii, 1995) during synthesis of the backbone.

Pectins are synthesized in the Golgi apparatus by the sequential addition of nucleotide sugars by glycosyltransferases, following which they are secreted to the apoplast as highly methylesterified structure, where they form various types of bonds, including covalent, ionic and hydrogen bonds and deesterified *in muro* by pectin methylesterases (PMEs) (Willats *et al*., 2001; Pelloux *et al*., 2007). During secretion into intercellular spaces pectins may form a jelly like matrix that is intercalated with cellulose/hemicellulose structure (Carpita and Gibeaut, 1993). Due to its cohesive and interacting properties, pectin is critical for tissue integrity, porosity and accessibility to cell wall degrading enzymes (CWDEs).

2. Pectic enzymes

Pectin is a dynamic structure continuously remodeled by the action of enzymes. Pectin enzymes are heterogeneous group of related enzymes that hydrolyze the pectic substances, present mostly in plants, and are widely distributed in higher plants and microorganisms (Whitaker, 1990). Pectin enzymes can be classified based on their preferential substrate, highly methylesterified pectin or low methylesterified pectin and polygalacturonic acid (pectate). Pectinases are of prime importance for plants as they help

in cell wall extension and softening of some plant tissues during maturation and storage. They also aid in maintaining ecological balance by causing decomposition and recycling of waste plant materials. Plant pathogenicity and spoilage of fruits and vegetables by rotting are some other major manifestations of pectinolytic enzymes (Jayani *et al*., 2005).

The action of pectin enzymes and, in particular, of endopolygalacturonases (endo-PGs) on cell walls appears to be a prerequisite for cell wall degradation by other CWDEs: cellulases and hemicellulases (Karr and Albersheim, 1970). During plant development and infection by pathogens, pectin can be degraded by an array of enzymes that can either depolymerize pectin i.e. polygalacturonases (endo-PG; EC 3.2.1.15 or exo-PG; EC 3.2.1.67), pectin lyases (PNL; EC 4.2.2.10) and pectate lyases (PEL; EC 4.2.2.2) (Kars and van Kan, 2004), or alter its structure i.e. pectin acetylesterases (PAE; EC 3.1.1.6) and pectin methylesterases (PME; EC 3.1.1.11) (Figure 1). When the degree of methylation (DM) of pectin decreases, pectin becomes more accessible to depolymerizing enzymes (Koch and Nevins, 1989).

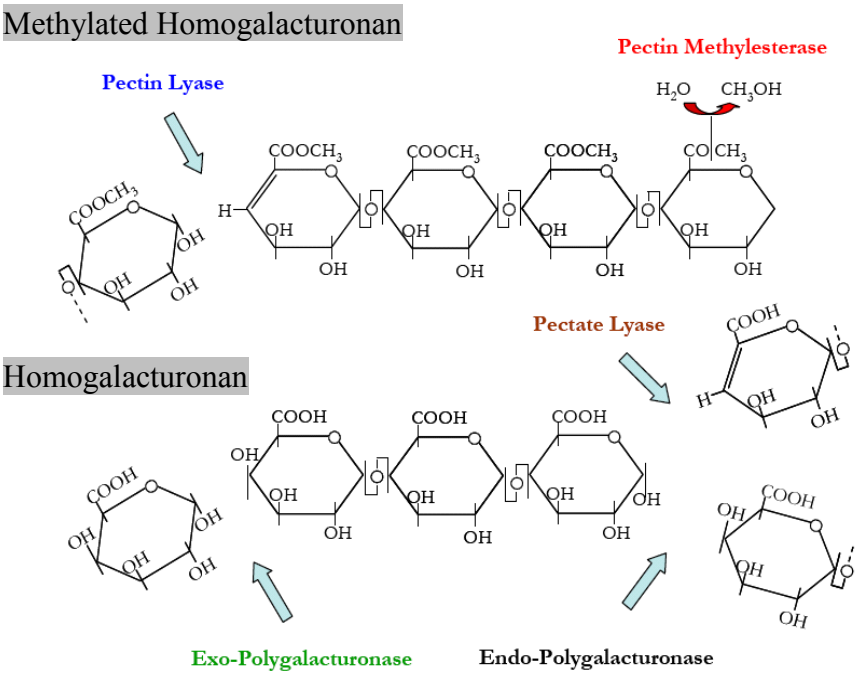

Figure 1. Pectinolytic enzymes. Endo-PGs catalyze the hydrolytic cleavage of α-(1,4)-galacturonan linkages between non-methylated D-galacturonic acid residues whereas Exo-PGs follow the same mode of action but at the non-reducing end of chain. PNLs catalyze β-elimination cleavage of methylesterified polygalacturonic acid in methylated pectin rather than pectate which is the preferred substrate of PEL. PMEs catalyze the demethylesterification of the C6-linked methyl-ester groups of HGA, releasing methanol and protons and creating free carboxyl groups.

3. PMEs and plant defences

Pectin methylesterases belong to class 8 (CE8) of the carbohydrate esterases (CAZy website, http://www.cazy.org/fam/CE8.html) (Henrissat, 1991). Plant PMEs belong to large gene families, for example, in *Arabidopsis thaliana* 67 *PME* genes have been predicted (The Arabidopsis Genome Initiative, 2000; Markovič and Janeček, 2004). Plant PMEs are mostly encoded as large pre-pro-proteins. The pre-region or signal peptide is necessary for protein targeting to the endoplasmic reticulum (ER). PME is thought to be secreted to the apoplasm via the Golgi network together with highly methylated pectin. In addition to its role in PME targeting in the cell wall, the pro-region may act as an intramolecular chaperone, which could either prevent the correct folding of PME or directly inhibit the

enzyme activity, in order to prevent premature demethylesterification of pectins before their insertion in the cell wall (Micheli, 2001; Giovane *et al.*, 2004). Most of the purified plant PMEs have neutral or alkaline isoelectric points (p*I*), and are bound to the cell wall via electrostatic interactions.

Thus far, plant PMEs have been involved in diverse processes associated with growth and development, such as cell wall remodelling, root tip elongation, seed germination, fruit ripening, loss tissue integrity and pollen tube growth (Micheli, 2001; Giovane *et al.*, 2004; Pelloux *et al.*, 2007). Moreover PMEs have been found to be involved in defence responses against pathogens and abiotic stresses. Since cell wall provides a physical barrier between pathogens and the internal content of plant cells, its modification are often associated with plant defence responses (Vorwerk *et al*., 2004).

The action of PME is required to make pectin susceptible to hydrolysis by enzymes such as PGs and PELs, allowing the possible generation of biologically active oligogalacturonides (OGs) (Moloshok *et al*., 1992, Cote *et al*., 1998). The induction of plant defences by OGs and their efficacy was shown to be due to the degree of polymerization and methylesterification (Boudart *et al*., 1998; Vorwerk *et al*., 2004). It has been observed in wild strawberry (*Fragaria vesca*) transgenic plants, over-expressing a fruit-specific PME isoform (FaPE1), a constitutive activation of pathogen defence responses, resulting in higher resistance to *Botrytis cinerea* probably due to the presence of partially de-methylated OGs (Osorio *et al.*, 2008). Moreover, Tobacco and Arabidopsis plants, expressing an attenuated version of PGII of *Aspergillus niger*, have a reduced content of homogalacturonan (Capodicasa *et al*., 2004) and are more resistant to *B. cinerea* and *Pseudomonas syringae*. It has been shown that these plants have enhanced defence responses possibly due to an increased accumulation of OGs or to perturbation of cellular defence status due to the altered cell wall architecture (Ferrari *et al*., 2008).

PMEs are important in controlling the degree of methylesterification of pectins. The decreases and increases in PME activities, observed in antisense or overexpressing plant, associated changes in the DM of cell wall pectins, have been correlated with changes in the susceptibility of plants to pathogens. For example, in *Solanum tuberosum*, differences in susceptibility to *Erwinia carotovora* correlate with the DM of pectin, with resistant genotypes having a higher DM of pectin than the susceptible genotypes (McMillan *et al.*,

1993; Marty *et al.*, 1997). Likewise, *A. thaliana* plants with reduced PME activity have a higher DM of pectin and are more resistant to fungal infection by *B. cinerea*. The reduced symptoms caused by the fungus were related to its impaired ability to grow on methylated pectins (Lionetti *et al.*, 2007).

PMEs are also involved in different processes during viral infection. *Nicotiana tabacum* PMEs promote the movement of *Tobacco mosaic virus* (TMV) by binding to the virus-encoded movement proteins necessary for the propagation of the virus. From inoculated non-vascular tissues, virions are loaded into the phloem in a PME-independent manner and subsequently spread systematically from source to sink organs. The viral exit out of the host phloem was demonstrated to be PME-dependent and the reduced expression of PME in antisense plants delayed the TMV egress from the vascular system (Dorokhov *et al.*, 1999; Chen *et al.*, 2000; Chen and Citovsky, 2003).

PMEs also participate in plant-nematode interaction during parasitism. AtPME3 isoform from Arabidopsis is required to aid cyst nematode parasitism by interacting with a secretory cellulose binding protein (CBP) from nematode *Heterodera schachtii*. It has been demonstrated that transgenic plants overexpressing *AtPME3* exhibited enhanced susceptibility to the nematode (Hewezi *et al*., 2008).

Furthermore, there are indication that PMEs may also play a role in defence against herbivores. In several plant species, herbivore attack increase PME expression and activity (Divol *et al*., 2005; Schmidt *et al*., 2005; Giri *et al*., 2006; von Dahl *et al*., 2006). Also the emissions of methanol (MeOH) are increased during herbivory and demethylation of pectin by PMEs was suggested to be the source of the emitted MeOH (von Dahl *et al*., 2006). These observations suggest that PME may participate in the induction of defence responses during insect herbivory either by generating MeOH as a signal molecule or via changes in the structural properties of the cell wall. In a recent work, by silencing Na*PME1*, a herbivore-inducible *PME* gene in *Nicotiana attenuate*, it has been demonstrated that this enzyme participates in the enhanced production of MeOH and in the elicitation of anti-herbivore responses (Körner *et al*., 2009).

4. Regulation of PME activity

HGA, the main pectic component, is deposited in the cell wall in a highly (70–80%) methylesterified form (O'Neill *et al*., 1990) and demethylesterified (Figure 2) by PME that catalyzes the hydrolysis of a methylester from pectic polyuronides and produce long stretches of free carboxylic groups (negatively charged) which lead to the formation of the so-called "egg-box" model structures as a results of formation of calcium cross-bridges between different HGA chains (Liners *et al*., 1989; Moustacas *et al*., 1991; Ridley *et al*., 2001; Willats, *et al*., 2001; Jayani *et al*., 2005). At least 10 contiguous unesterified galacturonyl residues are required to build a stable cross-link between chains. (Liners *et al*., 1992; Vincken *et al*., 2003) (Figure 3). PME was identified in carrot root juice from about two centuries ago, transformed the pectin in pectic acid (Fremy, 1840). In addition deesterification by PMEs makes HGA susceptible to the degradation by pectic enzymes, such as endo-PG and pectate lyases, affecting the texture and rigidity of the cell wall (Christgau *et al*., 1996). Thus, PMEs has major roles in pectin remodeling *in muro*.

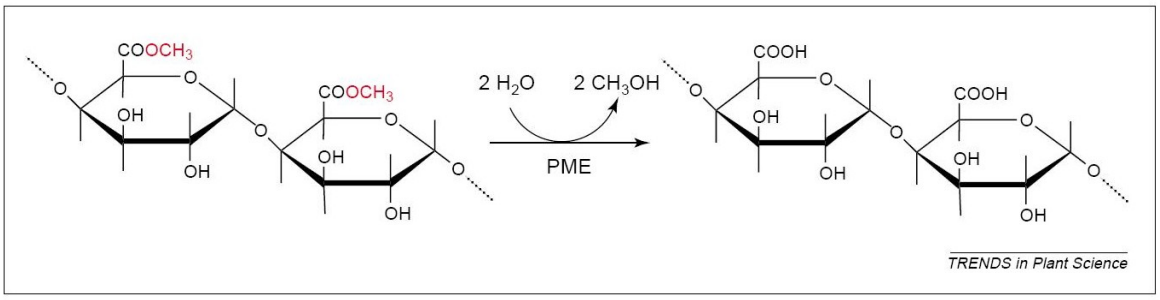

Figure 2. De-methylesterification of pectins by pectin methylesterases (PME). (Micheli, 2001).

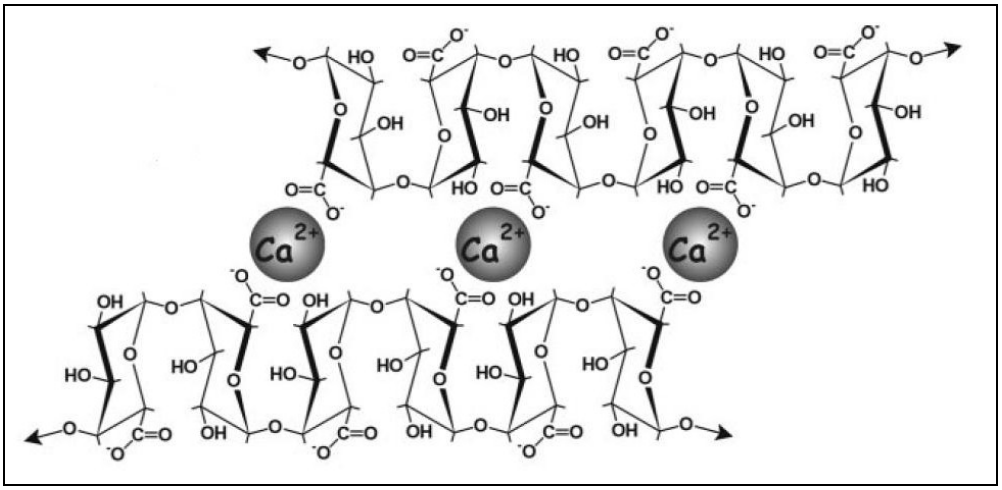

Figure 3. Calcium-pectin-crosslink as egg box model structure formed through insertion of Ca^{2+} ions between the unesterified carboxyl groups of the galacturonosyl residues of two HGA chains (Vincken *et al*., 2003).

It is conceivable that both the level of expression of plant PME and its enzymatic activity are finely regulated *in planta*. PME activity is regulated in different manners; e.g. [1] differential expression of isoforms (i.e., in *Arabidopsis thaliana* 67 open reading frames {ORFs} have been annotated as putative PMEs), [2] apoplastic pH variation (the enzyme optimal activity occurs at pH close to neutrality and is reduced by the local decrease of pH generated by protons released as a consequence of the reaction) or [3] proteinaceous inhibitors.

Numerous organisms, including plants, fungi, bacteria and nematodes, produce PMEs that may differ with respect to their pH optimum, isoelectric point values and requirement for calcium (Versteeg *et al*., 1978). In general, fungal PMEs have an optimal activity between pH 4 and 6, while plant and bacterial PMEs have an optimal activity between pH 6 and 8 (van Alebeek *et al*., 2003). Another notable difference that has been reported is that fungal PMEs have been shown to hydrolyze the methylesters in a random way, while plant and bacterial PMEs de-esterify in a 'block-wise' manner (linearly) (Markovič and Kohn, 1984; Kester *et al*., 1999; Benen *et al*., 2002). However, recent studies have shown that the DM of pectin and the pH of the environment influence the PME activity. At a given pH, some isoforms are more effective than others on highly methylated pectin. Also some isoforms have the ability to act randomly at acidic pH and

'block-wise' at alkaline pH (Catoire *et al*., 1998; Denès *et al*., 2000; Micheli, 2001). In recent years, increasing amounts of data have been generated concerning the functions of PMEs in plants; they include a wealth of genomic data showing that PMEs belong to large multigene families and that their primary and quaternary structures are conserved among plant taxa (Pelloux *et al*., 2007).

5. Pectin methylesterase inhibitors (PMEIs)

PME inhibitors of different chemical nature have been discovered in various plant species. Isolation of a PMEI was obtained during the purification of a PME isozyme from potato tuber. The potato inhibitor was mainly composed of uronic acid and neutral sugars (McMillan and Pérombelon, 1995) and was active against plant PME, whereas did not affect microbial PMEs. An inhibitory activity was also found in rubbery banana fruit (*Musa sapientum* L.) active against its own banana PME and the pea pod (*Pisum sativum*) PME (Wu *et al*., 2002). The chemical nature of this inhibitor has not been elucidated. Another inhibitory activity was discovered in jelly fig (*Ficus awkeotsang*) achenes and was found to consist of polypeptides (Jiang *et al*., 2002).

The first proteinaceous inhibitor of PME was found in kiwi (*Actinidia chinensis*) fruit. The kiwi inhibitor (AcPMEI) consists of 152 amino acid residues, accounting for a molar mass of 16.277 kDa, and effective in inhibiting PME in the pH range 3.5-7.5 (Balestrieri *et al*., 1990). AcPMEI has been shown to be active against several plant PMEs (Balestrieri *et al*., 1990; Giovane *et al*., 1995; Camardella *et al*., 2000; Ly-Nguyen *et al*., 2004) whereas does not inhibit fungal (Duvetter *et al*., 2005) and bacterial PME (D'Avino *et al*., 2003). Then, two genes encoding functional inhibitors of pectin methylesterases were identified in *Arabidopsis thaliana* (AtPMEI-1 and AtPMEI-2) (Wolf *et al*., 2003; Raiola *et al*., 2004) and, recently, another inhibitor of protein nature has been found in pepper (CaPMEI1) (An *et al*., 2008).

To date, the structures of only three PMEs, one from the bacterium *Erwinia chrysanthemi* (Jenkins *et al*., 2001), one from carrot (*Daucus carota*) (Johansson *et al*., 2002) and one from tomato (Di Matteo *et al*., 2005) have been solved. The three-dimensional structures of PMEI from Kiwi (AcPMEI) and from Arabidopsis (AtPMEI-1) have been elucidated (Hothorn *et al*., 2004; Di Matteo *et al*., 2005). Also the structure of the PME-PMEI complex has been determined (Di Matteo *et al*., 2005). The structure of the

PME-PMEI complex provides a possible explanation for the lack of inhibition of PMEIs on non plant-derived PMEs so far analysed (Giovane *et al.*, 2004; Raiola *et al.*, 2004). Superimposition of the known structures of PME from carrot and from the bacterium *Erwinia crysanthemi* with the PME-1 from tomato reveals that the plant-derived PMEs are almost identical, whereas in the bacterial enzyme the active site cleft in which the putative active site is located, is much deeper than in the enzymes of plant origin (Di Matteo *et al*., 2005). It is likely that the external loops of the bacterial enzyme create a steric impediment that prevents the interaction with the inhibitor. Furthermore PMEI is inactive towards PME form the phytopathogenic fungus *Aspergillus aculeatus*. Interestingly, almost all of the residues important for the interaction of tomato PME with the inhibitor are conserved in plant PMEs but not in the fungal enzyme thus furnishing a reason for the observed lack of interaction (Di Matteo *et al*., 2005).

AcPMEI is a strong inhibitor of PME and forms a reversible 1:1 non-covalent complex (Mattei *et al*., 2002; Di Matteo *et al*., 2005) (Figure 4). The stability of the complex is strongly influenced by pH, indicating that PME activity can be modulated by pH either directly or by modulation of the affinity between the enzyme and its inhibitor (Giovane *et al*., 2004). The analysis of the binding kinetics between AcPMEI and tomato PME reveals that the affinity between PME and PMEI increases as pH decreases, in the range 6.0–7.5 (Mattei *et al*., 2002; D'Avino *et al*., 2003).

