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INTERNSHIP REPORT

Impact of polygalacturonases on cell wall remodeling and plant development

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IMPACT OF POLYGALACTURONASES ON CELL WALL REMODELING AND PLANT DEVELOPMENT

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Abstract: Pectins are major components of the plant primary cell wall and the most heterogeneous cell wall polysaccharides. They comprise several domains, the most abundant being homogalacturonan (HG), built on a simple Gal A backbone which can be methyl esterified and acetylated. HG constant remodeling is mediated through the action of HG-modifying enzymes that are acting during plant development. As such, these enzymes play a key role in the maturation and degradation of HG, therefore controlling cell wall rheology. Polygalacturonases (PGs) are an essential class of HG-modifying enzymes as they degrade HG chains and produce oligogalacturonides (OGs) of various size and structure. As PG are encoded by a large multigenic family of 69 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 role during plant development. During this thesis, two PGs were studied, AtPGAZAT and AtPGLR, which genes are both expressed in roots. Both proteins were heterogously produced in *Pichia pastoris* as active enzymes and their biochemical properties and mode of action were compared. Their roles *in planta* was assessed through exogenous application of the enzymes and analysis of the phenotypical consequences on light- and dark-grown *Arabidopsis thaliana* seedlings. On the basis of the results, the main differences between these endo-PGs lie in their substrate specificity, as AtPGAZAT shows higher activity on highly methyl esterified pectins compared to AtPGLR. Furthermore, size exclusion chromatography coupled to mass spectrometry revealed difference in their mode of actions. Indeed, OG profiles obtained after hydrolysis of hypocotyl cell wall by AtPGAZAT generates higher abundance of methyl esterified, while AtPGLR generates acetyl esterified OGs. These subtle distinctions in their mode of action could be connected to differences in observed phenotypes after exogenous application on light grown plants and dark-grown hypocotyls.

Keywords: *Arabidopsis thaliana*, cell wall, homogalacturonan, oligogalacturonides, pectins, polygalacturonase

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Contents

PRESENTATION OF THE LAB AND LOCALISATION OF THE INTERNSHIP 1
Abbreviations 2
1. INTRODUCTION 3
2. BIBLIOGRAPHIC STUDY AND SUBJECT 3
2.1. PLANT CELL WALLS 3
2.2. PECTINS 5
2.3. HG-MODIFYING ENZYMES 7
2.4. POLYGALACTURONASES 8
2.5. OBJECTIVES 9
3. MATERIALS AND METHODS 12
3.1. RECOMBINANT PROTEIN PRODUCTION AND CHARACTERIZATION 12
3.1.1. Production of recombinant proteins 12
3.1.2. Purification of recombinant proteins 12
3.1.3. SDS-PAGE 12
3.1.4. De-glycosylation of AtPGAZAT 13
3.1.5. Western blot 13
3.1.6. Concentrating and buffer exchange 14
3.1.7. Determination of protein concentration 14
3.2. ENZYMATIC ACTIVITY 14
3.2.1. Activity assay 14
3.2.2. Determination of kinetic parameters for AtPGAZAT 14
3.2.3. Determination of optimal pH for AtPGAZAT 15
3.2.4. Sample preparation for oligoprofiling by mass spectrometry 15
3.3. PLANT MATERIAL 16
3.3.1. Seed sterilization and stratification 16
3.3.2. Growth conditions 16
3.3.3. Seedling analysis 16
4. RESULTS 17
4.1. PRODUCTION AND PURIFICATION OF AtPGAZAT AND AtPGLR 17
4.2. DE-GLYCOSYLATION OF AtPGAZAT 17
4.3. BIOCHEMICAL CHARACTERIZATION OF AtPGAZAT 18
4.3.1. Substrate and pH specificities of AtPGAZAT 18
4.3.2. Determination of kinetic parameters 19
4.3.3. Released OGs after application of AtPGAZAT on hypocotyl cell walls 20

4.4. EXOGENOUS APPLICATION OF AtPGAZAT AND AtPGLR AND EFFECT ON THE DEVELOPMENT OF PLANT 20
4.4.1. Effect of AtPGAZAT on dark-grown hypocotyl 20
4.4.2. Screening of EMS collection of mutants 21
4.4.3. Effect of AtPGAZAT and AtPGLR on light grown plants 22
5. DISCUSSION AND CONCLUSIONS 24
5.1. DISCUSSION 24
5.2. CONCLUSIONS AND PERSPECTIVES 27
6. REFERENCES 29
7. ANNEXE 33
Abstract 34
Résumé 35

PRESENTATION OF THE LAB AND LOCALISATION OF THE INTERNSHIP

The "Plant Biology & Innovation" research unit BIOPI EA3900 is part of the University Picardie Jules Verne based in Amiens, France. The research activities of the lab are focused on the roles of cell wall and phenylpropanoid modifications during plant development. Three main research topics, are developed in three teams. The research of Team 1 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 plant 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. During the course on my internship, I was part of the Team 1, so called „Pectin Dynamic“, led by Professor Jérôme Pelloux. 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).

Amiens, a historic city located north of Paris, is the capital of the Somme department in Hauts-de-France. Its most famous feature, the cathedral of Notre-Dame is the largest gothic church in France and a World Heritage Site declared by UNESCO. The famous author Jules Verne lived in Amiens and his house, turned into a museum, is another landmark of Amiens. Another notability are the Hortillonages, also known as „floating gardens of Amiens“, which are gardens on small islands surrounded by a network of river Somme and man-made canals.

Abbreviations

BMGY	Buffered glycerol complex medium
BMMY	Buffered methanol complex medium
DA	degree of acetylation
DM	degree of methyl esterification
DNS	3,5-dinitrosalicylic acid
DP	degree of polymerization
Gal A	galacturonic acid
HG	homogalacturonan
HGME	homogalacturonan-modifying enzymes
LC	liquid chromatography
MS	mass spectrometry
NaP	sodium phosphate
OG	oligogalacturonide
PAE	pectin acetylesterase
PG	polygalacturonase
PGA	polygalacturonic acid
PL	pectate lyase
PLL	pectate lyases-like
PME	pectin methylesterase
PMEI	pectin methylesterase inhibitor
PNL	pectin lyase
RG I	rhamnogalacturonan I
RG II	rhamnogalacturonan II
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TEMED	tetramethylethylenediamine
XyG	xyloglucan

1. INTRODUCTION

The plant cell wall is a dynamic structure which has important roles in modulating plants growth and development as well as their interactions with the environment. In dicotyledonous species, such as the model plant of this study, *Arabidopsis thaliana*, the primary cell wall consists of a hydrogen-bonded network of cellulose microfibrils and xyloglucans (XyGs) embedded in a complex pectic and protein matrix (Carpita and Gibeaut, 1993). The synthesis, differentiation, maturation and degradation of the cell wall are essential for development of the plant. Polygalacturonases (PG), which function in the last phase of pectin degradation in the cell wall, are a crucial class of pectin-modifying enzymes and are the main focus of this thesis.

2. BIBLIOGRAPHIC STUDY AND SUBJECT

2.1. PLANT CELL WALLS

A cell wall is a rigid, semi-permeable, complex protective structure that surrounds plant cells. The cell wall determines cell shape and has variety of functions throughout the plant lifecycle. It helps maintaining structural integrity by resisting to turgor pressure, but also provides flexibility to support cell division and has a key role in intercellular communication, water movement and defence. In addition to their physical/mechanical roles in architecture and protection, walls or selected wall components have chemical roles as ion exchangers, as bacterial agglutinins, and as sources of messages (the oligosaccharins) (Varner and Lin, 1989). Plant cell wall structure/composition must be finely regulated during cell expansion/differentiation which can determine the changes in cell shape and size that occur during plant growth and development (Cosgrove, 2005). The wall is a metabolically active compartment that is continually modified to face the various developmental stages of the plant as well as the environmental conditions (Farrokhi *et al.*, 2005). The interlacing molecules are cleaved by enzymes to loosen the cell wall and new microfibrils and polymers are deposited on the inner side of the wall (Cosgrove, 2005).

In the plant cell wall, up to three distinct layers can be found (***Figure 1 A***): the middle lamella, the primary cell wall, and in some cells the secondary cell wall. The middle lamella is an outer layer rich in pectins which helps cells to bind to one another (Varner and Lin, 1989). Primary cell wall, a thin and flexible layer which is formed while the cell is growing, is composed of carbohydrates such as cellulose, hemicellulose and pectin, as well as proteins, which can

contribute up to 5-10% of Arabidopsis wall's dry mass (Jamet et al., 2008) (***Figure 1 B***). In addition to the structural proteins, primary cell walls contain many enzymes (Varner and Lin, 1989; Rose and Lee, 2010). The secondary cell wall contains less pectins and more cellulose and is formed in mature, fully grown cells. It consists of a thick and rigid layer reinforced with lignin (Endler and Persson, 2011), which straightens and supports the cell.

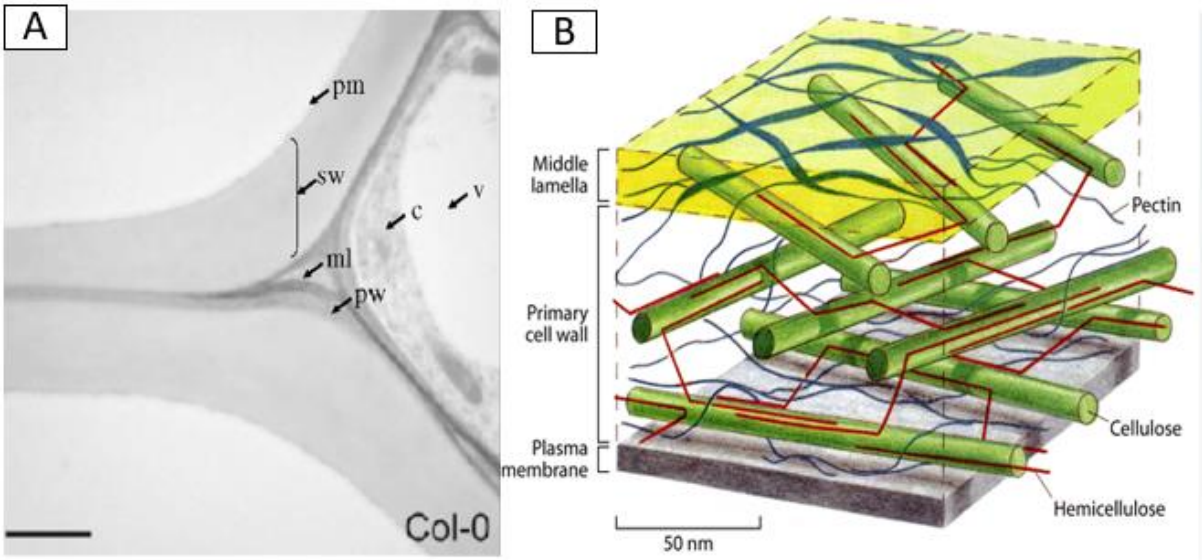

Figure 1.(A) The cell walls of Arabidopsis thaliana. Transmission electron micrograph of WT Arabidopsis thaliana Columbia-0 transverse root section showing the middle lamella (ml), primary wall (pw), and secondary wall (sw) of the metaxylem. Additional labeled features of the cell are the plasma membrane (pm), cytosol (c) and vacuole (v). Scale bar 2 µm. Adapted from Persson et al. (2007). (B) Simplified representation of polymers in primary cell wall. Scale bar represents 50 nm. Adapted from Scheller and Ulvskov (2010).

