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DYNAMICS OF CELL WALL PROTEINS DURING CELL ELONGATION IN ETIOLATED HYPOCOTYLS OF ARABIDOPSIS THALIANA AS SHOWN BY PROTEOMIC AND TRANSCRIPTOMIC SURVEYS

Muhammad Irshad

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Université
de Toulouse



THÈSE

pour obtenir le grade de

DOCTEUR DE L'UNIVERSITÉ DE TOULOUSE

Délivré par : **Université Toulouse III-Paul Sabatier**

UFR Sciences de la Vie et de la Terre (SVT)

Spécialité : **Biosciences Végétales**

présentée et soutenue par

Muhammad IRSHAD

le 9 juillet 2008

**DYNAMIQUE DES PROTÉINES PARIÉTALES
AU COURS DE L'ÉLONGATION CELLULAIRE
DANS DES HYPOCOTYLES ÉTIOLÉS
D'ARABIDOPSIS THALIANA :
APPROCHES PROTÉOMIQUE ET TRANSCRIPTOMIQUE**

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DYNAMICS OF CELL WALL PROTEINS DURING CELL ELONGATION IN ETIOLATED HYPOCOTYLS OF *ARABIDOPSIS THALIANA* AS SHOWN BY PROTEOMIC AND TRANSCRIPTOMIC SURVEYS

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“If you detect any mistakes of mine, I rely on your superior knowledge to excuse them; for who has ever avoided errors in the wide-extended field of Nature? Who is furnished with sufficient stock of observations? I shall be thankful for your friendly corrections, I have done what I could myself” .

Linnaeus,
Father of modern Taxonomy

DEDICATION

I dedicate this humble effort of mine
to my loving parents, my brother Israr and
my sweet wife Lubna, without the love, devotion,
sincerity, guidance and encouragement of whom,
I would have been lost.

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List of abbreviations

1D-E/2D-E: one or two dimensional electrophoresis
4CL: 4-coumarate CoA ligase
ACN: acetonitrile
AG: arabinogalactan
AGP: arabinogalactan protein
AGP-PRP: arabinogalactan protein–proline-rich protein
AGRIKOLA: *Arabidopsis* genomic rnai knock-out line analysis
BSA: bovine serum albumin
BY2: bright yellow (cv of *Nicotiana tabacum*)
C3H: p-coumarate 3-hydroxylase
C4H: cinnamate 4-hydroxylase
CAD: cinnamyl alcohol dehydrogenase
CATMA: complete *Arabidopsis* transcriptome microarray
CBB: Coomassie brilliant blue
CCoAOMT: caffeoyl-CoA O-methyltransferase
CCR: hydroxycinnamoyl-CoA reductase
cDNA: complementary DNA
CESA: cellulose-synthase
Col 0: ecotype Columbia 0 of *A. thaliana*
COMT: caffeic acid/5-hydroxyferulic acid O-methyltransferase
CSC: cellulose-synthesizing complex
CSL: cellulose-synthase like
CWG: cell wall gene
CWP: cell wall protein
dNTP: deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP)
DTT: dithiotheritol
EDTA: ethylenediamine tetra acetic acid
EGase: endo-1,4- β -D-glucanase
Endo-PG: endopolygalacturonase
ER: endoplasmic reticulum
F5H: ferulate 5-hydroxylase
FLA: fasciclin-like arabinogalactan protein

FPLC: fast protein liquid chromatography
FWER: Family Wise Error Rate
G: guaiacyl (unit of lignin)
GalA: galacturonic acid
GAX: glucuronoabinoxylan
GFP: green fluorescent protein
GH: glycoside hydrolase
GPI: glycosylphosphatidylinositol
GRP: glycine-rich protein
GST: gene-specific sequence tag
GT: glycosyl transferase
H/PRP: hydroxyproline/proline-rich protein
H: p-hydroxyphenyl (unit of lignin)
HCT: p-hydroxycinnamoyl-CoA:quininate shikimate p-hydroxycinnamoyl-CoA transferase
HG: homogalacturonan
HGA: homogalacturonic acid
HRGP: hydroxyproline-rich protein
kDa: kilo Dalton
LAE: late-abundant embryogenesis protein
LB medium: Luria-Bertani medium
LC: liquid chromatography
LC-MS/MS: liquid chromatography-tandem mass spectrometry
LRR: leucine-rich repeat
LRX: leucine-rich repeat extensin
LTP: lipid transfer protein
MALDI-TOF: matrix-assisted laser desorption ionization-time of flight
MS medium: Murashige and Skoog medium
MS: mass spectrometry
nptI: neomycin phosphotransferase
PAGE: polyacrylamide gel electrophoresis
PAL: phenylalanine ammonia-lyase
PCR: polymerase chain reaction
PG: polygalacturonase
PGA: polygalacturonic acid

PGIP: poly galacturonase-inhibiting protein
PL: pectate lyase
PL: polysaccharide lyase
PM: plasma membrane
PME: pectin methylesterase
PRP: proline-rich protein
PTM: post-translational modification
PVDF: polyvinylidene fluoride
QI: semi-quantitative index
RG-I: rhamnogalacturonan-I
RG-II: rhamnogalacturonan-II
ROS: reactive oxygen species
rpm: round per min
RT-qPCR: reverse transcription-quantitative PCR
S: syringyl (unit of lignin)
SDS: sodium dodecyl sulphate
SPG: secretory pathway gene
TE buffer: Tris EDTA-buffer
Tris: Trishydroxymethylaminomethane, or 2-amino-2-hydroxymethyl-1,3-propanediol
U: unit
UHQ water: Ultra High Quality Water
XG: xyloglucan
XTH: xyloglucan endotransglucosylase-hydrolase

CHAPTER 1
INTRODUCTION

Chapter summary (French)

L'existence d'une paroi autour des cellules végétales est l'un des caractères qui les différencie des cellules animales. Cette paroi joue des rôles importants au cours du développement des plantes, et au cours de leurs interactions avec l'environnement qu'il s'agisse de stress biotiques ou abiotiques. Elle est également essentielle pour le port dressé des végétaux et permet la circulation de molécules signal entre cellules distantes. La croissance des végétaux fait intervenir deux processus cellulaires, qui sont la division cellulaire et l'augmentation de volume qui peut-être être soit anisotrope (grossissement), soit directionnelle (élongation). Ce travail porte sur la recherche de protéines importantes pour l'élongation des cellules végétales, et plus particulièrement pour les modifications des parois au cours de ce processus.

La première partie de cette introduction décrit les constituants pariétaux principaux : les polysaccharides (cellulose, hémicelluloses, pectines), les protéines, les lignines, les lipides, les cires et les ions. La description des protéines est volontairement succincte puisqu'elle reprend l'état des connaissances avant le début de mes travaux de thèse, *i.e.* avant l'essor des approches protéomiques qui ont permis d'avoir une vue globale des protéines pariétales.

Dans les deux parties suivantes sont présentés successivement les protéines dont le rôle dans l'élongation cellulaire est connu ainsi que le mécanisme particulier de l'élongation cellulaire dans les hypocotyles d'*Arabidopsis thaliana*. En effet, ces hypocotyles présentent la particularité de s'allonger environ 100 fois en un temps très court par simple élongation cellulaire.

Enfin, les objectifs de ma thèse sont développés. Il s'agissait d'avoir une vue globale de la régulation des gènes impliqués dans la biogenèse des parois au cours d'un processus d'élongation cellulaire chez *A. thaliana*. Le modèle expérimental retenu a été celui des hypocotyles étiolés. Les questions posées étaient les suivantes :

- Quelles sont les protéines présentes dans les parois des hypocotyles étiolés à deux stades de leur développement (croissance active *vs* croissance terminée) ?
- Existe-t-il des différences entre les protéomes pariétaux à ces deux stades développement ?
- Quelles sont les fonctions possibles des protéines identifiées ?

Deux approches complémentaires ont été menées : une approche protéomique impliquant la mise au point de protocoles de purification de parois (chapitre 3) et de séparation des protéines pariétales (chapitre 4) ; et une approche de transcriptomique (chapitre 5). L'ensemble des résultats obtenus a été interprété grâce à la bioinformatique non seulement pour la prédiction de la localisation sub-cellulaire des protéines, mais encore pour celle de domaines fonctionnels.

Land plants are among the largest organisms. This achievement is based largely on the growth, and mechanics of their cell wall, a structure that encases cells of plants, algae, fungi and bacteria like an armour. Plant cell wall is remarkable complex and dynamic entity and is one of the most sticking features that differentiate plants from animals and other eukaryotic cells. Cell wall of higher plants is of high economic importance to human especially as raw material of human and animal food, textiles, wood, paper, thickeners, biofuels and other products.

Cell wall was first observed by Robert Hooke in 1665 when he examined thin slices of cork under his microscope. In fact, he found small tiny boxes that he called cells and being dead material, consisted only of cell walls.

Cell wall of higher plants is a complex but organized molecular composite that may comprise many different polysaccharides, lignin, suberin, wax, proteins, aromatic substances, calcium, boron and water. Owing to the diversity of cell shapes and functions, the molecular composition and arrangement of cell wall exhibits a great diversity. Generally the cell wall consists of three parts: middle lamella, primary wall and secondary wall. Middle lamella is the first layer formed between the adjacent cells at the time of cytokinesis. Primary wall is simultaneously laid inner to the middle lamella, and is present in all type of cells and allows changes in cell size and shape. Secondary wall is impregnated on the inner surface of the primary cell wall in some type of cells (*e.g.* conducting cells) after achieving their final shape and size.

The cell wall of higher plants performs a variety of functions during growth and development as well as in plant defense, including maintenance of the osmotic pressure, water movement, rate and direction of cell growth, cell differentiation, intercellular communication and signalling, structural support and morphology of plant, cohesion among the cells of a tissue, storage, protection against pathogens and abiotic stresses. They also contribute to the functional specialization of cell types.

Here a detailed description of the cell wall components, their properties, structural organization and interactions among them, will be given. In addition, the involvement of different cell wall components especially the proteins in growth and development will be elaborated. Finally, we will discuss how cell wall proteins contribute to cell elongation by interacting with other cell wall components which makes the subject of this work.

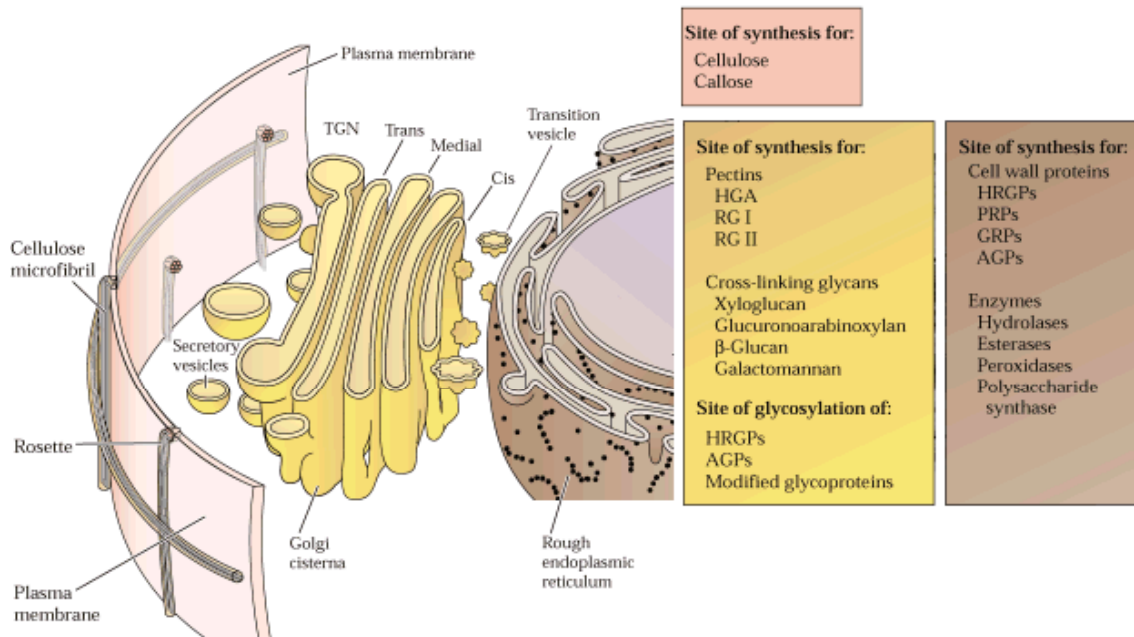


Figure 1.1: Biosynthesis of cell wall components (after Buchanan *et al.*, 2000)
 Cell wall biogenesis takes place in a series of coordinated steps inside or outside the cell. Synthesis of cellulose microfibrils and callose occurs at the plasma membrane surface. Synthesis and glycosylation of cell wall proteins and wall-modifying enzymes occur at the rough ER while synthesis of non-cellulosic polysaccharides at the Golgi apparatus. From here, the materials destined for cell wall are transported to cell surface in the form of secretory vesicles formed at the *trans*-Golgi network.

1.2. Cell wall components

About 10% of the plants genome is devoted to cell wall biogenesis (<http://cellwall.genomics.purdue.edu/>). Cell wall biogenesis has been divided into six distinct stages: substrate generation, polymer synthesis, secretion, assembly, rearrangement during development, and disassembly. These steps are taking place both inside and outside the cell. [Figure 1.1](#) illustrates the cell compartment where synthesis and processing or modifications of major cell wall components occur.

The molecular composition and arrangement of wall polymers differ among species, among tissues of a single species, among individual cells, and even among regions of the wall around a single cell (Buchanan *et al.*, 2000). Following are the major cell wall components.

1.1.1. Polysaccharides

1.1.2.1. Cellulose

Cellulose is the most abundant plant polysaccharide that accounts for 15-30% of the dry mass of the primary cell wall and even more in the secondary walls. In plants, cellulose occurs in paracrystalline assemblies, *i.e.* microfibrils of about 36 parallel arranged (1→4) β -D-Glc chains hydrogen-bonded to one another along their length ([Figure 1.2](#)). Many microfibrils combine to form a cellulose fiber laid down on the cell surface in several layers distinguished by the different orientation of their fibers (Buchanan *et al.*, 2000). Cellulose synthesis (reviewed in Reiter (Reiter, 2002); and Cosgrove (Cosgrove, 2005) occurs at rosette-like structures, the cellulose-synthesizing complex (CSC) that consists of six hexagonally-arranged subunits (CESA) that are embedded in the plasma membrane ([Figure 1.3](#)). Different CESA genes are assumed to be required to make a functional CSC, and different sets of genes are involved in the formation of the primary and secondary walls. CESA1, CESA3 and CESA6 or CESA6-like (CESA2, CESA9) are required for biosynthesis of the primary wall, whereas CESA4, CESA7 and CESA8 are required to form secondary walls. The most convincing proof of this comes from the study on the CESA1 and CESA3 mutants that result in gametophyte lethality and CESA6 mutant causing cellulose deficiency and growth defects (Persson *et al.*, 2007). In other such studies, the catalytic domain of cotton Cesa was immunolocalized at and near the CSCs (Kimura *et al.*, 1999) and the mutant *atcesA1* (*rsw1*) was shown to cause disassembly of CSC, reduced cellulose accumulation and accumulation of non-crystalline (1→4) β -D-Glc (Arioli *et al.*, 1998). CSCs probably contain other proteins. KORRIGAN is assumed to have some role in polymerization or crystallization of microfibrils

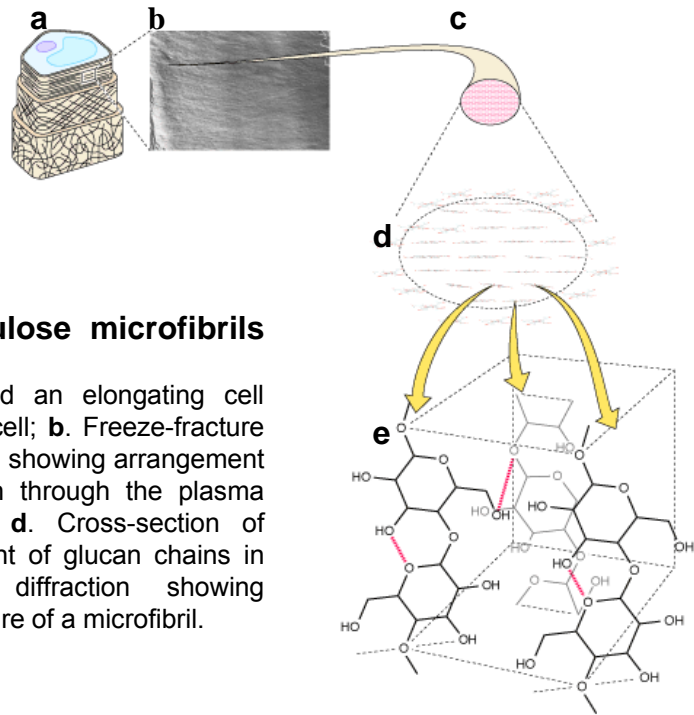


Figure 1.2: Structure of cellulose microfibrils

(after Buchanan *et al.*, 2000)

a. Helically-winded microfibrils around an elongating cell almost transverse to the long axis of cell; **b.** Freeze-fracture replica of an elongating maize root cells showing arrangement of cellulose microfibrils as impression through the plasma membrane; **c.** A single microfibril; **d.** Cross-section of microfibril showing spatial arrangement of glucan chains in the core of the microfibril; **e.** X-ray diffraction showing arrangement of atoms in the unit structure of a microfibril.

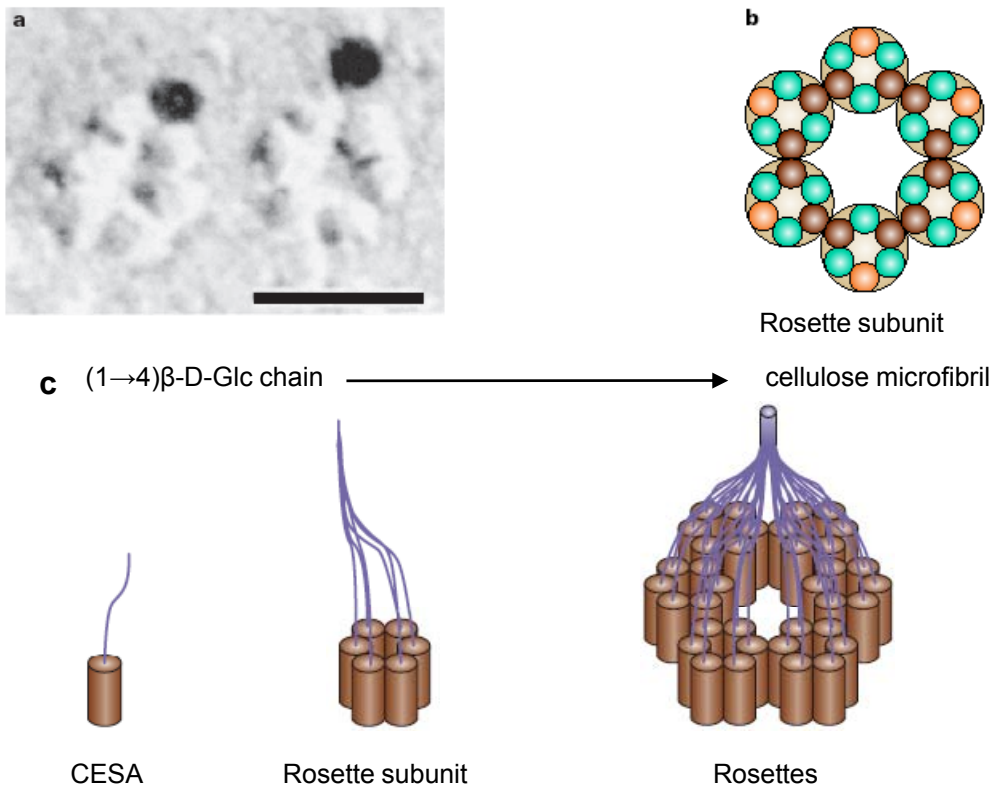


Figure 1.3: The cellulose-synthesizing machinery of the cell (after Cosgrove 2005)

a. Immunogold labelling of CESA, localized to hexameric cellulose synthesizing complex (CSC) in the plasma membrane. The black circles represent gold nanoparticles that are attached to antibody against CESA. Scale bar, 30 nm; **b.** A model showing how three different CESA proteins (shown in three different colours: orange, brown, green) might be organized into subunits and then into a hexameric CSC; **c.** A model of how CESA complexes synthesize a cellulose microfibril. Each CESA protein can synthesize a single (1→4)β-D-Glc chain. In this model, microfibril is shown to consist of 36 (1→4)β-D-Glc chains, synthesized by a CSC, which is composed of a hexamer of CESA hexamers.

Figure 1.4: Structure of xyloglucan (after Reiter 2002)
 XLFG type xyloglucan of Arabidopsis where the solid arrows indicate linkages that are always present, where as dashed arrows denote substitution patterns.

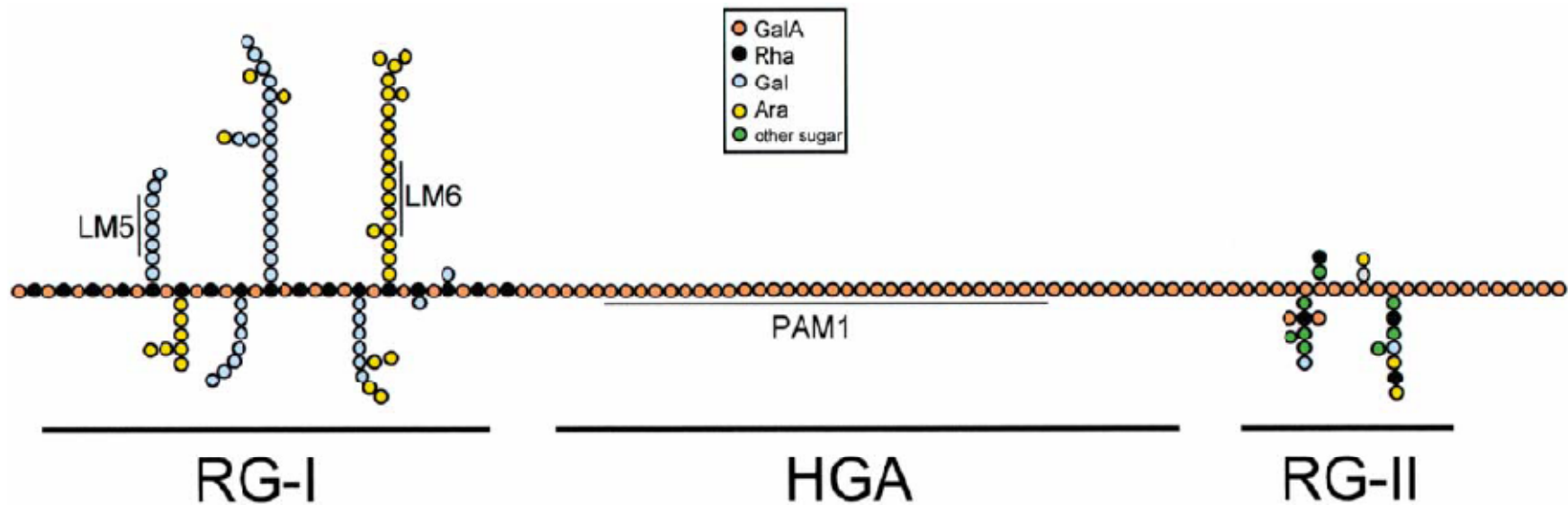
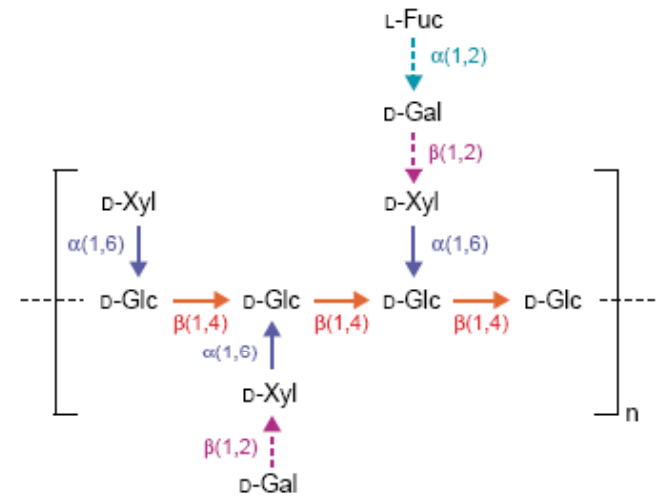


Figure 1.5: Structure of pectins (after Willats *et al.*, 2001)

Simplified schematic diagram to indicate some of the features of the three major types of pectins: HGA, RG-I and RG-II. Oligosaccharide epitopes recognized by anti-HGA monoclonal antibody PAM1 and anti-RG-I monoclonal antibodies LM5 and LM6 are also indicated.

or in recycling of sitosterol (Nicol *et al.*, 1998). COBRA is thought to link the complexes to nearby microtubules for guidance along the membrane (Roudier *et al.*, 2005). KOBITO1 is assumed to take part in the cellulose synthesis machinery or to play a role in the coordination between cell elongation and cellulose synthesis (Pagant *et al.*, 2002).

Cellulose is present in cell wall of all types, provides strength and rigidity to cell wall, and prevents the swelling of the cell wall and rupture of the plasma membrane that might occur when osmotic conditions favour water entry into the cell.

1.1.1.2. Cross-linking glycans

The cell wall polysaccharides that can hydrogen-bond to cellulose microfibrils are classified as cross-linking glycans. Most of them are also called hemicelluloses. In type I cell wall, characteristic of dicots, the major cross-linking glycans of primary cell wall are xyloglucans (XGs). XGs typically consist of a (1→4)β-D-Glc backbone carrying (1→6)α-D-Xyl moieties on three consecutive Glc residues. As shown in Figure 1.4, the Xyl residues attached to the second and third Glc residues of the backbone can carry D-Gal in (1→2)β-linkage, and the second of these Gal residues is usually substituted by L-Fuc in (1→2)α-linkage (Reiter, 2002) to make XLFG xyloglucan according to the nomenclature of Fry *et al.* (Fry *et al.*, 1993). The structure and distribution of the side branch (chains) vary in different tissues and species (forming XXFG, XXXG, XXLG, XLLG or XLXG) which seem important for bonding to cellulose. Glucuronoabinoxylans (GAXs) are the major cross-linking polymers of type II cell wall, characteristic of commelinoide monocots and some dicots. The structure of GAXs varies considerably with respect to degree of substitution and position of attachment of α-L-Ara residues (Carpita and Gibeaut, 1993). The other types of cross-linking glycans are “mixed linkage” (1→3, 1→4)β-D-glucan, glucomannans, galactoglucomannans and galactomannans (Carpita and Gibeaut, 1993).

1.1.1.3. Pectic matrix

Pectins (reviewed in Ridley *et al.* (Ridley *et al.*, 2001) and Willats *et al.* (Willats *et al.*, 2001)) are a mixture of heterogenous, branched and highly hydrated polysaccharides (Figure 1.5). They are rich in polygalacturonic acid (PGA) and account for 30% total cell wall mass (Carpita and Gibeaut, 1993). GalA occurs in two major structural features (homo and heteropolymers) that form the backbone of three polysaccharide domains found in almost all pectins: homogalacturonan (HGA), rhamnogalacturonan-I (RG-I), and rhamnogalacturonan-II

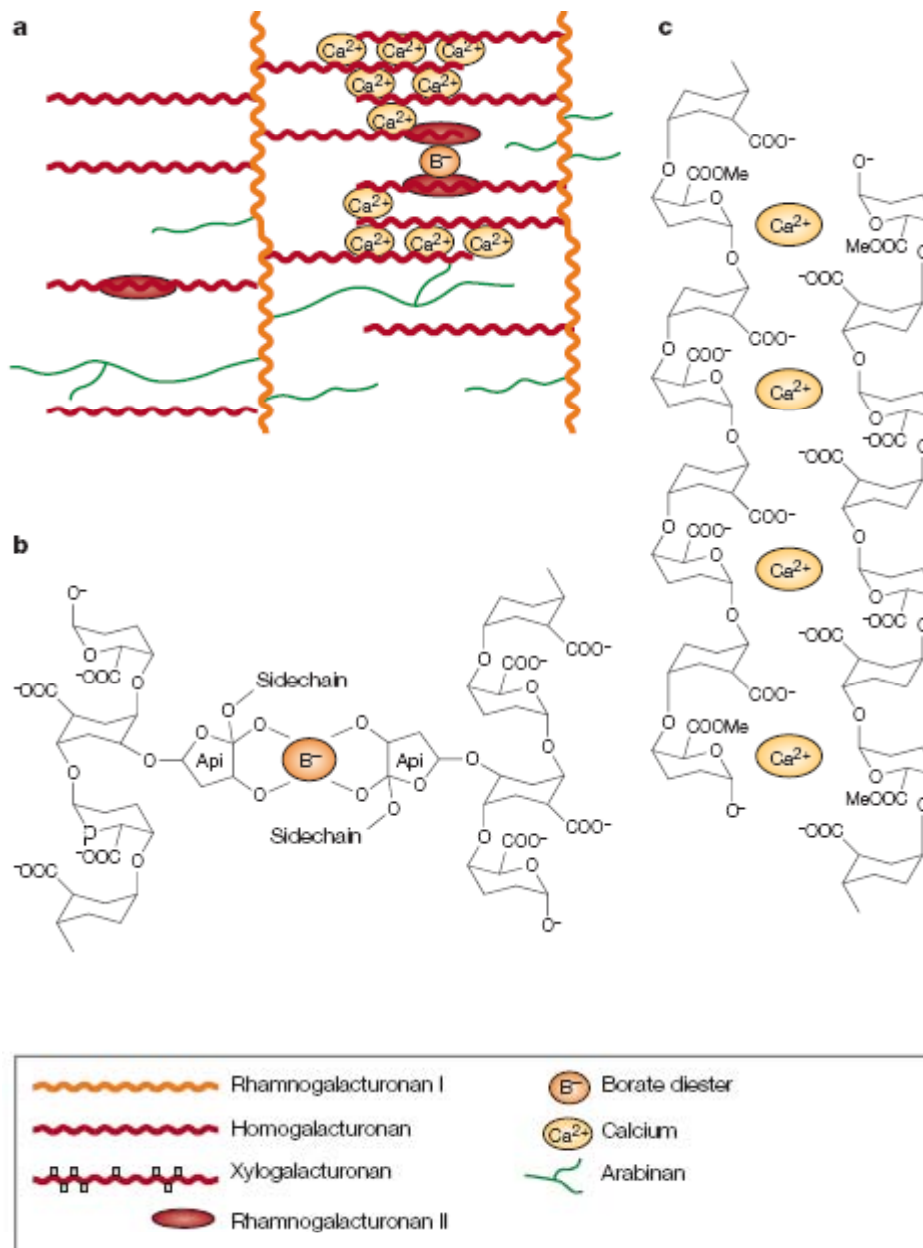


Figure 1.6: Formation of pectin networks by covalent and ionic bonds
(after Cosgrove 2005)

a. A model of how the different pectins may be covalently linked together to form a macromolecular pectin network. In this model, RG-I serves as the backbone and the other pectin domains are attached as branches; **b.** RG-II chains are cross-linked to form dimers through a borate ester bond; **c** HGA forms stiff gels through Ca^{2+} -mediated crosslinking of its carboxyl groups through ionic and coordinate bonds.

(RG-II). These three types of GalA covalently bond to each other to form a pectic network throughout the primary cell wall matrix and middle lamellae. HGA is a linear, unbranched (unsubstituted) homopolymer containing about 100–200 GalA residues, and is called smooth region of pectins. HGA is an abundant and widespread kind of pectin which is synthesized in the Golgi apparatus and deposited in the cell wall. It has 70–80% GalA residues methylesterified at the C-6 carboxyl. The demethylesterified HGA in the cell wall matrix can be cross-linked by Ca^{2+} to form supramolecular assemblies and gels (Figure 1.6). Other modifications and substitutions of HGA are not as widespread as methyl esterification. RG-I (known as the hairy region of pectin) is an acidic pectic domain consisting of as many as 100 repeats of the disaccharide L-Rha-D-GalA. RG-I is abundant and heterogenous and generally glycosidically-attached to HGA domains. Arabinogalactan and arabinan side chains commonly include D-Gal and L-Ara residues. Type I arabinogalactans are (1→4) β -linked D-Gal with non-reducing terminal-Ara (*t*-Ara) substituted at the O-3 of some of the Gal units (Carpita and Gibeaut, 1993). Arabinogalactans of type II with (1→3) β - and (1→6) β -linked-D-Gal residues also occur on pectic backbones. Arabinans can become branched by links through O-2 and O-3. Another major, widespread highly conserved pectic domain is RG-II which is similar to HGA by possessing homopolymer backbone, but is branched like RG-I. It consists of around 9 GalA residues backbone substituted by 4 heteropolymeric side chains each consisting of eleven different sugars including Api, aceric acid and 2-keto-3-deoxy-D-manno-octulosonic acid (kdo). RG-II can dimerize by means of borate ester links through Api residues.

In plants, several enzymes like pectin methylesterases (PMEs) (Micheli, 2001) polygalacturonase (PGs) (Tanaka *et al.*, 2002) and pectate lyases (PLs) (Marin-Rodriguez *et al.*, 2002) modify or degrade pectins in relation with changes in cellular adhesion and plasticity of the cell wall, a prerequisite to elongation and development.

1.1.2. Cell wall proteins

Until the last decade cell wall was considered to be made mainly of polysaccharides and structural proteins. But recent studies, especially proteomic studies have reported the presence of many other proteins including enzymes that perform a wide range of functions. The present study has further contributed to this area. Here cell wall proteins (CWPs) will be treated as have been considered before this study: structural proteins, that can form networks and other proteins that contribute to a wide range of functions from growth and development

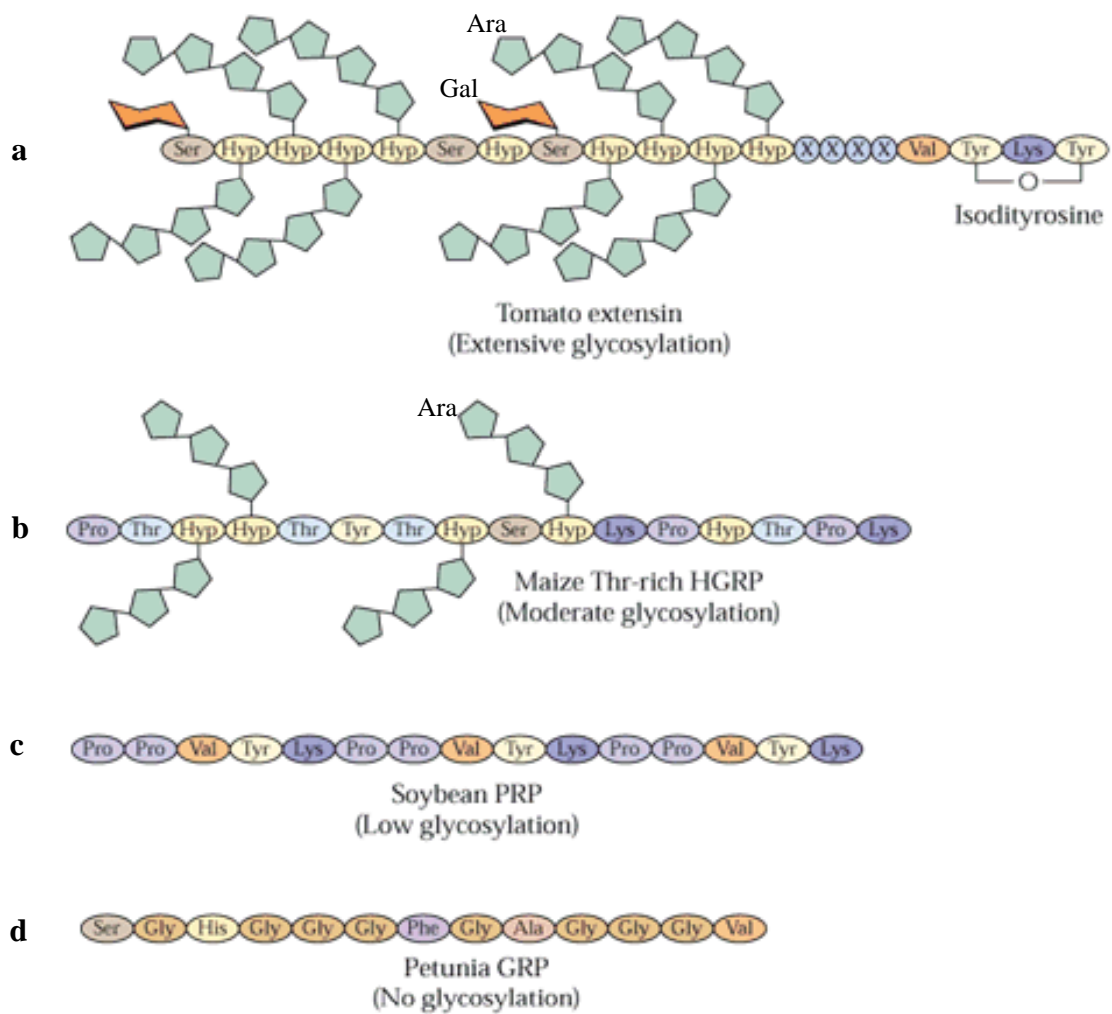


Figure 1.7: Cell wall structural proteins, motifs and glycosylation

(after Buchanan *et al.*, 2000).

a. Tomato extensin possessing Ser (Hyp)₄ or related motif with high glycosylation with tetra-Ara as well as Gal at Ser. Isodityrosine linkage is also shown on the Tyr-Lys-Tyr motif, a likely position for this linkage; **b.** Maize extensin like Thr-rich moderately-glycosylated protein; **c.** A Soybean PRP that lacks contiguously hydroxylated Ser, Thr, and Hyp residues (a likely position for glycosylation with Ara) are not heavily glycosylated; **d.** *Petunia* GRP with no glycosylation site.

to response to biotic and abiotic stresses. All CWPs possess a signal peptide and are targeted to secretory pathway (Buchanan *et al.*, 2000).

1.1.2.1. Structural proteins

Although all types of cell wall are mainly composed of polysaccharides, cellulose-crosslinking glycans network embedded in pectic matrix, another network is formed by cell wall structural proteins. Cell wall structural proteins can be grouped into three principal classes on the basis of their sequence rich in some particular type of amino acids: the extensins, hydroxyproline/proline-rich proteins (H/PRPs) and glycine rich proteins (GRPs). Examples of such proteins are given in [Figure 1.7](#). All these proteins are encoded by large multigene families and are developmentally-regulated. Extensins and H/PRPs are assumed to be cross-linked to make the wall less extendable when the cells achieve their final size. Their relative amount varies among tissues and species.

Extensins are well studied-structural proteins, which are rod-shaped distinguished by their amino acid sequence rich in Pro, Tyr, Lys, Ser, His, Val where Pro may reach up to 50%. They possess the repeating motif Ser-(Pro)_n ($n \geq 3$) and Tyr-Lys-Tyr sequences that are important for their secondary and tertiary structure making inter- and intra-molecular bonding. They are highly *O*-glycosylated and have basic pIs (Cassab, 1998; Sommer-Knudsen *et al.*, 1998; Buchanan *et al.*, 2000). Normally extensins are *O*-glycosylated with chains of Ara on contiguous Hyp residues and Gal on Ser (Kieliszewski and Lamport, 1994; Kieliszewski, 2001).

H/PRPs represent another large multigene family of structural proteins. Because of their similarity to extensins they are also thought to be rod-shaped (Buchanan *et al.*, 2000). H/PRPs contain the repetitive pentamere motif (Pro-Hyp-Val-Tyr-Lys)_n or its variants but lack Ser and are *O*-glycosylated (Cassab, 1998).

GRPs (reviewed in Ringli *et al.* (Ringli *et al.*, 2001)) that belong to another major cell wall structural protein family, should be distinguished from intracellular GRPs that are assumed to bind RNAs. Cell wall GRPs have repetitive sequences which may contain more than 60% Gly arranged in short repeating units. The GRP protein sequences often follow the motif (Gly-X)_n in the Gly-rich region, where X is often Gly, but can also be another amino acid. Ala, Ser, Val, His, Phe, Tyr and Glu are common at the X position. In some cases, the

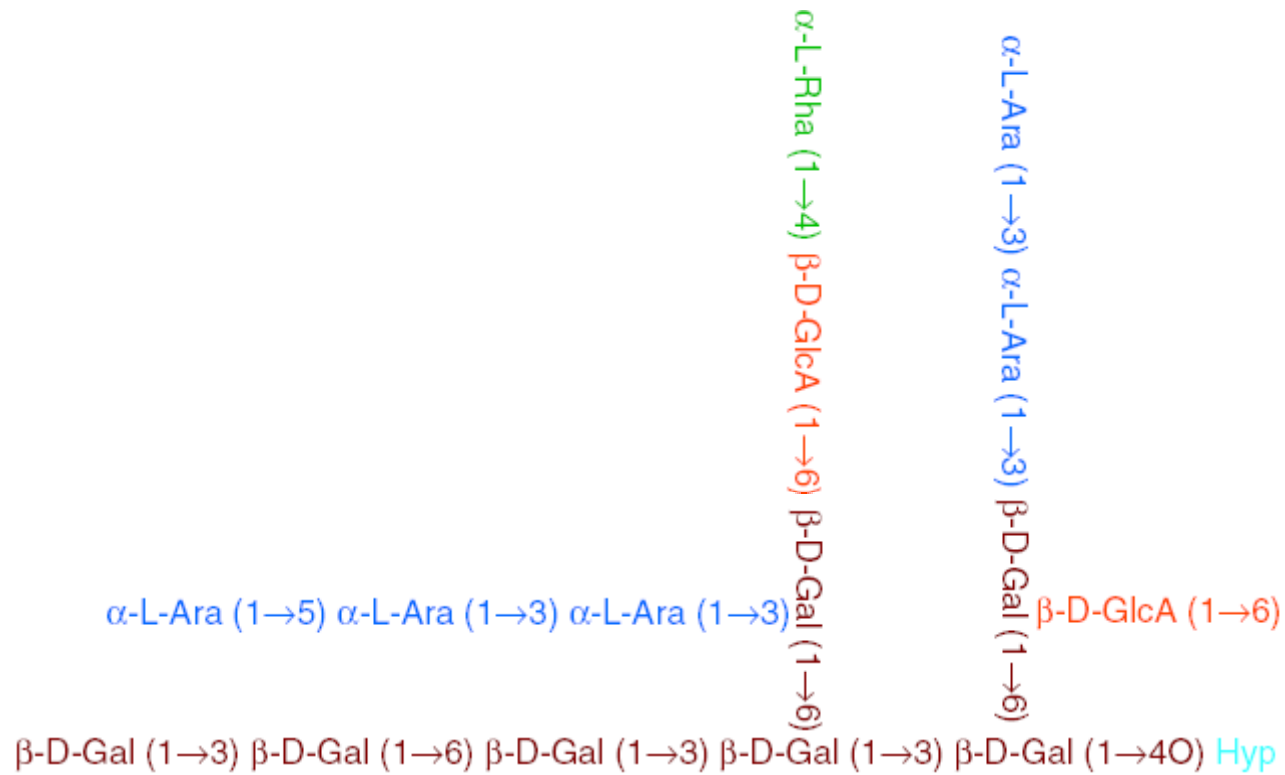


Figure 1.8: Structural complexity of an arabinogalactan (Seifert and Robert 2007)
 The first complete structure of an arabinogalactan glycan, derived from synthetic green fluorescent protein (GFP):(Ala-Pro)₅₁, expressed in tobacco BY2 cells.

motif varies (Ringli *et al.*, 2001). Beside the (Gly-X)_n motif, higher-order repetitive sequences that are rarely perfect are sometimes found and were proposed to be important for the formation of the secondary structure of the proteins. GRPs are believed to form plate-like structure rather than rod-shape conformation, on the plasma membrane-cell wall interface. They are thought to crosslink to cell wall polysaccharides are therefore difficult to extract.