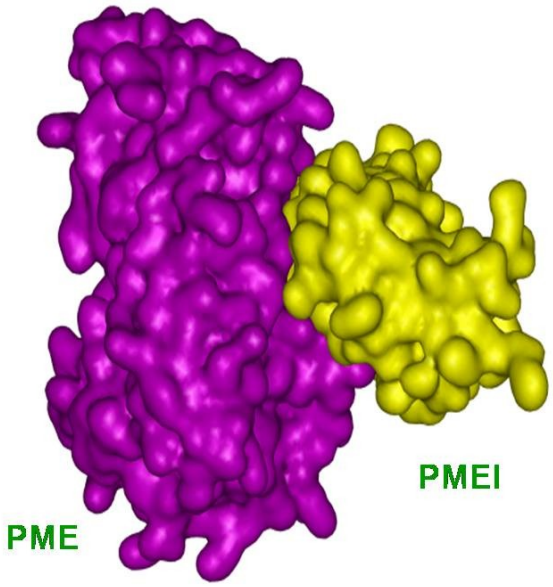

Figure 4. Molecular surface of the PME-PMEI complex. The picture show the 3D crystal structure complex of PME enzyme isolated from tomato fruit (*Lycopersicon esculentum*) and PMEI inhibitor from kiwi (*Actinidia deliciosa*) (Di Matteo *et al*., 2005).

The functional PME inhibitors, identified in Arabidopsis, are encoded by two members, *AtPMEI-1* and *AtPMEI-2*, located in different chromosomes (locus At1g48020; locus At3g17220, respectively). AtPMEI-1 and AtPMEI-2, which share about 47% identity at the amino acid level, consist of 151 amino acids (molecular mass of 16.266 kDa, predicted p*I* 7.7) and 148 amino acids (molecular mass 15.615 kDa, predicted p*I* 9.0), respectively (Wolf *et al*., 2003; Raiola *et al*., 2004). In comparison to AcPMEI, AtPMEI-1 and AtPMEI-2 share about 38% identity at the amino acid level and both Arabidopsis proteins show a conserved C-terminal hydrophobic region (CxIxLVISN) and five conserved Cys residues, the first four of which have been shown to be engaged in disulfide bridges in AcPMEI. Like AcPMEI, AtPMEIs specifically recognize PMEs of plant origin and not microbial ones (Camardella *et al*., 2000; Raiola *et al*., 2004). Both *AtPMEIs* are expressed in flowers, while *AtPMEI-1*, which shows a considerable higher expression level than *AtPMEI-2*, is also expressed in seedling, stems and mature leaves (Wolf *et al*., 2003; Raiola *et al*., 2004). Analysis of genomic data showed that PMEIs belong to large multigene families in all plant species analysed. For instance, in *Arabidopsis thaliana*, 69 ORFs have been annotated as putative PMEIs (Wolf *et al*., 2009).

Arabidopsis inhibitors are active in a wide pH range and perform high thermal stability. Both AtPMEIs were active against PME from several plants, including tomato, kiwi and apricot fruits, carrot roots, tobacco leaves, Arabidopsis leaves and flowers whereas they were inactive against PMEs of *E. chrysanthemi*, *Aspergillus niger*, *A. aculeatus* and *B. cinerea* (Raiola *et al*., 2004; Lionetti *et al*., 2007). However, not all plant PME isozymes can undergo inhibition by PMEI. Recently, it has been found an atypical Arabidopsis PME (AtPME31) able to escape kiwi PMEI inhibition (Dedeurwaerder *et al*., 2009).

It is likely that the specific co-regulation and interaction of given PME and PMEI isoforms are of utmost importance in controlling the degree of methylesterification of pectin and thus modifying cell wall mechanical properties during development.

Chapter I

An Increase in Pectin Methylesterification Restricts Bacterial Infection by *Pectobacterium carotovorum* in Arabidopsis

1.1. Introduction

Soft rot pathogens, like *Botrytis cinerea* and *Pectobacterium carotovorum* subsp. *carotovorum* (formerly, *Erwinia carotovora* subsp. *carotovora*; Gardan *et al.*, 2003), are widely distributed in the world and cause serious damage on a wide variety of plants. The ability of these pathogens (together with other soft rot *Pectobacterium* species) to macerate plant tissues is dependent by the coordinate production of high levels of multiple exoenzymes, including pectinases, cellulases and proteases (CWDEs), which breakdown plant cell walls and release nutrients for bacterial growth. In particular, pectinases are the main exoenzymes involved in disease development (Pérombelon, 2002).

B. cinerea contains at least six endo-PG genes (Wubben *et al.*, 1999), among which two genes, Bc*PG1* and Bc*PG2*, are required for its virulence on several host plants (van Kan, 2006). The role of PMEs in pathogenesis was also studied. It is considered that endo-PGs cannot efficiently depolymerize highly methylated pectin, hence demethylation by PMEs presumably precedes and facilitates the action of endo-PGs. The contribution of PME to virulence of *B. cinerea* has been demonstrate for the Bd90 strain on different host plants (Valette-Collet *et al.*, 2003) although virulence of the B05.10 strain on tomato (*Solanum lycopersicon*) and grape (*Vitis vinifera*) leaves is unaffected when both *Bcpme1* and *Bcpme2* are disrupted (Kars *et al.*, 2005).

In addition to pectinases, other types of cell-wall-degrading enzymes produced by *B. cinerea* have been studied. Deletion of a cellulase gene did not affect virulence (Espino *et al.*, 2005), whereas the deletion of a β-1,4-xylanase gene delayed lesion formation and reduced lesion development by more than 70% (Brito *et al.*, 2006).

Pectobacterium carotovorum mainly affects crops in temperate and subtropical regions and has probably the widest host range among soft rot bacteria, including brussels sprout, carrot, celery, cucumber, capsicum, turnip, chicory and potato and can also cause severe losses in many other crops during post-harvest (Pérombelon and Kelman, 1980; Pérombelon, 2002; Toth *et al*., 2003).

Many of pectinases exist in multiple forms (isoenzymes) encoded by independent genes that are clustered and appear to be derived from successive rounds of gene

duplication (Barras *et al*., 1987; McMillan *et al*., 1994). The exact nature of this diversity is not known, however there is some evidence to support both a wide substrate diversity and independent regulation (Nachin and Barras, 2000). The expression of pectic enzymes occurring during pathogenicity in the soft rot erwinias is sequential (Yang *et al*., 1992; Masclaux *et al*., 1996), as for fungal pathogens (Cooper, 1983).

PELs are the main pectinases in pathogenesis and, as with other exoenzymes, their number varies between species, subspecies and strains (Toth *et al*., 2003). For example, in *E. chrysanthemi* different pectinases are required for virulence on different hosts, e.g. a mutation in pelD reduced virulence in chicory leaves but not potato tubers, while a mutation in pelE actually increased virulence on both hosts. This production of exoenzymes and other pathogenicity factors at a precise stage in the infection process is necessary to overcome plant host defences and lead to disease development (Hugouvieux-Cotte-Pattat *et al*., 1996; Thomson *et al*., 1999).

In the case of *P. carotovorum*, it has been reported that an endo-PG gene (*pehA*) and at least four pectate lyase genes (*pelA* to *pelD*) contribute mostly to its pathogenicity (Flego *et al*., 2000).

Since highly methylesterified pectins are less susceptible to the action of endo-PG and PEL (Willats *et al*., 2001), it is conceivable that a reduction of endogenous PME activity *in planta*, by inhibitors or by gene disruption of selected PME isoforms, could confer a reduced susceptibility to soft rot pathogens.

1.2. Materials and Methods

1.2.1. Plant lines and growth conditions

Arabidopsis thaliana, accession Columbia-0, wild-type (WT) and transformed lines over-expressing AtPMEI-1 (locus At1g48020; lines 1-1 and 1-5) or AtPMEI-2 (locus At3g17220; lines 2-7 and 2-9) in addition to knockout line in the *AtPME3* gene (SwissProt accession Q9LUL7; locus At3g14310, Gabi-Kat line 002A10) were kindly obtained from Department of Plant Biology, University of Rome ‘‘La Sapienza’’, Italy. Plants were grown in a growth chamber maintained at 22 °C, 70% relative humidity, with a 12-h photoperiod (100µmol m^{-2} s^{-1} fluorescent light) and nutrient solution (5 mM KNO_3, 2.5 mM KH_2PO_4, 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, pH 6.5; according to Ruegger *et al*., 1998) was supplied every two weeks.

1.2.2. Bacterial growth and infection

Pectobacterium carotovorum subsp. *carotovorum* (Gardan *et al.,* 2003) was obtained from DSMZ GmbH Germany (strain DSMZ 30169). Bacterial strain was stored at -70°C in medium containing 40% glycerol. A single bacterial colony, obtained after streaking (on LB agar medium) and growing an aliquot of the stock culture (for 24 h at 28°C), was used for plant inoculation. The inoculum was obtained by inoculating the colony in 10 ml of LB liquid medium. After 16 h of growth at 28°C on rotary shaker at 250 rpm, the culture was harvested by centrifugation at 4500 x *g* for 10 min and the pellet was resuspended in 50 mM of potassium phosphate buffer (pH 7.0) at 5 x 10^7 CFU ml^{-1}.

For infection test, fully developed leaves of 4-week-old Arabidopsis plants were detached and placed on a moist filter paper in Petri dishes. A small hole was made with a needle in each leaf and 5µl of the bacterial suspension were spotted. Symptoms were scored measuring the area of macerated tissue after 16 h post inoculation at 22°C and 12-h photoperiod. Lesion areas were measured by ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2008). The experiments was repeated three times with different plant batches, and statistically analysed.

1.2.3. Cell wall isolation

Four weeks old Arabidopsis leaves (500 mg) were homogenized using mortar and pestle in liquid nitrogen. The ground tissue was immersed in a boiling (80°C) 85% ethanol (1:3, w/v). After 15 min of boiling, the mixture was centrifuged at 10000 x *g* for 10 min and the pellet was resuspended in 1.5 ml of 85% ethanol and incubated again for 15 min at 80°C. This procedure was repeated twice. The pellet was resuspended in 1.5 ml of chloroform:methanol mixture (1:1, v/v). After centrifugation and washing once with absolute ethanol (1.5 ml), the final pellet was resuspended in 1.5 ml of 80% acetone. Acetone was removed by centrifugation and remained residues were evaporated under vacuum for 30 min and cell wall stored at -20°C for analysis. The yield of cell wall material was consisted of about 25 mg.

1.2.4. Bacterial growth on different pectic substrates

P. carotovorum was grown in 50 ml-flask in 20 ml of a minimal medium (MM) containing (g l^{-1}): $NaNO_3$, 1.5; $MgSO_4.7H_2O$, 0.3; NaCl, 2.3; K_2HPO_4, 4; KH_2PO_4, 1.5; pH 7.2 (Fraaije, *et al.*, 1997), supplemented as sole carbon sources with either 0.2% (w/v) PGA (Sigma) or 0.2% (w/v) lime pectin with 81% or 60% degree of methylesterification (E81 and P60; DANISCO). Bacterium was also grown on cell walls, isolated from transformed and wild-type leaves, dispersed as dried material in 5 ml of MM (in 50 ml-Falcon tube) at a final concentration of 1 mg ml^{-1}. Cultures were inoculated at a final concentration of 4 x 10^6 CFU ml^{-1} and incubated at 28°C on rotary shaker at 250 rpm. Bacterial growth on PGA, E81 or P60 was measured spectrophotometrically (OD_{600nm}) at 6, 10 and 24 h post-inoculation. The bacterial growth on different cell wall preparations was determined 12 h after inoculum by plating serial dilutions on LB agar plates and counting colonies after 24 hours of incubation at 28°C.

1.2.5. RT-PCR analysis

Total RNA was extracted from 100 mg of leaf tissues with TRIzol reagent (Sigma) (1 ml) using a Mixer Mill Homogenizer (Qiagen, type MM300). After grinding in an microcentrifuge tube with one stainless steel bead (5 mm in diameter) for 1 min at 25 Hz, the homogenate was centrifuged at 12000 x *g* for 10 min at 4°C to remove the insoluble

material. After transferring the clear supernatant to a fresh tube, 0.5 ml of isopropanol was added and RNA precipitated by centrifugation at 12000 x *g* for 10 min at 4°C. After removing the supernatant the RNA pellet was washed by adding 75% of ethanol and was resuspended in 30 µl of RNase/DNase-free water. RNA concentrations was determined both spectrophotometrically and by densitometric analysis of RNA bands following agarose gel electrophoresis. Total RNA (2 µg) was DNase-treated by adding 2 µl of RQ1 DNase Reaction buffer 10X (Promega), 2 µl of RQ1 RNase-free DNase 1 u / µl (Promega), nuclease-free water to a final volume of 20 µl and incubated 30 min at 37°C. The reaction was stopped with 1 µl of RQ1 DNase Stop Solution (Promega) at 65°C for 10 min.

For cDNA synthesis, DNase-treated RNA (1 µg) was reverse-transcribed using the Improm-II™ Reverse Transcription System according to manufacturer's instructions (Promega). Reactions were brought to a final volume of 40 µl containing random hexamer primers to a final concentration of 0.5 µM. Synthesis conditions of cDNA were: 5 min at 25°C for primers annealing; 60 min at 42°C for first strand cDNA synthesis; 15 min at 70°C to inactivate reverse transcriptase. For DNA amplification, 2 µl of synthesized cDNA were amplified using the following oligonucleotide primers (sense and antisense, respectively) specific for each genes: 5'-TCGCTGGCATAACACTATGG-3' and 5'-TTGGGAGCAAGAGTGGAGTT-3' for PAD3, 5'-CGCACCGGCAATGGTGG-3' and 5'-ATCCATGTTTGGCTCCTTC-3' for PDF1.2, 5'-GTAGGTGCTCTTGTTCTTCCC-3' and 5'-CACATAATTCCCACGAGGATC-3' for PR-1, 5'-GGAAGAAGAAGACTTAC ACC-3' and 5'-AGTCCACACTTACCACAGTA-3' for UBQ5.

The PCR experiments were performed in a MyCycler^{TM} thermal cycler (Bio-Rad Life Science) and amplification conditions consisted of 25 cycles as follows: 94°C for 1 min, 60°C for 1 min, 72°C for 1 min.

1.3. Results

1.3.1. Overexpression of *AtPMEI* genes and *AtPME3* gene knockout reduce susceptibility to *Pectobacterium carotovorum* in Arabidopsis

Overexpression of *AtPMEI-1* and *AtPMEI-2* genes in *Arabidopsis thaliana*, able to encode efficient inhibitors of plant PMEs, has been demonstrated to determine a significant decrease of the overall PME activity, an increase of the degree of pectin methylesterification (about 16%) and a reduced susceptibility to the necrotrophic fungus *B. cinerea* with respect the untransformed wild-types (Lionetti *et al*., 2007). In order to extend this study to soft rot bacterial pathogens, the resistance of transformed *AtPMEI* plants to *P. carotovorum* was assessed.

Arabidopsis *AtPMEI-1* (line 1.1) and *AtPMEI-2* (line 2.7) leaves were inoculated with *P. carotovorum* and the susceptibility to the bacterium was compared to the wild-type. Sixteen hours post-inoculation wild-type leaves exhibited typical soft rot symptoms characterized by water-soaked lesions, whilst *AtPMEI-1* and *AtPMEI-2* plants displayed lesions with reduced size (about 30% and 47%, respectively) (Figure 1.1). No difference in the ratio between the number of expanding lesion and the number of inoculated spots were observed among AtPMEIs and wild-type plants.

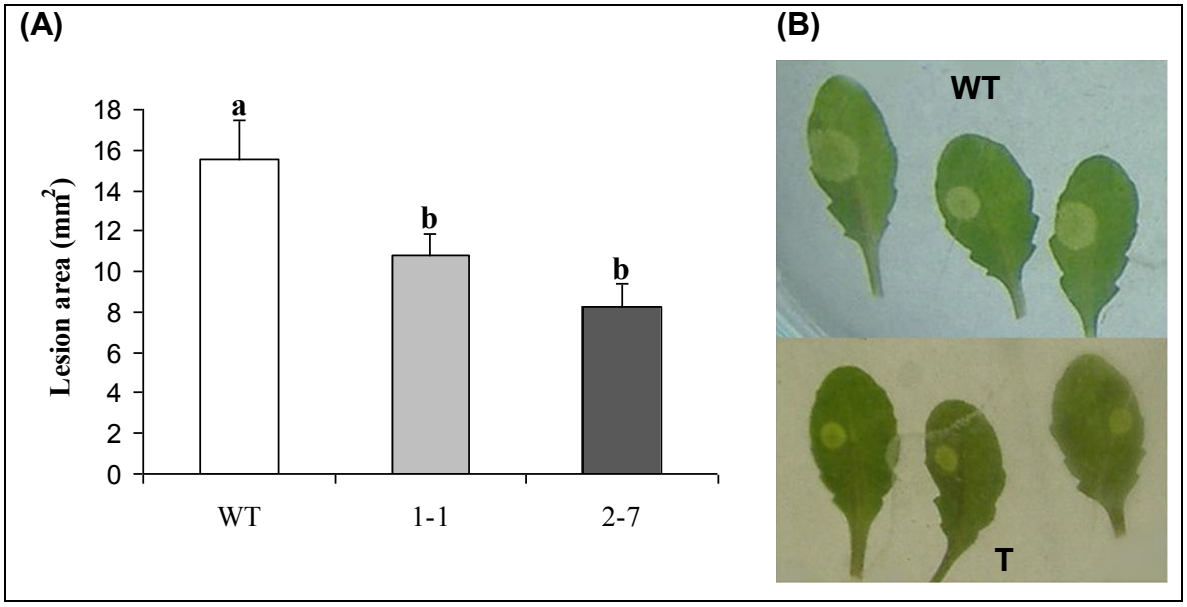

Figure 1.1. Resistance of *AtPMEI-1* and *AtPMEI-2* plants to *P. carotovorum* infection. (A & B) Detached leaves from wild-type (WT) and *AtPMEI* transformed lines (T) were inoculated with 5µl of a bacterial suspension (5 x 10^7 CFU ml^{-1}) and the lesion area was determined 16 h post infection. Bars represent the average lesion area ± SE (n>12). Different letters represent data set significantly different, according to ANOVA analysis followed by Tukey's test ($P > 0.95$). The experiment was repeated three times with similar results.

Resistance to *P. carotovorum* was also assessed in a T-DNA-tagged *AtPME3* insertional mutant (*Atpme3*) (Gabi-Kat line 002A10, At3g14310-knockout). *AtPME3* encodes a PME isoform mainly expressed in Arabidopsis leaves (Micheli *et al*., 1998) and has been shown to interact with both AtPMEIs (Lionetti *et al*., 2007). Since these Arabidopsis mutant plants show less PME activity in their leaf tissues (Figure 1.2 A; determination of PME activity is described in material and methods of chapter II) and cell wall with pectin methylesterification level significantly higher than the wild-type plants (about 30%; Dr. Vincenzo Lionetti, University "La Sapienza" Rome, unpublished results), these plants were also tested for susceptibility to bacterial infection. The *Atpme3* mutant displayed a significant reduced lesion size (about 40%) after *P. carotovorum* infection compared to control wild-type plants ($P < 0.01$; Figure 1.2 B & C).

