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First indication of the role of Pectate Lyase-Like enzymes (PLLs) in the control of vegetative growth in Arabidopsis

by

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FIRST INDICATION OF THE ROLE OF PECTATE LYASE-LIKE ENZYMES (PLLS) IN THE CONTROL OF VEGETATIVE GROWTH IN ARABIDOPSIS

Irin Antun Stošić, 1013/MB

Abstract: Pectin is the most complex polysaccharide of the primary plant cell wall that contributes to complex processes as cell growth and differentiation. It is composed mostly of homogalacturonan (HG), a homopolymer of galacturonic acid residues that can be methylesterified and acetylated. According to those modifications, pectin can rigidify or be digested by pectin-degrading enzymes such as Pectate Lyase-Like (PLLs). Activity of PLLs in has been linked to fruit ripening, reproductive organs elongation and pathogen susceptibility. However, PLLs have not been linked to vegetative growth of *Arabidopsis thaliana*. In this study we show that the *PLL21* loss-of-function mutant exhibits shorter light-grown roots and dark-grown hypocotyls than the wild type. A change in pectin content has also been noticed, however with different trends in roots and in hypocotyls. Surprisingly, we have also found that presumably safe green light leads to loss of phenotype in hypocotyls, indicating a putative role of light in PLL21 regulation. Furthermore, *PLL21* and *PLL20* double mutant showed no difference in length of dark-grown hypocotyls, and a less substantial difference in light-grown root length. Overall increased PLL expression in the double mutant and pectin content more similar to the WT, with lack of these trend in the single mutant, point to a compensatory mechanism triggered by the absence of two enzymes, which could explain the smaller changes observed in the double mutant.

Keywords: Arabidopsis, cell wall, pectin, pectate lyase-like, functional genomics, growth

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To my friends, I am happy we became who we are together.

Dear Dušan, thank you for being close, while far away.

Mojoj obitelji, voli vas vaš Irin.

Table of Contents

Presentation of the Laboratory and Localisation of the Internship

The EA 3900 BIOPI - “Biologie des Plantes et Innovation” research unit is a part of University of Picardy Jules Verne (UPJV), located in Amiens. Three collaborating and complementing teams of BIOPI aim to understand the impact of changes in cell wall structure and secondary metabolism. Team I is focused on the remodelling of pectin in plant cell wall and its impact on plant development. Team II studies phenylpropanoid metabolism and related biosynthetic pathways including effects of stress and elicitation on plant metabolism. Team III is engaged in studying specialized metabolic pathways in roots, root molecular engineering and development of bioprocess for molecule production. Besides a well-equipped laboratory, BIOPI has access to state-of-the-art equipment of the UPJV platforms such as electronic and confocal microscopy, chromatography and mass spectrometry for analytical chemistry and proteomic, instruments and robots dedicated to molecular biology, and phenotyping, as well as greenhouses.

During this internship, I was a member of team I, called « Pectin Dynamic » led by Professor Jérôme Pelloux. This team has strong expertise in functional genomic approaches, biochemistry, quantitative PCR as well as heterologous protein expression and purification. Integration of these approaches is used to research roles and regulation of pectin remodelling enzymes, as well as the effects of stress on plant cell wall dynamics of flax and *Arabidopsis thaliana* plants. The team has participated in a number of ANR-funded projects, either as the coordinator (GROWPEC, GALAPAGOS and WALLMIME) or as a partner (NOSTRESSWALL and PECTOSIGN).

Amiens is the capital of Somme department in Hauts-de-France region, located 120 km north of Paris. The city is famous for its 13th century Gothic cathedral, the largest one in France, and listed as UNESCO World Heritage site. Moreover, Amiens takes pride in being home to a famous writer Jules Verne nearly half of his life, and his final resting place. The city is crisscrossed by canals stretching from Somme river, making a lot of small water-surrounded gardens – Les Hortillonages, cultivated since the 12th century.

1. Introduction

The plant cell is surrounded by a cell wall whose major role is to withstand the osmotic pressure of the cell and to provide rigidity that in the end defines the whole shape of the plant. It also provides protection against pathogens and it plays a role in controlling the differentiation and growth of cells (Lodish *et al.*, 2012). Furthermore, cell walls contain molecules that participate in signalling, and molecules that mark a cell's position within the plant (Carpita *et al*., 2015). In order for the cell wall to be solid, but at the same time to allow the growth of the cells and the whole plant, the plant cell wall is a complex and dynamic matrix. While the cell is growing, material is incorporated into a thin and flexible primary cell wall. When the cell no longer grows, the primary cell wall can be retained without modification, but some plant cells form a more rigid and permanent structure known as the secondary cell wall by depositing new material layers between the plasma membrane and the primary wall. Adjacent plant cells are cemented together by the outermost layer of the cell wall called middle lamella (Alberts, 2008).

2. Bibliographic Study and Subject

2.1. Plant cell wall

The primary cell walls of dicotyledonous and non-commelinoid species, such as the model plant *Arabidopsis thaliana*, are predominately constituted of cellulose, β-(1→4)-D-glucan chains, hydrogen-bonded together to form 3-4 nm wide microfibrils (**Figure 1**). The second constituent is xyloglucan, present in a slightly lower amount than cellulose (Vogel, 2008). Xyloglucans are also made up of β-(1→4)-D-glucan chains but differ from cellulose by having three out of four backbone chain glucose residues substituted with α-D-xylose (Carpita and Gibeaut, 1993). Some of the xylosyl units can further be substituted with alternative monosaccharides, typically α-L-arabinose, β-D-galactose, or less commonly α-l-fucose, to improve water solubility. Xyloglucans, together with the less abundant non-cellulosic polysaccharides, such as gluco- and galactoglucomannans, galactomannans, (1→3)-D glucans and glucuronoarabinoxylans form crosslinking glycans, often called hemicelluloses (Carpita *et al.*, 2015). Through hydrogen bonds, hemicelluloses bind with cellulose microfibrils and coat them allowing them to be cross-linked to 10-25 nm wide fibril aggregates (macrofibrils) by pectins, a highly hydrated network of polysaccharides rich in galacturonic acid (GalA) (Gibson, 2012; Alberts, 2008). Cellulose and xyloglucans (about 50% of the wall mass) form the first out of three intertwined but structurally different plant cell wall networks. The first network is incorporated into the second network (about 30% of wall mass) made out of pectin (Carpita and Gibeaut, 1993). Albeit

polysaccharides make up the vast majority of the plant cell wall, proteins are present and make up the third network domain (Alberts, 2008). Many of the proteins are enzymes and related inhibitors that modify the plant cell wall and allow the growth, while the others are structural proteins. The most abundant type of structural proteins consists of the hydroxyproline-rich glycoproteins (HGPRs) that include three main families: lightly glycosylated proline-rich proteins (PRPs), moderately glycosylated proteins such as extensins (EXTs) and hyperglycosylated arabinogalactan proteins (AGPs) (Showalter *et al.* 2010).

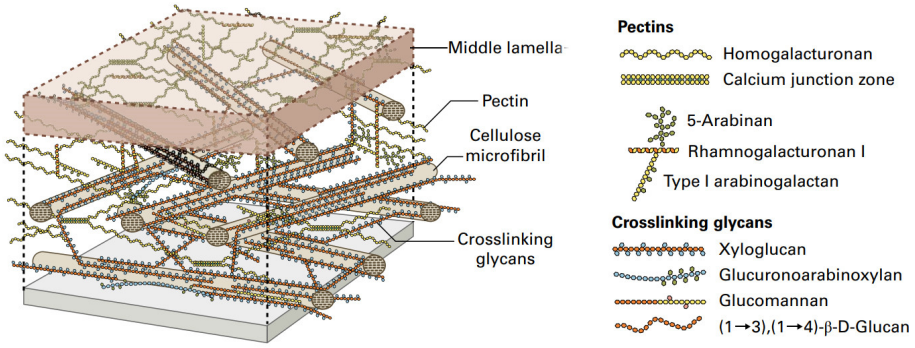

Figure 1 *Model of the type I plant cell wall showing polysaccharide composition (Carpita, et al., 2015, pg 77, fig 2.25)*

Secondary cell walls are secreted upon the completion of cell growth (Alberts, 2008). Because their role is highly specified, their structure can be significantly different according to the plant but also the type of cell that secretes them (Carpita *et al*., 2015). However, secondary cell walls usually consist of cellulose, hemicelluloses (xylans and glucomannans), and the additional aromatic polymer called lignin that provides mechanical strength, rigidity, and hydrophobicity (Zhong and Ye, 2014).

2.2. Pectins

As mentioned, pectin is present throughout the cell walls of growing and dividing cells, as well in the middle lamella, and cell corners. It makes around 35 % of primary cell walls in dicots, and it is structurally and functionally the most complex polysaccharide of the plant cell walls (Mohnen, 2008). Functions of pectins are numerous, they determine wall porosity and provide a charged surface that modulates wall pH and ion balance, some pectic fragments serve as molecules that signal the presence of symbiotic organisms and pathogens and regulate cell-cell adhesion in middle lamella (Carpita *et al*., 2015).

Chemically, pectins are defined as a family of complex polysaccharides that are rich in α-D-GalA residues (70 % of pectin) (Ridley *et al.*, 2001). They consist of four main macromolecular domains: homogalacturonans (HGs), rhamnogalacturonans type I (RG-I),

rhamnogalacturonans type II (RG-II), and xylogalacturonans (XGA) (Figure 2) (Hocq *et al.*, 2017). HG is the most abundant constituent of pectin and in *Arabidopsis thaliana* primary cell wall it makes up 50 % to 70 % of pectins (Anderson, 2015). It is a linear homopolymer of 100 to 200 α-1,4-linked GalA residues (Willats *et al.*, 2001). HG is synthesized in the Golgi apparatus and can be methylesterified until 70-80 % at the C6 position on GalA residues (O'Neill *et al.*, 1990). Methylesterification is the most present modification, but GalA residues can also be O-acetylated at O3 (more often) or at O2 (less often) positions (Mohnen, 2008). The second most abundant domain (20-35 %) is RG-I (Mohnen, 2008). It consists of up to 100 repeats of the disaccharide (1→2)-α-L-rhamnose-(1→4)-α-D-GalA (Willats *et al.*, 2001). Rest of the pectin is made up of XGA and RG-II. XGA is formed by substituting GalA of HG at C3 position with residues of xylose (Willats *et al.*, 2001). RG-II is the most diverse domain that consists of a HG backbone of at least 8 1,4-linked α-D-GalA residues that have side branches consisting of 12 different types of sugars in over 20 different linkages (Mohnen, 2008). They include apiose, aceric acid, 2-O-methyl fucose, 2-O-methyl xylose and the others making a complex, but the highly conserved structure in flowering plants (Carpita *et al.*, 2015).