1.1.2.2. Arabinogalactan proteins (AGPs)

AGPs (reviewed in Seifert and Roberts (Seifert and Roberts, 2007)) consist of a core protein of highly varying length and domain complexity, and one or more arabinogalactan (AG) side chains (Figure 1.8). They often contain a glycosylphosphatidylinositol (GPI) lipid anchor (Borner *et al.*, 2005). The relative ratio of glycan to protein is sometimes higher than 9, but can vary strongly for the same AGP core protein isolated from the same tissue. AGPs are precipitated by treatment with the β -Yariv reagent. In some AGPs, the peptide backbones are 10-13 residue-long and are called AG peptides. Most AGP sequences consist of a single central domain rich in Pro, Ala, Ser, and Thr. Most AGPs are *O*-glycosylated at one or more Hyp residues by AG type II (found in dicots) groups. These consist of (1 \rightarrow 3) and (1 \rightarrow 6) β -linked Gal chains connected to each other by (1 \rightarrow 3, 1 \rightarrow 6)-linked branch points, O-3 and O-6 positions substituted with terminal Ara residues. Type I AGs (characteristic of monocots and some dicots) are (1 \rightarrow 4) β -linked D-Gal with non-reducing terminal-Ara (*t*-Ara) substituted at the O-3 of some of the Gal units (Carpita and Gibeaut, 1993). AGPs are involved in a variety of functions, *e.g.* embryonic and post-embryonic patterns, pollen tube guidance, growth, secondary wall deposition, abscission and interaction with growth regulators and microbes.

1.1.2.3. Other proteins

Recently it has become clear that cell wall is very dynamic and contains many different enzymes and other agents (reviewed in Cosgrove (Cosgrove, 1999) and Fry (Fry, 2004)) conferring it dynamic properties like plasticity and extensibility. Among these proteins were expansins (*e.g.* α - and β -expansins), hydrolases (*e.g.* cellulases, xylanases, PMEs, PGs), transferases (*e.g.* xyloglucan endotransglycosylase hydrolases (XTHs), lyases (*e.g.* pectate lyases) oxido-reductases (*e.g.* peroxidases and laccases). These non-structural proteins perform a wide range of biological roles. A few examples of these functions are cited below while others, concerning especially the modification of cell wall and regulation of cell elongation are given in section 1.2.

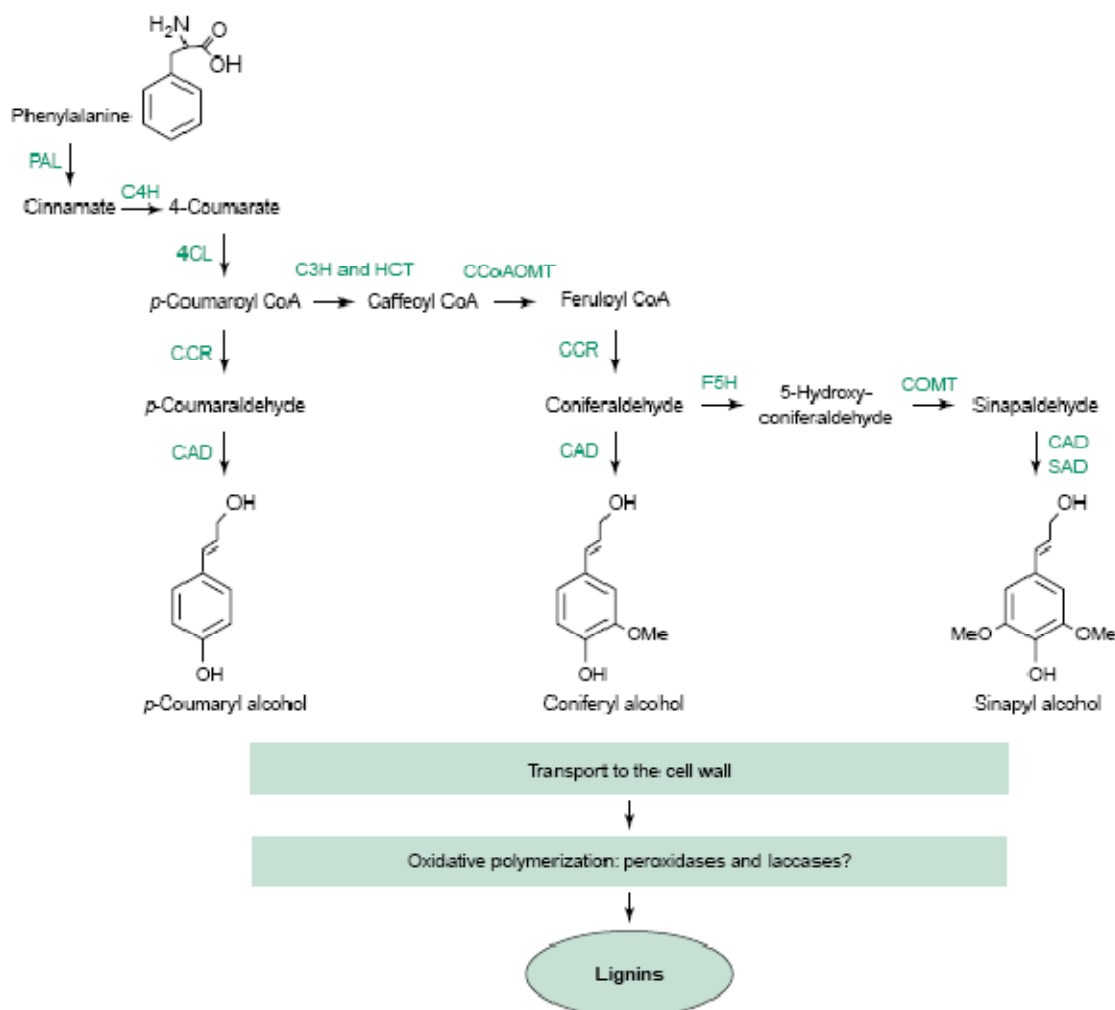


Figure 1.9: Lignin biosynthesis in dicots (after Boudet *et al.*, 2003)

The figure shows a simplified view of the most favoured lignin biosynthetic pathway in angiosperms where the implication of laccases in the oxidation of monolignols is still not known.

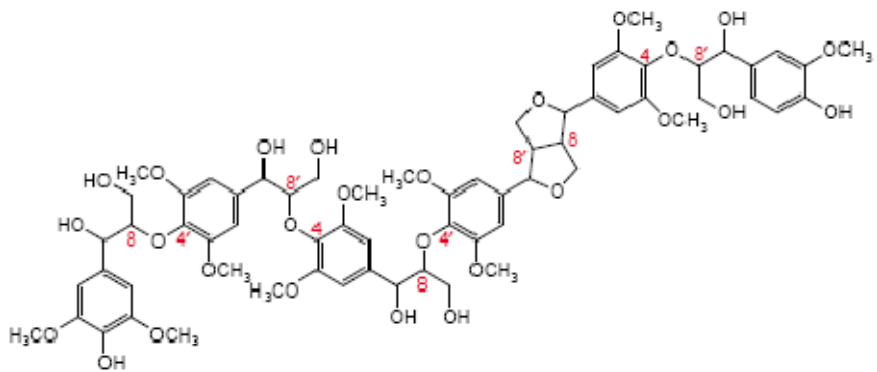
Abbreviations: CAD, cinnamyl alcohol dehydrogenase; CCoAOMT, caffeoyl-CoA O-methyltransferase; C3H, p-coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CCR, hydroxycinnamoyl-CoA reductase; 4CL, 4-coumarate CoA ligase; COMT, caffeic acid/5-hydroxyferulic acid O-methyltransferase also known as AldOMT, 5-hydroxyconiferaldehyde O-methyltransferase; F5H, ferulate 5-hydroxylase also known as Cald5H, coniferaldehyde 5-hydroxylase; HCT, p-hydroxycinnamoyl-CoA:quininate shikimate p-ydroxycinnamoyl-CoA transferase; PAL, phenylalanine ammonia-lyase; SAD, sinapyl alcohol dehydrogenase.

Inside cells, proteases are involved in all aspects of the plant life cycle (Schaller, 2004), but their role in cell wall is not very clear. Outside cells, some are thought to be involved in cell-cell communication by generating local-signals (Matsubayashi and Sakagami, 2006). The subtilisin-like serine protease *Stomatal Density and Distribution 1 (SDD1)* is thought to control stomata distribution and density in *Too Many Mouths (TMM)* dependent way (Berger and Altmann, 2000; Nadeau and Sack, 2003). The cell wall HRGP, *Root-Shoot-Hypocotyl-Defective (RSH)*, is essential for normal embryo development in *A. thaliana* and the mutant fails to develop normal embryo because mal-positioning of the cell plate at the time of division leading to the formation of abnormal embryo (Hall and Cannon, 2002). Reviewing the role of the cell wall in embryogenesis (Malinowski and Filipecki, 2002), the authors have highlighted that chitinases, XTHs, and peroxidases participate in embryogenesis regulation by involvement in signal transduction, and by influencing cell shape and division plane. AGPs are considered source of signals in a variety of ways (Seifert and Roberts, 2007). They may bind directly to a receptor that activate a signal transduction cascade or may release lipid signals by cleavage of GPI anchor by phospholipase or oligosaccharide as signals by endoglucanases. *Inflorescence Deficient in Abscission (IDA)* gene encoding an AGP playing a role in the abscission where its mutant delayed floral organ abscission and its over-expression produced opposite effect. Pectate lyases play important role in softening ripening fruits (Marin-Rodriguez *et al.*, 2002). Peroxidases that rigidify cell wall by cross-linking of wall compounds are expressed to cope with biotic and abiotic stresses like wound, pathogen interaction, and climatic aggression (Passardi *et al.*, 2004; Passardi *et al.*, 2005). Polygalacturonase-inhibiting proteins (PGIPs) protect plant against fungal attack by inhibiting fungal PGs (De Lorenzo *et al.*, 2001).

1.1.3. Other cell wall components

1.1.3.1. Lignins

Lignins (reviewed in Boerjan *et al.* (Boerjan *et al.*, 2003), Boudet *et al.* (Boudet *et al.*, 2003) and Davin and Lewis (Davin and Lewis, 2005)) are the 2nd most abundant plant substances in vascular plants. They are usually deposited in the secondary cell wall with few lignins exceptionally occurring in primary cell wall (Buchanan *et al.*, 2000). Lignins are assumed to be obtained by the oxidative polymerization of monolignols in the cell wall by peroxidases and laccases. The first step of lignin biosynthesis is the deamination of phenylalanine (dicots) or tyrosine (monocots) by ammonia lyases, yielding cinnamic or p-coumaric acids, respectively (Figure 1.9). Successive steps of hydroxylation, methylation,



S-(8-O-4')-S-(8-O-4')-S-(8-O-4')-S-(8-8')-S-(8-O-4')-G

Figure 1.10: Lignin structure (after Evtuguin and Amado 2003, in Davin and Lewis 2005)
An example of putative primary sequence structure of a syringyl lignin-derived hexamer fragment from *Eucalyptus globulus*. The radical-radical coupling linkages between the sinapyl alcohol monomers are shown in red.

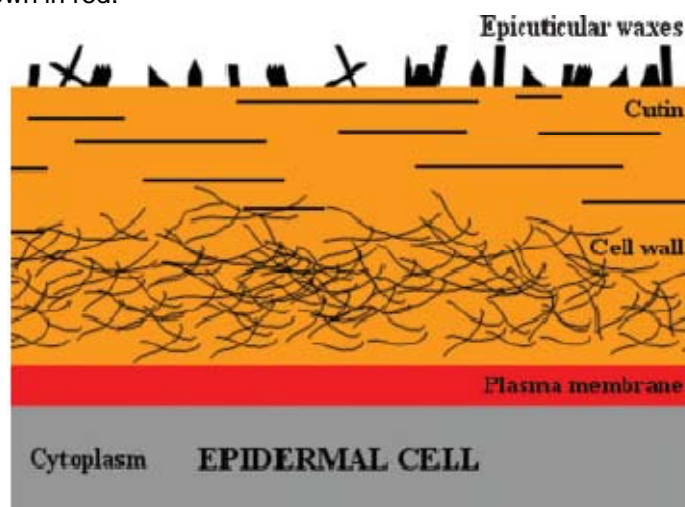
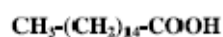


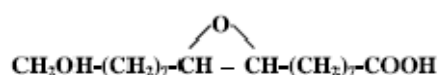
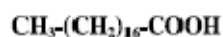
Figure 1.11: Plant cuticle (after Heredia-Guerrero *et al.*, 2008)
Current microscopic model of plant cuticle that shows the epicuticular wax crystals deposited on an amorphous and dense matrix of cutin polymer, containing some intracuticular waxes.

FAMILY C₁₆:



$$x, y = 6, 7; x+y = 13$$

FAMILY C₁₈:



$$x, y = 7, 8; x+y = 15$$

Figure 1.12: Major monomers of plant cutin (after Heredia-Guerrero *et al.*, 2008)
Chemical structure of the major monomers present in plant cutin. These monomers are derived from C₁₆ saturated fatty acid and from C₁₈ unsaturated fatty acids. Some plants have mainly C₁₆ family of monomers, whereas others have a mixture of both C₁₆ and C₁₈ families of monomers.

formation of hydroxycinnamoyl-CoA thioesters, reduction of hydroxycinnamoyl-CoA thioesters to hydroxycinnamaldehydes, and reduction of hydroxycinnamaldehydes lead to three p-hydroxycinnamyl alcohols (monolignols), p-coumaryl, coniferyl, and sinapyl alcohols which are transported from the cytosol to the apoplast. When reaching the apoplast, monolignols undergo dehydrogenative polymerization via oxidases, forming lignins. They comprise two major components namely guaiacyl (G), derived from coniferyl alcohol, and syringyl (S), derived from sinapyl alcohol (Figure 1.10) and one minor component p-hydroxyphenyl units (H), derived from p-coumaryl alcohol. Although polymers of only three types of monomers, the composition and structure of lignins vary significantly among different plants or within the same plant (Billa *et al.*, 1998).

1.1.3.2. Lipids

Lipids constitute the impermeable hydrophobic outer portion of the cell wall called cuticle, a continuous layer that covers aerial parts of leaves, fruits and young non-woody stems. Cuticle weight ranges from 2000 $\mu\text{g}/\text{cm}^2$ (in fruits) to 450-800 $\mu\text{g}/\text{cm}^2$ (in leaves). Forty to 80% of this is constituted by cutin, the extracellular lipids that are interconnected by ester bonds and can be studied after hydrolysing the polyesters (Heredia, 2003). Waxes are soluble extracellular lipids and can be extracted with organic solvents. The structure and composition of the cuticle vary in different plants, plant organs and growth stages but basically comprise a cutin matrix developed on the epidermal plant cell wall with waxes. Waxes are solid, partially crystalline aggregates at room temperature (Schreiber, 2005). They appear either embedded in the matrix (intracuticular) or deposited on its surface (epicuticular) as shown in Figure 1.11 (Riederer and Müller, 2005). Epicuticular wax may exist as a smooth film in some species or as wax crystals in other species (Buschhaus *et al.*, 2007). Chemically, cutin is a polymeric network of polyhydroxylated C₁₆ and C₁₈ fatty acids cross-linked by ester bonds (Figure 1.12). Waxes are generally described as mixtures of homologous long-chain aliphatic compounds, like alkanes, alcohols, aldehydes, fatty acids and esters with the addition of varying proportions of cyclic compounds like triterpenoids and hydroxycinnamic acid derivatives (Heredia, 2003).

Cuticle controls non-stomatal water loss, protects plants against ultraviolet radiation. It does not allow the water to stand easily on surface of the plant thus minimizing accumulation of dust, pollen and air pollutants. In addition, surface wax is thought to play important roles in plant defense against bacterial and fungal pathogens (Kunst and Samuels,

2003). Surface wax has also been shown to participate in a variety of plant-insect interactions (Eigenbrode and Espelie, 1995). For example epicuticular lipid extracts and individual lipid component enhance or deter oviposition, movement, and feeding.

1.1.3.3. Suberin

Suberin is a biopolymer making a barrier between plants and the environment in specialized plant tissues (*e.g.* periderm, bark and tuber skin) that protects the internal living tissues from dehydration, injuries, and pathogens (Soler *et al.*, 2007). Suberized cells are also found in the epidermis and hypodermis of roots, the endodermis and the bundle sheath of grasses (Graca and Santos, 2007). Suberin is a complex polyester made of glycerol and long-chain diacids and hydroxyacids (Schreiber *et al.*, 1999; Groh *et al.*, 2002). In suberized cells, suberin represents up to 50% of the mass of the cell wall (Pereira, 1988).

In the outermost tissues of plants, suberized cells play a vital role affording protection against environmental aggressions and pathogens, and controlling temperature and water loss (Schreiber *et al.*, 1999; Groh *et al.*, 2002).

1.1.3.4. Inorganic salts

Calcium and boron are the two major minerals localized in the cell wall. Calcium is also an essential plant nutrient and is required for various structural roles in the cell wall and membranes. Plants take up calcium from soil through roots and calcium is delivered to the shoots via xylem. Calcium enters the plant cells through specific ion channels in their plasma membranes (White and Broadley, 2003). In cell wall, Ca^{2+} participates in cross-linking the demethylesterified HGA to form supramolecular assemblies and gels (see Figure 1.6) (Willats *et al.*, 2001) that modify cell wall physical and chemical properties.

Boron is an essential nutrient for vascular plants. In cell wall, it cross-links RG-II to form dimers by borate ester bond between two apioses (see Figure 1.6) (Ridley *et al.*, 2001). Boron deficiency makes the tissues brittle or fragile, while plants grown on high boron levels may have unusually flexible or resilient tissues (Loomis and Durst, 1992). Boron helps establishing an effective legume–*Rhizobium* symbiosis (Bolanos *et al.*, 1994) and is required for the maintenance of nodule cell wall structure (Bonilla *et al.*, 1997).

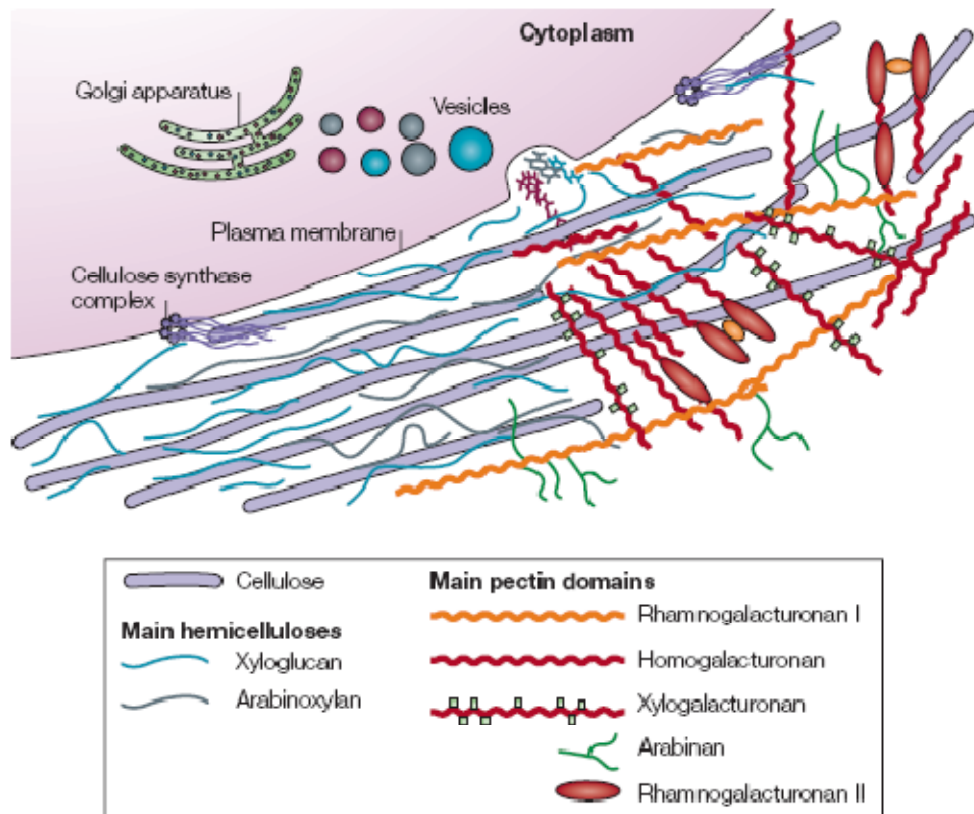


Figure 1.13 Structure of the primary cell wall (after Cosgrove 2005)

Cellulose microfibrils (purple rods) are synthesized by large hexameric complexes in the plasma membrane, whereas hemicelluloses and pectins, which compose the matrix polysaccharides, are synthesized in the Golgi apparatus and are deposited to the wall surface by vesicles. Xyloglucans are tightly bound to microfibrils by hydrogen bonds and form cross-links between them, thus constituting a load-bearing network. For clarity, the hemicellulose–cellulose network is shown on the left part of the cell wall without pectins, which are emphasized on the right part of the figure. In most plant species the main hemicellulose is xyloglucan (blue), while hemicelluloses such as arabinoxylans (grey) and mannans (not shown) are found in lesser amounts. The main pectin polysaccharides include rhamnogalacturonan I and homogalacturonan, with smaller amounts of xylogalacturonan, arabinan, arabinogalactan I (not shown) and rhamnogalacturonan II. Pectin domains are believed to be covalently linked together and to bind to xyloglucan by covalent and non-covalent bonds. Neutral pectin polysaccharides (green) are also able to bind to cellulose surfaces. pH of the cell wall and the action of different enzymes (not shown) like expansins, endo-1,4- β -D-glucanases, XTHs, PME and peroxidases on the cell wall components modify the properties of cell wall resulting in wall loosening or rigidification.

1.2. Cell wall proteins in relation to cell elongation and growth

Growth can be described as an irreversible increase of volume. In plants, growth is the outcome of cell division, enlargement of the new cells and their differentiation into different types of tissues. These processes of growth are accompanied by permanent change in size (usually an increase in length or volume) and an increase in dry mass of the growing part.

Enlargement of cells may be polarized, expanding more in one axis than the other (elongation) or it may be uniform in all directions as in isodiametric cells. Furthermore polarized growth takes place in two ways. In the first case, called tip growth, in which the growth is focused on a single specialized region, the apex of the tip growing cell. This type of growth is found in pollen tube, root hair and fungal hyphae. The other type is called diffuse growth and takes place at any point of a meristematic cell mostly in the cell wall parallel to the axis of elongation. This latter type of elongation generally occurs in the apical meristem of root and shoot or the intercalary meristem. This study is focused exclusively on diffuse polarized cell growth.

Before maturity, plant cells usually enlarge 10- to 1000-fold in volume by the process of vacuolation and irreversible cell wall expansion (Cosgrove, 1997). According to this review, there are two major concepts that account for the molecular basis of expansion. The first concept couples wall expansion to biosynthesis and secretion of wall polymers but did not explain the wall stress relaxation which is essential expansion of the cell *via* water uptake. The second concept considers wall expansion as result of biochemical loosening of the wall which permits turgor-driven extension of the wall polymer network. Though convincing, this latter neglects the need for integration of new polymers into the expanding wall.

To explain cell wall expansion based on rheological properties of the growing wall several potential mechanisms of stress relaxation and expansion can be imagined. Figure 1.13 represents the organization of various cell wall components and the various types of coordinated interactions between them that allow the cell to elongate but maintain cell wall integrity as intact structure. These interactions are described below in slight detail.

The acid-growth theory of cell wall expansion (Hager, 2003), which states that auxin, in an activated form ($\sim A$) activates a H^+ -pumping ATPase at the plasma membrane (PM) which utilizes respiratory energy (ATP) to raise the proton concentration in the

apoplast. This triggers cell elongation by activating cell wall enzymes. The transport of protons into the cell wall is compensated by a flow of cations into the cytoplasm, which maintains turgor pressure during cell elongation.

In elongating cells, the cellulose microfibrils are deposited perpendicular to the axis of elongation, forming a spring-like structure (Green, 1962). Such an arrangement reinforces lateral walls on one side and allows directional expansion (elongation) of the cell on the other hand through loosening of cellulose-hemicellulose networks. Expansins that can restore acid-induced creep of denatured walls, also participate this process. In fact, expansins are thought to transiently displace short stretches of hemicelluloses that are bonded to the surface of cellulose microfibril. It makes polymers to creep dragging along other structural components if the wall is in tension (Cosgrove, 1998).

Endo-1,4- β -D-glucanases (EGases) are assumed to cleave the β -1,4-glycosidic bonds between glucose of xyloglucans relaxing wall polysaccharides to move apart during cell elongation.

XTHs also perform cell wall modifications required for the process of wall assembly and cell expansion, by either wall loosening or incorporating new xyloglucan chains into extending walls (Chanliaud *et al.*, 2004). They cleave xyloglucans and rejoin the newly generated reducing ends to others by acting on the xyloglucans attached to cellulose microfibrils (Vissenberg *et al.*, 2005).

PMEs can cause wall loosening or wall stiffening according to their mode of action on HGs (Micheli, 2001). Once integrated into the cell wall, they may act randomly (as in fungi) or linearly (as in plants) on HGs. When PMEs act randomly on HGs (at acidic pH), the demethylesterification releases protons that promote the action of endopolygalacturonases (Endo-PGs) and contribute to cell wall loosening. When PMEs act linearly on HGs (at basic pH), PMEs give rise to blocks of free carboxyl groups that could interact with Ca^{2+} , so creating a pectate gel. Because the action of Endo-PGs in such a gel is limited, this action pattern of PMEs contributes to cell wall stiffening.

Peroxidases (reviewed in Passardi *et al.* (Passardi *et al.*, 2004)) also play a dual role in this context. By peroxidative cycle, they oxidize various substrates such as tyrosine

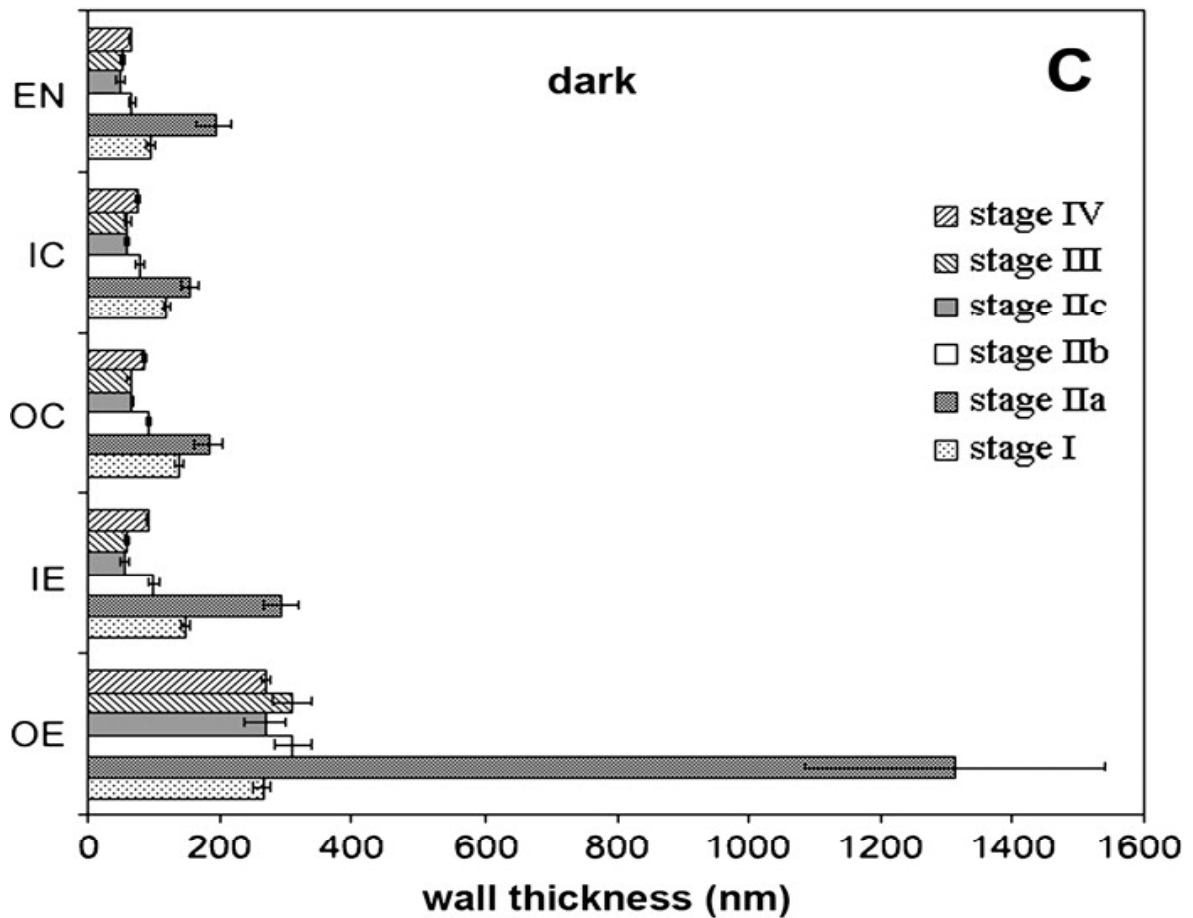


Figure 1.14. Changes in thickness of cell walls of dark-grown hypocotyls

(after Derbyshire *et al.*, 2007)

Cell wall thicknesses of different tissues [outer epidermis (OE), inner epidermis (IE), outer cortex (OC), inner cortex (IC), and endodermis (EN)] of hypocotyls freeze-fractured at their mid-points at different developmental stages of dark-grown hypocotyls:

stage I: at the embryo stage prior to germination

stage II: at the onset of germination

stage III: at 50% of their final length

stage IV: immediately after cessation of growth

residues, monolignols, suberin units and ferulic acids. The radicals produced by the peroxidative cycle, if linked to other polymers can cross-link cell wall polymers like extensins or lignins that block any further wall loosening and hence cell expansion. Inversely, by hydroxylic cycle they produce reactive oxygen species (ROS) like $\cdot\text{OH}$ that can cleave various polysaccharides non-enzymatically and contribute to wall loosening. Wall loosening is also caused by regulating H_2O_2 concentration by both peroxidative and hydroxylic cycles.

Listed above are the proteins potentially involved directly or indirectly in cell wall elongation or its arrest, but still a huge investment is needed in this field to completely understand the process.

1.3. *A. thaliana* etiolated hypocotyls as a model for cell elongation

To understand the mechanism of cell elongation, hypocotyls of *A. thaliana* are widely used as model (Gendreau *et al.*, 1997). During hypocotyl elongation, almost the entire cell machinery is devoted to synthesize, export and reorganize cell walls. The cell wall remains dynamic in real sense provided with the required plasticity and elasticity. Etiolation of hypocotyls makes them to elongate more than hypocotyls of light-grown seedlings. In dark-grown seedlings, hypocotyls elongate along a spatially and temporally steep acropetal gradient. This growth takes place mainly by cell elongation (100-fold elongation as compared to embryo cells) and does not involve significant cell divisions. Elongation follows an acropetal gradient and takes place in two distinct, time-separated phases: synthesis and deposition of new cell wall polymers and addition and re-organization of the existing ones (Gendreau *et al.*, 1997; Derbyshire *et al.*, 2007). During the first 3 days after germination, synthesis and deposition of cell wall material are the main processes, producing cells with thick walls. In the following days (until 7 days), hypocotyls grow mainly by extensive polymer disassembly and rearrangement resulting in thinner cell walls (Figure 1.14). Furthermore they respond to growth hormones as normal plant parts do. The model plant *A. thaliana* have the advantage of the existence of mutants with altered hypocotyl growth facilitating gene functional study (Mouille *et al.*, 2003). Owing to these characteristics, etiolated hypocotyls of *A. thaliana* could be considered an ideal model for the study of cell elongation.

1.4. Objectives of the project

As discussed above, CWPs contribute to several physiological functions. But these correspond to only a small part of the cell wall genes and role of most of the CWPs is still hidden. This project is aimed at identifying cell wall genes involved in cell elongation or its arrest. Here we have tried to identify the members of the multigene families known to be involved in elongation or elongation arrest in etiolated hypocotyls of *A. thaliana* as well as other proteins that could contribute to these processes.

The questions raised during this work are:

- i) Which are the proteins present in cell wall of elongating hypocotyls and fully-grown hypocotyls that have stopped elongation?
- ii) Do there exist differences between the cell wall proteomes of elongating organs and those that have stopped elongation? What are these differences?
- iii) What are the functions of these proteins?

To achieve these goals, comparative cell wall proteomics was used as primary approach in etiolated hypocotyls of *A. thaliana*: a fast growing stage (5-day-old) and a stage when there is no more elongation (11-day-old) will be compared. Since previous studies showed no defined correlation between the mRNAs and protein concentrations (Moritz and Meyer, 2003), working directly with proteins was preferred here. Proteomics has been defined as a global qualitative analysis of complex protein mixtures, including the post-translationally modified proteins as well as those encoded by alternatively spliced transcripts, and appears as a complementary approach to genomic and transcriptomic studies (Hunter *et al.*, 2002). Comparative proteomics provides an overview of the proteome and detects proteins which are altered between different stages (Moritz and Meyer, 2003) like the two physiological stages in this study. In addition, a comparative transcriptomic study was performed on the same material. Microarrays are a powerful, sensitive, versatile, and easy-to-use genomic tool that can simultaneously determine expression levels for thousands of genes at reasonable cost (Meyers *et al.*, 2004; Trevino *et al.*, 2007). Furthermore, combining the two types of data will give information about the post-transcriptional regulations of genes encoding CWPs. Here a global view of the functions of different genes representing these two physiological stages will be given.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant material

Arabidopsis thaliana ecotype Columbia 0 (Col 0) was used for *in vitro* and *in vivo* cultures for obtaining the hypocotyls, other plant material, and for transformation with the desirable constructs.

2.1.2. *In vitro* culture for obtaining etiolated hypocotyls

One hundred and thirty mg seeds of *A. thaliana* were weighed in an Eppendorf tube, soaked for 2-3 h in 1 mL tap water, sterilized by treating with 4 times diluted Javel (sodium hypochlorite) for 45 min. To remove the sterilizing solution, the seeds were washed 6 times with 1 mL of sterilized ultra high quality (UHQ) water each time. Finally, the seeds were sowed in Magenta boxes containing 50 mL of Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) pH 5.8, supplemented with 2% (w/v) sucrose and 0.8-1.2% (w/v) agar. Synchronization of germination was obtained by 2-4 days chill treatment and 4 h light treatment of the seeds. Seedlings were grown at 23°C in the dark for 5 or 11 days.

2.2. Methods

2.2.1. Isolation of cell walls from hypocotyls (after Feiz *et al.* (Feiz *et al.*, 2006))

For one experiment, hypocotyls were collected from 18 and 36 Magenta boxes for the 11 day-old and 5 day-old samples respectively. Hypocotyls of *A. thaliana* were collected by cutting the seedlings with scissors below the cotyledons and above the crown measuring ~0.7 cm for 5 day-old and ~1.2 for 11 day-old seedlings. The hypocotyls thus obtained were transferred to 5 mM sodium acetate buffer (pH 4.6), 0.4 M sucrose in a large Petri dish kept on ice. To remove the cut cotyledons and testa, hypocotyls were extensively washed on a 2 mm mesh, with 0.8-3.0 L of 5 mM sodium acetate buffer (pH 4.6), 0.4 M sucrose depending on the weight of the hypocotyls. Afterwards, the hypocotyls were transferred to a Moulinex®-type grinder containing 600 mL of the same buffer supplemented with 1 mL of protease inhibitor cocktail (Sigma Aldrich, Saint Louis, USA) and Polyclar® at the rate of 1 g/10 g fresh weight of hypocotyls in order to complex the phenolic compounds (Charmont *et al.*, 2005). The mixture was ground in cold room at full speed for 5 min. Cell walls were separated from soluble cytoplasmic materials by centrifugation for 15 min at 1000 × g at 4°C using the Beckman J2-HC centrifuge and a JA 14 rotor. They were further purified by two additional centrifugations in 150 mL per tube of 5 mM sodium acetate buffer (pH 4.6) containing 0.6 M and 1 M sucrose respectively. The cell wall containing pellet was hold at

each step. To ensure the removal of cytosolic proteins and other soluble material, the pellet was extensively washed on nylon net (25 μm pore size) with 3-5 L of 5 mM sodium acetate buffer (pH 4.6). Finally it was ground in liquid nitrogen with mortar and pestle in order to obtain very fine powder and to have intimate contact with the protein extraction buffer. The cell wall fraction was lyophilized and stored at -20°C .

2.2.2. Sequential extraction of proteins from purified cell walls

Generally, 0.6-0.9 g of lyophilized cell walls were taken per tube for protein extraction. Proteins were extracted in 4 successive steps by using 10 mL/g of salt solutions in this order: 2 extractions with 6-9 mL 5 mM sodium acetate buffer (pH 4.6), 0.2 M CaCl_2 , followed by 2 extractions with 6-9 mL 5 mM sodium acetate buffer (pH 4.6), 2 M LiCl. Protease inhibitor cocktail (Sigma Aldrich, Saint Louis, USA), was added at a concentration of 15 $\mu\text{L/g}$ cell walls, during the first extraction with each salt. Cell walls were resuspended by vortexing during 5-10 min at room temperature and then centrifuged for 15 min, at $40000 \times g$ and 4°C . The protein content of each extract was measured using the Bradford method (Bradford, 1976) with the Coomassie[®] protein assay reagent kit (Pierce, Perbio Science, Rockford, USA) taking bovine serum albumine (BSA) as a standard. Proteins were desalted using Econo-Pac[®] 10DG columns (Bio-Rad Laboratories, Inc. Hercules, CA, USA) equilibrated with 0.2 M ammonium formate. Depending on the volume of each extract and of protein concentration, protein solutions could be concentrated by successive centrifugations using the Centriprep[®] centrifugal filter device (YM-10 kDa membrane for volumes greater than 6 mL or 5 kDa for smaller volumes) (Millipore, Billerica, MA, USA) at $4000 \times g$. Finally, proteins were lyophilized.

2.2.3. Protein fractionation by cation exchange chromatography

All the lyophilized extracts were combined and redissolved in a total volume of 2 or 3 mL of water. They were again quantified as described above. One mg of proteins was used for chromatographic fractionation on a 1 mL HiTrap[™] SP FF column (Amersham Biosciences, Uppsala, Sweden) equilibrated with 50 mM MES (pH 5.6m adjusted with NaOH) operated with an FPLC[™] System (Amersham Biosciences, Uppsala, Sweden) controlled by FPLCdirector[™] version 1.0 (Amersham Biosciences, Uppsala, Sweden). The protein solution was adjusted to 50 mM MES (pH 5.6) and 20 μL protease inhibitor cocktail (Sigma Aldrich, Saint Louis, USA) were added before loading onto the column. Loading onto

the column was accomplished at a flow rate of 0.5 mL/min. A 10 mL unfixed fraction was collected at the same rate. Three mL of first wash with 50 mM MES (pH 5.6) were collected at a flow rate of 1 mL/min. Fixed proteins were eluted by a gradient from 0 to 0.8 M NaCl in 50 mM MES (pH 5.6) and 24 fractions (1 mL each) were collected at a flow rate of 1 mL/min. A modified gradient was applied when enrichment of certain proteins was required. Finally the column was successively washed with 3 mL of 1.2 M and 3 mL of 1.5 M NaCl in 50 mM MES (pH 5.6) at the same flow rate. These washes were also collected as 6 fractions (1 mL/tube). Two $\mu\text{L/mL}$ Protease inhibitor cocktail (Sigma Aldrich, Saint Louis, USA) was added to all the 1 mL collecting tubes. Quantity of proteins in each fraction was measured by the Bradford method (Bradford, 1976). The fractions were combined in groups of 2 or 3 depending on their protein concentration and were desalted as previously described prior to lyophilization.

2.2.4. 1D-E (SDS-polyacrylamide gel electrophoresis) and staining procedures

Each lyophilized chromatography fraction (group of 2 or 3 fractions) was redissolved in 200 μL water and electrophoresis of proteins was performed according to Laemmli (Laemmli, 1970). Samples were loaded on 12 \times 15 cm polyacrylamide gel with a concentration of 12.5% and a thickness of 1.5 mm (Annex I). For transfer, 8% polyacrylamide gels of 8 \times 6 cm and 1.5 mm thickness were used (Annex II).

Gels were stained with any one of the following staining procedures:

2.2.4.1. Staining with Coomassie Brilliant Blue (CBB)

CBB staining was performed according to Scheler *et al.* (Scheler *et al.*, 1998). After electrophoresis, the gel was fixed by overnight dipping and gentle shaking in 50% methanol. It was then rinsed 3 times with UHQ water by changing the water after each 30 min. Then, the gel was sensitized by dipping it in sensitising solution (34% methanol, 2% H_3PO_4 17% ammonium sulfate) for 1 h. Finally, the gel was put in the staining solution (34% methanol, 2% H_3PO_4 17% ammonium sulfate, CBB G (Sigma Aldrich, Saint Louis, USA) 0.66 g/L for 2-3 days. For clearing the background, the gel was rinsed 1-2 times in 2% H_3PO_4 before scanning the gel. The gel was stored at 4°C in 2% H_3PO_4 .