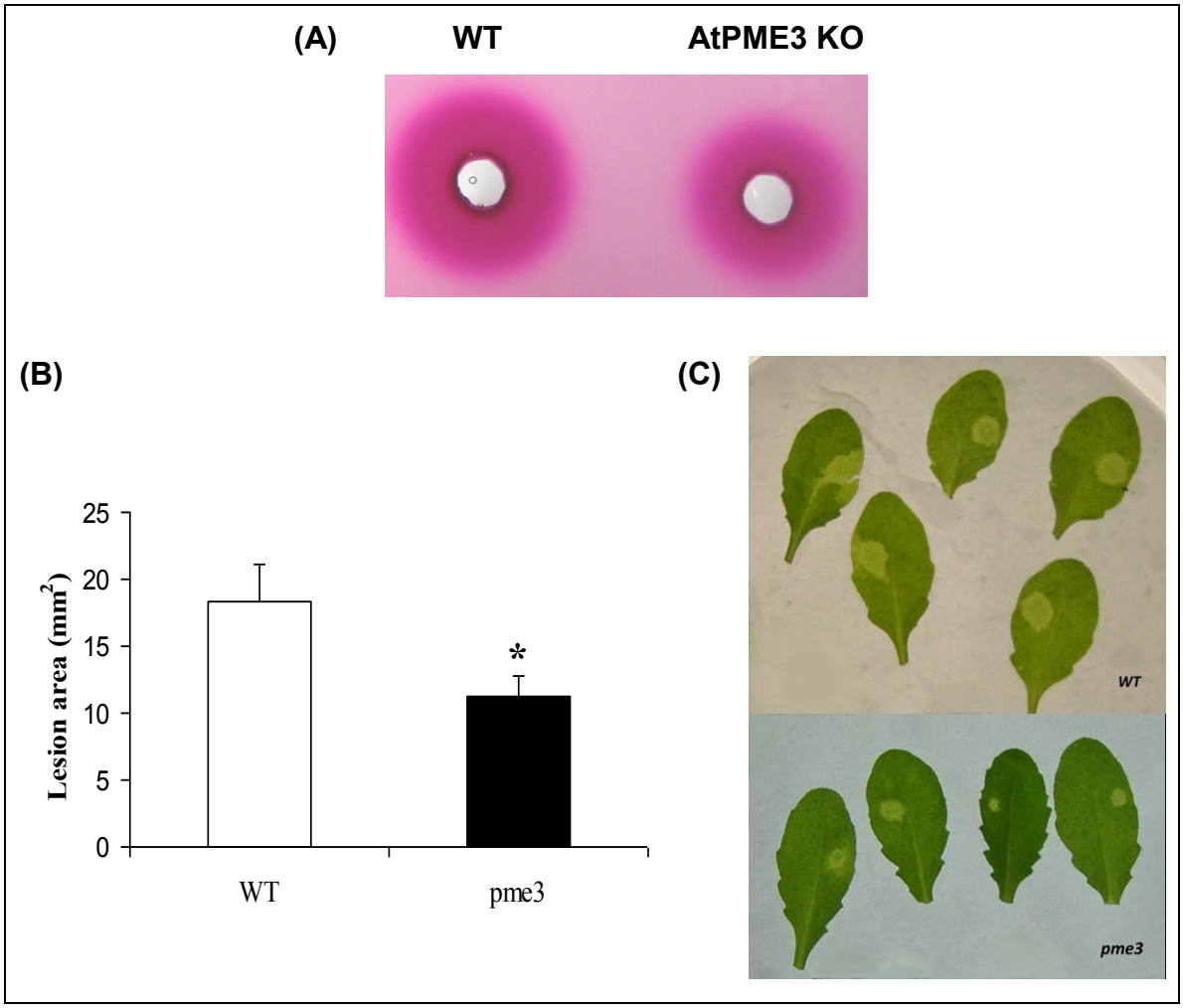

Figure 1.2. Resistance of *Atpme3* to *P. carotovorum*. (A) Gel diffusion assay showing the pectin methylesterase activity detectable in wild-type (WT) and *Atpme3* leaves protein extracts prepared from equal amounts of fresh weight tissues. (B & C) Detached leaves from wild-type and *Atpme3* plants were inoculated with *P. carotovorum* cells and the lesion size detected 16 h post-infection. Bars represent the average lesion area ± SE of three independent experiments (n>12 in each experiment). Asterisk represents data sets significantly different, according to Student's *t* test ($P < 0.01$).

1.3.2. Bacterial growth is impaired by pectin methylesterification

To ascertain if pectin methylesterification affects the growth of *P. carotovorum*, bacterial growth was measured on liquid synthetic media containing, as sole carbon source, polygalacturonic acid (PGA), 81% (E81) or 60% (P60) methylesterified pectins. After 10

hours of growth, *P. carotovorum* grew significantly better on PGA than on E81 or P60 (Figure 1.3), suggesting that *P. carotovorum* PMEs does not perform an efficient pectin demethylesterification. Consistently, no PME activity was detected by radial gel diffusion assay in the culture filtrates. After 24 h from inoculum, bacteria continued to show a strongly reduced growth on E81 pectin substrate (data not shown).

To determine if cell wall composition of transformed plants (*AtPMEI-1*, *AtPMEI-2* and *Atpme3*) affects *P. carotovorum* growth, also the ability of the bacterium to grow on a liquid medium containing cell wall isolated from these plants as a carbon source was measured. After 12 h of culture a consistent reduction of bacterial growth (about 70%) was observed on cell walls isolated from *AtPMEI-1, AtPMEI-2* and *pme3* plants in comparison with the wild-type (Figure 1.4). These data suggest that the reduced symptoms observed on *AtPMEI* and *Atpme3* plants are related to an impaired ability of this bacterium to grow on methylesterified pectins.

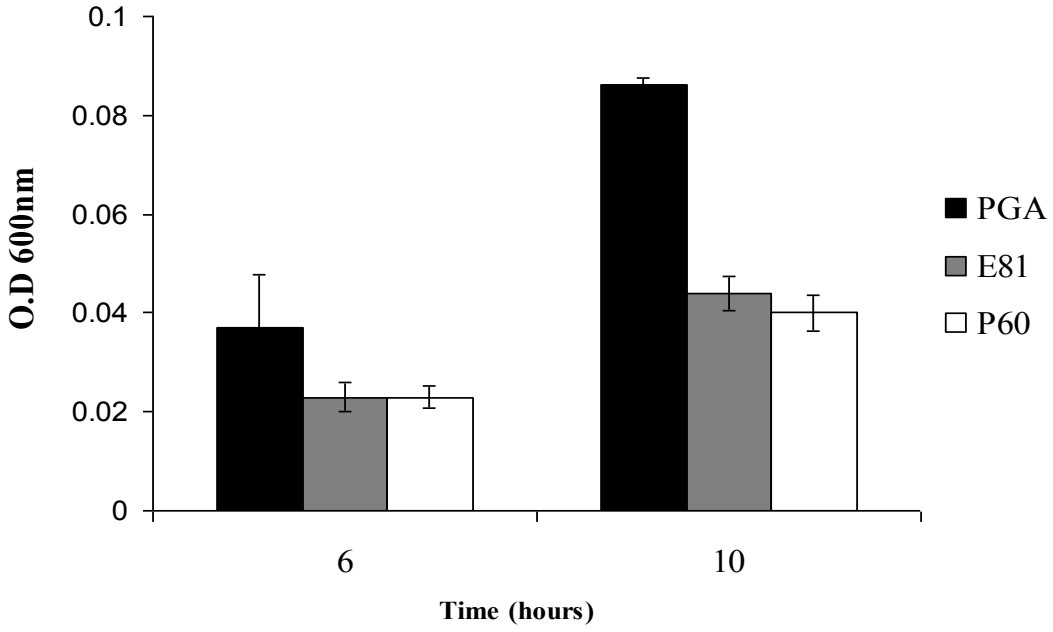

Figure 1.3. Bacterial growth on liquid synthetic media containing PGA or two series of model pectins (E81 and P60) was measured spectrophotometrically as culture at $O.D_{600nm}$ after 6 h and 10 h of incubation in a 250 rpm rotary shaker at 28°C. Data indicate the average values from triplicate readings ± SD. The experiment was repeated three times with similar results.

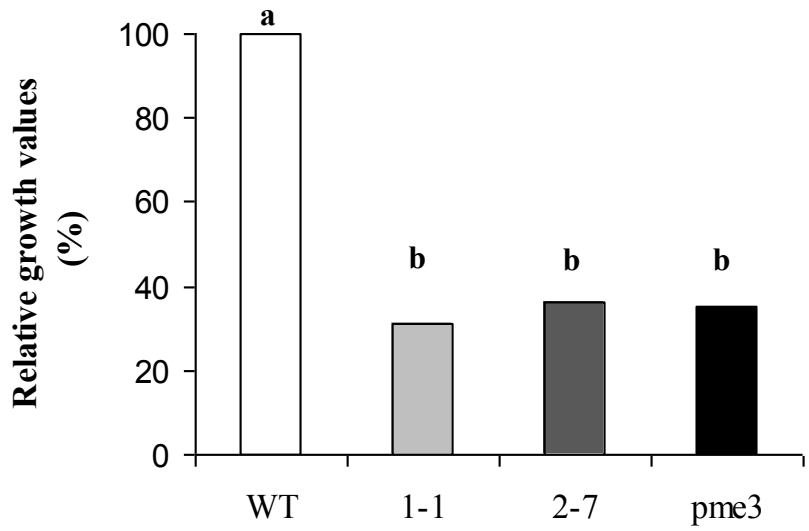

Figure 1.4. Reduction of bacterial growth on cell walls isolated from transformed *AtPMEI* plants and *Atpme3* mutant after 12 h of culture. Bars represent relative percentage of growth and different letters indicate data sets significantly different at $P < 0.01$ (ANOVA followed by LSD test). The number of bacterial colonies was determined by 1000-fold dilution from 3 independent experiments with n=3 each.

1.3.3. Defence genes expression in *AtPMEI* and *Atpme3* plants after *P. carotovorum* infection

Finally, the expression of a number of defence genes, including *PAD3*, *PDF1.2*, *PR-1*, during *P. carotovorum* infection were analysed in *AtPMEI* and *pme3* plants, in comparison with wild-type (Figure 1.5). *PDF1.2* gene, known as a marker for jasmonate/ethylene dependent defence responses (Thomma *et al*., 2001) which encode an antifungal defensive peptide, was similarly induced in all plant during bacterial infection. Also the salicylic acid regulated *PR-1* gene (Thomma *et al*., 2001) and *PAD3* gene, a gene required for camalexin biosynthesis (Zhou *et al*., 1999), were strongly induced in inoculated leaves with similar kinetics among wild-type and transformed plants. These data indicate that defence responses typically induced by necrotrophic pathogens are not altered in *AtPMEIs* and *Atpme3* plants in comparison with wild-type and the reduced susceptibility to *P. carotovorum* is mainly due to alteration of cell wall structure which *per se* causes a reduced growth of the pathogen.

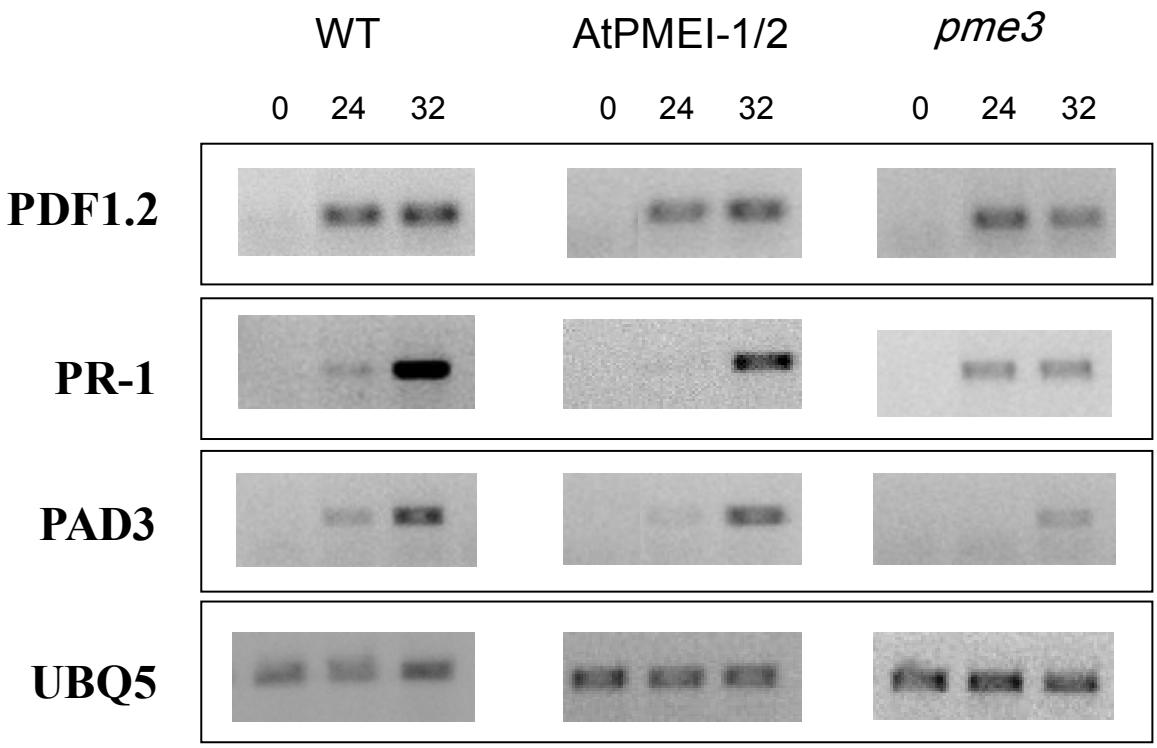

Figure 1.5. Gene expression in response to *P. carotovorum* infection. RT-PCR was performed on total RNA extracted from rosette leaves of WT and transformed (*AtPMEIs* and *Atpme3*) plants 24 and 32 hours after inoculation with *P. carotovorum*. The UBQ5 gene was used as internal standard (data in collaboration with Dr. Lionetti, University "La Sapienza" Rome).

1.4. Discussion

A role of pectin degree of methylesterification (DM) in plant disease resistance has been reported in several pathosystems. For example, a higher amount of methylated and branched pectins was observed in potato cultivars resistant to soft-rots Erwinias (McMillan *et al*., 1993; Marty *et al*., 1997). A higher degree of esterification has been found in bean cultivars resistant to the anthracnose fungus *Colletotrichum lindemuthianum* compared with that found in susceptible cultivars (Boudart *et al*., 1998). A different methylester distribution has been found in wheat cultivars resistant and susceptible to the stem rust fungus *Puccinia graminis* (Wiethölter *et al*., 2003). Therefore, due to the possible protective effect of methylesterification in pectin degradation by fungal and bacterial enzymes, resistance of Arabidopsis plants constitutively expressing the PME inhibitors, AtPMEI-1 and AtPMEI-2 (Lionetti *et al*., 2007), and of an Arabidopsis mutant, lacking in a PME isoform, to the bacterial necrotroph, *P. carotovorum*, was assessed. The soft rot pathogen *P. carotovorum*, known to cause serious losses in a large number of crop species, is characterized by the production of large quantity of CWDEs among which pectinases are the main extracellular enzymes. It has been reported that an endo-PG and PEL are virulence factors of *P. carotovorum* (Flego *et al*., 2000). Endo-PGs and PELs cannot efficiently depolymerize highly methylated pectins, hence demethylation by PME presumably precedes and facilitates the action of these enzymes (Christgau *et al*., 1996). It is therefore conceivable that a reduction of endogenous PME activity *in planta*, maintaining pectin polymer in a highly methylated form, could confer a reduced susceptibility to soft rot pathogens. Arabidopsis AtPMEI-1 (line 1.1) and AtPMEI-2 (line 2.7), showing the highest level of AtPMEI transcripts and proteins and a concomitant lower level of PME activity and higher DM than in wild-types (Lionetti *et al*., 2007), exhibited a reduced spreading of disease symptoms after inoculation with two soft rot pathogens, *B. cinerea* and *P. carotovorum*. This analysis was extended to a knockout mutant in *AtPME3* gene, encoding a PME isoform mainly expressed in Arabidopsis leaves. These mutant plants, exhibiting a significant increase of DM in cell walls extracted from rosette leaves, resulted more resistant to *P. carotovorum* infection. Growth data in liquid cultures indicate that the bacterial biomass on methylesterified pectin is lower than on PGA, suggesting that

P. carotovorum PME does not perform an efficient pectin de-methylesterification and indicating that the bacterium prefers unesterified pectins as carbon source. Also cell wall composition affects bacterial growth as observed during growth of bacterium in liquid medium containing cell walls isolated from *AtPMEI-1*, *AtPMEI-2* and *pme3* plants when compared to the wild-type. The possibility that AtPMEI or PME KO, modifying pectin polymer, may influence the type of oligogalacturonides (OGs) released from the host cell wall and their elicitor activity was investigated. For example, pectic fragments with higher DM and superior elicitor activity have been reported to accumulate in bean (*Phaseolus vulgaris*) cultivars resistant to *C. lindemuthianum* (Boudart *et al*., 1998). It was recently demonstrated that transgenic expression of a fungal endo-PG confers plant resistance to microbial pathogens because of release of OGs or other wall-derived elicitors (Ferrari *et al*., 2008). The expression of a number of defense genes, during *P. carotovorum* infection was analysed in AtPMEI and pme3 plants. However, no significant differences were observed in the timing and level of defence gene expression in transformed plants with respect the untransformed controls, indicating that the reduced susceptibility of transformed plants to this necrotroph is mainly due to the alteration of cell wall structure which *per se* causes a reduced growth of the pathogen.

Chapter II

Alterations of the *Turnip Vein-Clearing Virus* Spreading in *Arabidopsis thaliana* Expressing Pectin Methylesterase Inhibitors

2.1. Introduction

2.1.1. Plant viruses and their control

Plant viruses are responsible for a significant number of commercially relevant plant diseases. Viruses control is mostly preventive and directed to eradicate the virus inoculum sources (plants or/and vectors). There are very few efficient control measures for viral diseases, but the use of genetic resistance appears to be promising strategy (Gómez *et al*., 2009). But resistance genes to control viral diseases are few and efficient only against a few viruses. Genetically modified resistance plants to virus have been obtained using gene sequences from viral genome, these exogenous genes are able to activate in the transformed plants, through a gene silencing mechanism, the resistance to the donor virus when it challenges plants. This technology has been successfully used to obtain resistant plants (Lindbo and Dougherty, 1992**;** Waterhouse *et al*., 1998).

A possible alternative to obtain virus resistant plants is to block viral replication or distribution within plant tissues. On this regards plant cell components usually involved in virus life cycle could be the molecular target to counter viral infections.

2.1.2. Plant virus movement

The capacity of a virus to move within a plant is a prerequisite for successful infection. Plant viruses, differently from animal viruses, must traverse plant cell wall. The initial entry of viruses into plant cells occurs by physical penetration of the wall by mechanical wounding of leaf hairs or epidermal cells, or by insects, mites or nematodes that feed on the plant. Then transport of virus material (nucleic acid or virus particle) from the initially infected cells to the adjacent cells occurs through the protoplasmic bridges, called plasmodesmata (PD) (Carrington *et al*., 1996; McLean *et al*., 1997; Waigmann *et al*., 2004). In general the opening of the plasmodesmata are too small (2.5 nm in diameter; Ding *et al*., 1992) for passive passage of the whole virus particles (10 - 200 nm) (Scholthof, 2005). To circumvent this physical restriction, plant viruses encode one or more movement proteins (MPs), that expand the plasmodesmata openings, to enable the passage of viral nucleic acid or, sometimes, even intact virions (Lazarowitz and Beachy 1999; Waigmann

et al., 2004; Boevink and Oparka, 2005). Some viruses also need the coat protein (CP), as an auxiliary protein, for cell-to-cell movement. Also a great variation of host encoded proteins can be involved in cell to cell transport and a precise MP-host-protein combination is decisive in determining virus host compatibility (Scholthof, 2005). The phase following cell-to-cell movement involves systemic virus spread from the local infection sites to other plant parts and for this purpose most viruses move along with the flux of the photosynthesis products through the phloem and viral CP is often a major factor for the long distance transport of viruses (Callaway *et al*., 2001).

2.1.3. Movement of TMV: a model system

Both movements, cell-to-cell and long-distance, are complex processes involving a variety of virus-encoded proteins and host-encoded factors. MPs are thought to require compatible interactions with host plant components (McLean *et al*., 1997, Lazarowitz and Beachy 1999; Waigmann *et al*., 2004, Lucas, 2006). To date, many host plant proteins that bind to MPs have been identified (Waigmann *et al*., 2004) and several of them have been shown to influence viral movement.