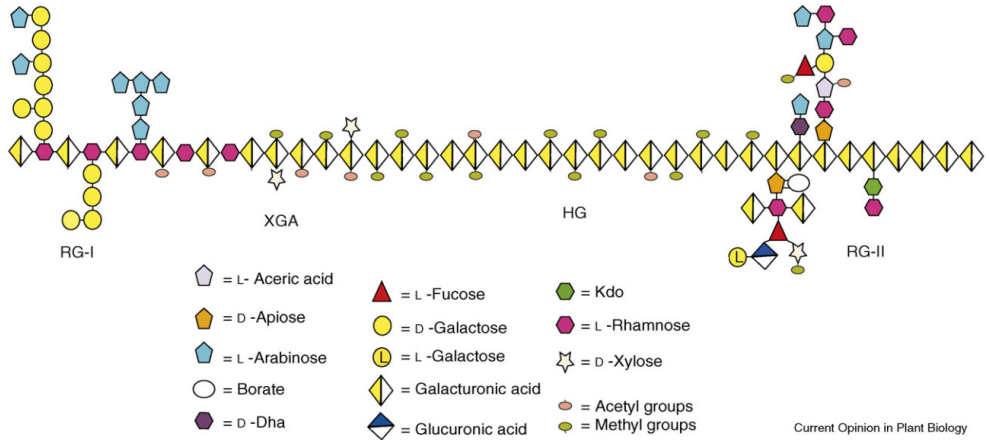

Figure 2 *Schematic structure of four pectic domains: homogalacturonan (HG), xylogalacturonan (XGA), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) (Mohnen, 2008).*

2.3. HG synthesis and HG remodelling

Being the most abundant cell wall pectic domain (up to 65 % of pectins), HG has a crucial role in changing the mechanical properties of the cell wall that affects the growth of the cells. HG backbone is polymerized by GAlactUronosyl Transferases (GAUTs) and GAuTs-Like (GATLs). It is additionally methylesterified (up to 80 %) by HG MethylTransferases (HGMTs) before its secretion into the cell wall. Then its degree of methylesterification (DM) can be selectively modulated by Pectin MethylEsterase enzymes (PMEs), themselves regulated by proteins called Pectin MethylEsterase Inhibitors (PMEIs) (Hocq *et al.*, 2017). Depending on

the mode of action of PMEs, partially demethylesterified HGs could be targeted by degrading enzymes contributing to the cell wall loosening or cross-linked through calcium bridges, leading to the formation of so-called "egg-boxes" structures and cell wall stiffening (Cabrera *et al*., 2008; Peaucelle *et al*., 2012). Similarly, Pectin AcetylEsterases (PAEs) deplete HG of acetyl groups that can impair formation of the above-mentioned Ca^{2+} - HG bonds, thus also altering the cell wall viscosity (Sénéchal *et al.*, 2014). Those two families of enzymes fall into "desterifying" enzymes, one of the two pectin-modifying types of enzymes. The other type are pectin-depolymerizing enzymes, which degrade HG backbone. This allows loosening of the pectin and release of pectic fragments (oligogalacturonides, OGs) putatively involved in signalling and modulation of cell growth (Sinclair *et al.*, 2017). Pectin-degrading enzymes are comprised of PolyGalacturonases (PGs) and Pectate Lyase-Like (PLLs) which both cleave α-(1–4) bond between GalA residues, but through a different type of action. Their activity depends on the DM and the methylesterification pattern of pectins, hence it is modulated by PMEs (Sénéchal *et al.*, 2014; Babu and Bayer, 2014). PGs are glycosyl hydrolase enzymes divided into endo-PGs, which hydrolase HG randomly with a diversity of mode of action depending of the methylesterification/acetylation patterns of HG (Xiao *et al.*, 2014) and exo-PGs, which reduce the overall length of polymer chain by attacking the de-methylesterified/de-acetylated ends of HG (Babu and Bayer, 2014; Bonnin *et al.*, 2013). PLLs cleave the glycosidic α-(1–4) bond by β-elimination. They are divided into pectin and pectate lyases depending on their preference for substrate and optimal conditions, which will be discussed in the next chapter.

2.4. Pectate lyase-like enzymes

2.4.1. Biochemical information and diversity in microorganisms

Pectate Lyase-Like enzymes (PLLs) cleave the glycosidic α-(1–4) bond by β-elimination, creating 4, 5 unsaturated GalA (**Figure 3**) (Mutenda *et al.*, 2002).

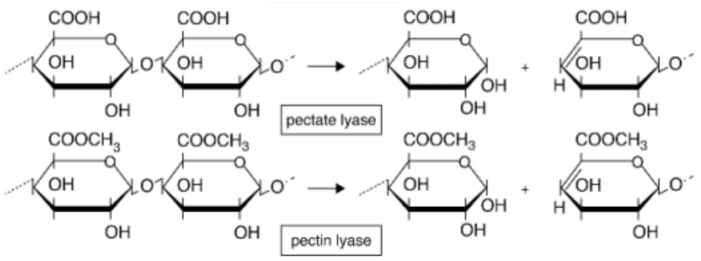

Figure 3 *PL and PNL mechanism of action (Wen, et al., 2007)*

The family comprises of pectate lyases (PLs; E.C. 4.2.2.9 and E.C. 4.2.2.10) and pectin lyases (PNLs; E.C. 4.2.2.10). PLs show higher activity on fully de-methylesterified pectin or pectin

with low DM. They require Ca^{2+} ions for their optimal activity, and most of them have the pH optimum at around 8.5. Also, they show both endo- and exo-activities. On the contrary, PNLs preferentially cleave highly methylesterified form of pectins, do not require Ca^{2+}, have the pH optimum close to 5.5, and until now, only endo-activity has been found (Mayans *et al.*, 1997). PLLs are found in plants, bacteria, fungi, and nematodes (Sénéchal *et al.*, 2014). PNLs are predominately produced by fungal microorganisms, such as *Aspergillus*, *Penicillium,* and *Fusarium* species, and some yeast but they are also present in some bacteria. However, bacteria primarily produce PLs (Cao, 2012; Yadav *et al.*, 2009). Best-studied PLs are those from bacterium *Erwinia chrysanthemi*, but they are also present in other genera as *Bacillus* (Marin-Rodriguez, 2002) and *Paenibacillus* (Li *et al.*, 2014). In pathogenic microorganisms, PLLs are virulence factors that allow penetrating the plant cell wall and living of the host (Mayans *et al.*, 1997).

2.4.2. Functions in vegetative and reproductive events in plants

In plants, PLL genes constitute one of the largest and most complex families (Sun and van Nocker, 2010). Their presence was firstly documented in pollen and then flowers and anthers of the tomato by finding sequences similar to *Erwinia* PL (Marin-Rodriguez, 2002). Furthermore, PLL expression has been reported in ripening fruits such as banana (Payasi and Sanwal, 2003; Dominguez-Puigjaner *et al.*, 1997), mango (Chourasia *et al.*, 2006), grape (Nunan *et al.*, 2001) and raspberry (Simpson *et al.*, 2016). In strawberry, more research was done discovering that PL action on the primary wall and middle lamella is important for fruit ripening (Santiago-Domenech *et al.*, 2008). Also, a genetically altered raspberry was constructed with antisense expression of one PL gene which reduced the softening during the transition from the white to the red stage (Jimenez-Bermudez, 2002). In tomato, the function of PL on altering the texture of tomato, without affecting other aspects of ripening, has been confirmed by the construction of a transgenic tomato with a silenced PL gene (Uluisik *et al.*, 2016). Concerning elongation, cotton fibre elongation, achieved by cell wall loosening, was found to be highly impaired in cotton with a silenced *PLL* gene. Silencing also heavily affected the degradation of de-esterified pectin in the primary cell wall, further connecting normal elongation with PLLs (Wang *et al*., 2009). Furthermore, high expression of all HG Modifying Enzymes (HGMEs), including PLs in latex of opium poppy suggests an important role in the development of elongated, latex-excreting cells (Bečka *et al.*, 2014). In Japanese willow, expression of a PL in reproductive organs was restricted to elongating filaments of stamens, the secretory tissues of pistils and the xylem parenchyma cells of catkins (Futamura *et al.*, 2002).

Regarding vegetative growth, in stems of *Zinnia elegans,* it has been found that auxin upregulates the expression of a PL gene, connecting it to elongating and differentiating cells (Domingo *et al.*, 2002) and possible involvement in tracheary elements development (Milioni *et al.*, 2001). Furthermore, role of PLLs in mediating plant growth has been documented in rice (Leng *et al.*, 2017).

2.4.3. PLLs in *Arabidopsis thaliana*

The model plant *Arabidopsis thaliana* has 26 *PLL* genes that have evolved through multiple gene duplication events, which suggests that individual enzymes have a particular function. However, expression data suggest a general function in growth and development and specialization by some of *PLL* genes (Sun and van Nocker, 2010). Majority of PLLs are expressed in roots, stems and leaves, with varying intensity. All PLLs are expressed in flowers (some exclusively), suggesting an important role there, and some lack expression just in stems and leaves. Fourteen of 26 *PLL* genes are expressed in pollen, with four showing high expression (Palusa *et al.*, 2007) suggesting a function in pollen germination and tube growth (Pina, 2005). According to promotor activity, abscission-related role of PLLs was proposed (Sun and van Nocker, 2010). Furthermore, a major number of PLL isoforms present in roots, as well as the high expression in auxin-treated roots, indicate a function in root differentiation (Sénéchal *et al.*, 2014). One of *PLL* genes, *PLL13* (also known as *PMR6* - Powdery Mildew Resistance6), is confirmed to be involved in disease susceptibility (Vogel, 2002).

Similarity of promoter activity between PG and PLL families and their common substrate specificity implies close function association between PLLs and PGs (Sun and van Nocker, 2010). For PGs, role in vegetative development of Arabidopsis has been documented, for example, overexpression of *PGX1* enhanced hypocotyl elongation (Xiao *et al*., 2014), and effect on stomata development in cotyledons (Rui *et al.*, 2017). Furthermore, roles in vegetative growth for PME and PMEI, which mediate DM and patterns of methylesterification, were identified. In transgenic lines, PME activity has been linked to root growth (Sénéchal *et al.*, 2014) and PMEI4 overexpression to the delay of growth acceleration in dark-grown hypocotyls (Pelletier *et al.*, 2010). In contrast, the role of PLLs in vegetative growth of Arabidopsis has not been characterized well and stays an open question for further research.

2.5. Objectives

The first objective of this thesis was to investigate the possible role of *PLL* genes in the vegetative growth of plant using the model *Arabidopsis thaliana*. It has been hypothesized that

activity of PLLs is involved in cell elongation and differentiation (Palusa *et al.*, 2007), and to assess that, two experimental growth models and loss-of-function mutants were used. Firstly, dark-grown hypocotyls were used because their lengthening is caused by cell elongation, rather than cell division (Gendreau *et al.*, 1997). Cell elongation in dark-grown hypocotyls involves two growth phases: an initial slow phase, and a faster phase (Figure 4). Moreover, a feature of dark-grown hypocotyls is the thinning and extensive cell wall remodelling that includes modulation of genes encoding pectin modifying enzymes (Figure 4; Refregier, 2004).

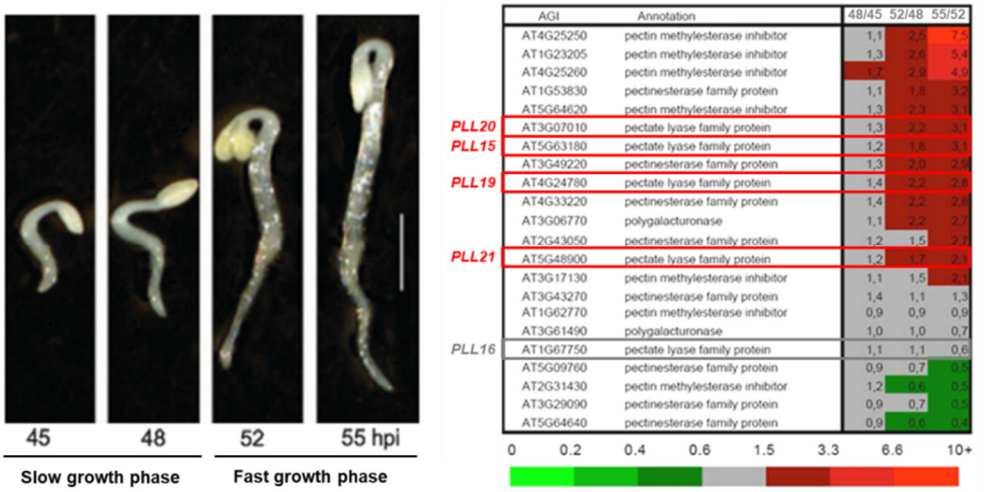

Figure 4 *Two phases of hypocotyl growth in dark and gene expression of pectin modifying enzymes in different hypocotyl growth phases, with PLLs showing upregulation marked in red (adopted from Refregier, 2004).*

As the other model, light-grown roots were used, a well-established model for developmental studies because of their cell expansion in the elongation zone and cell division in the meristematic zone especially the apical meristem (Scheres and Wolkenfelt, 1998).

The focus was on two Arabidopsis genes At3g07010 and At5g48900 coding for PLL enzymes PLL20 and PLL21, respectively (Figure 4, **Figure 5**A). These two proteins are closely related and show 85.1 % of sequence identity that could suggest common substrate specificities on cell wall pectin and similar function in plant regulation. Their promotor activity has been reported in roots of light-grown-seedlings (Sun and van Nocker, 2010) (**Figure 5**B) and, according to the publicly available transcriptomic data, those genes are two of 11 PLL genes detected to be co-expressed in seedlings, hypocotyl, leaf and root organs (Sénéchal *et al.*, 2014). Moreover, increased expression of PLL20 and PLL21 has been noticed in transition from a slow to fast growth phase in dark-grown hypocotyls (Figure 4B, Pelletier *et al.*, 2010), which could have an impact on cell wall remodelling that enables hypocotyl elongation.