2.2.4.2. Staining with silver nitrate

For silver nitrate staining, the protocol of Shevchenko *et al.* (Shevchenko *et al.*, 1996) was followed. The gel was first fixed in 45% ethanol, 5% acetic acid, 55% UHQ water for 30 min to one night. Then, the gel was rinsed with 30% ethanol and UHQ water, one after the other (10 min \times 3 each). The gel was sensitized by treating with 0.02% Na₂S₂O₃. Then, it was rinsed 2 times for 1 min each, with UHQ water. Lastly, stain was developed on gel with 0.04% formaldehyde, 0.2% Na₂CO₃. It was left on a shaker and stopped when enough stained by taking it out and immersing in 1% acetic acid and stored in it at 4°C.

2.2.4.3. Staining with Amido Black

Amido Black staining was used for staining polyacrylamide gel and PVDF transfer membrane (0.2 μ m, Schleicher & Schuell, Dassel, Germany) after transferring proteins on it. The protocol was provided by Mr. Jacques D'Alayer, Head of "Le Plateau d'Analyse et de Microséquençage des Protéines", Pasteur Institute, Paris.

The gel was fixed in 50% methanol, 10% acetic acid solution for 30 min \times 2. The gel was stained by overnight treatment with 50% methanol, 10% acetic acid and 0.003% (3 mg/100 mL) Amido black (Naphthol Blue Black, Sigma Aldrich, Saint Louis, USA). The gel was rinsed 3 times with UHQ water to remove methanol, acetic acid and excess of colour.

For the PVDF membrane, once the proteins were transferred on it, it was first rinsed with UHQ water and stained in staining solution (40% methanol, 1% acetic acid) for 15-60 s. Water was used for destaining gel background. The membrane was dried in folds of blotting paper. Pieces of 2 \times 8 mm were cut for N-terminal sequencing.

2.2.4.4. Staining with Gelcode[®] Glycoprotein staining kit

Pierce kit (Perbio Science, Rockford, USA) was used to detect the glycoproteins present in the crude extract of hypocotyls. After electrophoresis, the gel was completely immersed in 50% methanol for 30 min. Then, the gel was washed twice with 3% acetic acid. The gel was transferred to "Oxidizing Solution" and let to agitate for 15 min. The gel was again washed twice with 3% acetic acid for 5 min. Now, the gel was treated with "Reducing Solution" for 5 min with gentle agitation. Finally, the gel was washed extensively with 3% acetic acid and then with UHQ water. The gel was stored in 3% acetic acid.

2.2.5. Transfer of proteins from gel on PVDF membrane

For this, tank transfer method using Mini Trans-Blot[®] Electrophoretic Transfer Cell (Bio-Rad Laboratories, Inc. Hercules, CA, USA), 8% polyacryamide gel, PVDF membrane was followed. PVDF membrane (0.2 μm , Schleicher & Schuell, Dassel) measuring 8×10 cm, was first immersed for a while in pure methanol, rinsed 2 times for 10 min with UHQ water and finally equilibrated in cold transfer buffer (0.025 M Tris, 0.192 M Glycine, 0.01% SDS). The fiber pads and Whatman 3 mm filter paper were also soaked in cold transfer buffer. The transfer sandwich (also named gel cassette) was prepared inside the transfer buffer contained in a tray. From cathode towards anode, the order was as follows: first fiber pad, 2 folds of Whatman[®] 3 mm filter paper, gel, PVDF membrane, 2 folds of Whatman[®] 3 mm filter paper and fiber pad. The sandwich and an ice cube were inserted into the tank which was completely filled with the transfer buffer. Transfer was carried out in cold room at constant 75 V during 2 h 15 min.

2.2.6. Protein identification by mass spectrometry

Protein bands were excised from stained gels with a 2 mm inner diameter Pasteur pipette. Each gel piece was washed twice with 100 μL of acetonitrile (ACN)/25 mM ammonium bicarbonate (50/50 v/v), for 10 min. After drying under vacuum for 15 min, gel pieces were rehydrated with 10 μL of sequencing grade a trypsin (Promega Corporation, Madison, WI, USA) solution (10 $\mu\text{g}/\text{mL}$) in 25 mM ammonium bicarbonate and digested overnight at 37°C. After addition of 1 μL ACN, a 5 min sonication was performed. An aliquot of 1 μL was deposited on the sample plate of the mass spectrometer with 1 μL of the matrix solution (6 g/L of α -cyano-4-hydroxycyannamic acid in 50% ACN/0.1% trifluoroacetic acid).

All mass spectrometry (MS) analyses were performed on the “Plate-Forme de Protéomique”, Toulouse Midi-Pyrénées, France. Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometer (Voyager-DeTM STR, Perseptive Biosystems, USA), was operated in positive reflector mode at the following parameters: accelerating voltage 20 kV; grid voltage 68%; extraction delay time, 200 ns. An external calibration was realized with a mixture of known masses of peptides (monoisotopic MH^+ 904.4681, 1296.6853, 1570.6774, 2093.0867, 2465.1989). Acquisition mass was between 750 and 4000 Da. Mass data were analysed by Data Explorer software (Applied Biosystems/MDS Sciex, Foster City, CA, USA). For each sample, an internal mass calibration was performed

using trypsin autolysis peaks (monoisotopic MH^+ 842.5100, 1045.5600 and 2211.1046). Peptide mass fingerprinting data were analyzed by MS-FIT Protein Prospector (<http://prospector.ucsf.edu>). The retained parameters were mass tolerance of 20 ppm, one missed cleavage, NCBI non redundant database (2005.01.06), 6 open reading frames and oxidation of methionine (or in some cases hydroxylation of proline). In some cases peptides were analyzed using, the hybrid linear ion trap mass spectrometer (Q TRAP[®] MS), LC/MS/MS system (Applied Biosystems/MDS Sciex, Foster City, CA, USA) as described in Boudart *et al.* (Boudart *et al.*, 2005). Data were processed by the Analyst software (Applied Biosystems/MDS Sciex, Foster City, CA, USA) and submitted to the search software MASCOT (<http://www.matrixscience.com/home.html>).

2.2.7. Extraction of RNAs

RNAs were extracted from *A. thaliana* hypocotyls for comparative transcriptomics. Hypocotyls were cut below cotyledons and above crown with scissors and grinded in liquid nitrogen using a mortar and pestle and stored at -80°C.

RNAs were extracted using “SV total RNA extraction kit” (Promega) following their protocol for “Isolation of Total RNA from Plant Tissue” and “RNA purification by centrifugation (spin)” with minor changes required. The ground tissue was mixed with “RNA Lysis Buffer” (175 μ L/30 mg). Then added to it “RNA Dilution Buffer” (350 μ L/30 mg) and mixed by inversion. Now, the mixture was distributed in 1.5 mL Eppendorf tubes (1 tube/30 mg ground tissue). The tubes were centrifuged at 13,000 rpm in a microcentrifuge for 10 min. The cleared lysate solution was transferred to a fresh microcentrifuge tube by pipetting. To this, 200 μ L of 95% ethanol were added, and mixed. The mixture was then transferred to the “Spin Column Assembly” (SCA) and centrifuged at 13,000 rpm for 1 min. The flow-through was discarded and 600 μ L of “RNA Wash Solution” were added to the “SCA”. It was again centrifuged at 13,000 rpm for 1 min. Then 50 μ L of freshly prepared “DNase Incubation Mixture” (40 μ L Yellow Core Buffer, 5 μ L 0.09 M $MnCl_2$ and 5 μ L of DNase I enzyme per sample) was directly added to the membrane inside the “SCA”. After application of the mixture, “SCA” was incubated for 15 min at 20-25°C. After incubation, 200 μ L of “DNase Stop Solution” were added to the “SCA”, and centrifuged at 13,000 rpm for 1 min. Now the membrane inside the “SCA” was washed successively with 600 μ L and 250 μ L “RNA Wash Solution” by centrifugation at 13,000 rpm for 1 and 2 min respectively. For eluting RNA, the basket was transferred from the “Collection Tube” to the “Elution Tube” and 100 μ L

“Nuclease-Free Water” were added to the membrane before centrifugation at 13,000 rpm for 1 min. The RNAs were collected in “Elution Tube” and stored at -80°C. Generally 108 µg and 118 µg RNAs were extracted from 800 mg of powder of 5 and 11 days hypocotyls respectively.

2.2.8. RT-PCR and semi-quantitative PCR

RT-PCR was used for comparison of transcript levels of selected genes in 5-day-old hypocotyls to those in 11-day-old hypocotyls of wild type plants. The RNAs extracted from the hypocotyls were used as starting material. Complementary DNA (cDNA) first strand were obtained from mRNA templates through reverse transcription. For this, 1 µL dNTPs (10 mM each), 1 µL oligo (dT)₁₅ primers (Promega) and 1 µL pork desmin mRNA were added to 1 µg (10 mL) total RNAs (treated with DNase). In order to denature RNAs, the reaction mixture was heated at 65°C for 5 min and immediately put on ice. Then 4 µL of 5× First Strand Buffer (Invitrogen), 2 µL of 0.1 M DTT (Invitrogen) and 1 µL RNasine Plus RNase Inhibitor (Promega) were added to the reaction tube. Then the mixture was incubated at 42°C for 2 min before addition of 1 µL of SuperScript™ II reverse transcriptase (Invitrogen). Again the tube was incubated at 42°C for 1 h. At the end, the enzyme was denatured by incubating the tubes at 70°C for 15 min. The cDNAs obtained in this way were kept at -20°C. The details of the reaction are given in Annex III.

For comparing RNA quantity of selected genes in 5- and 11-day-old hypocotyls, qPCR was performed on their respective first strand cDNA using a Roche lightcycler system (Roche Diagnostics, Meylan, France) according to manufacturer’s recommendations and gene specific oligonucleotide primers (Table 2.1). Using the results from quantitative PCR to determine the number of amplification cycles required to be in a linear range for all genes of interest, semi-quantitative PCR was performed to compare relative amounts of the corresponding mRNAs. The amplified fragments were analyzed by electrophoresis in polyacrylamide gels in standardized conditions. In each case, the presence of a fragment of the expected size was checked after staining with ethidium bromide.

Table 2.1 Primers used for RT-qPCR amplifications

| Accession number | Primer sequence (5'→3', sense orientation) | Primer sequence (5'→3', antisense orientation) |
|---------------------------|--|--|
| <i>At1g05570</i> | CACCTCCCTCTGATAACAATCAAAGAC | GCCTTACACGCTTGTGCAATG |
| <i>At1g10550</i> | CAATTCAGTAAAGATCGCCATTG | TTTGACACCAACCCAGCTC |
| <i>At1g28290</i> | AACCATAAGACTCAAACCCTTC | TGAGGGTGGTGGTGATGAG |
| <i>At1g49240</i> | CACCCGAGAGGAAGTACAGTG | CATACTCTGCCTTAGAGATCCACA |
| <i>At1g66180</i> | ACCACAAGCTCAACAAATGGT | GGAGGAAGCTTTTTACGATGAC |
| <i>At1g68560</i> | GTCGCCGTTAAATGTTGTTG | CCCATCTACATTGATGAAATCCT |
| <i>At1g69530</i> | GCATCGCTCAATACAGAGC | GAGTGTCCGTTTTATCGTAAACCTT |
| <i>At2g21140</i> | TCTCATCCTTGATAAAGATGC | TCTACGTAGAAATCAACAAAGC |
| <i>At2g28790</i> | GTGCCACTACAACGGAAAA | TGAGCTAGAGAAGCTGGTGGT |
| <i>At3g07130</i> | GGGATCGGCAACCTGATTA | GCCCATGTCTCGTTCCTCAT |
| <i>At3g16850</i> | GTAGCCTCAGAACCACCAGA | TCAACGCTTGTGAGATCGAC |
| <i>At3g43270</i> | TTCCGATCTGCTACATTGCT | GACCGCTTGGTGTTTTTCC |
| <i>At4g12880</i> | GGCATGAAGCTAGATGTTTTAGTTG | ACATAAAATTTGATTTCTTATTGTGCTG |
| <i>At4g18670</i> | CACCACCTCCAATCTACGAAG | GCCCTTTTGAGAACATTCTG |
| <i>At5g05850</i> | TGGTACCCTGGAGGAAAGC | TTGCAACTCCATAGCCACAG |
| <i>At5g11420</i> | GTCTCTCTCTTTACTTTGGTCTGC | AGTCGCCGTTTGGTAAACATC |
| <i>At5g44360</i> | AGGAGCTTATTGAATTACCGAGA | TCGCATCTTCAAAGCTCGTA |
| <i>At5g64100</i> | GCAAGACTTCGCTGCTAAAAAC | GCCGTTGAAGTTAACGAACC |
| <i>desmin^a</i> | CAGCCTCAGTCTCCAAATCACA | TAGGCCTGAGGTCACAGAGGT |

^a : Pig desmin RNA was used as an internal control for reverse transcription

2.2.9. Microarray analysis

Microarray analysis was carried out at the Unité de Recherche en Génomique Végétale (URGV), Evry, France, by Mrs. Ludivine Soubigou-Taconnat and Dr. Jean-Pierre Renou, using the CATMA array (Crowe *et al.*, 2003; Hilson *et al.*, 2004), containing 24,576 gene-specific sequence tags (GSTs) from *Arabidopsis*. RNA samples from 3 independent biological replicates were pooled. For each comparison, one technical replication with fluorochrome reversal was performed for each RNA sample. The reverse transcription of RNAs in the presence of Cy3-dUTP or Cy5-dUTP (Perkin-Elmer-NEN Life Science Products), the hybridization of labeled samples to the slides, and the scanning of the slides were performed as described in Lurin *et al.* (Lurin *et al.*, 2004).

2.2.10. Statistical analysis of microarray data

Experiments were designed with the statistics group of the URGV. Statistical analysis was based on 2 dye swaps (*i.e.* 4 arrays, each containing 24,576 GSTs and 384 controls) as described in Lurin *et al.* (Lurin *et al.*, 2004). Controls were used for assessing the quality of the hybridizations, but were not included in the statistical tests or the graphic

representation of the results. For each array, the raw data comprised the logarithm of median feature pixel intensity at wavelengths 635 (red) and 532 nm (green). No background was subtracted. In the following description, log ratio refers to the differential expression between two conditions. It is either \log_2 (red/green) or \log_2 (green/red) according to the experimental design. Array-by-array normalization was performed to remove systematic biases. First, spots that were considered badly formed features were excluded. Then, global intensity-dependent normalization using the LOESS procedure was performed to correct the dye bias. Finally, for each block, the \log_2 ratio median calculated over the values for the entire block was subtracted from each individual \log_2 ratio value to correct print tip effects on each metablock. To determine differentially expressed genes, a paired t test was performed on the \log_2 ratios, assuming that the variance of the \log_2 ratios was the same for all genes. Spots displaying extreme variance (too small or too large) were excluded. The raw p-values were adjusted by the Bonferroni method, which controls the Family Wise Error Rate (FWER). We considered as being differentially expressed the genes with an FWER of 5%. The Bonferroni method (with a type I error equal to 5%) was used in order to keep a strong control of the false positives in a multiple-comparison context (Ge *et al.*, 2003).

2.2.11. Data Deposition

Microarray data from this article were deposited at Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>; accession No. E-MEXP-789) and at CATdb (<http://urgv.evry.inra.fr/CATdb/>; Project RS05-11_Hypocotyls) according to the “Minimum Information About a Microarray Experiment” standards.

2.2.12. Bioinformatic analysis

DNA sequences related to each protein or to each gene were collected using data available in the Unigene database (<http://www.ncbi.nlm.nih.gov>). The *Arabidopsis* Genome Initiative (AGI) nomenclature was used (<http://mips.gsf.de/proj/thal/>, <http://www.tigr.org/tdb/tgi/agi/>). Sub-cellular localization and length of signal peptides were predicted using PSORT (<http://psort.nibb.ac.jp>) and TargetP (<http://www.cbs.dtu.dk/services/TargetP/>) (Nielsen *et al.*, 1997; Emanuelsson *et al.*, 2000). Prediction of transmembrane domains was done using Aramemnon (<http://aramemnon.botanik.uni-koeln.de/>) (Schwacke *et al.*, 2003). Molecular mass and pI value were calculated using the aBi program (http://www.up.univ-mrs.fr/~wabim/d_abim/compo-p.html). Homologies to other proteins were searched using

BLAST programs (<http://www.ch.embnet.org/software/BottomBLAST.html>) [Altschul *et al.*, 1990]. Identification of protein families and domains was performed using InterProScan (<http://www.ebi.ac.uk/InterProScan/>) (Quevillon *et al.*, 2005). GHs and CEs were classified according to the CAZy database (<http://www.cazy.org/CAZY/>) (Coutinho and Henrissat, 1999). The GT77 family was annotated according to Egelund *et al.* (Egelund *et al.*, 2004). XTHs and expansins were named according to <http://labs.plantbio.cornell.edu/xth/> and <http://www.bio.psu.edu/expansins/index.htm> respectively. AGPs and FLAs were named according to Schultz *et al.* (Schultz *et al.*, 2002), Johnson *et al.* (Johnson *et al.*, 2003), Van Hengels and Roberts (van Hengel and Roberts, 2003) and Liu and Mehdy (Liu and Mehdy, 2007). Proteins homologous to COBRA, LRXs and Hyp/Pro-rich proteins were annotated according to Roudier *et al.* (Roudier *et al.*, 2002), Baumberger *et al.* (Baumberger *et al.*, 2003), and Fowler *et al.* (Fowler *et al.*, 1999) respectively. The lignin toolbox was proposed by Raes *et al.* (Raes *et al.*, 2003). Peroxidases were named as in the PeroxiBase (<http://peroxidase.isb-sib.ch/index.php>) (Bakalovic *et al.*, 2006). Laccases were annotated as in Pourcel *et al.* (Pourcel *et al.*, 2005) and McCaig *et al.* (McCaig *et al.*, 2005). SKU-like proteins and phytocyanins were described in Jacobs and Roe (Jacobs and Roe, 2005), and Nersissian and Shipp (Nersissian and Shipp, 2002) respectively. Subtilases are listed at <http://csbdb.mpimp-golm.mpg.de/csbdb/dbcawp/psdb.html>.

CHAPTER 3

**EVALUATION OF CELL WALL PREPARATIONS FOR
PROTEOMICS: A NEW PROCEDURE FOR PURIFYING
CELL WALLS FROM *ARABIDOPSIS* HYPOCOTYLS**

Chapter summary (French)

Le premier objectif de ce travail étant d'identifier les protéines présentes dans les hypocotyles en élongation et après l'achèvement de leur croissance, il nous fallait mettre en œuvre une étude de protéomique. En effet, la protéomique est un outil de choix pour une étude globale des protéines. Essentiellement deux types de stratégies, appelées non-destructive ou destructive, ont été mises en œuvre jusqu'à présent, aucune d'elles n'étant capable de révéler l'ensemble des protéines pariétales (Lee *et al.*, 2004 ; Jamet *et al.*, 2008). Dans le premier cas, l'intégrité des cellules est respectée puisqu'il s'agit soit d'analyser des protéines secrétées dans des milieux de culture, soit de pratiquer des infiltrations dans des espaces intercellulaires avec des solutions salines. Dans le second cas, les tissus sont broyés avant de procéder à la purification des parois et à l'extraction des protéines avec des solutions salines. Cette dernière approche présente un certain nombre de contraintes qui affectent les résultats qualitativement et quantitativement :

- i) les protéines pariétales peuvent être perdues au cours de la purification des parois puisque ce compartiment extracellulaire n'est pas entouré d'une membrane ;
- ii) les protéines extracellulaires peuvent être contaminées par des protéines intracellulaires qui se lient de manière non-spécifique aux polysaccharides des parois au cours de leur purification.

Dans les études protéomiques précédemment publiées, ces problèmes ont rarement fait l'objet d'une grande attention. C'est ainsi qu'un grand nombre de protéines connues ou prédites par bioinformatique pour être intracellulaires (parfois plus de 50%) ont été considérées comme pariétales. Pour pratiquer une étude protéomique des parois végétales, la qualité de la purification des parois apparaît donc essentielle. Avant de commencer la mise au point de notre protocole de purification de parois, nous avons analysé en détail les résultats de

plusieurs études publiées précédemment. Nous avons retenu plusieurs points qui nous paraissaient importants et avons essayé d'apporter des solutions :

- i) Perte de protéines au cours de la purification des parois en présence de tampon à forte force ionique : nous avons employé un tampon de faible force ionique (tampon acétate de sodium 5 mM) pendant toute la procédure de purification des parois.
- ii) Contamination des parois par des organelles ou des vésicules membranaires : nous avons employé des centrifugations successives en présence de concentrations croissantes de saccharose (0.4 M, 0.6 M, et 1.0 M). La forte densité des parois permettait ainsi de les recueillir sous forme de culot après chaque centrifugation.
- iii) Contamination des parois par des protéines intracellulaires : nous avons introduit une étape de lavage extensif des parois purifiées avec le tampon à faible force ionique (tampon acétate de sodium 5 mM).
- iv) Protéolyse : des inhibiteurs de protéases ont été ajoutés tout au long de la procédure de purification des parois et des protéines.
- v) Contaminations intracellulaires dues à la présence de détergents dans le tampon d'extraction : aucun détergent n'a été ajouté dans les tampons utilisés.
- vi) Méthodes de vérification de la qualité des préparations de parois : les méthodes traditionnelles se sont révélées inappropriées pour vérifier la présence de contaminants intracellulaires par manque de sensibilité. Nous avons employé systématiquement les outils de la bioinformatique pour prédire l'adressage sub-cellulaire des protéines ainsi que les données de la littérature. Il s'agissait de s'assurer de la présence d'un peptide signal et de l'absence de signal de rétention des protéines dans le reticulum endoplasmique.

Le protocole de purification retenu a permis d'obtenir des préparations de parois de bonne qualité, comme en témoigne la grande proportion de protéines dont la localisation sub-cellulaire prédite est extracellulaire (environ 80%). Cette proportion n'a été atteinte dans aucune autre étude publiée précédemment. Les résultats sont détaillés dans l'article qui suit publié dans *Plant Methods*.

As a first objective of the work to identify the proteins that are present in the elongating and fully-elongated hypocotyls, a proteomic study was needed. Proteomics is considered an appropriate tool for large scale studies. Several methods classified as destructive and non-destructive are used (Lee *et al.*, 2004; Jamet *et al.*, 2008) since no single method is sufficient alone to study complete cell wall proteome. In the non-destructive methods living cells are used, and proteins secreted in the culture medium or extracted with washing of cells or vacuum infiltration of tissues with salt solutions are analysed. In other cases, destructive methods are employing the purification of cell wall from the tissue followed by extraction of the proteins with salts. However, the latter approach has several constraints that affect the results qualitatively and quantitatively:

- i) loss of CWPs during cell wall purification due to the lack of a surrounding membrane
- ii) contamination of CWPs by intracellular proteins that non-specifically binds to the cell wall polysaccharides in the cell wall preparation procedure.

In previous cell wall proteomic studies, these problems were not being given due attention and thus resulted in the identification of high number (up to 50% or more) of known or predicted intracellular contaminants. For performing a proteomic study on cell wall, purity of cell wall is of utmost importance. To establish a protocol that could purify a proteomic-grade cell wall, the existing protocols of cell wall preparation, and the results obtained with them were analysed. This analysis brought into our notice some steps that seem to be responsible for these problems. Keeping in mind these points, we have established a new protocol for cell wall preparation. These problems and distinguishing features of our protocol to overcome these problems are listed below:

- (i) Loss of CWPs by using high ionic strength buffers in cell wall preparation: We replaced these buffers by low ionic strength buffer (5 mM acetate buffer) throughout cell wall preparation steps in this new protocol.
- (ii) Contamination of cell wall by cell organelles and other vesicles: To purify organelles-free cell wall, our protocol uses increasing sucrose concentration (0.4 M, 0.6 M and 1.0 M) during three successive centrifugations.
- (iii) Lack of extensive washes of the cell wall to wash away the soluble intracellular contaminants: To eliminate soluble intercellular contaminants, our protocol

proposes extensive washing of the cell wall pellet with the low ionic strength buffer (5 mM acetate buffer).

- (iv) Proteolytic degradation of CWPs: For preventing CWP proteolytic degradation, we use protease inhibitors.
- (v) Intracellular contamination caused by the use of detergents in the extraction buffer: Our new method avoids use of detergents in the protein extraction buffer from the purified cell wall that contribute to minimizing the number of intracellular proteins in CWP extract.
- (vi) Use of inappropriate methods to judge the purity of cell wall and to distinguish true CWPs from the false ones: Traditional methods used were not enough sensitive to distinguish between pure and impure cell walls and all the proteins identified were considered as CWPs. We analyse the identified proteins with bioinformatic tools to check for the presence of signal peptide and absence of ER retention signal.

During this study, the above protocol was used and satisfactory results were obtained. CWPs extracted from cell wall purified with this protocol allowed the identification of high number (about 80 %) of predicted CWPs with few intracellular contaminants. None of the previously published studies has reported such high proportion. The detailed results are given in the article published in *Plant Methods*.

The supplementary data files are given as annexes at the end of the report.

Evaluation of cell wall preparations for proteomics: a new procedure for purifying cell walls from *Arabidopsis hypocotyls*

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Abstract

Background: The ultimate goal of proteomic analysis of a cell compartment should be the exhaustive identification of resident proteins; excluding proteins from other cell compartments. Reaching such a goal closely depends on the reliability of the isolation procedure for the cell compartment of interest. Plant cell walls possess specific difficulties: (i) the lack of a surrounding membrane may result in the loss of cell wall proteins (CWP) during the isolation procedure, (ii) polysaccharide networks of cellulose, hemicelluloses and pectins form potential traps for contaminants such as intracellular proteins. Several reported procedures to isolate cell walls for proteomic analyses led to the isolation of a high proportion (more than 50%) of predicted intracellular proteins. Since isolated cell walls should hold secreted proteins, one can imagine alternative procedures to prepare cell walls containing a lower proportion of contaminant proteins.

Results: The rationales of several published procedures to isolate cell walls for proteomics were analyzed, with regard to the bioinformatic-predicted subcellular localization of the identified proteins. Critical steps were revealed: (i) homogenization in low ionic strength acid buffer to retain CWP, (ii) purification through increasing density cushions, (iii) extensive washes with a low ionic strength acid buffer to retain CWP while removing as many cytosolic proteins as possible, and (iv) absence of detergents. A new procedure was developed to prepare cell walls from etiolated hypocotyls of *Arabidopsis thaliana*. After salt extraction, a high proportion of proteins predicted to be secreted was released (73%), belonging to the same functional classes as proteins identified using previously described protocols. Finally, removal of intracellular proteins was obtained using detergents, but their amount represented less than 3% in mass of the total protein extract, based on protein quantification.

Conclusion: The new cell wall preparation described in this paper gives the lowest proportion of proteins predicted to be intracellular when compared to available protocols. The application of its principles should lead to a more realistic view of the cell wall proteome, at least for the weakly bound CWP extractable by salts. In addition, it offers a clean cell wall preparation for subsequent extraction of strongly bound CWP.

Background

Cell walls are natural composite structures, mostly made of high molecular weight polysaccharides, proteins, and lignins, the latter found only in specific cell types. They are dynamic structures contributing to the general morphology of the plant. Cell walls are involved in cell expansion and division, and they are sources of signals for molecular recognition within the same or between different organisms [1-5]. Cell wall proteins (CWP) represent a minor fraction of the wall mass: 5–10% in primary cell walls of dicots, as reported for cell suspension cultures, but accurate determinations in various plant organs are still lacking [6]. Despite their low abundance, CWP contribute, at least in part, to the dynamic of cell walls. CWP can be involved in modification of cell wall components, wall structure, signalling, and interactions with plasma membrane proteins at the cell surface [7].

Proteomics appears to be a suitable method to identify a large number of CWP thus providing information for many genes still lacking a function. Recent publications on cell wall proteomics have shown that more than 50% of the identified proteins were known to be intracellular proteins in higher plants [8,9], green alga [10] and fungi [11]. Different techniques unrelated to proteomics, such as biotinylation of cell surface proteins, or immunoelectron microscopy, also suggested a cell wall location for some glycolytic enzymes, proposing that they are *bona fide* components of the yeast cell wall [11]. However, the reliability of protein profiling for a compartment like the cell wall, strongly depends on the quality of the preparation. Unfortunately, the classical methods to check the purity of a particular fraction are not conclusive for proteomic studies, since the sensibility of the analysis by mass spectrometry is 10 to 1000 times more sensitive than enzymatic or immunological tests using specific markers. Our experience in the field has shown that the most efficient way to evaluate the quality of a cell wall preparation is (i) to identify all the proteins extracted from the cell wall by mass spectrometry, and (ii) to perform extensive bioinformatic analysis to determine if the identified proteins contain a signal peptide, and no retention signals for other cell compartments [12-15]. It is then possible to conclude about the quality of the cell wall preparation by calculating the proportion of predicted secreted proteins to intracellular ones.

The aim of the present study is to present a comparative analysis of different methods previously published to prepare cell walls for proteomic studies. These methods will be evaluated by the proportion of proteins predicted to be secreted after bioinformatic analysis as stated above. A new method is presented, based on classical cell wall preparations, but adapted to the new technologies. The

results indicate that such a method significantly reduces the number of proteins without predicted signal peptide.

Results and discussion

Several strategies have been designed to gain access to CWP. The most labile CWP, *i.e.* those having little or no interactions with cell wall components, can be recovered in culture media of cell suspension cultures [12] or liquid cultured seedlings [14]. Extracellular fluids can be harvested from cell suspension cultures [12,16] or intact organs such as leaves [13]. However, such analysis cannot be done in all cases. It is then necessary to isolate cell walls starting with a drastic mechanical disruption of the material of interest. Consequently, labile CWP may be lost, and intracellular proteins or organelle fragments may contaminate cell wall preparations.

To design a procedure for cell wall isolation and subsequent protein extraction, several general features should be kept in mind. Plant and fungal cell walls are mainly built up with highly dense polysaccharides. This property can be used to purify them through density gradients by centrifugation. The biochemical structure of walls is complex, and CWP can be bound to the matrix by Van der Waals forces, hydrogen bonds, and hydrophobic or ionic attractions. Such interactions can also be modulated by the composition of the isolation medium. Commonly, a low ionic strength is preferred to preserve ionic bonds, but also to dilute the ionic strength of the cell wall itself. An acidic pH is chosen to maintain the interactions between proteins and polysaccharides as *in planta*. Once isolated, cell walls are classically treated by CaCl_2 buffers to release proteins, and by LiCl buffers for extraction of glycoprotein [17,18]. The use of detergents has also been reported to extract proteins strongly embedded in the polysaccharide matrix, like wall associated kinases [19]. Finally, CWP can be covalently bound to cell wall components so that they are resistant to salt-extraction. At present, there is no satisfactory procedure to isolate them. We analyze recent publications using different methods to isolate cell walls from plants or yeast prior to proteomic analysis [8,9,20].

Analysis of early methods

Chivasa et al. [8] used *A. thaliana* cell cultures to purify cell walls. The procedure is schematically represented in Figure 1A. The first step consisted in cell grinding in water. The homogenate was layered onto 10% glycerol and let to sediment for several hours. The cell wall pellet was resuspended in water and washed 3 times by repeated centrifugations. The proteins were sequentially extracted with 0.2 M CaCl_2 , and urea buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 2% Pharmalytes 3–10). The extracted proteins were separated by 2D-GE and identified by MALDI-TOF mass spectrometry (MS). The identified proteins were analyzed with several bioinformatic programs

A. thaliana cell suspension culture

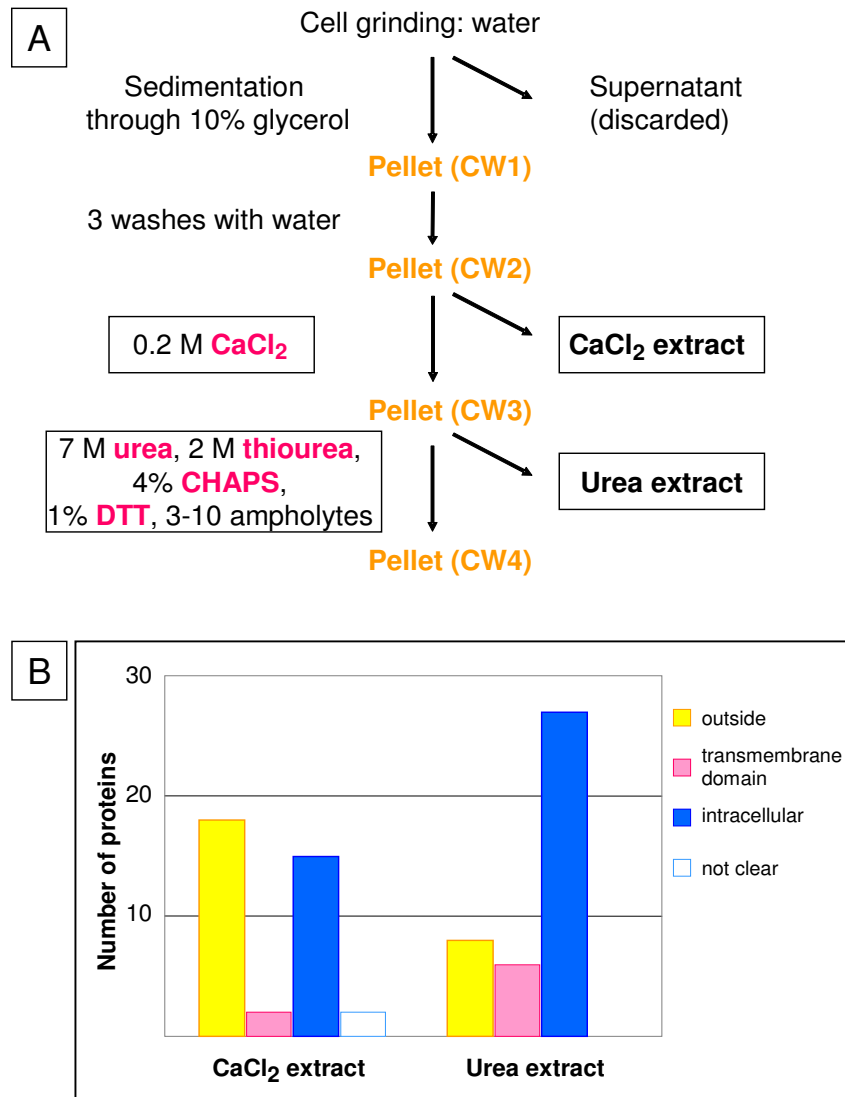


Figure 1

Cell wall preparation from *A. thaliana* cell cultures. **A** – Schematic representation of the purification of cell walls from *A. thaliana* cell suspension cultures, and of the different extracts obtained [8]. **B** – Number of proteins identified in each extract after separation by 2D-GE and MALDI-TOF MS analysis. After bioinformatic analysis, proteins were classified as outside (proteins containing a signal peptide and no other targeting sequences), having at least one trans-membrane domain and intracellular (predicted to be located in any intracellular compartment). Proteins for which predictions by different bioinformatic programs are in conflict are classified as "not clear". Twenty-four different proteins predicted to be secreted were identified in this study (see Additional file 1).

M. sativa stems

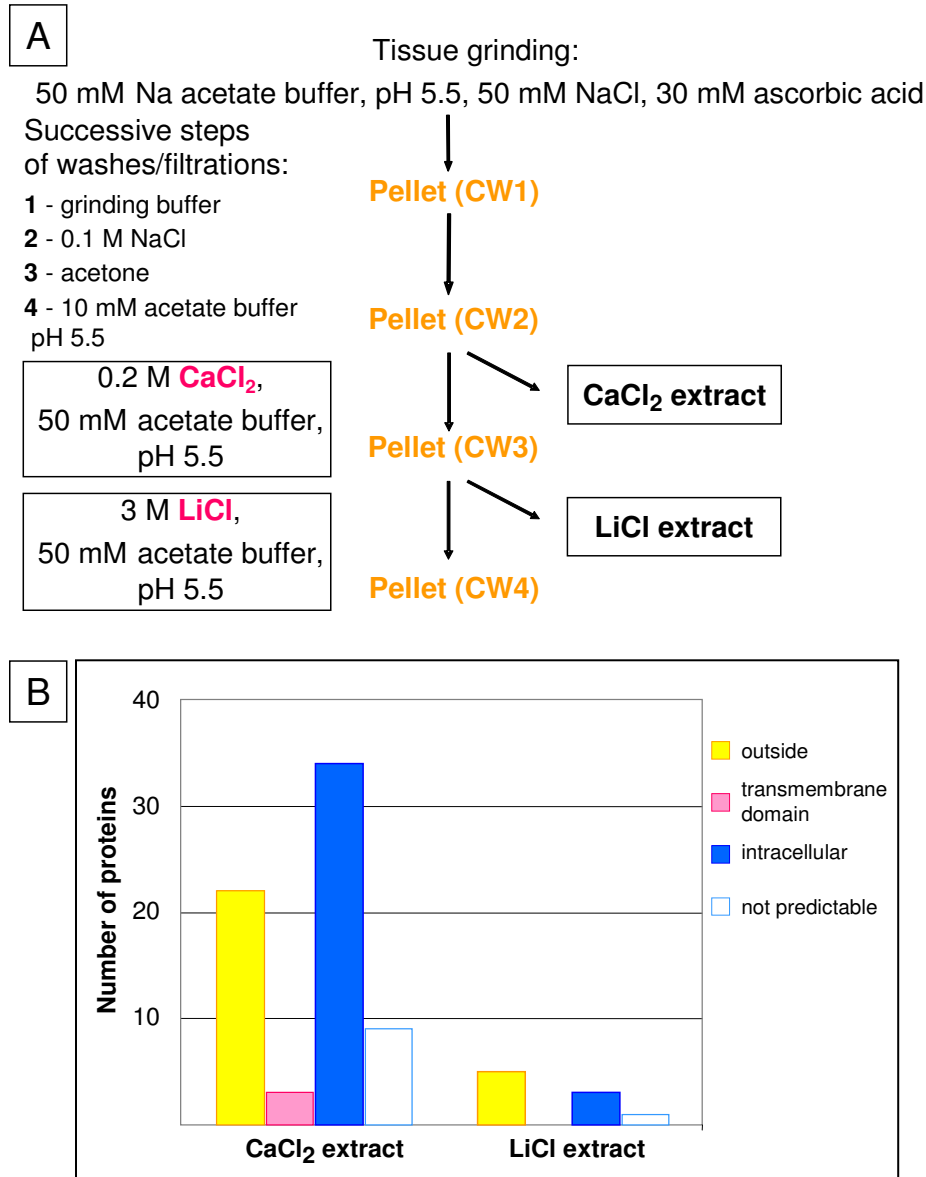


Figure 2

Cell wall preparation from *M. sativa* stems. **A** – Schematic representation of the purification procedure of cell walls from *M. sativa* stems, and of the different extracts obtained [9]. **B** – Number of proteins identified in each extract after 2D-GE separation and LC-MS/MS analysis. Proteins were classified as indicated in legend to Figure 1. Since the *M. sativa* genome is not fully sequenced, the sequence of the N-terminus of some proteins is not known. They were classified as "not predictable". Twenty-five different proteins predicted to be secreted were identified in this study (see Additional file 2).

C. albicans yeast and hyphae

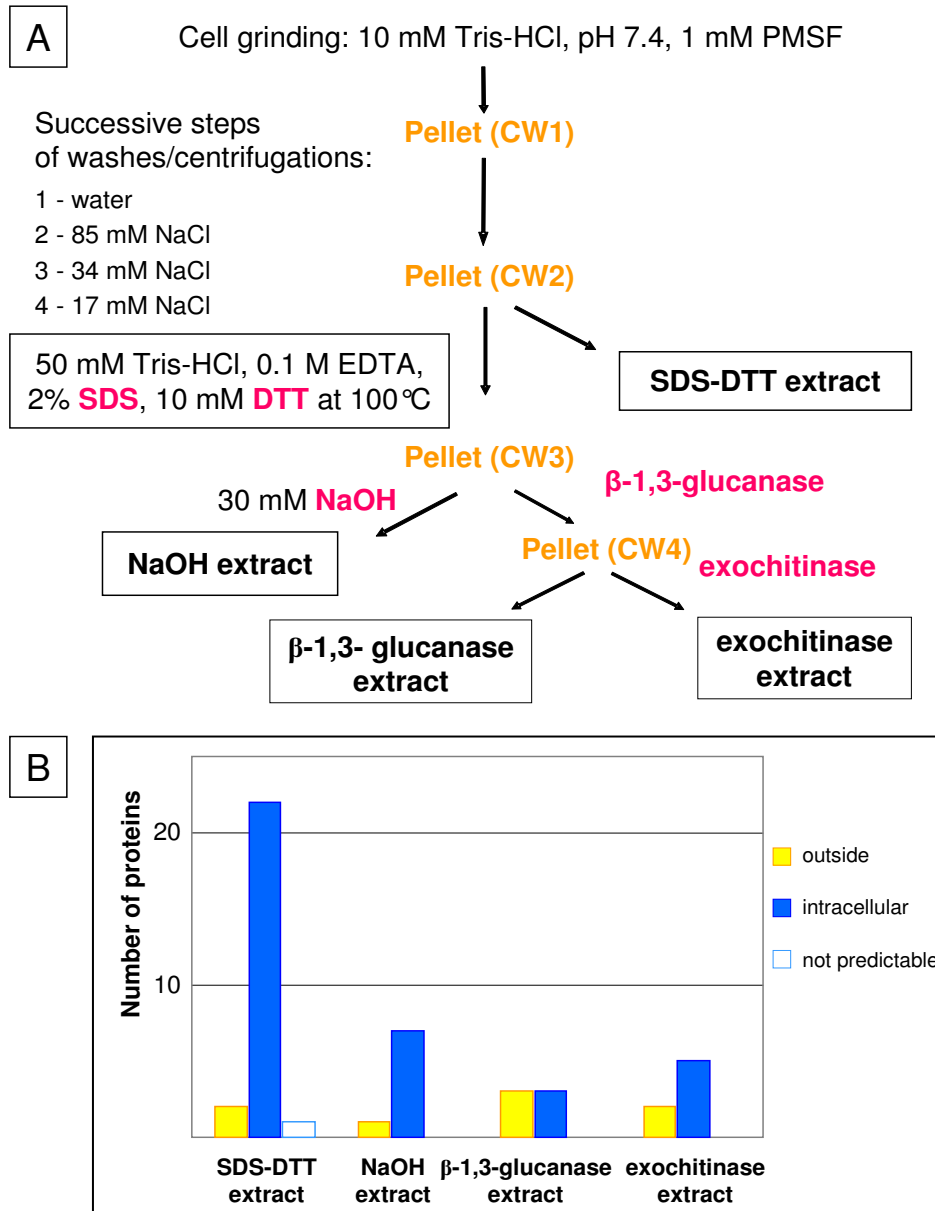


Figure 3

Preparation of cell walls from *C. albicans* (yeast and hyphae). **A** – Schematic representation of the purification procedure of cell walls from the dimorphic fungus *C. albicans* (yeast and hyphae), and of the extracts obtained [20]. **B** – Number of proteins identified in each extract after 2D-GE and analysis by MALDI-TOF or MALDI-TOF/TOF MS. Proteins were classified as indicated in legend to Figure 1. Four different proteins predicted to be secreted were identified in this study (see Additional file 3).

and classified as: (i) outside (proteins predicted to be secreted since they contain a signal peptide and no other targeting sequence), (ii) having trans-membrane domain(s), or (iii) intracellular (proteins not fulfilling these criteria). Figure 1B represents the results of both extractions. It appears that the CaCl₂ extract contains the highest proportion of proteins predicted to be secreted (50%), and that the use of detergents and chaotropic agents brings out mostly intracellular proteins, even if 20% of this fraction corresponds to predicted secreted proteins. Twenty-four different proteins predicted to be secreted were identified with this method.

