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Martinić-Cezar, Tea

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Master Sciences du Vivant

***Spécialité*: Biotechnologies, Biologie Moléculaire et Cellulaire**

INTERNSHIP REPORT

Similar but different: Determining the mode of action and effects of pectin methylesterases (PME)

by

Tea Martinić-Cezar

(February, 2021- July, 2021)

Biologie des plantes et innovation EA 3900 33, rue Saint Leu 80039 Amiens Cedex 1

Mentor: Valérie Lefebvre, Assistant professor

Supervisor: Jérôme Pelloux, Professor

Firstly, I would like to express my gratitude to Professor Jérôme Pelloux for giving me an opportunity to work in his research team and for the supervision of my internship.

Special thanks to my mentor Assist. Prof. Valérie Lefebvre for her excellent guidance and mentorship. Thank you for all your patience, knowledge, optimism and laughs you shared with me!

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My deepest and sincerest gratitude goes to my family. Thank you for believing in me and giving me the strength to pursue my goals.

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Hvala vam!

PRESENTATION OF THE LABORATORY

The UMR INRAe 1158 BioEcoAgro -BIOPI (Plant Biology & Innovation) unit is located in Amiens (France) and it is a part of University of Picardie Jules Verne. It is directed by Professor François Mesnard. The research activities of the laboratory are focused on the roles of modifications of cell wall and phenylpropanoid metabolism during plant development. Both model plants, *Arabidopsis thaliana,* and industrially important species, like flax (*Linum usitatissimum L*.) are used.

BIOPI research is divided into 3 teams, depending on the topics. During my internship stay I was working in Team 1, 'Pectin Dynamic', led by Professor Jérôme Pelloux, that is focused on the roles of pectin-modifying enzymes during plant development and in response to biotic and abiotic stresses. Team 2's main focus is understanding phenylpropanoid metabolism in flax and Team 3 develops biotechnological processes for metabolites and recombinant proteins produced using plants' hairy roots. BIOPI has access to all the equipment from the University platforms (Analytical, Microscopy, Molecular Biology, Greenhouses, Phenotyping robots), with state- of-the-art facilities.

The "Pectin Dynamic" team has notably a strong expertise in functional genomics approaches, biochemistry, immunocytochemistry and analytical chemistry, gene (qPCR) and protein expression, protein production and purification. They pioneered approaches to express pectin remodeling enzymes from plants in heterologous system such as *Escherichia coli* and *Pichia pastoris*, to characterize their biochemical specificities and their roles in plant development. Over the recent years, the team has participated in a number of ANR-funded projects, either as coordinator (GROWPEC, GALAPAGOS and WALLMIME) or as partners (NOSTRESSWALL and PECTOSIGN).

ABBREVIATIONS

4-MU	4-methylumbelliferone
BMGY	buffered glycerol complex medium
BMMY	buffered methanol complex medium
DA	degree of acetylation
DM	degree of methylesterification
DP	degree of polymerization
EDTA	ethylenediaminetetraacetic acid
GalA	galacturonic acid
GUS	β-glucuronidase
HG	homogalacturonan
LC	liquid chromatography
MS	mass spectrometry
MUG	4-methylumbelliferyl-β-D-galactopyranoside
NaP	sodium phosphate buffer
OG	oligogalacuronides
PAE	pectin acetylesterase
PDF	plant defensin
PG	polygalacturonase
PL	pectate lyases
PM	processive motif
PME	pectin methylesterases
PMEI	pectin methylesterase inhibitor
PNL	pectin lyase
RGI	rhamnogalacturonan I
RGII	rhamnogalacturonan II
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SP	signal peptide
TEMED	tetramethylethylenediamine
TM	transmembrane domain
XG	xylogalacturonan

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1. INTRODUCTION

Pectins are complex branched polysaccharides present in plant primary cell walls among which the most present is homogalacturonan. The structure of homogalacturonan can be modified by homogalacturonan-modifying enzymes including pectin methylesterases (PMEs) causing changes in pectin chemistry and cell wall properties. PMEs are encoded by large multigenic families in plants, and although, protein structure is highly conserved between isoforms, recent data suggest that structural variations could translate into distinct processivities.

1.1 PLANT CELL WALL

Plant cell wall is a rigid, semi-permeable protective structure that surrounds plant cells and connects cells into tissues, enabling signalization and controlling the shape of plant organs (Lodish *et al*., 2000). Plant cell wall plays a fundamental role in plant morphogenesis, mechanical strength and support, conduction of water and nutrients as well as defense responses. In addition, plant cell wall is the most abundant renewable resource on Earth, which represents enormous resources for the various needs of mankind including heat production, building material, natural fibers for textile and paper production and biofuels. On the other hand, it is the least understood cellular structure in plants (Zhang *et al*., 2020).

Morphologically, plant cell wall can be divided into three zones. From the outermost zone, they are identified as the middle lamella, primary cell wall, and secondary cell wall (**Figure 1**). Middle lamella is composed almost exclusively of pectins. Pectins control cell adhesion by enabling cell walls of nearby cells to bind one to another. Primary cell wall is mainly composed of cellulose microfibrils contained within a gel-like matrix of hemicellulose fibers and pectin polysaccharides. It provides strength and flexibility to the cell and controls growth. Secondary cell wall is present only in some specialized mature cells. It has predominantly mechanical function and consist of three layers (S1, S2 and S3) distinguished by differences in the orientation of cellulose microfibrils (Heredia *et al*., 1995). The most common additional polymer in secondary walls is lignin, a complex network of phenolic compounds found in the walls of the xylem vessels and fiber cells of woody tissues (Alberts *et al*., 2002).

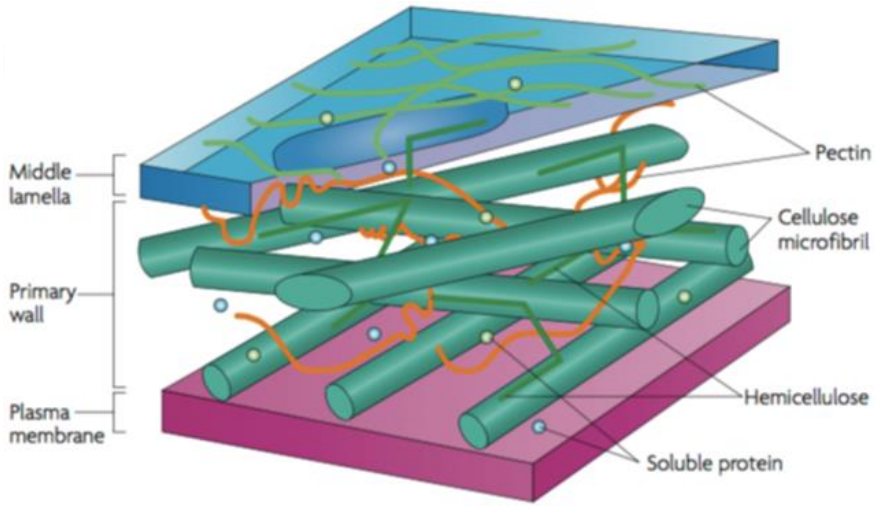


Figure 1. **A simplified model of the cell wall structure.** The cell wall surrounds the plasma membrane and provides the cell protection. It is composed of cellulose microfibrils, hemicellulose, pectin, lignin and soluble proteins which are organized into 3 major layers; middle lamella, primary cell wall and secondary cell wall (not shown on the figure). Figure is adapted from the reference (Sticklen, 2008).

Plant cell wall is composed of a complex mixture of polysaccharides, proteins, lignin and water, as well as other substances such as cutin, suberin and other inorganic compounds that can vary from one plant to another (Heredia *et al*., 1995). Classically, cell wall polysaccharides have been grouped into cellulose, hemicelluloses, and pectins (Scheller and Ulvskov, 2010). Cellulose is the most abundant polysaccharide in the cell wall, that provides tensile strength to the primary cell wall. One molecule of cellulose contains at least 500 glucose residues connected in a linear chain by covalent β-1,4-glycosidic bonds forming a ribbonlike structure that is stabilized by hydrogen bonds within the chain. In addition, the structure is further strengthened with intermolecular hydrogen bonds forming structure called cellulose microfibrils (Alberts *et al*., 2002). Hemicelluloses are characterized by β-1,4-linked backbone of sugars such as xyloglucans, xylans, mannans and glucomannans, and β-(1→3,1→4)-glucans. The main role of hemicelluloses is to bind cellulose microfibrils, thereby strengthening the cell wall (Scheller and Ulvskov, 2010). Pectins are highly heterogenous polysaccharides composed of large amount of negatively charged galacturonic acid (GalA) units. They play roles in cell expansion, strength, porosity, adhesion, and intercellular signaling (Houston *et al*., 2016). In addition to polysaccharides, there are numerous proteins in the cell wall. Some of them have a

structural role such as glycine-rich and hydroxyproline-rich glycoproteins, while others, enzymes, modify the structure of cell wall during growth, fruit ripening or organ abscission (Wolf-Dieter, 1998).

In flowering plants, two types of primary cell walls can be distinguished on the basis of their composition and physical properties (Carpita and Gibeaut, 1993). Type I cell wall, characteristic for dicotyledons (*Arabidopsis thaliana*), consists of cellulose fibers encased in a network of xyloglucan (XyG), pectin and structural proteins. In this type of cell wall pectins represent approximately 35% of cell wall dry weight. Type II cell wall, specific for some monocotyledons (family *Poaceae*), are composed of cellulose fibers encased in glucuronoarabinoxylans (GAX) network, with high levels of hydroxycinnamates, and very low levels of pectin and structural proteins (Vogel, 2008; Voragen *et al*., 2009).

1.2 PECTINS

Pectins are one of the main components in plant cell wall and one of the most complex macromolecules in nature, as they may be composed of up to 17 different monosaccharides linked by more than 20 different bonds (O'Neill *et al*., 2004; Ridley *et al*., 2001). In plants, up to 35% of the primary cell wall is composed of pectins that carry 70% of all negatively charged groups (Jarvis, 1984). Pectins are involved in processes as diverse as cell growth and cell differentiation, defense mechanisms against plant pathogens and wounding, regulation of ion transport and control of cell wall permeability for enzymes (Voragen *et al*., 2009).

Pectins are defined as a heterogeneous group of galacturonic acid-rich polysaccharides including homogalacturonan (HG), rhamnogalacturonan I (RGI), rhamnogalacturonan II (RGII) and xylogalacturonan (XG) domains (**Figure 2**). Homogalacturonan, the most abundant pectic domain, is a homopolymer of α-1,4-linked galacturonic acid that can be methylesterified at the C-6 carboxyl or *O*-acetylated at *O*-2 or *O*-3 positions (Mohnen, 2008). HG is synthesized in the Golgi apparatus and secreted to the cell wall in a highly methylesterified form (up to 80%) (Krzesłowska, 2011). The control of the degree of methylesterification (DM) has been under focus as it significantly affects the physical properties of pectins. For example, Ca^{2+} ions can bind to blocks that contains more than 10 consecutive non-esterified GalA units, promoting the formation of so called 'egg box' structure enabling the formation of pectic gels, or become a target for enzymatic modifications (Sénéchal *et*

al., 2014). Structurally, the most complex pectic polysaccharide is rhamnogalacturonan II (RGII), that makes up 10% of all pectins. It is highly conserved between plant species and consists of HG backbone of at least 8 α-1,4-linked -D-GalA residues branched with side chains consisting of 12 different types of sugars in over 20 different linkages (O'Neill *et al*., 2004). Xylogalacturonan (XG) is a β-linked D-xylose substituted HG at *O*-3 position. Research has shown that substituted HGs, such as XG, have a role in protecting against pathogens as they cannot be degraded by pathogenic polygalacturonases (PG) (Jensen *et al*., 2008). The backbone of rhamnogalacturonan-I (RGI) is composed of disaccharide repeats [-α-D-GalA-1,2-α-L-Rha-1-4-]$_n$. Rhamnosyl residues in RGI have side chains containing individual linear or branched α-L-Ara and β-D-Gal residues (Mohnen, 2008).