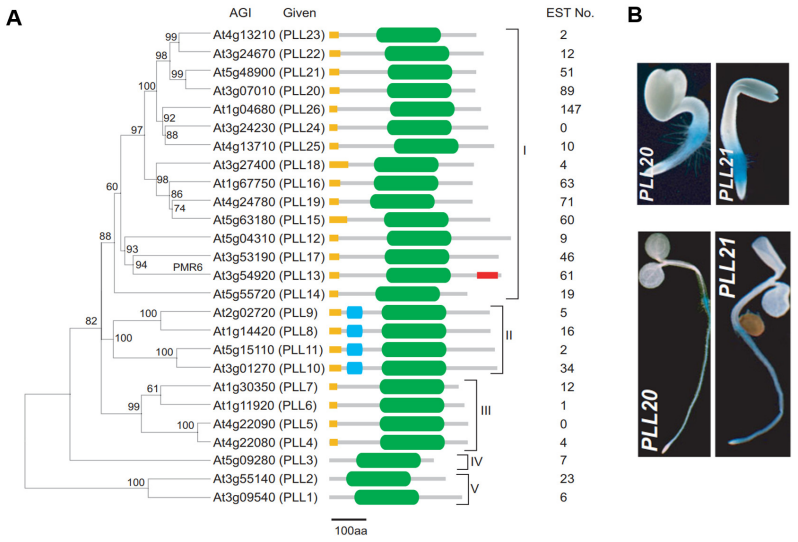

Figure 5 A) *Phylogenetic analysis of Arabidopsis PLL amino acid sequences* ***B)*** *PLL::GUS expression in seedlings (Sun and van Nocker, 2010)*

Since the biochemical properties of plant PLL enzymes have not been reported, their production in high-yield expression systems as yeast *Pichia pastoris* and bacteria *E. coli* were the second objective of the thesis. The goal was to determine if plants such as Arabidopsis show PL or PNL activity. In the host laboratory, expression of PLL12, 15, 16, 19, 20 and 21, which all belong to the same cluster (**Figure 5**A), has been tried in *Pichia pastoris* without success. In this study we tried expression of PLL4 that shows 48.5 % of sequence identity to PLL20 and PLL21. Its different structure positions it in another cluster, making its sequence possibly more adapt for expression in yeast. In parallel, previously attempted expression of PLL21 was retried in another strain of *E. coli*.

3. Results

3.1. Expression of PLLs in dark-grown seedlings and light-grown roots

To characterize the temporal expression of PLLs in organs growing, RT-qPCR was performed using RNA extracted from 2, 3, and 4-day-old dark-grown seedlings, as well as 4, 7 and 10 day-old light-grown roots. In dark conditions, hypocotyl is the organ showing a strong elongation. However, it was not dissected for the RT-qPCR because roots and cotyledons are less developed than the hypocotyl in the dark, and it is accepted that the quantified transcripts in the whole seedling mainly reflect hypocotyl. Under the light, the abundance of transcripts in root, hypocotyl and cotyledons are more important. Thus for investigating PLL expressions, especially in root, hypocotyl and cotyledons were discarded.

In the dark, the highest and the most constant relative expression was noted in At1g67750 (*PLL16*) (Figure 6A). Genes At3g55140 (*PLL2*), At1g04680 (*PLL26*), At3g24670 (*PLL22*) and At3g53190 (*PLL17*) showed around 1 order of magnitude lower, but also a relatively constant expression comparing all time points. Genes At4g22090 (*PLL5*), At3g27400 (*PLL18*) and At4g22080 (*PLL4*) showed the highest expression in two-day-old seedlings, which proportionally declined over three- and four-day time points. Regarding the genes whose loss-of-function mutants were studied in this report, At3g07010 (*PLL20*) was more expressed than At5g48900 (*PLL21*), but both showed similar expression patterns, with the expression at the second day slightly lower than those of the third and fourth day.

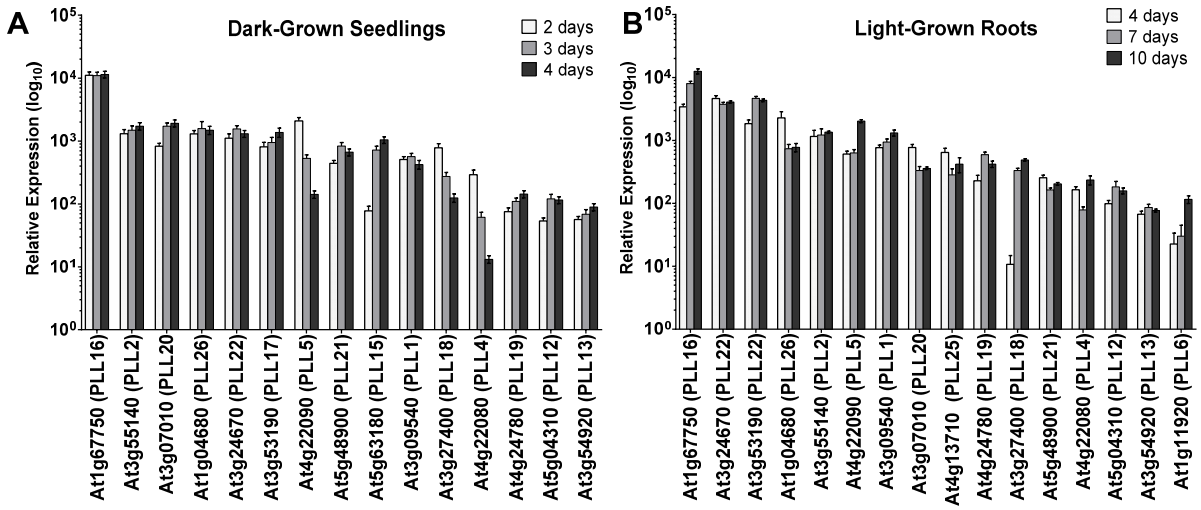

Figure 6 *PLL expression in* ***A)*** *dark-grown seedlings and* ***B)*** *light-grown roots; n=2, means ± confidence interval; normalization according to At4g26410 in dark-grown seedling and At5g25760 in light-grown roots, the same trends observed with another reference gene*

In light-grown roots, as in dark-grown seedlings, At1g67750 (*PLL16*) showed the highest expression (**Figure 6**B), but with a slight increase over time. Other PLLs showed relatively comparable expressions over time, with the expressions of At4g22090 (*PLL5*) and At1g11920 (*PLL6*) maxing out at 10 days, and the expression of At1g04680 (*PLL26*) being the highest on the fourth day. The most prominent difference was noticeable in the expression of At3g27400 (*PLL18*), which was about 3 orders of magnitude lower on the fourth, than on the seventh and tenth day. Interestingly, *PLL20* and *PLL21* showed opposite trends in their root expression patterns, in comparison with the dark-grown seedlings, with a slight decrease of the expression from the fourth to the tenth day.

3.2. Structure of the selected genes and information about the T-DNA insertions lines

In this study three allelic mutations of *PLL21* were used. Insertion line Salk_128674 was named *pll21-1*, Salk_017091 was named *pll21-2* and Sail_674G05 was named *pll21-3* (**Figure 7**A). The double mutant was named *pll20/pll21* and it carried insertion Salk_128674 present in the *pll21-1* mutant, and Sail_148H03 (*pll20*) insertion. All insertion lines were checked to assure presence of the insertion and homozygosity by PCR genotyping.

RT-qPCR, using RNA extracted from 4 day-old dark-grown seedlings, was done for *pll21* allelic mutants. Relative expression values show that *pll21-1* and *pll21-2* are knock down mutants with *pll21-1* showing significantly lower expression compared to the wild type, while *pll21-2* shows less prominent difference (**Figure 7**B). Mutant *pll21-3* has the insertion in the promotor (**Figure 7**A) that caused a slight overexpression compared to the wild type. Study would be more complete with a single mutant that carries the same *pll20* mutation as the double mutant, however, seeds of that mutant failed to germinate.

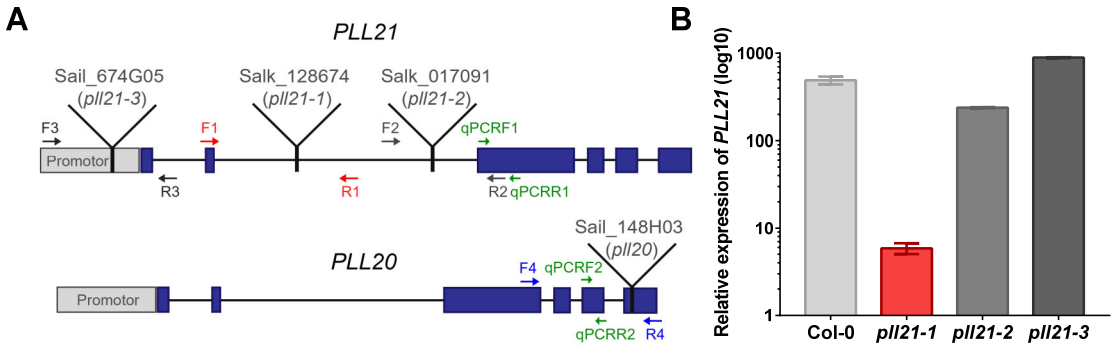

Figure 7 A) *Scheme of T-DNA insertion lines and primers used for genotyping and qPCR;* ***B)*** *RT-qPCR in 4-day-old dark-grown seedlings, normalization according to At4g26410, the same trends observed with another reference gene*

3.3. Growth of hypocotyl and primary root in *pll21* and *pll20/pll21* mutants

3.3.1. Lengths of 4 day-old dark-grown hypocotyls and 10 day-old light-grown roots

Since etiolated hypocotyls undergo cell extension without the cell division, accompanied by a substantial cell wall remodelling (Pelletier *et al.*, 2010; Refregier *et al.*, 2004), dark-grown hypocotyls were used to study the impact of *PLL* mutations on the growth. Plants were grown in the dark at 21 °C and after 4 days photographed and hypocotyls were measured. Hypocotyls of the Arabidopsis seedlings carrying *pll21-1* mutation showed 5,3 % shorter length (**Figure 8**A) when compared to the wild type Col-0. Other allelic mutants, *pll21-2* and *pll21-3,* did not show significant difference from the wild type (**Figure 8**A). Interestingly, the *pll20/pll21*double

mutant also did not show significant change in the hypocotyl length when compared to the wild type (**Figure 8**A), possibly implying an opposite role in cell elongation of *PLL20* which should be further investigated with *pll20* single mutant.

Rate of cell expansion is a major factor in morphogenesis and it dictates the shape of the cell, but also of the organism. Expansion in the elongation zone of Arabidopsis is well described and used in genetic studies on cell expansion (Scheres and Wolkenfelt, 1998). Consequently, Arabidopsis primary roots were used as another model to investigate the impact of the *PLL* mutations on the growth. Plants were grown in long day photoperiod (16 h day/8 h night) at 21 °C, after 10 days they were photographed and primary roots were measured. Overall, phenotype was more prominent in roots than in hypocotyls (**Figure 8**B). As in hypocotyls, *pll21-1* mutant showed the most prominent phenotype, namely a decrease of 20 % in the length of 10-day-old roots. Mutant *pll21-3* did not show significant difference, while *pll21-2* mutant had ~11 % shorter roots than the wild type with less statistical significance (**Figure 8**B). These results were in accordance with the level of *PLL21* expression in the *pll21* mutants (**Figure 7**BS). In contrast to the lack of phenotype in the hypocotyls, the double mutant *pll20/pll21* showed a length decrease of ~7.5 % compared to the wild type (**Figure 8**B).