Stems of *Medicago sativa* (alfalfa) were used for cell wall protein profiling [9]. In this case, a different procedure was used to isolate cell walls (Figure 2A). Tissues were frozen and ground in cold grinding buffer (50 mM Na acetate buffer, pH 5.5, 50 mM NaCl, and 30 mM ascorbic acid) with PVPP. Cell walls were isolated by filtering through a 47 μm² mesh nylon membrane and washed sequentially with grinding buffer, 0.1 M NaCl, acetone and 10 mM Na acetate pH 5.5. The proteins were sequentially extracted with 0.2 M CaCl₂, and 3 M LiCl buffers. The data obtained in this publication was analyzed as mentioned above, and the results are presented in Figure 2B. The proportion of intracellular proteins in the CaCl₂ extract is quite high (50%). It seems that the first washes do not eliminate such proteins. It is also possible that the wash performed with 0.1 M NaCl eliminates part of the secreted proteins. Twenty-five different proteins predicted to be secreted were identified in this study.

The procedure for isolation of cell walls from the dimorphic fungus *Candida albicans* used by Pitarch et al [20] was based on previous methods designed to isolate proteins covalently linked to the polysaccharide matrix [21,22]. Yeast and hyphae were collected by centrifugation and filtration, washed several times with lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM PMSF), and mechanically disrupted in lysis buffer (Figure 3A). After centrifugation, the pellet was successively washed with cold water and decreasing concentrations of NaCl (85, 34 and 17 mM) in 1 mM PMSF. Proteins were extracted with boiling SDS-extraction buffer (50 mM Tris-HCl, pH 8.0, 0.1 M EDTA, 2% SDS, 10 mM DTT). The residue was separated in two fractions, one was extracted with alkali (30 mM NaOH), and the other was submitted to sequential digestions by a β-1,3-glucanase followed by an exochitinase to break down the polysaccharide matrix. Each of the four samples were separated by 2D-GE, digested with trypsin and the peptides identified by MALDI-TOF or MALDI-TOF/TOF MS. The proteins identified in this publication were submitted to bioinformatic analysis and the results are represented in Figure 3B. Only four proteins predicted to be

secreted were identified. All the others are predicted to be intracellular proteins (78%).

Altogether, this evaluation of three procedures to isolate cell walls from plants or fungi prior to proteomic analyses shows that they all produce a material containing a high proportion of proteins predicted to be intracellular, suggesting they are contaminants. Even if a careful bioinformatic analysis allows the discrimination between secreted and intracellular proteins, the time and effort consumed is not satisfactory.

A modified method to prepare plant cell walls

From the analysis of the presented methods and other classical cell wall preparations used for the purification of cell wall enzymes [23,24], several points appear to be essential for the purification of cell walls. First, the presence of NaCl at early steps of cell wall preparations of *M. sativa* and *C. albicans* in grinding or washing buffers might induce a release of CWP even at a low concentration [12]. This might indirectly increase the proportion of intracellular proteins sticking non-specifically to cell wall polysaccharides. The use of a low ionic strength buffer for tissue grinding and subsequent washes to purify cell walls appears as an interesting alternative to prevent loss of CWP. Second, the protocol used for *A. thaliana* cell suspension cultures is the only one including a purification of cell walls through a dense medium, *i.e.* sedimentation in 10% glycerol. Proteins predicted to be secreted represented 50% of the identified proteins. It seems that a series of sedimentations/centrifugations in solutions of increasing densities would help in eliminating organelles and other vesicles less dense than cell wall polysaccharides [23,24]. Third, despite repeated washes, the cell wall preparation from *A. thaliana* cell suspension cultures still contained a high proportion of proteins predicted to be intracellular. A way to eliminate soluble contaminants such as intracellular proteins is to perform extensive washes of cell walls with a low ionic strength buffer [24]. Finally, the addition of polyvinyl polypyrrolidone (PVPP) to trap plant phenolic compounds [14] as well as anti-proteases to limit protein degradation during the manipulations [13], improves the quality of protein identification by mass spectrometry. Our procedure was established on the basis of these requirements.

Eleven day-old etiolated hypocotyls were ground in a low ionic strength buffer, 5 mM acetate buffer pH 4.6 in 0.4 M sucrose (Figure 4). PVPP and anti-proteases were added to the homogenate, centrifuged, and the resulting pellet resuspended in 5 mM acetate buffer pH 4.6 with increasing concentrations of sucrose (0.6 M and 1 M) and centrifuged. The residue (CW3) was extensively washed on a nylon membrane (25 μm pore size) with large amounts of 5 mM acetate buffer pH 4.6 (3 L for 16 g fresh material).

A. thaliana hypocotyls: cell wall preparation

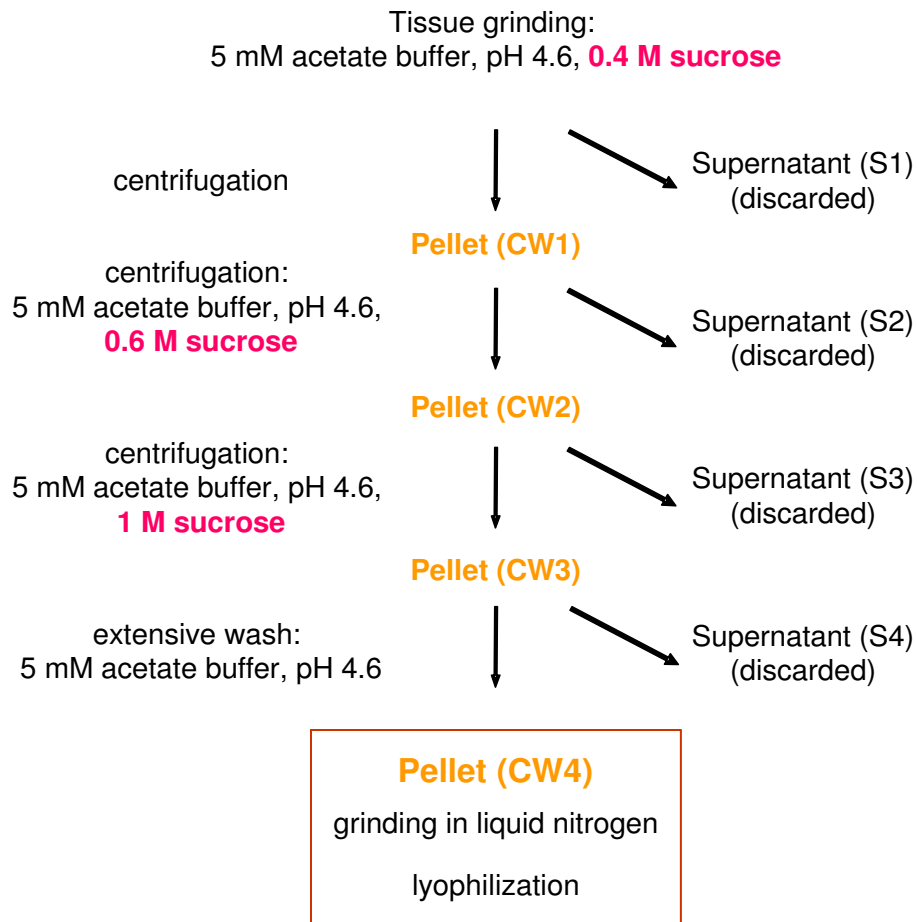


Figure 4

Cell wall preparation from *A. thaliana* hypocotyls. Schematic representation of the purification procedure of cell walls of *A. thaliana* etiolated hypocotyls. Sixteen g of fresh etiolated hypocotyls were ground. All supernatants were discarded after each centrifugation. The CW3 residue was extensively washed on a nylon net (25 μ m pore size) with 3 L of 5 mM acetate buffer, pH 4.6. The CW4 pellet was ground in liquid nitrogen in a mortar with a pestle in order to reduce the size of the fragments, and lyophilized, obtaining 1.3 g of dry powder.

A. thaliana hypocotyl cell walls: extraction of proteins with salts

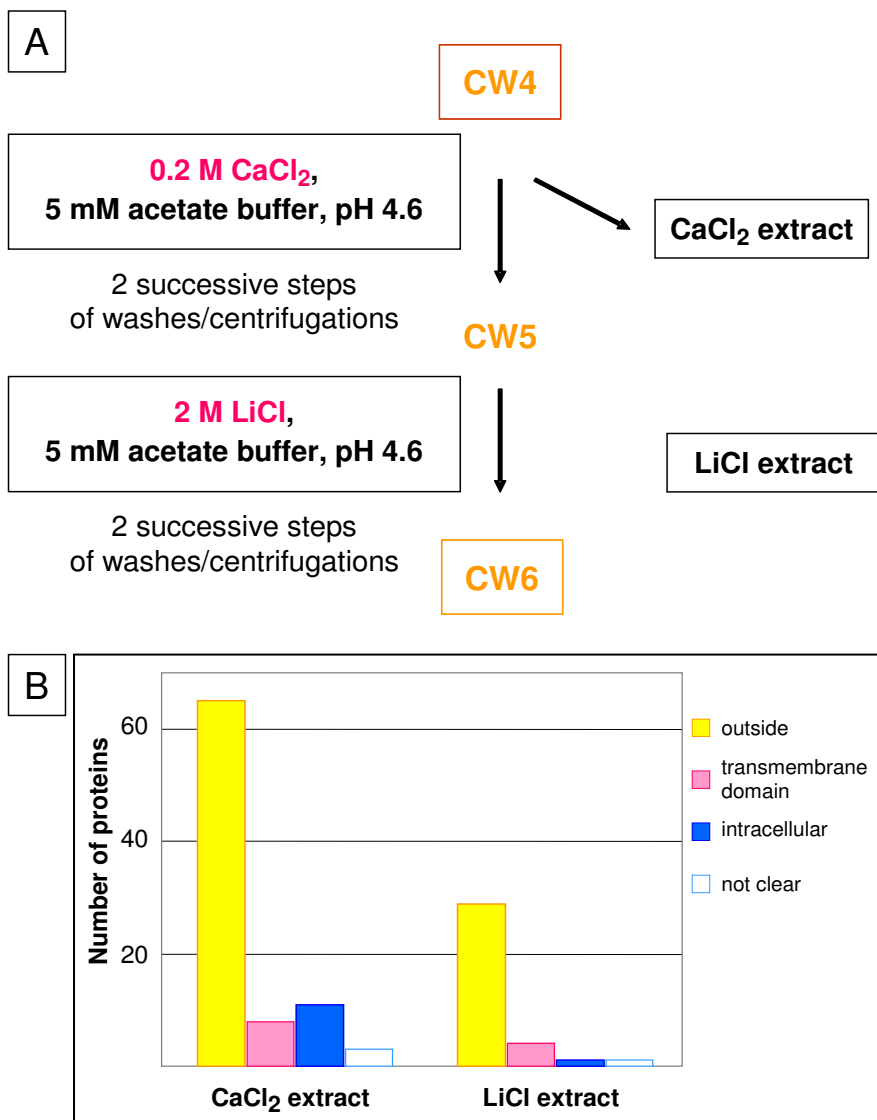


Figure 5

Extraction of proteins from *A. thaliana* hypocotyls with salts. **A** – Half of the CW4 lyophilized powder (0.65 g) was successively suspended in 0.2 M CaCl₂, 5 mM acetate buffer pH 4.6, and in 2 M LiCl, 5 mM acetate buffer pH 4.6. The CaCl₂ extract contained 400 µg of proteins. The LiCl extract contained 40 µg of proteins. **B** – Number of proteins identified in each extract after 1D-GE separation, and analysis by MALDI-TOF MS or LC-MS/MS. Proteins were classified as indicated in legend to Figure 1. Seventy-three different proteins predicted to be secreted were identified in this study (see Additional file 4).

A. thaliana hypocotyl cell walls: extraction of proteins with boiling SDS and DTT

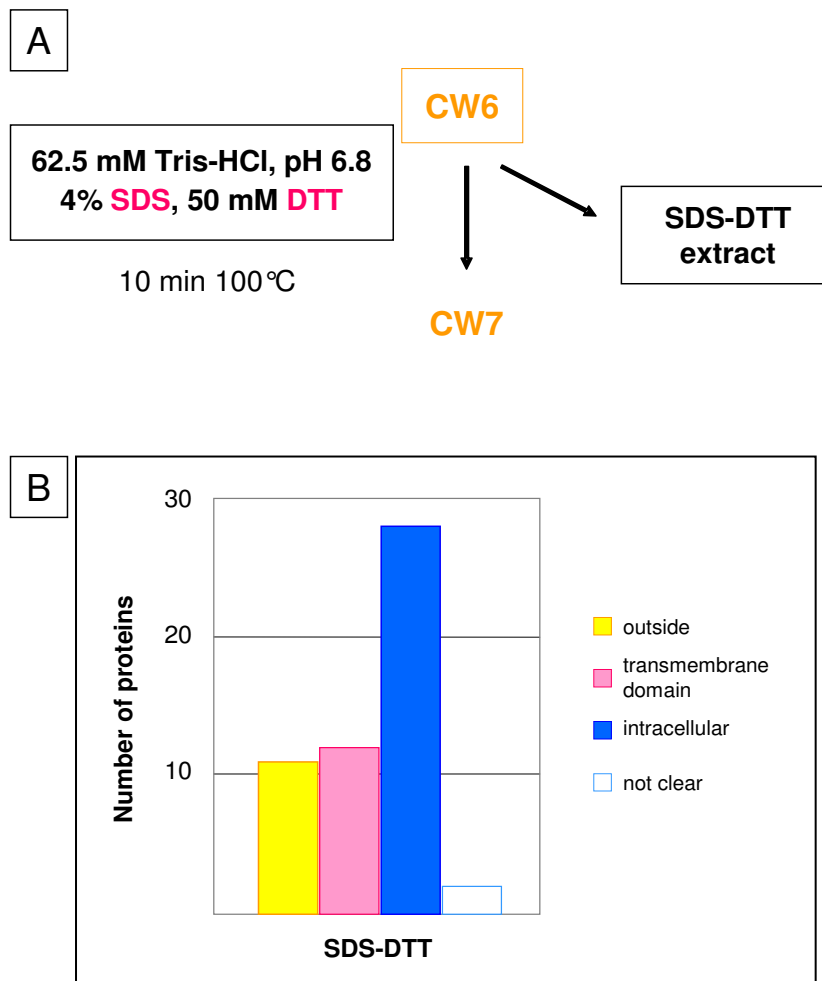


Figure 6

Extraction of proteins from *A. thaliana* hypocotyls cell walls with boiling SDS and DTT. **A** – The CW6 pellet described in Figure 5 was used for further extraction of proteins using boiling 4% SDS and 50 mM DTT in 62.5 mM Tris-HCl buffer, pH 6.8. The SDS-DTT extract contained 10 µg of proteins. **B** – Number of proteins identified in each extract after ID-GE separation, and analysis by MALDI-TOF MS or LC-MS/MS. Proteins were classified as indicated in legend to Figure 1 (see Additional file 5).

The cell wall fraction was ground in liquid nitrogen in a mortar to reduce the size of the particles, which improves the subsequent extraction of proteins (CW4). In a typical cell wall preparation from *A. thaliana* hypocotyls, 16 g of fresh material resulted in 1.3 g of lyophilized powder.

Sequential salt extraction of proteins from cell walls

The lyophilized powder (CW4) was sequentially extracted with salts that can extract proteins ionically bound to the polysaccharide matrix. Calcium chloride has been reported as an efficient salt for the extraction of cell wall proteins [8,9,13], and was used in this procedure (Figure 5A) to identify 65 secreted proteins (see Additional file 4). From 0.65 g of lyophilized powder, 400 µg of proteins were routinely obtained. LiCl was known as potent salt to extract hydroxyproline-rich glycoproteins from *Chlamydomonas reinhardtii* cells, and was successfully used on *M. sativa* cell walls and *A. thaliana* rosettes [9,13,18]. LiCl only extracted 40 µg of proteins, 29 were identified as predicted to be secreted, but only 8 were specifically extracted by this salt (see Additional file 4).

A recent review on 281 CWP identified in proteomic studies by mass spectrometry [7] concluded that more than 60% of them have a basic pI, and around 80% in salt-extractable fractions. This is a serious problem for the separation of CWP by the classical 2D-GE, since it is well known that basic glycoproteins are poorly resolved by this technique [7]. We have then used 1D-GE for the separation, each band stained with Coomassie™ blue was digested by trypsin and further analyzed by MALDI-TOF MS or LC/MS/MS. Each protein was analyzed using several bioinformatic programs as described above. Seventy-three different proteins predicted to be secreted were identified in this study, whereas only 12 proteins predicted to be intracellular and 11 proteins predicted to have trans-membrane domains were found.

The protocol of CWP extraction we used is almost the same as the one employed with *M. sativa* stems [9]. But comparison of Figures 2B and 5B shows big differences in the proportion of proteins predicted to be intracellular or having trans-membrane domains (50% for *M. sativa* vs 27% for *A. thaliana*). Since the main difference between the two protocols is the addition of centrifugations through a dense medium, it shows that this step is critical for the purity of cell walls.

The proteins predicted to be secreted identified in this study belong to the same functional classes as those described in previous cell wall proteomes established from cell wall preparations [8,9,25]. Shortly, these functional classes comprise proteins acting on polysaccharides (e.g. glycoside hydrolases, carbohydrate esterases, expansins), proteases, proteins with interacting domains

(e.g. lectins, leucine-rich repeat proteins, enzyme inhibitors), oxido-reductases (e.g. peroxidases, berberine-bridge enzymes), proteins involved in signaling processes (e.g. arabinogalactan proteins), structural proteins, proteins of unknown function and miscellaneous proteins [7]. The new protocol appears to be more efficient since a large proportion of identified proteins are predicted to be targeted to the compartment of interest.

Since we noticed that the use of detergents described in previous protocols increased the proportion of proteins predicted to be intracellular or having trans-membrane domains [8,20], we wanted to test it on our cell wall preparation. The CW6 pellet (Figure 6) was treated with boiling SDS-DTT buffer (62.5 M Tris-HCl, pH 6.8, 4% SDS, 50 mM DTT). Less than 10 µg of proteins were obtained. As for the other fractions, the proteins were concentrated, separated by 1D-GE, and the Coomassie™ blue stained bands were analyzed by mass spectrometry. Fifty-three proteins were identified in this fraction, among which 11 (20%) were predicted to be secreted, 12 (23%) were predicted to have trans-membrane domains and 30 (57%) were predicted to originate from intracellular compartments including cytoplasm, endoplasmic reticulum, microbodies, and chloroplasts. Comparison of these results with those shown in Figures 1B and 3B, in which detergents such as CHAPS-Urea-DTT or SDS-DTT were also used, confirms that treatment with detergents mainly extract intracellular proteins as well as membrane proteins trapped within the polysaccharide matrix: respectively 66% and 88% of proteins predicted to be intracellular, and 15% of proteins having trans-membrane domain in the case of *A. thaliana* cell walls. Unless looking for specific proteins only extractable in those conditions, this step should be avoided for a large-scale cell wall proteomic study. It can rather be a good method to get rid of contaminants and to have a cleaner preparation for subsequent extraction of strongly bound CWP.

Conclusion

The new cell wall preparation procedure followed by salt extraction of proteins described in this paper gives the lowest proportion of proteins predicted to be intracellular when compared with other available protocols and allows the identification of proteins fitting in the same functional classes. Addition of a step including detergent treatment revealed the presence of minor amounts of a few additional proteins predicted to be secreted, but of many proteins predicted to be intracellular. Prediction of the sub-cellular localization of proteins by different bioinformatic programs appeared as an essential tool to evaluate cell wall purification procedures. However, it should not be considered satisfactory to determine the sub-cellular localization of any protein identified by a proteomic analysis. Additional experiments performed *in planta*, such as

immunolocalization or localization of fluorescent protein fusions, are required to confirm it. The application of the principles of cell wall purification described in this paper should lead to a more realistic view of the cell wall proteome, at least for the weakly bound CWP extractable by salts. In addition, it offers a clean cell wall preparation for subsequent extraction of strongly bound CWP.

Methods

Plant material and isolation of cell walls

One hundred and fifty mg of *A. thaliana* seeds (ecotype Columbia 0) were sowed per Magenta box containing Murashige and Skoog medium [26] supplemented with 2% w/v sucrose and 1.2% w/v agar. Seedlings were grown at 23°C in the dark for 11 days. For one experiment, hypocotyls from 16 Magenta boxes were collected. One cm long hypocotyls were cut below the cotyledons and above the root, washed with distilled water and transferred into 500 mL of 5 mM acetate buffer, pH 4.6, 0.4 M sucrose and protease inhibitor cocktail (Sigma) 1 mL per 30 g of hypocotyl fresh weight. The mixture was ground in a blender at full speed for 15 min (Figure 4). After adding PVPP (1 g per 10 g fresh weight of hypocotyls), the mixture was incubated in cold room for 30 min while stirring. Cell walls were separated from soluble cytoplasmic fluid by centrifugation of the homogenate for 15 min at 1000 × g and 4°C. The pellet (CW1 in Figure 4) was further purified by two successive centrifugations in 500 mL of 5 mM acetate buffer, pH 4.6, respectively 0.6 M and 1 M sucrose. The residue (CW3) was washed with 3 L of 5 mM acetate buffer, pH 4.6, on a nylon net (25 μm pore size). The resulting cell wall fraction (CW4) was ground in liquid nitrogen in a mortar with a pestle prior to lyophilization. Starting with 16 g fresh weight of hypocotyls, this process resulted in 1.3 g dry powder.

Sequential proteins extraction and identification

Typically, 0.65 g of lyophilized cell walls was used for one experiment. Proteins were extracted by successive salt solutions in this order: two extractions each time with 6 mL CaCl₂ solution (5 mM acetate buffer, pH 4.6, 0.2 M CaCl₂ and 10 μL protease inhibitor cocktail), followed by two extractions with 6 mL LiCl solution (5 mM acetate buffer, pH 4.6, 2 M LiCl and 10 μL protease inhibitor cocktail). Cell walls were resuspended by vortexing for 5–10 min at room temperature, and then centrifuged for 15 min at 4000 × g and 4°C. Supernatants were desalted using Econo-Pac® 10 DG columns (Bio-Rad) equilibrated with 0.2 formic acid ammonium salt. The extract were lyophilized and resuspended in sample buffer for separation of proteins by 1D-GE, as previously described [12].

The next extraction was carried out by SDS and DTT. The cell wall preparation was treated with 12 mL solution containing 62.5 mM Tris, 4% SDS, 50 mM DTT, pH 6.8

(HCl). The mixture was boiled for 5 min and centrifuged for 15 min at 40000 × g and 4°C. The supernatant was dialyzed against 1 L H₂O in Spectra/Por® membrane 10 kDa MWCO bags (Spectrum Medical Industries) at room temperature, then concentrated by successive centrifugation using the Centriprep® centrifugal filter devices (YM-10 kDa membrane) (Millipore) at 4000 × g followed by speed vacuum centrifugation.

The protein content of each extract was measured using the Bradford method [27] with the Coomassie™ protein assay reagent kit (Pierce) using bovine serum albumin (BSA) as standard.

Gels were stained with Coomassie™ Brilliant Blue-based method [28]. Colored bands were digested with trypsin and MALDI-TOF MS or LC-MS/MS analyses were performed as previously reported [12,13].

The sequences of the identified proteins were subsequently analyzed with several bioinformatic programs to predict their sub-cellular localization [29-31]. In some cases, predictions were not the same with the three programs. Results are then indicated as "not clear". Data are described in Tables 1–5 (additional data).

Abbreviations

1D/2D-GE: one or two dimensional gel electrophoresis

CWP: cell wall proteins

CHAPS: 3- [(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid

DTT: dithiothreitol

EDTA: ethylene diamino tetraacetic acid

LC-MS/MS: liquid chromatography-tandem mass spectrometry

MALDI-TOF: matrix-assisted laser desorption ionization-time of flight

MS: mass spectrometry

PMSF: phenylmethylsulfonyl fluoride

PVPP: polyvinyl polypyrrolidone

SDS: sodium dodecyl sulphate

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

LF and MI carried out cell wall preparations, protein extractions, protein separations and mass spectrometry analyses. HC designed the cell wall preparation procedure and contributed to MALDI-TOF analyses and manuscript writing. EJ conceived data evaluation, performed bioinformatic analyses and was involved in manuscript writing. RPL participated in discussions all along the study, in literature screening, conceived the manuscript, and coordinated its writing. All authors read and approved the final manuscript.

Additional material

Additional data file 1

Table 1 – Bioinformatic analysis of proteins extracted from cell walls of *A. thaliana* cell suspension cultures [8].

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[<http://www.biomedcentral.com/content/supplementary/1746-4811-2-10-S1.pdf>]

Additional data file 2

Table 2 - Bioinformatic analysis of proteins extracted from cell walls of *M. sativa* stems [9]

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Additional data file 3

Table 3 – Bioinformatic analysis of proteins extracted from cell walls of *C. albicans* [20].

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Additional data file 4

Table 4 - Bioinformatic analysis of proteins extracted from cell walls of *A. thaliana* etiolated hypocotyls with salts.

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[<http://www.biomedcentral.com/content/supplementary/1746-4811-2-10-S4.pdf>]

Additional data file 5

Table 5 - Bioinformatic analysis of proteins extracted from cell walls of *A. thaliana* etiolated hypocotyls with SDS and DTT.

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[<http://www.biomedcentral.com/content/supplementary/1746-4811-2-10-S5.pdf>]

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CHAPTER 4

**A NEW PICTURE OF CELL WALL PROTEIN DYNAMICS
IN ELONGATING CELLS OF *ARABIDOPSIS THALIANA*:
KNOWN PLAYERS AND NEW COMERS**

Chapter summary (French)

La croissance des cellules végétales est accompagnée par celle de leurs parois qui nécessite d'une part la synthèse de constituants pariétaux, d'autre part leur ajout dans les parois et le remaniement des réseaux existants. Les protéines pariétales sont importantes pour cette dernière phase. Obtenir une image globale des protéines pariétales dans des hypocotyles en cours d'élongation (plantules âgées de 5 jours) et après la fin de leur croissance (plantules âgées de 11 jours) devrait contribuer à la compréhension des mécanismes de réarrangement des constituants pariétaux ainsi que les processus de régulation de la croissance pariétale.

Après avoir surmonté la première difficulté de ce travail consistant à obtenir des préparations de parois de bonne qualité (chapitre 3), je me suis consacré à l'étude protéomique des parois des hypocotyles en élongation comparée à celle des hypocotyles après la fin de leur croissance. Les protéines pariétales ont été extraites avec du CaCl_2 , puis du LiCl à chaque stade de développement. La difficulté suivante est apparue au niveau de la séparation des protéines. En effet, les méthodes existantes ne se sont pas révélées très efficaces dans la mesure où les protéines pariétales sont majoritairement basiques et glycosylées, donc difficiles à séparer avec la méthode traditionnellement utilisée en protéomique, *i.e.* l'électrophorèse bi-dimensionnelle. Par ailleurs, l'électrophorèse mono-dimensionnelle était un outil insuffisamment performant puisque de nombreuses protéines comigraient et devenaient ainsi difficiles à identifier par cartographie peptidique massique (spectrométrie de masse de type MALDI-TOF). Ce problème a été surmonté en effectuant une séparation des protéines en deux étapes. La première étape a consisté en une chromatographie d'échange de cations à haute performance, utilisant le caractère basique des protéines pariétales. La seconde étape a consisté en une électrophorèse mono-dimensionnelle des fractions recueillies le long du gradient d'éluion par une solution saline. Ensuite, les protéines colorées au bleu de Coomassie ont été systématiquement identifiées par cartographie peptidique massique et bioinformatique. C'est ainsi (i) que le nombre de protéines identifiées a été doublé, comparé à ce qui avait été identifié après une simple séparation par électrophorèse mono-dimensionnelle, (ii) que la qualité des identifications a été largement améliorée, et (iii) qu'il a été possible d'effectuer une semi-quantification des protéines.

Cette étude a permis d'identifier 137 protéines pariétales (120 à 5 jours, et 101 à 11 jours) parmi lesquelles 51 n'avaient pas encore été identifiées par protéomique. La prédiction

de domaines fonctionnels par bioinformatique a permis de les classer en neuf groupes : des protéines agissant sur les polysaccharides pariétaux, des oxydo-réductases, des protéases, des protéines ayant des domaines d'interaction avec des polysaccharides ou d'autres protéines, des protéines impliquées dans la signalisation, des protéines structurales, des protéines liées au métabolisme des lipides, diverses protéines et des protéines de fonction encore inconnue.

La comparaison des deux stades de développement a montré des différences de profil. Bien que beaucoup de protéines extracellulaires soient communes (84), 36 et 17 protéines ont été identifiées de manière spécifique à 5 et 11 jours respectivement. De plus, la comparaison des niveaux relatifs des 84 protéines communes montre que 61 et 26 protéines sont plus abondantes à 5 et à 11 jours respectivement. L'ensemble de ces résultats montre que les parois hypocotyles en élongation active (5 jours) semblent plus métaboliquement plus actives que celles des hypocotyles dont la croissance est achevée (11 jours).

L'analyse par bioinformatique des fonctions des protéines identifiées dans les hypocotyles au cours de leur élongation ou après la fin de leur croissance a permis d'obtenir les résultats suivants :

- i) Les protéines agissant sur les polysaccharides (XTH, PG, PME et expansines) sont plus nombreuses dans les hypocotyles en cours d'élongation, ce qui est cohérent avec leurs rôles supposés dans le réarrangement des polysaccharides ou l'insertion de nouveaux polymères dans les parois.
- ii) Cependant, ces protéines ainsi que les peroxidases sont trouvées aux deux stades de développement. Ceci indique soit que ces protéines ont des demi-vies importantes, soit qu'elles pourraient jouer des rôles différents. Par exemple, les XTH pourraient intervenir non seulement pour le réarrangement des xyloglucanes au cours de la phase d'élongation cellulaire, mais aussi au cours de la différenciation d'éléments trachéaires plus tardivement. Les peroxidases pourraient d'une part contribuer à la formation de radicaux libres participant à la fragmentation des polysaccharides au cours de l'élongation cellulaire, et d'autre part assurer la formation des réseaux de protéines et de polyphénols après son arrêt.
- iii) La présence d'inhibiteurs d'enzymes telles que les protéases, PME, PG et xyloglucane endoglucanases (XEGIP) montre que la présence d'une enzyme ne signifie pas nécessairement qu'elle est active. L'interaction avec un inhibiteur peut

moduler son activité biologique. De même, 14 protéases ont été trouvées : elles pourraient soit assurer la maturation de protéines pour les rendre actives ou au contraire assurer leur dégradation. L'apport d'une approche protéomique est ici essentiel pour envisager ces types de régulation.

- iv) Six protéines liées au métabolisme des lipides ont été identifiées. Leur rôle n'est pas clairement établi. Cependant, on peut imaginer qu'il s'agit de candidats qui pourraient être impliqués dans la formation de la cuticule particulièrement épaisse des hypocotyles ayant poussé à l'obscurité.
- v) Seize protéines de fonction inconnue ont été identifiées. Ces protéines pourraient jouer des rôles importants qui restent à découvrir au cours de la croissance des hypocotyles.

L'ensemble de cette étude a permis de donner une image globale des protéines présentes dans les parois des hypocotyles étiolés d'*Arabidopsis*. Cette étude permet d'identifier des gènes potentiellement importants pour l'élongation cellulaire. Elle permet aussi d'envisager des mécanismes de régulation de l'activité biologique des protéines dans les parois en rapprochant des partenaires potentiels (enzymes et leurs inhibiteurs, protéases). Des études fonctionnelles fines sont encore nécessaires pour préciser le rôle de ces protéines au cours du développement des hypocotyles. Les résultats sont détaillés dans l'article qui suit, actuellement soumis pour publication.

In plants cell, elongation and plant overall growth are accompanied by the synthesis, addition and re-arrangement of cell wall components. CWPs are important for proper assembly and reorganization of cell wall components. A global picture of the CWPs of elongating (5-day-old) and fully elongated (11-day-old) hypocotyls may help to uncover their interactions with other cell wall components and to understand the regulatory mechanisms of cell elongation.

After overcoming the first problem of obtaining good quality cell wall (chapter 3), I focused on comparative proteomic study of cell wall of etiolated hypocotyls during and after elongation. CWPs were extracted with CaCl_2 and LiCl from the cell wall purified from hypocotyls of both stages according to the method described in chapter 3. Good quality resolution of CWPs was a problem:

- i) the CWP extract resolved with one dimensional electrophoresis (1D-E), more than one protein were identified from the same band of CBB-stained polyacrylamide gel because of the complexity of the sample with several proteins of same molecular weight.
- ii) 2D-E is not a suitable resolving tool for CWPs because most of them are glycosylated and have basic PIs.

This problem was overcome by first separating CWP extract by cation exchange chromatography using Fast Protein Liquid Chromatography (FPLC), followed by 1D-E of the salt eluted fractions. The profile of the proteins on CBB-stained polyacrylamide gel and their identification by mass spectrometry and bioinformatics justified the use of FPLC followed by 1D-E as a powerful alternative to 2D-E for CWPs. Use of this technique (i) doubled the number of identified proteins as compared to 1D-E alone, (ii) gave higher quality identification by higher numbers of peptides for identification and repetition of identification in succeeding fractions, and (iii) allowed semi-quantification of proteins.

Overall 137 CWPs (120 and 101 in 5- and 11-day-old respectively) were identified in this study, among which 51 are reported for the first time in a proteomic study. The bioinformatics prediction of functional domains allowed their classification into nine categories: proteins acting on carbohydrates, oxido-reductases, proteins with interaction domains, proteases, structural proteins, proteins involved in signaling, proteins related to lipid metabolism, proteins with miscellaneous functions, and proteins of unknown function.

Comparison of the two physiological stages revealed differences regarding presence/absence or abundance of several proteins in one or the other samples. Although most CWP (84) were common to both stages, 36 and 17 were identified only in 5- and 11-day-old hypocotyls respectively. The comparison of relative amount showed 61 and 26 proteins are more abundant at 5- and 11-day-old stages respectively. These results suggest that the cell wall of 5-day-old hypocotyls undergoing active elongation is metabolically more active than at 11-day-old hypocotyls that are fully grown.

The main results obtained from the bioinformatic analysis of the functions of the proteins identified in etiolated hypocotyls during and after elongation are listed below:

- i) Proteins expected to be acting on carbohydrates (XTHs, PGs, PMEs, and expansins) are numerous in elongating hypocotyls where they can be involved in the rearrangement of wall polysaccharides or integration of the new wall polymers.
- ii) However such proteins involved in elongation (XTHs, expansins, PGs, PMEs and peroxidases) are found in both stages. This gives clues about long half-lives of these proteins or their involvement in other processes. For example the XTHs could be involved in rearrangement of xyloglucans not only during elongation, but also later during treacher elements differentiation. The peroxidases produce free radicals that can break cell wall polysaccharides during elongation or cross-link structural proteins and polyphenols after elongation.
- iii) It seems that the proteases found at both stages regulate these proteins by proteolytic degradation.
- iv) Presence of Inhibitors of enzymes like proteases, PME, PGs and xyloglucan endoglucanases (XEGIPs) suggests that the presence of a protein does not mean its active functioning. Thus the interaction of an inhibitor may regulate the biological activity of the enzyme. Fourteen proteases were also found that may be involved in the maturation of proteins or in their degradation. The contribution of proteomic approach is therefore essential to consider such regulations.
- v) The function of proteins related to lipid metabolism is not clear. Six members of this group identified during this study might be involved in the formation of thick cuticle, characteristic of etiolated hypocotyls of *A. thaliana*.
- vi) Sixteen proteins of unknown function were found in both stages that could perform important roles in hypocotyls thus requiring special attention.

This study gives a global picture of proteins present in the cell wall of etiolated hypocotyls of *A. thaliana* and helped to identify genes potentially important for cell elongation. By identifying the proteases and enzyme inhibitors, it pointed out the mechanisms that may regulate the biological activity of proteins in the cell wall. Despite all these, functional characterization of these proteins is needed to know their exact roles during hypocotyle development. The detailed results are given in the following article, recently submitted for publication.

The supplementary data files are given as annexes at the end of the report.

A new picture of cell wall protein dynamics in elongating cells of***Arabidopsis thaliana*: known players and new comers**

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Summary

- To figure out the role of cell wall proteins (CWPs) in the addition and rearrangement of plant cell wall components during cell elongation, the identification, quantification, and comparison of the secreted proteins of *Arabidopsis thaliana* half- (active growth) and fully-grown (after growth arrest) etiolated hypocotyls was performed.
- A comparative proteomic study was performed at the two developmental stages of hypocotyls starting from purified cell walls and using a new strategy for CWP separation including high performance cation exchange chromatography and mono-dimensional electrophoresis
- In total, 137 proteins predicted to be secreted were identified, among which 51 were not previously found by proteomic approaches. Expected CWPs known to be involved in cell wall extension such as XTHs, expansins, PGs, PMEs and peroxidases were identified as well as new CWPs such as proteases, proteins related to lipid metabolism and proteins of unknown function.
- This work highlights a great dynamics of CWPs between the two developmental stages. The presence of proteins well-known to be related to cell wall extension after growth arrest showed that they might play other functions in cell walls. From this global view of cell wall proteins, putative regulatory mechanisms of protein biological activity are discussed.

Keywords: protein dynamics, cell elongation, *Arabidopsis thaliana*, cell wall, etiolated hypocotyl, proteome.

Abbreviations

AGP: arabinogalactan protein; CBB: Coomassie brilliant blue; CWP: cell wall protein; 1D-E: mono-dimensional electrophoresis; 2D-E: two-dimensional electrophoresis; FLA: fasciclin arabinogalactan protein; GH: glycoside hydrolase; GRP: glycine-rich protein; LAE: late-abundant embryogenesis protein; LTP: lipid transfer protein; LRR: leucine-rich repeat; LRX: leucine-rich repeat extensin; MALDI-TOF: matrix-assisted laser desorption ionization – time of flight; PG: polygalacturonase; PL: polysaccharide lyase; PME: pectin methylesterase; PTM: post-translational modification; XTH: xyloglucan endotransglucosylase-hydrolase.

Introduction

Plant cell walls are dynamic compartments which composition and structure vary during development and response to environmental stresses. Variability has been observed in developing roots at the level of glycoproteins in carrot (Smallwood *et al.*, 1994), and of polysaccharides in *Arabidopsis thaliana* (Freshour *et al.*, 1996). In both articles, antibodies against specific epitopes showed irregular distribution between cell types, as well as changes during development. To characterize cell wall plasticity, elongation is a particularly appropriate process in which cell walls need to enlarge and to be re-organized. The cell machinery is almost completely dedicated to synthesize, export, and reorganize cell walls that are mainly composed of polysaccharide networks (Cosgrove, 2005).

To gain information on the genes and derived proteins involved in cell wall elongation, it is important to dissect the different phases of this process. *Arabidopsis* hypocotyls are a suitable material, since almost no cell division occurs, and only the cells present in the embryo undergo elongation (Gendreau *et al.*, 1997; Saibo *et al.*, 2003; Refrégier *et al.*, 2004). Another advantage is that in etiolated hypocotyls, synthesis, addition, and reorganization of the cell wall material occur in time-separate phases (Gendreau *et al.*, 1997; Derbyshire *et al.*, 2007). During the first 3 days after germination, synthesis and deposition of cell wall material are the main features, producing cells with thick cell walls. Through the following days, the hypocotyl will grow mainly by extensive polymer disassembly and rearrangement.

Understanding the molecular mechanisms responsible for rearrangement of cell wall polymers during hypocotyl growth requires the identification of proteins present *in muro*. Previous studies identified gene families involved in rearrangement of cell wall components during cell elongation (Nicol and Hofte, 1998; Micheli, 2001; Fry, 2004; Passardi *et al.*, 2004). On the other hand, plant cell wall proteomics has emerged a few years ago providing information on cell wall proteins (CWPs) present in many different types of cells including cell suspension cultures, roots, rosette leaves, and stems (Jamet *et al.*, 2008; Lee and Saravanan, 2004). In most cases, limitations were encountered, *e.g.* presence of intracellular contaminants (Feiz *et al.*, 2006), poor quality of separation of CWPs by two-dimensional electrophoresis (2D-E) (Minic *et al.*, 2007). Based on bioinformatic prediction of functional domains, main families of CWPs were identified (Jamet *et al.*, 2006).

In this paper, we analyzed the cell wall proteome of half- (active elongation) and fully-grown (after growth arrest) etiolated hypocotyls identifying the proteins present at both stages. A new strategy has been established for CWP separation, and semi-quantification. The comparison of these two proteomes revealed significant dynamics of CWPs. The presence of CWPs expected to be involved in polysaccharide rearrangement and modification was confirmed in growing hypocotyls. However, some of them were also present in fully-grown hypocotyls, suggesting other functions. Finally, the presence of unexpected proteins suggested that unknown features of cell elongation should be investigated.

Material and Methods

Plant material

One hundred and thirty mg of *Arabidopsis thaliana* seeds (ecotype Columbia 0) were cultivated in Magenta box as described (Feiz *et al.*, 2006). Magenta boxes were maintained at 4°C in the dark during 48 h and subsequently exposed for 4 h to light to synchronize beginning of germination. Finally, seedlings were grown at 23°C in the dark for 5- or 11-days. Hypocotyls were cut with scissors below the cotyledons and above the roots of seedlings. Typically, 36 and 18 Magenta boxes were required for 5- and 11-day-old seedlings respectively.

Cell wall purification and protein extraction

Cell walls were purified as previously described (Feiz *et al.*, 2006). Proteins were extracted from purified cell walls with two successive extractions with CaCl₂ solution (5 mM sodium acetate buffer, pH 4.6, 0.2 M CaCl₂ and 10 µL protease inhibitor cocktail, Sigma), followed by two extractions with LiCl solution (5 mM sodium acetate buffer, pH 4.6, 2 M LiCl and 10 µL protease inhibitor cocktail). Finally, proteins were desalted and lyophilized.