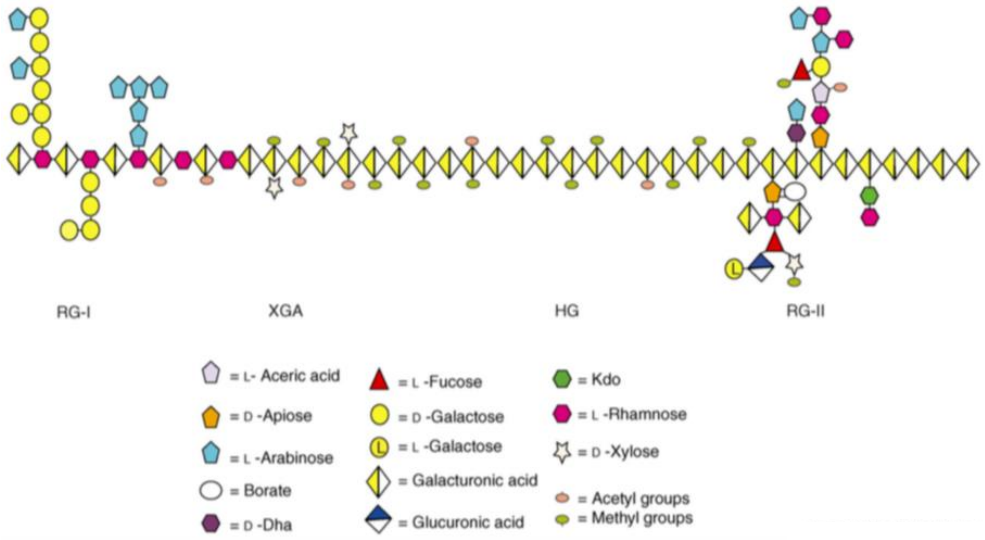


Figure 2. **Schematic structure of pectin.** Pectic polysaccharides homogalacturonan (HG), xylogalacturonan (XG), rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII) linked to each other. Figure is modified from Scheller *et al*., 2007.

1.3 HOMOGALACTURONAN-MODIFYING ENZYMES

The structure of HG can be modified to homogalacturonan-modifying enzymes (HGME) that are synthetized in the endoplasmic reticulum, post-translationally modified in Golgi apparatus and secreted in the cell wall. HGMEs are divided into various classes, including polygalacturonases (PGs; EC 3.2.1.15), pectin methylesterases (PMEs; EC 3.1.1.11), pectin acetylesterases (PAEs; EC 3.1.1.6), pectin lyases (PNLs; EC 4.2.2.10) and pectate lyases (PLs; EC 4.2.2.2) (**Figure 3**). In

Arabidopsis thaliana 66 PMEs, 12 PAEs, 68 PGs, and 26 PNLs and PLs isoforms are present, questioning the rationale for such abundance (Sénéchal *et al*., 2014). These enzymes are ubiquitous not only in plants but also in phytopathogenic organisms, where they play a role in the colonization of the host plant (Bonnin *et al*., 2014).

HG can undergo two different types of degradation; de-esterification and depolymerization. The degree of methylesterification (DM) and acetylation (DA) can be fine-tuned by the activities of PMEs and PAEs, respectively. PAEs have a great impact on functional properties of pectin since acetyl groups hinder the association abilities of pectin (Ralet *et al*., 2003) and can impair the activity of pectin-depolymerizing enzymes (Chen and Mort 1996; Benen *et al.* 1999; Bonnin *et al*. 2003). The action of PMEs will be described in the next chapter (1.4). The newly formed products (after PME and PAE activity) are substrates for further degradation, by four classes of enzymes, thus decreasing their degree of polymerization (DP). HG depolymerization is achieved through hydrolysis by the action of endopolygalacturonases (endo-PG), and exopolygalacturonases (exo-PG), and through β-elimination reaction by pectin and pectate lyases. While PNLs are specific for highly methylesterified forms of HG, PLs prefer non-methylated substrates (Bonnin *et al*., 2014.). Both reactions result in releasing of small fragments of HGs called oligogalacuronides (OGs) with different DM, DA and DP which have a role in signaling pathways.

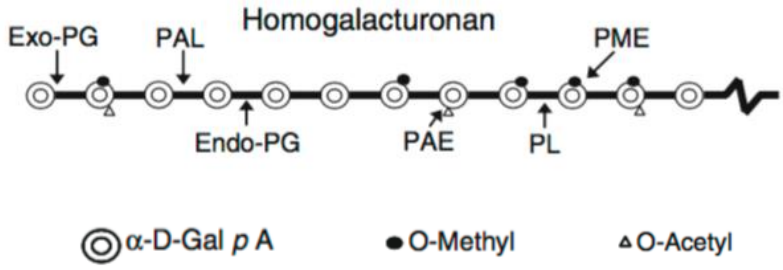


Figure 3. **Mode of action of pectinases involved in the degradation of homogalacturonans.** Exo-PG: exopolygalacturonases, PAL: pectate lyases, Endo-PG: endopolygalacturonases, PAE: pectin acetylesterases, PL: pectin lyases, PME: pectin methylesterases. Figure has been adapted from Hilz, 2007.

1.4 PECTIN METHYLESTERASES

Pectin methylesterases belong to the class 8 (CE8) of the carbohydrate esterases (CAZy website, http://www.cazy.org/fam/CE8.html) (Coutinho *et al*., 2003), and catalyze the reaction of demethylesterification, releasing methanol and protons from methylesterified GalA (**Figure 4**). The reaction consists of nucleophilic attack and an acid/ base catalysis by conserved aspartate catalytic residues on the carbonyl carbon of the C-6 methylester of GalA (Fries *et al.*, 2007). The action of PMEs appears to be sensitive to small pH changes (Catoire *et al.*, 1998; Denès *et al.*, 2000; Ly-Nguyen *et al.*, 2004; Do Amaral *et al.*, 2005; Verlent *et al.*, 2007; Jolie *et al.*, 2009; Dixit *et al.*, 2013). The impact of pH on PMEs' activity can be related to the pI of the isoforms, which are mostly neutral to alkaline for plant and bacterial PMEs and acidic to neutral for fungal PMEs (Senechal *et al*., 2014). In addition, PME isoforms may differ in their substrate specificity and processivity.

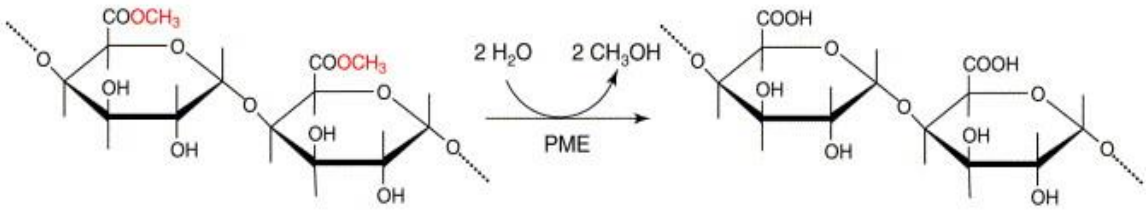


Figure 4. **Schematic representation of PME reaction.** Pectin methylesterase catalyzes hydrolysis of methylester bounds on HG in pectic substances, producing free carboxyl groups and methanol. Figure adapted from Micheli, 2001.

In *Arabidopsis thaliana* 66 ORFs have been identified as encoding putative full-length PMEs, which represents 6.81% of all carbohydrate-active enzymes (Coutinho *et al*., 2003). The structure of PME protein is organized into several domains. From the N-terminus, PME can harbor a signal peptide (SP) or transmembrane domain (TM) (role in positioning proteins in cell wall), a PMEI domain (inhibitory PRO domain that prevents PME activity), a processive motif (PM, cleavage site specific for subtilisin-like serine proteases), and lastly a PME domain (responsible for enzymatic activity). Based on the presence or absence of the PMEI domain, the PME isoforms in *Arabidopsis thaliana* are divided into Group 1 (without PMEI domain) and Group 2 (with PMEI domain) (**Figure 5**). PMEs of Group 1 (or type 2) have a molecular mass of about 27-45 kDa (250-400 amino acids) while non-processed PME of Group 2 (or type 1) have

size of 52-105 kDa (500-900 amino acids) and can harbor 1-3 PMEI domains (Pelloux *et al*., 2007). The processing of PMEI domain in Group 2 PME releases active forms of enzymes at the cell wall. Comparing the amino acids sequences of catalytic (PME) domains, it has been shown that five characteristic sequence fragments (44_GxYxE, 113_QAVAL, 135_QDTL, 157_DFIFG, 223_LGRPW; carrot numbering), six strictly conserved residues, and several highly conserved aromatic residues are functionally important (Markovic and Janecek, 2004).

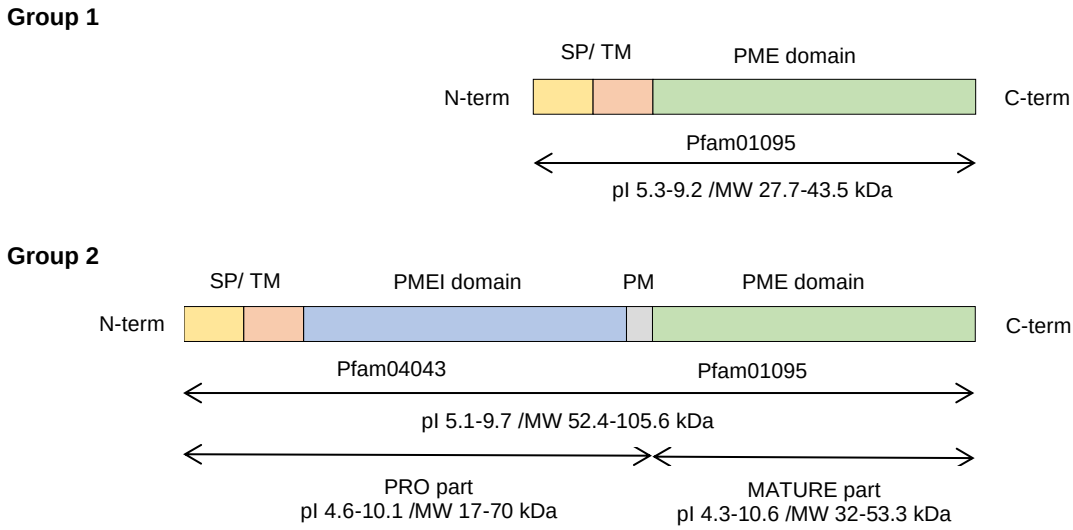


***Figure 5*. Differences in the protein structure between Group 1 and 2 PMEs.** All PME proteins (Group 1 and 2) have conserved PME domain (Pfam01095) and signal peptide/ transmembrane domain (SP/TM), while only proteins from Group 2 have PRO region which shares similarities with the PMEI domain (Pfam04043) and a processive motifs (PM). Figure adapted from Pelloux *et al*., 2007.

It is known that PMEs can have two different mode of actions: (*i*) continuously removing methylesters on HG substrates without dissociating from a single chain (highly processive or blockwise) and (*ii*) randomly removing one by one methylesters (random or non-blockwise) (Senechal *et al*., 2014) (**Figure 6**). While randomly demethylesterified HGs can become substrates for PGs and PNLs, releasing OGs and causing cell wall loosening, block-wise demethylesterified HG can cross-link with Ca^{2+} creating 'egg-box' structures and increasing cell wall stiffness. It is been suggested that 'acidic' PMEs (most of the fungal PMEs) act randomly while 'alkaline' PMEs (most of the plant PMEs) act block-wise, but now is accepted that the processivity may vary

between the isoforms, and depending on pH (Kim *et al*., 2005). Accordingly, a lot of research is needed to unravel the substrate specificity, mode of action, pH dependence and the effects on plants of each individual isoform (Pelloux *et al*., 2007).