The most characteristic component found in plant primary cell walls is cellulose. It consists of polymer of β-1,4-linked glucan chains that interact with each other via hydrogen bonds to form condensed microfibrils (Somerville, 2006). In addition to cellulose, primary cell walls contain several matrix polysaccharides that are grouped into two general categories: (1) the pectic polysaccharides including homogalacturonans (HGs), and rhamnogalacturonans I and II (RG I, RG II) (Harholt et al., 2010) and (2) the hemicellulosic polysaccharides including xyloglucans, glucomannans, xylans, and mixed-linkage glucans (Scheller and Ulvskov, 2010). Hemicelluloses are usually characterized by having a β-1,4-linked backbone of mannose, glucose, or xylose (Malinovsky *et al*., 2014). Their central role is to strengthen the cell wall by interacting with cellulose (Scheller and Ulvskov, 2010; Endler and Persson, 2011). Although most primary cell walls are composed of these three main polymers, they can considerably differ in their relative abundance, their cross-linking, the abundance of proteins and phenolic compounds as well as their three dimensional architectures (Vogel, 2008; Malinovsky et al., 2014). Two types of primary cell walls have been described: Type 1, present in *Dicotyledonae*,

such as our model *Arabidopsis thaliana*, and some *Monocotyledonae*, and type 2, which can be found in *Poaceae* and closely related monocotyledonous families (Carpita and Gibeaut, 1993). In type 1 primary wall, non-cellulosic polysaccharides mainly consist of xyloglucans that interlock the cellulosic framework (Carpita and Gibeaut, 1993). The xyloglucans are linear chains of 1,4-β-D-glucan substituted with xylosyl units in O-6 position. This cellulose-xyloglucan framework makes about 50% of the wall mass and is embedded in a matrix of pectic polysaccharides which represents about 30% of the total mass (Carpita et al., 1993). In type 2 primary cell wall the principal polymers that interlock cellulose microfibrils in dividing cells are glucuronoarabinoxylans, linear chains of β-1,4-D-xylose with single arabinose or glucosyluronic acid (GlcA) units substitutions on the backbone (Carpita and Gibeaut, 1993). Those side groups not only prevent hydrogen bonding between two unbranched xylan chains or xylan to cellulose, but also allow water-solubility of glucuronoarabinoxylans. Unlike type I, type II walls are poor in pectins, with only 2-10%, (Voragen *et al*. 2009) and structural proteins.

2.2. PECTINS

Pectins, which are the most complex and heterogeneous cell wall polysaccharides, are predominantly present in the primary cell wall and in the middle lamella where they have roles in expansion, strengthening, porosity, cell-to-cell adhesion, and intercellular signalling (Houston *et al*., 2016). Among other functions they provide charged surfaces that modulate wall pH and ion balance, and are involved in signalling during developmental responses to symbiotic organisms, pathogens, and insects (McNeil *et al*., 1984). Furthermore, pectins form a dense aqueous wall matrix and connect cell wall polymers around and between cells (Somssich *et al*. 2016). Pectin synthesis, constant remodeling and degradation influence tissue elongation, pollen development, fruit ripening and organ abscission (Yang *et al*., 2018). Synthesis of pectins occurs in the Golgi apparatus, where they are packed into vesicles and delivered to the wall. The two fundamental pectic backbones of all flowering plant pectins are homogalacturonan (HG), which is a homopolymer of α-1,4-D-galacturonic acids (GalA), and rhamnogalacturonan I (RG I), which is a heteropolymer of repeating α-1,2-L-rhamnosyl- α-1,4 -D-GalA disaccharide units (Jarvis, 1984) ***(Figure 2***).

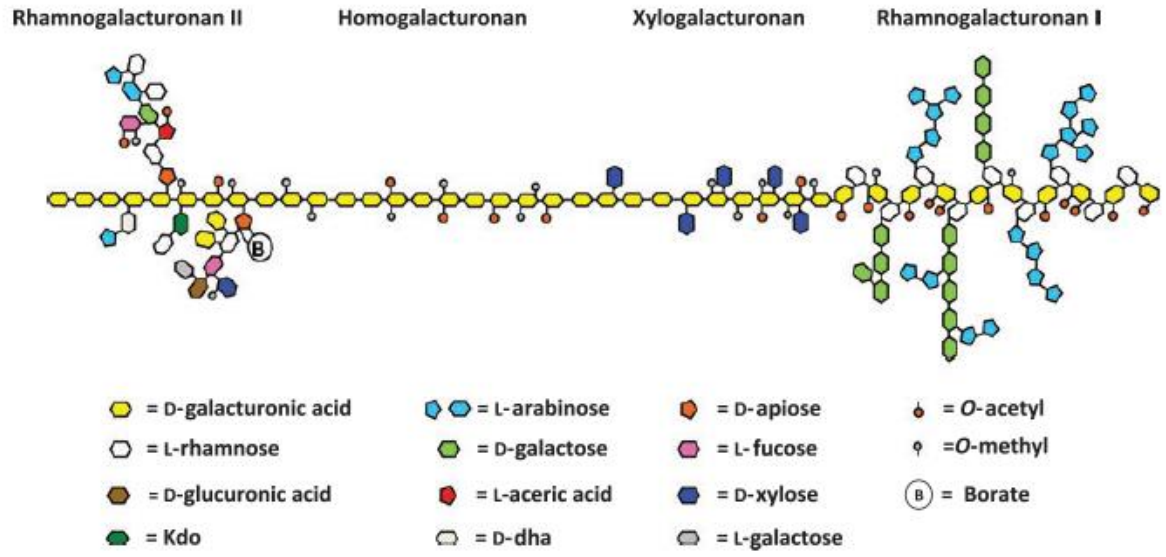

Figure 2. Schematic representation of pectin (Scheller et al., 2007)

RG-I can be substituted with galactan, arabinnan and arabinogalactan side chains. On the other hand, RG-II polymer, based on an HG backbone, can form highly complex and diverse polymers and has the richest diversity of linked sugars as it consists of at least 12 different monosaccharides in more than 20 different linkages (Scheller *et al.*, 2007). The sugar substitutions and the side chains on the backbones influence solubility, viscosity and interactions with other components of the cell wall. The main focus of research at BIOPI is homogalacturonan which can make up to 60% of pectins in the plant cell wall thus representing the most abundant pectic polysaccharide (Ridley *et al.*, 2001). It is often methyl esterified on C6 and can be acetylated at O2 and/or O3 positions of the galacturonic acid residues. It is secreted into the cell wall in highly methyl esterified form where its structure can be modified by enzymes present in the cell wall (Sterling *et al.*, 2001). The extent and pattern of methyl esterification of HG affects the characteristics of the polysaccharide (Wolf *et al.*, 2009). Growing cells usually synthesize homogalacturonan in which ~75% of the carboxyl groups are methyl esterified. This modification removes the negative charge of the carboxylate ion and blocks its ability to undergo Ca^{2+} crosslinking (Crosgrove, 2005) and the formation of so called egg-box structure that contributes to cell wall strength by complexing-methyl esterified HG chains into a tightly packed formation (Willats *et al.*, 2001; Caffall and Mohnen, 2009). Alternatively, low-methylesterified HG domains can become a target for pectin-degrading enzymes. Overall, the degree of methyl esterification (DM) of HG can affect cell wall rheology as, in vitro, low stiffness is correlated to the highest DM. The degree of polymerization (DP) of homogalacturonan is another important factor involved in the control of cell wall rheology.

Although less investigated, the degree of acetylation (DA) also modulates the properties of pectin molecules and contributes to the structural complexity of the pectin network. De-acetylation of pectin increases its solubility in water and makes it more accessible to pectin-degrading enzymes (Vercauteren *et al*., 2002). DA of pectin also changes during growth and differentiation of plant tissues and in response to the environmental conditions (Liners *et al*., 1994; Gou *et al*., 2008; Gou *et al*., 2012).

2.3. HG-MODIFYING ENZYMES

HG-modifying enzymes (HGME) are synthesized in the endoplasmic reticulum, post-translationally modified in the Golgi apparatus and then secreted in the cell wall where they act. They all belong to large multigenic families in all species sequenced to date (Sénéchal *et al*., 2014). As previously shown, HG structure is determined by its degree of methyl/acetyl esterification (DM/DA), as well as its degree of polymerisation (DP). After synthesis, DM and DA are controlled by pectin methylesterases (PMEs, under regulation of endogenous PMEIs (pectin methylesterase inhibitors (PMEIs)) (Pelloux *et al*., 2007; Wolf *et al*., 2009) and pectin acetylesterases (PAEs) whose activity is increased when the substrate has previously been de-methyl esterified (Williamson, 1991; Bordenave *et al*., 1995; Oosterveld *et al*., 2000). Other enzymes act in the degradation of the polymers, controlling their DP, such as polygalacturonases (PGs) and pectate lyases-like (PLLs), that include pectate lyases (PLs) and pectin lyases (PNLs) (***Figure 3***).

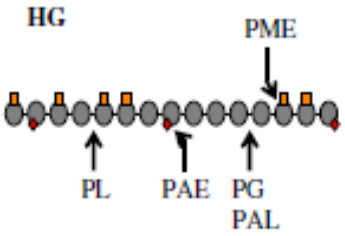

Figure 3. Main pectin degrading enzymes and their mode of action (HG-homogalacturonan; PL-pectin lyase; PAE-pectin acetylesterase; PG-polygalacturonase; PAL-pectate lyase; PME- pectin methylesterase). (Bonnin et al., 2014)

PMEs and PAEs modify HG locally by removing methyl and acetyl ester groups from Gal-A residues, respectively. Together, they could have a central role in the first step of wall degradation by creating substrates for pectin-degrading enzymes: randomly de-methyl esterified and de-acetylated HGs are indeed primary targets for PG and pectin lyases (PNLs). PLLs cleave the α-1,4- linkage of methyl esterified or non-methyl esterified D-Gal A units from HG backbone by β-elimination, thus forming unsaturated bonds, while PGs cleave the α-

1,4-D-Gal A linkage by hydrolysis (Brummell and Harpster, 2001). Their action leads to release of oligogalacturonides (OGs), cell wall loosening and rapid growth (Sénéchal *et al*., 2014). Released OGs can have a signalling function: They can act as potent defence response elicitors (Galletti *et al*., 2009) or as a hormone-like compound counteracting the effects of auxin during plant development (Ridley *et al*., 2001). It has been shown that auxin regulates the expression of several *PME*, *PAE*, *PG* and *PLL* genes during different developmental events such as lateral root emergence (Vanneste *et al*., 2005; Laskowski *et al*., 2006; Swarup *et al*., 2008). It is likely that other phytohormones are also directly or indirectly involved in control of *HGME* gene expression.

2.4. POLYGALACTURONASES

During cell expansion and separation, pectin is degraded mainly in the primary cell wall and middle lamella which decreases cell wall stiffness and increases wall fluidity (Yang *et al*., 2018). Based on differences in HG-hydrolysing activity, PGs can be divided into two main types: exo-PGs and endo-PGs (Yang *et al*., 2018) (***Figure 4***) and their activity in general positively correlated with the decrease in degree of methyl esterification of HG (Verlen *et al*., 2005). Endo- PGs catalyse the hydrolytic cleavage of α-1,4- linkage between at least two de-methyl esterified Gal A residues of the HG backbone with a random action pattern, leading to the formation of OGs with various DP, DM and DA (Sénéchal *et al*., 2014). Exo-PGs bind to the non-reducing ends of pectins due to a difference in the active site (Abbott and Boraston, 2007) and remove galacturonic acid residues one by one.