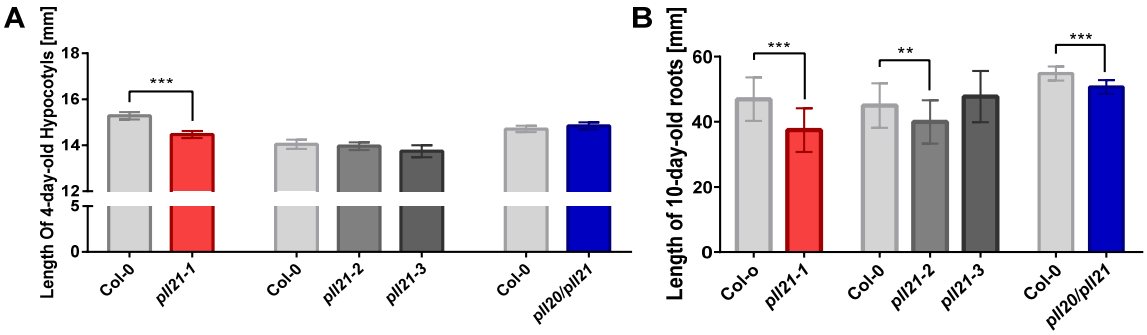

Figure 8 A) *Length comparison of wild type and mutant:* ***A)*** *4-day-old dark-grown hypocotyls, 160<n<277;* ***B)*** *10-day-old light-grown roots 21<n<54; means ± confidence interval; t-test: without annotation – p>0.05; ** p<0.01; *** p<0.001*

3.3.2. Growth kinetics of dark-grown hypocotyls and light-grown roots

Measuring plants at one time point does not provide conclusive evidence about the difference between mutants and the wild type because they could have different growth rates over time and/or because the phenotype could be due to delayed germination. To have a better insight of the growth kinetics of the mutants, automated plant phenotyping system called PhenoBox was used. In this and further experiments, *pll21-2* and *pll21-3* were not studied because of the less significant difference in expression from wild type (**Figure 7**B), lack of phenotype in hypocotyls (**Figure 8**A), and less difference in root length (**Figure 8**B).

To phenotype dark-grown hypocotyls, the same growth conditions as used in classical incubators (dark and constant 21 °C), were used in PhenoBox. The robot was set to make measurements every two hours, what required illuminating plates with green light to acquire pictures. Hypocotyl growth curves of *pll21-1* and the wild type were almost identical (**Figure 9**), what was in contrast with the previous shorter length observed in 4-day-old hypocotyls grown in a classic incubator. The double mutant *pll20/pll21* had a hypocotyl growth curve no different from the wild type, correlating with the previous phenotyping results (**Figure 8**A).

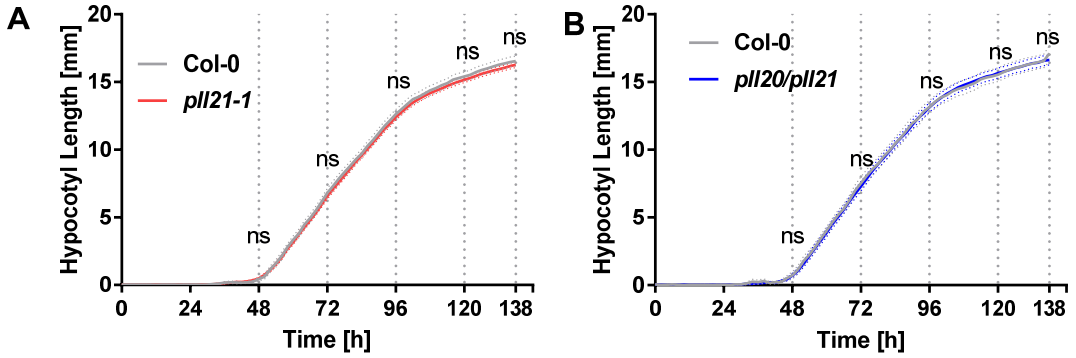

Figure 9 *Growth curves of dark-grown hypocotyls of* ***A)*** *pll21-1 mutant, 53<n<180; and* ***B)*** *pll20/pll21 mutant, 28<n<56; means ± confidence interval; t-test: ns – p>0.05*

Mutant *pll21-1* showed overall slower light-grown root growth rate than the wild type with a statistically significant difference starting immediately at the first chosen time point of 24 hours (**Figure 10**A). From the 48th hour to the end of the experiment, the percentage of difference progressively increased, being ~17 % at the 48th h, ~22 % at the 72nd h, ~30 % at the 120th and then growing by ~1% every 24 hours, reaching ~36 % in the end, more than ~20 % observed in usual phenotyping after 10 days (240 hours). The double mutant did not show so conclusive data in the time points up to 192 hours, but then in the 216th hour it had around 8 % and in the final 240th hour 10 % shorter length compared to the wild type (Figure 10B) which is similar to the decrease of 7.5 % noticed with manual measuring after 10 days (**Figure 8**B).

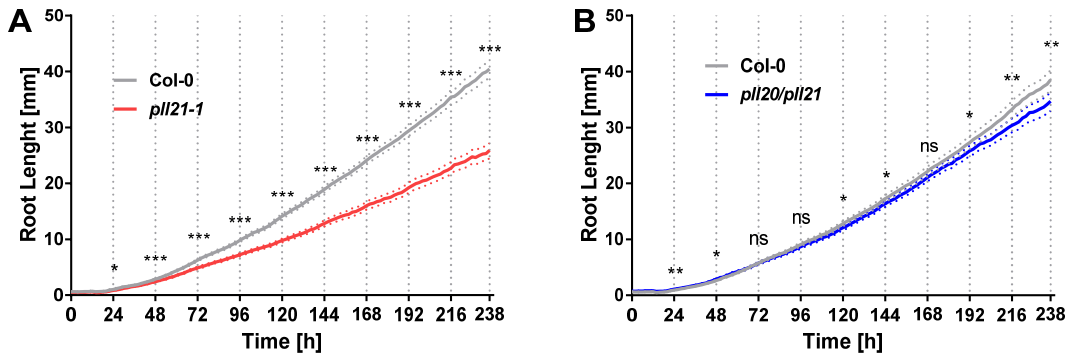

Figure 10 A) *pll21-1 and* ***B)*** *pll20/pll21 growth curves of light-grown roots; 55<n<68; means ± confidence interval; t-test: ns – p>0.05; ** p<0.01; *** p<0.001;*

3.3.3. Comparison of the dark-grown hypocotyl length in the classical incubator and PhenoBox for the *pll21-1* single mutant

As visible from previous data, mutant *pll21-1* showed shorter dark-grown hypocotyls when grown in an incubator, but not when grown in PhenoBox. The only apparent difference between the two growing systems is the number of seedlings per plate and the illumination of green light every two hours in PhenoBox. Thus, an experiment was conducted to assess the impact of these differences, which could explain the lack of the phenotype for *pll21-1* in the PhenoBox. Fifteen seeds of the *pll21-1* mutant and the related WT were sown on individual plates for growth in both systems, but now the plates dedicated for PhenoBox were wrapped in aluminium foil as the ones dedicated for the classic incubator.

When wrapped in foil, *pll21-1* mutant showed shorter length in PhenoBox, indicating that the presumably safe green light could have caused the loss of the phenotype (**Figure 11**A). Relative expression of PLL21 in dark and light-grown seedlings show upregulation of expression in the dark (**Figure 11**B).

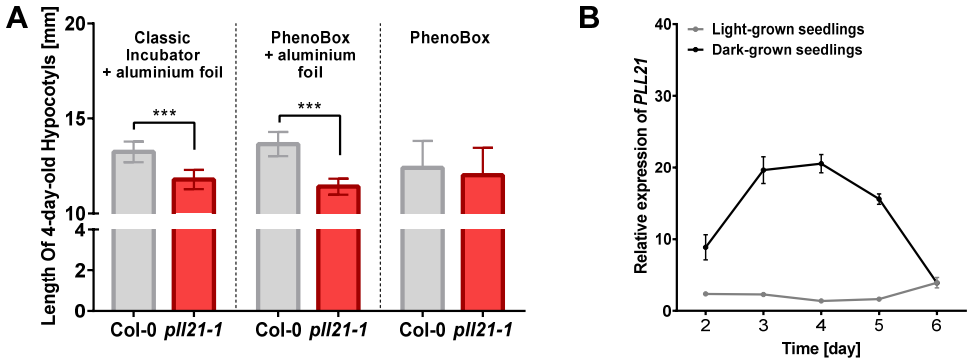

Figure 11 A) *Length comparison of 4-day-old hypocotyls grown wrapped in aluminium foil in a classic incubator and PhenoBox (28<n<30) and in PhenoBox without aluminium foil (53<n<180) (data extracted from the growth curve); means ± confidence interval; t-test: not annotated – p>0.05;* ***B)*** *Relative expression of PLL21 in dark and light grown seedlings, means* ± SEM, normalization according to At4g26410, the same trends seen with At3g33380

3.4. Cell wall pectin fingerprint in 4 day-old dark-grown hypocotyls and 10 day-old light-grown roots for *pll21-1* and *pll20/pll21*

To correlate changes in growth with cell wall pectin modifications, they were investigated in the *pll21-1* and *pll20/pll21* mutants. From the seedlings used for measuring, hypocotyls or roots were dissected and subjected to digestion by Aspergillus PG, generating oligosaccharides, which were isolated and analysed using LC-MS. The pectin structure determines the OGs produced by the AspPG based on its ability to cut HG if at least two consecutive de-methyl esterified GalA are present (Kim *et al.,* 2017). This enables us to obtain the pectin fingerprint giving information about the amount and the type of pectin. Since this experiment included just

three or less technical replicates, statistical analysis was not made, rendering these results just an indication of the trends.

In comparison with the wild type, more OGs were generated in the *pll21-1* hypocotyls (**Figure 12**A), that indicates that AspPG had more substrate to digest, pointing to a greater amount of pectins present in *pll21-1* which could be due to less activity of pectin-degrading enzymes. The double mutant does not show difference compared to the wild type, correlating with no difference in length previously observed (**Figure 8**A and **Figure 9**B).

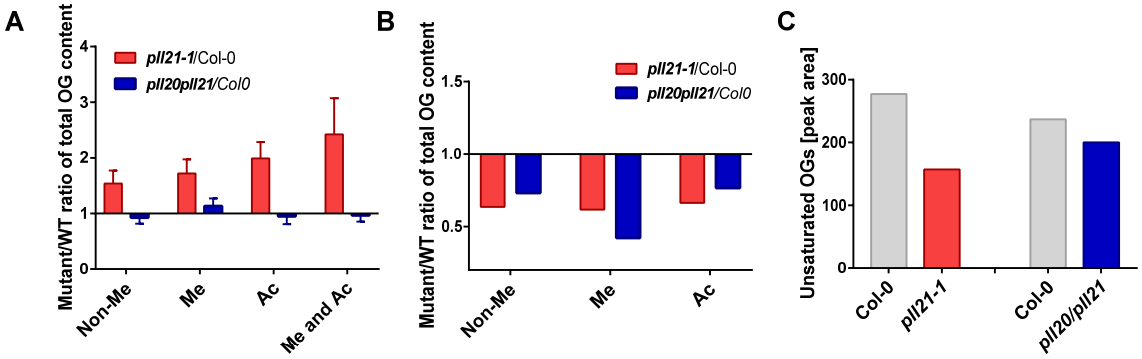

Figure 12 *Difference in OG content between mutants and wild type expressed as ratio in* ***A)*** *4 day-old dark-grown hypocotyls (2<n<3) means ± confidence interval* ***B)*** *10-day-old light-grown roots (n=1), means; Non-Me – non-methylesterified OGs, Me – methylesterified OGs Ac – acetylated* ***C)*** *Difference in unsaturated OGs between mutants and wild type in 10-day-old light-grown roots; means; n=1*

In light-grown roots, pattern is opposite to the hypocotyls. Here, we see a decrease in total amount of OGs both in the single and the double mutant pointing to a decrease of the available pectic substrate for AspPG, putatively due to an increase in endogenous activity of pectin-degrading enzymes (**Figure 12**B). Interestingly, the decrease in methylesterified OGs in the double mutant is the most prominent one, while in *pll21-1* decrease between different kind of OGs is comparable. These results suggest differences in the endogenous activity of pectin remodelling enzymes between *pll21-1* and *pll20/pll21*. In roots, compared to the hypocotyls, the amount of OGs containing an unsaturated bound produced by PLL activity, was high enough for quantification (**Figure 12**C). Both mutants showed a decrease in unsaturated OGs, suggesting a decreased PLL activity, more notable in *pll21-1* than *pll20/pll21,* reflecting the difference of root length (**Figure 8**B, **Figure 10**). However, these decreased activities are in contradiction with the lower pectin content obtained by digestion with AspPG.