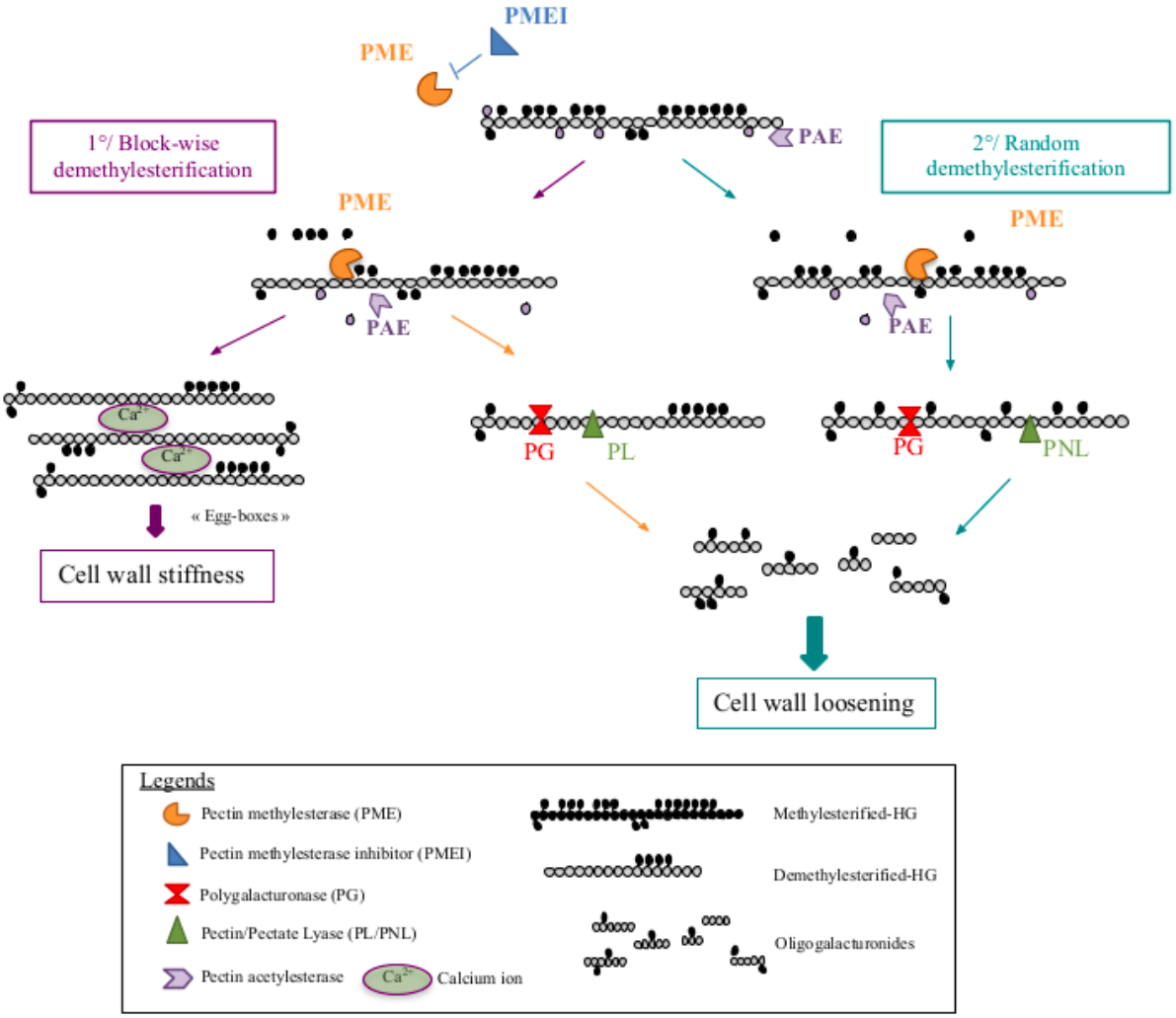


***Figure 6*. Theoretical mode of action of homogalacturonan-modifying enzymes on homogalacturonan.** Two different modes of action are shown. (A) Block-wise demethylesterification leads to large chains of negatively charged GalA that can interact with cations, such as calcium ions, and form 'egg-box' structures. (B) Random demethylesterification. Both modes of activity can generate a substrate for polygalacturonases (PGs) and pectin/pectate lyases (PLs/PNLs) that can form oligogalacturonides, which can lead to cell wall loosening. Figure is adapted from Hocq *et al*., 2017.

1.5 OBJECTIVES

In order to understand the rationale of such abundance of PMEs in plants, it is necessary to determine their biochemical specificities, and compare with that of plant pathogens. For this purpose, we investigated the specificities and effects of 5 PME isoforms from *Arabidopsis thaliana* that belong to Group 2 (PME-32, PME-2, TT-PME, PME-41 and PME-20) and one PME from plant fungal pathogen *Verticillium dahliae* (VdPME).

The objectives of the work were to *(i)* express and purify the PME isoforms in the heterologous system *Pichia pastoris (ii)* determine the potential difference in the pH-dependence of their mode of action using a recently-developed LC-MS/MS oligoprofiling method *(iii)* relate any differences in processivity of the isoforms to their phenotypical effects on plants. For this purpose, purified enzymes were applied exogenously on dark-grown hypocotyls.

Overall, this Master's project should allow sampling the diversity of PME function.

2. MATERIALS AND METHODS

2.1 PRODUCTION AND PURIFICATION OF RECOMBINANT PROTEINS

2.1.1 Production of PME-32, PME-2, PME-TT, PME-41, VdPME and PME-20

Production of recombinant PME-32 (At3g43270), PME-2 (At1g53830), TT-PME (KF696665.1), PME-41 (At4g02330), VdPME (VDAG_05799) and PME-20 (At2g47550) proteins was conducted in heterologous *Pichia pastoris* system after cloning of the protein-coding genes in methanol-inducible expression vector pPICZαB (Invitrogen) (**Figure A1**). Molecular constructs allowing the production of recombinant proteins were already available in the laboratory.

Under the sterile conditions, single colony of transformed X-33 yeast strain was inoculated into 125 mL Erlenmeyer flask filled with 12.5 mL BMGY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate, pH 6.0, 1.34% (w/v) yeast nitrogen base, 0.00004% (w/v) biotin, 1% (v/v) glycerol) with 100 µg/ml of zeocin. After 24h (30 °C, shaking at 250 rpm), 200 mL culture was launched at 1 OD_{600nm}/mL in BMMY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate buffer pH 6.0, 1.34% (w/v) YNB, 0.0004% (w/v) biotin, and 0.5% (v/v) methanol) in a 2 L Erlenmeyer flask (30 °C, shaking at 250 rpm). After 24 h and 48 h, 0,5% of sterile methanol was added in order to continuously induce production of recombinant protein. Finally, after 72 h production, supernatant was harvested after centrifugation (1500*g* for 5 min at 4 °C) in 50 mL falcon tubes and stored at 4 °C.

2.1.2 Protein purification

PME-2, TT-PME and PME-41 were purified using ion-exchange chromatography (for column refereces, see **Table 1**) after precipitation and dialysis. First, protein precipitation was achieved by adding 65 g of ammonium sulfate to 100 mL of supernatant. After addition of ammonium sulfate, proteins were pelleted by the centrifugation at 13000*g* for 10 min at 4 °C. Pellets were re-suspended in 1 mL of storage buffer (**Table 1**) and transferred into dialysis column (Gebaflex, Gene Bio-application) for overnight dialysis in 1 L of storage buffer. After the dialysis, ion-exchange purification was carried out. The peristaltic pump was set to 1 mL/min pumping rate, column (**Table 1**) was rinsed with 10 V of MiliQ water and equilibrated

with 10 V of storage buffer. Then, dialyzed protein sample was loaded onto the column and the bound proteins were eluted out by utilizing a gradient of linearly increasing salt concentration (storage buffer containing 50mM NaCl, 100mM NaCl, 250 mM NaCl and 1 M NaCl; 10 V each). Eluates were collected and kept on ice. The columns were finally washed with 10 V MilliQ water and stored in 20% (v/v) ethanol.

VdPME and PME-20 were purified performing the affinity chromatography (for column references, see **Table 1**) after filtration of 100 mL supernatant using 0.45 µm pore size PES filter (GE Healthcare). The peristaltic pump was set to 1 mL/min pumping rate. After washing the column with 10 V of MilliQ water, 100 mL of filtered supernatant was loaded. For VdPME the column was directly washed with 10 V of washing buffer 1 (50 mM sodium phosphate buffer (NaP), 250 mM NaCl, 10 mM imidazole, pH 7.4), followed by 10 V of washing buffer 2 (50 mM sodium phosphate buffer (NaP), 250 mM NaCl, 25 mM imidazole, pH 7.4) and finally 10 V of eluate (50 mM NaP, 250 mM NaCl, 500 mM imidazole, pH 7.4) was collected and kept on ice. On the other hand, for PME-20 after loading filtrated supernatant, proteins were eluted with the elution buffer (50 mM sodium phosphate buffer (NaP), 250 mM NaCl, 10 mM imidazole, pH 7.4), collected and stored on ice. The columns were washed with 10 V MilliQ water and stored in 20% (v/v) ethanol.

PME-32 is not purified but only precipitated and dialyzed as previously described for PME-2, TT-PME and PME-41.

***Table 1*. List of used storage buffers and purification columns for each enzyme.**

Enzyme	Storage buffer	Purification column (GE Healthcare column)
PME-32	50 mM NaP pH 7.5	/
PME-2	50 mM NaP pH 7	HiTrap™ CM-FF
TT-PME	50 mM Tris-HCl pH 9	HiTrap™ Q HP
PME-41	50 mM Tris-HCl pH 9	HiTrap™ Q FF
VdPME	50 mM Tris-HCl pH 7	HisTrap™ Excel
PME-20	50 mM NaP pH 5.7	HisTrap™ Excel

2.1.3 Concentrating and buffer exchange

Eluates were concentrated using 10 kDa centrifugal filters (Amicon® Ultra-4). The filter was firstly rinsed with distilled water through centrifugation at 7500*g*, 4°C and

then the samples were loaded for concentration until the final volume reached 140 µL (7500*g* at 4 °C).

The enzymes were transfered to appropriate storage buffers (**Table 1**) using a PD SpinTrap™ G-25 (GE Healthcare) according to the manufacturer's instructions.

2.1.4 Gel diffusion activity assay

The activity of purified, concentrated and buffer exchanged proteins was confirmed with gel diffusion activity assay (Downie *et al.,* 1998) with some modifications (Ren and Kermode, 2000). 10 µL of each enzyme, boiled (100 °C, 10 min) and native, was loaded on the gel containing 0.1 % citrus pectin (DM 55-70 %, Sigma, Cat. No. P9436) and prepared at pH5, pH6.3 and pH7.6, and reaction (ie demethylesterification of the pectins contained in the gel) was conducted overnight at 37 °C. Next day, the gel was rinsed with distilled water and 25 mL of 0.02% (w/v) ruthenium red (Sigma-Aldrich-R2751) was poured on the gel. After 1 h incubation, ruthenium red was removed, and the picture of gel was taken. Diameters of the red halos correspond to demethylesterified/acidic pectins and therefore to PME activity. Commercial PME from orange peel (Sigma-Aldrich P5400) was used as a positive control (**Figure 7**).

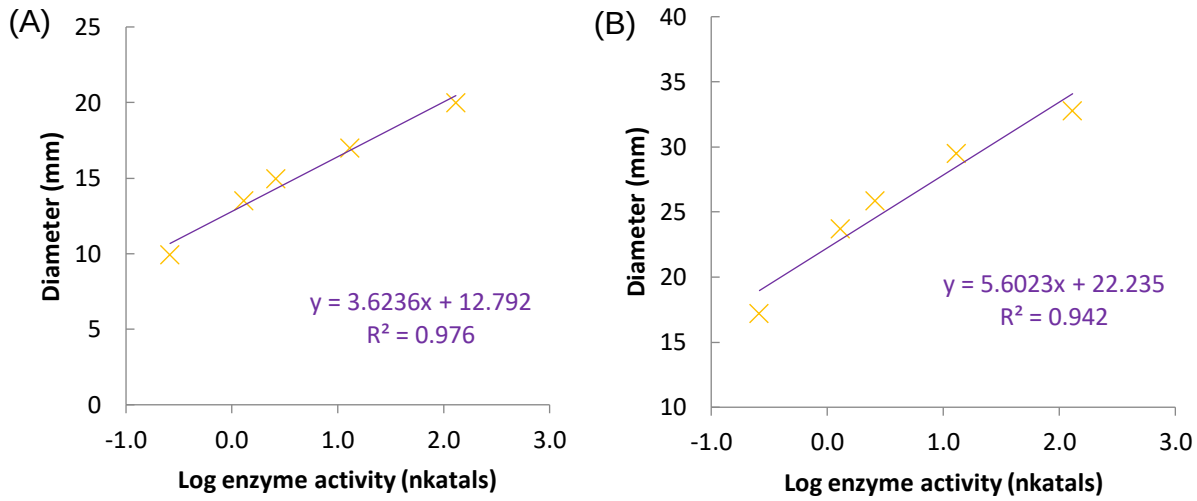


Figure 7. **Standard curve showing the dependence of hallows diameter (mm) corresponding to demetylestirified pectin and the logarithmic value of the commercial PME activity (nkatals) at *(A)* pH5 *(B)* pH7.6.**

2.1.5 Determination of protein concentration

Enzyme concentration was determined thanks to Bradford method (Bradford, 1976) in 96-well plate. As a standard 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 µg of bovine serum albumin (BSA) in appropriate buffer in the volume of 100 µL were used. The absorbance was measured at 595 nm on the microplate reader.

2.1.6 SDS-PAGE and Coomassie blue staining

To assess the quality of the purification, SDS-PAGE (12%) (resolving gel: 0.375 M Tris-HCl pH 8.8, 0.08% (v/v) TEMED, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate, 12/0.3% (w/v) Acrylamide/Bis-acrylamide; stacking gel: 0.125 M Tris-HCl, pH6.8, 0.1% (v/v) TEMED, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate, 2/0.05% (w/v) Acrylamide/Bis-acrylamide) was conducted. 10µL (6 µg) of sample was mixed with 2.5 µL of 5x protein loading buffer (313 mM Tris-HCl pH 6.8, 50% (v/v) glycerol, 10% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 0.02% (w/v) bromophenol blue) and denatured 10 min at 100 °C. Samples were loaded on the gel and electrophoresis (Bio-Rad PowerPac Basic Mini Electrophoresis System) was conducted in 1x TGS buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS) at 25 mA for 15 min then 35 mA until loading dye has reached the bottom of the gel. Gel was stained with Coomassie blue stain (Page Blue Protein Staining Solution, Thermo Scientific) according to manufacturer's recommendations and destaining was performed in water on a shaker.