In the case of *Tobacco mosaic virus* (TMV), a tobacco cell wall enzyme, pectin methylesterase, is involved in the cell-to-cell movement via plasmodesmata and in systemic movement via its regulation of viral unloading from the phloem tissue (Dorokhov *et al*., 1999; Chen *et al*., 2000; Chen and Citovsky 2003).

It is established that the binding of plant PMEs to the movement proteins (MPs) of some viruses is required for cell-to-cell movement through plasmodesmata (Chen *et al*., 2000). Although it is known that, PMEs are associated with cell wall-embedded plasmodesmata, MPs co-localize with plasmodesmata and PME-like proteins bind to MPs in cell wall extracts, the mechanism allowing PMEs to facilitate viral movement remains unclear (Pelloux *et al*., 2007). Different hypotheses, based on experimental evidences, can be suggested: PME may act as (i) a carrier of viral MP (TMV MP association with unprocessed PME may provide the ER translocation signal to facilitate viral transport through plasmodesmata), (ii) host cell receptor or (iii) make changes in plasmodesmal permeability allowing virus to move (Dorokhov *et al*., 1999; Chen *et al*., 2000; Tzfira *et al*., 2000).

Moreover, it was shown that co-agroinjection of functional Pro-PME cDNA and a viral vector stimulated plant virus defence via virus-induced gene silencing mechanisms that repress virus reproduction in tobacco (Dorokhov *et al*., 2006). The mechanisms by which PME participate to the different aspects of viral infections is still under debate, but tight regulation of different tissue-specific isoforms, and the balance between PME and MP levels are likely to be involved.

Virus translocation through plasmodesmata channels is a virus-coded function regulated by plant. Targeting plant factors involved in viral movement could be a promising approach to defend plant against viral pathogens. For instance, elevation of the intracellular levels of calreticulin in *Nicotiana benthamiana* substantially impaired cell-to-cell movement of the TMV (Chen *et al.*, 2005). Furthermore, Kaido *et al.* (2007) have identified in *Nicotiana benthamiana* a host factor (NbNACa1) involved in the cell-to-cell movement of *Brome mosaic virus* (BMV). Downregulation of *NbNACa1* expression determined a reduced cell-to-cell movement of the virus.

2.1.4. *Turnip vein-clearing virus* (TVCV): a tobamovirus infecting cruciferous

TVCV was isolated for the first time in Oklahoma from turnip plants (*Brassica rapa*) (Lartey *et al*., 1993). TVCV is a member of subgroup 3 of *Tobamovirus* genus (Figure 2.1.), that encode one MP of 30 kDa for its transport locally and systemically (Melcher, 2003) and TVCV MP has been found to interact "*in vitro*" with the tomato and citrus PME (Chen *et al.*, 2000). The particles encapsidate a single positive sense RNA (6.3 knt RNA which accounts for 5% of the virion mass). Virions, typical of tobamoviruses, are rod-shaped and its capsid protein has a molecular mass of 17.6 kDa (Lartey *et al*., 1995). TVCV virions are stable for years. Members of the crucifer family are hosts of this virus and also the virus is able to infect *Arabidopsis thaliana*. No vectors of transmission are known for this virus (Melcher, 2003). TVCV shares many characteristics with TMV, the member of the *Tobamovirus* genus as well. Symptoms of infection in *Arabidopsis thaliana* are diverse and dependent on the landrace of Arabidopsis used (Martín Martín *et al*., 1997). The prevalent symptom caused by TVCV on susceptible ecotypes of *A. thaliana* is a reduced growth and also bolting is delayed (Lartey *et al*., 1997).

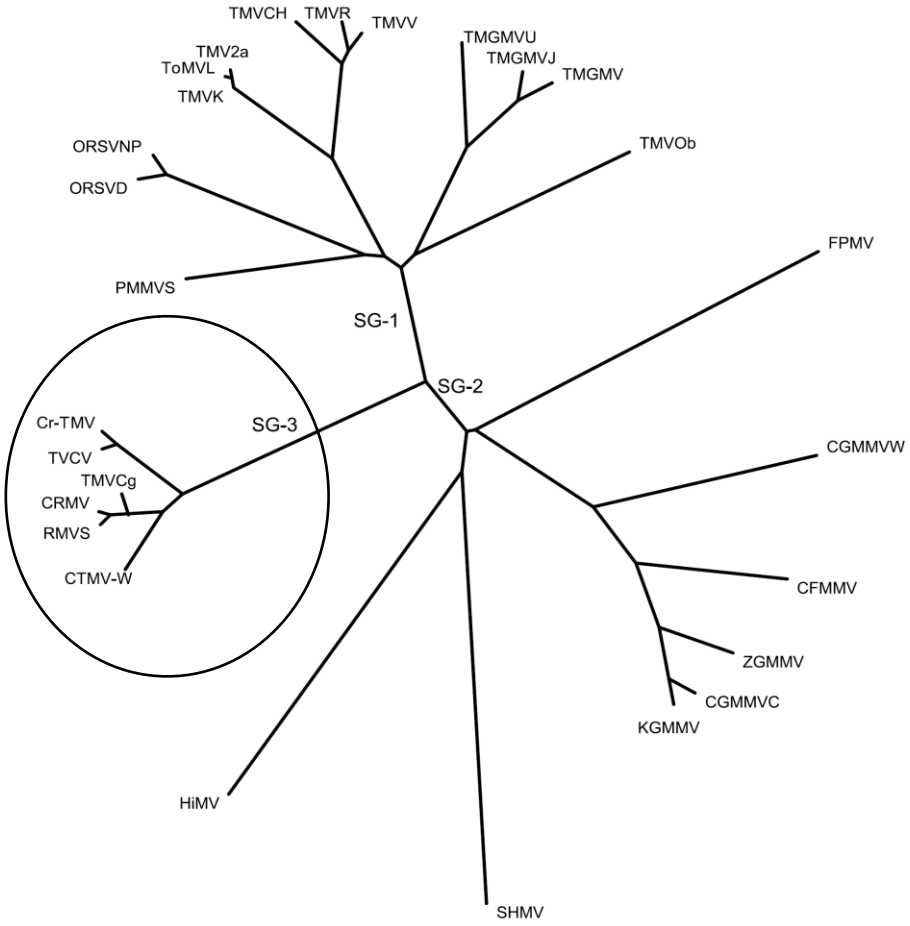

Figure 2.1. Tobamovirus phylogenetic analysis derived from alignment of MP amino acid sequences shows the existence of three separated groups. Viruses in subgroup 3, that includes TVCV, were preferentially isolated by crucifers (Melcher, 2003).

To extend the knowledge of the emerging role of plant PME played in the systemic virus infection by binding viral MP, infection process of TVCV in AtPMEIs and wild-type Arabidopsis plants has been investigated. In addition, studies on the interaction of viral movement protein with PME using biochemical technique has been performed to better clarify the complex relationship underlying virus spread through plant tissues.

2.2. Materials and Methods

2.2.1. Virus purification

A TVCV isolate (strain PV-0361 from DSMZ GmbH, Germany) was propagated in turnip plants (*Brassica rapa* L.). Three weeks after inoculation, the symptomatic systemic leaves were harvested and stored at -80°C. The virus was purified according to the method of Gooding and Herbert, 1967. Infected leaves (5 g) were homogenized in 1 : 4 (w/v) of 0.5 M phosphate buffer (pH 7.4) containing 0.1% (v/v) β-mercaptoethanol and the filtered crud extract was clarified with *n*-butanol (10%; v/v) and stirred for 15 min at room temperature. The mixture was centrifuged at 10000 x *g* for 30 min at 4°C and polyethylene glycol (PEG, MW 6000) was added to the supernatant (4%; w/v). After complete dissolving of PEG the mixture was stand 30 min in ice, then virus precipitated by centrifugation at 10000 x *g* for 15 min at 4°C. Pelleted virus was resuspended in 0.01 M phosphate buffer pH 7.4 (volume equal to 1/5 of initial extract). Resuspended solution was clarified by centrifugation at the same conditions, and supernatant subjected to further purification by an additional precipitation with 4% (w/v) of PEG and 4% (w/v) of NaCl. Finally, virus was precipitated (10000 x *g* for 15 min at 4°C) and resuspended in 0.01 M phosphate buffer (pH 7.4) as storage buffer in a volume equal to 1/50 of the initial extract. Virus concentration was estimated spectrophotometrically using an assumed extinction coefficient (260nm) of 3.0 (Dijkstra and De Jager, 1998; Lockhart, *et al*. 2008). TVCV preparation was aliquoted and stored at -80°C.

2.2.2. Virus infection of Arabidopsis plants

Fully developed rosette leaves of 4-week-old Arabidopsis plants (3 for each plant) were mechanically inoculated with 5 µl of a virus suspension at 40 µg ml^{-1} in 50 mM potassium phosphate buffer, pH 7 and Celite at 4 mg ml^{-1}. The inoculated plants were maintained in growth chamber at the Arabidopsis growth conditions previously described in chapter I.

2.2.3. TVCV CP assay

Leaf samples (about 100 mg) were collected daily up to 7 days post-inoculation from uninoculated rosette leaves (systemic). Leaf tissues were homogenized in an buffer composed of 75 mM Tris/HCl, pH 6.8, 9 M Urea, 4.3 % SDS and 7.5 % β-mercaptoethanol (Lartey *et al*., 1997) in a ratio of 1 : 2 (w/v). The homogenate was centrifuged at 10000 x *g* for 10 min to remove insoluble material. The supernatant was boiled at 100°C for 5 min and 4 µl were analysed by SDS-PAGE (Laemmli, 1970) on 0.75 mm-thick 16% gels. Following electrophoresis, gels were stained with colloidal Coomassie Blue G (Sigma).

2.2.4. Expression and purification of TVCV MP

2.2.4.1. cDNA preparation of TVCV MP

The coding sequence of the TVCV movement protein was derived from pTVCV50 plasmid (this plasmid contains the full-length TVCV cDNA described in Lartey *et al*., 1995 and was kindly obtained from Prof. Ulrich Melcher, Oklahoma State University) by PCR with *pfu* DNA polymerase (Promega) using the following primer pairs: 5'-ATGCGGATCCATGTCGATAGTCTCGTACGA-3' and 5'-ATGCGGTACCTTAAGC ATTGGTATGGGCTC-3'. The PCR conditions were as follows: 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 7 min. The amplification was performed in C1000^{TM} Thermal Cycler (BIO-RAD). The amplicon obtained was cut from 1% agarose gel, purified and then cloned between the *Bam*H I and *Kpn* I sites (underlined in the above primer sequences) into the N-terminal six-histidine tag expression vector pQE30 (The QIA*expressionist*™, Qiagen) (Figure 2.2).

2.2.4.2. Construction of the expression vector

The full length MP gene and pQE30 vector, both digested with *Bam*H I and *Kpn* I, were ligated with T4 DNA ligase (Promega) following the manufacturer's instruction, to obtain enough amount for transformation. The reaction mixture was used to transform *E. coli* strain M15[pREP4] (Qiagen) made competent using rubidium chloride (Hanahan, 1983). This bacterial strain contains pREP4 plasmid which encodes the *lac* repressor (required for regulated recombinant gene expression) and gives kanamycin resistance to the

cell. Aliquots of transformed cells were plated out on LB-agar medium containing kanamycin (25 µg/ml) and ampicillin (100 µg/ml). A positive clone was identified by restriction analysis of mini-prep DNA and following analysis on a 1% agarose TAE gel. The obtained construct, containing a full length TVCV MP cDNA, was named TVCVMP-pQE30 and subjected to sequencing.

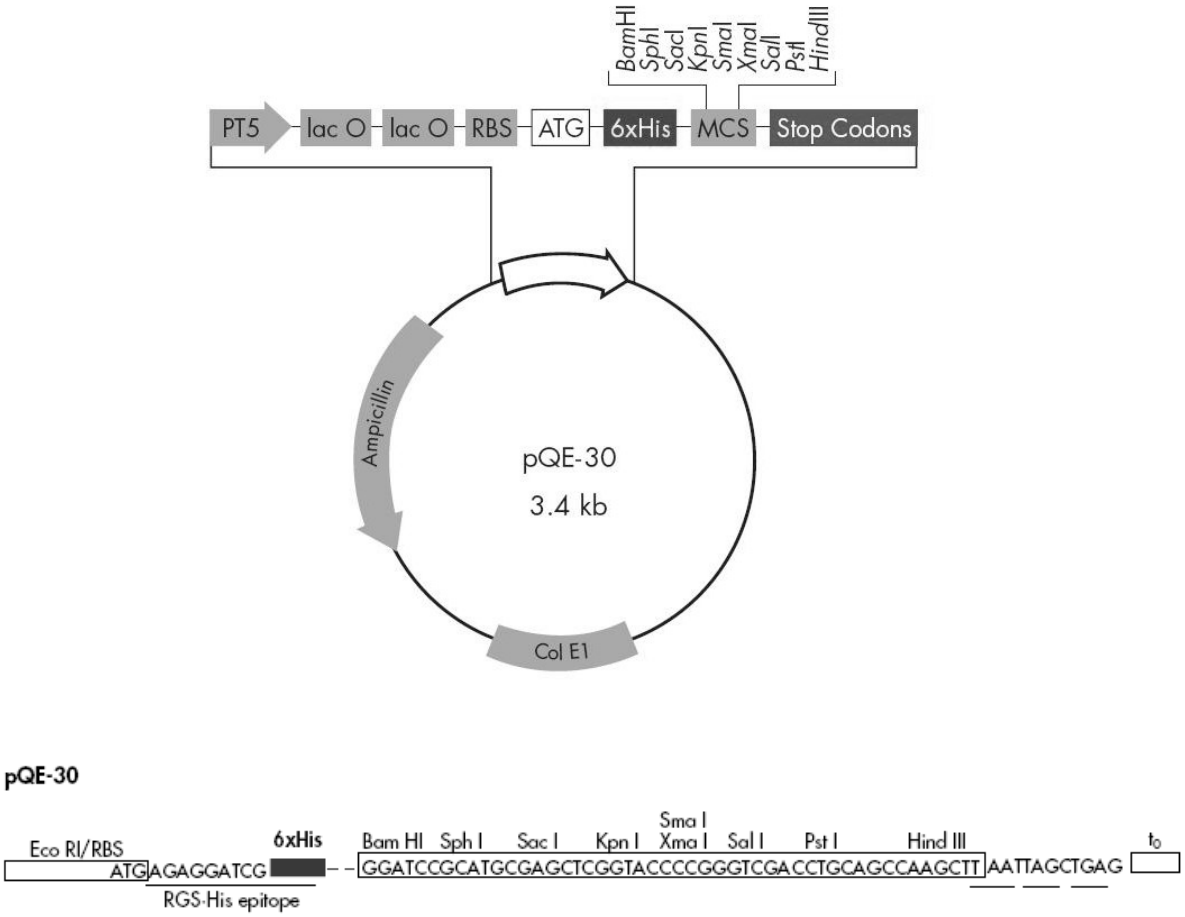

Figure 2.2. pQE30 vector with multiple cloning site (www.qiagen.com)

2.2.4.3. Expression of 6xHis-tagged TVCV MP

The 6xHis tagged MP of TVCV was expressed in *E. coli*. A single colony of *E. coli* harboring the expression vector TVCVMP-pQE30 was grown overnight in 6 ml of LB medium, containing ampicillin (100 µg/ml) and kanamycin (25 µg/ml), at 37°C on a rotary shaker (250 rpm). The overnight culture was added to 125 ml of prewarmed LB medium containing both antibiotics and grown at 37°C until an OD_{600} of 0.6 was reached (30 – 60

min). The expression of His-tagged MP was induced by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1 mM. The culture was left to grow for an additional 4 – 5 h and the cells harvested by centrifugation at 4500 x *g* for 10 min and resuspended in 10 ml of Milli-Q sterile water (Sartorius, Germany). To lyse the cells, 0.5 g of CelLytic powder was added to the solution (0.05 g of the CelLytic™ Express lysis powder for each milliliter of culture, according to the technical bulletin from Sigma C1990) together with protease inhibitor mixture 1:100 (Sigma, P8849). After incubation at room temperature for 20 min, the lysed cell solution was clarified by low speed centrifugation (3000 x *g* for 5 min) and an aliquot of the total lysate analysed on SDS-PAGE and Western blotting to verify the MP expression.

2.2.4.4. Purification of 6xHis-tagged TVCV MP

Purification of 6xHis-tagged MP was obtained by Ni-NTA affinity chromatography. 300 µl of Ni-NTA agarose resin (His-Select® HF Nickel Affinity Gel from Sigma) was used to immobilize the recombinant protein with histidine tag. The matrix was equilibrated twice with 4 bed volumes of equilibration buffer (EB: 50 mM sodium phosphate, pH 8.0, 0.3 M NaCl and 10 mM imidazole) in a microcentrifuge tube (batch mode). Clarified recombinant protein solution (2 ml) was added to nickel resin and incubated for 30 minutes with gently mixing. Mixture was centrifuged at 5000 x *g* for 30 sec to remove the supernatant. The affinity gel was washed with 6 bed volumes of EB, gently mixed and centrifuged at 5000 x *g* for 30 sec. This step was repeated (four or five times) until the OD_{280nm} of the supernatant was less than 0.01. To elute bound protein, affinity gel was treated three times with about 1 bed volume of EB containing 250 mM imidazole and 0.1 % Tween 20 and centrifuged at 5000 x *g* for 30 sec to save supernatant. Eluted fractions were analysed by SDS-PAGE (0.75 mm-thick 12% gels) and Western Blot.

2.2.4.5. Western blot analysis

Aliquots (12µl) of total lysate and eluted MP fractions were subjected to SDS-PAGE (12%) and electrotransferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore corporation, USA, P-15552) using a semidry transfer apparatus (Trans-Blot® SD, BIO-RAD) at 8 V for 30 min. The membrane was blocked with 3% BSA in Phosphate buffered Saline (PBS buffer; 140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4,

pH 7.4) for 2 h at room temperature with agitation. After three washing (5 min each) with PBST buffer (PBS + 0.05% Tween 20), the membrane was incubated with a Monoclonal Anti-polyHistidine Clone HIS-1 (mouse IgG2a isotype; Sigma, H1029) diluted 1:3000 (as per manufacturer's instructions) for 2 h at 37°C followed by 3 washes, 5 min each, with PBST buffer. The membrane was incubated with the secondary antibody (Anti-Mouse IgG, Fab specific, conjugated to Alkaline Phosphatase obtained from Sigma, A2179) diluted 1:30000 (as per manufacturer's instructions) for 1 h at 37°C. After washing away the unbound antibodies (3 times with PBST), the membrane was equilibrated with alkaline phosphate buffer (TBS: 0.1 M Tris/HCl, 0.1 M NaCl and 5 mM $MgCl_2$, pH 9.5) and stained with NBT/BCIP solution (0.5% 4-nitro blue tetrazolium chloride [NBT], 0.4% 5-bromo-4-chloro-3-indolyl phosphate [BCIP] in TBS). The reaction was stopped and de-stained using water and membrane was air dried.

2.2.5. Study of TVCV MP-PME interaction

2.2.5.1. Intercellular washing fluid (IWF) isolation

IWFs were collected from Arabidopsis leaves by centrifugation as described by Lionetti *et al*., 2007 and according to Lohaus *et al*., 2001. Rosette leaves were excised from 4-week-old wild-type plants and vacuum-infiltrated with 25 mM Tris/HCl pH 7.0, 1 mM EDTA containing 1 M LiCl for 1 h. Leaves were stacked in the bottom of a 5-ml plastic syringe then IWF was recovered by centrifuging at 1000 x *g* for 5 min at 4°C. The amount of IWF obtained from 1 g of tissue (fresh weight) was 0.4 - 0.5 ml.