Protein separation by cationic exchange chromatography

Lyophilized proteins were dissolved in a total volume of 2 mL of water. They were quantified with the Coomassie® protein assay reagent kit (Pierce) taking bovine serum albumin (BSA) as a standard (Bradford, 1976). One mg of proteins was used for chromatographic fractionation on a 1 mL HiTrap™ SP FF column (Amersham Biosciences) equilibrated with 50 mM MES (pH 5.6) operated with an FPLC™ System (Amersham Biosciences) controlled by FPLCdirector™ version 1.0 (Amersham Biosciences). The protein solution was adjusted to 50 mM MES (pH 5.6) and 20 µL protease inhibitor cocktail (Sigma) was added before loading onto the column at a flow rate of 0.5 mL.min⁻¹. A 10 mL unfixed fraction was collected at the same rate. Three mL of first wash with 50 mM MES (pH 5.6) were collected at a flow rate of 1 mL.min⁻¹. Fixed proteins were eluted by a gradient from 0 to 0.8 M NaCl in 50 mM MES (pH 5.6) and 24 fractions (1 mL each) were collected at a flow rate of 1 mL.min⁻¹. Finally the column was successively washed with 3 mL of 1.2 M NaCl and 3 mL of 1.5 M NaCl in 50 mM MES (pH 5.6) at the same flow rate. These washes were also collected as 6 fractions (1 mL per tube). To prevent protein degradation, 2 µL of protease inhibitor cocktail (Sigma) were added to all the 1 mL fractions, 6 µL to the first wash and 20

μL to the unfixed fractions. The fractions were combined by 2 or 3 depending on their protein concentration. Unfixed proteins were concentrated by successive centrifugations using the Centriprep® centrifugal filter device (YM-10 kDa membrane for volumes greater than 6 mL or 5 kDa for smaller volumes, Millipore) at $4000 \times g$. All protein fractions were desalted using Econo-Pac® 10DG columns (Bio-Rad) equilibrated with 0.2 M ammonium formate prior to lyophilization

Protein separation by mono-dimensional electrophoresis (1D-E) and identification

Each lyophilized fraction was dissolved in 200 μL water and electrophoresis of proteins was performed according to Laemmli (Laemmli, 1970). Samples were loaded on 22×15 cm SDS-polyacrylamide gel with a concentration of 12.5% of acrylamide and a thickness of 1.5 mm. The staining was carried out by a Coomassie Brilliant Blue (CBB)-based method (Scheler *et al.*, 1998). Colored bands were excised from gels and digested with trypsin. MALDI-TOF MS analyses were performed as previously reported (Borderies *et al.*, 2003; Boudart *et al.*, 2005). MALDI-TOF-TOF MS analysis was performed using a MALDI TOF-TOF Voyager 4700 (AppliedBiosystems/MDS Sciex, USA). N-terminal sequencing of the protein encoded by *At5g14920* was performed at *Plate-Forme d'Analyse et de Microséquençage des Protéines* at Institut Pasteur (Paris, France).

Semi-quantification

Peptide mass fingerprints were compared to the non-redundant database of *Arabidopsis* of NCBI (<ftp://ftp.ncbi.nih.gov/blast/db>) using ProteinProspector (MS-FIT: <http://prospector.ucsf.edu/cgi-bin/msform.cgi?form=msfitstandard>). A quantification index (QI) was calculated for each protein. It was calculated by adding the percentages of coverage by peptide mass mapping (ratio between the number of amino acids in peptides detected by MS and the total number of amino acids of the protein) in all the bands of the FPLC profile in which the protein was identified.

Bioinformatic analyses

Sub-cellular localization, length of signal peptides, prediction of transmembrane domains, homologies to other proteins and protein functional domains were predicted as described (Minic *et al.*, 2007). GHs and CEs were classified according to the CAZy database (<http://www.cazy.org/CAZY/>) (Coutinho and Henrissat, 1999). XTHs and expansins were

named according to <http://labs.plantbio.cornell.edu/xth/> and <http://www.bio.psu.edu/expansins/index.htm> respectively. AGPs and FLAs were named according to Schultz *et al.* (Schultz *et al.*, 2002), Johnson *et al.* (Johnson *et al.*, 2003) and van Engels and Roberts (van Hengel and Roberts, 2003). Proteins homologous to COBRA, LRXs and Hyp/Pro-rich proteins were annotated according to Roudier *et al.* (Roudier *et al.*, 2002), Baumberger *et al.* (Baumberger *et al.*, 2003), and Fowler *et al.* (Fowler *et al.*, 1999) respectively. Peroxidases were named as in the PeroxiBase (<http://peroxidase.isb-sib.ch/index.php>) (Bakalovic *et al.*, 2006). Laccases were annotated as in Pourcel *et al.* (Pourcel *et al.*, 2005) and McCaig *et al.* (McCaig *et al.*, 2005). SKU-like proteins and phytocyanins were named according to Jacobs and Roe (Jacobs and Roe, 2005), and Nersissian and Shipp (Nersissian and Shipp, 2002) respectively. Protease annotation was done according to the MEROPS database (<http://merops.sanger.ac.uk/>).

Results

Establishment of methods for efficient proteomic analysis of hypocotyl cell walls

This proteomic study aimed at comparing two developmental stages of *Arabidopsis* hypocotyls and required high amounts of material. It required establishing appropriate culture conditions to get large amounts of homogenous material. They include synchronization of germination, high seedling density on culture medium, and control of culture conditions *in vitro*. Two stages of development were compared: 5-day-old elongating hypocotyls and 11 day-old fully-grown hypocotyls (Gendreau *et al.*, 1997; Derbyshire *et al.*, 2007). First, purified cell walls were prepared avoiding intracellular contamination and loss of proteins (Feiz *et al.*, 2006). To collect high amounts of proteins, two successive extractions were performed with 0.2 M CaCl₂ and 2 M LiCl (Feiz *et al.*, 2006). Both extracts were combined and used for further analysis. Typically, about 1 mg of proteins was obtained from 1 g of dry purified cell walls.

Finally, proteins were separated prior to identification using matrix-assisted laser desorption ionization - time of flight (MALDI-TOF) mass spectrometry (MS) and bioinformatics. Since 2D-E is not appropriate for the separation of CWPs (Jamet *et al.*, 2008), these proteins were separated using mono-dimensional gel electrophoresis (1D-E). About 60 bands could be stained with Coomassie Brilliant Blue (CBB). Fifty-two and 67 proteins could be identified in the extract from 5- and 11-day-old hypocotyls respectively (Figures 1 and 2, and Tables S1 and S2, supplementary material). Because of the limited resolution of 1D-E, many proteins were identified in each band, some of them with a low number of peptides. In order to improve separation and identification of proteins, we introduced an additional step before 1D-E. Since most CWPs are basic proteins (Jamet *et al.*, 2006), a cationic exchange chromatography was performed using a Fast Performance Liquid Chromatography (FPLC) device (Figures S1 and S2, supplementary material). Fractions were collected and combined prior to separation by 1D-E (Figures 1 and 2, S1b and S2b, supplementary material). Now, about 500 bands could be stained with CBB and further analyzed by MALDI-TOF MS. The percentage of successful protein identification from stained bands was about 70%: 141 and 109 proteins were identified in extracts from 5- and 11-day-old hypocotyls respectively. Many of those proteins were identified in several bands, reinforcing their identification. A great improvement of the quality of the analysis was observed: (i) the number of identified

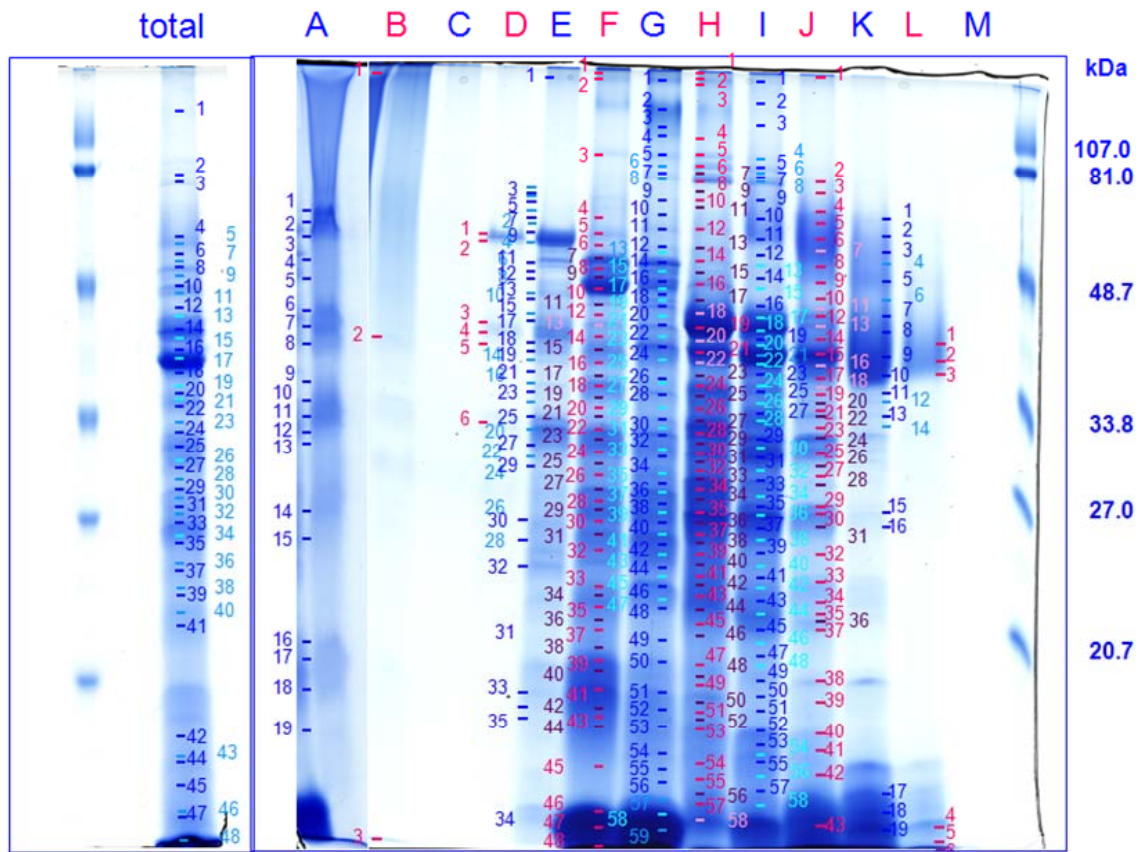


Figure 1. Analysis of proteins extracted by CaCl_2 and LiCl from purified cell walls of 5-day-old etiolated hypocotyls.

Total. Separation by 1D-E of the total extract of proteins. **Fractions A to M.** Separation by 1D-E of 13 fractions (A to M) obtained after cation exchange chromatography (see Figure S1, supplementary material). Molecular mass markers are on the right. Numbers refer to bands analyzed by MALDI-TOF MS with successful identification (see Table S1, supplementary material).

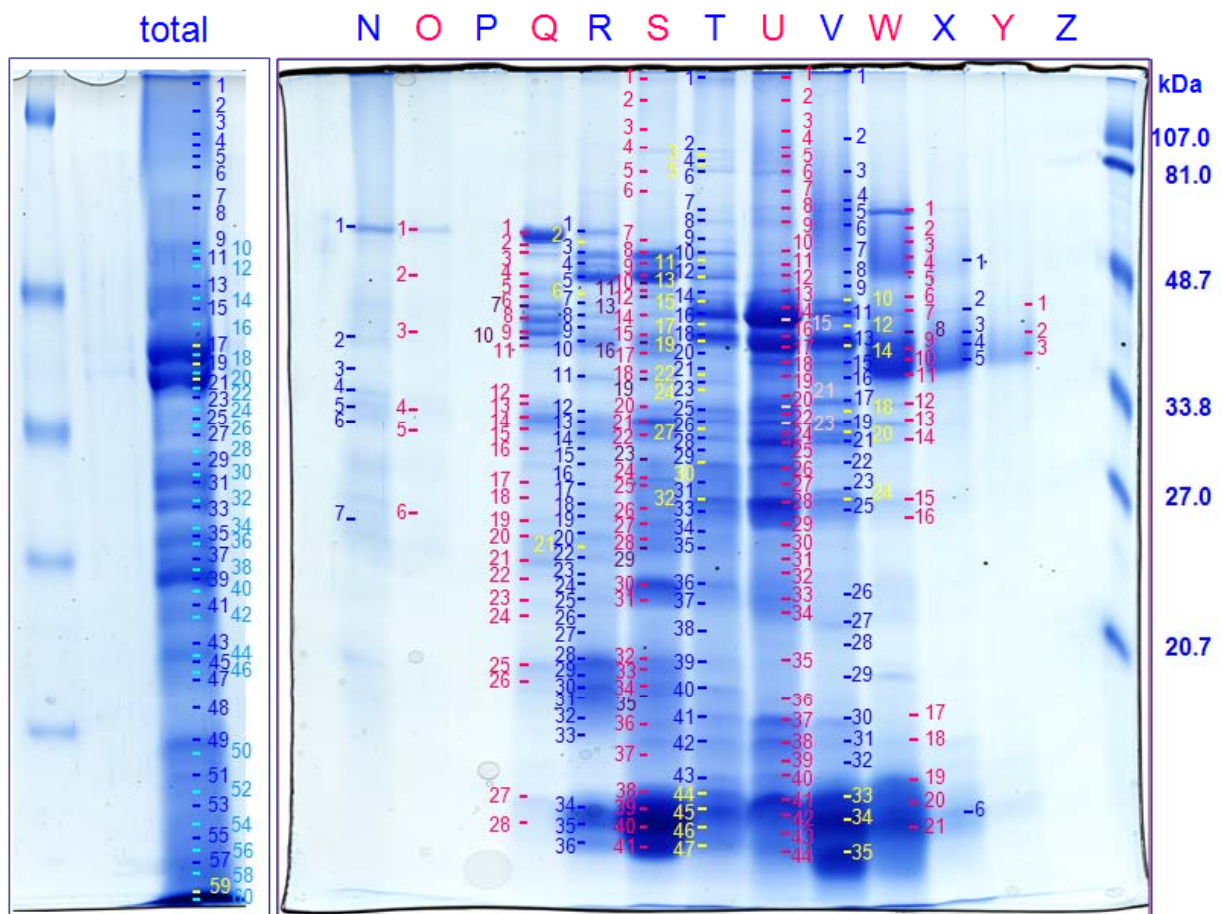


Figure 2. Analysis of proteins extracted by CaCl₂ and LiCl from purified cell walls of 11-day-old etiolated hypocotyls.

Total. Separation by 1D-E of the total extract of proteins. **Fractions N to Z.** Separation by 1D-E of 13 fractions (N to Z) obtained after cation exchange chromatography (see Figure S2, supplementary material). Molecular mass markers are on the right. Numbers refer to bands analyzed by MALDI-TOF MS with successful identification (see Table S2, supplementary material).

Table 1. Number of proteins identified in purified cell walls of 5- and 11-day-old etiolated hypocotyls of *Arabidopsis*.

| | 5-days | 11-days | altogether |
|--|--------|---------|------------|
| total number of identified proteins ^a | 147 | 126 | 173 |
| number of predicted secreted proteins ^a | 120 | 101 | 137 |
| number of predicted secreted proteins identified only in 5-or 11-day-old etiolated hypocotyls ^a | 36 | 17 | |
| number of predicted intracellular proteins ^a | 27 | 25 | 36 |

^a Proteins are listed in Table S3, supplementary material.

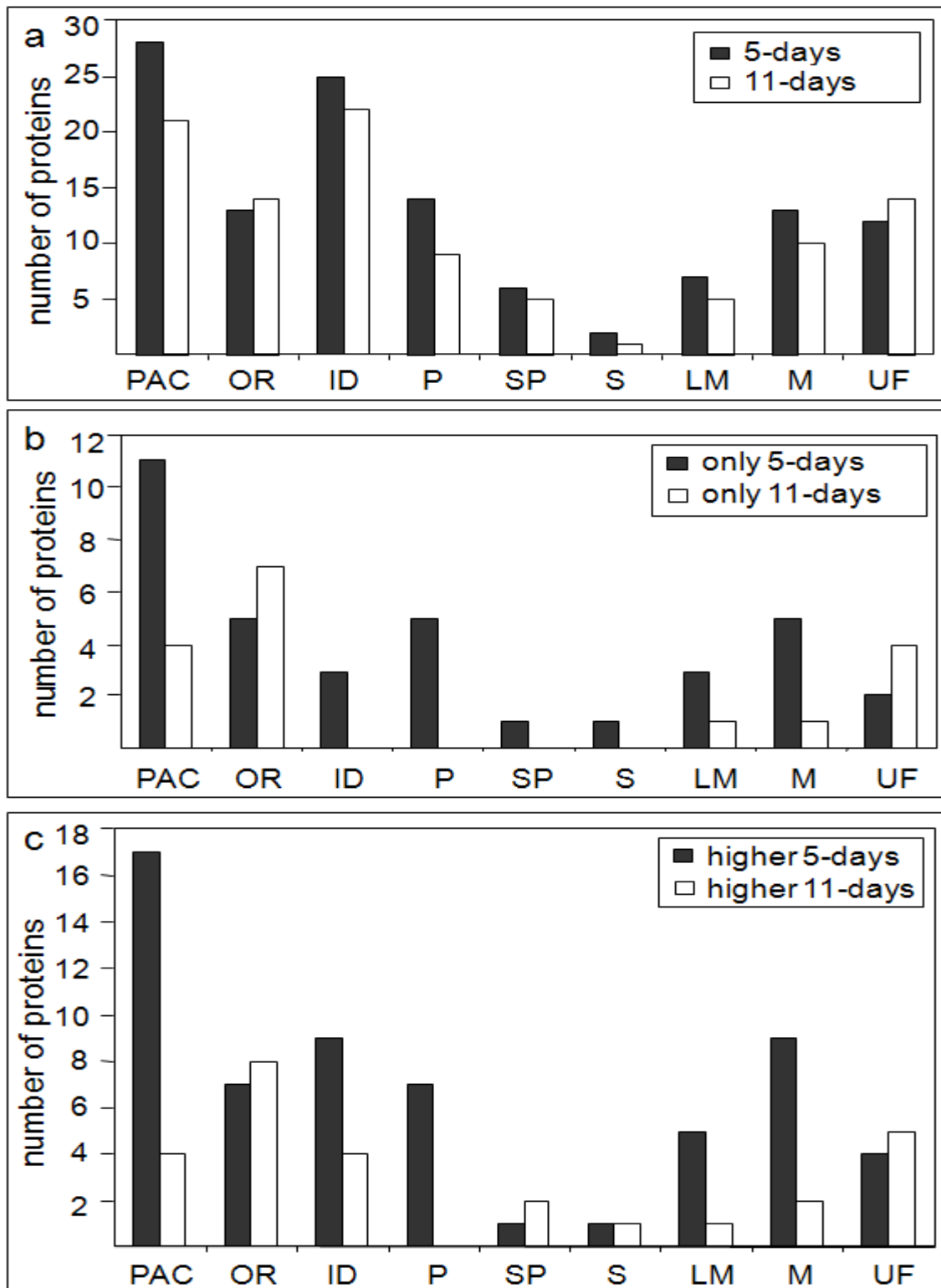


Figure 3. Sorting of CWPs identified from purified cell walls of 5- and 11-day-old etiolated hypocotyls in functional classes.

Proteins were classified according to their functional domains as described in Experimental procedures: proteins acting on carbohydrates (PAC), oxido-reductases (OR), proteins with interaction domains (ID), proteases (P), structural proteins (SP), proteins involved in signaling (S), proteins related to lipid metabolism (LM), proteins with miscellaneous functions (M), and proteins of yet unknown function (UF). **a.** Number of proteins found in the nine functional classes in 5- (5-days), and in 11-day-old hypocotyls (11-days). **b.** Number of proteins found only in 5- (only 5-days), or in 11-day-old (only 11-days) hypocotyls. **c.** Number of proteins present in higher amount in 5- (higher 5-days) or in 11-days-old (higher 11 days) hypocotyls.

proteins was doubled; (ii) the quality of the identifications was increased with higher numbers of peptides for identification of most proteins (Figures S3 and S4, supplementary material); (iii) it was possible to make a semi-quantification of proteins to compare the two samples.

Proteins identified in cell wall extracts of Arabidopsis etiolated hypocotyls

Combining results from 1D- and 2D-separation, 147 and 126 proteins were identified in 5- and 11-day-old etiolated hypocotyls respectively (Table 1). Bioinformatic prediction of their sub-cellular localization indicated that 120 (82%) and 101 (79%) proteins were secreted in 5- and 11-day-old hypocotyls respectively. On the other hand, 27 and 25 proteins (in 5- and 11-day-old hypocotyls respectively) did not have any predicted signal peptide and were considered as intracellular contaminants. Altogether, 173 proteins were identified in hypocotyls among which 137 (79%) were expected to be secreted, indicating the good quality of cell wall preparations. Those proteins will be called CWPs in the following. Although many CWPs (84) were found in both samples, 36 and 17 were identified only in 5- and 11-day-old hypocotyls respectively.

A second bioinformatic analysis of CWPs allowed their classification according to functional domains. For several protein families, nomenclature of experts was used as described in Experimental procedures. The nine functional classes defined by Jamet *et al.* (2008) were employed (Figure 3): proteins acting on carbohydrates, oxido-reductases, proteins with interaction domains, proteases, structural proteins, proteins involved in signaling, proteins related to lipid metabolism, proteins with miscellaneous functions, and proteins of unknown function. Some protein classes were more represented at 5-days than at 11-days, *e.g.* proteins acting on carbohydrates (28 *vs* 21), proteins with interaction domains (25 *vs* 22), proteins related to lipid metabolism (7 *vs* 5), and proteases (14 *vs* 9) (Figure 3a). In each functional class, some CWPs were only identified at 5- or 11-days (Figure 3b). Differences appeared among proteins acting on carbohydrates (respectively 11 and 4 were found only at 5- or 11-days), among proteases (5 were only found at 5-days), proteins with domains of interactions with proteins or carbohydrates (3 were only found at 5-days), miscellaneous proteins and proteins of unknown function. In particular, the pattern of oxido-reductases appeared to be very different with 5 and 6 proteins being only found at 5- and 11-days respectively. On the contrary, signaling and structural proteins showed minor changes. Due to their specific structural characteristics, they are poorly represented in both proteomes.

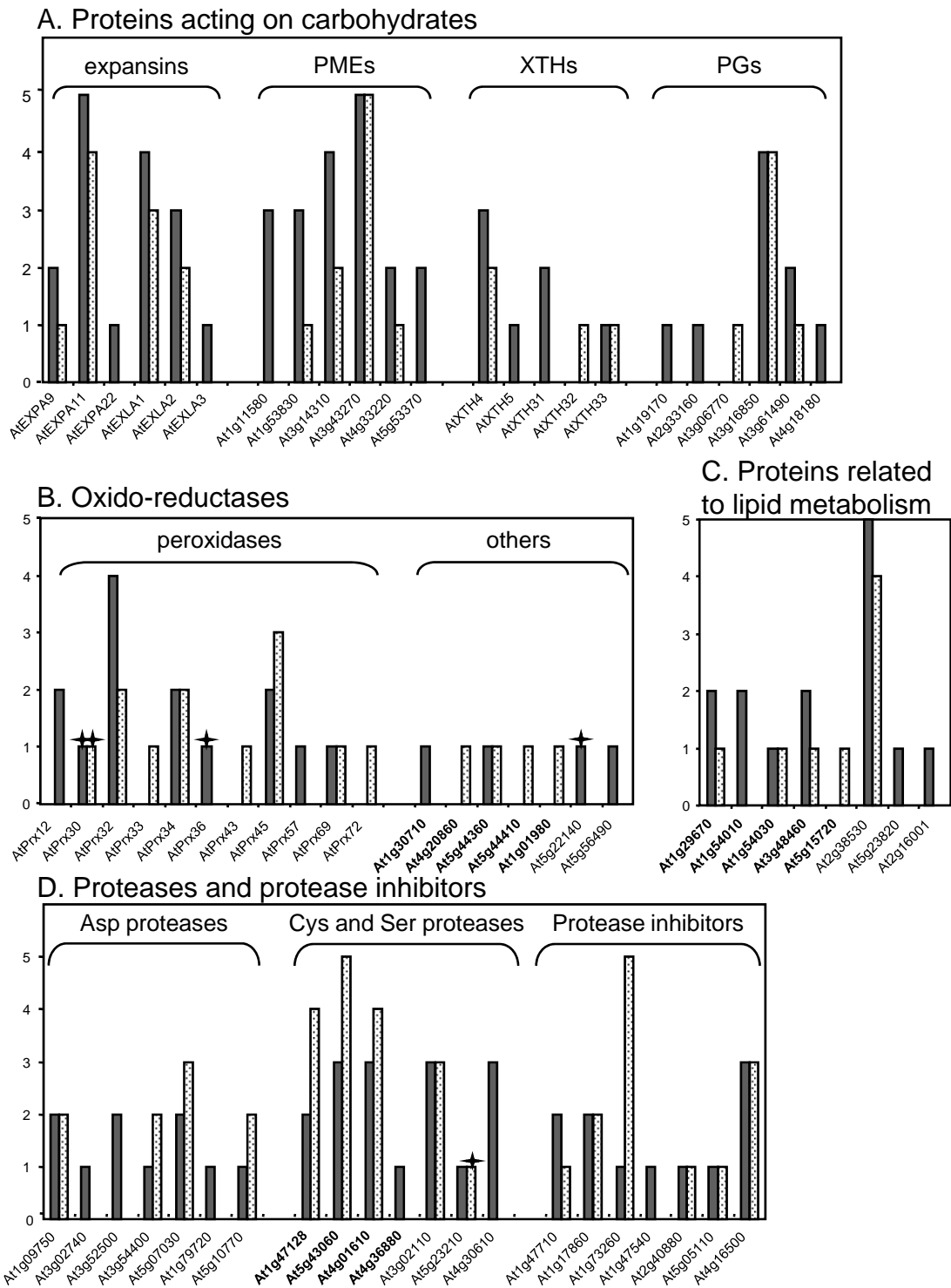


Figure 4. Occurrence of proteins belonging to several families among proteins extracted with CaCl₂ and LiCl from purified cell walls of 5- and 11-day-old etiolated hypocotyls.

a. Families of proteins predicted to encode expansins, PMEs, XTHs and PGs. **b.** Protein families predicted to encode oxido-reductases. Berberine-bridge enzymes are in bold. **c.** Protein families predicted to encode proteins related to lipid metabolism. Proteins having a GDSL Lipase/Acylhydrolase domain are in bold. **d.** Families of proteins predicted to encode proteases or protease inhibitors. Cys proteases are in bold. The number of FPLC fractions in which each protein was identified was counted: black and white bars stand for 5- and 11-day-old etiolated hypocotyls respectively. Stars indicate proteins that have been only identified after separation by 1D-E. Detailed information about the distribution of proteins in total extract and in FPLC fractions are given in Table S3 (supplementary material).

Structural proteins are difficult to extract when they are cross-linked, and to identify because of numerous post-translational modifications (PTMs) (Shpak *et al.*, 2001). Proteins involved in signaling like arabinogalactan proteins (AGPs) may also have many PTMs (Schultz *et al.*, 2000), and proteins with trans-membrane domains are not usually extracted in our working conditions.

Finally, 51 proteins reported in this work were not identified in previous cell wall proteomic studies (Table 2). There are 11 CWPs acting on carbohydrates, 8 oxido-reductases, 5 proteases, 8 carrying interacting domains, 1 possibly involved in signaling, 1 structural protein, 4 proteins related to lipid metabolism, 5 proteins with diverse functions, and 7 without known function.

Semi-quantitative comparative analysis of CWPs

The comparison between the two physiological stages done in the previous section was based on the single criterion of presence/absence of a protein among proteins identified by MS in our working conditions. A more precise comparison would require quantification of the proteins. However, CBB staining of the gels did not allow such quantification for several reasons. Despite improvement in the separation of proteins by liquid chromatography followed by 1D-E as compared to 1D-E alone, most proteins were found in several FPLC fractions and in several bands of the same FPLC fractions. This was probably due to PTMs, proteolytic maturation or degradation of proteins. Moreover, MALDI-TOF MS analyses are not quantitative due to differences in ionization efficiency of diverse peptides, and to variations related to competitive desorption of peptides at the time of ionization. We propose alternative ways to compare the proteins between both samples. In a first approach, FPLC fractions in which proteins were identified were counted to give a first criterion based on the following rationale: an abundant protein is more difficult to resolve and will be distributed in more fractions than a rare protein. Such a calculation done on members of several gene families gave an overview on the relative abundance of each of them (Figure 4). Two additional criteria were then considered to evaluate the relative amount of each protein at both stages of development: the number of bands per fraction, and the percentage of coverage of the amino acid sequence by peptide mass mapping. A correlation was observed between the abundance of a protein and the number of matching peptides expressed as percentage of coverage (results not shown). The addition of all values for a given protein provided a semi-

Table 2. CWPs identified in salt extracts of purified cell walls of 5- and 11-day-old etiolated hypocotyls of *Arabidopsis*.

Accession numbers of genes encoding proteins predicted to have a GPI anchor or trans-membrane domains are in bold or in grey boxes respectively. Accession numbers of genes encoding proteins newly identified in etiolated hypocotyls through cell wall proteomics are underlined. Details of functional annotation are in Tables S1 and S2 (supplementary material).

| accession | annotation | 5-days | 11-days |
|---|---|--------|---------|
| AGI | | | |
| proteins acting on carbohydrates | | | |
| At2g06850 | GH family 16 (AtXTH4) | ✓ | ✓ |
| At5g13870 | GH family 16 (AtXTH5) | ✓ | |
| <u>At3g44990</u> | GH family 16 (AtXTH31) | ✓ | |
| <u>At2g36870</u> | GH family 16 (AtXTH32) | | ✓ |
| <u>At1g10550</u> | GH family 16 (AtXTH33) | ✓ | ✓ |
| At4g16260 | GH family 17 | | ✓ |
| At4g19810 | GH family 18 (chitinase) | ✓ | |
| At3g12500 | GH family 19 (chitinase) | ✓ | ✓ |
| At3g55260 | GH family 20 (beta-hexosaminidase) | ✓ | |
| <u>At1g19170</u> | GH family 28 (polygalacturonase) | ✓ | |
| At2g33160 | GH family 28 (polygalacturonase) | ✓ | |
| <u>At3g06770</u> | GH family 28 (polygalacturonase) | | ✓ |
| At3g16850 | GH family 28 (polygalacturonase) | ✓ | ✓ |
| <u>At3g61490</u> | GH family 28 (polygalacturonase) | ✓ | ✓ |
| <u>At4g18180</u> | GH family 28 (polygalacturonase) | ✓ | |
| At1g68560 | GH family 31 (alpha-xylosidase) (AtXYL1) | ✓ | ✓ |
| At3g13790 | GH family 32 (beta-fructofuranosidase) | ✓ | ✓ |
| At5g34940 | GH family 79 (endo beta-glucuronidase/heparanase) | ✓ | ✓ |
| At1g11580 | CE family 8 (pectin methylesterase) | ✓ | |
| At1g53830 | CE family 8 (pectin methylesterase) (AtPME2) | ✓ | ✓ |
| At3g14310 | CE family 8 (pectin methylesterase) (AtPME3) | ✓ | ✓ |
| At3g43270 | CE family 8 (pectin methylesterase) | ✓ | ✓ |
| At4g33220 | CE family 8 (pectin methylesterase) | ✓ | ✓ |
| At5g53370 | CE family 8 (pectin methylesterase) | ✓ | |
| <u>At4g37950</u> | PL family 4 (rhamnogalacturonate lyase) | | ✓ |
| At1g05570 | GT family 48 (callose synthase) (AtCalS1) | ✓ | ✓ |
| At5g02260 | alpha-expansin (AtEXPA9) | ✓ | ✓ |
| At1g20190 | alpha-expansin (AtEXPA11) | ✓ | ✓ |
| <u>At5g39270</u> | alpha expansin (AtEXPA22) | ✓ | |
| At3g45970 | expansin-like A (AtEXLA1) | ✓ | ✓ |
| <u>At4g38400</u> | expansin-like A (AtEXLA2) | ✓ | ✓ |
| At3g45960 | expansin-like A (AtEXLA3) | ✓ | |
| oxido-reductases | | | |
| At1g71695 | peroxidase (AtPrx12) | ✓ | ✓ |
| At3g21770 | peroxidase (AtPrx30) | ✓ | ✓ |
| At3g32980 | peroxidase (AtPrx32) | ✓ | ✓ |
| At3g49110 | peroxidase (AtPrx33) | | ✓ |
| At3g49120 | peroxidase (AtPrx34) | ✓ | ✓ |
| <u>At3g50990</u> | peroxidase (AtPrx36) | ✓ | |
| <u>At5g25980</u> | peroxidase (AtPrx43) | | ✓ |

| | | | |
|------------------|--|---|---|
| At4g30170 | peroxidase (AtPrx45) | ✓ | ✓ |
| At5g17820 | peroxidase (AtPrx57) | ✓ | |
| At5g64100 | peroxidase (AtPrx69) | ✓ | ✓ |
| At5g66390 | peroxidase (AtPrx72) | | ✓ |
| At2g30210 | homologous to laccase (AtLAC3) | ✓ | ✓ |
| <u>At1g30710</u> | berberine-bridge enzyme homologue | ✓ | |
| <u>At4g20860</u> | berberine-bridge enzyme homologue | | ✓ |
| <u>At5g44360</u> | berberine-bridge enzyme homologue | ✓ | ✓ |
| <u>At5g44410</u> | berberine-bridge enzyme homologue | | ✓ |
| <u>At1g01980</u> | berberine-bridge enzyme homologue | | ✓ |
| At2g02850 | plantacyanin ARP (blue copper binding protein) | | ✓ |
| At4g12880 | early nodulin AtEN20 (blue copper binding protein) | ✓ | ✓ |
| <u>At5g22140</u> | expressed protein (oxido-reductase domain) | ✓ | |
| <u>At5g56490</u> | expressed protein (FAD binding domain) | ✓ | |

proteins with interacting domains

| | | | |
|------------------|---|---|---|
| At1g53070 | lectin homologue (legume lectin domains) | ✓ | ✓ |
| <u>At1g78820</u> | lectin homologue (curculin-like) | ✓ | |
| At1g78830 | lectin homologue (curculin-like) | ✓ | ✓ |
| At1g78850 | lectin homologue (curculin-like) | ✓ | ✓ |
| At1g78860 | lectin homologue (curculin-like) | ✓ | ✓ |
| At5g06860 | PGIP1 (LRR domains) | ✓ | ✓ |
| T23B7.10 | PGIP1 homologue (LRR protein FLR1) | ✓ | ✓ |
| At5g12940 | <i>Phaseolus vulgaris</i> PGIP2 homologue (LRR domains) | ✓ | ✓ |
| At1g33590 | expressed protein (LRR domains) | ✓ | ✓ |
| At2g34930 | expressed protein (LRR domains) | ✓ | ✓ |
| At3g20820 | expressed protein (LRR domains) | ✓ | ✓ |
| At2g17120 | expressed protein (LysM domain) | ✓ | ✓ |
| At1g03220 | carrot EDGP and tomato XEGIP homologue | ✓ | ✓ |
| At1g03230 | carrot EDGP and tomato XEGIP homologue | ✓ | ✓ |
| <u>At5g19110</u> | carrot EDGP and tomato XEGIP homologue | ✓ | ✓ |
| <u>At1g47710</u> | serpin homologue (serine protease inhibitor) | ✓ | ✓ |
| At1g17860 | inhibitor family I3 (Kunitz-P family) | ✓ | ✓ |
| At1g73260 | inhibitor family I3 (Kunitz-P family) | ✓ | ✓ |
| At1g47540 | inhibitor family I18 (mustard trypsin inhibitor-2 family) | ✓ | ✓ |
| <u>At2g40880</u> | inhibitor family I25 (phytostatin) | ✓ | ✓ |
| <u>At5g05110</u> | inhibitor family I25 (phytostatin) | ✓ | ✓ |
| At4g16500 | inhibitor family I25 (cystatin family) | ✓ | ✓ |
| <u>At4g25260</u> | invertase/ pectin methylesterase inhibitor homologue | ✓ | ✓ |
| <u>At5g46940</u> | invertase/pectin methylesterase inhibitor homologue | ✓ | |
| <u>At5g46960</u> | invertase/pectin methylesterase inhibitor homologue | ✓ | |

proteases

| | | | |
|------------------|---|---|---|
| At1g09750 | aspartic protease homologue (pepsin family) | ✓ | ✓ |
| <u>At3g02740</u> | aspartic protease homologue (pepsin family) | ✓ | |
| At3g52500 | aspartic protease homologue (pepsin family) | ✓ | |
| At3g54400 | aspartic protease homologue (pepsin family) | ✓ | ✓ |
| At5g07030 | aspartic protease homologue (pepsin family) | ✓ | ✓ |
| <u>At1g79720</u> | aspartic protease homologue (CND41 peptidase) | ✓ | |
| At5g10770 | aspartic protease (CND41 peptidase) | ✓ | ✓ |
| At1g47128 | cysteine proteinase homologue (papain family) | ✓ | ✓ |
| At5g43060 | cysteine proteinase homologue (papain family) | ✓ | ✓ |

| | | | |
|---|--|---|---|
| At4g01610 | cysteine proteinase homologue (papain family) | ✓ | ✓ |
| <u>At4g36880</u> | cysteine proteinase homologue (papain family) | ✓ | |
| <u>At3g02110</u> | serine carboxypeptidase D (SCPL25) | ✓ | ✓ |
| At5g23210 | serine carboxypeptidase (SCPL34) | ✓ | ✓ |
| <u>At4g30610</u> | carboxypeptidase homologue (BRS1 - Brassinosteroid-Insensitive BRI suppressor 1) | ✓ | |
| structural proteins | | | |
| At1g28290 | proline-rich protein | ✓ | ✓ |
| <u>At5g14920</u> | proline-rich protein | ✓ | ✓ |
| At2g05580 | glycine-rich protein | ✓ | ✓ |
| At4g13340 | LRR-extensin (AtLRX3) | ✓ | |
| At3g24480 | LRR-extensin (AtLRX4) | ✓ | ✓ |
| At4g18670 | LRR-extensin (AtLRX5) | ✓ | ✓ |
| signaling | | | |
| <u>At4g05200</u> | receptor kinase homologue (RLK, DUF26-1b subfamily) | ✓ | |
| At5g55730 | fasciclin-like arabinogalactan protein (AtFLA1) | ✓ | ✓ |
| proteins related to lipid metabolism | | | |
| At1g29670 | lipase acylhydrolase homologue (GDSL family) | ✓ | ✓ |
| At1g54010 | lipase/acylhydrolase homologue (GDSL family) | ✓ | |
| <u>At1g54030</u> | lipase/acylhydrolase homologue (GDSL family) | ✓ | ✓ |
| <u>At3g48460</u> | lipase/acylhydrolase homologue (GDSL family) | ✓ | ✓ |
| <u>At5g15720</u> | lipase/acylhydrolase homologue (GDSL family) | | ✓ |
| At2g38530 | non-specific lipid transfer protein type 1 (LTP2) | ✓ | ✓ |
| At5g23820 | expressed protein (ML domain - MD-2-related lipid recognition domain) | ✓ | |
| <u>At2g16001</u> | expressed protein (lipid recognition domain) | ✓ | |
| miscellaneous functions | | | |
| At2g27190 | purple acid phosphatase homologue (PAP1) | ✓ | ✓ |
| <u>At3g07130</u> | purple acid phosphatase homologue | ✓ | ✓ |
| At5g34850 | purple acid phosphatase homologue | ✓ | |
| At4g29270 | acid phosphatase homologue | ✓ | ✓ |
| <u>At4g24340</u> | phosphorylase homologue homologue | ✓ | |
| <u>At3g02870</u> | myo-inositol monophosphatase homologue | ✓ | |
| At5g09440 | <i>Nicotiana tabacum</i> phi-I homologue | ✓ | ✓ |
| At5g64260 | <i>Nicotiana tabacum</i> phi-I homologue | ✓ | ✓ |
| <u>At5g66590</u> | <i>Nicotiana tabacum</i> pathogenesis-related protein PR1 homologue | ✓ | ✓ |
| At2g28790 | <i>Lycopersicon esculentum</i> osmotin homologue | ✓ | ✓ |
| At5g15230 | gibberellin-regulated protein (GASA4) | ✓ | |
| At4g27110 | homologous to COBRA (AtCOBL10) | ✓ | |
| At1g09560 | germin (subfamily 2, member 1, GLP5) | | ✓ |
| unknown function | | | |
| <u>At3g56750</u> | expressed protein | | ✓ |
| <u>At3g22000</u> | expressed protein (DUF26) | | ✓ |
| <u>At1g26850</u> | expressed protein (DUF248) | | ✓ |
| At1g80240 | expressed protein (DUF642) | ✓ | ✓ |
| At3g08030 | expressed protein (DUF642) | ✓ | ✓ |
| At4g32460 | expressed protein (DUF642) | ✓ | ✓ |
| At5g11420 | expressed protein (DUF642) | ✓ | ✓ |

| | | | |
|------------------|--|---|---|
| At5g25460 | expressed protein (DUF642) | ✓ | ✓ |
| <u>At1g78460</u> | expressed protein (SOUL heme binding domain) | | ✓ |
| <u>At2g04690</u> | expressed protein (homologous to a human brain CREG protein) | ✓ | ✓ |
| At2g15220 | expressed protein (Plant Basic Secreted Protein domain) | ✓ | ✓ |
| At2g34700 | expressed protein (Ole e1 allergen domain) | ✓ | ✓ |
| At3g20370 | expressed protein (MATH domain) | ✓ | |
| <u>At2g28490</u> | expressed protein (cupin domain) | ✓ | ✓ |
| At3g22640 | expressed protein (cupin domain) | ✓ | ✓ |
| <u>At4g36700</u> | expressed protein (cupin domain) | ✓ | |

quantitative index (QI) allowing comparison of the relative amount of each protein between the two samples (Table S4, supplementary material).

Taking into account QIs, 63% of the proteins showed differences between the two developmental stages: 61 proteins (42%) are more abundant at 5-days and 26 (17%) at 11-days. Figure 3c presents the results ordered by functional classes. Differences observed considering only the presence/absence of proteins are enhanced especially for proteins acting on carbohydrates with 17 out of 32 being more abundant at 5- than at 11-days as for proteins having domains of interactions or related to lipid metabolism, and miscellaneous proteins. These results were fully consistent with those described above only taking into account the presence of a protein in FPLC fractions (Figure 3b).