2.2 LC-MS/MS ANALISYS

The mode of action of PME-32, PME-20 and VdPME was determined using the following strategy: firstly, a pool of OGs (which differ in their degrees of polymerization (DP) and methylesterification (DM)) was generated by treating on 0.4% (w/v) citrus pectin (DM 55-70 %, Sigma, Cat. No. P9436) samples with 2.9 U/ mL of *Aspergillus acuelatus* endo-polygalacturonase M2 (Megazyme, Bray, Ireland) in 100 mM ammonium acetate buffer pH5 at 37 °C. After 1.5 h of digestion, pectins and proteins precipitation was achieved with addition of 1V of absolute ethanol and centrifugation (5 min, 5000*g*, 20°C). Upper part containing OGs was divided into two tubes and evaporated using speed vac (Eppendorf Concentrator Plus) then re-suspended either in 100 mM ammonium acetate pH 5, or in 20 mM Tris-HCl pH8. In order to be sure that all PGs are removed from samples, centrifugal filtration was preformed using 3 kDa centrifugal filters (Amicon® Ultra-0.5) and the pH was adjusted using 1N NaOH. 200 µL

aliquots of each filtrate was separated into Eppendorf tubes and treated with VdPME, PME-32 or PME-20 overnight at 40 °C. The quantity of enzymes for digestion was determined using a gel diffusion activity test performed at pH5 and pH7.6 (**Table 2**). PME digestion was stopped by the addition of 1V of absolute ethanol and centrifugation (5 min, 5000*g*, 20°C). The upper part of each sample was transferred to inserts and evaporated using a speed vac (Eppendorf Concentrator Plus). The dried sample was resuspended in 200 µL of MiliQ water and analyzed by high performance size-exclusion chromatography (HP- SEC) and MS/MS analysis to separate and detect released OGs. It is an analytical method based on the separation of oligosaccharides combined with accurate determination of their size and methylesterification patterns using MS/MS. Samples were diluted at 1 mg/mL in ammonium formate 50 mM, formic acid 0.1%. Chromatographic separation was performed on an ACQUITY UPLC Protein BEH SEC Column (125Å, 1.7 µm, 4.6 mm X 300 mm, Waters Corporation, Milford, MA, USA). Elution was performed in 50 mM ammonium formate, formic acid 0.1% at a flow rate of 400 µL/min and a column oven temperature of 40 °C. The injection volume was set to 10 µL. MS-detection was performed with SYNAPT G2-Si in negative mode with the end plate offset set voltage to 500 V, capillary voltage to 4000 V, Nebulizer 40 psi, dry gas 8 L/min and dry temperature 180°C. Obtained spectra were analyzed by MassLynx software using the library 110 different oligogalacturonides.

Table 2. **Quantity (µg) of VdPME, PME-32 and PME-20 used for LC-MS/MS analysis.**

Enzyme	$m_{pH\ 5}$ (µg)	$m_{pH\ 8}$ (µg)
VdPME	2	0.2
PME-32	70	30
PME-20	3.5	0.6

2.3 EXOGENOUS APPLICATION OF PROTEINS ON COL-0 SEEDS

2.3.1 Seed sterilization and stratification

Around 50 µL of *Arabidopsis thaliana* seeds ecotype Columbia (Col-0) were placed into 1.5 mL Eppendorf tube and 950 µL of the sterilization solution (70% (v/v) ethanol, 0.05% Triton X-100) was added and shaken for 15 min. The sterilization solution was removed and replaced with 950 µL of 95% ethanol under the sterile conditions. After 15 min of shaking ethanol was removed and the seeds were left to dry overnight under a flow hood.

Next day, dry sterile seeds were sowed in 24-well plate. Between 20-30 seeds were placed on the top of 400 µL liquid sterile Duchefa medium whose pH is adjusted to 5.5 or 7.5 (Duchefa Biochemie, MILIEU ARABIDOPSIS) in each well. Plate was wrapped around with textile tape and put in dark for 3 days at 4 °C for cold-wet stratification.

2.3.2 Growth conditions

After 3 days of stratification, plate was put in growth room (21°C ± 2 °C). For etiolated hypocotyl phenotyping, seeds were flashed for 6 hours, then plate was wrapped with 3 layers of aluminium foil in order to stimulate the growth of the hypocotyl. After 24 hours from the start of growth, 20 µg (VdPME and PME-32) or 7 µg (PME-20) of filter-sterilized (13 mm PES filters, GE Healthcare) enzymes (either boiled or native) was added in the medium under green light and sterile conditions and plates were put back in the growth room for further etiolation. Plant growth was stopped after 4 days in total and phenotyped.

2.3.3 Seedling analysis

For cell adhesion observation, growth medium was replaced with 1 mL of 0.05% (w/v) ruthenium red (Sigma-Aldrich-R2751) for 2 min. Plantlets were rinsed 2 times with MiliQ water and observed under the binocular microscope (Leica EZ4) for better visualization of possible cell detachment. The plantlets were then fixed by the addition of 1.5% (v/v) formaldehyde and spreaded next day on square plates with solid Duchefa medium (Duchefa Biochemie, MILIEU ARABIDOPSIS). The pictures of plates were taken using a camera attached to a stand and the lengths of hypocotyls were measured using ImageJ software with NeuronJ plugin.

2.4 EXOGENOUS APPLICATION OF VdPME ON PDF1::GUS SEEDS

PDF1::GUS seeds were sterilized and stratified in a Duchefa medium (Duchefa Biochemie, MILIEU ARABIDOPSIS) pH7.5 and exposed to the action of 20 µg VdPME as it is previously described in chapter 2.3.1 and 2.3.2. As a negative control, native VdPME was replaced with same amount of storage buffer (50 mM Tris-HCl pH 7). After 4 days of growing in dark conditions, plants were transferred into 1.5 mL Eppendorf tubes and proteins were extracted by grinding plant material in presence of 200 µL extraction buffer (phosphate buffer 10X (Na_2HPO_4 500mM, EDTA 100mM, pH 7.2), 0.1% (w/v) triton and 0.1% (w/v) β-mercaptoethanol), followed by two successive centrifugations (12000*g*, 10', 4°C) of the upper parts containing proteins. Protein concentration was determined by the Bradford method. To indirectly determine β-glucuronidase activity, 2 µg of extracted proteins were mixed with 25 µL of substrate (MUG solution, phosphate buffer 10X, 0,2 mM MUG (Duchefa Biochemie), and filled to a final volume of 50 µL with phosphate buffer 10X. Reactions were performed at 37°C for 0, 15, 30 and 60 minutes. The reactions were stopped by addition of 950 µL stop solution (0.2M Na_2CO_3) and the concentration of the formed fluorescent 4-MU (excitation at 365 nm, emission at 455 nm) product was measured on a fluorimeter (VersaFluorTM Fluorometer, BIO-RAD). For fluorescence quantification, solutions of increased concentration of 4-MU (0, 5, 25, 50 and 100 pmol/mL mixed with 25 µL of MUG solution and 950 µL of stop solution) were used as standards.

3. RESULTS

3.1 RECOMBINANT PROTEIN PRODUCTION AND CHARACTERIZATION

Pichia pastoris was used as a host for a heterologous production of PME-32, PME-2, TT-PME, PME-41, VdPME and PME-20. Full length protein-coding sequences were cloned into pPICZαB expression vector (**Figure A1**) containing α-factor that enables secretion of the proteins into the medium and easier further purification due to presence of His-tag on C-terminus of the protein sequence. Previously performed experiments showed that for PME-2, TT-PME and PME-41, affinity chromatography based on His-tag is not possible probably due to degradation of the His-tag during protein production. For those enzymes, ion-exchange purification was performed. Concerning PME-32, simple protein precipitation was used as no purification protocol could be established.

After production and purification, protein concentration was determined by Bradford assay for all enzymes. The following numbers are representative of the different experiments conducted: 18.4 µg/µL for PME-32, 0.16 µg/µL for PME-2, 1.303 µg/µL for TT-PME, 1.72 µg/µL for PME-41, 1.98 µg/µL for VdPME and 0.49 µg/µL for PME-20.

Purity of proteins was checked using SDS-PAGE followed by Coomassie blue staining and PME activity was confirmed by gel diffusion assay at pH6.3 (**Figure 8**). Black boxes on SDS-PAGE denote the band corresponding to the processed PME isoforms as these positions on the gel were previously assessed by Western blot analyzes with specific PME antibody in the laboratory. The size of the proteins is larger than predicted (except for PME-2 and VdPME), due to the numerous glycosylation sites (detected by the NetNGlyc server) (**Figure 8A** and **Table 3**) and due to the fact that *Pichia pastoris* posttranslationally overglycosylates the produced proteins (Cereghino *et al*., 2002). According to the performed gel diffusion assay, all PMEs in their native form were active, while for denatured proteins (100°C, 10 min) no activity was measured (**Figure 8B**).

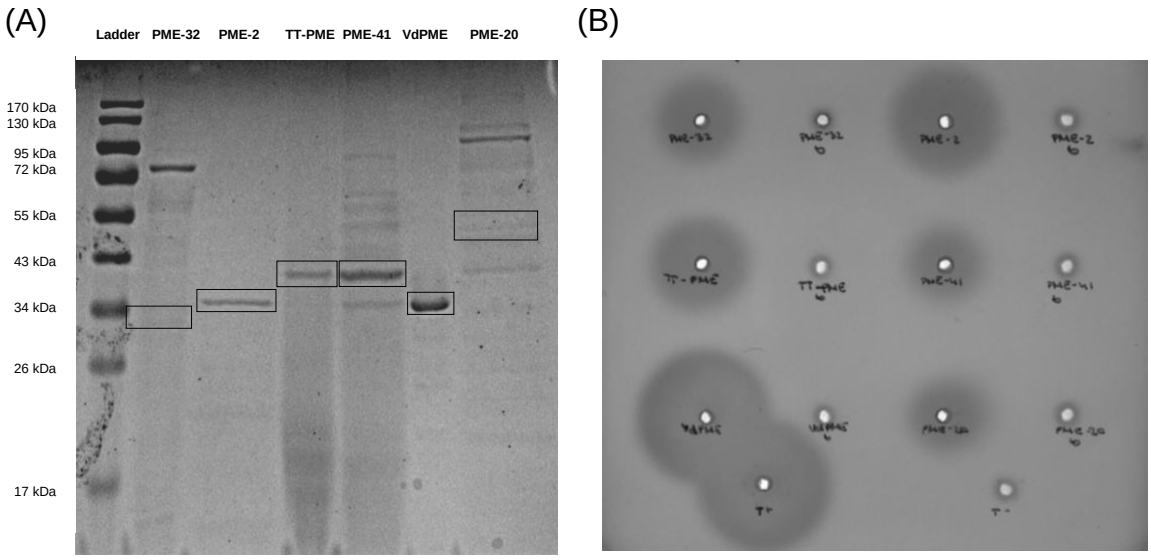


Figure 8. **12% SDS-PAGE stained with Coomassie blue *(A)*; gel diffusion activity assay pH6.3 of native and boiled (b, 100°C, 10 min) purified PMEs *(B).*** As controls 1/10 diluted commercial PME orange was used (T^+: native, T^-: boiled).

Comparison of PME isoforms was performed using online tools. It was shown that amino acids in the area of active site are highly conserved (**Figure A2** and **Figure A3**) despite variable identity percentage (**Table A1**). According to the calculation of the Clustal Omega program, it was shown that the highest percentage of identity is between PME-41 and PME-20 isoforms (75.54%), while the lowest matching is between *Verticillium dahliae* PME and plant PMEs (less than 32%). Data of molecular mass, number of glycosylation sites and pI are summarized in **Table 3**.

Table 3. **Theoretical characteristics of mature PME proteins**. Values are taken from Expasy (https://www.expasy.org) and NetNGlyc server (http://www.cbs.dtu.dk/services/NetNGlyc/).

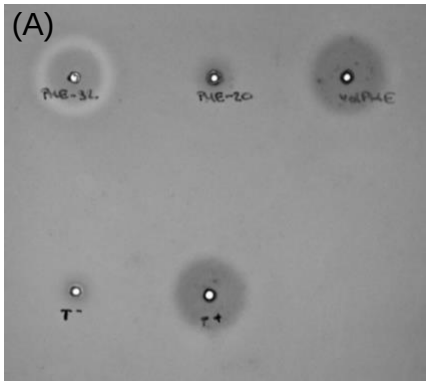
PME isoform	Molecular mass (kDa)	Number of glycosylation sites	pI
PME-32	35.65	6	7.84
PME-2	34.56	0	9.10
TT-PME	35.62	9	9.18
PME-41	36.64	10	4.63
VdPME	33.20	2	9.14
PME-20	35.61	10	4.96

3.2 DETERMINING THE MODE OF ACTION USING LC-MS/MS

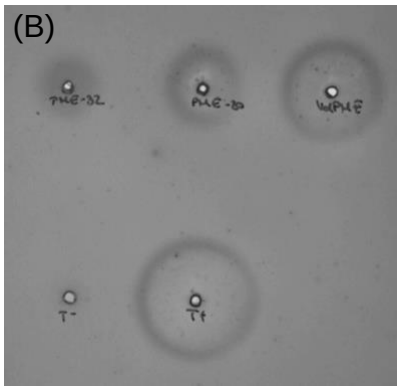
After preliminary tests, we decided to focus on 3 out of the 6 initial enzymes: VdPME, PME-32 and PME-20, and assess their processivity on a pool of OGs of mean DM 55-70% obtained from PG-hydrolyzed commercial pectins.