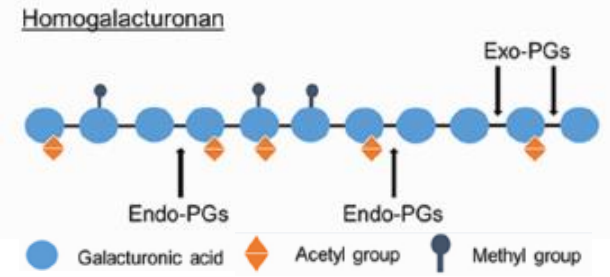

Figure 4. Modes of action of PGs (adapted from Yang et al., 2018).

As previously mentioned, prior action of PME is necessary to enable the degradation of HGs by PGs, as they were described to be more active on low-esterified segments of HG chain. PG degradation of HG backbones will lead to reduction of HG-mediated cell adhesion that is maintained by Ca^{2+} cross-linking (Yang *et al*., 2018). Interestingly, for the same DM, the pattern of de-methyl esterification can affect the action of plant PGs (Verlent *et al*., 2005).

In the model plant *A. thaliana* 69 genes were annotated, by comparison of their sequence with previously characterized fungal enzymes, as putative PG. The transcripts of these *Arabidopsis* PGs accumulate in roots, leaves, pollen tubes, flowers, and siliques throughout the development of the plant (Torki *et al*., 1999). PGs are involved in numerous developmental processes like vegetative growth, pollen development (Pressey and Reger, 1989), root emergence, organ abscission (Kumpf *et al*., 2013; González-Carranza *et al*., 2007), anther and fruit dehiscence (Meain and Roberts, 1991), leaf shedding and fruit maturation (Babu and Bayer, 2014). If both endo- and exo-PGs appear to be involved in cell elongation and cell separation events, their enzymatic activity doesn't directly correlate with one or another process, a combination of both activities seems to be necessary in both events (Babu and Bayer, 2014). Their activity has also been detected in rapidly growing tissues, indicating that it may be involved in cell expansion (Hadfield and Bennett, 1998). Furthermore, the endo-PG POLYGALACTURONASE INVOLVED IN EXPANSION1 (PGX1) was shown to regulate hypocotyl elongation, floral patterning (Xiao *et al*. 2014) while PGX2 regulates leaf expansion, stem lignification, mechanical stiffening and lodging (Xiao *et al*., 2017). This show that the diversity of PG sequences, and their potential biochemical specificities, might be of importance for the regulation of dedicated developmental processes.

The action of PGs may release OGs that can inhibit auxin-related responses, establishing a negative feedback loop on PG gene expression (Ferrari *et al*., 2013). As PGs are usually encoded by multigene families, the function of given isoforms in the regulation of cell wall dynamics and development as well as the regulatory pathways is still poorly understood.

2.5. OBJECTIVES

Considering the extent of the PG gene family, enzymes may differ in their biochemical properties, such as mode of hydrolysis or substrate specificity (Hadfield and Bennett, 1998). The existence of multiple proteins to carry out similar functions in each developmental step provides the basis for complex regulation of gene expression by a number of developmental and environmental signals. For many Arabidopsis PG genes, the lack of an obvious developmental phenotype in loss-of-function mutants might be the result of genetic redundancy (Hadfield and Bennett, 1998). To overcome this compensation effect and the challenge of studying mutants, we decided to use purified enzymes and to apply them exogenously on *Arabidopsis thaliana* in order to observe their effect on the remodeling of cell wall in the context of plant development. This strategy will allow determining more direct effect of PGs on the cell wall, their mode of action and their role in the fine-tuning of plant development.

Plant PGs were applied on two developmental models: plantlets developed under long day conditions and dark-grown hypocotyls of *Arabidopsis thaliana*, ecotype Columbia (Col-0). Etiolated hypocotyl has a simple anatomy, and elongates in the absence of cell division from 10 µm up to 1 mm length (Gendreau *et al*., 1997). It is therefore used as a system to analyse the consequences of cell wall modulation on cell elongation. The relationship between the DM of HG in primary cell wall and hypocotyl elongation has been shown to be of importance (Wolf *et al*., 2009). Changes in the pectic network also affect the development of light-grown plantlets, as it has been demonstrated that HG methyl esterification affects root growth (Hewezi et al., 2008) which is related to cell wall relaxation in meristems (Levesque-Tremblay *et al*., 2015) or changes in cell size in the root expansion zone (Lionetti *et al*., 2007).

Two plant polygalacturonases were chosen for this project, PG LATERAL ROOT AtPGLR (At5g14650) which is one of the key enzymes studied at BIOPI and PG ABSCISSION ZONE ARABIDOPSIS THALIANA AtPGAZAT (At2g41850), a recombinant protein which was expressed and characterized during the course of my internship. These enzymes belong to different phylogenetic clusters which may be indicative of distinct roles during plant development (***Figure 16***-annex) (Kim *et al*., 2006).

The two enzymes show a different expression pattern as revealed by transcriptomic analyses and activity of the promoters. While AtPGLR shows the highest expression in roots, AtPGAZAT has increased expression in dark-grown seedlings and receptacle (***Figure 5 A***). On the other hand, Kumpf *et al*. demonstrated that promoter of both genes were active in the same tissues and developmental processes: above lateral root emergence sites, and in the abscission zone of flowers (***Figure 5 B-E***, Kumpf *et al*., 2013). AtPGAZAT is assumed to be involved in floral organ abscission, fruit and anther dehiscence (Ogawa *et al*., 2009) while AtPGLR could have a function in cell expansion (Zhang *et al*. 2007), cell wall organization and lateral root emergence (Kumpf *et al*., 2013).

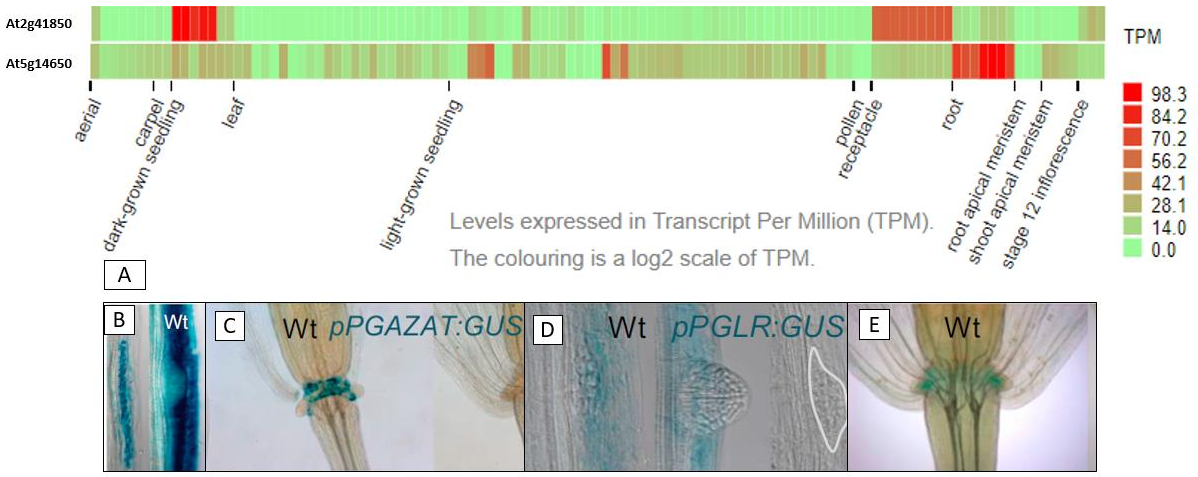

Figure 5. Levels of expression of AtPGAZAT (At2g41850) and AtPGLR (At5g14650) in plant organs (A). (public data, araport.org). Promoter's activity of AtPGAZAT (B, C) and AtPGLR (D, E) in sites of lateral root emergence and in floral organ abscission zones in WT Arabidopsis thaliana Col-0 obtained by GUS staining of promoter-GUS fusion lines (adapted from Kumpf et al., 2013).

Previous study in the laboratory (Maša Boras, Master thesis; Wafae Tabi, PhD) allowed the biochemical characterisation of AtPGLR. Its exogenous application has been tested on dark-grown hypocotyls, causing cell detachment as well as reduced hypocotyl elongation (***Figure 6***). Focus of this internship was the study of AtPGAZAT in comparison with AtPGLR. Indeed, as the two genes are expressed in the same tissues, we might hypothesize that both enzymes may be involved in the same developmental and physiological processes. AtPGAZAT was expressed in *P. pastoris*, purified and its activity characterized *in vitro* and compared to AtPGLR. The consequences of the exogenous application of AtPGAZAT on organ growth and cell adhesion, was assessed on dark-grown and light-grown seedlings, and compared to that of AtPGLR.

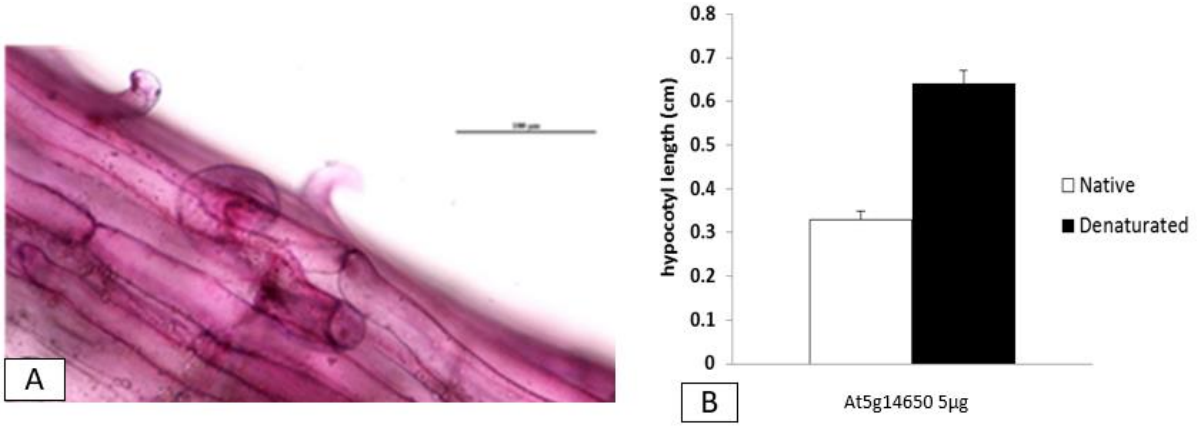

Figure 6. Cell detachment caused by AtPGLR (A). Scale bar 100 µm. Effect of 5 µg of AtPGLR on hypocotyl length (B). Maša Boras, Wafae Tabi

3. MATERIALS AND METHODS

3.1. RECOMBINANT PROTEIN PRODUCTION AND CHARACTERIZATION

3.1.1. Production of recombinant proteins

Production of recombinant AtPGLR (At5g14650) and AtPGAZAT (At2g41850) was conducted in heterologous system *Pichia pastoris* after cloning of the protein-coding genes in methanol-inducible expression vector pPICZαB (Invitrogen). Under sterile conditions a single colony of transformed yeast strain X-33 containing a gene for recombinant protein was placed in 125 mL Erlenmeyer flask containing 12,5 mL of 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 one night of culture (250 rpm/30°C) 100 mL of culture 1 U OD_{600nm}/mL was induced in BMMY 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, 0.5% (v/v) methanol). Culture was agitated at 250 rpm/30°C. 24 h and 48 h after, production of recombinant proteins was subsequently induced by the addition of sterile methanol in final concentration of 0.5% (v/v). After 72 h, supernatant, containing the protein of interest, was harvested in 50 mL falcons after pelleting of yeast cells using centrifugation at 1500 g for 10 minutes at 4°C. Supernatants were stored at 4°C until purification was performed.