For proteins acting on carbohydrates (Figure 4a), all the identified expansins were found to be more represented at 5- than at 11-days. The situation was similar for PME with the exception of At3g43270 that was found in the same number of FPLC fractions at 5- and 11-days. On the contrary, XTHs and PGs presented a more complex pattern. Three XTHs were more represented at 5- than at 11-days (AtXTH4, AtXTH5, and AtXTH31), AtXTH33 was equally represented at both stages, and AtXTH32 was only found at 11-days. Three PGs were only found at 5-days (At2g33160, At1g19170, and At4g18180), At3g15850 was equally represented at 5- and 11-days, and At3g06770 was only found at 11-days.

The distribution of oxido-reductases between the two stages looked as complicated as that of XTHs (Figure 4b). AtPrx36 and AtPrx57 were only identified at 5-days whereas AtPrx12, AtPrx43, and AtPrx72 were only identified at 11-days. AtPrx32 was more represented at 5-days whereas AtPrx45 was more represented at 11-days. Finally, AtPrx30, AtPrx34, and AtPrx69 were equally represented at both stages. Among other proteins predicted to be oxido-reductases, berberine-bridge enzymes were distributed between the two stages of development.

Most proteins related to lipid metabolism were more represented at 5- than at 11-days (Figure 4c). This is especially the case for proteins containing a GDSL-Lipase/Acylhydrolase domain (At1g29670, At1g54010, and At3g48460).

In the case of proteases (Figure 4d), the situation was contrasted depending on the protease family. Three Asp proteases were only found at 5-days (At1g79720, At3g02740, and At3g52500), whereas 3 of them were more abundant at 11- than at 5-days (At3g54400, At5g10770, and At5g07030). At1g09750 was equally represented at the two stages. Cys proteases were more represented at 11- than at 5-days (At1g47128, At5g43060, and At4g01610). On the contrary, a Ser protease was only found at 11-days (At4g30610). Finally, protease inhibitors (Figure 4d) were distributed between the two stages of development with 4 of them equally represented at both stages, 2 of them only found at 5-days, and 1 more represented at 11-days.

Discussion

This work provides a global picture of the cell wall proteome during elongation of etiolated hypocotyls of *Arabidopsis*. It shows the dynamics of CWPs during two phases of hypocotyls development, *i.e.* active elongation and after growth arrest. Expected CWPs known to be involved in cell wall extension such as XTHs, expansins, PGs, PMEs and peroxidases were identified as well as new CWPs such as proteases, proteins predicted to be related to lipid metabolism and proteins of unknown function. In addition, the occurrence of CWPs well-known to be related to cell wall extension after growth arrest showed that such proteins probably play other functions in mature cell walls.

A major advance in plant cell wall proteomics was brought by setting up a new separation method for CWPs. Separation of plant CWPs for proteomic purposes was difficult using 2D-E (Jamet *et al.*, 2008). The window of protein separation is optimal for pIs between 3 and 10 and for molecular masses between 120 and 10 kDa. Since most CWPs are basic glycoproteins, they tend to migrate as a smear on the basic side of 2D-gels (Minic *et al.*, 2007). Alternative methods were proposed that consisted in separation of CWPs into an acidic and a basic fractions by cationic exchange chromatography followed by 2D-E and 1D-E respectively (Borderies *et al.*, 2003; Boudart *et al.*, 2005). The new method includes a first step of separation by cationic exchange chromatography at acidic pH, and a second step of separation by 1D-E. It gives more information on the physico-chemical properties of the proteins, allows comparative semi-quantification between different samples, as well as a better identification through MALDI-TOF MS. In the case of etiolated hypocotyls of *Arabidopsis*, it allowed doubling the number of proteins identified as compared to separation by 1D-E alone. In addition, since many CWPs can now be visualized, this work provided preparative tools for developing biochemical studies on CWPs, *e.g.* for further purification or structural characterization.

Altogether, 137 CWPs were identified in this study among which 51 were not yet identified through cell wall proteomics. This work also presents an overview of the dynamics of CWPs during cell elongation. More CWPs were identified at 5- than at 11-days, suggesting a higher metabolic activity in cell walls during active elongation. Many differences were observed between elongating and full-size hypocotyls. When considering only presence/absence of a CWP, these changes concerned 53 out of the 137 identified CWPs

(38%). When taking into account the proposed semi-quantification method, this percentage even increased to 63% (34 additional proteins). Changes in the same gene family can reflect regulation of gene expression at different stages of development and/or differences in biological activity as discussed below for XTHs, PGs, expansins, PMEs, and peroxidases. Proteins acting on carbohydrates are more numerous and more abundant in elongating hypocotyls than in full-size hypocotyls. This was expected since rearrangements of cell wall polysaccharides are very important during cell elongation (Derbyshire *et al.*, 2007). More surprising is the fact that proteases are more numerous and in higher amounts at 5- than at 11-days. Nothing is known about their targets in cell walls. Are they contributing to release peptides involved in signaling (Matsubayashi and Sakagami, 2006)? Are they involved in protein maturation (Lee *et al.*, 2004) or in protein degradation? Conversely, two protease inhibitors are much more abundant at 11- than at 5-days. Altogether, it seems that proteolytic activities are more involved when elongation is active than during elongation arrest. Among oxido-reductases, five berberine-bridge enzymes were identified among which three were only present at 11-days. The role of such proteins in cell walls is unknown. For proteins with interacting domains, changes are found in lectins and PME inhibitors that are more abundant at 5-days. Among miscellaneous proteins, the amount of CWPs containing phosphatase domains was found to be higher at 5- than at 11-days. Such proteins were shown to be associated to regeneration of protoplast cell walls (Kaida *et al.*, 2003) and pollen tube growth (Ibrahim *et al.*, 2002), but their precise roles are unknown. A protein homologous to COBRA (AtCOBL10) was only found at 5-days. Although the function of AtCOBL10 is not known, it should be noted that COBRA was shown to participate in orientation of cellulose microfibrils, and dark-grown hypocotyls of *cob-4* mutant have a 95% reduction in length compared to wild-type (Roudier *et al.*, 2005). AtCOBL10 might play such role during elongation of hypocotyl cells.

Many proteins expected to participate in cell wall extension were found such as XTHs, expansins, PGs, PMEs and peroxidases (Micheli, 2001; Passardi *et al.*, 2004; Cosgrove, 2005; Zhang *et al.*, 2007). But such proteins, *i.e.* same proteins or proteins of the same family were also found after completion of elongation. Several hypotheses can be proposed. Although many proteases were identified at both stages of development suggesting a regulation of CWPs by proteolytic degradation, these proteins can have a long half-life. Some of these proteins probably also participate in differentiation of tracheary elements. XTHs belonging to phylogenetic group 3 such as AtXTH31-33 have been assumed to have

xyloglucan endo-hydrolysis activity (Rose *et al.*, 2002). They might be involved in rearrangement of cell walls of differentiating vessels elements. Such elements can be observed using microscopy (not shown). In the same way, some expansins were found in differentiating tracheary elements (Li *et al.*, 2003). Finally, at least PME and peroxidases were assumed to play roles both during the elongation process and elongation arrest. The enzymatic activity of PMEs might be modulated depending on the pH of the extracellular matrix and on the structure of pectic homogalacturonans. They could have either a local activity favoring the enzymatic activity of endo-PGs thus producing fragments of pectin, or a processing activity leading to de-esterification of stretches of GalA and formation of the so-called egg-boxes that tend to rigidify the pectic network (Micheli, 2001). Moreover, the degree of pectin methyl-esterification was shown to be positively correlated to hypocotyl growth (Derbyshire *et al.*, 2007). The activity of peroxidases is also versatile (Passardi *et al.*, 2004). During the hydroxylic cycle, peroxidases can produce reactive oxygen species that can break cell wall polysaccharides in a non-enzymatic way thus favoring cell wall extension (Fry, 1998; Passardi *et al.*, 2004). On the contrary, during the peroxidative cycle, peroxidases can promote cross-linking of cell wall components such as structural proteins or lignins. In addition, members of most of these protein families were identified in apoplastic fluids of rosette leaves (Boudart *et al.*, 2005). Since leaf cells are surrounded by mature walls, it means that those CWPs could play house-keeping roles.

Proteomics provides information about possible regulatory mechanisms of CWPs. As previously discussed in (Fry, 2004), the presence of a protein does not mean that it has full biological activity. Proteins with putative enzymatic activities are numerous, but inhibitors of these activities are also present. This is the case for proteases (14) and protease inhibitors (7), PMEs (6) and PME inhibitors (3 PMEIs). Some PMEs have a pro-domain consisting in a PMEI. However, such domains are assumed to be cleaved during or just after protein export since they were never found in purified PMEs (Micheli, 2001). In the same way, no peptide matching the PMEI domains were found during identification of PMEs by peptide mass mapping (data not shown). Other enzyme inhibitors are assumed to be involved in defense reaction such as PG inhibiting proteins (3 PGIPs) and inhibitors of xyloglucan endoglucanases (3 XEGIPs) since some of them were shown to be specifically active against fungal enzymes (Juge, 2006). But this was not proven for all of them. Other regulatory mechanisms include variations in pH of extracellular matrix that occur during growth arrest

(Juge, 2006), physical contact between enzymes and their substrates (Fry, 2004) and proteolytic degradation.

Eight proteins predicted to be related to lipid metabolism were identified at both stages of hypocotyl development. At present, little is known about the functions of such proteins in cell walls. Since etiolated hypocotyls have a thicker cuticle than light-grown hypocotyls (Gendreau *et al.*, 1997), presence of proteins involved in cuticle formation is expected. Several genes encoding proteins from the same families have been found to be up-regulated in 35S::*AtMYB41* plants having defects in cell expansion and leaf surface permeability (Cominelli *et al.*, 2008). Two mutants affected in genes encoding proteins related to lipid metabolism have been described. *GLIP1* encodes a predicted lipase/acylhydrolase that was shown to have a lipase activity *in vitro* and to disrupt fungal spore integrity at the level of cell wall and/or membrane (Oh *et al.*, 2005). Although none of the proteins of the GDSL family was shown to have an activity towards natural lipids *in vitro*, it cannot be excluded that such proteins are hydrolases acting on cutin or suberin lipids (F. Beisson, personal communication). *BODYGUARD* encodes a protein predicted to belong to an α/β -hydrolase fold superfamily (Kurdyukov *et al.*, 2006). The *bodyguard* mutant shows defects in cuticle formation that could result from incomplete polymerization of carboxyl esters of the cuticle. The function of LTPs is still a matter of debate. They were shown to bind fatty acids and to transfer phospholipids between membranes *in vitro* (Kader, 1997). *At2g38530* encoding LTP2 was found to be up-regulated in the epidermis of stems and assumed to contribute to active cuticle formation during stem elongation (Suh *et al.*, 2005). Apart from this role in cuticle formation, many roles were proposed for LTPs including systemic resistance signaling (Maldonado *et al.*, 2002), ability to promote cell wall expansion through binding to a hydrophobic partner in cell walls (Nieuwland *et al.*, 2005), and activation of a PG (Tomassen *et al.*, 2007). CWPs predicted to be related to lipid metabolism identified in this study are candidates for playing roles in cuticle formation.

This proteomic survey provides tools for biochemical studies of CWPs, identifies members of multigene families involved in cell elongation, and gives clues for unraveling the function of many CWPs in etiolated hypocotyls. It also shows a great dynamics of CWPs between the two developmental stages. This is illustrated by changes in protein amount, presence/absence of specific members of multigene families, and presence of many enzymes including proteases and their inhibitors. Interestingly, many CWPs were only found at one

stage of development, either active elongation or after growth arrest. Conversely, different CWPs from the same gene families were found at both stages of development showing stage-specific regulation and suggesting diverse roles in cell walls. A particular attention should be paid to proteins of unknown function, some of which are very abundant. Additional functional studies are now required to shed light on the roles of the identified CWPs during the elongation of hypocotyls in the dark.

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Additional data files

Additional data files1.

Table S1. Identification of proteins extracted from purified cell walls of 5-day-old Arabidopsis hypocotyls by CaCl₂ and LiCl solutions.

Additional data files2.

Table S2. Identification of proteins extracted from purified cell walls of 11-day-old Arabidopsis hypocotyls by CaCl₂ and LiCl solutions.

Additional data files3.

Table S3. Classification of proteins extracted from purified cell walls of 5- and 11-day-old Arabidopsis hypocotyls in FPLC fractions A to Z.

Additional data files4.

Table S4. Semi-quantification of CWPs identified by MALDI-TOF MS from 5- and 11-day old etiolated Arabidopsis hypocotyls.

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CHAPTER 5

CELL WALL BIOGENESIS OF *ARABIDOPSIS* ELONGATING CELLS: TRANSCRIPTOMICS COMPLEMENTS PROTEOMICS

Chapter summary (French)

Pour mieux comprendre la biogenèse des parois, une étude transcriptomique a été réalisée sur le même matériel que l'étude protéomique décrite dans le chapitre 4. Il s'agissait d'une part d'apporter des éléments concernant les niveaux de régulation des gènes codant des protéines impliquées dans la mise en place de la paroi, de rechercher des nouveaux gènes importants pour l'élongation des parois, et de comparer les résultats obtenus à ceux de la protéomique. Les puces CATMA (*Complete Arabidopsis Transcriptome MicroArray*) ont été utilisées dans le cadre d'une collaboration avec l'équipe de Jean-Pierre Renou de l'URGV d'Evry. Les résultats ont été analysés en se focalisant sur des gènes codant des protéines impliquées dans la biogenèse des parois et des gènes codant des protéines pariétales.

Dans un premier temps, le niveau de transcrits d'un ensemble de 846 gènes appelés CWGs (Cell Wall Genes) et annotés par des experts a été analysé. Ces gènes codent pour des protéines qui participent d'une part à la synthèse et au transport des constituants pariétaux, d'autre part à leur assemblage dans les parois ainsi qu'au remodelage des réseaux de polysaccharides, de protéines et de lignine. Environ 55% de ces gènes ont des niveaux de transcrits détectables sur les puces CATMA aux deux stades développement des hypocotyles. Ceci suggère que les processus de synthèse et d'assemblage des constituants pariétaux sont importants à la fois au cours de l'élongation cellulaire et après son arrêt. Les niveaux de transcrits d'autres gènes tels ceux qui codent pour des AGP et des FLA ont également été analysés. Certains d'entre eux ont des niveaux de transcrits très élevés. Cependant leur rôle dans les parois n'est pas encore connu.

Dans un second temps, nous avons recherché des gènes codant pour des protéines secrétées dont les niveaux de transcrits seraient modérés ou élevés. L'idée était d'identifier de nouveaux gènes importants pour les remaniements pariétaux au cours de l'élongation cellulaire. Les 278 gènes identifiés ont été appelés SPGs (*Secretory Pathway Genes*). Ils appartiennent aux mêmes classes fonctionnelles que celles qui ont été définies au cours des études protéomiques. Parmi les SPGs, figurent 55 gènes codant des protéines agissant sur les polysaccharides pariétaux, 6 peroxydases. Après avoir retiré ces gènes déjà identifiés parmi les CWPs, ce sont plus de 200 nouveaux gènes qui pourraient jouer des rôles dans l'élongation cellulaire parmi lesquels 70 codent des protéines de fonction inconnue.

Moins de 100 gènes codant des protéines secrétées ont des niveaux de transcrits significativement variables entre les deux stades de développement des hypocotyles (43 ont un niveau de transcrits supérieur à 5 jours, et 38 à 11 jours). Une réduction du niveau de transcrits entre 5 et 11 jours était attendue pour des gènes codant des GT et des GH, mais pas pour des gènes codant des PME dont l'activité de déméthylation des pectines semblait plutôt décrite après la phase d'élongation cellulaire pour favoriser la formation de complexes avec Ca^{2+} (« boîtes à œufs »). Par contre, une augmentation du niveau de transcrits de gènes codant des GRP était attendue à 11 jours, puisque les GRP ont été associées à la formation de protoxylème. Les différences entre les résultats attendus et ceux obtenus par ces analyses pourraient être expliquées soit par des nouveaux rôles des gènes concernés, soit par des régulations particulières de leur expression. Il pourrait exister un délai entre la synthèse de certains transcrits et leur traduction en protéine, ou encore, certaines protéines pourraient être particulièrement stables. Enfin, il faudrait prendre en compte l'activité des protéases qui pourraient intervenir dans l'activation de certaines enzymes ou dans leur dégradation.

Enfin, les données de transcriptomique ont été comparées aux données de protéomique. Trois cas ont été relevés : (i) la protéine est identifiée, et le niveau de transcrits du gène correspondant est détectable sur les puces CATMA; (ii) le gène a un niveau de transcrit modéré ou élevé, mais la protéine n'est pas identifiée ; (iii) la protéine est identifiée, mais le niveau de transcrits du gène correspondant n'est pas détectable sur les puces CATMA. Plusieurs explications peuvent être proposées pour le second cas du point de vue de la protéomique. Certaines protéines sont difficiles à extraire des parois, peu abondantes, difficiles à colorer avec le bleu de Coomassie, ou encore difficiles à identifier par spectrométrie de masse du fait de leurs nombreuses modifications post-traductionnelles. Il se pourrait aussi que les protéines aient une demi-vie très courte ou encore, comme évoqué précédemment, qu'il existe un délai entre transcription et traduction d'un ARNm. Dans le troisième cas, les transcrits pourraient avoir une courte demi-vie, et les protéines au contraire être stables. En conclusion, nos travaux ont montré qu'il n'y a pas de cohérence systématique entre les données de transcriptomique et de protéomique. La régulation post-transcriptionnelle semble être importante dans le cas des gènes codant des protéines pariétales dans les hypocotyles étiolés. Les données de transcriptomique et de protéomique semblent donc complémentaires pour étudier la régulation des gènes codant des protéines pariétales. L'ensemble de ces résultats est détaillé dans l'article qui suit actuellement soumis pour publication.

To better understand cell wall biogenesis by complementing the results of proteomic study (chapter 4) and to know about the transcriptional regulation during hypocotyl elongation, a transcriptomic study was performed on same material used for proteomic study in chapter 4. For transcript profiling, Complete *Arabidopsis* Transcriptome MicroArray (CATMA) was used at URGV, Evry in collaboration with the research team of Jean-Pierre Renou. This chapter provides an analysis of:

- i) the transcript level of genes that could possibly take part in the biogenesis of cell wall
- ii) the transcript level of the genes for which the proteins were identified in the proteomic study (chapter 4)

In a first selection, the level of a set of 846 genes called cell wall genes (CWGs) and known to be involved in cell wall biogenesis was measured. At both stages of hypocotyl development, about 55% of these genes had detectable transcript level on CATMA. These genes are participating in synthesis, transport, assembly and rearrangement of cell wall polysaccharides (nucleotide-sugar inter-conversion pathway, monolignol biosynthesis, vesicle trafficking, structural proteins, GTs, GHs, XTHs, PMEs, PLs, expansins, peroxidases) suggesting that these two processes are very active during elongation. Levels of transcripts of other genes like those encoding AGPs and ALFs have also been analysed. Some of them have very high levels of transcripts, however, their role in cell wall is not yet known.

In a second step, we looked for genes that encode secretory proteins with moderate or high level of transcripts. The idea was to identify new genes important for assembly and reorganization of cell wall during elongation. The 278 genes identified in this way were called Secretory Pathway Genes (SPGs). They belong to the same functional classes to which belonged the proteins identified in the proteomic study. Among the SPGs, 55 genes encode proteins acting on cell wall polysaccharides and 6 genes encode peroxidases. After removing the genes encoding CWPs identified in the proteomic study, more than 200 new genes remained including 70 genes encoding proteins of unknown function that may play roles in cell elongation.

Less than 100 genes encoding secretory proteins had significant differences in their transcript levels between the two stages of hypocotyl development (43 and 38 having higher level in 5- and 11-day-old hypocotyls respectively). Higher level of transcripts in 5-day-old

hypocotyls was expected for genes encoding GTs and GHs, but not for genes encoding PMEs, that seems important after elongation to perform demethylation of pectins and promote the formation of "egg boxes" with Ca^{2+} . Conversely, higher level of transcripts of genes encoding GRPs was expected in 11 days, since they have been involved in repair mechanism of elongating protoxylem primary cell wall. The differences between the expected results and those obtained during this work could be explained either by new roles of the concerned genes, or by special regulations of their expression. It could be a delay between the synthesis of mRNAs and their translation, or certain proteins could be particularly stable. Finally, the activity of proteases should also be considered since they may be involved in the activation of certain enzymes or in their degradation.

Comparison of the transcriptomic data to the proteomic data (chapter 4) revealed three different cases: (i) protein identification with detectable level of transcripts of the corresponding gene on CATMA; (ii) high level of transcript but protein was not identified; (iii) Protein identified while the transcripts of the corresponding gene was not detectable on CATMA.

Several explanations can be proposed for the second case from the viewpoint of proteomics; some proteins are difficult to extract from cell wall; others are difficult to stain with Coomassie blue; still others are difficult to identify by mass spectrometry because of their post-translational modifications or absence of trypsin cut sites. It is also possible that these proteins have a very short half-life or as mentioned above, there is a delay between the synthesis mRNA and active protein. In the third case, the transcripts might have a short half-life, and proteins rather are stable.

In brief, this study revealed: (i) that there exists no systematic correlation between the abundance of transcripts and the presence of the corresponding proteins; (ii) that the post-transcriptional regulation of genes encoding CWPs in etiolated hypocotyles seems to be important; (iii) the transcriptomic and proteomic data seem complementary to study the regulation of genes encoding CWPs.

The supplementary data files are given as annexes at the end of the report.

**Cell wall biogenesis of Arabidopsis elongating cells:
transcriptomics complements proteomics**

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Abstract

Background

Plant cell growth is a complex process involving cell division and cell elongation. *Arabidopsis thaliana* hypocotyls undergo a 100-fold length increase mainly by cell elongation. Cell enlargement implicates significant changes in the composition and structure of cell walls. In order to understand cell wall biogenesis during cell elongation, mRNA profiling was made on half- (active elongation) and fully-grown (after growth arrest) etiolated hypocotyls of *Arabidopsis*.

Results

Transcriptomic studies were performed on two sets of genes. The first set of 846 genes called cell wall genes (CWGs) included genes involved in cell wall biogenesis, that is in synthesis and transport of cell wall components and in their assembly and rearrangements in cell walls. A significant proportion of them have detectable levels of transcripts (54.5%), suggesting that these processes are important throughout hypocotyl elongation and after growth arrest. A second set of 3039 genes named secretory pathway genes (SPGs) was studied to search for new genes encoding secreted proteins possibly involved in cell wall expansion. Based on transcript level, about 200 genes were selected. Seventy of them encoded proteins of yet unknown function. Finally, comparison with results of a proteomic study on the same material revealed that 35 out of the 137 identified proteins were products of the genes having high or moderate level of transcripts. It was surprising to find that about 15% of the genes encoding proteins identified by proteomics showed levels of transcripts below background.

Conclusions

Members of multigenic families known to be involved in cell wall biogenesis were identified during growth of etiolated hypocotyls as well as about 200 genes that might be required for cell wall assembly and/or rearrangement. No clear correlation was found between the abundance of transcripts (transcriptomic data) and the presence of the proteins (cell wall proteomic data) demonstrating (i) the importance of post-transcriptional events for the regulation of genes during cell elongation and (ii) that transcriptomic and proteomic data are complementary.

Background

Plant cell growth occurs mainly by cell elongation, and may or may not be accompanied by cell division. A meristematic cell might enlarge as much as 50000-fold its initial volume. In this process, membrane surface area and amount of cell wall material increase. The primary cell wall plays an essential role since it should allow turgor-driven increase in cell volume by permitting the incorporation of new cell wall material and rearrangement of existing cell wall. Several plant organs including coleoptiles (*poaceae*), internodes (legumes), and hypocotyls (mung bean, sunflower, and *Arabidopsis thaliana*) have been used to study cell elongation [1]. Environmental signals such as light temperature, and hormones, regulate hypocotyl growth [2-5]. *Arabidopsis* seedlings grown in continuous darkness are a material of choice to analyze the cell elongation process. Indeed, cells of hypocotyls undergo a 100-fold length increase compared to embryo cells [6]. Growth occurs mostly by cell expansion, with little cell division [4, 6-8]. Changes in wall thickness during elongation of *Arabidopsis* hypocotyls were investigated using cryo-field-emission scanning electron microscopy [1]. At the germination stage, cell wall thickening occurs and involves high rates of biosynthesis and deposition of cell wall components. During the elongation stage, cell walls undergo remarkable thinning, requiring extensive polymer disassembly and rearrangement.

Many genes are assumed to be involved in cell wall synthesis and rearrangement to support growth of plant cell walls [9]. They encode cellulose synthases (CESAs), cellulose synthases-like (CSLs), endo-glucanases, xyloglucan endotransglucosylase/hydrolases (XTHs) and expansins. They belong to multigenic families, but the members of each family involved in elongation of hypocotyl cells have not been precisely identified. It is also likely that other genes are important for cell elongation.

In this paper, *Arabidopsis* etiolated hypocotyls were compared at two developmental stages, half- and fully-grown, *i.e.* active elongation *vs.* elongation arrest. Transcript profiling was carried out using CATMA (Complete *Arabidopsis* Transcriptome MicroArray) [10]: (i) to look at the level of transcripts of cell wall genes (CWGs) belonging to families known to be involved in cell wall biogenesis; (ii) to identify genes encoding secreted cell wall proteins (SPGs) having high or moderate level of transcripts; (iii) to reveal differential gene expression between half- and fully-grown etiolated hypocotyls; (iv) and to look at the correlation

between transcript abundance and protein presence as revealed by a proteomic study performed on the same material (Irshad *et al.*, submitted for publication).

Results and Discussion

Level of transcripts of cell wall genes (CWGs) during hypocotyl elongation

Etiolated hypocotyls were compared at two developmental stages. Five-day-old hypocotyls were approximately half the final size. Growth propagated following an acropetal gradient. After 5-days, the bottom cells were fully elongated, whereas the top cells were only starting elongation [8]. Eleven-day-old hypocotyls had reached their maximum size and the cells were more than 100-fold their initial length [6]. CATMA was used for mRNA profiling. Since one of the major modifications during cell elongation is the addition and rearrangement of cell wall components, a selection of genes possibly involved in cell wall biogenesis was made (Additional data file 1). This selection was called “Cell Wall Genes” (CWGs). It was mainly based on the knowledge of gene families known to be involved in biogenesis of cell walls, that means synthesis and transport of cell wall components and their assembly or rearrangement in cell walls (see Methods). It includes genes encoding proteins involved in substrate generation (nucleotide-sugar inter-conversion pathway, monolignol biosynthesis), polysaccharide synthesis (mainly glycosyl transferases), vesicle trafficking, assembly/disassembly of the wall (glycosyl hydrolases, expansins, carbohydrate esterases, carbohydrate lyases), structural proteins, oxido-reductases involved in cross-linking of wall components (mainly peroxidases and laccases). A few gene families encoding cell wall proteins were also included such as arabinogalactan proteins (AGPs), fasciclin AGPs (FLAs), phytocyanins, multicopper oxidases, pectin methylesterase (PME) inhibitors, and subtilases. Only genes annotated by experts were retained (see Methods) and 26 gene families are represented.

Altogether, 1021 genes were selected among which only 846 were analyzed through CATMA for technical reasons. Some genes had no gene-specific tag (GST) on the microarray, others were not considered because of a poor signal of hybridization to the RNA probe, or of inaccurate duplicates. The level of transcripts was expressed as \log_2 of the mean signal intensity. Values of \log_2 below 6.75 were considered as background. Values between 6.75 and 9 corresponded to a low level of transcripts (1 to 4-fold the background level),

Table 1. Cell Wall Genes (CWGs) with high levels of transcripts in either 5- or 11-day-old hypocotyls.

CWGs were selected according to several papers and databases as mentioned in Methods. The intensity of the signal is expressed as \log_2 , the ratios between the levels of transcripts at 11-days and 5-days as well as the Bonferroni p-values are indicated. CWGs are classified by gene family.

| Functional classes | AGI number | Predicted or known gene function | 5-days | 11-days | ratio 11-days / 5-days | p-value |
|--|------------|---|--------|---------|------------------------|----------|
| Substrate generation | | | | | | |
| nucleotide-sugar interconversion pathway | AT5G39320 | UGD1 | 11.43 | 11.03 | -0.40 | 1.00 |
| | AT1G08200 | AXS2 | 10.34 | 9.98 | -0.36 | 1.00 |
| lignin toolbox | AT4G39330 | Arath;CAD1(cinnamyl alcohol dehydrogenase) | 11.03 | 10.33 | -0.70 | 7.14E-02 |
| | AT2G30490 | Arath;C4H (CYP73A5) (trans-cinnamate 4-hydroxylase) | 10.84 | 10.43 | -0.41 | 1.00 |
| | AT1G76790 | Arath;COMT-like8 (caffeic acid O-methyltransferase) | 10.37 | 9.48 | -0.89 | 4.45E-05 |
| | AT5G48930 | Arath;HCT (hydroxycinnamoyl-CoA:shikimate/quinic acid hydroxycinnamoyl transferase) | 10.24 | 10.28 | 0.04 | 1.00 |
| Synthesis of polysaccharides | | | | | | |
| glycosyl transferases (GTs) | AT2G22900 | GT34 | 10.09 | 9.72 | -0.36 | 1.00 |
| | AT2G28110 | GT47 (Group E) (FRA8) | 11.39 | 10.73 | -0.67 | 1.86E-01 |
| | AT5G09870 | GT2 (AtCesA5) | 10.01 | 9.11 | -0.90 | 3.26E-05 |
| | AT5G05170 | GT2 (AtCesA3) | 10.36 | 9.89 | -0.47 | 1.00 |
| Vesicle trafficking | | | | | | |
| | AT1G69460 | emp24/gp25L/p24 family protein | 10.17 | 10.55 | 0.37 | 1.00 |
| Modification of polysaccharides | | | | | | |
| glycosyl hydrolases (GHs) | AT1G64390 | GH9 (endoglucanases) | 10.68 | 10.19 | -0.49 | 1.00 |
| | AT4G02290 | GH9 (endoglucanases) | 11.10 | 10.08 | -1.02 | 1.57E-07 |
| | AT2G06850 | GH16 (AtXTH4) | 12.98 | 12.15 | -0.83 | 6.40E-04 |
| | AT4G14130 | GH16 (AtXTH15) | 12.72 | 11.98 | -0.74 | 1.62E-02 |

| | | | | | | |
|------------------------------|-----------|--------------------------------------|-------|-------|-------|----------|
| | AT4G30290 | GH16 (AtXTH19) | 11.37 | 11.40 | 0.03 | 1.00 |
| | AT4G30270 | GH16 (AtXTH24) | 10.84 | 11.64 | 0.80 | 1.63E-03 |
| | AT1G32170 | GH16 (AtXTH30) | 10.06 | 9.85 | -0.21 | 1.00 |
| | AT5G56870 | GH35 (beta galactosidases) (AtBGAL4) | 13.18 | 11.97 | -1.22 | 5.60E-12 |
| | AT1G45130 | GH35 (beta galactosidases) (AtBGAL5) | 11.21 | 10.42 | -0.78 | 3.24E-03 |
| carbohydrate esterases (CEs) | AT2G43050 | CE8 (pectin methylesterases) | 12.56 | 10.76 | -1.80 | 0.00 |
| | AT3G49220 | CE8 (pectin methylesterases) | 11.81 | 10.99 | -0.82 | 8.65E-04 |
| | AT3G14310 | CE8 (pectin methylesterases) AtPME3 | 10.79 | 9.90 | -0.90 | 4.05E-05 |
| | AT3G05910 | CE13 (pectin acylesterases) | 10.44 | 10.14 | -0.3 | 1.00 |
| carbohydrate lyases (CLs) | AT5G48900 | PL1 (pectate lyases) (Group A) | 10.92 | 10.23 | -0.70 | 6.69E-02 |
| | AT1G04680 | PL1 (pectate lyases) (Group A) | 10.73 | 10.22 | -0.52 | 1.00 |
| | AT1G67750 | PL1 (pectate lyases) (Group A) | 10.66 | 10.42 | -0.24 | 1.00 |
| | AT3G07010 | PL1 (pectate lyases) (Group A) | 10.26 | 9.56 | -0.70 | 6.16E-02 |
| expansins | AT5G02260 | AtEXPA9 (alpha expansin) | 12.71 | 12.44 | -0.28 | 1.00 |
| | AT1G69530 | AtEXPA1 (alpha expansin) | 10.62 | 10.86 | 0.24 | 1.00 |
| | AT2G37640 | AtEXPA3 (alpha expansin) | 10.34 | 10.52 | 0.18 | 1.00 |
| | AT4G28250 | AtEXPB3 (beta expansin) | 9.07 | 10.38 | 1.31 | 0.00 |

Structural proteins

| | | | | | | |
|-------------------------------|-----------|--------|-------|-------|-------|------|
| Hyp/Pro rich proteins (H/PRP) | AT2G21140 | AtPRP2 | 11.67 | 11.15 | -0.51 | 1.00 |
|-------------------------------|-----------|--------|-------|-------|-------|------|

Oxido-reductases

| | | | | | | |
|---|-----------|------------------------|-------|-------|-------|----------|
| peroxidases | AT2G37130 | AtPrx21 | 14.28 | 12.86 | -1.42 | 0.00 |
| | AT4G21960 | AtPrx42 | 13.17 | 12.40 | -0.77 | 5.29E-03 |
| | AT1G05260 | AtPrx03 | 10.68 | 10.18 | -0.51 | 1.00 |
| | AT4G33870 | AtPrx48 | 10.52 | 9.25 | -1.27 | 0.00 |
| | AT1G71695 | AtPrx12 | 10.17 | 9.54 | -0.63 | 4.86E-01 |
| | AT5G64120 | AtPrx71 | 10.03 | 9.85 | -0.18 | 1.00 |
| phytoeyanins | AT4G12880 | AtEN20 (early nodulin) | 10.84 | 11.19 | 0.36 | 1.00 |
| | AT2G32300 | AtUCC1 (uclacyanin) | 10.61 | 10.60 | -0.02 | 1.00 |
| SKU-like proteins (multi-copper oxidases) | AT1G76160 | SKS5 | 10.33 | 9.91 | -0.43 | 1.00 |

Proteins possibly involved in signaling

| | | | | | | |
|---------------------------------|-----------|--------|-------|-------|------|------|
| arabinogalactan proteins (AGPs) | AT2G14890 | AtAGP9 | 11.71 | 11.82 | 0.12 | 1.00 |
|---------------------------------|-----------|--------|-------|-------|------|------|

| | | | | | | |
|-----------------------|-----------|----------------------|-------|-------|-------|----------|
| | AT5G11740 | AtAGP15 (AG peptide) | 11.03 | 11.71 | 0.68 | 1.10E-01 |
| | AT5G10430 | AtAGP4 | 10.84 | 10.65 | -0.19 | 1.00 |
| | AT3G13520 | AtAGP12 (AG peptide) | 10.77 | 11.51 | 0.74 | 1.50E-02 |
| | AT1G28290 | AtAGP31 | 10.97 | 11.42 | 0.46 | 1.00 |
| fasciclin AGPs (FLAs) | AT1G03870 | AtFLA9 | 12.49 | 12.25 | -0.24 | 1.00 |
| | AT2G45470 | AtFLA8 | 11.96 | 11.53 | -0.43 | 1.00 |
| | AT4G12730 | AtFLA2 | 10.85 | 10.44 | -0.41 | 1.00 |

Proteases

| | | | | | | |
|------------|-----------|------------------|-------|-------|-------|----------|
| subtilases | AT3G14067 | AtSBT1.4 | 11.85 | 10.71 | -1.15 | 2.80E-10 |
| | AT2G05920 | AtSBT1.8 | 11.54 | 10.90 | -0.64 | 4.50E-01 |
| | AT5G67360 | AtSBT1.7 (ARA12) | 11.45 | 11.12 | -0.33 | 1.00 |
| | AT5G51750 | AtSBT1.3 | 11.04 | 10.80 | -0.24 | 1.00 |
| | AT5G59090 | AtSBT4.12 | 10.47 | 9.49 | -0.99 | 7.99E-07 |

values between 9 and 10 to a moderate level (4 to 8-fold the background level) and values higher than 10 to a high level (more than 8-fold, and up to 64-fold the background level). The level of transcripts of the 846 genes analyzed was below background for 369 genes (45.8%), low for 343 (40.5%), moderate for 53 (6.3%) and high for 55 (6.5%). Most families are represented by genes with low, moderate or high levels of transcripts.

At both stages, of the 55 genes with high levels of transcripts (Table 1), 11 are involved in the synthesis or transport of cell wall components and 27 are involved in modifications of cell wall components. Altogether, 64.6% and 45.5% respectively of the genes related to synthesis or transport of cell wall components and to their modifications in cell walls respectively have detectable level of transcripts. Two separate phases of growth have been described in *Arabidopsis* dark-grown hypocotyls: an early phase of active synthesis of cell wall polysaccharides up to 3-days after beginning of germination, and a late phase of cell expansion [8]. The former phase results in thicker cell walls which later on become thinner as hypocotyls elongate [1]. Our results show that both synthesis and rearrangement of cell wall components are probably required throughout hypocotyl elongation, and even after growth arrest.

Most of these genes were expected to be transcribed during cell elongation. Genes involved in rearrangement of cell wall components encode GHs such as endoglucanases, XTHs, and beta-galactosidases; carbohydrate esterases such as PMEs; pectin acylesterases; PLs; expansins of the alpha- or beta-type; and peroxidases. However, 21 genes encoding PMEs and 6 genes encoding pectin acylesterases have detectable levels of transcripts among which 4 genes have high levels of transcripts (Additional file 1), which seems contradictory with previous results showing that a low level of pectin esterification, associated to abundance of PMEs and acylesterases, restricted cell elongation in *Arabidopsis* hypocotyls [11]. It should be noted that 21 genes encoding PME inhibitors also have detectable levels of transcripts. The interplay between PME and their inhibitors could regulate the activity of PMEs. Nine genes encoding proteins possibly involved in oxido-reduction reactions have high levels of transcripts (peroxidases, phycocyanins, and protein homologous to SKU5). Six peroxidase genes fall in this category, among which *AT2G37130* (AtPrx21) has one of the highest levels of transcripts. The multiple roles of peroxidases during growth have been reviewed [12]. They could either release reactive oxygen species able to cut polysaccharides

or restrict growth by cross-linking structural proteins, monolignols, or polysaccharides and ferulate.

Other CWGs are also well-represented such as those encoding arabinogalactan proteins (AGPs), fasciclin AGPs (FLAs) and subtilases (Table 1). Even if AGPs and FLAs have been shown to be associated with wood formation in poplar [13], their role in cell expansion is not very clear at present. Likewise, nothing is known about the role of proteases during cell elongation. Finally, genes of the COBRA gene family have level of transcripts low or below background. However, COBRA was shown to play an important role in microfibril deposition during rapid elongation and in the orientation of cellulose microfibrils [14]. It may mean that such proteins are stable during hypocotyl elongation.

This work gives clues for understanding the function of members of multigene families either during cell elongation or after its arrest. Indeed, general functions have been proposed for most of these gene families, but only scarce information is available for specific members.

Genes encoding secreted proteins with high or moderate level of transcripts in etiolated hypocotyls

Most of the gene families described above was already known to be involved in cell wall biogenesis. In order to identify new genes that might be involved in cell expansion, a second selection was made based on sub-cellular location of proteins, choosing all the genes encoding proteins with predicted signal peptide thus targeted to the secretion pathway. The 3039 selected genes were ranked by level of transcripts, producing a profile similar to the one obtained with CWGs, namely 1341 genes (44.1 %) above background level, 1363 (44.9%) with a low transcript level, 185 (6.1%) with a moderate level, and 150 (4.9%) with at high level. From this selection, only genes encoding proteins predicted to be located either outside the cell or in the plasma membrane were retained (see Methods). In Additional data file 2, 278 genes named “Secretory Pathway Genes” (SPGs) with moderate or high levels of transcripts are listed and grouped in families according to their predicted functional domains (see Methods). All these protein families were already described in cell wall proteomic studies [15-17]: proteins acting on carbohydrates (55 genes); proteases (25 genes); proteins possibly involved in signaling (23 genes); structural proteins (9 genes); proteins possibly involved in oxido-reduction reactions (20 genes); proteins with interacting domains (20 genes); proteins

related to lipid metabolism (25 genes); miscellaneous proteins (31 genes); proteins of unknown function (70 genes). Main differences lie in the genes encoding proteins possibly involved in signaling since they comprise AGPs, FLAs, and plasma membrane proteins that are difficult to isolate, separate or identify through proteomics [15]. In the same way, the group of proteins of unknown function is very important because 42% of them are predicted to have trans-membrane domains. On the contrary, the group of structural proteins is probably under-represented because of the lack of appropriate GSTs for many of them. Indeed, their repetitive amino acid sequences make the design of specific probes difficult. One should note the abundance of proteases that can be assumed to be essential for protein turnover in tissues undergoing rapid elongation followed by elongation arrest within a short time. They may also be involved in signaling as shown for SDD1 (STOMATAL DENSITY AND DISTRIBUTION 1) and ALE1 (ABNORMAL LEAF EPIDERMIS 1) [18, 19] or in protein maturation as assumed for AtSBT1.7, thus contributing to regulation of cell wall enzyme activities [20]. In addition, there are probably interactions between proteases and protease inhibitors to regulate the proteolytic activities in cell walls. Several of the 70 proteins of yet unknown function have known structural domains (29 genes). Others share domains with other proteins, such as domains of unknown function (DUF), or belong to the so-called uncharacterized protein families (UPF). Many are of particular interest, since they are only present in plants.

Among these 278 SPGs, only the 55 encoding proteins acting on carbohydrates, and the 8 encoding peroxidases or laccases were shown or assumed to contribute to assembly or rearrangement of cell wall components. It means that this study allowed identifying about 200 genes encoding proteins that are candidates to play roles during growth of *Arabidopsis* etiolated hypocotyls. Their functional characterization will be paramount to understand cell wall architecture and assembly during an elongation process.

Are there variations in the level of transcripts between half- and fully-grown hypocotyls?