After subsequent PME digestion, the pattern of released OGs was compared with that of non-PME treated samples (enzyme is replaced with an equal volume of storage buffer). Quantity of PME required for substrate digestion was tested on gel diffusion activity assay at pH5 and pH7.6 (**Figure 9A** and **9B**). The exact activity was calculated by measuring the diameter of the hallow, that corresponds to demethylesterified pectins on the plate using ImageJ and correlating the values to the PME orange standard (**Figure 7**). The obtained values are expressed in nkatals and shown in the table (**Figure 9C**).

(A)



(B)



(C)

	pH5	pH7.6
PME-32	19.86 nkat	0.03 nkat
PME-20	0.16 nkat	0.15 nkat
VdPME	6.53 nkat	1.45 nkat

Figure 9. **Gel diffusion activity assay at pH5 *(A)* and pH7.6 *(B)* and calculated PME activities using commercial PME orange as a standard *(C).*** Values were calculated using the calibration curve of the dependence of the hallow diameter on the logarithmic activity value expressed in nkatals. The values represented in the table were applied for the LC-MS/MS analysis.

Samples were subjected to LC-MS analysis and spectra were analyzed by MassLynx software and using the method developed in the laboratory allowing the detection of 110 different OGs. The following results show oligogalacturonides up to DP10 present in the sample in more than 0.5% of total OGs detected.

In the samples treated with VdPME, we observed that for each individual DP there is almost exclusively OGs in the unmethylated form (GalA5, GalA6, GalA7, GalA8, GalA9), while methylated forms of the same DP are in the negligibly low

concentration, which leads to the conclusion that VdPME completely demethylesterifies the substrates, in a processive manner. The same observation was noticed for both conditions, pH5 (**Figure 10A**) and pH8 (**Figure 10B**) (i.e. 92% and 99% of released GalA6 in total of DP6 (**Figure 10A** and **10B**, insets)), showing that the mode of action for VdPME does not change depending on the pH value.

Opposite to VdPME, PME-32 (**Figure 11**) and PME-20 (**Figure 12**) digested samples show a tendency to reduce the average degree of methylation, shifting towards lower DM but not leading to complete demethylesterification. This random demethylesterification can be observed focusing on DP9 (**Figure 11** and **12**, insets), where it is noticeable that for both enzymes at pH8 (**Figure 11B, 12B**) there is an increase in GalA9Me1 and GalA9Me2 compared to control, while at pH5 (**Figure 11A, 12A**) this also includes GalA9Me3. For both enzymes, a reduced activity at pH8 was detected, so in further studies increased enzyme quantity should be applied.

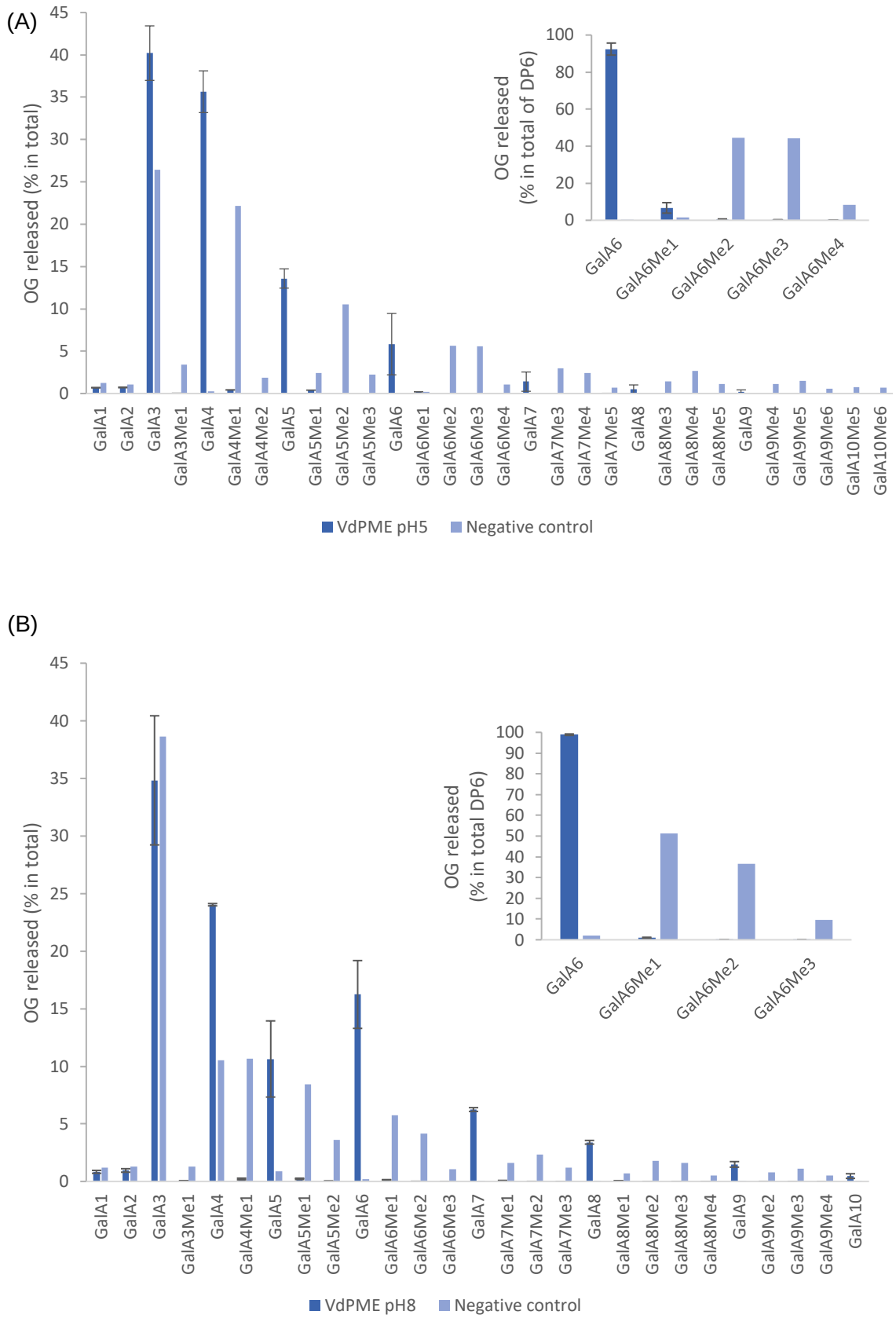


Figure 10. **Determination of VdPME mode of action using a LC-MS/MS.** Previously PG-digested pectins (DM 55-70%) are treated with (***A***) 2 µg (6.53 nkat) of VdPME at pH5 (***B***) 0.2 µg (1.45 nkat) of VdPME at pH8 and compared with non-digested samples at same pH.

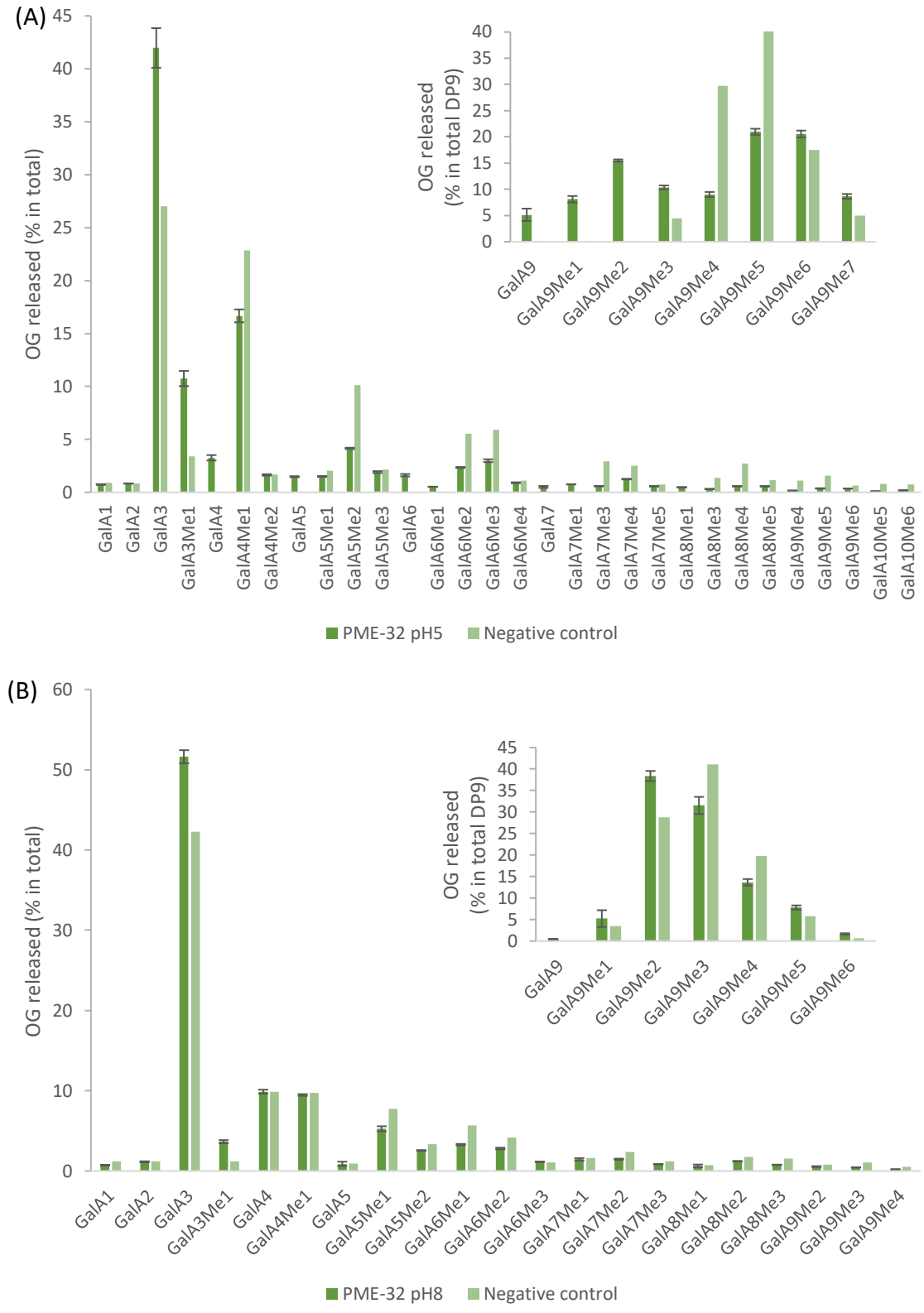


Figure 11*. Determination of PME-32 mode of action using a LC-MS/MS.** Previously PG-digested pectins (DM 55-70%) are treated with (A***) 70 µg (19.86 nkat) of PME-32 at pH5 (***B***) 30 µg (0.03 nkat) of PME-32 at pH8 and compared with non-digested samples at same pH.