2.2.5.2. Detection of PME activity

PME activity was detected by the radial gel diffusion assay as described by Downie *et al*., 1998 with some modifications. A gel was prepared with 0.1% (w/v) of 81% methylesterified lime pectin (E81 Danisco A/S Copenhagen, Dk), 0.8% (w/v) agarose, 12.5 mM citric acid and 50 mM Na_2HPO_4, pH 6.3. The gel was cast into agar plates (13 ml per plate) and allowed to polymerize at room temperature. Wells with a diameter of 4 mm were made and aliquots of IWFs were loaded in each well. Plates were incubated at 28°C for 16 h. The gels were stained with 0.05% (w/v) ruthenium red for 45 min to visualize the

red-stained areas, resulting from the hydrolysis of esterified pectin by the action of PME in the gel.

2.2.5.3. MP-PME binding by affinity chromatography

After swelling with 10 ml of cold 1 mM HCl for 30 min and equilibration with 5 volumes of $NaHCO_3$/NaCl coupling buffer (0.1 M $NaHCO_3$ pH 8.3 containing 0.5 M NaCl), 50 mg of cyanogen bromide (CNBr)-activated resin (CNBr-Sepharose® 4B, Sigma) was mixed with about 50 µg of bacterially expressed 6xHis tagged TVCV MP (1 ml), previously dialyzed against $NaHCO_3$/NaCl coupling buffer, and incubated with gentle mixing for 4 h at room temperature. After washing with $NaHCO_3$/NaCl coupling buffer to remove unreacted ligand, the MP Sepharose was treated with 1 M ethanolamine at pH 8.0 for 2 h at room temperature and subjected to extensively washing cycles (four or five times) with high and low pH buffer solutions (basic coupling buffer at pH 8.3 and 0.1 M acetate buffer at pH 4.0, containing 0.5 M NaCl). Finally the resin was equilibrated in a buffer (EB) containing 25 mM Tris/HCl (pH 8.0) 1 mM EDTA and 50 M LiCl . Adsorption of PME was performed in the batch mode with 2 h of contact of IWFs (300 µl dialyzed against EB buffer) with MP Sepharose. After extensively washing with EB until OD_{280nm} was close to zero, stepwise elution of proteins was performed with 100 µl of elution buffer, 25 mM Tris/HCl (pH 8.0), 1 mM EDTA, containing 0.5 M and 1 M LiCl, according to Dorokhov *et al*. (1999). Sepharose matrix without ligand (TVCV MP) was prepared as described and used as control. IEF-PAGE gels were used to detect PME activity in eluted fractions.

2.2.5.4. Immunodetection of MP immobilized to the Sepharose matrix

MP immobilized to the Sepharose matrix was detected following incubation of an aliquot of the gel with Monoclonal Anti-polyHistidine antibody (Sigma) for 2 h at 37°C and with the secondary antibody conjugated to Alkaline Phosphatase (Anti-Mouse IgG, Sigma) for 1 h followed by the color reaction with NBT/BCIP solution as described in western blot analysis.

2.2.5.5. Isoelectrofocusing and gel detection of PME

A 0.8 mm thick polyacrylamide gel (PAA) polymerized on a GelBond® PAG film (GE Healthcare Bio-Science), containing 5% (v/v) carrier ampholytes in the pH range 3.0-10.0 (Sigma), was prefocused for 30 min and loaded with aliquots (25µl) of affinity-eluted fractions. The run was carried out horizontally on a Flat Bed Apparatus FBE 3000 (Amersham Pharmacia) at a constant power of 5 W for 2 h. After the run, a 1% agarose gel (on GelBond® film, Amersham Bioscience) prepared with 0.05% (w/v) of 81% methylesterified pectin (E81, Danisco) at pH 6.3 was overlaid on the PAA gel. After 2 h at 28°C the agarose gel was stained for 1 h with ruthenium red (0.05% w/v in water) and then rinsed thoroughly with water until red coloured bands were distinct from the colourless background. Finally, the gel was air dried.

2.3. Results

2.3.1. Pectin methylesterase and virus movement

The ability of viruses to cross the plant cell wall to propagate infection throughout a plant is based on interactions among viral and host factors. The cell wall-located pectin methylesterase (PME) is one of these factors. It is established that PME can act as host-cell receptor of Tobacco mosaic virus movement protein (TMV MP) and also the PME-MP interaction is required for cell-to-cell movement of the virus through plasmodesmata. Plant PMEs have also been shown to interact with MP from other viruses such as *Turnip vein clearing virus* (TVCV) and *Cauliflower mosaic virus* (CaMV) suggesting this interaction as possibly being involved in the cell-to-cell movement of the virus. However, the role of the PME-MP interaction to virus movement has not been elucidated. PME activity in plant is regulated by several factors including the apoplastic pectin methylesterase inhibitors (PMEIs) (Balestrieri *et al*., 1990; Wolf *et al*., 2003; Raiola *et al*., 2004; An *et al*., 2008). With the aim to better clarify the mechanism of virus movement *in planta* and to possibly counteract the viral infection we have tested the ability of the invading virus to move systemically within host plants over-expressing PMEI. As host we have used Arabidopsis plants over-expressing AtPMEI-1 (two independent lines; lines 1-1 and 1-5) and AtPMEI-2 (lines 2.7 and 2.9) and as challenged virus the TVCV, a tobamovirus infecting crucifers. In these plants we have analysed, during the time of infection, the TVCV spreading in comparison with untransformed plants.

2.3.2. Infection of Arabidopsis by TVCV

TVCV is a member of subgroup 3 of the *Tobamovirus* genus infecting *Arabidopsis thaliana*. Purified virus (Figure 2.3) was used to mechanically inoculate 4-weeks-old Arabidopsis plants with a viral suspension of 200 ng / leaf (3 rosette leaves / plant). Inoculated plants showed markedly reduced growth after two-three weeks compared to uninoculated ones (Figure 2.4).

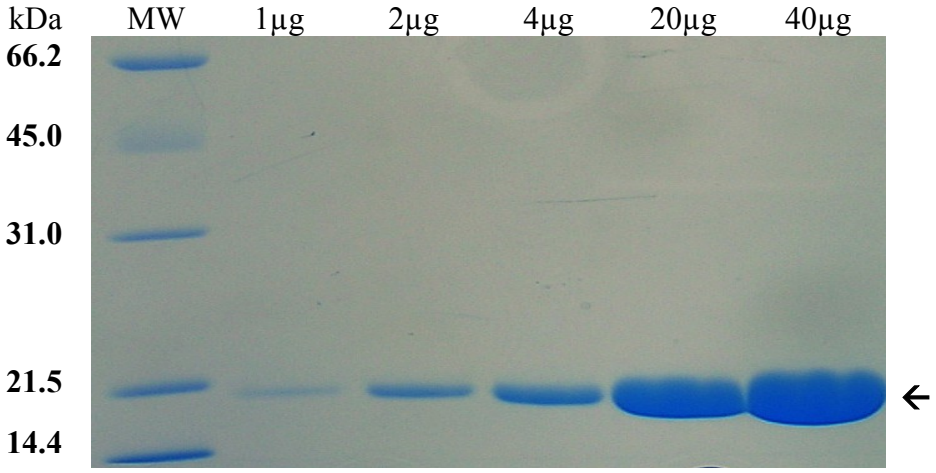

Figure 2.3. Purified TVCV. MW, molecular weight marker. Other lanes represent different amounts of purified virus. Arrow indicates TVCV CP (17.6 kDa; Lartey *et al*., 1995).

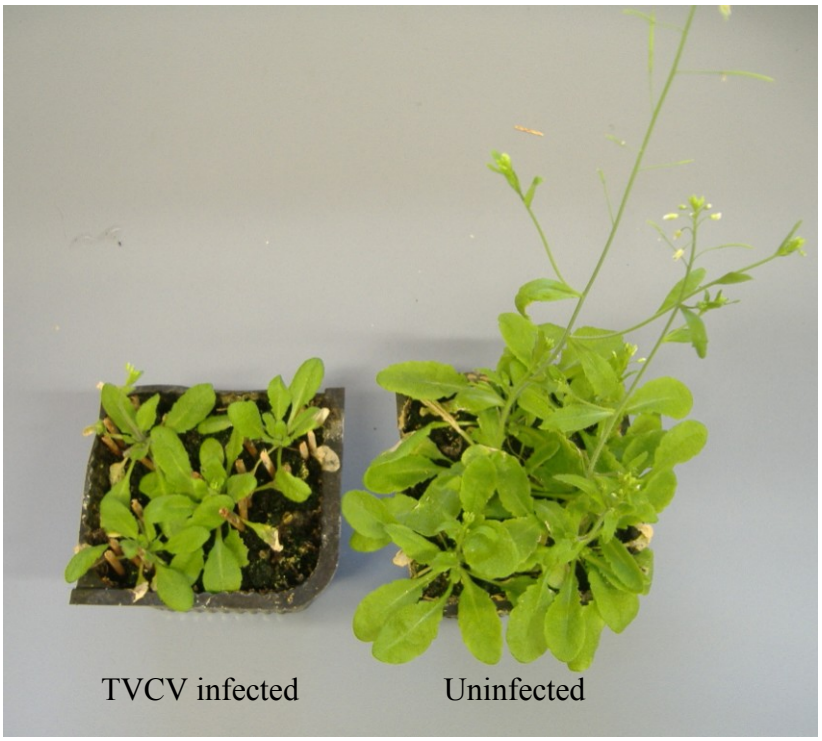

Figure 2.4. Symptoms of TVCV infection in Arabidopsis plants.

2.3.3. Viral coat protein detection

Viral movement in host plants is an important factor for development of disease. Following initial infection, plant viruses move through plasmodesmata (cell junctions in plants) to spread cell-to-cell until they reach vasculature. Viruses then move systemically through the vasculature and establish disease. As indicator of the presence of the virus, SDS-polyacrylamide gels were used to detect the presence of the coat protein (CP). Because very few proteins migrate on SDS-PAGE to the position of tobamoviral coat proteins and also because CP is the most abundant viral protein, the intensity of this band was used as an indicator of virus concentration (Lartey *et al*., 1997; Hii *et al*., 2002). SDS-PAGE analysis of total proteins isolated from Arabidopsis leaves challenged with TVCV shows the presence of TVCV CP band allowing a direct detection and quantification of viral infection (Figure 2.5).

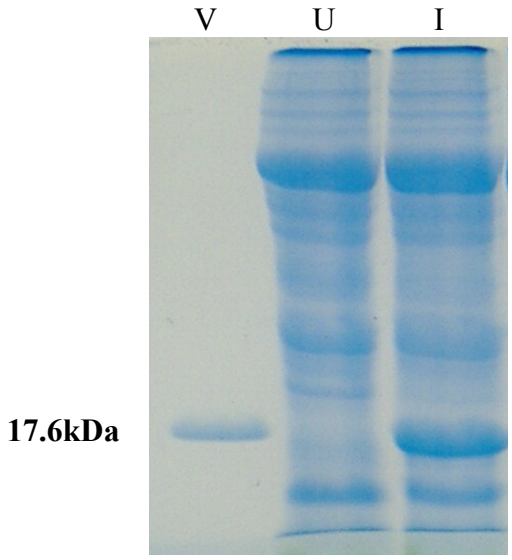

Figure 2.5. SDS-PAGE analysis of total proteins isolated from Arabidopsis wild-type rosette leaves. Lane V, purified TVCV (CP, 17.6 kDa); lane U, total protein extract from uninfected leaves; lane I, total protein extract from infected leaves.

2.3.4. Systemic accumulation of TVCV in rosette leaves of wild-type and overexpressing AtPMEI-1 and AtPMEI-2 Arabidopsis plants

The kinetics of viral spread was determined in AtPMEI-1 (lines 1-1 and 1-5) and AtPMEI-2 (lines 2-7 and 2-9) plants in comparison with untransformed plants (WT).

TVCV systemic infection was monitored by examining the appearance of TVCV CP in uninoculated rosette leaves, reaching its maximum accumulation within six - seven days post-infection (Lartey *et al*., 1997). Differently from WT and AtPMEI-1 plants, no viral CP was detected in systemic leaves of AtPMEI-2 plants (lines 2-7 and 2-9) at 6 days post infection (DPI) (Figure 2.6). On the other hand, viral CP was detected in all inoculated leaves at 6 DPI (Figure 2.7), indicating that TVCV efficiently infects Arabidopsis plants, transformed and untransformed, locally.

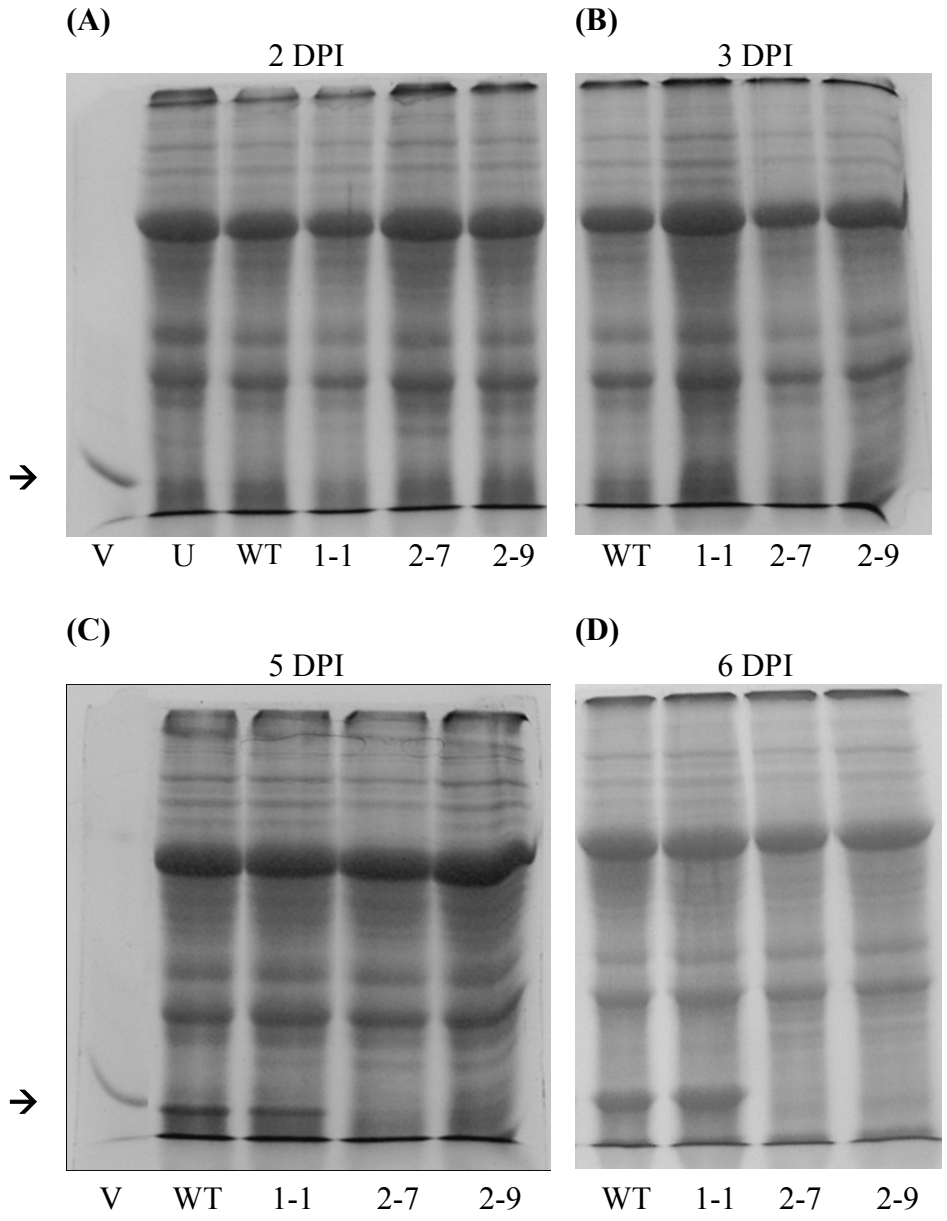

Figure 2.6. Infection kinetics following the TVCV CP accumulation in uninoculated Arabidopsis rosette leaves (systemic infection) in wild-type and AtPMEI plants. Panels (A), (B), (C) and (D) show 2, 3, 5 and 6 days post-infection (DPI), respectively. Lanes V, purified TVCV (CP, 17.6 kDa); lane U, total protein extract from uninfected plants; other lanes, total protein extracts from systemic leaves of wild-type, AtPMEI-1 (line 1-1) and AtPMEI-2 (lines 2-7 and 2-9) TVCV-infected plants, respectively. Arrows indicate the position of TVCV CP.

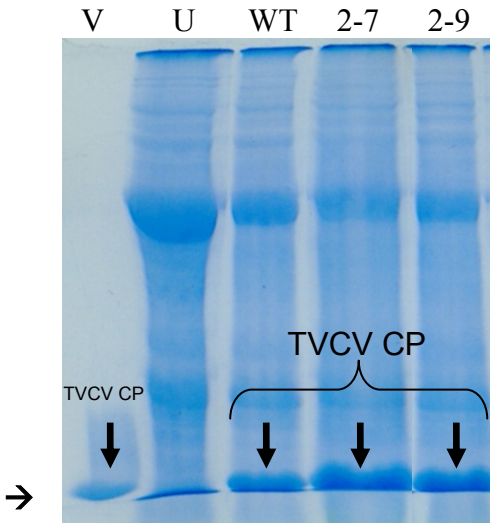

Figure 2.7. Electrophoretic protein pattern obtained from directly TVCV-inoculated leaves (local infection) 6 DPI. Lane V, purified TVCV CP (17.6 kDa); lane U, protein extract from control plants; other lanes refer to protein extracts from inoculated leaves of wild-type and AtPMEI-2 plants (lines 2-7 and 2-9), respectively. Arrows indicate TVCV CP.

This analysis was extended to follow the kinetics of viral spread in AtPMEI overexpressing plants in comparison with untransformed. Data showed a delay in the systemic movement of TVCV in Arabidopsis plants over-expressing AtPMEI-2 (two independent lines; 2-7 and 2-9). In these AtPMEI-2 transformed plants the virus was detected from 7th day post infection differently from WT and AtPMEI-1 plants (lines 1-1 and 1-5) where the virus was already accumulated at high level (Figure 2.8).

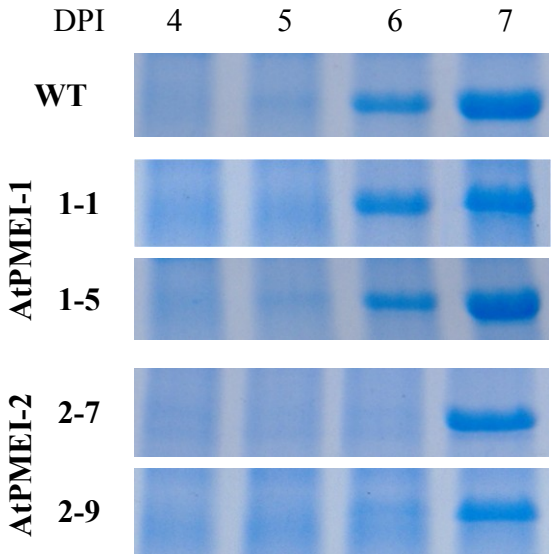

Figure 2.8. Accumulation of TVCV CP determined after seven days post-infection. Lanes show 4, 5, 6 and 7 days post-infection (DPI) in wild-type, AtPMEI-1 (lines 1-1 and 1-5) and AtPMEI-2 (lines 2-7 and 2-9) plants.