3.1.2. Purification of recombinant proteins

Since recombinant proteins were constructed with 6xHis tag in the C-terminus, protein purification was conducted using immobilized metal ion affinity chromatography (IMAC). The peristaltic pump was set to 1 mL/min flow rate and the 1 mL column (Histrap^{TM} GE Healthcare) was attached to the pump. Before purification, supernatant was filtered using sterile GD/XPES filter 0.45 μm pore size. The column was first rinsed with 10 mL of MilliQ water and then 100 mL of supernatant was loaded. Column was then washed with 50 mM NaP, 250 mM NaCl, 25 mM Imidazole pH 7.5 and proteins attached to the column were eluted with 50 mM NaP, 250 mM NaCl, 500 mM Imidazole pH 7.5. Column outlets were kept in 15 mL falcons positioned in the ice. After the elution the column was washed with 10 mL of MilliQ water followed by 10 mL of 20% (v/v) ethanol and stored at 4°C.

3.1.3. SDS-PAGE

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, pH 6.8, 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 to assess the quality of the purification. 15µL of sample was mixed with 3.5 µL 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 95°C. Samples were loaded on the gel and electrophoresis (Bio-Rad PowerPac Basic Mini Electrophoresis System) was conducted in 1x running 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 overnight on a shaker.

3.1.4. De-glycosylation of AtPGAZAT

1 µL of purified AtPGAZAT (1.33 µg/µL) was subjected to de-glycosylation in denatured conditions. De-glycosylation was performed using PNGase F (New England BioLabs, 500 000 U/mL) according to the supplier's instructions. Reaction mix without PNGase F was used as a control. Samples were further subjected to SDS-PAGE and Western blot analysis.

3.1.5. Western blot

Western blot analysis was conducted in order to check for the presence of 6xHis tag on the protein. After the SDS page, 1.5 mm gel was placed in the cathode buffer (25 mM TRIS pH 9.4, 40 mM glycine, 10% (v/v) ethanol) for 15 minutes. During that time PVDF (polyvinylidene difluoride) membrane was placed for 15 seconds in 100% ethanol, 2 minutes in distilled water and 5 minutes in anode 2 buffer (25 mM TRIS pH 10.4, 10% (v/v) ethanol). Semi dry system (Trans-blot Turbo, BIO-RAD) with layers of seven Whatman 3M papers soaked in cathode buffer (4x), anode 2 buffer (1x) and anode 1 buffer (2x) (3000 mM TRIS pH 10.4, 10% (v/v) ethanol) was used for transfer of proteins from the gel onto the PVDF membrane. Transfer was conducted at 60 mA for 30 minutes. The membrane was then placed in 96% ethanol for 1 minute and then dried and stored. For immunodetection, membrane was incubated in 4% (w/v) milk solution for 30 minutes on shaker and then hybridized with monoclonal anti-His antibody conjugated with horse radish peroxidase (Sigma A7058) for 1 hour (0.5% in 0.025%(w/v) milk solution). Membrane was then washed 2x for 15 minutes in 1X TBS Tween 20 (5 mM Tris, 15 mM NaCl, pH 7.6), then 5 minutes in 1X TBS Tween 20 and 5 minutes in distilled water. Detection of protein of interest was performed covering the membrane with 1X DAB substrate (300 µL 10X DAB, 2.7 mL 1X peroxidase solution buffer (DAB substrate kit, Thermo Scientific)).

3.1.6. Concentrating and buffer exchange

Amicon Ultra-4 Centrifugal filters Ultracel 10 kDa were used to concentrate the enzymes obtained in the eluates of purification. After rinsing of the column with water, concentration of samples was achieved by repetitive loading and centrifugation (7500g, 15 min at 4°C) until final volume of about 150 µL of the sample was obtained. The buffer exchange to 50 mM sodium acetate pH 5.2 was performed using PD SpinTrap^{TM} G25 column (GE Healthcare) according to manufacturer's instructions.

3.1.7. Determination of protein concentration

Concentration of enzymes was determined by Bradford method (Bradford, 1976) in 96-well plate. Solutions of 0, 1, 2, 3, 5 µg/mL of bovine serum albumin (BSA) in 50 mM sodium acetate pH 5.2 in the volume of 100 µL were used as standards. Measures of absorbance at 595 nm were performed on microplate reader.

3.2. ENZYMATIC ACTIVITY

3.2.1. Activity assay

Activity of enzymes was determined by quantifying reduced sugars' concentration using 3,5-dinitrosalicylic acid (DNS) colorimetric method. It is based on a redox reaction between the 3,5- dinitrosalicyclic acid and the reducing sugars present in the sample. During the reaction yellow DNS is reduced to red 3-amino-5-nitrosalicylic acid which can be quantified by spectrophotometry at 550 nm. To form a standard curve, solutions of 0, 1, 2, 5, 10 and 20 nmol/µL of galacturonic acid (Sigma 48280) in 50 mM sodium acetate pH 5.2 were used. 4 µg of enzyme was incubated in the presence of polygalacturonic acid at final concentration of 0.4% (w/v) (Sigma P-81325) in the appropriate buffer. Reaction was performed at 50°C for 60 minutes before addition of 150 µL of DNS reagent (NaOH 1% (w/v), DNS 1% (w/v), potassium sodium tartarate tetrahydrat 30% (w/v), sodium sulfite 0.05% (w/v)) was added and incubated at 95°C for 8 minutes and 4°C for 10 minutes. Aliquots of 100 µL were placed in 96-well plate and absorbance was measured at 550 nm. Activity was expressed as nmol of galacturonic acid produced per µg of enzyme per minute.

3.2.2. Determination of kinetic parameters for AtPGAZAT

In order to choose optimal parameters for determination of kinetic constants (Vmax and Km), enzymatic reactions were performed when 0.025, 0.011 or 0.0055 µg/µL of enzyme was incubated at 25°C for 5, 10, 15, 20, 45 and 60 minutes and product formation (Gal A) was quantified using DNS method. The activity of the enzyme (expressed as nmol of GalA produced per µg of enzyme) was plotted against time. On the basis of obtained results,

appropriate reaction time and enzyme concentration were chosen, so 0.0148 µg of enzyme was incubated in the presence of 1, 2, 3, 4, 5, 6 ,7 and 8 mg/mL of PGA. Reaction was performed in triplicates at 25°C for 5 minutes. Velocity of the reaction was calculated from the standard curve of Gal A and expressed as nmol of Gal A produced per minute and per µg of enzyme. The Lineweaver-Burk plot of 1/V against 1/[S] was constructed, Vmax was calculated from the y-intercept which corresponds to 1/Vmax and Km was calculated from the x-intercept which corresponds to -1/Km.

3.2.3. Determination of optimal pH for AtPGAZAT

To obtaine solutions with various pH, 50 mM sodium acetate buffer and 80 mM phosphate citrate buffer were prepared, and pH was adjusted with acetic acid (100%) and NaOH 1M respectivly to prepare solutions in pH range from 4 to 7. Reaction mix contained 0.0148 µg of enzyme and 0.4% (w/v) PGA prepared in the appropriate buffer. To form a standard curve, solutions of galacturonic acid in 50 mM sodium acetate pH 5.2 were prepared as described earlier. Duration of DNS reaction was 5 minutes at 25°C, and rate of the reaction was expressed as nmol of Gal A produced per minute and per µg of enzyme.

3.2.4. Sample preparation for oligoprofiling by mass spectrometry

Cell wall extraction was performed by addition of 1 mL of absolute ethanol to 40 etiolated hypocotyls in 1.5 mL Eppendorf tube and samples were incubated overnight at room temperature. Ethanol was removed and samples were incubated in absolute acetone 2 times for 5 minutes and allowed to dry overnight at room temperature. After rehydration (2 h at 40°C with 142 µL of 100 mM amonium acetate pH 5), 10 µg of AtPGAZAT was added and samples were incubated overnight at 40°C. The digestion was stoped by addition of 150 µL of absolute ethanol. Samples were centrifuged for 5 minutes at 5000 g and supernatants containing OGs were harvested and dried in the speedvac (Eppendorf Concentrator Plus). Before the analysis the samples were resuspended by 200 µL of MilliQ water.

Samples were later on subjected to 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 acetylesterification 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 method that detects 110 different oligogalacturonides.

3.3. PLANT MATERIAL

3.3.1. Seed sterilization and stratification

100 µl seeds of *Arabidopsis thaliana* (Col-0) were placed in 1.5 mL Eppendorf tube and sterilized in 950 µL of sterilization solution (70% (v/v) ethanol, 0.05% Triton X-100) under agitation for 15 minutes and briefly vortexed. Sterilization solution was removed and replaced with 950 µL of absolute ethanol and shaked for 15 more minutes. Ethanol was removed and open tubes were left in sterile conditions to dry overnight. The next day 950 µL of sterile water was added and seeds were placed at 4°C for 3 days in the dark conditions for cold-wet stratification. Aternative is to wash the seeds 8 times with sterile water, leave them in 950 µL of sterile water and place them for cold-wet stratification.

3.3.2. Growth conditions

Enzymes were filter sterilized in an aliquot of liquid medium with 13 mm PES filters (GE Healthcare) and added to either semi-solid half strength Murashige and Skoog (Murashige and Skoog, 1962) medium (light-grown condition) or in liquid Arabidopsis growth medium (Duchefa, dark-grown condition). 30-40 stratified *Arabidopsis thaliana* Col-0 seeds were sowed in each condition in sterile 24-well plates and grown for 7 days in long day condition (16 hours light/8 hours dark, 21°C), or for 4 days in the dark (plates wraped in 3 layers of aluminum foil, 21°C) after 6 hours of flash. As a negative controls, heat-denatured filter-sterilized enzymes (100°C/15min) were applied in growth medium or plants were grown in the absence of the enzyme.

3.3.3. Seedling analysis

Seedlings were observed under binocular microscope (Leica EZ4) and photographed. A few hypocotyls of each condition were dyed with ruthenium red 0.05% (w/v) (Sigma R-2751) for better visualization of cell detachment. Plantlets were fixed with 4% (v/v) formaldehyde and spread on square plates with solid media the next day. Images of plates were taken using a

camera on a fixed rack in order to measure the length of roots and hypocotyls using ImageJ software with NeuronJ plugin.