The comparison of the transcript levels of genes encoding secreted proteins between 5- and 11-day-old hypocotyls showed that 81 genes have a significant differential expression (Additional data file 3). Forty-three and 38 genes have a higher level of transcripts in 5- and 11-day-old hypocotyls respectively. The highest increase (13.8-fold) was found at 11-days for a gene encoding a GRP (*AT2G05440*). Conversely, the largest decrease (3.5-fold) was

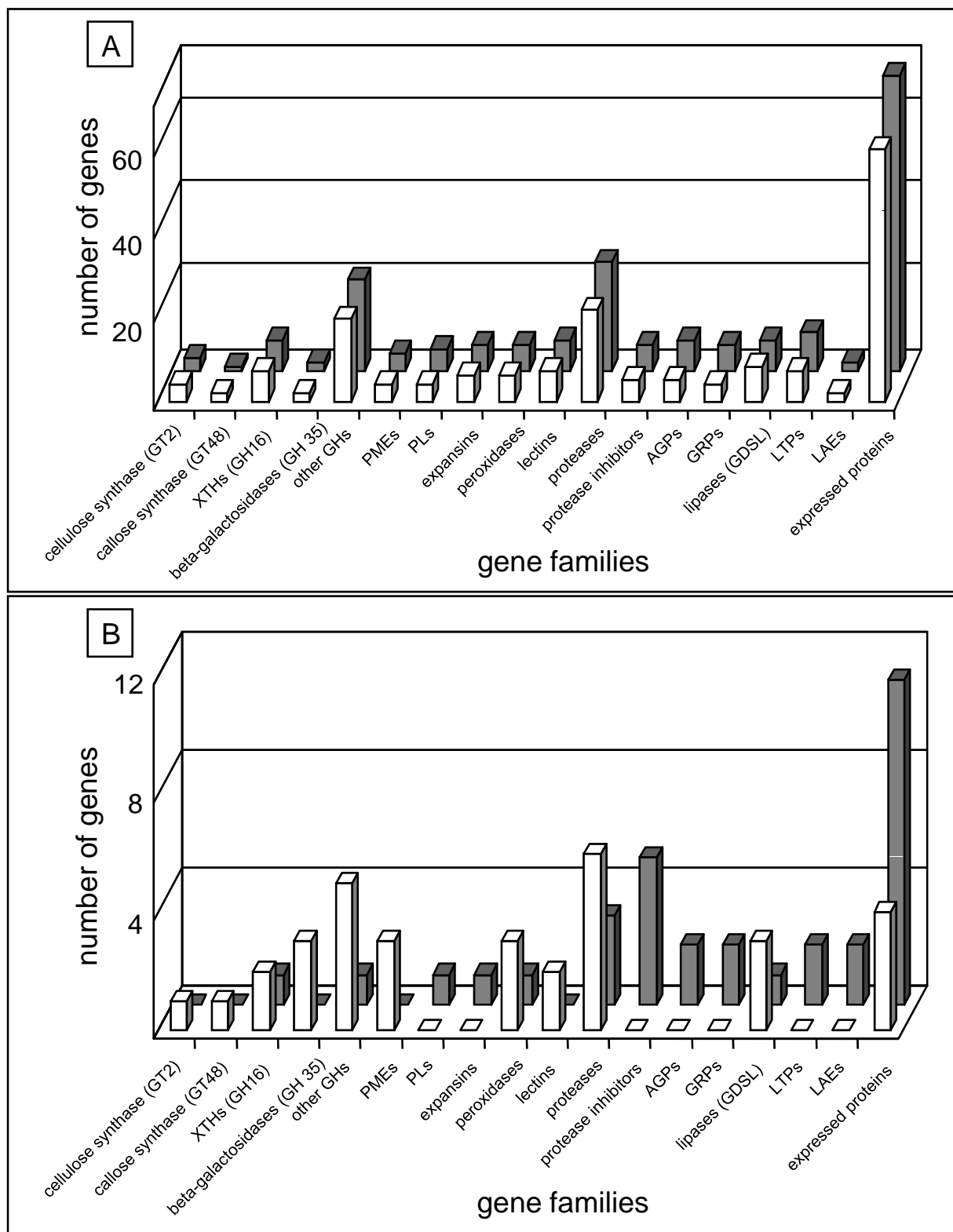


Figure 1. Overview of SPGs expressed in 5- and 11-day-old hypocotyls.

A. The number of genes belonging to several families of SPGs with moderate and high level of transcripts is shown: white and grey bars are used for 5- and 11-day-old hypocotyls respectively. **B.** Genes of the same families showing significant differences in expression between 5- and 11-day-old hypocotyls are counted: genes with higher level of transcripts in 5-day-old hypocotyls are represented by white bars; genes with higher level of transcripts in 11-day-old hypocotyls are represented by grey bars. AGP: arabinogalactan protein; GRP: glycine-rich protein; GT: glycosyl transferase; GH: glycosyl hydrolase; LAE: late embryogenesis abundant; LTP: lipid transfer protein; PL: polysaccharide lyase; PME: pectin methylesterase; XTH: xyloglucan endotransglucosylase/hydrolase.

observed at 11-days for a gene encoding a putative Asp protease (*AT5G10770*). The number of genes of selected families expressed differentially in both samples is represented in Figure 1. For comparison, the number of genes of the same families having high or moderate levels of transcripts is also represented. All the selected gene families are represented by almost the same number of genes at both developmental stages (Figure 1A). However, there are striking differences when the comparison is done with genes which level of transcripts varies significantly (Figure 1B).

Several genes encoding GTs, GHs, and PMEs have higher levels of transcripts at 5-days than at 11-days, *i.e.* at a time hypocotyls undergo active elongation. Although GTs and GHs are expected to be expressed during elongation, the role of PMEs appears to be more complex [21]. They have been described as enzymes involved in the arrest of cell elongation acting at the level of esterified pectins to de-esterify methyl residues and to allow binding to Ca^{2+} ions, thus promoting the formation of egg boxes. But they could also play roles during elongation in combination with polygalacturonases to favor the fragmentation of pectin chains. The proportion of cells already elongated could also be significant after 5-days of growth. Alternatively, there might be a delay between synthesis of mRNAs, and production of an active protein. This might be the case for PMEs that are usually produced as polyproteins comprising an inhibitor at their N-terminus and an active enzyme at their C-terminus [21].

Genes belonging to other gene families have higher levels of transcripts at 11-days: PLs, expansins, protease inhibitors, AGPs, GRPs, lipid transfer proteins (LTPs), and late-embryogenesis abundant proteins (LEAs). Higher levels of transcripts were not expected for several of them, *e.g.* PLs that are enzymes involved in the deconstruction of pectin backbones [22], expansins that are assumed to be involved in cell expansion [9], and LTPs that have recently been related to cell elongation [23]. On the contrary, GRPs have been shown to be associated to cell walls of xylem and phloem by tissue printing [24]. The great increase in the amount of transcripts of *AT2G05440* is consistent with the development of protoxylem elements that contain GRPs [25]. Concerning AGPs, they are very well represented in the transcriptome of hypocotyls with 12 AGPs having detectable levels of transcripts (Additional data file 1). AGPs are candidates for cell-to-cell communication [26], and were found to be associated to wood formation in poplar [13]. Nothing is known about the role LEAs could play in fully-developed hypocotyls.

Table 2. Genes with high levels of transcripts in either 5- or 11-day-old hypocotyls for which the encoded proteins were identified in a proteomic study performed on the same plant material.

Genes are classified by predicted functional domains as described in Methods. The intensity of the signal is expressed as \log_2 , the ratios between the levels of transcripts at 11 and 5 days as well as the Bonferroni p-values are indicated.

| Functional class | Gene family | AGI number | Predicted or known gene function | 5-days | 11-days | ratio 11-days / 5-days | p-value |
|---|---|------------|----------------------------------|--------|---------|------------------------|----------|
| Proteins acting on carbohydrates | glycoside hydrolase family 16 (xyloglucan endotransglycosidases/hydrolases) | AT2G06850 | AtXTH4 | 12.98 | 12.15 | -0.83 | 6.40E-04 |
| | glycoside hydrolase family 31 | AT1G68560 | XYL1 (alpha-xylosidase) | 10.60 | 10.13 | -0.47 | 1.00 |
| | expansin | AT5G02260 | AtEXPA9 | 12.71 | 12.44 | -0.28 | 1.00 |
| | carbohydrate esterase family 8 (pectin methylesterases) | AT3G14310 | AtPME3 | 10.79 | 9.9 | -0.90 | 4.05E-05 |
| Proteases | cysteine protease (papain family) | AT4G01610 | | 12.4 | 12.38 | -0.02 | 1.00 |
| | aspartyl protease | AT1G11910 | | 12.95 | 12.35 | -0.60 | 1.00 |
| | aspartyl protease | AT5G10770 | | 12.46 | 9.97 | -2.49 | 0.00E+00 |
| | asparaginyl peptidase | AT4G32940 | | 12.35 | 10.82 | -1.53 | 0.00E+00 |
| Structural proteins | proline-rich protein (PRP) | AT1G28290 | | 10.97 | 11.42 | 0.46 | 1.00 |
| Proteins involved in oxidation-reduction reactions | peroxidases | AT1G71695 | AtPrx12 | 10.17 | 9.54 | -0.63 | 4.86E-01 |
| | protein homologous to germin | AT1G09560 | GLP5 (subfamily 2, member 1) | 10.19 | 9.79 | -0.40 | 1.00 |
| | phytoeyanin (proteins homologous to blue copper binding proteins) | AT4G12880 | plastocyanin | 10.84 | 11.19 | 0.36 | 1.00 |
| Proteins with interacting domains | protein homologous to lectins | AT1G78850 | curculin-like, mannose binding | 11.95 | 11.42 | -0.53 | 1.00 |

| | | | | | | | |
|--------------------------------|--|-----------|--|-------|-------|-------|----------|
| | protein with leucine-rich domains (LRRs) | AT3G20820 | expressed protein (LRR domains) | 10.49 | 9.99 | -0.50 | 1.00 |
| | enzyme inhibitor | AT1G73260 | serine protease inhibitor | 12.00 | 12.5 | 0.50 | 1.00 |
| <hr/> | | | | | | | |
| Miscellaneous functions | | AT2G22170 | homologous to <i>Brassica napus</i> dehydration stress-induced protein | 10.57 | 10.54 | -0.03 | 1.00 |
| | | AT5G09440 | homologous to phosphate-induced proteins (phi) | 11.87 | 10.73 | -1.14 | 3.41E-10 |
| | | AT5G15230 | gibberellin regulated protein (GASA4) | 12.58 | 13.42 | 0.84 | 4.08E-04 |
| | | AT2G27190 | homologous to purple acid phosphatase | 10.67 | 10.42 | -0.25 | 1.00 |
| <hr/> | | | | | | | |
| Unknown functions | expressed proteins | AT5G11420 | DUF642 | 11.97 | 11.68 | -0.29 | 1.00 |
| | | AT3G08030 | DUF642 | 12.03 | 11.69 | -0.33 | 1.00 |
| | | AT3G20370 | MATH domain | 10.31 | 10.33 | 0.01 | 1.00 |
| | | AT3G22640 | cupin domain | 9.52 | 10.52 | 1.00 | 4.06E-07 |
| | | AT2G28490 | cupin domain | 10.09 | 10.69 | 0.59 | 1.00 |
| | | AT2G34700 | pollen proteins Ole e I family | 10.14 | 8.35 | -1.80 | 0.00E+00 |

Finally, some gene families have members which level of transcripts is higher either in 5- or in 11-day-old hypocotyls. This is the case of XTHs, peroxidases and proteases. XTHs are expected to play roles in cell elongation by cutting and linking fragments of xyloglucans [27]. However, they have also been identified by proteomics in mature tissues such as rosette leaves [28]. Peroxidases are assumed to cross-link networks of structural proteins and to polymerize monolignols in secondary walls at the end of elongation, thus being involved in the arrest of cell elongation [12]. But they could also be involved in the generation of free radicals able to cut polysaccharides, thus favoring wall extension [12]. The role of proteases in cell walls during elongation has not yet been described. As mentioned above, it can be assumed that they contribute to protein turnover in tissues where many physiological processes occur within a short time. It should be noted that 5 genes encoding protease inhibitors are up-regulated in 11-day-old hypocotyls, suggesting complex regulations of proteolytic activities in cell walls at the end of the arrest of hypocotyls elongation.

These differences in transcript abundance between the two samples should be taken carefully with regard to the possible functions carried out by the proteins, since many other genes from the same families are transcribed in half- and fully-grown hypocotyls, but without significant differences.

Transcriptome vs. proteome

In order to look for the consistency between levels of mRNAs and presence of the corresponding proteins, which are the molecules present in cell walls, a proteomic analysis has been performed on cell walls and the results were compared to those of this transcriptomic analysis. The cell wall proteomes of 5- and 11-days-old hypocotyls were achieved and a total of 137 proteins predicted to be secreted were identified (Irshad *et al.*, submitted for publication). When these 137 proteins were compared to the 278 SPGs with moderate and high levels of transcripts (Additional data file 2), only 35 proteins matched (12.8 %). From the 150 SPGs having high levels of transcripts in etiolated hypocotyls, only 25 (16.6 %) showed the corresponding proteins (Table 2). It was expected that proteomic profiling identified at least the proteins encoded by the highly-transcribed genes. The great inconsistency between the abundance of mRNAs and the presence of the corresponding proteins was surprising, but several reasons may explain this disparity. It is known that CWP extraction and identification can be challenging [15, 16]. Many proteins can remain linked to the polysaccharide matrix, such as the structural proteins [29, 30], or some peroxidases that

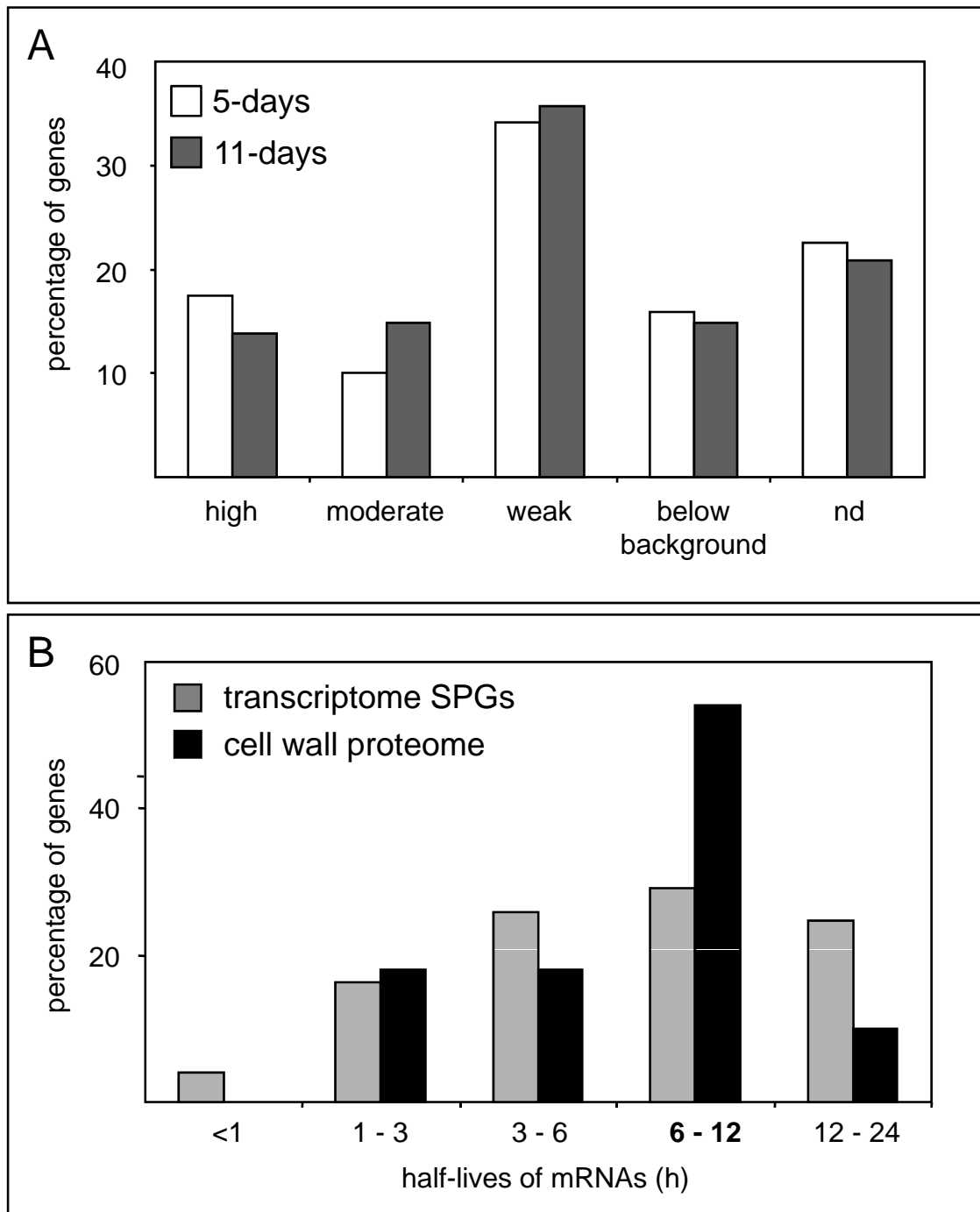


Figure 2. Level of transcripts of genes encoding CWPs identified through proteomics.

A. The levels of transcripts of genes were determined by the CATMA analysis in 5- and 11-day-old hypocotyls (white and dark grey bars respectively). Percentage of genes falling in the three following categories are represented: high transcript level corresponds to \log_2 values of the mean signal intensity higher the 10, moderate to values between 9 and 10, and weak to values between background (6.75) and 9. nd means not determined. **B.** Half-lives of mRNAs (h) corresponding to SPGs having high or moderate levels of transcripts (grey bars) or to proteins identified through cell wall proteomics (black bars). Percentage of genes in each range of half-lives is indicated for each set of gene. Half-lives of mRNAs in cell suspension cultures were from Narsai et al. (2007) [36].

might be strongly bound to pectins [31]. Others are difficult to identify because of their structure, *e.g.* a high level of *O*-glycosylation as for AGPs, which require a special deglycosylation step [32]. Proteins containing few linkages sensitive to trypsin digestion can also escape identification by peptide mass mapping. Finally, low-abundant proteins elude proteomic analyses. For the proteins that were identified without particular problems such as GHs, expansins and proteases, only a few of them correspond to highly-transcribed genes. It indicates that a high level of transcripts is not always correlated with the presence of the protein.

We then looked at the level of transcripts of the 137 genes encoding the proteins identified through proteomics (Figure 2A). The transcript level of 21 genes was not found in the CATMA experiment since some of them have no GST or were eliminated because of poor signals of hybridization to the RNA probe, or of inaccurate duplicates. The levels of transcripts of the 105 remaining genes were surprising, since 34.2 (resp. 35.6%) had low levels of transcripts and 15.8% (resp. 14.9%) had levels of transcripts below background at 5-days (resp. 11-days). However, all the identified proteins are assumed to be the most abundant. This shows that the transcripts could have short half-lives and/or that the proteins could have a low turnover.

To confirm the results obtained with the CATMA analysis, the level of transcripts was estimated by semi-quantitative RT-PCR for several genes corresponding to the three cases described (Figure 3): high or moderate level of transcripts and proteins identified; high level of transcripts and proteins not identified; low or below background level of transcripts and proteins identified. The results obtained for genes having high or moderate level of transcripts are consistent with CATMA results. When the number of PCR cycles was increased to visualize transcripts of some genes having levels of transcripts below background in CATMA analysis (Figure 3C), no band (*At5g44360*) or bands of weak intensity were detected, as compared with *At2g21140* which has a high level of transcripts (Figure 3B). It means that CATMA results are consistent with semi-quantitative RT-PCR results.

Altogether, these results show that there is not a clear correlation between the presence of CWPs as shown by cell wall proteomic analysis and the amount of transcripts of the corresponding genes. The quality of this correlation may depend on genes and/on environmental conditions. For example, the quantification of soluble proteins of yeast at mid-

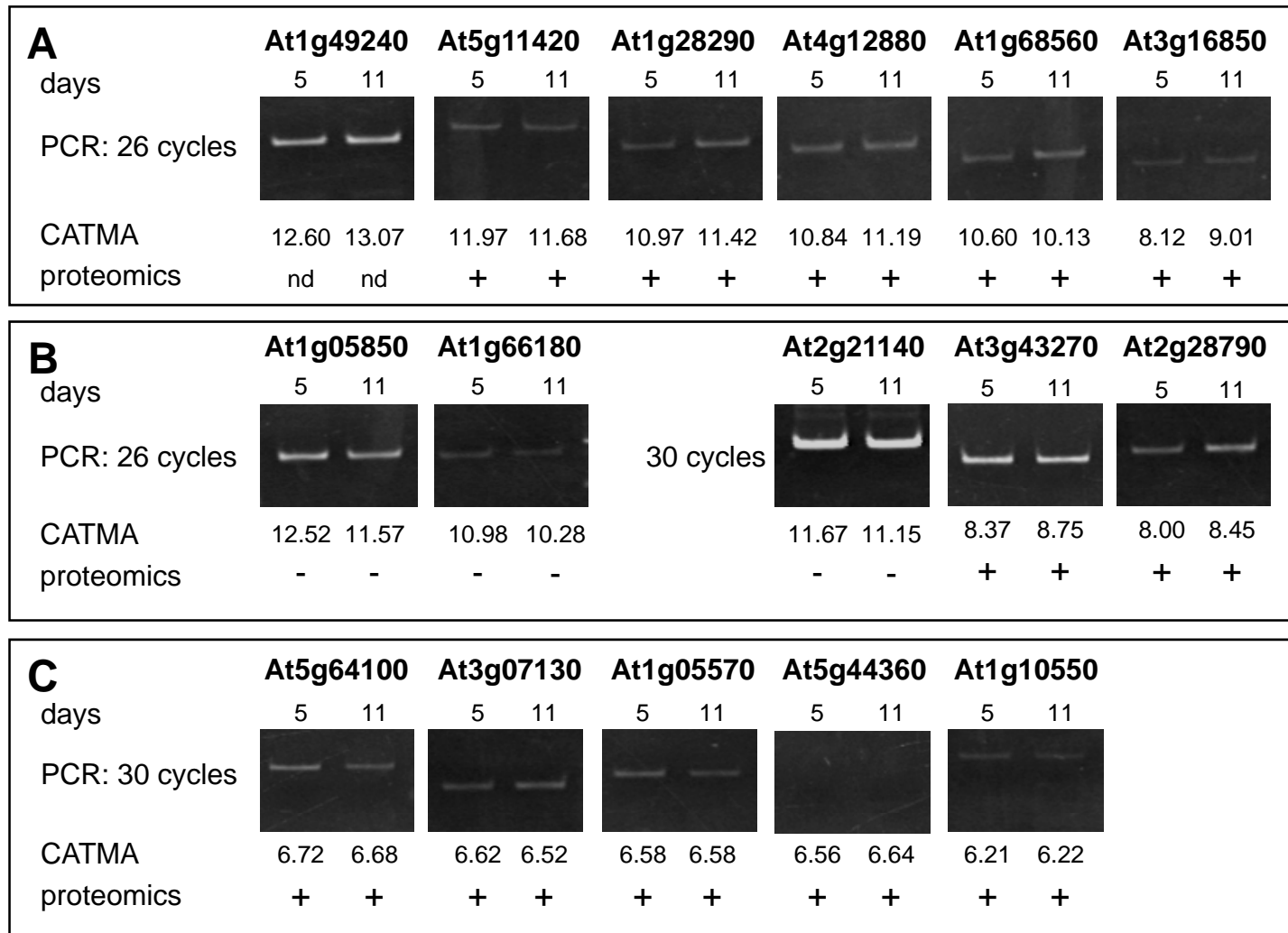


Figure 3. RT-PCR analysis of the transcripts of some genes encoding CWPs identified through proteomics.

A. Genes with high or moderate level of transcripts in CATMA analysis and for which the protein was identified by proteomics (+). At1g49240 encoding an actin was used as a control. **B.** Genes with high level of transcripts in CATMA analysis but for which the protein was not identified by proteomics (-); or with low level of transcripts in CATMA analysis for which the protein was identified by proteomics (+). **C.** Genes with transcript level under background in CATMA analysis (lower than 6.75) and for which the proteins were identified by proteomics (+). PCR was run for 26 cycles for genes having high or moderate levels of transcripts in CATMA analysis (A and B), or for 30 cycles for genes having level of transcripts lower than background in CATMA analysis (B and C).

log phase showed that for a given transcript level, protein levels were found to vary by more than 20-fold, whereas for a given protein level, transcript levels were found to vary 30-fold [33]. However, up-regulation of yeast genes in response to glucose or nitrogen limitation was found to be controlled at the transcriptional or post-transcriptional level respectively [34]. In *Arabidopsis* and rice, changes observed in the soluble proteome in response to bacterial challenge were not strictly correlated to changes in transcript levels [35]. These results show that quantitative analysis of transcript levels is not sufficient to infer protein levels. Multilevel analysis must take into account the stability of transcripts, their availability for active translation, as well as the stability of proteins that is certainly essential considering the high number of proteases in cell walls. With regard to transcript stability, data from a recent study aiming at measuring mRNA decay rates in *Arabidopsis* cell suspension cultures [36] were used to look for half-lives of gene transcripts identified through proteomics (Figure 2B). It can be seen that more than half of the proteins (64%) identified by cell wall proteomics correspond to genes having transcripts with rather long half-lives (6-24 h). Conversely, no gene corresponding to proteins identified by cell wall proteomics has transcripts with half-life shorter than 1 h. This distribution differs from that of transcripts of genes having high or moderate level of transcripts since 46% of these genes have half-lives shorter than 6h.

For the particular case of *Arabidopsis* etiolated hypocotyls, only 25 genes both showed a high level of transcripts during cell elongation and after its arrest and encoded proteins identified by cell wall proteomics. They might be considered either as house-keeping genes in this organ or good markers for cell elongation of dark-grown hypocotyls.

Conclusions

mRNA profiling of the genes potentially involved in cell wall biogenesis (CWGs) in elongating etiolated hypocotyls showed that more than half of them present a detectable level of transcripts. All gene families are expressed. The results suggest that both synthesis and rearrangement of wall components are required throughout hypocotyl elongation. The transcriptomic analysis of genes encoding secreted proteins showed that around 200 new genes might be implicated in this process. Understanding the biochemical and biological functions of these genes might reveal new mechanisms of cell wall expansion or growth arrest, or new functions for the cell wall.

Less than 100 genes encoding secreted proteins had significantly different levels of transcripts between growing and full-elongated hypocotyls. As expected, genes acting on

polysaccharides (GTs or GHs) had higher levels of transcripts at 5-days, whereas others encoding PME or peroxidases were not supposed to have higher level of transcripts during active elongation. Their function during cell elongation should be revisited. On the contrary, several genes encoding LTPs, LEAs, lipases, and protease inhibitors had higher levels of transcripts at 11-days. Their functions remain to be found at the end of the elongation process. Finally, as expected, some genes encoding GRPs were found to have much higher levels of transcripts in fully-grown hypocotyls at a time lignification is an active process. However, since all these genes belong to multigene families, one cannot rule out the fact that a similar function can be shared by several genes.

Finally, a proteomic analysis of the cell walls of half- and fully-grown hypocotyls identified 137 proteins. When the transcript levels of the corresponding genes were analyzed, 15% presented levels of transcripts below CATMA background. On the contrary, only 13% of the genes encoding secreted proteins with high or moderate levels of transcripts corresponded to proteins identified through proteomics. Thus, the comparison between transcript levels and presence of the corresponding proteins suggested that many genes encoding proteins secreted in cell walls are regulated at a post-transcriptional level during the elongation of etiolated hypocotyls. In conclusion, transcriptomic and proteomic data appeared to be complementary to describe the regulation of gene activity.

Methods

Plant material

Arabidopsis thaliana seedlings (ecotype Columbia 0) were grown in continuous dark on Magenta box on Murashige and Skoog [37] medium supplemented with 2% sucrose. Etiolated hypocotyls were collected after 5- and 11- days of culture.

Total RNA extraction

Three independent RNA extractions were performed for each sample (5- and 11-day-old hypocotyls). Hypocotyls were cut below cotyledons and above crown with sterile scissors. They were ground in liquid nitrogen in a mortar with a pestle. Extraction of total RNAs was performed using the SV Total RNA Isolation kit according to manufacturer's instructions (Promega France, Charbonnières, France). For each RNA extraction, 750 mg of ground hypocotyls were used. Typically, about 110 µg of total RNAs were obtained.

Transcriptome studies

Microarray analysis was carried out at the Unité de Recherche en Génomique Végétale (Evry, France), using the CATMA array [10, 38], containing 24,576 GSTs from *Arabidopsis*. RNA samples from three independent biological replicates were pooled. For each comparison, one technical replication with fluorochrome reversal was performed for each RNA sample (*i.e.* four hybridizations per comparison). The reverse transcription of RNA in the presence of Cy3-dUTP or Cy5-dUTP (Perkin-Elmer-NEN Life Science Products), the hybridization of labeled samples to the slides, and the scanning of the slides were performed as described in Lurin *et al.* [39].

Statistical analysis of microarray data

Experiments were designed with the statistics group of the Unité de Recherche en Génomique Végétale. Statistical analysis was based on two dye swaps (*i.e.* four arrays, each containing 24,576 GSTs and 384 controls) as described [39]. Controls were used for assessing the quality of the hybridizations, but were not included in the statistical tests or the graphic representation of the results. For each array, the raw data comprised the logarithm of median feature pixel intensity at wavelengths 635 (red) and 532 nm (green). To estimate the expression level of each gene, we considered a background value obtained by addition of the average background value to 2 background standard deviation. This background was not subtracted from the data presented in this paper, but was considered for the interpretation of the results. In the following description, \log_2 ratio refers to the differential expression between two conditions. It is either \log_2 (red/green) or \log_2 (green/red) according to the experimental design. Array-by-array normalization was performed to remove systematic biases. First, we excluded spots that were considered badly formed features. Then, we performed global intensity-dependent normalization using the LOESS procedure to correct the dye bias. Finally, for each block, the log ratio median calculated over the values for the entire block was subtracted from each individual log ratio value to correct print tip effects on each metablock. To determine differentially expressed genes, we performed a paired t test on the log ratios, assuming that the variance of the log ratios was the same for all genes. Spots displaying extreme variance (too small or too large) were excluded. The raw p-values were adjusted by the Bonferroni method, which controls the FWER. We considered as being differentially expressed the genes with an FWER of 5%. We use the Bonferroni method (with

a type I error equal to 5%) in order to keep a strong control of the false positives in a multiple-comparison context [40].

Data deposition

Microarray data from this article were deposited at Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>; accession No. E-MEXP-789) and at CATdb (<http://urgv.evry.inra.fr/CATdb/>; Project RS05-11_Hypocotyls) according to the “Minimum Information About a Microarray Experiment” standards.

Semi-quantitative PCR

cDNA first strand were obtained from total RNAs using 1 µg total RNAs and SuperScript™ II reverse transcriptase (Invitrogen, Carlsbad, San Diego, CA, USA). As a control, the same amount of pig desmin RNA was added in each sample. Quantitative PCR was performed using a Roche lightcycler system (Roche Diagnostics, Meylan, France) according to manufacturer’s recommendations. The sequences of oligonucleotide primers used for amplification is provided in Additional data file 5. Using the results from quantitative PCR to determine the number of amplification cycles required to be in a linear range for all genes of interest, semi-quantitative PCR was performed to compare relative amounts of the corresponding mRNAs. The amplified fragments were analyzed by electrophoresis in polyacrylamide gels in standardized conditions. In each case, presence of a fragment of the expected size was checked after staining with ethidium bromide, and the comparison of staining intensities was used for semi-quantification.

Bioinformatic analyses

Sub-cellular localization and length of signal peptides were predicted using PSORT (<http://psort.nibb.ac.jp/>) and TargetP (<http://www.cbs.dtu.dk/services/TargetP/>) [41, 42]. Prediction of transmembrane domains was done with Aramemnon (<http://aramemnon.botanik.uni-koeln.de/>) [43]. Molecular masses and pI values were calculated using the aBi program (http://www.up.univ-mrs.fr/~wabim/d_abim/compo-p.html). Homologies to other proteins were searched for using BLAST programs (<http://www.ncbi.nlm.nih.gov/BLAST/>) [44]. Identification of protein families and functional domains was performed using MyHits (http://myhits.isb-sib.ch/cgi-bin/motif_scan) and InterProScan (<http://www.ebi.ac.uk/InterProScan/>) [45].

All the protein families chosen in our CWG list were annotated by experts. GHs and CEs were classified according to the CAZy database (<http://www.cazy.org/CAZY/>) [46] at the Cell Wall Genomics website (<http://cellwall.genomics.purdue.edu/intro/index.html>). The GT77 family was annotated according to Egelund *et al.* [47]. XTHs and expansins were named according to <http://labs.plantbio.cornell.edu/xth/> and <http://www.bio.psu.edu/expansins/index.htm> respectively. AGPs and FLAs were named according to Schultz *et al.* [48], Johnson *et al.* [49], Van Hengels and Roberts [50], and Liu and Mehdy [51]. Proteins homologous to COBRA, LRXs and Hyp/Pro-rich proteins were annotated according to Roudier *et al.* [14], Baumberger *et al.* [52], and Fowler *et al.* [53] respectively. The lignin toolbox was proposed by Raes *et al.* [54]. Peroxidases were named as in the PeroxiBase (<http://peroxidase.isb-sib.ch/index.php>) [55]. Laccases were annotated as in Pourcel *et al.* [56] and McCaig *et al.* [57]. SKU-like proteins and phytocyanins were described in Jacobs and Roe [58], and Nersissian and Shipp [59] respectively. Subtilases are listed at <http://csbdb.mpimp-golm.mpg.de/csbdb/dbcawp/psdb.html>.

List of abbreviations

AGP: arabinogalactan protein; CATMA: Complete *Arabidopsis* Transcriptome MicroArray; CESA: cellulose-synthase; CSL: cellulose-synthase like; CWG: cell wall gene; CWP: cell wall protein; FLA: fasciclin arabinogalactan protein; GH: glycoside hydrolase; GRP: glycine-rich protein; GST: gene-specific tag; GT: glycosyl transferase; LEA: late-embryogenesis abundant protein; LTP: lipid transfer protein; LRR: leucine-rich repeat; LRX: leucine-rich repeat extensin; PL: polysaccharide lyase; PME: pectin methylesterase; SPG: secretory pathway gene; XTH: xyloglucan endotransglucosylase-hydrolase

Authors' contributions

E.J. conceived the study, participated in its design, coordination, analysis of data, and drafted the manuscript. D.R. and M.I. carried out the culture of plants, RNA extractions and PCR-analysis. HSC was involved in bioinformatic analyses. L.S.-T. and J.-P.R. performed the microarray and statistical analyses of the results. R.P.-L. contributed to the analysis of data and to drafting of the manuscript. All authors read and approved the final manuscript.

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Additional data files

Additional data file 1.

Table 1. Cell Wall Genes (CWGs) with detectable levels of transcripts in 5- and 11-day-old hypocotyls.

Additional data file 2.

Table 2. Secretory Pathway Genes (SPGs) with moderate or high levels of transcripts in 5- and 11-day-old hypocotyls.

Additional data file 3.

Table 3. Secretory Pathway Genes (SPGs) with modulated levels of transcripts in 5- and 11-day-old hypocotyls.

Additional data file 4.

Table 4. Proteins extracted and identified by mass spectrometry from purified cell walls of 5- and 11-day-old etiolated hypocotyls of *A. thaliana*.

Additional data file 5.

Table 5. Nucleotide primers used for PCR amplifications

CHAPTER 6

**CONCLUSIONS
AND PERSPECTIVES**

French version

La première étape de ce travail a consisté en l'identification des protéines présentes dans les parois d'hypocotyles étiolés d'*Arabidopsis* en élongation ou après la fin de leur élongation en utilisant une approche de protéomique. Pour éviter la perte de protéines au cours de la purification des parois et la contamination par des protéines intracellulaires, nous avons dû établir un nouveau protocole de purification de parois. Les protéines ont été extraites avec des solutions salines avant d'être séparées par électrophorèse 1D. nous avons pu identifier un grand nombre de protéines par cartographie peptidique massique en utilisant la spectrométrie de masse de type MALDI-TOF. Une grande proportion de ces protéines avait un peptide signal prédit par bioinformatique. Une proportion aussi élevée de protéines secrétées n'avait jamais été obtenue dans les études de protéomique de la paroi précédemment publiées. Ces protéines ont été appelées protéines pariétales (CWP). Notre méthode de purification a été récemment utilisée au laboratoire avec succès pour des suspensions cellulaires de tabac (S. Muzammil, M2R, Université Toulouse III, 2007-2008). Ceci montre qu'il doit être possible de l'adapter à différents organes et à d'autres plantes. Des expériences préliminaires indiquent également qu'une fraction de protéines interagissant avec les pectines peut être extraite de telles parois (G. Boudart, résultats non publiés). Des expériences supplémentaires sont maintenant nécessaires pour caractériser ces protéines. Enfin, bien que toutes les protéines appelées CWP aient un peptide signal prédit, leur localisation sub-cellulaire doit encore être vérifiée par immunolocalisation ou par localisation de protéines fusion avec la GFP (*Green Fluorescent Protein*) *in planta*.

Concernant la protéomique des parois végétales, un autre apport majeur de ce travail est le développement d'une nouvelle méthode de séparation des protéines pariétales. Mettant à profit le fait que la plupart des CWPs sont basiques, nous avons utilisé une chromatographie échangeuse de cations à pH acide comme première étape de séparation, mimant ainsi les parois végétales qui sont chargées négativement. La seconde étape de séparation a consisté en une électrophorèse 1D. L'efficacité de cette nouvelle stratégie de séparation 2D a permis d'identifier deux fois plus de protéines que la simple électrophorèse 1D. De plus, les identifications par cartographie peptidique massique ont été de meilleure qualité puisque le pourcentage de recouvrement des séquences des protéines était significativement plus élevé. Enfin, il a été possible de procéder à une semi-quantification des protéines. Cette nouvelle stratégie pourra être employée pour des études biochimiques et structurales futures de

nombreuses protéines pariétales. En effet, il est maintenant possible de les visualiser après seulement deux étapes de séparation.

Cette analyse protéomique a permis l'identification de 137 protéines pariétales à partir des hypocotyles étiolés d'*Arabidopsis*. 51 de ces protéines ont été identifiées pour la première fois par protéomique, ce qui suggère une expression organe-spécifique des gènes correspondants. 84 de ces protéines étaient communes aux deux stades de développement des hypocotyles (élongation active vs élongation terminée) alors que 36 et 17 ont été identifiées seulement à 5 jours (élongation active) et à 10 jours (élongation terminée) respectivement. La semi-quantification des protéines a montré que le niveau de 34 protéines différaient entre les deux stades de développement. D'un point de vue quantitatif, les résultats de cette étude suggèrent que les parois des hypocotyles subissant une élongation sont métaboliquement plus actives que celles des hypocotyles dont l'élongation est terminée.

Parmi les protéines identifiées dans cette étude, celles qui étaient attendues car jouant un rôle soit dans le réarrangement des polysaccharides au cours de l'élongation cellulaire (XTH, PG, expansines), ou encore dans la formation des boîtes à œufs pectiques (PME), des réseaux de protéines structurales et de polyphénols (peroxydases, laccases) en fin d'élongation ont été trouvées. Cependant, certaines d'entre elles ont également été trouvées tout au long du développement des hypocotyles. Ce résultat inattendu suggère l'existence de mécanismes de régulation au niveau de la durée de vie des protéines, ou de l'activité biologique des protéines. Une autre hypothèse est que ces protéines seraient impliquées dans d'autres processus encore à découvrir. Plusieurs protéines liées au métabolisme des lipides ont été identifiées. Aucune fonction ne leur a encore été clairement associée bien qu'il a été suggéré qu'elles pourraient contribuer à la formation de la cuticule particulièrement épaisse des hypocotyles étiolés. De la même manière, les protéines de fonction inconnue sont des bons candidats pour contribuer à l'élongation cellulaire. Des études supplémentaires incluant l'obtention de plantes présentant des niveaux d'expression des gènes correspondants modifiés sont nécessaires pour comprendre la fonction de tous ces gènes.

En plus de l'identification de gènes candidats pour jouer des rôles au cours de l'élongation cellulaire, la protéomique permet d'obtenir une vision globale des protéines d'un compartiment cellulaire, et notamment d'identifier en même les partenaires d'interactions protéine-protéine. Par exemple, des enzymes et leurs inhibiteurs ont été trouvés tels que des

protéases et des inhibiteurs de protéases, des PME et des PME1, des PG et des PGIP. Ceci montre que l'activité biologique d'une protéine peut être régulée par une maturation par une protéase, une interaction avec un inhibiteur, et/ou la protéolyse. L'abondance des protéases et de leurs inhibiteurs dans les parois des hypocotyles doit être soulignée. Des tests fonctionnels devraient permettre de montrer l'existence de ces activités de régulation.

L'étude protéomique des hypocotyles étioles a été complétée par une étude de transcriptomique réalisée sur le même matériel. Dans un premier temps, des niveaux de transcrits significatifs ont été détectés pour plus de la moitié des gènes supposés être impliqués dans la biogenèse des parois (CWG) avec une proportion importante de gènes codant des protéines impliquées dans la synthèse et le transport des constituants pariétaux. Nos résultats indiquent que la synthèse, le transport et le réarrangement des constituants pariétaux interviendraient tout au long du développement des hypocotyles étioles. Ce résultat n'était pas attendu dans la mesure où deux phases successives de modification des parois avaient été décrites : une synthèse active de constituants pariétaux ayant pour conséquence une augmentation de l'épaisseur de parois, suivie par une phase de réduction de l'épaisseur des parois pendant l'élongation cellulaire. Dans un second temps, nous avons recherché des gènes codant des protéines secrétées ayant des niveaux de transcrits modérés ou élevés (SPG). C'est ainsi que nous avons identifié plus de 200 gènes qui pourraient jouer un rôle important pendant le développement des hypocotyles à l'obscurité. Comprendre leurs fonctions pourrait révéler de nouveaux mécanismes importants pour l'élongation cellulaire, l'arrêt de la croissance, ou de nouvelles fonctions pour les parois.

La dernière partie de l'étude a été consacrée à la comparaison entre les données transcriptomique et protéomique. A notre surprise, environ 15% des protéines identifiées par protéomique correspondent à des gènes dont les niveaux de transcrits sont inférieurs au bruit de fond CATMA. Au contraire, nous avons identifié des protéines correspondant à seulement 13% des gènes codant des protéines secrétées et ayant des niveaux de transcrits modérés ou élevés. Ceci montre que les données de protéomique et de transcriptomique ne sont pas systématiquement cohérentes. Une grande proportion de gènes codant des protéines secrétées semble être régulée à un niveau post-transcriptionnel dans les hypocotyles étioles. Ainsi, les niveaux de protéines ne peuvent pas être déduits des niveaux de transcrits. Les deux types de données devraient être utilisées pour mieux décrire la régulation de l'activité des gènes pendant l'élongation des hypocotyles. Des expériences supplémentaires devraient être effectuées pour

déterminer la demi-vie des transcrits, leur capacité à être transcrits, et déterminer la demi-vie des protéines. Ceci constitue un champ de recherches complètement nouveau dans lequel peu de données expérimentales existent.