2.3.5. Cloning and expression of TVCV encoded movement protein (TVCV MP)

Cell-to-cell movement requires the increase of plasmodesmata size exclusion limit (Wolf *et al*., 1989; Angell *et al*., 1996) to permit the movement of the viral nucleic acid and is mediated by the interaction of the virus encoded movement proteins (MPs) with several cellular components, among which plant pectin methylesterases (PMEs) associated with plasmodesmata. Tobacco mosaic virus (TMV)-MP has been shown to interact with various PMEs and in tobacco this interaction is required for cell–to-cell and systemic movement (Dorokhov *et al*., 1999; Chen *et al*., 2000; Chen and Citovsky, 2003). A specific TMV MP domain, between amino acid residues 130-185, has been shown to be involved in binding to PME (Chen *et al*., 2000). A comparison between the movement proteins of TMV and TVCV has revealed a higher amino acid identity (44%) of the region 130-185 with respect to the identity between the complete amino acid sequence of the two proteins (33%) (Figure 2.9). In order to verify the interaction between TVCV MP and PMEs of Arabidopsis, an heterologous expression of TVCV MP has been performed. To this aim, TVCV MP cDNA

was introduced into the pPICZαA vector to transform *Pichia pastoris* but the expression of the recombinant MP was unsuccessful. Therefore MP cDNA has been cloned in the pQE30 vector to be expressed and purified as a recombinant histidine-tagged protein in *E. coli*.

```
CLUSTAL 2.0.10 multiple sequence alignment


MP-TMV          MALVVKGKVNTNEFIDLTKMEKILPSMFTPVKSVMCSKVDKIMVHENESLSEVNLLKGVK 60
MP-TVCV         -MSIVSYEPKVSDFLNLSKKEEILPKALTRLKTVSISTKDIISVKESETLCDIDLLINVP 59
                   :*. : :..:*::*:* *:***. :* :*:*  *. * * *:*.*:*.:::** .*

MP-TMV          LIDSGYVCLAGLVVTGEWNLPDNCRGGVSVCLVDKRMERADEATLGSYYTAAAKKRFQFK 120
MP-TVCV         LDKYRYVGILGAVFTGEWLVPDFVKGGVTISVIDKRLVNSKECVIGTYRAAAKSKRFQFK 119
                * .  ** : * *.**** :**  :***::.::***: .:.*..:*:* :** .******

MP-TMV          VVPNYAITTQDAMKNVWQVLVNIRNVKMSAGFCPLSLEFVSVCIVYRNNIKLGLREKITN 180
MP-TVCV         LVPNYFVSTVDAKRKPWQVHVRIQDLKIEAGWQPLALEVVSVAMVTNNVVMKGLREKVVA 179
                :**** ::* ** :: *** *.*::::*:.**: **:**.***.:* .* :  *****:.

MP-TMV          VRDGGPMELTEEVVDEFMEDVPMSIRLAKFRSRTGKKS--DVRKGKNSSSDRSVPNKNYR 238
MP-TVCV         INDPD-VEGFEGVVDEFVDSVAAFKAVDNFRKRKKKVEERDVVSKYKYRPEKYAGPDSFN 238
                :.* . :*  * *****::.*.    : :**.*. * .  ** .  :  .:: .  ..:.

MP-TMV          NVKDFGGMSLKKNNLIDDDSEATVAESDSF 268
MP-TVCV         LKEENVLQHYKPESVPVLRSGVGRAHTNA- 267
                  ::      * :.:    * .  *.:::
```

Figure 2.9. Amino acids alignment between TMV MP and TVCV MP. The alignment indicates the match points between both MPs in the region 130-185 showing an identity of 44% (underlined).

To obtain TVCV MP, the coding sequence was amplified from the plasmid pTVCV50 and cloned into the pQE30 expression vector, to create the construct TVCVMP-pQE30 (Figure 2.10). The pQE vectors permit high-level of expression of recombinant proteins in *E. coli*. The vector pQE30 provides 6 histidine at the N-terminus of the cloned proteins, allowing purification of the recombinant His-tagged proteins by immobilized metal affinity chromatography. Figure 2.11 shows the TVCVMP-pQE30 vector after digestion with restriction endonucleases *Bam*H I and *Kpn* I and the resulting DNA fragment corresponding to MP gene (804 bp). The recombinant vector was used to transform *E. coli*.

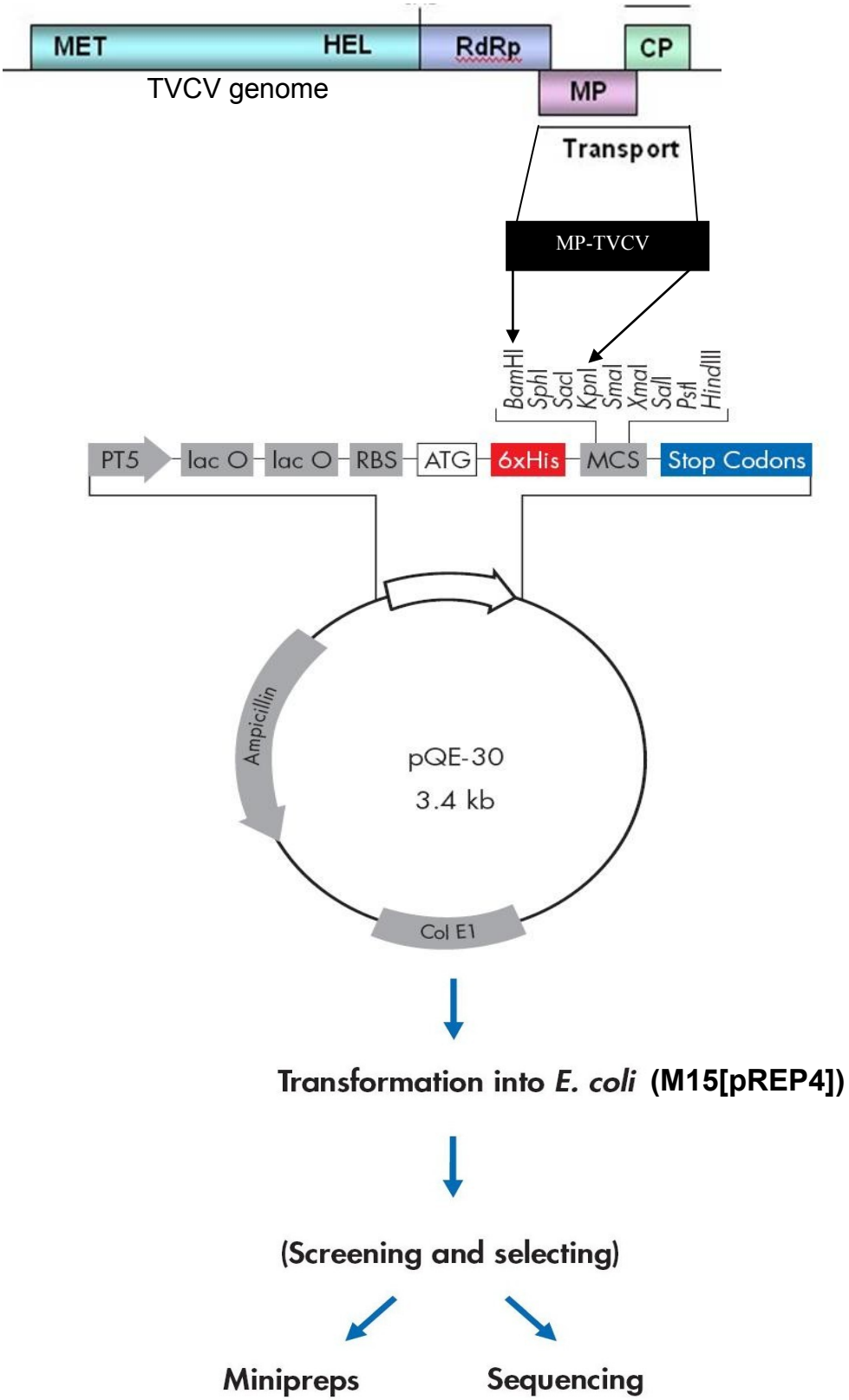

Figure 2.10. TVCVMP-pQE30 expression vector used to transform *E. coli*.

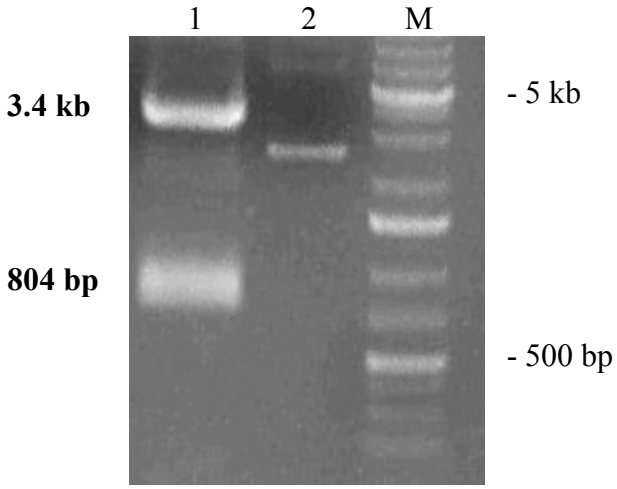

Figure 2.11. Agarose gel analysis of TVCVMP-pQE30 construct. Lane M, molecular weight marker; lane 1, TVCVMP-pQE30 digested with *Bam*H I and *Kpn* I. The resulting bands correspond to MP cDNA (804 bp) and to pQE30 linearized vector (3.4 kb); lane 2, uncut TVCVMP-pQE30 vector.

2.3.6. Purification and detection of 6xHis-tagged TVCV MP

The recombinant His-tagged MP was expressed in *E. coli* and purified by affinity chromatography using Ni-NTA agarose. After MP gene induction, cell extract from *E. coli* harboring TVCVMP-pQE30 plasmid displayed a protein band with an approximate apparent molecular mass of 35 kDa, as detected by SDS-PAGE, whereas bacterial cells untransformed or transformed with the empty vector did not show the same protein (Figure 2.12). The contaminating *E. coli* proteins were subsequently washed from the Ni-NTA matrix and 6xHis-MP was eluted by competition with imidazole. Collected fractions were applied to 12% SDS-PAGE (Figure 2.12).

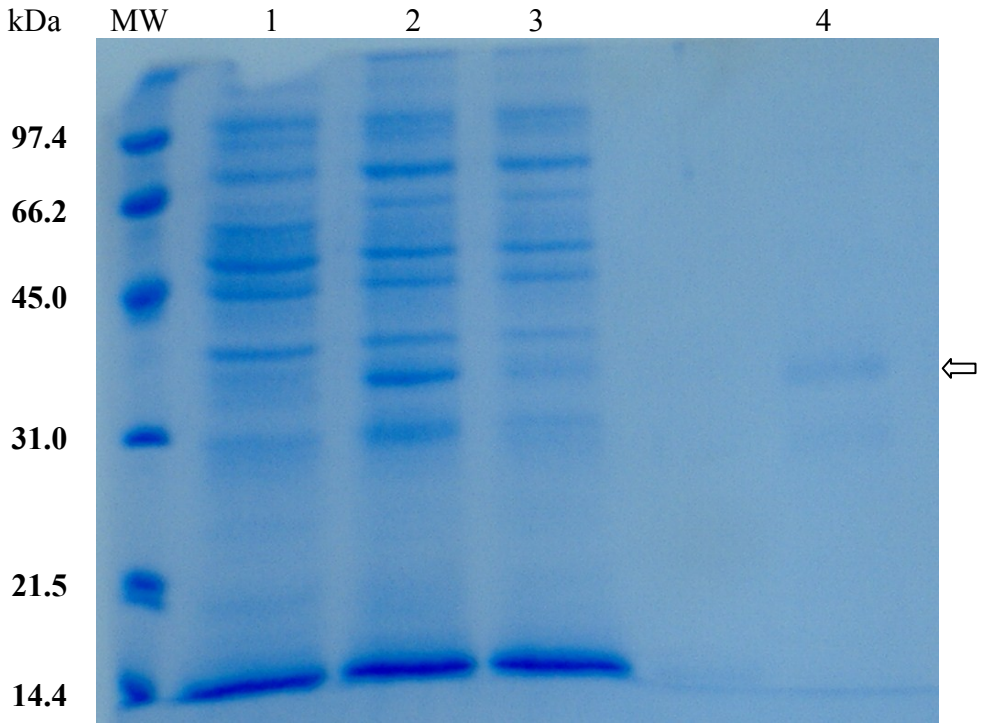

Figure 2.12. Expression and purification of His-tagged TVCV MP (30 kDa). Lane MW, molecular weight marker; lane 1, proteins from induced (IPTG) *E. coli* M15 cells harboring pQE30 empty vector (negative control); lane 2, proteins from induced (IPTG) *E. coli* M15 cells harboring the expression plasmid TVCVMP-pQE30; lane 3, flow-through from transformed *E. coli* after affinity chromatography; lane 4, eluted 6xHis-MP. Arrow indicates the MP band.

To verify the production of recombinant 6xHis tagged MP in *E coli*, a western blot detection was achieved by using an antibody against 6-histidine epitope tag. As shown in Figure 2.13, after blotting, the induced MP in transformed *E. coli* was targeted by a monoclonal anti-polyhistidine antibody, whereas any signal was present in the samples obtained from untransformed bacteria. The detection of other bands in western blot may represent truncated forms of the protein likely due to the presence in the coding sequence of several arginine codons (AGG and/or AGA), rarely used in *E. coli* (Rosenberg *et al*., 1993). Also potential proteolytically digested MP cannot be excluded. Yield of MP expression was estimated to be about 2 μg for ml of bacterial culture.

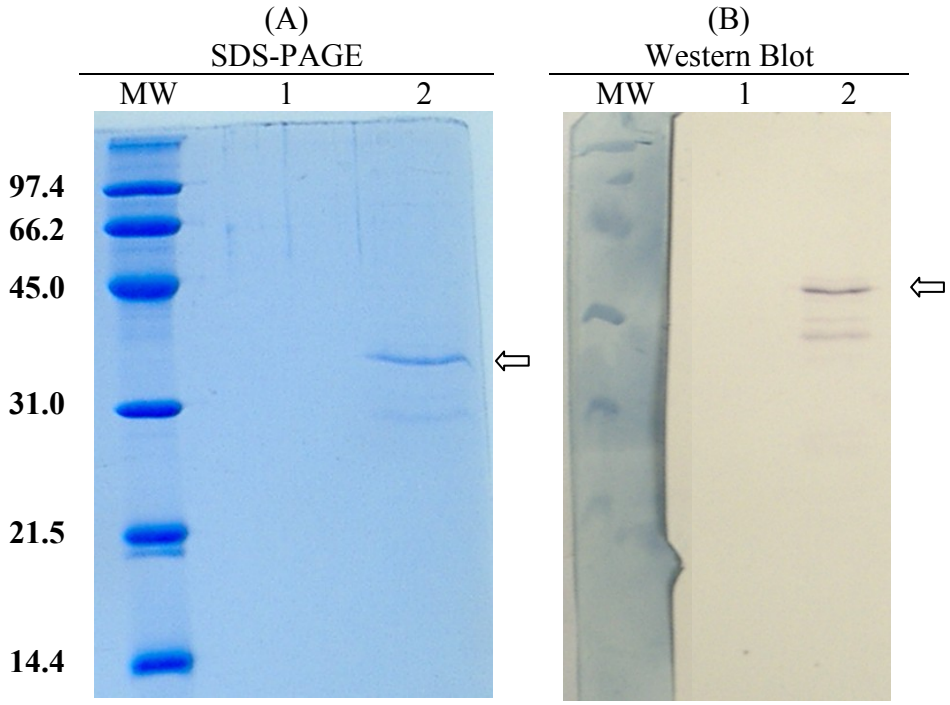

Figure 2.13. Western blot analysis for 6xHis tagged TVCV MP. (A) SDS-PAGE (B) PVDF membrane. Lane MW, molecular weight marker; lane 1, eluted fraction from induced *E. coli* M15 (negative control); lane 2, eluted MP. Arrow indicates the full-length MP.

2.3.7. Isolation of cell wall-associated Arabidopsis PMEs and examination of the binding to TVCV MP

2.3.7.1. Intercellular Washing Fluid (IWF) Isolation

To verify the ability of the cell wall-associated PME to specifically bind the TVCV encoded MP, intercellular fluids (IWFs) were isolated from mature rosette leaves and assayed using the quantitative PME activity radial gel diffusion assay (Downie *et al*., 1998). Figure 2.14 shows PME activity detected in the extracellular fluids after analysis on agarose gel and ruthenium red staining.

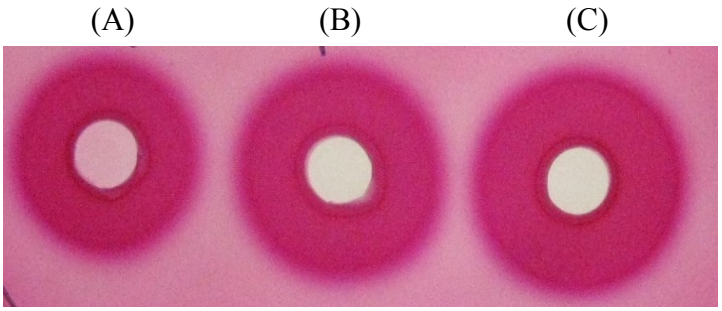

Figure 2.14. Gel diffusion assay showing the PME activity detectable in 5, 10, 15 µl (A, B and C, respectively) of IWF.

2.3.7.2. Binding of the TVCV MP to PME

Purified MP, containing a polyhistidine tag, was immobilized on a Sepharose matrix to isolate interacting PME by affinity chromatography. To confirm the binding of viral movement protein to Sepharose, an aliquot of the resin was probed with an anti-polyhistidine antibody. Following immunodetection, Sepharose matrix containing TVCV MP (Sepharose-MP) became colored, differently from resin without ligand (Sepharose) used as control (Figure 2.15). Both Sepharose matrixes, with and without MP, were incubated with IWFs to allow the tight and selective association with its proteins. Following incubation, the eluted bound proteins were analysed by isoelectrofocusing polyacrylamide gel electrophoresis (IEF-PAGE).

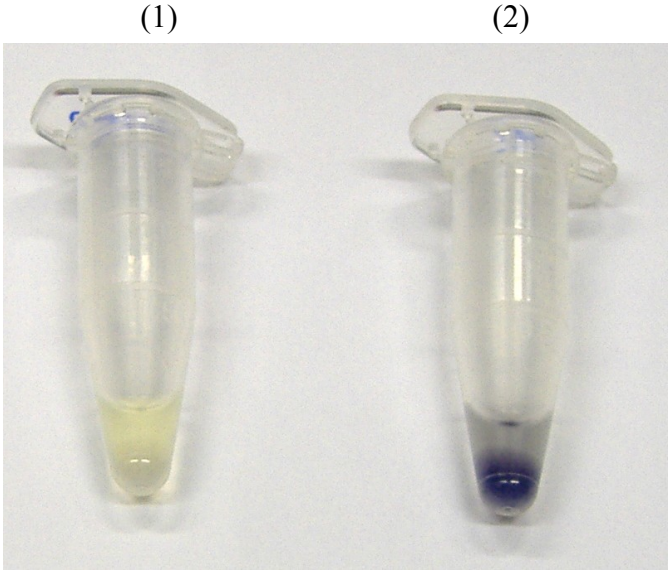

Figure 2.15. Immunodetection of MP immobilized to the Sepharose matrix using an anti-polyhistidine antibody. (1) Sepharose matrix without ligand (control), (2) Sepharose matrix with TVCV MP.

2.3.7.3. IEF gel detection of interacting PME

Analytical IEF-PAGE separation, agarose overlay technique and ruthenium red staining were used to detect PME activity in the fractions obtained by affinity chromatography using immobilized MP. IWFs were loaded on Sepharose-MP and, following an extensive wash, the bound protein was eluted at high ionic strength. Whilst no PME activity (Figure 2.16; lanes 6 and 7) was detected in the fractions eluted from the Sepharose matrix (control), the high-salt eluted fractions from Sepharose-MP showed PME activity (Figure 2.16; lanes 4 and 5), indicating its ability to bind MP.