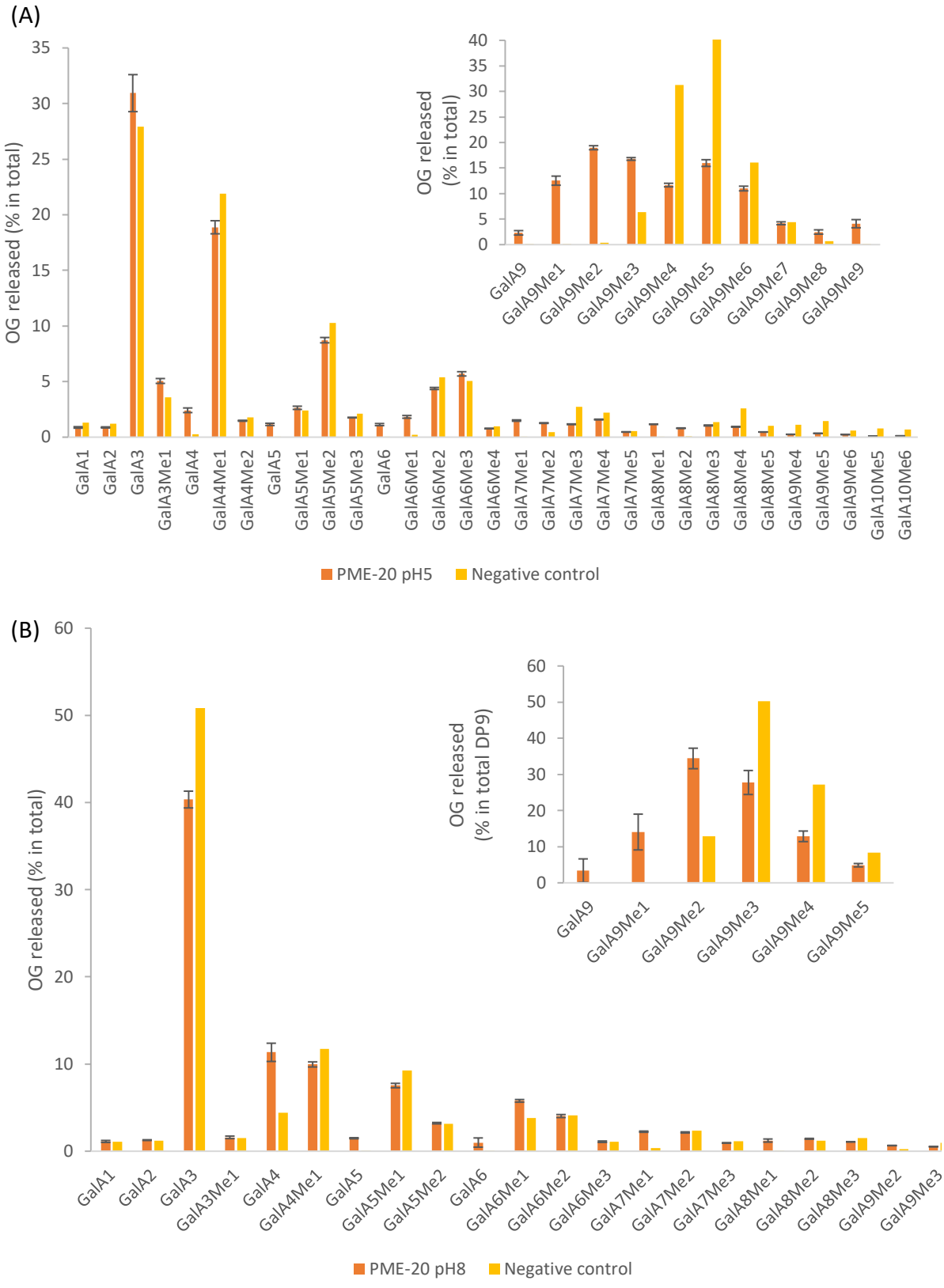


Figure 12. **Determination of PME-20 mode of action using a LC-MS/MS.** Previously PG-digested pectins (DM 55-70%) are treated with (***A***) 3.5 µg (0.16 nkat) of PME-20 at pH5(***B***) 0.6 µg (0.15 nkat) of PME-20 at pH8 and compared with non-digested samples at same pH.

3.3 EXOGENOUS APPLICATION OF VdPME, PME-32 and PME-20 AND EFFECT ON THE PLANT DEVELOPMENT

As a way of assessing the effects of PMEs in planta, previously filter-sterilized enzymes were added in the culture medium 24h after sowing. Plantlets were observed after 3 days in the dark (4 days of culture in total) to assess the effects of the enzymes on cell wall remodeling and plant development. Plants were observed under microscope and the length of roots (for VdPME) and hypocotyls (for VdPME, PME-32 and PME-20) were measured using ImageJ (NeuronJ plugin) to detect potential phenotypes caused by enzyme application. For each condition between 20-30 plants were examined. In order to perform this experiment 20 µg of VdPME, 20 µg of PME-32 or 7 µg of PME-20 was applied on *Arabidopsis thaliana* Col-0 seeds in native and heat-denaturated form.

We first observed that native VdPME causes a decrease in hypocotyl and root lengths at both pH5.5 and pH7.5. An opposite effect was monitored for PME-32 and PME-20, as an increase in length for both pH was measured (**Figure 13**). Carrying on with statistical analysis, significant difference in length was confirmed for VdPME in all conditions (for hypocotyl and root length and for both pH) while in cases of PME-32 and PME-20 it was only confirmed for hypocotyl length at pH5.5 (**Figure 14**).

Monitoring of the cell adhesion defects was done by the addition of ruthenium red solution and observation of the bound red color on the hypocotyl. Cell detachment rate was calculated by dividing the number of hypocotyls with visible cell adhesion defect with the total number of hypocotyls for one given condition. An increase in cell detachment was observed for all native proteins compared to boiled, but the most present was for VdPME pH5.5 (70.73%) and pH7.5 (37.21%) (**Figure 13**).

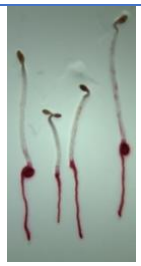
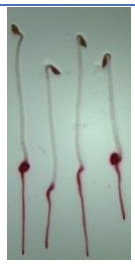
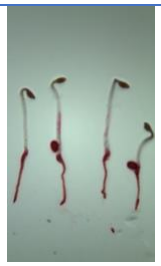
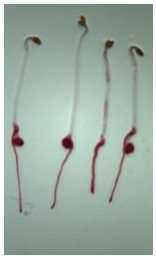
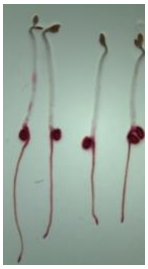
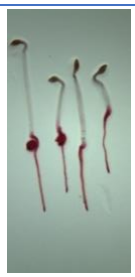
	pH5.5		pH7.5	
	Boiled	**Native**	**Boiled**	**Native**
VdPME				
Average length of hypocotyls (cm)	0.616 ± 0.131	0.512 ± 0.133	0.575 ± 0.121	0.417 ± 0.118
Average length of roots (cm)	0.593 ± 0.117	0.343 ± 0.060	0.400 ± 0.076	0.256 ± 0.046
Cell detachment rate (%)	6.90	70.73	7.89	37.21
PME-32				
Average length of hypocotyls (cm)	0.546 ± 0.176	0.662 ± 0.131	0.556 ± 0.174	0.621 ± 0.133
Cell detachment rate (%)	9.09	15.38	3.85	6.67
PME-20				
Average length of hypocotyls (cm)	0.428 ± 0.107	0.536 ± 0.100	0.449 ± 0.165	0.504 ± 0.112
Cell detachment rate (%)	2.38	14.71	6.67	21.88

Figure 13. **The effect of PMEs on dark-grown hypocotyls and roots.** 4-day old plants grown in the 3 days presence of 20 µg (VdPME or PME-32) or 7 µg (PME-20) boiled or native enzyme. Length was measured using ImageJ and the standard deviation was calculated for each condition. Cell detachment rate was estimated after staining hypocotyls with ruthenium red 0.05% (w/v).

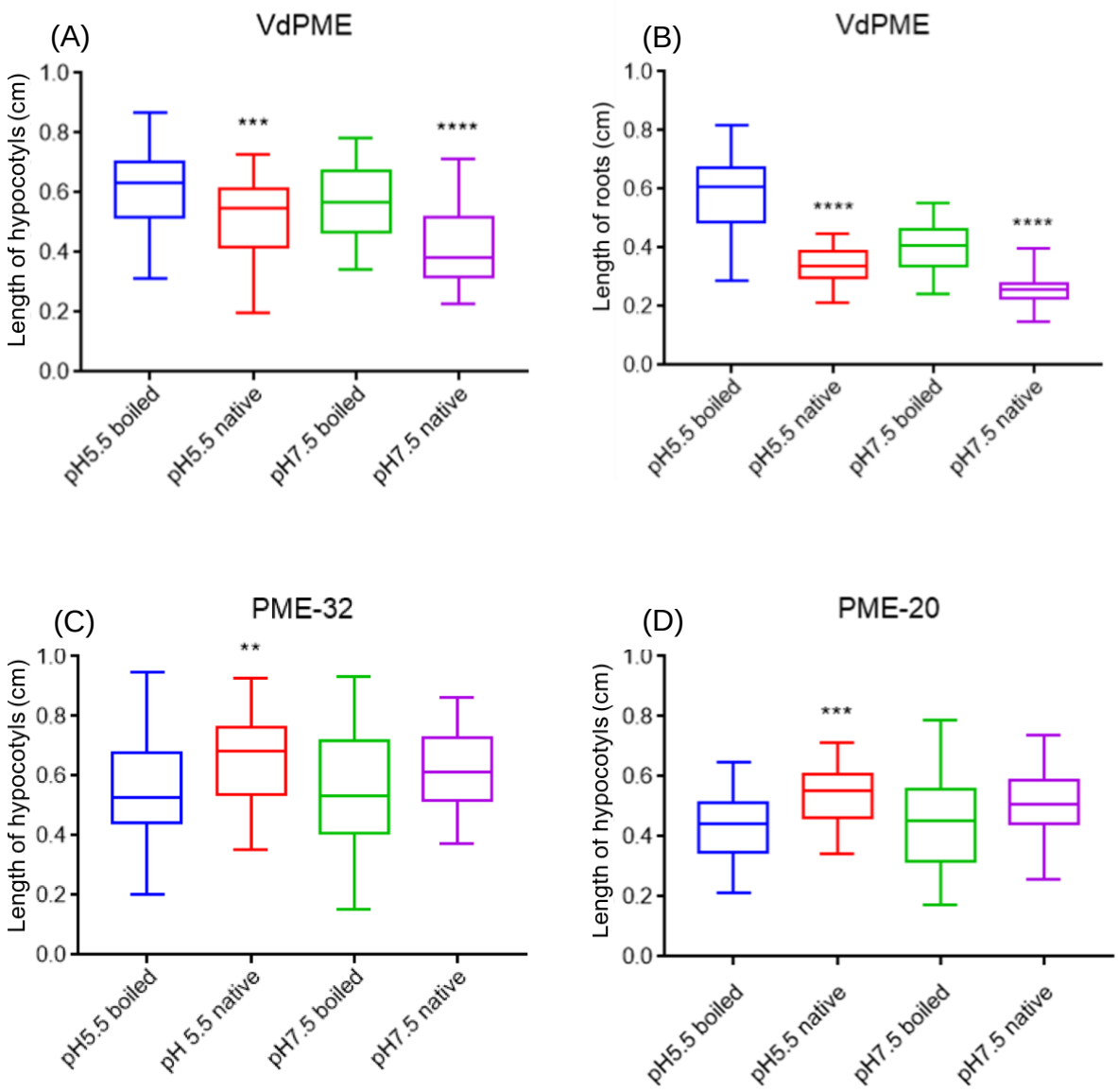


Figure 14. **Length of measured hypocotyls and roots.** Asterisks indicate a significant difference obtained using ANOVA test. ***(A)*** ***p=0.0003, ****p<0.0001, p<0.0001, ***(B)*** p****<0.0001, p<0.0001 ***(C)*** **p=0.0023, p= 0.0025 ***(D)*** ***p=0.0004, p=0.0008.

In order to test if exogenous application of PME enzyme is sensed as a pathogenic attack by the plant, we used transgenic PDF1(At1g75830)::GUS *Arabidopsis thalinana* lines, in which a structural β-glucoronidase gene was inserted under the control of a plant defensin promoter. Under proper conditions, the promoter of the plant defensin is activated leading to transcription then translation of the β-glucoronidase gene. β-glucoronidase proteins, extracted from the different samples, transform the MUG substrate into a fluorescent 4-MU product quantifiable by a

fluorimeter. Therefore, measure of the fluorescence after reaction is an indirect quantification of the activity of PDF's promoter in each experimental condition.

The conditions under which the experiment was performed correspond to those of exogenous application of VdPME to Col-0 seeds at pH7.5. The obtained results show that, after one hour of β-glucoronidase reaction, the concentration of fluorescent product is visible between seeds treated with VdPME and non-treated seeds (higher concentration under VdPME exposure), while for 0, 15 and 30 minutes of reaction the difference is negligible (**Figure 15**). The results indicate the activation of the defense system in plants during exposure to VdPME, as it is reported to be the case upon fungal infection (Bar.utoronto.ca/efp).

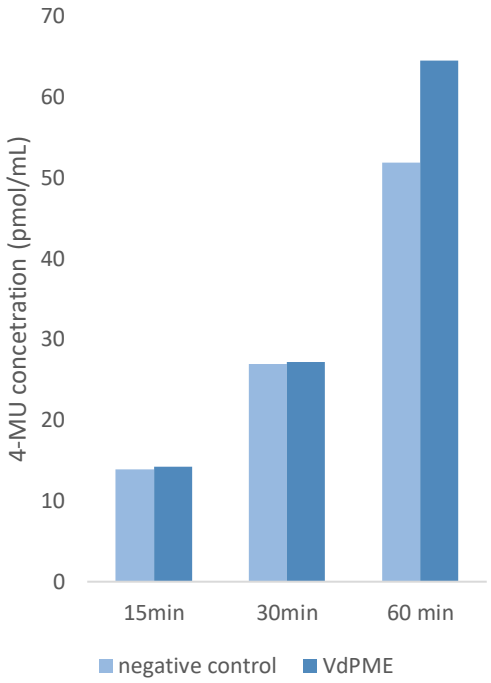


Figure 15. **Indirectly measured activity of plant defensin's promotor showed as a dependence of formed 4-MU product and reaction time (15, 30 and 60 min).** PDF1::GUS transgenic lines are exposed to 20 µg of VdPME for 3 days in dark grown conditions. MUG substrate was added to the plant protein extract, which was converted to fluorescent 4-MU by glucuronidase activity. As a negative control an equal volume of storage buffer corresponding to a volume of 20 µg VdPME was used.