3.5. Relative expression of PLLs in 3 day-old dark grown hypocotyls and 7 day-old light roots for *pll21-1* and *pll20/pll21*

To reveal changes of *PLL* expressions in the *pll* mutants showing interesting modifications of pectin fingerprint and growth, RT-qPCR was made to investigate the difference in the

expression of *PLLs* in the wild type and *pll* mutants. For the dark grown seedlings, the 72-hour time point was chosen for comparison, to capture the expression profile that could have led to shorter length observed after 96 hours in phenotyping. When comparing data of relative expression, ratios of the mutant over wild type expression greater than 2 and lower than 0.5 are regarded as significant. In the single mutant *pll21-1*, only the mutated *PLL21* gene showed a significant difference, with the expression about 40 times lower than the wild type. The double mutant showed the expression of *PLL21* around 10 times lower, while the impact of the *pll20* mutation was not visible because the qPCR primers used to amplify *PLL20* are located before the insertion (**Figure 7**A, **Figure 13**). However, the mutant showed an overall higher expression of *PLLs* with the genes At4g22090 (*PLL5*), At4g22080 (*PLL4*), At1g11920 (*PLL6*), and At1g14420 (PLL8) having significantly higher expression, indicating a possible compensatory mechanism due to the lack of two *PLLs* in the double mutant.

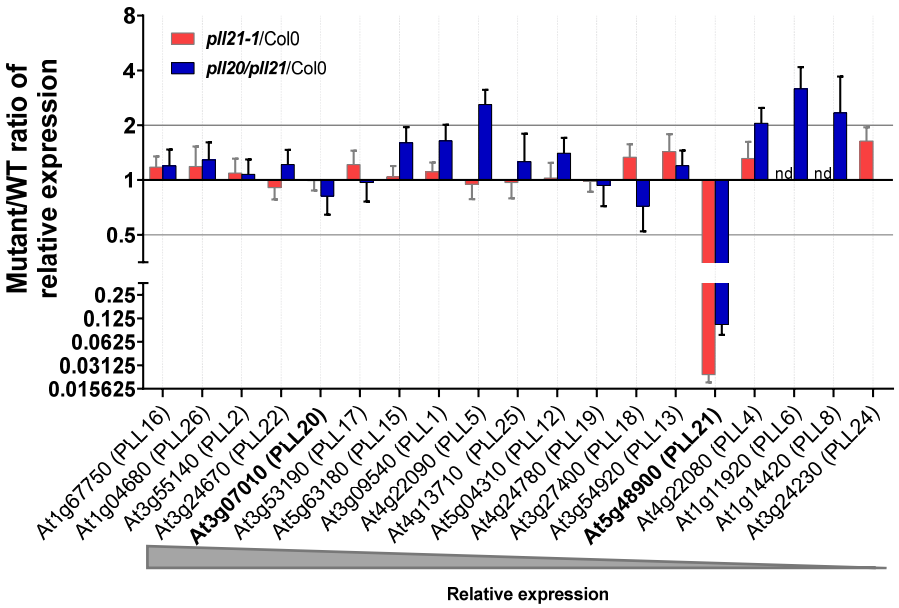

Figure 13 *Comparison of relative expression in mutants and wild type in 3-day-old dark-grown seedlings; means ± confidence interval; normalization according to At4g26410, the same trends observed with another reference gene*

For the same reasons as for the dark grown seedlings, the 7-day-time point was chosen for the light-grown roots. Here, *pll21-1* mutant showed a decrease in expression of the usually most expressed *PLL* gene, At1g67750 (*PLL16*) having the ratio just on the verge of significance (**Figure 14**). A decrease (of around 8 times) was also visible in At5g55720 (*PLL14*), a gene with the lowest relative expression. Relative expression of At5g48900 (*PLL21)* was around 10 times lower than in the wild type.

The double mutant showed a different trend with a significant increase of expression in as much as 6 *PLL* genes At3g53190 (*PLL17)*, At4g22090 (*PLL5)*, At3g27400 (*PLL18)*, At4g22080 (*PLL4),* At1g11920 (*PLL6*), and At3g24230 (*PLL24)*. Besides a decrease (around 12 times) in

the mutation affected *PLL21*, a smaller (2.7 times) decrease in the relative expression of At1g04680 (*PLL26)* gene was also visible (**Figure 14**).

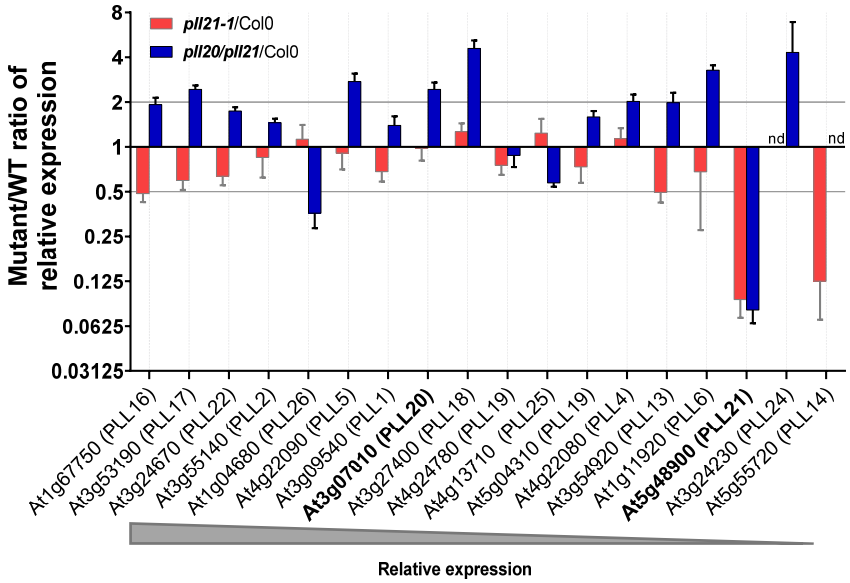

Figure 14 *Comparison of relative expression in mutants and wild type in 7-day-old light-grown roots; means ± confidence interval, normalization according At5g25760, the same trends observed with another reference gene*

3.6. Heterologous expression of PLLs in eukaryotic and prokaryotic systems

In order to biochemically characterize Arabidopsis PLLs for the first time, heterologous expressions were tried in two different expression systems: *Pichia pastoris* yeast and *Escherichia coli* bacterium.

3.6.1. Heterologous expression in *Pichia pastoris* yeast

To express *PLL4* gene using *Pichia pastoris* yeast, PLL4 CDS amplified from flower cDNA was cloned in pPICZα expression vector which carries a sequence for a signalling peptide, and a HIS-tag. The signalling peptide allows secretion of the heterologous protein in the medium, and the HIS-tag allows its detection using anti-HIS Western-blot, as well as purification using nickel affinity chromatography. Expression vector was amplified in *E. coli*, and then *P. pastoris* yeast was transformed with the linearized and purified plasmids. All five positive clones were tested for production of PLL4 by anti-HIS Western-blot using non-concentrated and concentrated supernatant of culture medium. Unfortunately, none of the clones showed production and consequently purification and activity tests were not performed.

3.6.2. Heterologous expression *Escherichia coli* bacterium

Expression has previously been tried in BL21 *E. coli* strain, but without success. Protein degradation was substantial and the amount of the expressed protein was too small to visualize

on SDS-PAGE with Coomassie staining. However, Western-blot using anti-HIS antibodies showed a band of the expected PLL21 size but no PLL activity was detected. In order to biochemically characterize PLL21, heterologous expression in a new *E. coli* strain Rosetta2 has been conducted, using the same expression vector. This strain has a plasmid that supplies tRNAs for rare codons and this could improve translation as well as PLL21 folding and ultimately yield (Novagen, 2004).

After overnight production, proteins in the total and soluble fractions were assessed using SDS-PAGE and western blot using anti-HIS. A signal at the expected size was only detected in the total and soluble fractions of the clone expressing PLL21, and not in the protein fractions from bacteria transformed with the empty vector. Thus, from the soluble fraction, a crude purification using Ni-NTA beads was performed with a concentration step during the elution of PLL21. Then, SDS-PAGE was done with elutions to assess success of the purification and the amount of PLL21. Coomassie staining of a SDS-PAGE gel did not show a band corresponding to the expected PLL21 size of 53.4 kDa (**Figure 15**A), so silver staining was performed on the same gel to check if PLL21 can be detected using a more sensitive staining method. A slight band at approximately the right size is visible in first elution lane (E1, **Figure 15**B) in the culture with the vector carrying PLL21 gene insert, but not in the culture with an empty vector (**Figure 15**B). In accordance with silver-staining, Western-blot showed anti-HIS signal only in the culture harbouring the vector carrying PLL21 gene insert (**Figure 15**C). The highest intensity band was present in the first purification elution (E1) along several other bands implying putative protein degradation due to over activities of proteases, which is a common problem in *E. coli* expression system (Murby *et al*., 1996) and/or some unspecific bindings of the anti-HIS antibody.

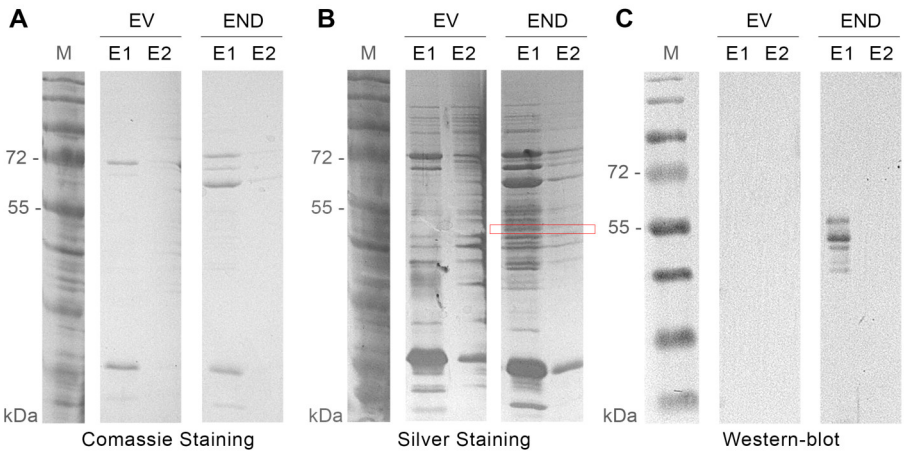

Figure 15 *Coomassie* ***(A)*** *and silver* ***(B)*** *staining gel of SDS-PAGE gel and* ***(C)*** *Western blot of elution fractions; EV – empty vector; END – pET30a(+)PLL21EXP-END; E1 – first purification elution with 250 mM imidazole: E2 – second purification elution with 500 mM imidazole*

Even though the amount of the expressed protein presumed to be PLL21 is minute (only detected with silver staining and western-blot), a PL activity assay was conducted with the first elution purified fraction and pectin with low DM (20-34%) as substrate. The results did not show any PL activity, while the control commercial PL showed a normal activity. The lack of activity is presumably due to the too small amount of the enzyme in the reaction, but also possible wrong conformation of the enzyme (not active PLL21) or the combination of the two.

4. Discussion, Conclusion and Perspectives

4.1. Discussion

The objective of this study was to investigate the possible role of PLLs in Arabidopsis vegetative growth with a focus on two closely related proteins: PLL20 and PLL21. Different patterns of expression in PLL family suggest a different role in the development. For example, dark-grown seedlings show the highest expression of *PLL4* and *PLL5* on the 2nd day indicating the involvement of these genes in the early stages of the development, possibly elongation of the hypocotyl responsible for the completion of germination (Sliwinska *et al*., 2009). On the other hand, a lower expression of *PLL20, PLL21, PLL12* and especially *PLL15* on the 2nd day followed by an increase on the 3rd day, could be involved with the transition from the slow to the fast growth phase in dark-grown hypocotyls (Refregier *et al.*, 2004).

Loss-of-function mutant *pll21-1*, compared to Col-0, showed a decrease in dark-grown hypocotyl length when grown in a classic incubator. However, when grown in the PhenoBox system, the phenotype was lost. A possible explanation could be that the green light used to illuminate plates for photo acquisition affected the growth trends. It has been found that green light promotes faster early hypocotyl growth, which however, results in the same hypocotyl length as the dark grown seedlings after 24 h. Authors explained this by proposing that seedlings reach terminal expansion for their developmental state earlier, but then stagnate (Folta 2004; McCoshum and Kiss, 2011). Also, authors hypothesized that green-light illuminated plants take on semi-skotomorphogenic strategy combining tendencies of dark and light development (Folta and Maruhnich, 2007).