4. RESULTS

4.1. PRODUCTION AND PURIFICATION OF AtPGAZAT AND AtPGLR

Pichia pastoris was used in the lab to produce recombinant AtPGAZAT and AtPGLR. The C-terminal His-tagged proteins were expressed as a secreted form in the culture media to enable easier and faster purification by using immobilized metal ion affinity chromatography. The production yield, obtained by quantification of recombinant proteins after purification, was on average 2,53 and 2,09 µg of protein per mL of culture, for AtPGLR and AtPGAZAT, respectively. SDS PAGE and Coomassie blue staining were used to assess the quality of purification and to verify the size of the produced recombinant protein. Predicted size of AtPGAZAT, extrapolated from the sequence of the protein, is 45,4 kDa which corresponds to the lowest band in the line 4 on the ***Figure 7 A***. The other bands are likely to correspond to differently glycosylated forms of AtPGAZAT, for which 2 N-glycosylation sites were predicted at positions 135 and 369 as inferred from bioinformatic analyses using dedicated programs (NetNGlyc software). For AtPGLR a major band at a molecular mass of 60 kDa was detected, while its predicted size is 46 kDa. Again this is likely due to the presence of one predicted N-glycosylation site.

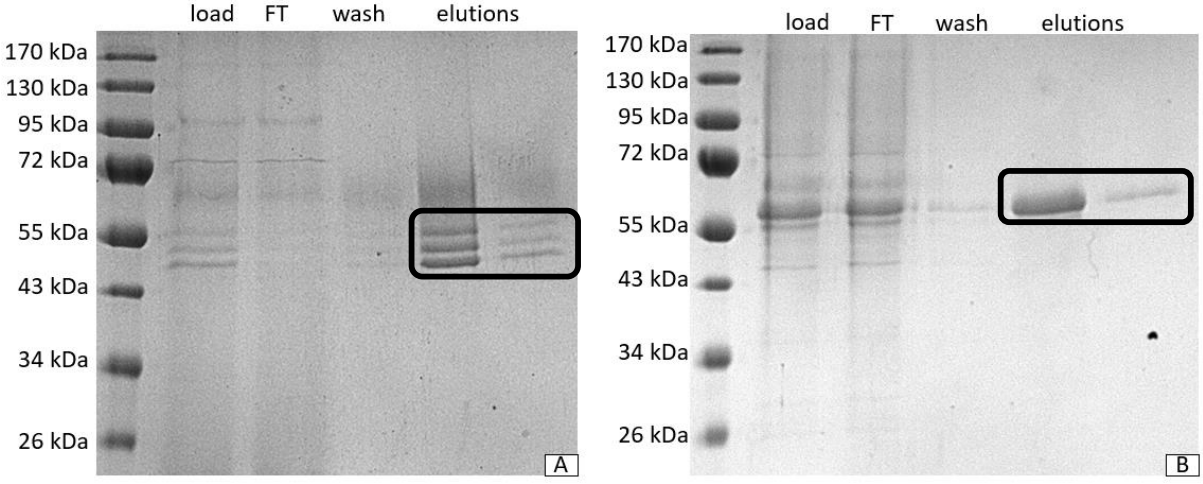

Figure 7. 12 % SDS PAGE, stained with Coomassie blue, of AtPGAZAT (A) and AtPGLR (B) with samples from different steps of purification.

4.2. DE-GLYCOSYLATION OF AtPGAZAT

De-glycosylation of purified AtPGAZAT was performed using PNGase F (New England BioLabs, 500 000 U/mL) according to the manufacturer's instructions to confirm the size of the non-glycosylated protein. On the ***Figure 8***, de-glycosylated proteins correspond to one

large band of 45 kDa in line 1, while two additional bands of molecular mass >45 kDa were detected for proteins that were not treated with PNGase F (line 2)

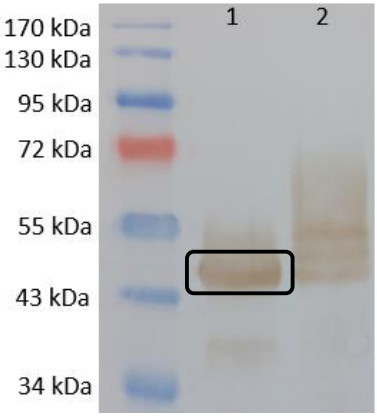

Figure 8. De-glycosylated (1) form of AtPGAZAT obtained after de-glycosylation by PNGase F corresponds to the lowest band of native sample (2). 12 % SDS PAGE and Western blot analysis.

4.3. BIOCHEMICAL CHARACTERIZATION OF AtPGAZAT

4.3.1. Substrate and pH specificities of AtPGAZAT

In order to determine substrate preference for AtPGAZAT, its activity was measured using the DNS method in the presence of polygalacturonic acid and a set of commercial pectins with increased DM extracted from citrus (Sigma, pectin esterified from citrus fruit with 30%, 55-70% and >85% degree of methyl esterification). As previously observed for other PGs (Bonnin et al., 2002), the activity of AtPGAZAT is the highest on PGA and decreases with increasing DM. It is noteworthy that for the highest DM tested, the activity of AtPGAZAT is slightly higher than that observed for DM 55-70% (***Figure 9 A***). In contrast, substrate specificity for AtPGLR, previously determined in the laboratory, shows no residual activity for high DM pectin (***Figure 9 B***). The optimal pH for AtPGAZAT activity was tested in a pH range from 4 to 7 using the DNS method. The highest activity obtained was 141,15 nmol/min/µg at pH 4,81. The activity decreased drastically above pH 6 (***Figure 9 C***).

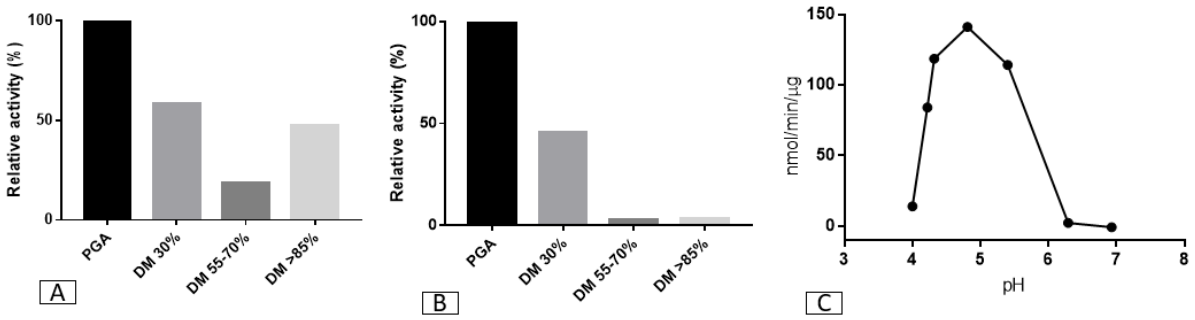

Figure 9. Relative activities of AtPGAZAT (A) and AtPGLR (B) with polygalacturonic acid (un-methylesterified pectins), and pectins with different DM (DM 30%, DM 55-70% and DM >85%). Duration of reaction was 60 minutes at 50°C, pH 5.2; (C) pH dependence of AtPGAZAT. Velocity was calculated using GalA standard curve and expressed in nmol GalA produced per minute and per µg of protein. Reaction was conducted at 25 °C for 5 minutes.

4.3.2. Determination of kinetic parameters

In order to biochemically characterize AtPGAZAT, maximal velocity (Vmax) and Michaelis constant (Km) were assessed. As expected, AtPGAZATs' activity increases with PGA concentration, but is inhibited above 4 mg/mL of substrate, revealing substrate inhibition (***Figure 10 A***). Double reciprocal Lineweaver-Burk plot was constructed to calculate Vmax and Km from y- and x-intercept. The area of linearity on the plot was taken into consideration when calculating Vmax and Km. Maximal velocity of AtPGAZAT was 149 nmol/min/µg and Km obtained was 8,6 mg/mL. The kinetic parameters previously obtained for AtPGLR showed a Vmax of 27,9 nmol/min/µg and Km of 11,0 mg/mL. Unlike AtPGAZAT, AtPGLR was not prone to substrate inhibition.

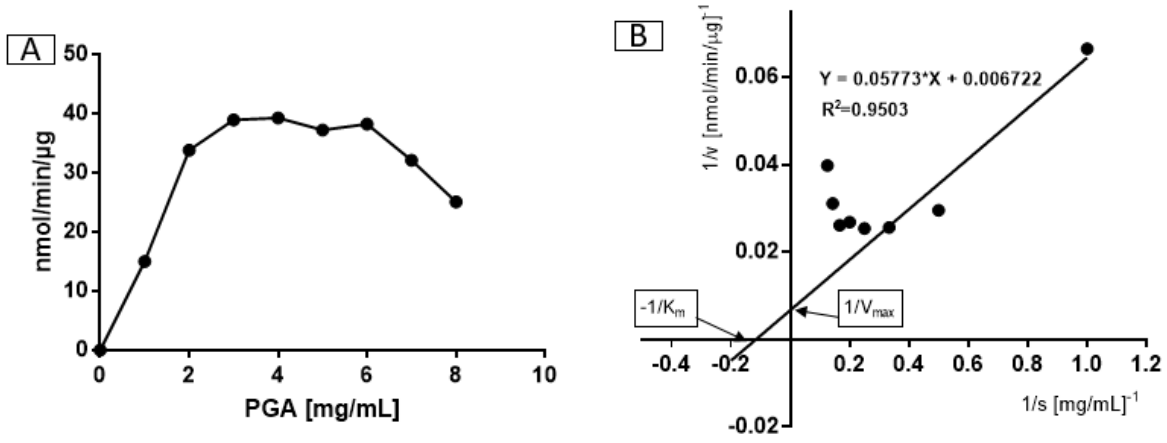

Figure 10. Activity of AtPGAZAT with different concentrations of PGA (A) expressed in nmol of GalA produced per min and per µg of protein, velocity was calculated from Gal A standard curve; Lineweaver-Burk plot for AtPGAZAT (B). Duration of reaction was 5 minutes at 25°C, pH 5.2 and activity determined using the DNS method.

4.3.3. Released OGs after application of AtPGAZAT on hypocotyl cell walls

In order to determine the digesting sites of AtPGAZAT on cell walls and determine its mode of action, 10 µg of AtPGAZAT was applied on the extracted cell walls of etiolated hypocotyls and released OGs were analysed. Samples were subjected to LC-MS analysis and spectra were analysed by MassLynx software. These results were compared to those previously obtained for AtPGLR (Maša Boras, Wafae Tabi). Profiles of OGs released after the digestion of cell wall were different when comparing AtPGLR and AtPGAZAT (***Figure 11 A***). AtPGLR was more efficient in releasing acetylated OGs (22% of total relative abundance) than AtPGAZAT (15%). In contrast, AtPGAZAT can release higher proportion of methylated OGs (21%) compared to AtPGLR (5%). Digestion of hypocotyl cell wall by AtPGAZAT resulted in release of OGs that are both acetylated and methylated, while AtPGLR did not (***Figure 11 B***). These results undoubtedly show that the two PGs differ in their mode of action.