4. DISCUSSION AND CONCLUSIONS

4.1 DISCUSSION

The aim of this study was to point out the similarities and diversity of PME proteins. Although they belong to the same protein family, have a highly conserved 3D structure (**Figure A2**) and amino acid residues in the vicinity of the active site (**Figure A3**), the modes of action of enzymes and their impact on plant development may differ. Beginning with production in heterologous system and purification of single isoforms, we described how different mode of action might control plant development and even how PME activity can lead to activation of defense's response in plants.

In the laboratory, the modes of actions were already examined for PME-2 (pH5 and pH8) and for VdPME (pH8). It has been shown that PME-2 processivity can change depending on pH: at pH5 it behaves non-processively, while at pH8 it becomes fully processive (Hocq *et al*., 2021). On the other hand, despite the fact that most fungal PMEs are non-processive (Fries *et al*., 2007; Mercadante *et al*., 2013; Mercadante *et al*., 2014; Sénéchal *et al*., 2015; Kent *et al*., 2016), it was shown that VdPME has block-wise activity at pH8 (Šafran *et al*., 2021). Our study now confirms that VdPME has a similar processivity at pH5 as at pH8 (**Figure 10**). The difference in composition of the two mixes of OGs (pH5 versus pH8) produced to assess PME activities is likely to be the consequence of the adjustment to pH8 after PG digestion: addition of NaOH caused mild demethylesterification of OGs, resulting in a shift in DM of the OGs. In addition to the pH-independent processivity shown on the commercial substrate, VdPME was shown to cause reduction on the size of hypocotyls when applied exogenously on *Arabidopsis thaliana* seeds (**Figure 14A**). This is likely to be the consequence of increased cell wall stiffness, through cross-linking of blockwise-demethylesterified pectins with calcium ions following processive mechanism of VdPME at both pH (**Figure 10**). Such correlation between PME activity and cell wall stiffness was already reported (Hocq *et al*., 2021). Interestingly, a significant influence of VdPME on root length was also observed (**Figure 14B**). *Verticillium dahliae* is known as a pathogen that attacks plants through the roots (Fradin and Thomma., 2006; Blum *et al*., 2018), so our results could contribute to better understanding the way by which Verticillium can colonize the plants through cell wall degradation, and consequently in improving plant protection and preventing crop decay. Furthermore, in comparison to

other tested PMEs, VdPME showed the highest cell adhesion defects (**Figure 13**) which led to the investigation of whether the plant recognizes the action of VdPME as an attack or not. According to the results obtained on PDF1::GUS lines, it seems that plants are indeed activating their defensive response (**Figure 15**) even if they are exposed to the PMEs that are not causing complete degradation of HGs, like PGs. The fact that VdPME is of pathogen origin appears sufficient in triggering defenses' response.

In contrast to what observed for VdPME, the mode of action of PME-20 and PME-32 appears to be non-processive at both pH, generating a shift in the degree of methylation of the OGs for a given degree of polymerization (**Figure 11 and 12**). Such processivity is not common for plant PMEs (Johansson *et al.*, 2002; Willats *et al.*, 2006; Jolie *et al.*, 2010). Consequently, such processivity was also reflected in the effects on the length of the hypocotyl resulting in length increase, likely to be related to cell wall loosening (**Figure 14**). This confirms the theoretical model presented on **Figure 6**, where random demethylesterification would create cleavage sites for HG degrading enzymes. Hypocotyls' cell adhesion defects were slightly increased following application of PME-20 and PME-32, comparing to the hypocotyls treated with boiled enzymes, but were not as pronounced compared to that observed with VdPME (**Figure 13**).

Comparing the experimental data obtained by LC-MS/MS analysis (**Figure 10, 11 and 12**) and theoretical characteristics (**Table 3**) obtained using bioinformatics tools, it can be concluded that PME processivity cannot be predicted based on pI values. Despite previous suggestions that most of plant PMEs are alkaline and act block-wise (Jolie *et al*., 2010; Dixit *et al*., 2013), our results obtained for PME-20 shows completely opposite behavior (acidic pI and random mode of action). PME-32 expectedly has alkaline pI but its mode of action is random as for 'acidic' PME-20. On the other hand, most of the fungal PMEs were characterized as an acidic and non-processive (Senechal *et al.,* 2014), while VdPME has an alkaline pI and acts in processive way. Moreover, the activity of PME-32, PME-20 and VdPME isoforms was shown to be pH-independent in contrast to the previously analyzed PME-2 (Hocq *et al*., 2021).

In order to discover the cause of the diversity of PME activities despite the preserved protein structure (**Figure A2 and A3**), a new approach based on electrostatic potential measurements was developed (Mercadante *et al*., 2014).

Electrostatic properties were recognized as a significant determinant of substrate binding and of the processivity of PMEs. Their asymmetric charge distribution around the binding groove is essential for the sliding of a negatively charged, demethylesterified polysaccharides. Previous research (Hocq *et al*., 2021) has shown that pH-dependent PMEs (e.g. AtPME-2 and citrus PME-4) that change the mode of action from processive at pH8 to non-processive at pH5 vary in electrostatic similarity indices measured at both pH compared to PMEs that have pH-independent processivity. In comparison to PME-2 and CsPME-4, fully processive VdPME has a smaller differences of electrostatic similarity indices, whereas, non-processive PME-32 shows the largest differences of electrostatic properties across the pH range (**Figure A4**). The same calculations should be performed for PME-20 to determine if electrostatic potentials can indeed be used as a predictive tool of enzyme's processivity. This approach would significantly facilitate the understanding of the diversity of PME isoforms and simplify their characterization.

4.2 CONCLUSIONS AND PERSPECTIVES

The focus of this master's thesis was to get insights into PME diversity through their biochemical characterization, *in vitro,* and the assessment of their activity *in vivo.* This was notably realized by the determination of their mode of action using oligoprofiling of commercial substrates and by the analysis of the phenotypical consequences of exogenous application of enzymes on plants. After successful production and purification of 6 enzymes (PME-32, PME-2, TT-PME, PME-41, VdPME and PME-20) we decided to focus on 3 of them (VdPME, PME-32 and PME-20) for which the pH-dependence of the processivity and the impact on plant development was successfully determined. VdPME shows block-wise behavior and may cause cell wall stiffness in the hypocotyl, but also in the roots, at both pH. The effects of the application of the enzymes on roots has still to be carried out in light conditions, for which better root development occurs, and any effects of the enzyme on root architecture will be more easily noticeable. Differently, PME-32 and PME-20 trigger random demethylesterification and stimulate cell wall loosening at pH5. The mode of action of TT-PME and PME-41 as well as their effects on plants, together with that of PME-2, need further analyses.

Although it is currently difficult to predict the mode of action of PME enzymes based on their sequence and structure, our results undoubtedly show that PMEs are not all equal with regards to their processivity and pH-dependence. The apparent diversity of enzymes activities appears as a key for the fine modulation of pectin structure *in planta*, for instance in specific cell wall microenvironments. New insights into structure-processivity links might be provided by molecular dynamics simulations approach. Analysis of the electrostatic potential of the enzymes at given pH, which might determine the interaction between enzyme and substrate, give promising results that could greatly facilitate understanding the variability within the PME family.

5. REFERENCES

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insights into the specificity and processivity of two novel pectinases from *Verticillium dahliae*. *Int. J. Biol. Macromol.,* **176**, 165-176.

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6. ANNEXE

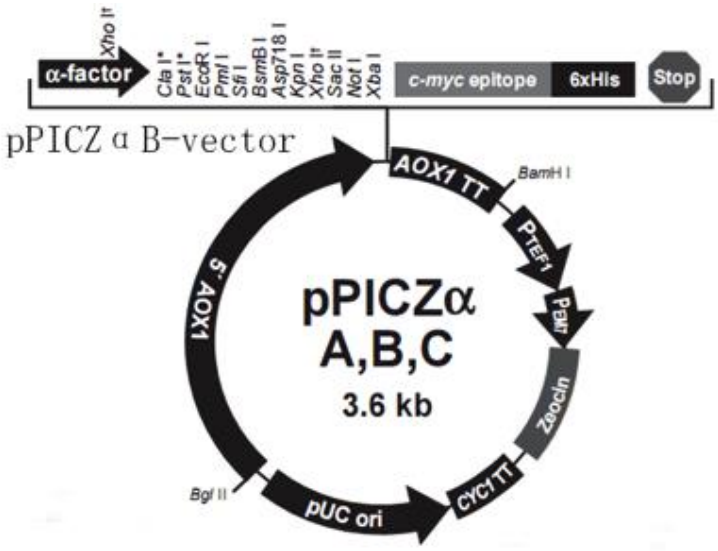

Figure A1. ***Pichia pastoris*** **protein expression vector**. The expressed recombinant PME protein is a fusion protein containing an N-terminal polypeptide encoding the secretory signal of α-factor from *Saccharomyces cerevisiae* and His-tag on C-terminus.

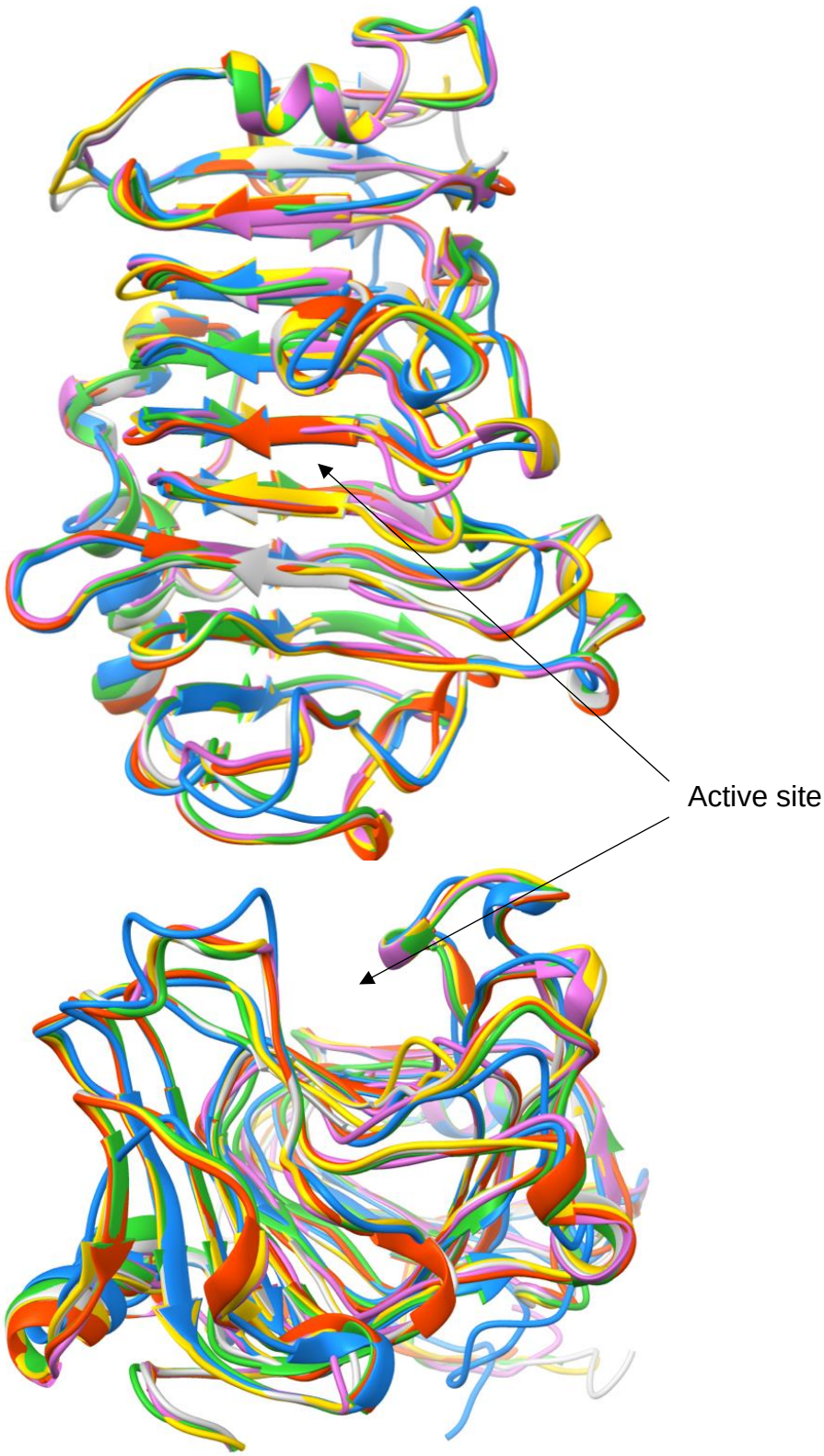

Figure A2. **Structural comparison of PME-32, PME-2, TT-PME, PME-41, VdPME AND PME-20.** PME-32: green, PME-2 red: TT-PME: purple, PME-41: grey, VdPME: blue, PME-20: yellow.