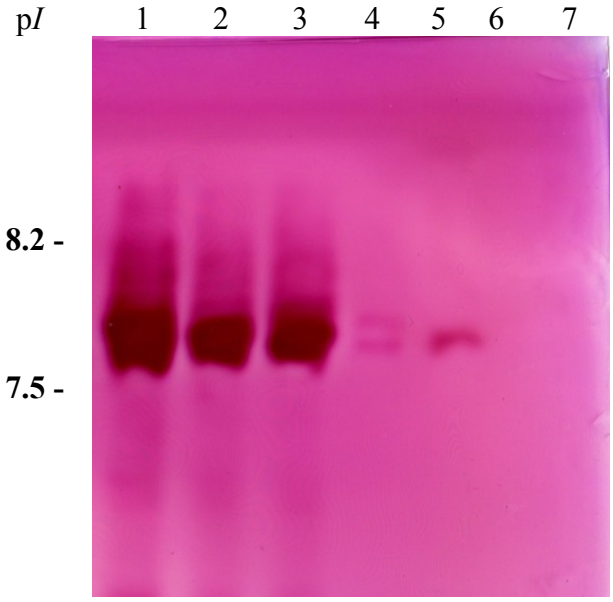

Figure 2.16. Thin layer IEF separation in the pH range 3-10 of PME before and after affinity chromatography. Lane 1, PME activity of the sample loaded on Sepharose with and without MP; lane 2, flow-through from Sepharose (control); lane 3, flow-through from Sepharose-MP; lanes 4 and 5, high-salt eluted fractions with 0.5 M LiCl from Sepharose-MP; lanes 6 and 7, high-salt eluted fractions from Sepharose (control).

2.4. Discussion

Plant viruses diffuse in host plants locally by cell-to-cell movement through plasmodesmata, and systemically through the host vascular system. Viral spread through the vascular tissue is mostly a passive process, occurring with the flow of photoassimilates (Leisner and Howell, 1993). Cell-to-cell movement is an active function, requiring specific interaction between the invading virus and plasmodesmata. Cell-to-cell movement is a function regulated by viral coded proteins and plant host factors (Whitham and Wang, 2004; Scholthof, 2005). Some of the interactions between viral and host proteins may be explained in several ways, suggesting that these interactions may be multifunctional (Boevink and Oparka, 2005). The host proteins known to have a role or able to interact with viral proteins are mostly localized intracellularly. It has been shown that an enzymatic protein that acts on cell wall pectins, the pectin methylesterase (PME), acts as a host-cell receptor of the Tobacco mosaic virus movement protein (TMV MP) (Chen *et al*., 2000). This interaction between PME and the virus movement protein is required for the translocation of the virus from cell to cell through plasmodesmata.

Regulation of PME activity can be obtained by specific protein inhibitors (PMEIs). Therefore PMEI interacting with plant PME may interfere with viral transport and then with viral systemic diffusion by influencing MP-PME binging. To verify if PMEI can be exploited to contain viral diseases, the level of viral resistance in plants containing high amount of PME inhibitor has been analysed.

Arabidopsis plants over-expressing AtPMEI-1 or AtPMEI-2 have been challenged with *Turnip vein clearing virus* (TVCV). In these plants we have analysed, during the time of infection, the TVCV spreading in comparison with untransformed plants. We examined TVCV systemic infection of Arabidopsis by monitoring the appearance of TVCV coat protein band (17.6 kDa) using SDS-PAGE analysis. Differently from WT and AtPMEI-1 plants, when viral CP appear in the uninoculated leaves, no CP was detected in systemic leaves of AtPMEI-2 plants. So a delay in the systemic TVCV infection was observed in Arabidopsis plants over-expressing high level of AtPMEI-2.

AtPMEI-1 and AtPMEI-2 have different biochemical properties: i.e. AtPMEI-2 has a stronger affinity respect to AtPMEI-1 for plant PME (Raiola *et al.*, 2004) and may interact differently with PME isoforms.

Three different glycoforms of AtPMEI-2 are present in the transformed plants (Lionetti *et al.*, 2007) and may form a complex with endogenous PME, consistent with the previous observation that the fully glycosylated recombinant AtPMEI-2, expressed in *Pichia pastoris*, inhibits plant PMEs (Raiola *et al.*, 2004).

AtPMEI-2 may compete during virus infection with MP, for the same plant PME, affecting viral spread.

Because TMV MP region between amino acid residues 130-185 has been demonstrated to be involved in binding PME from tobacco and the alignment between TMV MP and TVCV MP has revealed an higher amino acid identity of the region 130-185 with respect to the identity between the complete amino acid sequence of the two proteins, we have performed an heterologous expression of TVCV MP to study the molecular bases of protein-protein interactions involved in this viral diffusion process. To this aim, TVCV MP cDNA has been introduced in the pQE30 vector to be expressed and purified as a recombinant histidine-tagged protein. The recombinant protein was recognized by histidine-tag-specific monoclonal antibody in immunoblot analysis. Purified MP has been immobilized on a Sepharose matrix to be used to identify an interacting protein showing PME activity. PME interaction with TVCV MP suggests a role for this cell wall-associated enzyme in TVCV infection process. Its characterization may help to clarify the role of PME in plant viral movement and the behaviour showed by AtPMEI plants during infection.

Arabidopsis PME able to bind TVCV MP will permit to study the molecular bases of PME-MP interaction also in the presence of the PME inhibitors. One of the aims of this research is to extend the knowledge on the interaction between PME and virus movement protein to better understand the complex relationship underlying virus spread through the plant tissues.

CONCLUSIONS

CONCLUSIONS

Pectin, one of the main components of plant cell wall, is secreted in a highly methyl-esterified form and subsequently de-esterified *in muro* by pectin methylesterases (PMEs). Pectin is responsible for mechanical properties of the wall such as porosity, adhesion and rigidity and is the substrate of pectin-degrading enzymes such polygalacturonases and pectin methylesterases. The level and pattern of pectin methylesterification play a role in constitutive resistance to fungal and bacterial pathogens influencing the susceptibility of the wall to microbial pectin-degrading enzymes. Regulation of PME activity can be obtained by specific protein inhibitors (PMEIs). By inhibiting the endogenous PME and maintaining the pectin in a highly methylated form, PMEI can play a role in the resistance of plants against pathogens attack. In this thesis, AtPMEI plants, so far characterized for resistance to the necrotrophic fungus *Botrytis cinerea*, were also found to be more resistant to the necrotrophic bacterium *P. carotovorum*. Soft rot pathogens represent one of the most serious threats to agriculture. The exploitation of natural resistance or the production of transgenic resistant genotypes is required to develop a more environmentally safe protection. It is known that pectin degrading enzymes are produced by pathogens to macerate and spread into the plant tissue. This research demonstrates that by transgenic approach, based on PMEI overexpression, it is possible to significantly increase the level of resistance against two soft rot pathogens. On the other hand, another approach to reduce PME activity is the gene disruption of selected PME isoforms. The analysis of resistance of an Arabidopsis knockout mutant in *PME3* gene reveals that downregulating PME activity by reverse genetics approaches it is possible to increase resistance towards soft rot pathogens. The recent resolution of the 3D structure of PME/PMEI complex provides new information about the functional properties of these plant inhibitors and may permit to design more efficient inhibitors and improve plant protection against pathogens, possibly in combination with inhibitors belonging to other families.

Another aspect that has been investigated in this research is the involvement of PMEs and of their inhibitors during a viral infection process. While the expression of the viral message and the replication of viral nucleic acid are important phases of the infection

cycle, if the infection stops within the first infected cells, which are often small in number, the plant remain healthy. So the capacity of a virus to move within a plant is therefore an important component of a successful infection. PMEs are thought to play also a role in cell-to-cell and systemic movement of the tobamoviruses. It has been exploited the possibility to counteract viral propagation targeting PME. A delay in TVCV spreading in Arabidopsis plants overexpressing AtPMEI-2 has been observed. It is conceivable that the tight regulation of different tissue-specific PME isoforms and the balance between PME and MP levels are likely involved. Targeting plant factors involved in viral infections could be a promising approach to defend plants against viral pathogens. It may be alternative tool to pathogen-derived resistance, whose the major drawback is the extreme specificity of plant protection and the risk of formation and diffusion of new recombinant viral agents.

REFERENCES

REFERENCES

An, S. H., Sohn, K. H., Choi, H. W., Hwang, I. S., Lee, S. C., and Hwang, B. K. (2008). Pepper pectin methyl-esterase inhibitor protein CaPMEI1 is required for antifungal activity, basal disease resistance and abiotic stress tolerance. *Planta*, 228: 61–78.

Angell, S. M., Davies, C., and Baulcombe, D. C. (1996). Cell-to-cell movement of potato virus X is associated with a change in the size exclusion limit of plasmodesmata in trichome cells of Nicotiana clevelandii. *Virology*, 216: 197-201.

Balestrieri, C., Castaldo, D., Giovane, A., Quagliuolo, L., and Servillo, L. (1990). A glycoprotein inhibitor of pectin methylesterase in kiwi fruit (*Actinidia chinensis*). *European Journal of Biochemistry*, 193: 183–187.

Barras, F., Thurn, K. K., and Chatterjee, A. K. (1987). Resolution of four pectate lyase structural genes of *Erwinia chrysanthemi* (EC16) and characterization of the enzymes produced in *Escherichia coli*. *Molecular and General Genetics*, 209(2): 319–325.

Benen, J. A. E., Vincken, J. P., and van Alebeek, G. J. W. M. (2002). Microbial pectinases. *In*: G. Seymour and P. Knox, eds., Pectins and Their Manipulation. Sheffield Academic Press, Sheffield, pp. 174-221.

Boevink, P., and Oparka, K. J. (2005). Virus-host interactions during movement processes. *Plant Physiology*, 138: 1815–1821.

Boudart, G., Lafitte, C., Barthe, J. P., Frasez, D., Esquerré-Tugayé, M.-T. (1998). Differential elicitation of defense responses by pectic fragments in bean seedlings. *Planta*, 206: 86–94.

Brito, N., Espino, J. J., and González, C. (2006). The endo-β-1,4-xylanase Xyn11A is required for virulence in *Botrytis cinerea*. *Molecular Plant-Microbe Interactions*, 19(1): 25–32.

Callaway, A., Giesman-Cookmeyer, D., Gillock, E. T., Sit, T. L., and Lommel S. A. (2001). The multifunctional capsid proteins of plant RNA viruses. *Annual Review of Phytopathology*, 39: 419–460.

Camardella, L., Carratore, V., Ciardiello, M. A., Servillo, L., Balestrieri, C., and Giovane, A. (2000). Kiwi protein inhibitor of pectin methylesterase amino-acid sequence and structural importance of two disulfide bridges. *European Journal of Biochemistry*, 267(14): 4561–4565.

Capodicasa, C., Vairo, D., Zabotina, O., McCartney, L., Caprari, C., Mattei, B., Manfredini, C., Aracri, B., Benen, J., Knox, J. P., De Lorenzo, G., and Cervone, F. (2004). Targeted modification of homogalacturonan by transgenic expression of a fungal polygalacturonase alters plant growth. *Plant Physiology*, 135: 1294–1304.

Carpita, N. C. and Gibeaut, D. M. (1993). Structural models of primary cell walls in flowering plants – consistency of molecular structure with the physical properties of the walls during growth. *The Plant Journal*, 3: 1–30.

Carrington, J. C., Kasschau, K. D., Mahajan, S. K. and Schaad M. C. (1996). Cell-to-cell and long-distance transport of viruses in plants. *The Plant Cell*, 8: 1669-1681.

Catoire, L., Pierron, M., Morvan, C., Hervé du Penhoat, C., and Goldberg, R. (1998). Investigation of the action patterns of pectin methylesterase isoforms through kinetic analyses and NMR spectroscopy. Implications in cell wall expansion. *The Journal of Biological Chemistry*, 273(50): 33150-33156.

Chen, M.-H., and Citovsky, V. (2003). Systemic movement of a tobamovirus requires host cell pectin methylesterase. *The Plant Journal*, 35: 386–392.

Chen, M.-H., Sheng, J., Hind, G., Handa, A. K., and Citovsky, V. (2000). Interaction between the tobacco mosaic virus movement protein and host cell pectin methylesterases is required for viral cell-to-cell movement. *The EMBO Journal*, 19(5): 913–920.

Chen, M.-H., Tian, G.-W., Gafni, Y., and Citovsky, V. (2005). Effects of calreticulin on viral cell-to-cell movement. *Plant Physiology*, 138: 1866–1876.

Christgau, S., Kofod, L. V., Halkier, T., Andersen, L. N., Hockauf, M., Dörreich, K., Dalbøge, H., and Kauppinen, S. (1996). Pectin methylesterase from *Aspergillus aculeatus*: Expression cloning in yeast and characterization of the recombinant enzyme. *Biochemical Journal*, 319(Pt3): 705-712.

Cooper, R. M. (1983). The mechanisms and significance of enzyme degradation of host cell walls by parasites. *In*: J. A. Callow, ed., Biochemical Plant Pathology. New York, John Wiley, USA, pp. 101-135.

Cote, F., Ham, K. S., Hahn, M. G., and Bergmann, C. W. (1998). Oligosaccharide elicitors in host-pathogen interactions: generation, perception, and signal transduction. *In*: B. B. Biswas and H. Das, eds., Cellular Biochemistry-Plant-Microbe Interactions, New York, Plenum Publishing Corp. 29: 385-432.

D'Avino, R., Camardella, L., Christensen, T. M., Giovane, A., and Servillo, L. (2003). Tomato pectin methylesterase: modeling, fluorescence, and inhibitor interaction studies-comparison with the bacterial (*Erwinia chrysanthemi*) enzyme. *Proteins*, 53: 830–839.

Dedeurwaerder, S., Menu-Bouaouiche, L., Mareck, A., Lerouge, P., and Guerineau, F. (2009). Activity of an atypical *Arabidopsis thaliana* pectin methylesterase. *Planta*, 229: 311–321.

Denès, J.-M., Baron, A., Renard, C. M. G. C., Péan, C., and Drilleau, J.-F. (2000). Different action pattern for apple pectin methylesterase at pH 7.0 and 4.5. *Carbohydrates Research*, 327(4): 385-393.

Di Matteo, A., Giovane, A., Raiola, A., Camardella, L., Bonivento, D., De Lorenzo, G., Cervone, F., Bellincampi, D., and Tsernoglou, D. (2005). Structural basis for the interaction between pectin methylesterase and a specific inhibitor protein. *The Plant Cell*, 17: 849–858.

Dijkstra, J., and De Jager, C. P. (1998). Practical plant virology: protocols and exercises. Springer- verlag, Berlin, 459 pp.

Ding, B., Turgeon, R., and Parthasarathy, M. V. (1992). Substructure of freeze substituted plasmodesmata. *Protoplasma*, 169: 28-41.

Divol, F., Vilaine, F., Thibivilliers, S., Amselem, J., Palauqui, J.-C., Kusiak, C., and Dinant, S. (2005). Systemic response to aphid infestation by *Myzus persicae* in the phloem of *Apium graveolens*. *Plant Molecular Biology*, 57: 517–540.

Dorokhov, Y. L., Frolova, O. Y., Skurat, E. V., Ivanov, P. A., Gasanova, T. V., Sheveleva, A. A., Ravin, N. V., Mäkinen, K. M., Klimyuk, V. I., Skryabin, K. G., Gleba, Y. Y., and Atabekov, J. G. (2006). A novel function for a ubiquitous plant enzyme pectin methylesterase: the enhancer of RNA silencing. *FEBS Letters*, 580: 3872–3878.

Dorokhov, Y. L., Mäkinen, K., Frolova, O. Y., Merits, A., Saarinen, J., Kalkkinen, N., Atabekov, J. G., and Saarma, M. (1999). A novel function for a ubiquitous plant enzyme pectin methylesterase: the host-cell receptor for the tobacco mosaic virus movement protein. *FEBS Letters*, 461: 223–228.

Downie, B., Dirk, L. M. A., Hadfield, K. A., Wilkins, T. A., Bennett, A. B., and Bradford, K. J. (1998). A gel diffusion assay for quantification of pectin methylesterase activity. *Analytical Biochemistry*, 264: 149–157.

Duvetter, T., Fraeye, I., van Hoang, T., van Buggenhout, S., Verlent, I., Smout C., van Loey, A., and Hendrickx, M. (2005). Effect of pectinmethylesterase infusion methods and processing techniques on strawberry firmness. *Journal of Food Science*, 70 (6), S383-S388.

Espino, J. J., Brito, N., Noda, J. and González, C. (2005). *Botrytis cinerea* endo-β-1,4-glucanase Cel5A is expressed during infection but is not required for pathogenesis. *Physiological and Molecular Plant Pathology*, 66(6): 213–221.

Ferrari, S., Galletti, R., Pontiggia, D., Manfredini, C., Lionetti, V., Bellincampi, D., Cervone, F., and De Lorenzo, G. (2008). Transgenic expression of a fungal endo-polygalacturonase increases plant resistance to pathogens and reduces auxin sensitivity. *Plant Physiology*, 146: 669-681.

Flego, D., Marits, R., Eriksson, A. R. B., Koiv, V., Karlsson, M.-B., Heikinheimo, R., and Palva, E. T. (2000). A two-component regulatory system, *pehR-pehS*, controls endopolygalacturonase production and virulence in the plant pathogen *Erwinia carotovora* subsp. *carotovora*. *Molecular Plant-Microbe Interactions*, 13(4): 447-455.

Fraaije, B. A., Bosveld, M., Van den Bulk, R. W. and Rombouts, F. M. (1997). Analysis of conductance responses during depolymerization of pectate by soft rot *Erwinia* spp. and other pectolytic bacteria isolated from potato tubers. *Journal of Applied Microbiology*, 83: 17–24.

Fremy, E. (1840). Sur la pectine et l'acid pectique, *J. Pharm*., 26: 368–393. (cited by Giovane *et al.*, 2004).

Fry, S. C. (2004). Primary cell wall metabolism: tracking the careers of wall polymers in living plant cells. *New Phytologist*, 161: 641–675.

Gardan, L., Gouy, C., Christen, R., and Samson R. (2003). Elevation of three subspecies of *Pectobacterium carotovorum* to species level: *Pectobacterium atrosepticum* sp. nov., *Pectobacterium betavasculorum* sp. nov. and *Pectobacterium wasabiae* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, 53: 381–391.

Giovane, A., Balestrieri, C., Quagliuolo, L., Castaldo, D., and Servillo, L. (1995). A glycoprotein inhibitor of pectin methylesterase in kiwi fruit: purification by affinity chromatography and evidence of a ripening-related precursor. *European Journal of Biochemistry*, 233: 926–929.

Giovane, A., Servillo, L., Balestrieri, C., Raiola, A., D'Avino, R., Tamburrini, M., Ciardiello, M. A., Camardella, L. (2004). Pectin methylesterase inhibitor. *Biochimica et Biophysica Acta*, 1696: 245–252.

Giri, A. P., Wünsche, H., Mitra, S., Zavala, J. A., Muck, A., Svatos, A., and Baldwin, I. T. (2006). Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. VII. Changes in the plant's proteome. *Plant Physiology*, 142(4): 1621–1641.

Gómez, P., Rodríguez-Hernández, A. M., Moury, B., and Aranda, M. A. (2009). Genetic resistance for the sustainable control of plant virus diseases: breeding, mechanisms and durability. *European Journal of Plant Pathology*, 125: 1-22.

Gooding, G. V. and Herbert, T. T., (1967). A simple technique for purification of tobacco mosaic virus in large quantities. *Phytopathology*, 57(11): 1285.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology*, 166: 557–580.

Henrissat, B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal*, 280 (Pt2):309-316.

Hewezi, T., Howe, P., Maier, T. R., Hussey, R. S., Mitchum, M. G., Davis, E. L., and Baum, T. J. (2008). Cellulose binding protein from the parasitic nematode *Heterodera schachtii* interacts with *Arabidopsis* pectin methyl-esterase: cooperative cell-wall modification during parasitism. *The Plant Cell*, 20: 3080–3093.

Hii, G., Pennington, R., Hartson, S., Taylor, C. D., Lartey, R., Williams, A., Lewis, D., and Melcher, U. (2002). Isolate-specific synergy in disease symptoms between cauliflower mosaic and turnip vein-clearing viruses. *Archives of Virology*, 147(7): 1371–1384.