Since the PhenoBox system uses a wide spectrum green light (with a small intensity of blue and yellow wavelengths), correlation with impacts of low intensity blue light (LBL) can be made. It has been shown that under LBL, some Cryptochromes (CRYs) could interact with some Phytochrome Interacting Factors (PIFs) such as PIF4, increasing transcription of genes that promote hypocotyl elongation. In our case, this is interesting because Chip-sequencing

analysis revealed that 50 % of *PLL* genes are putatively targeted by PIF4 (Pedmale *et al.*, 2016) suggesting a strong light dependency of this family. In addition to that, our RT-qPCR analyses for *PLL21* have shown that *PLL21* is highly expressed in the dark, while its expression stays limited under white light (**Figure 11**B) where it shows the same hypocotyl growth as the WT (data not shown). Under white light phytochrome B (phyB) protein leads to degradation of PIF4, preventing transcription of hypocotyl elongation promoting genes. The situation is opposite under shade containing low red/far red light ratio that inactivates phyB, activating PIF4, and allowing modulation of its activity by LBL, as mentioned before. PIF4 is also active in the complete darkness, when plants are highly sensitive to light, and a slight change in intensity and composition of light could affect them.

According to the results, it appears that the *pll21-1* mutant is highly sensitive to green light pulses and it is possible that they are enough to change the balance in light-dependent pathways of CRY and PIF4, resulting in the loss of phenotype. Also, recently it was shown that pectic fragments called OGs, generated by PLLs and PGs, could be involved in some signalling pathways, connected to the PIF regulation, to promoting skotomorphogenesis (Sinclair *et al.*, 2017). Theoretically, the *pll21-1* could lack the same kind of OGs, impairing this signalling pathway and leading to an oversensitivity for the green light in the mutant.

Double mutant *pll20/pll21* did not show differences in dark-grown hypocotyl length and OG content while the *pll21-1* mutant showed shorter hypocotyls and an increase in the OG content (in complete darkness in a classic incubator) pointing to a higher pectin amount (less endogenously degraded) available for digestion with the exogenous AspPG. Correlating that to expression, the only difference seen in *pll21-1* was a significant decrease of *PLL21* expression, while the double mutant showed a slight increase in the expression of some PLLs. Those variations could indicate the presence of a compensatory mechanism that explains both the lack of changes in pectin content (higher PLL activity degrading more pectin endogenously) and the lack of morphological phenotypes when PLL20 and 21 are mutated together. Lack of phenotype in Arabidopsis loss-of-function mutants is frequent and attributed to the ability of plants to adapt, and the redundancy of Arabidopsis genome (Bouché, 2001). Absence of compensatory trend in *pll21-1* and presence in *pll20/pll21* suggests that the double mutation triggered compensation, while the single mutation was not enough. Another explanation could be that the lack of PLL20 causes an opposite impact on hypocotyl length and OG content, making the double mutant more similar to the wild type.

Regarding light-grown roots, we can see a decrease in their length independently of the system used for phenotyping. The obtained growth curve shows a progressive decrease in length, proving that the difference in length arises from a defect in growth, rather than germination. Contrary to the hypocotyl, root growth in *pll21-1* does not appear to be affected by the green light pulses occurring only during the night period. Ten-day-old light-grown roots of *pll20/pll21* showed the length decrease less substantial than the one in *pll21-1* mutant and an overall increase of PLL expression (more prominent than in hypocotyls) which is not the case in *pll21-1*.

In the *pll20/pll21* mutant, that over-expresses some PLLs, the PLL activity should be higher than the one in *pll21-1*. Detection of the unsaturated OGs in the roots (Figure 8C) seems to validate this hypothesis with a reduction of the unsaturated OGs in the *pll* mutants compared to their associated WT, a smaller decrease in the double mutant suggests a higher PLL activity in *pll20/pll21* than in *pll21-1*. If the PLL activity appears to be decreased in such mutants, the amount of pectin should be increased, but here it is not the case. Thus, an overall decrease in the OG content both in single and double mutant indicates a more complex compensatory mechanism in roots than in hypocotyls; possibly including PG family that shares the same role of cleaving HG and it is confirmed to be involved in cell elongation (Xiao *et al.,* 2014). Changes in the PLL activity could induce overexpression of PGs and increase PG activity to regulate the pectin content.

By focusing on *pll21-1* mutant and comparing organs, contradictory changes can be highlighted. Namely, opposite variations in pectin content lead to the same growth consequences. This could be explained by the endogenously generated OGs (acting as a signal molecule) rather than the pectin amount itself. The decrease/increase of the pectin content reflects changes in the endogenous PLL/PG activities, which can qualitatively differ in such organs, thus generating various types of OGs and triggering different signalling pathways.

4.2. Conclusion and Perspectives

In this study, which aimed to show the connection of PLLs and vegetative growth, *pll21-1* mutation was shown to be associated with a defect in growth of light-grown roots, and hypocotyls grown in complete darkness. Surprisingly, presumably safe green light showed to have an impact on *pll21-1* mutant's hypocotyl growth, equalizing it with the with wild type. This finding opens questions about the regulation of PLLs by light and indicates that impacts of green light on plant growth should be taken into consideration in experiments conducted in

dark conditions. For deeper investigation of PLL21 regulation by light, some mutant backgrounds impaired in the early molecular events of the light regulation, such as *pif4*, should be investigated.

Furthermore, expression data and phenotyping of the double mutant *pll20/pll21* point to compensatory mechanisms between members of PLL family, and possibly in-between PG and PLL families. In this context, the expression of PGs and PG activity should be further investigated in *pll* mutants. Also, *pll20* single mutant needs to be investigated to elucidate the impact of lack of PLL20 on phenotype and possible compensatory mechanisms described.

Trends of decrease in the content of unsaturated OGs in mutants point to a possibility of quantifying PLL activity by quantifying OGs after digestion with AspPG. PGs are not able to generate unsaturated bonds but they can hydrolyse bigger unsaturated OGs that become detectable using LC-MS. More research on this topic will be done with the goal of developing a method that can quantify PLL activity *in vivo* by directly seeing their end-products. However, detecting the end-products does not inform us clearly about the substrate and pH specificity. Thus, an improved *in vitro* method for quantifying PLL activity from a cell wall-enriched protein extract on commercial pectic substrates should be used to determine if PLLs in Arabidopsis are PL and/or PNL.

Heterologous expression of PLLs proved to be very challenging, and in this study, neither *P. pastoris* nor *E. coli* expression systems showed to be effective. *E. coli* was a bit more successful with a signal in Western-blot, but the protein band was visible only with silver staining, and purified fraction did not show PL activity. Such a low amount of protein suggests that further optimization of expression could be made, however, production of PLL in the amount needed for extensive biochemical characterization, let alone crystallization, is highly unlikely in *E. coli*. Thus, the expression of PLL21 in other systems such as insect cells (coll. Julia Santiago, Univ. Lausanne) and Arabidopsis roots (coll. Team III, BIOPI) is in progress.

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6. Annexe

6.1.Abbreviations

AGP - ArabinoGalactan Proteins

AspPG - Aspergillus PolyGalacturonase

CDS - Coding Sequence

CRY - Chryptocrhome

DM - Degree of Methylesterification

EXT - extensins

GalA - Galacturonic Acid

GAUT - GAlactUronosyl Transferase

HG - HomoGalacturonan

HGMT - MethylTransferases

HGPR - Hydroxyproline-rich Glycoprotein

LBL - Low (intensity) Blue Light

OG - Oligogalacturonide

PAE - Pectin AcetylEsterase

PG - PolyGalacturonase

phyB - Phytochrome B

PIF - Phytochrome Interacting Factor

PL - Pectate Lyase

PLL - Pectate Lyase-Like

PME - Pectin MethylEsterase enzyme

PMEI - Pectin MethylEsterase Inhibitor

PNL - Pectin Lyase

PRP - Proline-Rich Protein

RG I - Rhamnogalacturonans type I -

RG-II - Rhamnogalacturonans type II

SDS PAGE -Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

XGA - Xylogalacturonans

6.2. Materials and Methods

Arabidopsis thaliana seeds sterilisation

Up to 50 mg of *Arabidopsis thaliana* seeds were shaken for 15 minutes in 1 mL of sterilization solution (70 % (v/v) ethanol, 0.05 % Triton X-100). The sterilization solution was removed, 1 mL of 96 % ethanol was added and tubes were again shaken for 15 minutes. Ethanol was removed in a laminar flow hood and the seeds were washed with 1 mL of sterile water three times, leaving the seeds in the last sterile water. Seeds were stored on 4 °C for one day to start the stratification.

Growth conditions in incubators

To induce hypocotyl growth, around 100 *Arabidopsis thaliana* seeds per genotype were sown on Arabidopsis medium plates (Duchefa Biochemie, Milieu Arabidopsis) stratified for three days at 4 °C, exposed to light for 6 h at 21 °C and grown in dark at 21 °C. For root growth around 9 *Arabidopsis thaliana* seeds per genotype were sown on half-strength Murashige and Skoog medium (Duchefa Biochemie, MS Basal salt mixture). Growth conditions were 16 h of photoperiod at 120 mol m^{-2} s^{-1} 1 and 21 °C for 10 days.

Measuring of hypocotyls and roots

After the specified time of growth, plates were photographed on a fixed rack with a reference scale. Measurement for hypocotyls was done using ImageJ software and the roots were measured using NeuronJ update for ImageJ. When generating seed stock, mutant and WT plants were grown in the same trays, ensuring exactly the same growth conditions. All mutants had Col-0 background and they were compared to the related WT Col-0 (from the seeds of plants grown in the same tray).

Automated plant phenotyping (PhenoBox)

The same growth conditions and medium as in classic incubators were used in PhenoBox, but here, 15 seeds per plate were sown for dark-grown hypocotyl measuring, and 12 for light-grown root measuring. Automated picture acquisition and measuring took place every two hours, which for dark conditions required illumination by green light (broad spectrum green light). Dark-grown hypocotyls were grown for 7 days, and dark-grown roots for 10 days.

Preparation and digestion of plant cell wall for the oligoprofiling by mass spectrometry

Plant material was harvested and incubated at least overnight in Eppendorf tubes containing 1 mL of 100 % ethanol. Ethanol was removed, the plant material was incubated two times for 5 minutes in 1 mL of acetone and left to dry overnight on room temperature. The next day 135 µL of 100 mM acetate ammonium at pH 5 was added and the samples were rehydrated at 40 °C for a couple of hours. The volume of 15 µL of PG from *Aspergillus aculeatus* (E-PGALUSP, MegaZyme) (enzyme buffer was changed to acetate ammonium pH 5 using PD SpinTrap G-25 (GE Healthcare) by manufacturer's instructions) was added, and the samples were incubated overnight at 40 °C. After the incubation, 1 volume (150 µL) of 100 % ethanol was added, samples were centrifuged 5 minutes at 5 000 rcf, 200 µL of the supernatant was transferred into glass inserts, and completely dried in speed-vac concentrator at 45°C (Concentration plus, Eppendorf). Inserts were stored until analysis when 200 µL of dH_2O was used to dissolve the pellet. 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 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. Chromatograms and mass spectra were analysed using Mass Lynx v4.1 software. OG detections and peak area integration were done using Target Lynx tool in the software. Each sample was compared to the WT grown in the same plate and digested in the same batch of reactions.

Rapid DNA extraction from *Arabidopsis thaliana*

A small leaf (about 5 x 2 mm) was harvested and placed in a 1.5 mL Eppendorf tube containing 40 µL of 0.5x Direct-N-Lyse buffer (1.5 % SDS, 100 mM Tris pH 8.0, 25 mM EDTA, 2 M NaCl, 3 % Tween). Using a pestle, the leaf was ground, the tube was centrifuged at 14 000 rcf for 10 s, and then incubated at 95 °C for 10 minutes. After incubation, the tube was again centrifuged 1 min at 14 000 rcf. Dilution was done by taking 5 µL of supernatant, avoiding the plant debris, and adding it to 45 µL of dH_2O. The undiluted extract was stored at -20 °C, and the dilution at 4 °C.

Genotyping of insertion lines

PCR primers flanking the insertion and amplifying a sequence of 300 to 500 bp of the wild type gene were constructed. Amplification of a wild type of the gene works normally, but the amplification of the mutated gene is not possible due to 5 to 25 kb size of the insertion between the primers. To further confirm the presence of the insertion, another PCR reaction is conducted. It contains a primer binding to the left border of the insertion and on the complementary DNA strain outside of the insertion, allowing amplification of the sequence comprised of a part of the insertion, and a part of the gene. Thus, a plant homozygous for the wild type gene will only show amplification with the primers flanking insertion, while the plant homozygous for mutation will show just the T-DNA amplification. A heterozygous plant will show both amplifications.