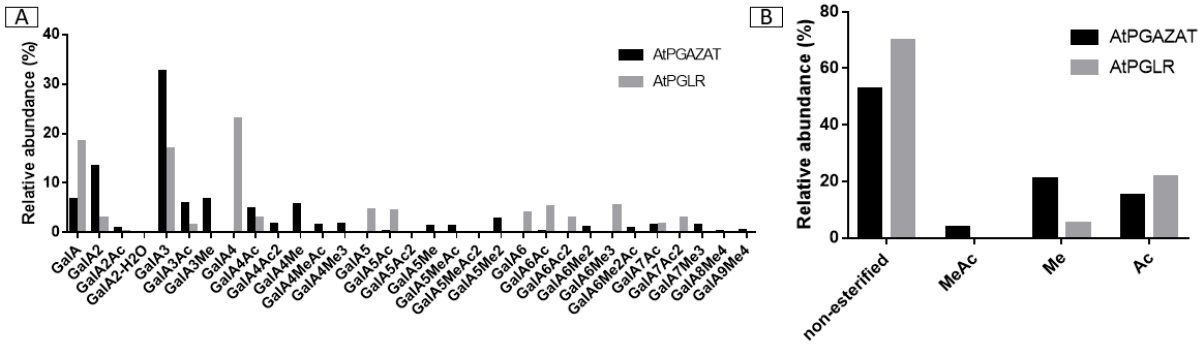

Figure 11. Mode of action of AtPGLR and AtPGAZAT on cell walls of hypocotyls determined by LC-MS. Release of total amount of differently esterified OGs (A) and in individual OGs (B). Reaction was performed at 40°C overnight, pH 5.2.

4.4. EXOGENOUS APPLICATION OF AtPGAZAT AND AtPGLR AND EFFECT ON THE DEVELOPMENT OF PLANT

Enzymes, previously filter-sterilized, were applied exogenously on *Arabidopsis thaliana* Col-0 seeds after addition in the germination medium, to observe their effect on cell wall remodeling and plant development. Two different developmental models were used: (i) plants grown in the presence of enzyme for 7 days in long day conditions and (ii) plants grown in the presence of the enzyme for 4 days in the dark. Length of roots and hypocotyls were measured and plants were observed under the microscope to detect putative phenotypes caused by application of enzyme.

4.4.1. Effect of AtPGAZAT on dark-grown hypocotyl

As the effect of AtPGLR on dark-grown hypocotyls has already been investigated in the laboratory (Maša Boras, Wafae Tabi, ***Figure 6***), the impact of AtPGAZAT was also studied in the same conditions. To this end, 10 µg and 20 µg of native AtPGAZAT was added in the

growth media for *Arabidopsis thaliana* Col-0 seeds. After 4 days in the dark, hypocotyl length of control plants reached 9.25 mm on average whereas a strong effect on cell elongation was visible in the presence of AtPGAZAT. Indeed, hypocotyl length was only 1.98 mm and 1.56 mm in the presence of 10 µg and 20 µg, respectively (***Figure 12 A-D***). In addition, as observed for AtPGLR, a strong effect on cell adhesion was noticed on the epidermis, and probably cortex of hypocotyls, but also cell detachment on the cotyledons (***Figure 12 G-J***).

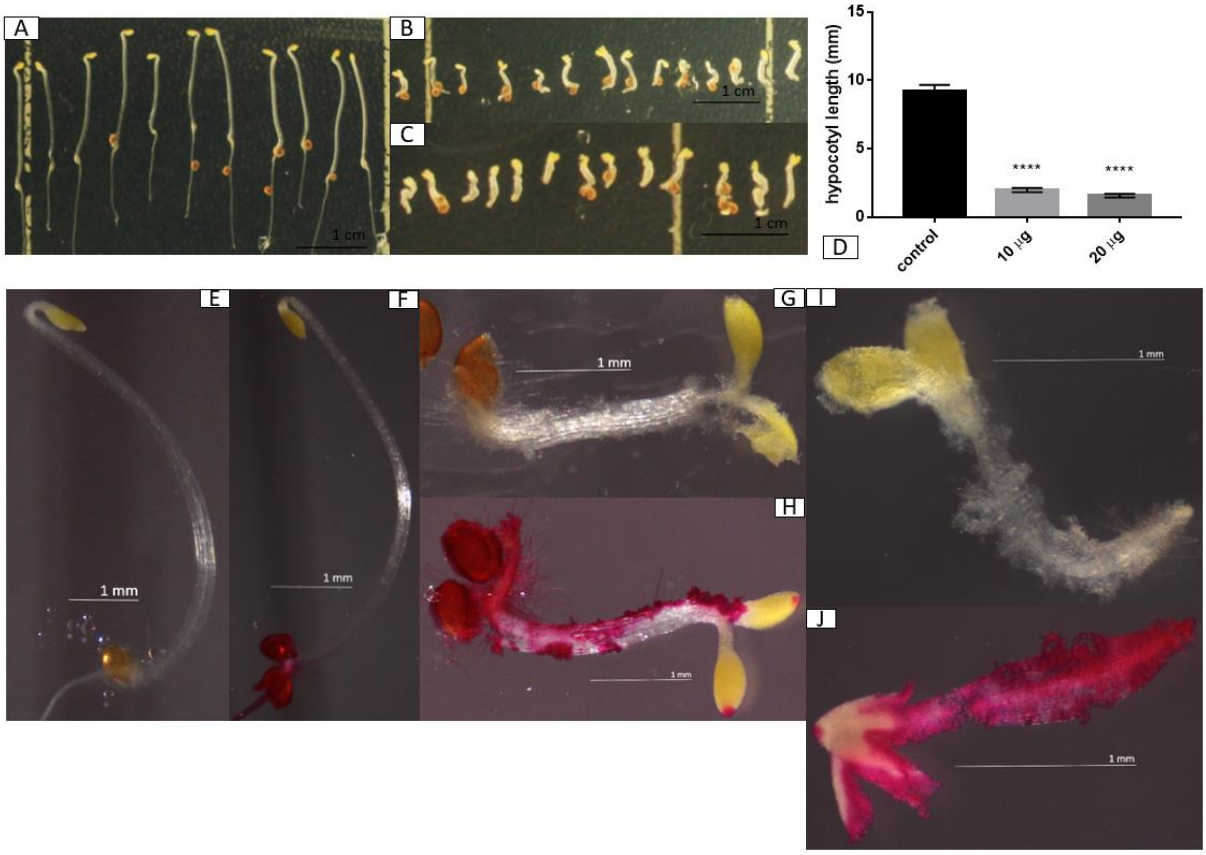

*Figure 12. The effect of AtPGAZAT on dark-grown hypocotyls. 4 days old plants grown without enzyme (control, A, E, F), and in the presence of 10 µg (B, G, H) or 20 µg (C, I, J) of AtPGAZAT. Hypocotyls (F, H, J) stained with ruthenium red 0.05% (w/v). Graph (D) showing reduced hypocotyl length in the presence of AtPGAZAT. Error bars show 95% CI; asterisks indicate a significant difference obtained using t-test (**** p<0.0001).*

4.4.2. Screening of EMS collection of mutants

In order to find mutants resistant to AtPGAZAT exogenous application, an EMS (ethyl methanesulphonate) mutant collection of *Arabidopsis thaliana* was screened. For that purpose, EMS lines were grown in the presence of AtPGAZAT (the equivalent activity as in previous experiment, known to induce visible phenotypes in term of length and cell adhesion on etiolated-hypocotyls). The identification of mutants at least partially resistant to applied PG may help in understanding the origin of the observed phenotype on wild type. Wild type *Arabidopsis thaliana* Col-0 seeds were used as controls (negative, without added enzyme;

positive, with added enzyme). After 4 days of growth in the dark, hypocotyls were visually checked for their length and stained with ruthenium red in order to reveal cell detachment.
Among 83 EMS lines tested, 3 of them (32, 70, 77 ***Figure 13***) showed partial resistance to the exogenous application of the enzyme, as hypocotyls are longer, and less staining than positive control and no cell detachment was visible on their epidermis. On the other hand, other mutants present similar or stronger phenotype than the positive control (e.g. line 66, ***Figure 13***).

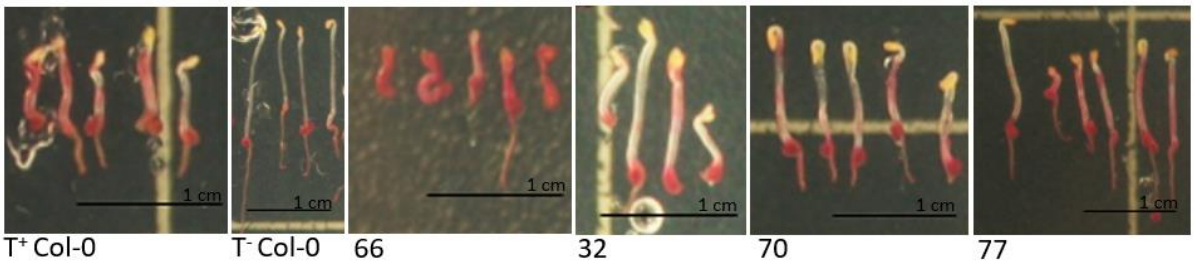

Figure 13. Effect of AtPGAZAT on EMS mutant lines showing similar (line 66) and different (line 32,70,77) phenotype as T+.

4.4.3. Effect of AtPGAZAT and AtPGLR on light grown plants

AtPGLR and AtPGAZAT were then applied on plantlets developed under long day conditions. The effect of AtPGAZAT on light grown plants was assessed in the presence of 10 µg and 20 µg of native enzyme in the growth media. Morphology of the cotyledons was severely affected: growth defects as well as cell disorganization and cell detachment of their surface were observed (***Figure 14***). Impaired growth of cotyledons was also noticed in the presence of 5 µg of AtPGLR as cotyledons of the plantlets were in general smaller size than control. In addition, they presented cell detachment and disorganization of the epidermis of the adaxial surface.
To further examine this effect, different quantities of AtPGLR (0.5, 1, 2, 5, 8, 10 µg) were applied in the germination media. The addition of 0.5 µg, 1 µg, and 2 µg of enzyme did not lead to any visible phenotype (***Figure 15 A-D***). In contrast, when 5 µg of enzyme was applied, cell detachment appeared on the cotyledons and the phenotype increased with the increasing of quantity AtPGLR (***Figure 15 E-G***). Interestingly, first leaves and trichomes development did not seem to be affected.
A strong impact on the development of roots was also observed in the presence of both enzymes. Indeed, root length of plants grown under control condition reached 16.85 mm, while it was strongly reduced in the presence of AtPGAZAT (4.16 mm and 2.4 mm for 10 µg and 20 µg of AtPGAZAT respectively, (***Figure 14 G***)). For some plants root growth was completely impaired, especially when 20 µg of enzyme was applied. As for AtPGLR, length of roots in control reached 21.35 mm whereas root development was reduced when increasing the amount

of the enzyme: with 8 µg and 10 µg of enzyme, root length reached only 16.5 mm (***Figure 15 H***).