Hothorn, M., Wolf, S., Aloy, P., Greiner, S., and Scheffzek, K. (2004). Structural insights into the target specificity of plant invertase and pectin methylesterase inhibitory proteins. *The Plant Cell*, 16: 3437–3447.

Hugouvieux-Cotte-Pattat, N., Condemine, G., Nasser, W., and Reverchon, S. (1996). Regulation of pectinolysis in *Erwinia chrysanthemi*. *Annual Review of Microbiology*, 50: 213–257.

Ishii, T. T. (1995). Pectic polysaccharides from bamboo shoot cell-walls. *Journal of the Japan Wood Research Society (Mokuzai Gakkaishi)*, 41(7): 669-676.

Jayani, R. S., Saxena, S., and Gupta, R. (2005). Microbial pectinolytic enzymes: A review. *Process Biochemistry*, 40: 2931–2944

Jenkins, J., Mayans, O., Smith, D., Worboys, K., and Pickersgill, R. W. (2001). Three-dimensional structure of *Erwinia chrysanthemi* pectin methylesterase reveals a novel esterase active site. *Journal of Molecular Biology*, 305: 951–960.

Jiang, C.-M., Li, C.-P., Chang, J.-C. and Chang, H.-M. (2002). Characterization of pectinesterase inhibitor in jelly fig (*Ficus awkeotsang* Makino) achenes. *Journal of Agricultural and Food Chemistry*, 50(17): 4890– 4894.

Johansson, K., El Ahmad, M., Friemann, R., Jornvall, H., Markovič, O., and Eklund, H. (2002). Crystal structure of plant pectin methylesterase. *FEBS Letters*, 514: 243–249.

Kaido, M., Inoue, Y., Takeda, Y., Sugiyama, K., Takeda, A., Mori, M., Tamai, A., Meshi, T., Okuno, T. and Mise, K. (2007). Downregulation of the *NbNACa1* gene encoding a movement-protein-interacting protein reduces cell-to-cell movement of *Brome mosaic virus* in *Nicotiana benthamiana*. *Molecular Plant-Microbe Interactions*, 20(6): 671-681.

Karr, A. L., and Albersheim, P. (1970). Polysaccharide-degrading enzymes are unable to attack plant cell walls without prior action by a "wall-modifying enzyme". *Plant Physiology*, 46: 69-80.

Kars, I., and van Kan, J. A. L. (2004). Extracellular enzymes and metabolites involved in pathogenesis of *Botrytis*. *In*: Y. Elad, B. Williamson, P. Tudzynski and N. Delen, eds., *Botrytis:* Biology, Pathology and Control. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 99-118.

Kars, I., McCalman, M., Wagemakers, L., and van Kan, J. A. L. (2005). Functional analysis of *Botrytis cinerea* pectin methylesterase genes by PCR-based targeted mutagenesis: *Bcpme1* and *Bcpme2* are dispensable for virulence of strain B05.10. *Molecular Plant Pathology*, 6(6): 641–652.

Kester, H. C. M., Benen, J. A. E., and Visser, J. (1999). The exopolygalacturonase from *Aspergillus tubigensis* is also active on xylogalacturonan. *Biotechnology and Applied Biochemistry*, 30: 53-57.

Koch, J. L., and Nevins, D. J. (1989). Tomato fruit cell-wall. I. Use of purified tomato polygalacturonases and pectinmethylesterase to identify developmental-changes in pectins. *Plant Physiology*, 91: 816-822.

Körner, E., von Dahl, C. C., Bonaventure, G., and Baldwin I. T. (2009). Pectin methylesterase Na*PME1* contributes to the emission of methanol during insect herbivory and to the elicitation of defence responses in *Nicotiana attenuata*. *Journal of Experimental Botany*, 60(9): 2631–2640

Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature*, 277: 680–685.

Lartey, R. T., Hartson, S. D., Pennington, R. E., Sherwood, J. L., and Melcher, U. (1993). Occurrence of a vein-clearing tobamovirus in turnip. *Plant Disease*, **77**: 21–24.

Lartey, R. T., Voss, T. C., and Melcher, U. (1995). Completion of a cDNA sequence from a tobamovirus pathogenic to crucifers. *Gene,* 166: 331–332.

Lartey, R., Ghoshroy, S., Ho, J., and Citovsky, V. (1997). Movement and subcellular localization of a tobamovirus in Arabidopsis. *The Plant Journal*, 12(3): 537–545.

Lazarowitz, S. G., and Beachy, R. N. (1999). Viral movement proteins as probes for intracellular and intercellular trafficking in plants. *The Plant Cell*, 11: 535–548

Leisner, S. M., and Howell, S. H. (1993). Long-distance movement of viruses in plants. *Trends in Microbiology*, 1(8): 314–317.

Lindbo, J. A., and Dougherty, W. G. (1992). Pathogen-Derived Resistance to a Potyvirus: Immune and Resistant Phenotypes in Transgenic Tobacco Expressing Altered Forms of a Potyvirus Coat Protein Nucleotide Sequence. *Molecular Plant-Microbe Interactions*, 5(2): 144-153.

Liners, F., Letesson, J. J., Didembourg, C., and van Cutsem, P. (1989). Monoclonal antibodies against pectin: recognition of a conformation induced by calcium. *Plant Physiology*, 91: 1419–1424.

Liners, F., Thibault, J.-F., and van Cutsem, P. (1992). Influence of the degree of polymerization of oligogalacturonates and of esterification pattern of pectin on their recognition by monoclonal antibodies. *Plant Physiology*, 99: 1099–1104.

Lionetti, V., Raiola, A., Camardella, L., Giovane, A., Obel, N., Pauly, M., Favaron, F., Cervone, F., and Bellincampi, D. (2007). Overexpression of Pectin Methylesterase Inhibitors in Arabidopsis Restricts Fungal Infection by *Botrytis cinerea*. *Plant Physiology*, 143: 1871–1880.

Lockhart, B. E., Swenson, A. S., Olszewski N. E., and Voth, P. (2008). Characterization of a Strain of *Turnip vein-clearing virus* causing red ringspot of penstemon. *Plant Disease*, 92(5): 725-729.

Lohaus, G., Pennewiss, K., Sattelmacher, B., Hussmann, M., and Muehling, K. H. (2001). Is the infiltration-centrifugation technique appropriate for the isolation of apoplastic fluid? A critical evaluation with different plant species. *Physiologia Plantarum*, 111: 457–465.

Lucas, W. J. (2006). Plant viral movement proteins: agents for cell-to-cell trafficking of viral genomes. *Virology*, 344(1): 169–184.

Ly-Nguyen, B., van Loey, A. M., Smout, C., Verlent, I., Duvetter, T., and Hendrickx, M. E. (2004). Effect of intrinsic and extrinsic factors on the interaction of plant pectin methylesterase and its inhibitor from kiwi fruit. *Journal of Agricultural and Food Chemistry*, 52: 8144–8150.

Markovič, O., and Janeček, S. (2004). Pectin methylesterases: sequence-structural features and phylogenetic relationships. *Carbohydrate Research*, 339(13): 2281–2295.

Markovič, O., and Kohn, R. (1984). Mode of pectin deesterification by *Trichoderma reesei* pectinesterase. *Cellular and Molecular Life Science (Experientia)*, 40(8): 842-843.

Martín Martín, A., Martínez-Herrera, D., Cabrera y Poch, H. L., and Ponz, F. (1997). Variability in the interactions between *Arabidopsis thaliana* ecotypes and oilseed rape mosaic tobamovirus. *Australian Journal of Plant Physiology*, 24(3): 275–281.

Marty, P., Jouan, B., Bertheau, Y., Vian, B., and Goldberg, R. (1997). Charge density in stem cell walls of *Solanum tuberosum* genotypes and susceptibility to blackleg. *Phytochemistry*, 44(8): 1435-1441.

Masclaux, C., Hugouvieux-Cotte-Pattat, N. and Expert, D. (1996). Iron is a triggering factor for differential expression of *Erwinia chrysanthemi* 3937 pectate lysases during pathogenesis on African violets. *Molecular Plant-Microbe Interactions*, 9: 198–205.

Mattei, B., Raiola, A., Caprari, C., Federici, L., Bellincampi, D., De Lorenzo, G., Cervone, F., Giovane, A., and Camardella, L. (2002). Studies on plant inhibitors of pectin modifying enzymes: polygalacturonase-inhibiting protein (PGIP) and pectin methylesterase inhibitor (PMEI). *In*: T. T. Teeri, B. Svensson, H. J. Gilbert, and T. Feizi, eds., Carbohydrate Bioengineering: Interdisciplinary Approaches. Royal Society of Chemistry, Cambridge, UK, pp 160–167.

McLean, B. G., Hempel, F. D., and Zambryski, P. C. (1997). Plant intercellular communication via plasmodesmata. *The Plant Cell*, 9: 1043-1054.

McMillan, G. P. and Pérombelon, M. C. M. (1995). Purification and characterization of a high p*I* pectin methyl esterase isoenzyme and its inhibitor from tubers of *Solanum tuberosum* subsp. *tuberosum* cv. Katahdin. *Physiological and Molecular Plant Pathology*, 46(5): 413–427.

McMillan, G. P., Barrett, A. M., and Pérombelon, M. C. M. (1994). An isoelectric focusing study of the effect of methyl-esterified pectic substances on the production of extracellular pectin isoenzymes by soft rot *Erwinia* spp. *Journal of Applied Microbiology*, 77(2): 175–184.

McMillan, G. P., Hedley, D., Fyffe, L., and Pérombelon, M. C. M. (1993). Potato resistance to soft-rot erwinias is related to cell wall pectin esterification. *Physiological and Molecular Plant Pathology*, 42: 279–289.

Melcher, U. (2003). *Turnip vein-clearing virus*, from pathogen to host expression profile. *Molecular Plant Pathology*, 4(3): 133–140.

Micheli, F. (2001). Pectin methylesterases: cell wall enzymes with important roles in plant physiology. *Trends in Plant Science*, 6(9): 414-419.

Micheli, F., Holliger, C., Goldberg, R., and Richard, L. (1998). Characterization of the pectin methylesterase-like gene AtPME3: a new member of a gene family comprising at least 12 genes in *Arabidopsis thaliana*. *Gene*, 220: 13– 20.

Mohnen, D. (2008). Pectin structure and biosynthesis. *Current Opinion in Plant Biology*, 11: 266–277.

Moloshok, T., Pearce, G., and Ryan, C. A. (1992). Oligouronide signaling of proteinase inhibitor genes in plants: structure-activity relationships of Di- and trigalacturonic acids and their derivatives. *Archives of Biochemistry and Biophysics*, 294(2): 731–734.

Mort, A. J., Qiu, F. and Maness, N. O. (1993). Determination of the pattern of methylesterification in pectin. Distribution of contiguous nonesterified residues. *Carbohydrate Research*, 247: 21-35.

Moustacas, A. M., Nari, J., Borel, M., Noat, G., and Ricard, J. (1991). Pectin ethylesterase, metal ions and plant cell-wall extension. The role of metal ions in plant cell-wall extension. *Biochemistry Journal*, 279: 351–354.

Nachin, L., and Barras, F. (2000). External pH: an environmental signal that helps to rationalize *pel* gene duplication in *Erwinia chrysanthemi*. *Molecular Plant-Microbe Interactions*, 13(8): 882–886.

O'Neill M. A., Albersheim P., and Darvill A. (1990**).** The pectic polysaccharides of primary cell walls. *In*: P. M. Dey and J. B. Harborne, eds., Methods in Plant Biochemistry, Vol. 2. Academic Press, London, pp. 415–441.

Osorio, S., Castillejo, C., Quesada, M. A., Medina-Escobar, N., Brownsey, G. J., Suau, R., Heredia, A., Botella, M. A., and Valpuesta, V. (2008). Partial demethylation of oligogalacturonides by pectin methyl esterase 1 is required for eliciting defence responses in wild strawberry (*Fragaria vesca*). *The Plant Journal*, 54: 43–55.

Pelloux, J., Rustérucci, C., and Mellerowicz, E. J. (2007). New insights into pectin methylesterase structure and function. *Trends in Plant Science*, 12(6): 267–277.

Pérombelon, M. C. M. (2002). Potato diseases caused by soft rot erwinias: an overview of pathogenesis. *Plant Pathology*, 51(1)**:** 1-12.

Pérombelon, M. C. M., and Kelman, A. (1980). Ecology of the soft rot *Erwinias*. *Annual Review of Phytopathology*, 18: 361-387.

Raiola, A., Camardella, L., Giovane, A., Mattei, B., De Lorenzo, G., Cervone, F., and Bellincampi, D. (2004). Two Arabidopsis thaliana genes encode functional pectin methylesterase inhibitors. *FEBS Letters*, 557: 199–203

Rasband, W. S. (1997-2008). ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, (http://rsb.info.nih.gov/ij/).

Ridley, B. L., O'Neill, M. A. and Mohnen, D. (2001). Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry*, 57: 929–967.

Rombouts, F. M., and Thibault, J. F. (1986). Chemistry and Function of Pectins. *In*: M. L. Fishman and J. J. Jen. *ACS Symposium Ser. 310*. American Chemical Society, Washington DC, pp. 46-60.

Rosenberg, A. H., Goldman, E., Dunn, J. J., Studier, F. W., and Zubay, G. (1993). Effects of consecutive AGG codons on translation in *Escherichia coli* demonstrated with a versatile codon test system. *Journal of Bacteriology*, 175(3): 716–722.

Ruegger, M., Dewey, E., Gray, W. M., Hobbie, L., Turner, J., and Estelle, M. (1998). The TIR1 protein of *Arabidopsis* functions in auxin response and is related to human SKP2 and yeast Grr1p. *Genes and Development*, 12: 198-207.

Schmidt, D. D., Voelckel, C., Hartl, M., Schmidt, S., and Baldwin, I. T. (2005). Specificity in ecological interactions. Attack from the same lepidopteran herbivore results in species-specific transcriptional responses in two solanaceous host plants. *Plant Physiology*, 138(3): 1763–1773.

Scholthof, H. B. (2005). Plant virus transport: motions of functional equivalence. *Trends in Plant Science*, 10(8): 376–382.

The Arabidopsis Genome Initiative (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature*, 408: 796–815.

Thomma, B. P. H. J., Tierens, K. F. M., Penninckx, I. A. M. A., Mauch-Mani, B., Broekaert, W. F., and Cammue, B. P. A. (2001). Different micro-organisms differentially induce Arabidopsis disease response pathways. *Plant Physiology and Biochemistry*, 39: 673-680.

Thomson, N. R., Thomas, J. D., and Salmond, G. P. C. (1999). Virulence determinants in the bacterial phytopathogen, *Erwinia*. *In*: Margaret C. M. Smith and R. Elizabeth Sockett, eds., Methods in Microbiology, Volume 29 - *Genetic Methods for Diverse Prokaryotes.* Chapter 12, pp. 347-426.

Toth, I. K., Bell, K. S. Holeva, M. C., and Birch, P. R. J. (2003). Soft rot erwiniae: from genes to genomes. *Molecular Plant Pathology*, 4(1): 17–30.

Tzfira, T., Rhee, Y., Chen, M.-H., Kunik, T., and Citovsky, V. (2000). Nucleic acid transport in plant-microbe interactions: the molecules that walk through the walls. *Annual Review of Microbiology*, 54: 187–219.

Valette-Collet, O., Cimerman, A., Reignault, P., Levis, C., and Boccara, M. (2003). Disruption of *Botrytis cinerea* pectin methylesterase gene *Bcpme*1 reduces virulence on several host plants. *Molecular Plant-Microbe Interactions*, 16(4): 360-367.

van Alebeek, G.-J. W. M., van Scherpenzeel, K., Beldman, G., Schols, H. A., and Voragen, A. G. J. (2003). Partially esterified oligogalacturonides are the preferred substrates for pectin methylesterase of *Aspergillus niger*. *Biochemical Journal*, 372: 211-218.

van Kan, J. A. L. (2006). Licensed to kill: the life style of a necrotrophic plant pathogen. *Trends in Plant Science*, 11: 247–253

Versteeg, C., Rombouts, F. M., and Pilnik, W. (1978). Purification and some characteristics of two pectinesterase isozymes from orange. *Lebensmittel-Wissenschaft und-Technologie (Food Science and Technology)*, 11: 267-274.

Vincken, J.-P., Schols, H. A., Oomen, R. J. F. J., McCann, M. C., Ulvskov, P., Voragen, A. G. J., and Visser, R. G. F. (2003). If homogalacturonan were a side chain of rhamnogalacturonan I. Implications for cell wall architecture. *Plant Physiology*, 132: 1781–1789.

von Dahl, C. C., Hävecker, M., Schlögl, R., and Baldwin, I. T. (2006). Caterpillar-elicited methanol emission: a new signal in plant–herbivore interactions?. *The Plant Journal*, 46: 948–960.

Vorwerk, S., Somerville, S., and Somerville, C. (2004). The role of plant cell wall polysaccharide composition in disease resistance. *Trends in Plant Science*, 9(4): 203–209.

Waigmann, E., Ueki, S., Trutnyeva, K., and Citovsky, V. (2004). The ins and outs of non-destructive cell-to-cell and systemic movement of plant viruses. *Critical Reviews in Plant Sciences*, 23(3): 195–250

Waterhouse, P. M., Graham, M. W., and Wang, M.-B. (1998). Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proceedings of the National Academy of Sciences (PNAS)*, USA, 95: 13959–13964.

Whitaker, J. R. (1990). Microbial pectinolytic enzymes. *In*: W. M. Fogarty and C. T. Kelly, eds., Microbial enzymes and biotechnology. 2nd ed. Elsevier Science Ltd., London, pp. 133–176.

Whitham, S. A., and Wang, Y. (2004). Roles for host factors in plant viral pathogenicity. *Current Opinion in Plant Biology*, 7: 365–371.

Wiethölter, N., Graessner, B., Mierau, M., Mort, A. J., and Moerschbacher, B. M. (2003). Differences in the methyl ester distribution of homogalacturonans from near-isogenic wheat lines resistant and susceptible to the wheat stem rust fungus. *Molecular Plant-Microbe Interactions*, 16: 945–952.

Willats, W. G. T., McCartney, L., Mackie, W., and Knox, J. P. (2001). Pectin: cell biology and prospects for functional analysis. *Plant Molecular Biology*, 47(1-2): 9–27.

Wolf, S., Deom, C. M., Beachy, R. N., and Lucas, W. J. (1989). Movement protein of tobacco mosaic virus modifies plasmodesmata size exclusion limit. *Science*, 246(4928): 377-379.

Wolf, S., Grsic-Rausch, S., Rausch, T., and Greiner, S. (2003). Identification of pollen-expressed pectin methylesterase inhibitors in Arabidopsis. *FEBS Letters*, 555: 551–555.

Wolf, S., Mouille, G., and Pelloux, J. (2009). Homogalacturonan Methyl-Esterification and Plant Development. *Molecular Plant*, 2(5): 851–860.

Wu, M. C., Tseng, K. C., Huang, T. H., and Chang, H. M. (2002). Pectinesterase inhibitor in rubbery banana (*Musa sapientum* L.). *Journal of Food Science*, 67(4): 1337–1340.

Wubben, J. P., Mulder, W., ten Have, A., van Kan, J. A. L., and Visser, J. (1999). Cloning and partial characterisation of endopolygalacturonase genes from *Botrytis cinerea*. *Applied and Environmental Microbiology*, 65 (4): 1596–1602.

Yang, Z , Cramer, C. L., and Lacy, G. H. (1992). *Erwinia carotovora* subsp. *carotovora* pectic enzymes: *in planta* gene activation and roles in soft-rot pathogenesis. *Molecular Plant-Microbe Interactions*, 5(1): 104-112.

Zhou, N., Tootle, T. L., and Glazebrook, J. (1999). Arabidopsis *PAD3*, a gene required for camalexin biosynthesis, encodes a putative cytochrome P450 monooxygenase. *The Plant Cell*, 11**:** 2419-2428.