PCR reaction consisted of 1 µL of 10 pmol/µL forward and reverse primer (Table 1), 2 µL of gDNA prepared by the described method, 4 µL of Pol 5x Master Mix Ready to Load (Euromedex) and water to 20 µL. Denaturation steps were conducted at 95 °C and elongation at 72 °C, annealing temperatures are noted in Table 1. Amplification was checked on 1 % agarose gel containing Midori Green Advance (NipponGenetics) and SmartLadder (Eurogentec) as a molecular weight marker.

RNA extraction and cDNA synthesis

Whole dark-grown seedlings grown for 2, 3 and 4 days at 21 °C were collected under green light, and roots from light grown-seedlings for 4, 7 and 10 days at 21 °C were excised. All surfaces and equipment were cleaned using RNAse away (Sigma). Plant material was frozen and ground in liquid nitrogen. The powder was added to 2 mL of TLES (100mMTris-HCl pH 8, 100 mM LiCl, 10 mM EDTA pH 8, 5 % SDS) buffer, vortexed, 200 µL of 20 % PEG 8000 was added followed by vortexing and incubating for 10 min at RT. The precipitate was lowered by centrifuging (10 min at 12 000 rcf) and the supernatant was collected. Two mL of phenol at 80 °C was added, tubes were vortexed for 30 s, then 2 mL of chloroform:isoamyl alcohol (24:1) was added and the tubes were again vortexed for 20 s. Centrifugation at 12 000 rcf at 4 °C for 10 minutes was performed and to the supernatant (aqueous phase), 2 mL of 4 M LiCl was added. After overnight incubation at 4 °C, centrifugation for 30 min at 4 °C was done. From this step, all manipulations were done on ice. The supernatant was removed and the pellet was dissolved in 125 µL of dH_2O, 12.5 µL of 3 M sodium acetate (pH 5.2) and 250 µL of 100 % ethanol was added, tubes were vortexed and incubated at -80 °C for an hour. After centrifugation (12 000 rcf, 4 °C for 20 min), ethanol was removed and the pellet was resuspended in 500 µL of 80 %

ethanol, and again centrifuged (12 000 rcf, 4 °C for 10 min). Ethanol was removed and when the pellet was completely dried, it was dissolved in 20 µL of dH_2O. Possible genomic DNA contamination was removed using TURBO DNA-free™ kit (Life Technologies) following the manufacturer's instructions. For cDNA synthesis, reverse transcription was performed using Transcriptor High Fidelity cDNA Synthesis Kit by the manufacturer's instructions using 4 µg of RNA as a template.

RT-qPCR

The mix for every different cDNA reaction contained 511 µL of SYBR Green Master Mix (ThermoScientific), 10 µL of cDNA, 279 µL of dH_2O. Pipetting was done by a robot, and each well in 384 well plate contained 7.2 µL of dedicated mix and 2 µL of 2.5 µM of dedicated primers. LightCycler® 480 system was used to conduct the reaction and measurement. The two most stable reference genes were chosen by using geNorm software (Vandesompele et *al.*, 2002). For roots At5g25760 (*PEX4)* and At4g34270 (*TIP41)* genes were used. For dark-grown seedlings At4g26410 (*RHIP1*) and At4g33380 genes were used. The first mentioned gene was used in graphs and second to confirm that the trends are the same.

High fidelity PCR

To amplify CDS of *PLL4* for cloning it into an expression vector, high-fidelity PCR amplification using a high-fidelity polymerase (Phusion Hot Start II DNA Polymerase, Thermo Fisher Scientific) was used. PCR amplification was conducted in 3 reactions of 50 µL containing 2 µL of cDNA, 10 µL of 5X Phusion HF Buffer, 0.4 µL of 10 mM dNTPs, 1 µL of forward and reverse primer (Table 2) and 14.5 µL of dH_2O. Denaturation steps were conducted at 98 °C and elongation at 72 °C, annealing temperatures are noted in Table 2.

DNA purification

DNA bands of the amplified CDS were excised from the agarose gel and purified using Gel Extraction Kit (NeoBiotech) according to manufacturer's instructions. Concentration of the samples was performed using speed-vac concentrator (Concentration plus, Eppendorf)

Digestion with restriction enzymes and ligation of the insert and the vector

After concentration, all of DNA was digested with 3 µl of NotI (#R01895) and EcoRI (#R0101S) in 1x CutSmart™ Buffer (New England Biolabs). Reaction lasted 2 h at 37 °C. Ligation with pPICZα B vector was conducted using 1 µl T4 DNA Ligase (Invitrogen), 1 µL of 10X T4 DNA Ligase Buffer and 100 ng already digested vector (1:3 ratio) in the total volume of 10 µL. Reaction was performed overnight at 4 °C.

E. coli TOP10 transformation and colony PCR

All steps were performed on ice. Aliquot of competent bacteria (45 µL) was added to 2 µL of the ligation mix. After incubation on ice for 20 min, heat-shock for 40 s at 42 °C was made, followed by 5 min incubation on ice, and addition of 250 µL of SOC medium (2 % (w/v) Tryptone 0.5 % (w/v) Yeast Extract. 10 mM NaCl, 2.5 mM KCl, 20 mM $MgSO_4$, 0.4 % (w/v) dextrose). Cultures were incubated at 37 °C for 1 hour, and they were spread on LB low salt plates (1 % (w/v) Tryptone, 0.5 % (w/v) NaCl, 0.05 (w/v) Yeast extract, 0.015 % (w/v) agar) plates containing Zeocin (25 µg/mL, Invitrogen) and incubated at 37 °C overnight. Grown colonies were picked on new plates, the tips used for that were placed in 20 µL of dH_2O and heated for 8 minutes at 95 °C. Colony PCR was performed to assure presence of the plasmid with the insert. PCR reaction contained 4 µL of Pol 5x Master Mix Ready to Load (Euromedex), 2 µL of DNA, 1 µL AOX5' and AOX3' primers and dH_2O to 20 µL. Denaturation steps were conducted at 95 °C and elongation at 72 °C, annealing temperatures are noted in Table 2.

MiniPrep and sequencing

A positive colony was inoculated in 5 mL of LB (LB Mix, Duchefa), and grown over-night. MiniPrep of the culture was done using illustra™ plasmidPrep Mini Spin Kit (GE Healthcare). Plasmid DNA was send to Eurofins Genomics for sequencing.

Pichia pastoris X33 transformation

MiniPrep of 40 mL of *E. coli* culture was prepared in the same way. Plasmid DNA was concentrated using speed-vac concentrator (Concentration plus, Eppendorf) and linearized with *PmeI* using the same protocol as above. Competent X-33 cells were prepared according to Pichia EasyComp protocole (Invitrogen). Electroporation was performed using Evaporator (Eppendorf). One transformation contained about 1 µg of linearized vector and 80 µL of competent X-33 cells. Cultures were spread on YPD plates ((1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) dextrose, 2 % (w/v) agar). Positive colonies were picked on new plates and inoculated in 5 mL of BMGY medium (1 % (w/v) yeast extract, 2 % (w/v) peptone, 100 mM KH_2PO_4, $4*10^{-5}$ % (w/v) biotin, 1% (w/v) glycerol) + Zeocin (100 µg/mL). They were incubated at 30 °C overnight. The next day, the volume of the culture needed to obtain OD_{600}=1 was centrifuged and the pellet was resuspended in 4 mL of BMMY medium (1 % (w/v) yeast extract, 2 % (w/v) peptone, 100 mM KH_2PO_4, $4*10^{-5}$ % (w/v) biotin, 0.5 % (w/v) methanol). Cultures, as well as a control (just BMMY, *P. pastoris* carrying PME vector (positive control), *P. pastoris* carrying an empty vector.) were incubated at 30 °C 250 rpm and every 24 h, during

three days, 25 % methanol to final concentration of of 0.5 % was added. On the fourth, day the cultures were centrifuged at 1500 rcf for 5 min and supernatant was tested with anti-HIS Western-blot.

Expression of heterologous PLL21 in Rosetta 2 strain of *E. coli*

Rosetta 2 strain transformed with pET30a(+) carrying an insert of *PLL21* CDS (**Figure 16**) that carries no start and stop codons (for translational fusion with HIS-tags in N- and C-term) was used for expression (primers used for construction in Table 2). As a negative control, the same strain transformed with the same vector without the insert was used.

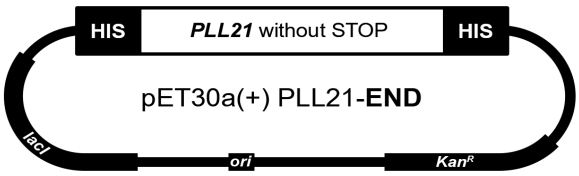

Figure 16 *Plasmid map of pET30a(+) with the insert used to express PLL21*

Five mL starter culture was grown over-night in LB containing 25 µg/mL of chloramphenicol and 50 µg/mL of kanamycin. The next day, the volume of starter needed to obtain OD_{600}=0.1 was added to 50 mL of LB with the same antibiotics. Expression was induced with IPTG (final concentration 0.6 mM) at the culture density of OD_{600}=0.4. Cultures were grown over-night at 30 °C and 150 rpm and harvested by centrifuging (2500 rcf, 5 minutes). Soluble proteins were extracted by resuspending the pellet in lysozyme solution (1 mg/mL of Lysozyme from chicken egg white (Sigma-Aldrich) in PS8 buffer (50 mM Na_2HPO_4, 300 Mm NaCl), incubating 15 min at RT and then sonicating 3x for 20 s with incubation on ice in-between sonication steps.

Purification of PLL21

Volume of 50 µL of Ni-NTA bead suspension (Quiagen) was washed two times in 1.5 mL Eppendorf tubes by adding 1 mL of dH_2O, centrifuging 15 s at 12 000 rpm, and removing the supernatant. The wash was repeated with 1 mL of 20 mM imidazole (prepared in PS8 buffer). Then, 1 mL of protein soluble fraction was incubated for 1 h at 4 °C with the beads. The beads were then washed with 1 mL of 20 mM, 40 mM, and 50 mM imidazole in the same way as before, and the washes were kept. For the first elution, the beads were incubated at 4 °C in 100 µL of 250 mM imidazole for 15 minutes and slow agitation, then they were sedimented by 30 s centrifugation at 12 000 rpm, and the supernatant was collected. The second elution was done in the same manner, with 500 mM imidazole.

SDS PAGE and coomassie/silver staining

Protein samples were loaded on the 12 % SDS polyacrylamide gel (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). The volume of 10 µL of samples was loaded in gels for Coomassie and silver staining, and 15 µL was loaded in the gel dedicated for Western-blot. As a molecular weight ladder, 5 µL of PageRuler™ Prestained Protein Ladder (ThermoScientific) was used. Electrophoresis was run in a mini-gel apparatus (Bio-Rad PowerPac Basic Mini Electrophoresis System) in 1x running buffer (25mM Tris, 192 mM glycine, 0.1 % (w/v) SDS), firstly 40 mA (for two gels) and then 60 mA when the dye entered the resolving gel. Electrophoresis was stopped when the dye leaked out from the bottom of the gel. Gels were microwaved at full power in water for one minute, shaken for 2 minutes, water was replaced with Coomassie blue stain (Page Blue Protein Staining Solution, Thermo Scientific), gels were again microwaved for 10 seconds and the staining was done for 20 minutes. Gels were destained in water overnight. For silver staining, PlusOne Silver Staining kit (Protein GE Healthcare) was used by following the manufacturer's instructions.