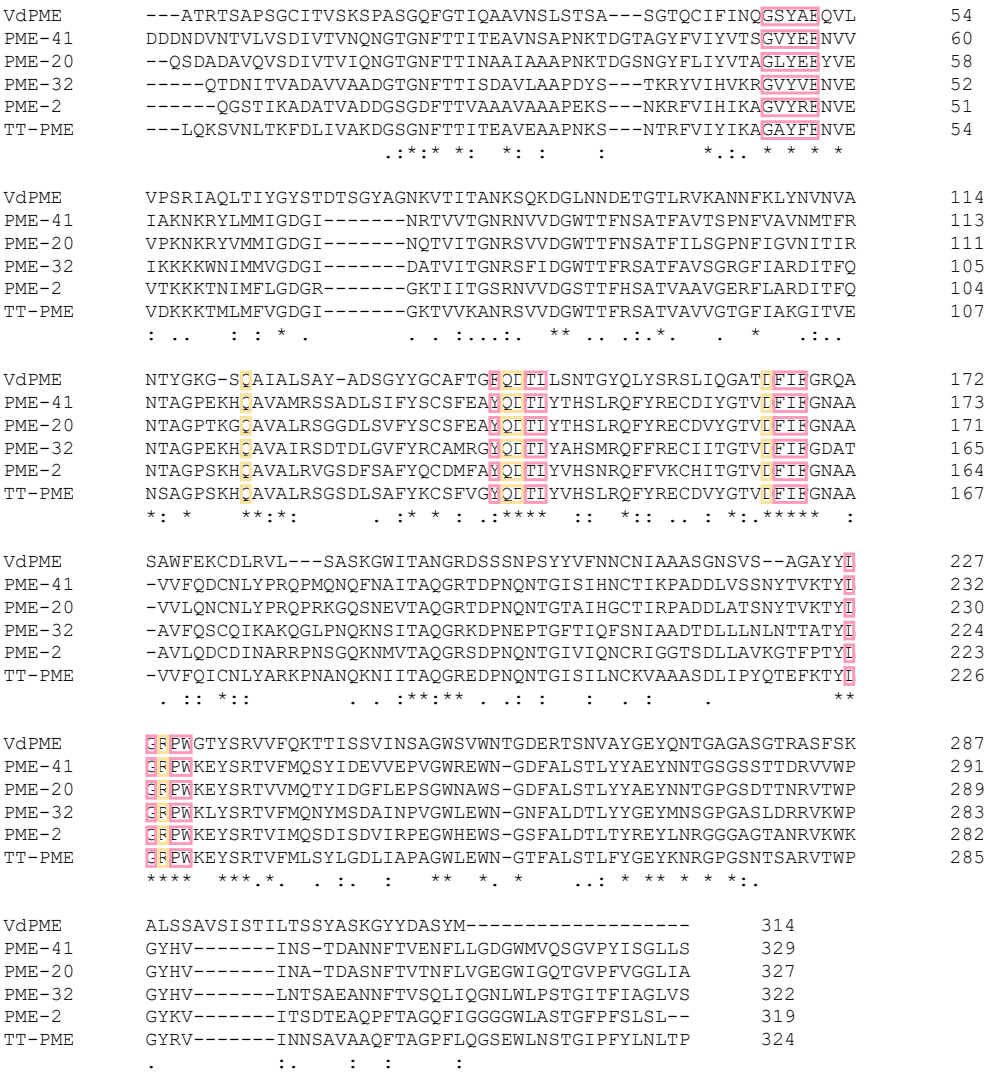

Figure A3. **Multiple sequences alignment of PME-32, PME-2, TT-PME, PME-41, VdPME AND PME-20.** The four highly conserved regions are pink-boxed and the amino acids of the active site are yellow-boxed. The alignment was performed using Clustal Omega.

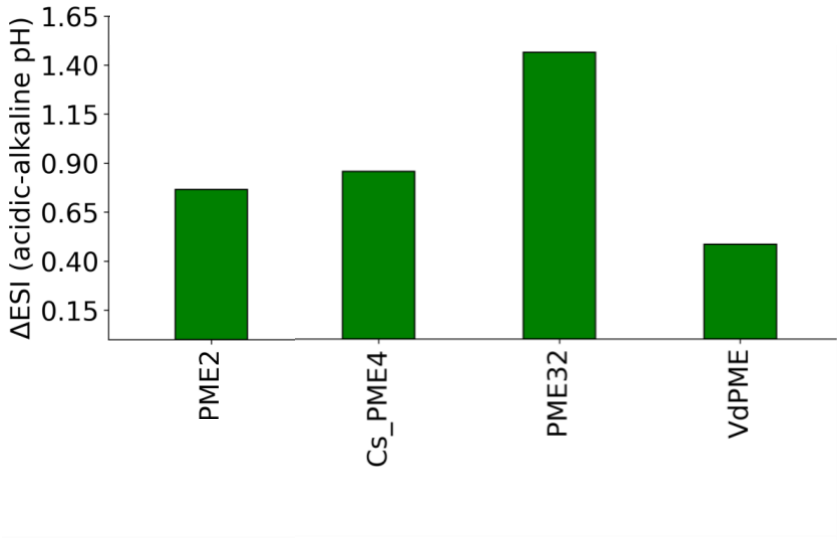

Figure A4. **Difference between the electrostatic similarity indices at pH5 (acidic) and pH8 (basic).** Figure adapted from Davide Mercadante (University of Auckland).

Table A1**. Percent identity matrix.** Quantitative measurement of the identity between PMEs made with Clustal Omega.

%	VdPME	PME-41	PME-20	PME-32	PME-2	TT-PME
VdPME	100.00	31.65	29.29	30.74	29.49	30.87
PME-41	31.65	100.00	75.54	56.07	54.40	60.99
PME-20	29.29	75.54	100.00	52.65	55.03	59.13
PME-32	30.74	56.07	52.65	100.00	58.62	59.01
PME-2	29.49	54.40	55.03	58.62	100.00	62.38
TT-PME	30.87	60.99	59.13	59.01	62.38	100.00

ABSTRACT

Pectins are the most abundant components in plant primary cell wall and the most heterogenous cell wall polysaccharides. The most common domain of pectins is homogalacturonan, built on a GalA backbone which can be methylesterfied and acetylated. The structure of homogalacturonan can be modified during plant development due to action of homogalacturonan modifying enzymes. As such, these enzymes play a key role in the maturation and degradation of homogalacturonans and therefore in the control of cell wall rheology. Pectin methylesterases (PMEs) can hydrolyze methyl groups from GalA residues, generating negatively charged groups and creating substrates for other HG-modifying enzymes that can hydrolyze pectins. As PMEs are encoded by a large multigenic family of 66 genes in Arabidopsis, this represents a major challenge for reverse genetic approaches. As an alternative; we chose exogenous application of recombinant enzyme to study their effects during plant development. During this thesis, five plant (PME-32, PME-2, TT-PME, PME-41 and PME-20) and one fungal (VdPME) PMEs were studied. All proteins were heterogeneously produced in *Pichia pastoris* as active enzymes and their biochemical properties were compared. For three of them (PME-32, PME-20 and VdPME), their mode of action and their roles *in planta* were assessed at acidic and alkaline pH. Mode of action was determined using size exclusion chromatography coupled to mass spectrometry, while their roles *in planta* were assessed through exogenous application of the enzymes and analysis of the phenotypical consequences on dark-grown *Arabidopsis thaliana* seedlings. This overall showed that the mode of action between PME isoforms differ but appears difficult to predict on the basis of sequences. On the other hand, new approach based on electrostatic potential measurements gives promising results that could greatly facilitate understanding the variability within the PME family.

Keywords: *Arabidopsis thaliana*, cell wall, homogalacturonan, oligogalacturonides, pectin, pectin methlyesterase, *Verticillium dahliae*

RÉSUMÉ

Les pectines sont les composants les plus abondants de la paroi végétale primaire et les polysaccharides les plus complexes. Le domaine le plus simple des pectines correspond aux homogalacturonanes, un enchainement linéaire d'acides galacturoniques qui peuvent être méthylés et/ou acétylés. La structure des homogalacturonanes peut être modifiée par l'action d'enzymes, qui vont jouer un rôle clé dans la maturation et la dégradation des pectines, contrôlant ainsi la rhéologie de la paroi. Les pectine méthylestérases (PMEs) peuvent hydrolyser le groupement methyl des résidus GalA, générant des charges négatives et créant des substrats pour d'autres enzymes qui peuvent hydrolyser les pectines. Les PMEs étant codées par une famille multigénique de 66 membres, cela rend difficile les approches de génomique fonctionnelle. Une alternative pour évaluer les effets des PMEs sur le développement des plantes consiste en l'application exogène d'enzymes produites de façon recombinante. Au cours de ce travail, cinq enzymes de plantes (PME-32, PME-2, TT-PME, PME-41 and PME-20), et une de champignon (VdPME) ont été étudiées. Toutes les protéines ont été produites sous forme active dans *Pichia pastoris*, et leurs propriétés biochimiques ont été comparées. Pour trois de ces enzymes (PME-32, PME-20 and VdPME), leurs modes d'action et leur effets in planta ont été déterminés à pH acides et alcalins. Le mode d'action des enzymes a été déterminé par chromatographie d'exclusion stérique couplée à la spectrométrie de masse, tandis que leurs effets sur le développement ont été mesurés grâce à des analyses phénotypiques après application exogène sur des hypocotyles étiolés d'Arabidopsis thaliana. Les résultats obtenus montrent que les modes d'action des PMEs diffèrent, mais il apparait difficile de prédire la processivité sur la base des séquences protéiques. Une nouvelle approche, basée sur l'analyse des potentiels électrostatiques des protéines pourrait permettre de prédire de manière plus fine le mode d'action des différentes isoformes au sein de la famille des PMEs.

Keywords: *Arabidopsis thaliana*, paroi végétale, homogalacturonans, oligogalacturonides, pectines, pectin methlyesterase, *Verticillium dahliae*

SAŽETAK

Pektini su najzastupljeniji i najheterogeniji polisaharidi biljne stanične stijenke. Homogalakturonan je najčešća domena pektina i izgrađen je od GalA okosnice koja je metilestrificirana i acetilirana. Struktura homogalakturonana može se modificirati tijekom razvoja biljaka djelovanjem homogalakturonan-modificirajućih enzima. Kao takvi, ti enzimi igraju ključnu ulogu u razgradnji homogalakturonana te posljedično kontroliraju reološka svojstva stanične stjenke. Pektin metilesteraze (PME) hidroliziraju metilne skupine homogalakturonana, ostavljajući za sobom negativno nabijene skupine koje postaju supstrati za druge HG-modificirajuće enzime, koji mogu hidrolizirati okosnicu homogalakturonana. Kako su PME kodirani velikom multigeničnom obitelji od 66 gena u *Arabidopsis thaliani*, to predstavlja veliki izazov za primjenu pristupa reverzine genetike. Kao alternativa; odabrali smo egzogenu primjenu rekombinantnog enzima kako bismo proučili njegove učinke na razvoj biljaka. Tijekom ovog rada proučeno je pet biljnih (PME-32, PME-2, TT-PME, PME-41 i PME-20) i jedan PME porijeklom iz *Verticilium dahliae* (VdPME). Svi proteini su heterogeno proizvedeni u *Pichii pastoris* kao aktivni enzimi te su uspoređena njihova biokemijska svojstva. Za tri od njih (PME-32, PME-20 i VdPME), određen je način djelovanja i njihova uloga u biljci pri kiselom i alkalnom pH. Način djelovanja određen je pomoću kromatografije (size exclusion chromatography) povezane sa spektrometrijom masa, dok je njihova uloga u biljci procijenjena egzogenom primjenom enzima i fenotipskom analizom hipokotila *Arabidopsis thaliane*. Pokazno je da se način djelovanja između izoformi PME razlikuje te je teško predvidljiv na temelju sekvenci. S druge strane, novi pristup temeljen na mjerenjima elektrostatičkog potencijala daje obećavajuće rezultate koji bi mogli uvelike olakšati razumijevanje varijabilnosti unutar PME obitelji.

Ključne riječi: *Arabidopsis thaliana*, stanična stijenka, homogalakturonan, oligogalakturonidi, pektin, pektin metilesteraze, *Verticillium dahliae*