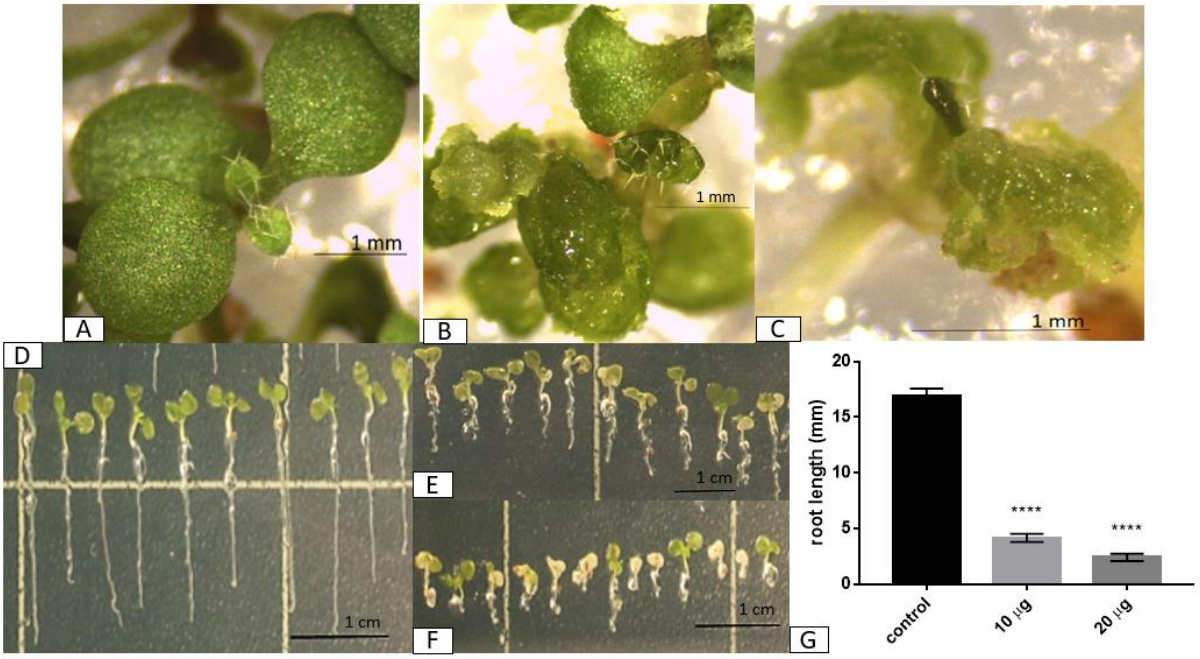

*Figure 14. Effect of AtPGAZAT on light-grown plantlets. 7 days old plants grown without enzyme (control, A, D) and in the presence of 10 µg (B, E) or 20 µg (C, F) of AtPGAZAT. Graph (G) showing reduced root length in the presence of AtPGAZAT. Error bars show 95% CI; asterisks indicate a significant difference obtained using t-test (**** p<0.0001).*

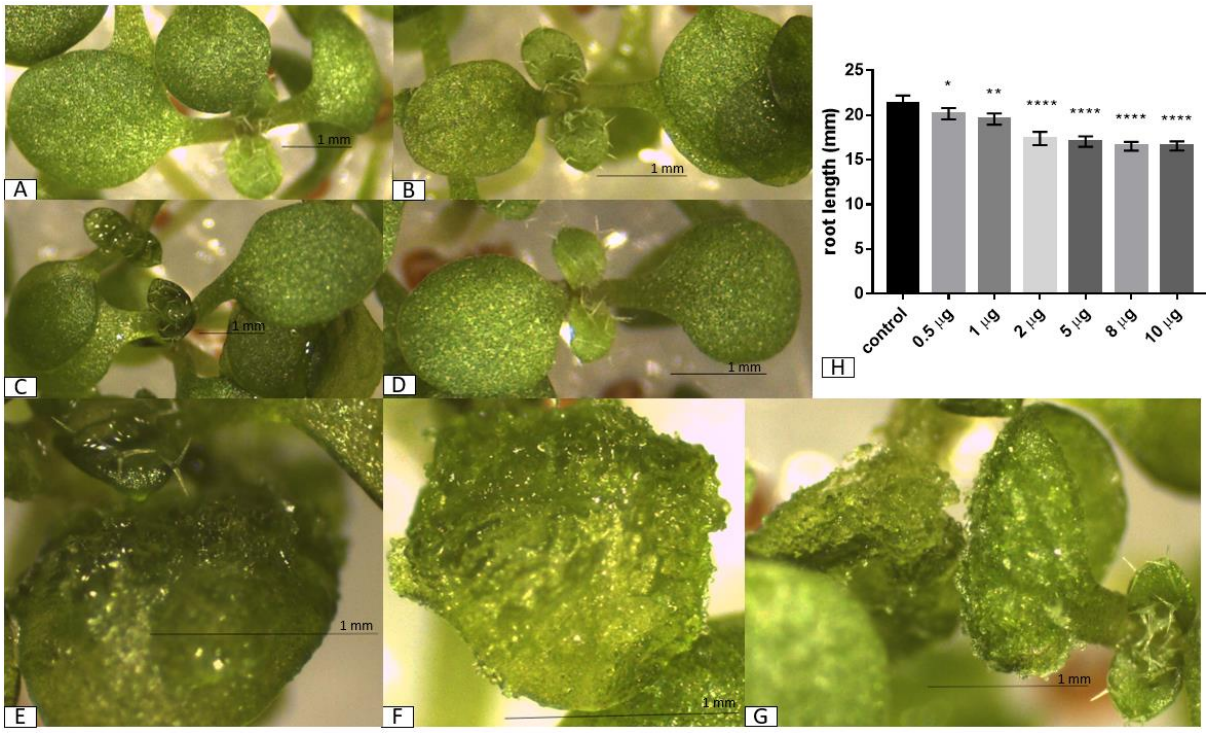

*Figure 15. Effect of different quantities of AtPGLR on cotyledons (A - without enzyme; B - 0.5 µg; C - 1 µg; D - 2 µg; E - 5 µg; F - 8 µg; G - 10 µg) and on root length (H). Error bars show 95% CI; asterisks indicate a significant difference obtained using t-test (*p=0.0288; **p=0.0010; **** p<0.0001).*

5. DISCUSSION AND CONCLUSIONS

5.1. DISCUSSION

Focus of this thesis was to get insights into PG diversity through their biochemical characterization, *in vitro* and their *in vivo* activity assessed by the phenotypical consequences of exogenous application of enzymes on plants. We chose to study AtPGAZAT, in comparison with already characterized AtPGLR, regarding its substrate/pH specificity, kinetic constants and mode of action. Further on, exogenous application of enzymes on plants developed under long day conditions and in the dark (for hypocotyl etiolation) was performed in order to observe and compare the effects of enzymes on cell wall remodeling and ultimately their consequences on plant development.

As AtPGAZAT was a newly produced enzyme in the research team, analysis of its biochemical characteristics was performed. After protein expression and purification, three distinct bands were observed on SDS PAGE indicating the presence of differently glycosylated forms of the protein. Although protein obtained after purification showed activity on polygalacturonic acid by using DNS method, question remains whether or not glycosylation affects its activity. Since de-glycosylation was performed in denaturing conditions to confirm the size of the protein, the determination of the activity of non-glycosylated AtPGAZAT could not be tested. In order to fully understand the impact of glycosylation on the activity of AtPGAZAT de-glycosylation would need to be realized in native conditions to allow activity to be tested.

As expected, activity of both enzymes, tested on commercial substrates, decreases with the increasing DM. However, AtPGAZAT shows higher activity on highly methylated pectins than AtPGLR and surprisingly presents slightly better capacity of degrading DM>85% pectins than DM 55-70% (***Figure 9***). As two other enzymes characterized in the laboratory showed the same trend, this is likely to represent a peculiar specificity. For the above mentioned experiment, commercial pectins extracted from citrus fruit were used, for which the exact pattern of methyl-esterification is unknown and distribution of methyl groups are not familiar. Possible explanation for such difference in the activity between pectins of DM>85% and DM 55-70% could be the distribution pattern of methyl groups on the HG backbone, that makes these pectins more or less digestible for one enzyme compared to another. Differences in OG profiles released after digestion of hypocotyl cell walls with either enzymes partially validate this hypothesis as it revealed a difference in the mode of action of the two enzymes. AtPGAZAT indeed shows higher tolerance to methylated stretches of HG compared to

AtPGLR as it produces a greater proportion of methylated OGs. (***Figure 11***) This analysis further demonstrated that AtPGLR can hydrolyse HG in regions that are acetylated as a number of acetylated OGs were released. In contrast, AtPGAZAT appeared to be less tolerant to acetylated streches.

When comparing kinetic constants for the two enzymes, lower Km value determined for AtPGAZAT indicates that it has a higher affinity for PGA than AtPGLR. In addition, higher Vmax measured for AtPGAZAT also points towards a better efficiency in degradation of PGA than AtPGLR. These differences could be related to subtle variations in active sites, or substrate binding interfaces, of the two enzymes. X-Ray diffraction crystallography performed on AtPGLR allowed the resolution of AtPGLR structure in the laboratory (PhD Josip Šafran). Crystallisation of AtPGAZAT and further analysis of the enzyme structure including substrate binding groove should be performed to understand how 3D structure might explain distinct mode of action.

Both AtPGAZAT and AtPGLR seem to affect the morphology of cotyledons developed under long day conditions and show a similar phenotype of cell detachment of their epidermis (***Figure 14,Figure 15***), but, surprisingly, no cell adhesion phenotype was observed on roots. A similar phenomenon of round detached cells, as observed cotyledons' surface, can be seen in the abscission zone of flowers (Merelo *et al.*, 2017) where AtPGAZAT is presumed to have a significant role (González *et al.*, 2002; González-Carranza *et al.*, 2007). It thus could be assumed that both enzymes, when in contact with early developing plantlets will have the same consequence as when they are locally acting in the abscission zones in planta.

AtPGAZAT and AtPGLR also caused a strong cell adhesion defect on dark-grown hypocotyls. One of the major difference lies in the fact that, in this growth condition, cell detachment on cotyledons is observed after application of only AtPGAZAT (***Figure 12***). Two hypothesis could explain the observed phenotypes on cotyledons and hypocotyls: (i) exogenously applied enzymes are primary actors and cause cell detachment through direct action on the cell wall or (ii) they are just mediators, releasing signalling OGs that will induce gene expression of other cell wall remodeling enzymes responsible for the observed phenotypes.

The cell detachment phenotype on cotyledons and hypocotyls could indeed come from cell wall loosening caused by direct action of the enzymes on HG in the middle lamella (first hypothesis). It is known that PGs are involved in depolymerisation of HG. After hydrolysis, the remaining chains could be too short for effective calcium binding, which can result in loss

of cohesion in the pectin gel and can lead to loss of HG-mediated cell adhesion (Yang *et al*., 2018). It could be that AtPGAZAT and AtPGLR affect the pectic network of middle lamella thus causing cells to detach one from another.

Presence of cell detachment phenotype on cotyledons, but not on roots, could derive from differences in cell wall composition between roots and cotyledons and thus the ability of degradation by enzymes. Interestingly, first leaves and trichomes were not influenced by the action of the enzymes. It is possible that cotyledons were affected in their early development phase or later on, if the enzyme was present on the surface of cotyledons, or the enzyme is no longer active when the development of leaves begins.

Since cotyledons or hypocotyls are not in direct contact with the growth medium and thus with applied enzyme, it is more likely that OG signalling could be the cause of the observed phenotype (second hypothesis). We could hypothesize that PGs added in growth medium could digest root cell walls, as they both are in direct contact. OGs released could then be the cause of the observed phenotype on above-grown organs through signalling, if signal transduces through the whole plantlet. It is indeed known that following HG-pectin degradation by fungal PGs OGs are released which can be involved in signalling and affect growth and development of Arabidopsis (Ferrari *et al*., 2013; Davidsson *et al*., 2017).