Western blot

SDS PAGE was done as described using a 1.5 mm thick gel. Upon the completion of electrophoresis, the gel was placed in the cathode buffer (25 mM Tris pH 9.4, 40 mM glycine, 10 % ethanol) for 15 minutes. Polyvinylidene difluoride membrane was treated with 100 % ethanol for 15 s, incubated 2 minutes in water, and 5 minutes in anode II buffer (25 mM Tris pH 10.4, 10 % ethanol). For the transfer, semi-dry system (Trans-blot Turbo, BIO-RAD) was used. In the apparatus tray Whatman 3M paper, SDS PAGE gel and the membrane were arranged from the bottom to the top in the following manner: three Whatman papers, first one soaked in anode I buffer (300 mM Tris pH 10.4, 10 % ethanol), and the other two soaked in anode II buffer, then the membrane followed by the SDS PAGE gel, and finally four Whatman papers soaked in the cathode buffer. All papers were cut to the exact size of the gel and the membrane. Transfer was conducted for 30 minutes at 100 mA. After completion, the membrane was placed in 96 % ethanol for 1 minute and dried. Immunodetection was done using anti-HIS antibodies (Sigma). The membrane was incubated in 4 % milk solution (1 g milk, 25 mL, TBS-Tween 20 (5 mM Tris, 15 mM NaCl, pH 7.6, 0.5 % Tween 20)) for 30 minutes, then transferred to a small plastic bag with 3 mL of 0.5 % milk with antibodies (0.1 g milk, 20 mL TBS-Tween

20, 0.75 µL of anti-HIS antibodies (1/4000 dilution) and incubated for 1.5 hours. After, two 15-minute washes and one 5-minute wash in TBS-Tween 20 were done, and in the end, the membrane was washed 5 minutes in dH_2O. For revelation, the volume of 3 mL of 1X DAB substrate (DAB substrate kit, Thermo Scientific) was applied over the membrane, and when bands no longer grew in intensity, the membrane was washed in water and photographed.

PLL activity assay

PLL activity was assessed spectrophotometrically, using the fact that the unsaturated bond in the products of the reaction absorb UV light at 232 nm. Positive control reaction mix consisted of 40 µL of pectins with low DM (Citrus pectin, Sigma P9311, 12 mg/mL) dissolved in Tris-HCl 50 mM buffer, pH 8, 40 µL of 1/2000, 1/4000 or 1/8000 dilution of commercial PL from *Aspergillus sp.* (E-PCLYAN, MegaZyme) and 40 µL of Tris-HCl 50 mM buffer at pH 8 with 0.3 mM $CaCl_2$. Tested samples contained purified elution (elution 1 or elution 2 as a negative control) of protein from *E. coli* instead of the commercial enzyme. The reaction was conducted at 40 °C for 1 h with spectrophotometric reads every 3 minutes.

Table 1 Primers used for genotyping

Associated gene	**Primer**	**5'-3' sequence**	**DNA template**	**Ta [°C]**
PLL21	PL21EXP-F	GCGGATCCACATGGCATGAACATGCAGT	RIKEN pda04081	65
	PL21EXP-Rstop	CGCTCGAGTTAACACCGGCGACCACCTC		
	PL21EXP-F	GCGGATCCACATGGCATGAACATGCAGT	RIKEN pda04081	65
	PL21EXP-Rend	CGCTCGAGACACCGGCGACCACCTCTGC		
PLL4	PLL4F	TCTAAGAATTCACGAAAATAATGGTTATTACGGGTATACC	Flower cDNA	55
	PLL4R	TGCACGCGGCCGCGCCCTGGAAGTACAGGTTCTCATAACAAGGGCCGTTGC		
Primer		5'-3' sequence	Ta [°C]	
AOX5'		GACTGGTTCCAATTGACAAG	55	
AOX3'		GCAAATGGCATTCTGACATCC		

Table 2 Primers used for cloning

Primers used to amplify WT gene				
T-DNA insertion	**Associated gene**	**Primer**	**5'-3' sequence**	**Ta [°C]**
Salk 128675 (*pll21-1)*	*PLL21*	F1	TGCAGTTGAGAATCCAGAGG	60
		R1	ATGGAAGCGAAGACAAATCG	
Salk 017091 (*pll21-2)*		F2	TTGGTGGACCAGACATGAAA	
		R2	GATTGCGCAATTGGCTAGAT	
Sail 674G05 (*pll21-3*)		F3	TGGTGAGAAGAATGAAAGTGC	
		R3	TGTGTTTGATCACATGGATCTCT	

Sail 148 H03 (*pll20*)	*PLL20*	F4	TTCTTCACCCACCACAATGA	
		R4	AATGGGTTGACTGGAGCAAG	
Primers used to amplify T-DNA insertion				
T-DNA insertion		Primer	5'-3' sequence	Ta [°C]
Salk lines		LBb1.3	ATTTTGCCGATTTCGGAAC	60
Sail lines		LB3	TAGCATCTGAATTTCATAACCAATCTCGATACAC	

Table 3 Primers used for RT-qPCR

Gene Name	Accession	Primer Fw (5'-3')	Primer Rev (5'-3')
PLL26	At1g04680	TCGTCGGAGCTATGGAGAACAC	TTCGGTTTCGTCGGATCTAGGG
PLL6	At1g11920	AGAATGCCTAGAGTGCGAAGAGG	GCTAGGGTCATCAGAAGCAACG
PLL8	At1g14420	ATGCTATTGACGGCTCCACTGC	AAACAACATCACCTCTTGGTGGTC
PLL7	At1g30350	TCCCAATGGAATGGATGG	CGAGAAAGGAAACAATGATCG
PLL16	At1g67750	TGGAGGAGCCTCTTCAAGCTATGC	ACGTGACTGAAGCCACCAATGAG
PLL9	At2g02720	TTAACTACCATGCCGCCTTAGC	ACGGAGATCCCTCCTTGTTTCG
PLL10	At3g01270	AGCTCGTAAACATACCCTTATGGC	GTCGTAATGACGCTCGTGGAAG
PLL20	At3g07010	TGCTGGTCCAACCATCAACAGTC	CACCTCCTTAGCAAATGGGTTGAC
PLL1	At3g09540	TTACGCTGTTGGTGCTGGTGTC	GGTTTCTCTTTATCAGCGGCCTTC
PLL24	At3g24230	AAACGGTTCCACTGCCATTACC	TGCCCTAGCAACATCACCTCATC
PLL22	At3g24670	TCGCCAAAGAGGTGACAAAGAGG	TCCAGTTCCATTGCTTCCATTCCC
PLL18	At3g27400	TGACGCACCATGATAAGGTCATGC	GCACCGTGGCATTCTTTGAACG
PLL17	At3g53190	TGGCGTTGGTGGCAGGAATAAC	AACCGCTAGTACCTTCCGATCCAG
PLL13	At3g54920	ATCCTAGCGCCAAAGAGGTGAC	TCGACCATTCACCATCGTCCTTC
PLL2	At3g55140	TGCTAGTGTAGAAGCGCAGGTG	TCTCGCTTCTTCCTTGTCAGCAG
PLL23	At4g13210	GATCCGGTTACACCAAAACC	AAGACGATCCATAGCGGTTC
PLL25	At4g13710	CAACAATGGCGGCGATAAGGAAG	AACGGCGTGTTGTTGTTGTTGTG
PLL4	At4g22080	TGTCATTCCTTGGTCCATGC	TTTCTCGGGGAGGTAGTTGG
PLL5	At4g22090	ACGTGACGAACGTCATTGTG	CCTCACCTTTCCAGATTTCC
PLL19	At4g24780	GATTGCAAGCAAGGTGGCAACG	ATCGTTCTCCACCCGAAATGCC
PLL12	At5g04310	TCCCAATGCCAAAGAGGTAACG	TCCAGTTCCATCCCGACCAATG
PLL3	At5g09280	TGCAACCAGAGAATGCCAAGAG	TTACACGAGGGCTCATGCTTCC
PLL11	At5g15110	CAAACCGCATTACAGAGAGGTGAC	TCCAGTGTTTCCACTCGTCCTC
PLL21	At5g48900	ATGAGCATTCGAAACAGCACAGC	ATCGATTGGATTTCCGGTTGAGC
PLL14	At5g55720	TGGTGGAAGTGCTAATCCAACG	TGCACTCTCTCGCTTTGTAACCTC
PLL15	At5g63180	ACCATCAACTCTCAAGGCAACCG	CGCATCCTCGTGCTTTGTTACCTC
REFERENCE GENES			
APT1	At1g27450	GAGACATTTTGCGTGGGATT	CGGGGATTTTAAGTGGAACA
At4g26410	At4g26410	TTTCCAATTGAATCGCAGTG	AGGAAGCAGGCAAAAACACA
At4g33380	At4g33380	GTGTTGTAACGGCTTGAGCA	TGTTTGCATCTTTGGTACGG
TIP41	At4g34270	GCTCATCGGTACGCTCTTTT	TCCATCAGTCAGAGGCTTCC
PEX4	At5g25760	CTTGGACGCTTCAGTCTGTG	TGAACCCTCTCACATCACCA
CLA	At5g46630	GTTTGGGAGAAGAGCGGTTA	CTGATGTCACTGAACCTGAACTG
PPR	At5g55840	GCAAGACAGTGAAGGTGCAA	CAGCGTTTATCAACCCACCT
EF1a	At5g60390	TGGTGACGCTGGTATGGTTA	TCCTTCTTGTCCACGCTCTT

Abstract

Pectin is the most complex polysaccharide of the primary plant cell wall that contributes to complex processes as cell growth and differentiation. It is composed mostly of homogalacturonan (HG), a homopolymer of galacturonic acid residues that can be methylesterified and acetylated. According to those modifications, pectin can rigidify or be digested by pectin-degrading enzymes such as Pectate Lyase-Like (PLLs). Activity of PLLs in has been linked to fruit ripening, reproductive organs elongation and pathogen susceptibility. However, PLLs have not been linked to vegetative growth of *Arabidopsis thaliana*. In this study we show that the *PLL21* loss-of-function mutant exhibits shorter light-grown roots and dark-grown hypocotyls than the wild type. A change in pectin content has also been noticed, however with different trends in roots and in hypocotyls. Surprisingly, we have also found that presumably safe green light leads to loss of phenotype in hypocotyls, indicating a putative role of light in PLL21 regulation. Furthermore, *PLL21* and *PLL20* double mutant showed no difference in length of dark-grown hypocotyls, and a less substantial difference in light-grown root length. Overall increased PLL expression in the double mutant and pectin content more similar to the WT, with lack of these trend in the single mutant, point to a compensatory mechanism triggered by the absence of two enzymes, which could explain the smaller changes observed in the double mutant.

Keywords: Arabidopsis, cell wall, pectin, pectate lyase-like, functional genomics, growth

Résumé

La pectine représente le polysaccharide pariétal le plus complexe d'un point de vu structural et est impliquée dans la régulation de divers processus tels que la croissance et la différentiation cellulaire. Elle est principalement composée d'homogalacturonane (HG), un homopolymère constitué d'acide galacturonique, qui peut être méthylestérifié et acétylé. En fonction des patrons de methylesterification et d'acetylation, les pectines peuvent se rigidifier ou être dégradées par des enzymes de dégradation des pectines telles que les Pectate Lyases-Like (PLL). Il a été montré que l'activité des PLL jouait un rôle dans la maturation du fruit, dans le développement des organes reproducteurs et dans la sensibilité vis-à-vis de certains organismes pathogènes. Cependant un rôle évident dans la croissance des organes végétatifs n'a pas été démontré, en particulier chez la plante modèle *Arabidopsis thaliana*. Dans cette étude, nous avons démontré, chez cette espèce, qu'un mutant perte de fonction pour le gène *PLL21* (*pll21-1*) présentait un raccourcissement de la racine primaire et de l'hypocotyle étiolé comparé à l'écotype sauvage. Des changements de contenu en pectine ont également été identifiés, cependant ceux-ci présentaient des variations opposées dans les deux organes étudiés. De façon surprenante, nous avons également découvert que la lumière verte présumée sans effet sur les plantes, conduisait à une perte du phénotype de croissance dans l'hypocotyle du mutant *pll21-1*, indiquant un rôle probable de la lumière dans la régulation de *PLL21*. En outre, un double mutant pour les gènes *PLL21* et *PLL20* (*pll20/pll21*) ne montrait pas de différences significatives dans la longueur de l'hypocotyle étiolé et une différence moins importante que le mutant *pll21-1* pour la longueur de la racine primaire. Globalement, une augmentation de l'expression des *PLL* dans le double mutant et un contenu en pectines similaire avec le sauvage ont été observé, contrairement au simple mutant, suggérant la mise en place de mécanismes compensatoires, déclenchés par la perte de fonction des deux gènes simultanément. Cela pourrait en partie expliquer la réduction des modifications morphologiques observées chez le double mutant.

Mots-clés: Arabidopsis, paroi, pectine, pectate lyase-like, génomique fonctionnelle, croissance

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.

Irin Antun Stošić