Although AtPGAZAT and AtPGLR didn't cause cell detachment or roots, their application lead to a decrease in root length (***Figure 14, Figure 15***). In relation to the above-mentioned first hypothesis, root elongation may be inhibited through direct subtle remodeling of the root cell wall which does not result in cell adhesion defect. On the other hand, root development may be impaired by release of signalling OGs upon digestion of root cell wall, which are known to interfere with auxin response. OGs have been hypothesized to be involved in the regulation of plant growth and development as they are generated by the action of endogenous PGs and inhibit the stimulation by auxin of the mitotic activity (Ferrari *et al*., 2013). OGs have been shown to inhibit auxin-induced root formation in tobacco and *Arabidopsis* leaf explants (Bellincampi *et al*., 2000; Savatin *et al*., 2011). Furthermore, Davidsson et al., presented data which indicated that treatment of *Arabidopsis* with mix of short OG down-regulates the expression of gene related to plant growth, organelle organization, cellular component biogenesis and photosynthesis which resulted in significant growth retardation (Davidsson *et al*., 2017). Interestingly, the same activity of AtPGAZAT and AtPGLR doesn't seem to have the same effect on root development with AtPGAZAT revealing more significant impact on

root elongation (***Figure 14, Figure 15***). Considering previous results, this could be related to substrate preference of each enzyme, resulting in generation of various OGs, which could further influence root development in a distinct, gradual way.

Screening of EMS collection of mutants was performed to find possible reversion in phenotype. The difference in observed phenotypes between positive control and partially resistant mutant lines could come from differences in composition of the cell wall and the inability of AtPGAZAT to act on it. On the other hand, if AtPGAZAT is not the primary actor of the phenotype, resistance could come from differences in signalling pathways between mutant lines and wild type. Under this scenario, mutation could affect the signalization pathway in a way that OG signalling is interrupted and doesn't lead to the usual phenotype observed on wild type. Identification of mutations in these revertant lines would allow determining the actors of this. However, we have to be cautious as these mutant lines were not grown without the presence of enzyme as an additional control, it is therefore hard to distinguish between the effect of the enzyme and the sole effect of the mutations.

5.2. CONCLUSIONS AND PERSPECTIVES

Although both enzymes studied in this master thesis belong to the PG family and show similarity in expression patterns, they differ in the mode of action, showing differences in released OGs and substrate specificity. Although they cause a similar phenotype when applied exogenously, it differed in its intensity, in particular considering root development. Two additional *Arabidopsis* PGs have been previously studied and characterized in the laboratory, and show a different mode of action and no visible phenotype following exogenous application. Considering these results we demonstrated that, although showing simmilarities in terms of sequence, gene expression and predicted roles during plant development, the biochemical diversity of plant PGs is tremendous. Certainly, still a lot of effort needs to be put into this research to fully understand subtile differences in their mode of action, which are probably conected to their distinct or complementary roles during the plant life cycle.

In order to better characterize the mode of action of AtPGAZAT and AtPGLR, further identification of released OGs after application of enzymes on commercial pectins with different DM and at different time points should be performed. These analyses would reveal primary products of degradation and further help understanding enzymes preferences for cleavege sites when binding on the substrate. Also, they would reveal the abundance and variety of released OGs (in terms of DM, DA and DP) during the reaction course. OG profiling

could also be performed on cell walls of roots and cotyledons to compare released OGs with the results obtained on hypocotyl cell walls. This should indirectly reveal differences in cell wall composition between these organs and help understanding the phenotypes. Interesting informations should come from OG profiling on etiolated hypocotyls and long-day roots or shoots of plantlets grown on each enzyme to assess consequences of application of the enzymes on cell wall structure.

The observed root phenotype also needs further characterization to examine if the cause of decrease in root length lies in the elongation zone of the root or in the root apical meristem structure. Cell wall remodeling could either impair elongation (as for etiolated hypocotyl), which could be observed by measuring the cells in the elongation zone, or affects the division and differentiation of cells of root apical meristem. Also, to further investigate involvement of OGs in root phenotype, expression of genes that are most likely involved in the signalization could be assessed, for instance auxin induced genes. All this data could help provide neccessary informations to further characterize the diversity of these enzymes.

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7. ANNEXE

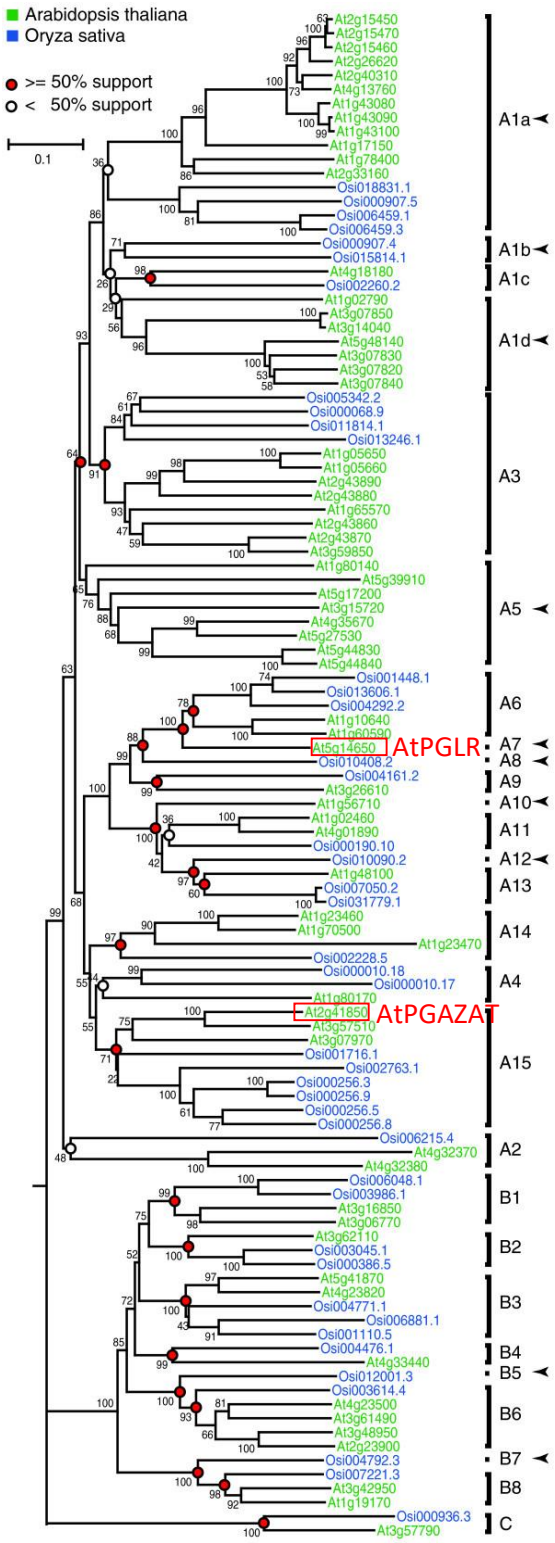

Figure 16. The phylogeny of Arabidopsis and rice PGs, showing AtPGLR and AtPGAZAT belong to different clusters. The phylogeny was generated using neighbor-joining algorithm with 1,000 bootstrap replicates. Figure adapted from Kim et al. 2006.

Abstract

Pectins are major components of the plant primary cell wall and the most heterogeneous cell wall polysaccharides. They comprise several domains, the most abundant being homogalacturonan (HG), built on a simple Gal A backbone which can be methyl esterified and acetylated. HG constant remodeling is mediated through the action of HG-modifying enzymes that are acting during plant development. As such, these enzymes play a key role in the maturation and degradation of HG, therefore controlling cell wall rheology. Polygalacturonases (PGs) are an essential class of HG-modifying enzymes as they degrade HG chains and produce oligogalacturonides (OGs) of various size and structure. As PG are encoded by a large multigenic family of 69 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 role during plant development. During this thesis, two PGs were studied, AtPGAZAT and AtPGLR, which genes are both expressed in roots. Both proteins were heterogously produced in *Pichia pastoris* as active enzymes and their biochemical properties and mode of action were compared. Their roles *in planta* was assessed through exogenous application of the enzymes and analysis of the phenotypical consequences on light- and dark-grown *Arabidopsis thaliana* seedlings. On the basis of the results, the main differences between these endo-PGs lie in their substrate specificity, as AtPGAZAT shows higher activity on highly methyl esterified pectins compared to AtPGLR. Furthermore, size exclusion chromatography coupled to mass spectrometry revealed difference in their mode of actions. Indeed, OG profiles obtained after hydrolysis of hypocotyl cell wall by AtPGAZAT generates higher abundance of methyl esterified, while AtPGLR generates acetyl esterified OGs. These subtle distinctions in their mode of action could be connected to differences in observed phenotypes after exogenous application on light grown plants and dark-grown hypocotyls.

Keywords: *Arabidopsis thaliana*, cell wall, homogalacturonan, oligogalacturonides, pectins, polygalacturonase

Résumé

Les pectines, polysaccharides majeur des parois primaires sont aussi les plus hétérogènes. Ils sont constitués de plusieurs domaines dont les plus simples sont les homogalacturonanes (HG), enchainements linéaires d'acide galacturonique (GalA) dont les unités peuvent être à la fois méthylées et acetylées. La structure des HGs est constamment modifiée au cours du développement des plantes par l'action d'enzymes de remodelage. En tant que tel, elles jouent un rôle de premier plan dans le remodelage des HG et participent ainsi au contrôle de la rhéologie pariétale. En particulier, les Polygalacturonases (PGs), une famille multigénique de 69 membres chez Arabidopsis, dégradent les HG en hydrolysant les liaisons glycosidiques entre deux GalA successifs pour produire des oligogalacturonides (OGs) de taille et structure variées. Ce nombre important d'isoformes complique leur étude par génétique inverse. Au laboratoire, une approche alternative est conduite, qui consiste en l'application exogène des enzymes recombinantes et l'étude de leurs conséquences sur le développement. Pendant ce stage, nous avons choisi d'étudier AtPGAZAT et AtPGLR, deux enzymes, dont les gènes sont exprimés aux sites d'émergence des racines latérales. Ces enzymes ont été produites en système hétérologue *Pichia pastoris* et caractérisées biochimiquement, de même que leur mode d'action. Leurs impacts dans le cadre de l'allongement de l'hypocotyle à l'obscurité et du développement des plantules à la lumière, a été évalué par analyse des phénotypes engendrés après application. Les principaux résultats ont permis de conclure que contrairement à AtPGLR, AtPGAZAT est capable d'hydrolyser les substrats hautement méthylestérifiés. Le mode d'action de ces endo-PGs, déterminé par chromatographie à exclusion de taille couplée à la spectrométrie de masse, confirme ces données puisque AtPGAZAT hydrolyse la paroi d'hypocotyle étiolé en générant une grande proportion d'OGs méthylés. En parallèle, AtPGLR digère préférentillement les HGs dans les regions acétylées. Ces différences dans leur mode d'action pourraient partiellement expliquer les différences phénotypiques observées lors des applications exogènes sur les deux modèles développementaux.

Mots-clés: *Arabidopsis thaliana*, paroi, pectines, homogalacturonanes, oligogalacturonides, polygalacturonase

STATEMENT OF ORIGINALITY

This is to certify, that the intellectual content of this thesis is the product of my own independent and original work and that all the sources used in preparing this thesis have been duly acknowledged.

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