English version

The first step of this work consisted in the identification of proteins present in cell walls of elongating and fully-elongated hypocotyls of *Arabidopsis* by a proteomic approach using a destructive method. To avoid losing CWPs during the purification of cell walls and contamination by intracellular proteins, we had to establish **a new protocol for purification of cell walls**. Proteins were extracted with salt solutions and separated by 1D-E. We were able to identify a high number of proteins by peptide mass mapping using MALDI-TOF mass spectrometry, and a high proportion of them were predicted to be secreted by bioinformatics. Such a high proportion of secreted proteins was never found before in cell wall proteomic studies. These proteins were called CWPs. This method of purification of cell walls has been recently successfully applied to tobacco cell suspension cultures in the laboratory (S. Muzammil, M2R, Toulouse III University, 2007-2008). This result shows that it should be possible to adapt it to different plant organs and to other plants. Preliminary experiments indicate that a fraction of proteins interacting with pectins can also be released from such purified cell walls (G. Boudart, unpublished results). Additional experiments are now required to characterize these proteins. Finally, although all the so-called CWPs are predicted to have a signal peptide, their sub-cellular localization should be confirmed either by immunolocalization or localization of Green Fluorescent Protein (GFP)-fusions *in planta*.

Another major achievement of this study is the development of **a new separation method for CWPs**. Since most CWPs are basic proteins, cation exchange chromatography with FPLC was used at acidic pH as a first step of separation, thus mimicking cell walls that are negatively charged *in planta*. The second step of separation consisted in 1D-E. The efficiency of this new 2D-separation strategy allowed the identification of twice as much proteins as 1D-E alone. In addition, it permitted a better identification with a higher number of matching peptide by MALDI-TOF MS peptide mass mapping, and semi-quantification of proteins. This method also proved to be a powerful tool for future biochemical and structural studies of CWPs.

This proteomic analysis allowed the identification of 137 CWPs from *Arabidopsis* etiolated hypocotyls, thus providing a global image of protein dynamics during cell elongation. Fifty-one CWPs were identified for the first time by cell wall proteomics, supporting the idea of organ-specific expression of CWPs. Eighty-four CWPs were common to both stages of hypocotyl development (active elongation *vs* after elongation arrest) whereas

36 and 17 were only identified at 5-days (active elongation) and 11-days (after elongation arrest) respectively. Semi-quantification showed that the amount of 34 CWPs was different at one or the other developmental stage. Altogether, from a quantitative point of view, the results of this study suggest that cell walls of hypocotyls undergoing elongation are metabolically more active than those of hypocotyls after elongation arrest.

Among the CWPs identified in this study, the proteins expected to play roles in rearrangements of cell wall polysaccharides during cell wall elongation (XTHs, PGs, expansins), in formation of pectin egg boxes (PME) or in cross-linking of proteins or polyphenols (peroxidases, laccases) after the end of elongation were found. However, some of them were also found all along the growth of hypocotyls. This unexpected result suggests the existence of regulatory mechanisms at the level of protein half-lives or protein biological activity. Alternatively, such proteins might be involved in other processes still to be discovered. Several proteins related to lipid metabolism were identified. They have yet no clear function in cell walls although it has been suggested that they could be involved in the synthesis of the thick cuticle that surround etiolated hypocotyls. In the same way, proteins of yet unknown function are good candidates to contribute somehow to cell elongation. Additional analyses including study of plants with modified level of expression of these genes should allow understanding the function of these genes.

Apart from identifying new candidates for performing roles in cell wall elongation, proteomics provides a unique way to get a global view of CWPs, thus putting together proteins able to interact. For example, enzymes and the corresponding inhibitors were found such as proteases and protease inhibitors, PMEs and PMEIs, PG and PGIPs. It suggests that the biological activity of a protein might be regulated by maturation by a protease, interaction with an inhibitor, and/or proteolysis. The great abundance of proteases and of their inhibitors in the cell wall proteome of hypocotyls should be emphasized. Functional tests should allow demonstration of these regulatory activities.

The proteomic study of etiolated hypocotyls was complemented by a **transcriptomic survey** performed with the same material. In a first analysis, significant transcript levels were detected for more than one half of the genes assumed to be involved in cell wall biogenesis (CWGs) with a significant proportion of genes involved in synthesis and transport of cell wall components. Our results indicate that synthesis, transport and

rearrangements of cell wall components occur during hypocotyl elongation and growth arrest. This result contrasts with what was previously reported about the existence of two phases of cell wall modification during hypocotyl elongation: active synthesis of cell wall polysaccharides with increase in cell wall thickness, followed by thinning down of cell walls during cell elongation. In a second analysis, we focused on genes encoding secreted proteins (SPGs) and we could identify more than 200 genes having moderate or high level of transcripts. All of them are candidates to play important roles, not yet described, during hypocotyl development. Understanding their function might reveal new mechanisms important for cell wall expansion, growth arrest, or new function for cell walls.

The last part of our study consisted in the **comparison between proteomic and transcriptomic data**. To our surprise, about 15% of the proteins identified by proteomics corresponded to genes having level of transcripts below CATMA background level. Conversely, we identified proteins corresponding to only 13% of the genes encoding secreted proteins and having moderate or high level of transcripts. It means that proteomic and transcriptomic data are not consistent in all cases. A great proportion of genes encoding secreted proteins seems to be regulated at a post-transcriptional level in etiolated hypocotyls. Hence, protein abundance cannot be inferred from transcriptomic data. Both kinds of data should be better used to describe regulation of gene activity during hypocotyl elongation. Additional experiments should be performed to know more about half-lives of transcripts, their ability to be translated, and about half-lives of proteins in cell walls. This is a completely new field of research in which there is no data.

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**ANNEXES AND
SUPPLEMENTARY DATA**

ANNEXES CHAPTER 2

Annex I. Composition of (12 × 15 cm) polyacrylamide gel for 1D-E of proteins

| Solutions and buffers | Nature, %age of acrylamide, and volume (mL) | | | | | | | |
|---------------------------------------|---|--------------------|--------------------|---------------------|---------------------|---------------------|-----------------------|-------------------|
| | Resolving gel (7.5%) | Resolving gel (8%) | Resolving gel (9%) | Resolving gel (10%) | Resolving gel (11%) | Resolving gel (12%) | Resolving gel (12.5%) | Stacking gel (4%) |
| Acrylamide 40% | 7.280 | 7.776 | 8.748 | 9.720 | 10.692 | 11.664 | 12.150 | 1.944 |
| Bis-acrylamide 2% | 4.000 | 4.288 | 4.824 | 5.360 | 5.896 | 6.432 | 6.700 | 1.072 |
| Buffer A 4x (Tris-HCl 1.5 M pH 8.8) | 10.000 | 10.000 | 10.000 | 10.000 | 10.000 | 10.000 | 10.000 | 0.000 |
| Buffer B 10x (Tris-HCl 1.25 M pH 6.8) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2.000 |
| SDS 10% (filtered) | 0.400 | 0.400 | 0.400 | 0.400 | 0.400 | 0.400 | 0.400 | 0.200 |
| UHQ water | 18.120 | 17.336 | 15.828 | 14.320 | 12.812 | 11.304 | 10.550 | 14.720 |
| Ammonium persulfate 10% | 0.200 | 0.200 | 0.200 | 0.200 | 0.200 | 0.200 | 0.200 | 0.100 |
| TEMED | 0.020 | 0.020 | 0.020 | 0.020 | 0.020 | 0.020 | 0.020 | 0.020 |
| Total | 40.020 | 40.020 | 40.020 | 40.020 | 40.020 | 40.020 | 40.020 | 20.056 |

Annex II: Composition of mini (6 × 8 cm) polyacrylamide gel for 1D-E of proteins

| Solutions and buffers | Nature, %age of acrylamide, and volume (mL) | | | | | | | |
|---------------------------------------|---|--------------------|--------------------|---------------------|---------------------|---------------------|-----------------------|-------------------|
| | Resolving gel (7.5%) | Resolving gel (8%) | Resolving gel (9%) | Resolving gel (10%) | Resolving gel (11%) | Resolving gel (12%) | Resolving gel (12.5%) | Stacking gel (4%) |
| Acrylamide 40% | 1.456 | 1.555 | 1.750 | 1.944 | 2.138 | 2.333 | 2.430 | 0.486 |
| Bis-acrylamide 2% | 0.800 | 0,858 | 0.965 | 1.072 | 1.179 | 1.286 | 1.340 | 0.268 |
| Buffer A 4x (Tris-HCl 1.5 M pH 8.8) | 2.500 | 2.500 | 2.500 | 2.500 | 2.500 | 2.500 | 2.500 | 0.000 |
| Buffer B 10x (Tris-HCl 1.25 M pH 6.8) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.500 |
| SDS 10% (filtered) | 0.100 | 0.100 | 0.100 | 0.100 | 0.100 | 0.100 | 0.100 | 0.050 |
| UHQ water | 3.089 | 2.932 | 2.631 | 2.329 | 2.027 | 1.726 | 1.575 | 3.680 |
| Ammonium persulfate 10% | 0.050 | 0.050 | 0.050 | 0.050 | 0.050 | 0.050 | 0.050 | 0.025 |
| TEMED | 0.005 | 0.005 | 0.005 | 0.005 | 0.005 | 0.005 | 0.005 | 0.005 |
| Total | 8.000 | 8.000 | 8.000 | 8.000 | 8.000 | 8.000 | 8.000 | 5.014 |

Annex III. Composition of RT-PCR buffer

| Components | Volume (μL) |
|---|--------------------------|
| Oligo (dT) ₁₅ primer (Promega Corporation, Madison, WI, USA) 500 $\mu\text{g}/\text{mL}$ | 1 |
| Total RNA (100 $\text{ng}/\mu\text{L}$) | 10 |
| dNTPs (10 mM each) | 1 |
| Desmine mRNA (Pork mRNA) | 1 |

After 5 min heating at 65°C and putting again on ice, the following components are added:

| | |
|---|---|
| 5× First Strand Buffer (Invitrogen, Carlsbad, San Diego, CA, USA) | 4 |
| DTT, 0.1 M (Invitrogen, Carlsbad, San Diego, CA, USA) | 2 |
| RNasine Plus RNase Inhibitor, 40 U/ μL (Promega Corporation, Madison, WI, USA) | 1 |

SUPPLEMENTARY DATA CHAPTER 3

Additional file 1: Bioinformatic analysis of proteins extracted from cell walls of *A. thaliana* cell suspension cultures [8].

Data are from [8]. Proteins were extracted from *A. thaliana* cell walls of cell suspension cultures as described in Figure 1. Two successive extractions were performed : step 1 using CaCl₂ and step 2 using urea. All proteins sequences were analyzed with three bioinformatic programs to predict their sub-cellular localization. Proteins for which predictions by different bioinformatic programs are in conflict are classified as "not clear".

| step 1: CaCl ₂ extract | Predicted subcellular localization | Gene (<i>A. thaliana</i>) | PSORT (a) | TargetP (b) | Predicted signal peptide (c) | Aramemnon (d) |
|-----------------------------------|------------------------------------|-----------------------------|--|--|------------------------------|---------------|
| | transmembrane domain | | Golgi (0.900), plasma membrane (0.790), chloroplast thylakoid membrane (0.762) | other (0.796) | | yes |
| | | At3g30810 At4g12420 | plasma membrane (0.919) | secretory pathway (0.989) | 1-20 | GPI anchor |
| | outside | At1g06870 | outside (0.638) | secretory pathway (0.850) | 1-21 | |
| | | At1g21670 | outside (0.820) | secretory pathway (0.973) | 1-21 | |
| | | At1g53070 | outside (0.528) | secretory pathway (0.972) | 1-17 or 1-23 | |
| | | At1g78830 | outside (0.820) | secretory pathway (0.962) | 1-22 | |
| | | At1g78850 | outside (0.820) | secretory pathway (0.973) | 1-22 | |
| | | At2g39700 | outside (0.810) | secretory pathway (0.961) | 1-20 | |
| | | At2g41800 | outside (0.370) | secretory pathway (0.369) | 1-21 | |
| | | At2g44450 | outside (0.820) | secretory pathway (0.853) | 1-22 | |
| | | At2g47050 | outside (0.820) | secretory pathway (0.928) | 1-20 | |
| | | At3g07320 | outside (0.790) | secretory pathway (0.482) | 1-19 | |
| | | At3g08030 | outside (0.820) | secretory pathway (0.985) | 1-21 | |
| | | At3g45960 | outside (0.805) | secretory pathway (0.744) | 1-20 | |
| | | At3g45970 | endoplasmic reticulum (0.820) | secretory pathway (0.854) | 1-20 | |
| | | At3g61820 | outside (0.628) | secretory pathway (0.828) | 1-26 or 1-25 | |
| | | At4g20830 | outside (0.820) | secretory pathway (0.540) | 1-30 | |
| | | At4g25900 | outside (0.820) | secretory pathway (0.924) | 1-20 or 1-24 | |
| | | At4g30270 | outside (0.800) | secretory pathway (0.975) | 1-21 | |
| | At5g06860 | outside (0.733) | secretory pathway (0.964) | 1-21 | | |
| | intracellular | At1g08450 | endoplasmic reticulum (0.910) (C-terminal HDEL) | secretory pathway (0.995) | 1-28 | |
| | | At1g13440 | microbody (0.539) | other (0.610) | | |
| | | At1g30580 | microbody (0.507) | other (0.579) | | |
| | | At2g39050 | microbody (0.515) | other (0.910) | | |
| | | At2g43710 | microbody (0.495) | chloroplast (0.977) | | |
| | | At3g02630 | chloroplast stroma (0.593) | chloroplast (0.779) | | |
| | | At3g26060 | mitochondry (0.679) | chloroplast (0.904) | | |
| | | At3g48380 | mitochondry (0.596) | mitochondry (0.308) | | |
| | | At3g58480 | nucleus (0.760) | other (0.802) | | |
| | | At4g17260 | cytoplasm (0.450) | other (0.435) | | |
| | | At4g37870 | nucleus (0.880) | other (0.849) | | |
| | | At5g08310 | mitochondry (0.789) | mitochondry (0.946) | | |
| | | At5g20080 | mitochondry (0.859) | mitochondry (0.855) | | |
| | | At5g46550 | nucleus (0.940) | other (0.504) | | |
| | At5g55990 | microbody (0.569) | secretory pathway (0.580), other (0.576) | | | |
| | not clear | At4g22410 | cytoplasm (0.450) | secretory pathway (0.922) | 1-26 | |
| | | At5g37990 | endoplasmic reticulum (0.550) | secretory pathway (0.523), chloroplast (0.307) | 1-15 | |

step 2: urea extract

| | | | | | |
|-----------------------------|-----------|---|--|--------------|-----|
| transmembrane domain | At1g61500 | plasma membrane (0.460) | secretory pathway (0.963) | 1-24 | yes |
| | At1g67880 | plasma membrane (0.790) | other (0.955) | | yes |
| | At3g25560 | plasma membrane (0.460) | secretory pathway (0.742) | 1-32 | yes |
| | At3g57400 | plasma membrane (0.830) | other (0.792) | | yes |
| | At5g08390 | plasma membrane (0.460) | secretory pathway (0.932) | 1-22 | yes |
| | At5g58640 | plasma membrane (0.640) | secretory pathway (0.326) | 1-17 | yes |
| outside | At1g71695 | outside (0.820) | secretory pathway (0.829) | 1-22 or 1-31 | |
| | At2g16430 | outside (0.820) | secretory pathway (0.963) | 1-25 | |
| | At2g44450 | outside (0.820) | secretory pathway (0.853) | 1-22 | |
| | At3g08030 | outside (0.820) | secretory pathway (0.985) | 1-21 | |
| | At3g45970 | endoplasmic reticulum (0.820) | secretory pathway (0.854) | 1-20 | |
| | At3g52500 | outside (0.820) | secretory pathway (0.649) | 1-18 | |
| | At4g08770 | outside (0.633) | secretory pathway (0.955) | 1-22 | |
| | At4g24890 | outside (0.820) | secretory pathway (0.925) | 1-20 or 1-26 | |
| intracellular | At1g24360 | mitochondry (0.613) | chloroplast (0.545), mitochondry (0.206) | | |
| | At1g30580 | microbody (0.507) | other (0.579) | | |
| | At1g43800 | nucleus (0.760) | mitochondry (0.430) | | |
| | At1g43800 | nucleus (0.760) | mitochondry (0.430) | | |
| | At1g52960 | nucleus (0.960) | chloroplast (0.630) | | |
| | At1g56070 | mitochondry (0.360) | other (0.823) | | |
| | At1g69290 | chloroplast (0.840) | chloroplast (0.528) | | |
| | At1g72560 | cytoplasm (0.450) | other (0.872) | | |
| | At2g17190 | cytoplasm (0.650) | other (0.797) | | |
| | At2g32920 | endoplasmic reticulum (0.910) (C-terminal KDEL) | secretory pathway (0.920) | 1-17 or 1-24 | |
| | At2g36530 | endoplasmic reticulum (0.600) | other (0.747) | | |
| | At2g43710 | microbody (0.495) | chloroplast (0.977) | | |
| | At2g44350 | mitochondry (0.883) | mitochondry (0.848) | | |
| | At3g04600 | nucleus (0.940) | other (0.954) | | |
| | At3g14440 | chloroplast (0.905) | chloroplast (0.719) | | |
| | At3g15730 | microbody (0.634) | other (0.614) | | |
| | At3g16857 | nucleus (0.700) | other (0.376) | | |
| | At3g57400 | plasma membrane (0.830) | other (0.792) | | |
| | At4g17260 | cytoplasm (0.450) | chloroplast (0.703) | | |
| | At4g31180 | chloroplast (0.890) | chloroplast (0.876) | | |
| | At4g36530 | microbody (0.594) | other (0.828) | | |
| | At4g37870 | nucleus (0.880) | other (0.849) | | |
| | At5g06450 | microbody (0.640) | other (0.954) | | |
| | At5g20080 | mitochondry (0.859) | mitochondry (0.855) | | |
| | At5g26710 | endoplasmic reticulum (0.550) | chloroplast (0.552) | | |
| | At5g28540 | endoplasmic reticulum (0.910) (C-terminal HDEL) | secretory pathway (0.993) | 1-27 | |
| | At5g41550 | chloroplast (0.520) | mitochondry (0.517) | | |

Colour code:

proteins found at both steps 1 and 2
 proteins found at both steps 1 and 2



(a) PSORT : <http://psort.nibb.ac.jp/form.html> [29]

(b) TargetP: <http://www.cbs.dtu.dk/services/TargetP/> [30]

(c) Two sizes are indicated when different signal peptides are predicted by PSORT and TargetP. The first one is predicted with PSORT.

(d) Aramemnon: <http://aramemnon.botanik.uni-koeln.de/> [31]

Additional file 2: Bioinformatic analysis of proteins extracted from cell walls of *M. sativa* stems [9].

Data are from [9]. Proteins were extracted from *M. sativa* cell walls of stems as described in Figure 2. Two successive extractions were performed : step 1 using CaCl₂, and step 2 using LiCl. All proteins sequences were analyzed with bioinformatic softwares to predict their sub-cellular localization. When only partial protein sequences or no sequence are available, proteins are classified as "not predictable".

| step 1: CaCl ₂ extract | Predicted subcellular localization | Gene (<i>A. thaliana</i>) | Protein | PSORT (a) | TargetP (b) | Predicted signal peptide (c) | Aramemnon (d) |
|-----------------------------------|------------------------------------|-----------------------------|----------------------------------|---|---------------------------|------------------------------|---------------|
| | transmembrane domain | | CAC34417 | plasma membrane (0.460) chloroplast (0.659), plasma membrane (0.650) | secretory pathway (0.986) | 1-20 | yes |
| | | | P16002 | plasma membrane (0.790), chloroplast (0.753) | chloroplast (0.924) | | yes |
| | | | P26291 | chloroplast (0.753) | chloroplast (0.797) | | yes |
| | outside | At1g09560 | AAB51577 | outside (0.609) | secretory pathway (0.960) | 1-23 | |
| | | At3g62020 | AAB51752 | plasma membrane (0.685) | secretory pathway (0.970) | 1-21 | no |
| | | | BAA81904 | plasma membrane (0.790) | secretory pathway (0.758) | 1-39 | no |
| | | | CAA09607 | outside (0.820) | secretory pathway (0.996) | 1-25 or 1-23 | |
| | | | CAA10167 | outside (0.790) | secretory pathway (0.963) | 1-16 or 1-22 | |
| | | | CAA10287 | outside (0.585) | secretory pathway (0.867) | 1-33 | |
| | | | CAC44501 | outside (0.820) | secretory pathway (0.985) | 1-24 | |
| | | | CAD29731 | outside (0.714) | secretory pathway (0.982) | 1-23 or 1-26 | |
| | | At1g49750 | NP_175397 | vacuole (0.750), outside (0.666) | secretory pathway (0.239) | 1-15 or 1-22 | |
| | | At3g20820 | NP_188718 | vacuole (0.861), outside (0.820) | secretory pathway (0.752) | 1-19 | |
| | | At4g34480 | NP_195174 | plasma membrane (0.460) | secretory pathway (0.942) | 1-22 | |
| | | At5g14440 | NP_196949 | outside (0.786) | secretory pathway (0.941) | 1-30 | |
| | | At5g51890 | NP_200002 | plasma membrane (0.685) | secretory pathway (0.991) | 1-24 | no |
| | | At1g09750 | NP_563851 | outside (0.456) | secretory pathway (0.899) | 1-21 or 1-23 | |
| | | At1g20850 | NP_564126 | vacuole (0.808), outside (0.786) | secretory pathway (0.987) | 1-26 | |
| | | At4g33490 | NP_567922 | outside (0.820) | secretory pathway (0.913) | 1-21 | |
| | | | P36907 | outside (0.757) | secretory pathway (0.985) | 1-23 | |
| | | | Q01806 | plasma membrane (0.685) | secretory protein (0.983) | 1-30 | no |
| | | | Q9S8P4 | outside (0.820) | secretory pathway (0.967) | 1-20 | |
| | | | S68805 | outside (0.781) | secretory pathway (0.961) | 1-24 | |
| | | T07086 | outside (0.743) | secretory pathway (0.771) | 1-19 | | |
| | | T09642 | vacuole (0.761), outside (0.657) | secretory pathway (0.981) | 1-30 or 1-24 | | |

| | | | | |
|------------------------|-----------|---------------------------------------|-------------------------------|--|
| intracellular | AAC49358 | mitochondry (0.555) | chloroplast (0.628) | |
| | AAG34872 | microbody (0.540) | other (0.568) | |
| | AAL77589 | mitochondry (0.679) | chloroplast (0.765) | |
| | CAA09177 | microbody (0.640) | other (0.884) | |
| | At1g16470 | NP_173096 | cytoplasm (0.450) | other (0.703) |
| | At1g23740 | NP_173786 | mitochondry (0.594) | chloroplast (0.568), mitochondry (0.442) |
| | At1g48590 | NP_175292 | mitochondry (0.480) | mitochondry (0.529) |
| | At1g67280 | NP_176896 | mitochondry (0.807) | chloroplast (0.895) |
| | At2g15570 | NP_179159 | chloroplast (0.919) | chloroplast (0.979) |
| | At2g24940 | NP_180066 | cytoplasm (0.450) | other (0.890) |
| | At2g32520 | NP_180811 | microbody (0.570) | other (0.896) |
| | At3g20390 | NP_188674 | mitochondry (0.644) | chloroplast (0.497), mitochondry (0.371) |
| | At3g26060 | NP_189235 | mitochondry (0.679) | chloroplast (0.904) |
| | At3g43810 | NP_189967 | cytoplasm (0.650) | other (0.894) |
| | At3g55330 | NP_191093 | chloroplast (0.647) | chloroplast (0.966) |
| | At4g09010 | NP_192640 | chloroplast (0.512) | chloroplast (0.737) |
| | At5g01650 | NP_195785 | chloroplast (0.890) | other (0.719) |
| | At1g09310 | NP_563841 | cytoplasm (0.650) | other (0.906) |
| | At3g17440 | NP_566578 | endoplasmic reticulum (0.850) | other (0.736) |
| | At3g63190 | NP_567141 | mitochondry (0.838) | chloroplast (0.933) |
| | At5g20080 | NP_568391 | mitochondry (0.859) | mitochondry (0.855) |
| | O65194 | O65194 | chloroplast (0.876) | chloroplast (0.710) |
| | O65198 | O65198 | chloroplast (0.921) | chloroplast (0.941) |
| | P10933 | P10933 | endoplasmic reticulum (0.550) | chloroplast (0.907) |
| | P16048 | P16048 | mitochondry (0.870) | mitochondry (0.645) |
| | P16059 | P16059 | mitochondry (0.750) | chloroplast (0.307), |
| | P29450 | P29450 | chloroplast (0.950) | chloroplast (0.977) |
| | P49231 | P49231 | cytoplasm (0.450) | other (0.818) |
| | P51615 | P51615 | plasma membrane (0.615) | other (0.941) |
| | Q02610 | Q02610 | cytoplasm (0.450) | other (0.404) |
| | Q42971 | Q42971 | endoplasmic reticulum (0600) | other (0.608) |
| | Q43636 | Q43636 | cytoplasm (0.450) | other (0.907) |
| | T06363 | T06363 | plasma membrane (0.650), | |
| T09286 | T09286 | chloroplast (0.539) | chloroplast (0.505) | |
| | | mitochondry (0.920) | mitochondry (0.766) | |
| not predictable | AAD34458 | encoded by a truncated cDNA | | |
| | AAL06644 | encoded by a truncated cDNA | | |
| | AAL15646 | encoded by a truncated cDNA | | |
| | JC4780 | data not available in usual databases | | |
| | JC4781 | data not available in usual databases | | |
| | JC4782 | data not available in usual databases | | |
| | S22489 | encoded by a truncated cDNA | | |
| | T05957 | encoded by a truncated cDNA | | |
| | T09165 | encoded by a truncated cDNA | | |

step 2: LiCl extract

| | | | | | |
|------------------------|-----------|-----------|---|---------------------------|--------------|
| outside | At1g49750 | NP_175397 | vacuole (0.850), outside (0.666) | secretory pathway (0.239) | 1-15 or 1-22 |
| | | P36907 | outside (0.757) | secretory pathway (0.985) | 1-23 |
| | | T07086 | outside (0.743) | secretory pathway (0.771) | 1-19 |
| | | T07171 | outside (0.820) | secretory pathway (0.981) | 1-23 |
| | | T09665 | outside (0.820) | secretory pathway (0.922) | 1-28 |
| intracellular | At5g41550 | AAD56659 | mitochondry (0.483) | mitochondry (0.839) | |
| | | NP_198970 | chloroplast (0.520) | mitochondry (0.517) | |
| | | T93508 | endoplasmic reticulum (0.910) (C-term HDEL) | secretory pathway (0.991) | 1-20 |
| not predictable | | P04353 | encoded by a truncated cDNA | | |

Colour code:

proteins found at both steps 1 and 2



(a) PSORT : <http://psort.nibb.ac.jp/form.html> [29]

(b) TargetP: <http://www.cbs.dtu.dk/services/TargetP/> [30]

(c) Two sizes are indicated when different signal peptides are predicted by PSORT and TargetP. The first one is predicted with PSORT.

(d) Aramemnon: <http://aramemnon.botanik.uni-koeln.de/> [31]

Additional file 3: Bioinformatic analysis of proteins extracted from cell walls of *C. albicans* [20].

Data are from [20]. Proteins were extracted from *C. albicans* cell walls as described in Figure 3. Four successive extractions were performed : step 1 using SDS-DTT, step 2 using NaOH, step 3 using a β -1,3-glucanase and step 4 using an exochitinase. All proteins sequences were analyzed with bioinformatic programs to predict their sub-cellular localization. Proteins for which predictions by different bioinformatic programs are in conflict are classified as "not clear".

| step 1: SDS-DTT extract | Predicted subcellular localization | Accession (a) | PSORT (b) | TargetP (c) | Predicted signal peptide (d) |
|-------------------------|------------------------------------|------------------|--|------------------------------------|------------------------------|
| | outside | CA1541 Phrp * | outside (0.370) | secretory pathway (0.946) | 1-18 |
| | intracellular | CA0362 | cytoplasm (0.650) endoplasmic reticulum (0.910) (C-term HDEL) | other (0.911) | 1-29 or 1-32 |
| | | CA0915 | mitochondry (0.541) | secretory pathway (0.977) | |
| | | CA1015 | nucleus (0.760) | other (0.694), mitochondry (0.271) | |
| | | CA1230 | mitochondry (0.360) | other (0.843) | |
| | | CA1691 | endoplasmic reticulum (0.910) (C-term HDEL) | other (0.789), mitochondry (0.227) | |
| | | CA1755 | microbody (0.300) | secretory pathway (0.955) | 1-23 |
| | | CA2474 | nucleus (0.760) | other (0.798) | |
| | | CA2857 | nucleus (0.980) | other (0.842) | |
| | | CA3081 | cytoplasm (0.650) | other (0.885) | |
| | | CA3208 | mitochondry (0.360) | mitochondry (0.663) | |
| | | CA3483 | mitochondry (0.450) | other (0.275), mitochondry (0.299) | |
| | | CA3534 | cytoplasm (0.450) | other (0.887) | |
| | | CA3874 | cytoplasm (0.450) | other (0.793) | |
| | | CA4671 | mitochondry (0.853) | other (0.834) | |
| | | CA4765 | mitochondry (0.471) | mitochondry (0.716) | |
| | | CA4844 | endoplasmic reticulum (0.550) | other (0.704) | |
| | | CA4862 | mitochondry (0.360) | other (0.934) | |
| | | CA4959 | nucleus (0.600) | other (0.923) | |
| | | CA5135 | cytoplasm (0.450) | other (0.928) | |
| | | CA5180 | cytoplasm (0.450) | other (0.797) | |
| | | CA5892 | mitochondry (0.471) | mitochondry (0.637) | |
| | | CA5950 | microbody (0.800) | other (0.604) | |
| | not predictable | CA2810 | not present in the database | | |
| step 2: NaOH extract | outside | Hsp150p/Pir2p* | | | |
| | intracellular | CA1691 | mitochondry (0.360) | other (0.789), mitochondry (0.227) | |
| | | CA2474 | microbody (0.300) | other (0.798) | |
| | | CA3483 | mitochondry (0.360) | other (0.275), mitochondry (0.299) | |
| | | CA3874 | cytoplasm (0.450) | other (0.793) | |
| | | CA4765 | mitochondry (0.853) | mitochondry (0.716) | |
| | | CA5180 | cytoplasm (0.450) | other (0.797) | |
| | | CA5892 | mitochondry (0.471) | mitochondry (0.637) | |

| | | | | |
|---|----------------------|--|---|--|
| step 3: β-1,3-glucanase extract | outside | putative β -1,3-glucanase* Hsp150p/Pir2p* Phrp * | | |
| | intracellular | CA1691 CA3874 CA5892 | mitochondry (0.360) cytoplasm (0.450) mitochondry (0.471) | other (0.789), mitochondry (0.227) other (0.793) mitochondry (0.637) |
| step 4: exochitinase extract | outside | Hsp150p/Pir2p* Phrp * | | |
| | intracellular | CA1691 CA2474 CA3874 CA5180 CA5892 | mitochondry (0.360) microbody (0.300) cytoplasm (0.450) cytoplasm (0.450) mitochondry (0.471) | other (0.789), mitochondry (0.227) other (0.798) other (0.793) other (0.797) mitochondry (0.637) |

colour code: proteins found in at least 2 fractions
 proteins found in at least 2 fractions

* proteins that failed to be identified to already annotated *C. albicans* proteins

(a) <http://genolist.pasteur.fr/CandidaDB/>

(b) PSORT : <http://psort.nibb.ac.jp/form.html> [29]

(b) TargetP: <http://www.cbs.dtu.dk/services/TargetP/> [30]

(d) Two sizes are indicated when different signal peptides are predicted by PSORT and TargetP. The first one is predicted with PSORT.

Additional file 4: Bioinformatic analysis of proteins extracted from cell walls of *A. thaliana* etiolated hypocotyls with salts.

Proteins were extracted from *A. thaliana* cell walls of etiolated hypocotyls as described in Figure 5. Two successive extractions were performed : step 1 using CaCl₂, and step 2 using LiCl. All proteins sequences were analyzed with bioinformatic programs to predict their sub-cellular localization. Proteins for which predictions by different bioinformatic programs are in conflict are classified as "not clear".

| step 1: CaCl ₂ extract | Predicted subcellular localization | Gene (<i>A. thaliana</i>) | PSORT (a) | TargetP (b) | Predicted signal peptide (c) | Aramemnon (d) | |
|-----------------------------------|------------------------------------|-----------------------------|-------------------------------|---------------------------|------------------------------|------------------|----|
| transmembrane domain | | At1g23480 | plasma membrane (0.919) | secretory pathway (0.875) | 1-29 | yes | |
| | | At1g54010 | plasma membrane (0.685) | secretory pathway (0.991) | 1-29 | yes | |
| | | At1g55850 | plasma membrane (0.600) | other (0.787) | | yes | |
| | | At1g62660 | plasma membrane (0.790) | other (0.822) | | yes | |
| | | At1g79680 | outside (0.820) | secretory pathway (0.929) | 1-26 or 1-24 | yes | |
| | | At3g20580 | plasma membrane (0.600) | secretory pathway (0.860) | 1-35 | yes (GPI anchor) | |
| | | At3g45970 | endoplasmic reticulum (0.820) | secretory pathway (0.854) | 1-20 | yes | |
| | | At4g15320 | plasma membrane (0.600) | other (0.879) | | yes | |
| | outside | | At1g02335 | plasma membrane (0.685) | secretory pathway (0.933) | 1-22 | no |
| | | | At1g03220 | outside (0.820) | secretory pathway (0.912) | 1-22 | |
| | | | At1g03230 | outside (0.820) | secretory pathway (0.910) | 1-23 | |
| | | | At1g09560 | outside (0.609) | secretory pathway (0.960) | 1-23 | |
| | | | At1g09750 | outside (0.456) | secretory pathway (0.899) | 1-21 or 1-23 | |
| | | | At1g11580 | outside (0.820) | secretory pathway (0.423) | 1-34 | |
| | | | mitochondry (0.850), plasma | | | | |
| | | At1g18970 | membrane (0.650) | secretory pathway (0.932) | 1-27 | no | |
| | | At1g20190 | outside (0.820) | secretory pathway (0.961) | 1-20 | | |
| | | At1g28290 | outside (0.681) | secretory pathway (0.483) | 1-24 | | |
| | | At1g29670 | outside (0.820) | secretory pathway (0.987) | 1-24 | | |
| | | At1g33590 | outside (0.738) | secretory pathway (0.963) | 1-24 | no | |
| | | At1g65590 | outside (0.819) | secretory pathway (0.948) | 1-24 | | |
| | | At1g68560 | outside (0.820) | secretory pathway (0.982) | 1-27 | | |
| | | At1g73260 | outside (0.542) | secretory pathway (0.985) | 1-26 | | |
| | | At1g76160 | outside (0.820) | secretory pathway (0.984) | 1-23 | | |
| | | At1g78830 | outside (0.820) | secretory pathway (0.962) | 1-22 | | |
| | | At1g78850 | outside (0.820) | secretory pathway (0.973) | 1-22 | | |
| | | At1g78860 | outside (0.652) | secretory pathway (0.981) | 1-22 | | |
| | | At2g05580 | outside (0.820) | secretory pathway (0.993) | 1-20 | no | |
| | | At2g18140 | outside (0.820) | secretory pathway (0.695) | 1-20 or 1-16 | | |
| | | At2g28790 | outside (0.705) | secretory pathway (0.990) | 1-24 or 1-21 | | |
| | | At2g30210 | outside (0.820) | secretory pathway (0.983) | 1-25 | | |
| | | At2g38530 | plasma membrane (0.685) | secretory pathway (0.967) | 1-23 | no | |
| | | At3g08030 | outside (0.820) | secretory pathway (0.985) | 1-21 | | |
| | | At3g13790 | outside (0.494) | secretory pathway (0.969) | 1-20 or 1-28 | | |
| | | At3g14220 | outside (0.609) | secretory pathway (0.926) | 1-20 or 1-28 | | |
| | | At3g14310 | plasma membrane (0.460) | secretory pathway (0.227) | 1-40 or 1-37 | no | |
| | | At3g16850 | outside (0.820) | secretory pathway (0.965) | 1-19 or 1-20 | | |
| | | At3g18080 | outside (0.370) | secretory pathway (0.374) | 1-23 | | |

| | | | | | |
|----------------------|------------|---|--|--------------|-----|
| | At3g20370 | outside (0.633) | secretory pathway (0.965) | 1-25 | |
| | At3g20820 | outside (0.820) | secretory pathway (0.752) | 1-19 | |
| | At3g21770 | outside (0.820) | secretory pathway (0.967) | 1-27 | |
| | At3g22640 | outside (0.820) | secretory pathway (0.996) | 1-22 | |
| | At3g24480 | outside (0.795) | secretory pathway (0.978) | 1-25 | |
| | At3g25700 | endoplasmic reticulum (0.820) | secretory pathway (0.425) | 1-23 | |
| | At3g43270 | outside (0.820) | secretory pathway (0.979) | 1-24 | |
| | At3g49120 | outside (0.609) | secretory pathway (0.604) | 1-28 or 1-29 | |
| | At3g54400 | outside (0.595) | secretory pathway (0.409) | 1-19 | |
| | At3g55260 | outside (0.738) | secretory pathway (0.805) | 1-22 or 1-20 | |
| | At4g08950 | outside (0.820) | secretory pathway (0.871) | 1-21 | |
| | At4g12880 | outside (0.820) | secretory pathway (0.952) | 1-18 or 1-26 | |
| | At4g13340 | outside (0.820) | secretory pathway (0.945) | 1-20 | |
| | At4g16500 | outside (0.820) | secretory pathway (0.929) | 1-22 | |
| | At4g18970 | plasma membrane (0.685) | secretory pathway (0.980) | 1-22 | no |
| | At4g29270 | outside (0.820) | secretory pathway (0.983) | 1-26 | |
| | At4g30170 | outside (0.695) | secretory pathway (0.980) | 1-25 | |
| | At4g33220 | outside (0.709) | secretory pathway (0.919) | 1-19 | |
| | At5g02260 | plasma membrane (0.685) | secretory pathway (0.969) | 1-20 | no |
| | At5g06230 | plasma membrane (0.790) | secretory pathway (0.744) | 1-38 | |
| | At5g06860 | outside (0.733) | secretory pathway (0.964) | 1-21 | |
| | At5g07030 | outside (0.820) | secretory pathway (0.559) | 1-19 | |
| | At5g09440 | outside (0.820) | secretory pathway (0.812) | 1-19 or 1-23 | |
| | At5g10770 | plasma membrane (0.760) | secretory pathway (0.276) | 1-25 | |
| | At5g11420 | outside (0.820) | secretory pathway (0.989) | 1-22 | |
| | At5g12940 | outside (0.820) | secretory pathway (0.985) | 1-29 or 1-26 | |
| | At5g23210 | outside (0.820) | secretory pathway (0.992) | 1-25 | |
| | At5g25460 | outside (0.820) | secretory pathway (0.979) | 1-19 | |
| | At5g26260 | outside (0.547) | secretory pathway (0.926) | 1-22 or 1-23 | |
| | At5g26280 | outside (0.528) | secretory pathway (0.963) | 1-22 or 1-23 | |
| | At5g34850 | outside (0.748) | secretory pathway (0.870) | 1-16 or 1-22 | |
| | At5g34940 | outside (0.820) | secretory pathway (0.996) | 1-21 | |
| | At5g43060 | outside (0.685) | secretory pathway (0.991) | 1-21 | |
| | At5g44380 | plasma membrane (0.811) | secretory pathway (0.439) | 1-32 | no |
| | At5g63810 | outside (0.819) | secretory pathway (0.887) | 1-29 | |
| | At5g64260 | outside (0.685) | secretory pathway (0.966) | 1-19 or 1-23 | |
| intracellular | At1g67090* | chloroplast (0.923) | chloroplast (0.769) | | |
| | At3g09260 | endoplasmic reticulum (0.910) (C-term KDEL) | secretory pathway (0.952) | 1-24 | |
| | At3g16880 | cytoplasm (0.450) | other (0.625) | | |
| | At4g15440 | microbody (0.540) | other (0.891) | | |
| | At4g22165 | microbody (0.640) | other (0.493) | | |
| | At4g23670 | microbody (0.560) | other (0.847) | | |
| | At4g28520° | outside (0.820) | secretory pathway (0.962) | 1-23 | |
| | At5g38410* | outside (0.370) | chloroplast (0.741) | | |
| | At5g38420* | endoplasmic reticulum (0.550) | chloroplast (0.806) | | |
| | At5g38430* | outside (0.370) | chloroplast (0.807) | | |
| | At5g44120° | outside (0.700) | secretory pathway (0.975) | 1-24 or 1-23 | |
| not clear | At2g21540 | plasma membrane (0.700) | other (0.945) | | no |
| | At3g26380 | outside (0.690) | other (0.355), secretory pathway (0.168) | 1-32 | yes |
| | At3g32980 | mitochondry (0.660) | secretory pathway (0.356) | 1-29 | |

step 2: LiCl extract

| | | | | | |
|-----------------------------|-----------------|--|---------------------------|--------------|-----|
| transmembrane domain | At1g54010 | plasma membrane (0.685) | secretory pathway (0.991) | 1-29 | yes |
| | At1g73620 | Golgi (0.900), plasma membrane (0.790) | other (0.532) | | yes |
| | At4g16590 | plasma membrane (0.600) | other (0.879) | | yes |
| | At5g20630 | plasma membrane (0.460) | secretory pathway (0.922) | 1-20 | yes |
| outside | At1g03220 | outside (0.820) | secretory pathway (0.912) | 1-22 | |
| | At1g03230 | outside (0.820) | secretory pathway (0.910) | 1-23 | |
| | At1g09750 | outside (0.456) | secretory pathway (0.899) | 1-21 or 1-23 | |
| | At1g17860 | outside (0.690) | secretory pathway (0.910) | 1-19 | |
| | At1g29670 | outside (0.820) | secretory pathway (0.987) | 1-24 | |
| | At1g47128 | outside (0.771) | secretory pathway (0.993) | 1-21 | |
| | At1g68560 | outside (0.820) | secretory pathway (0.982) | 1-27 | |
| | At1g73260 | outside (0.542) | secretory pathway (0.985) | 1-26 | |
| | At1g78850 | outside (0.820) | secretory pathway (0.973) | 1-22 | |
| | At2g05580 | outside (0.820) | secretory pathway (0.993) | 1-20 | |
| | At2g22170 | outside (0.820) | secretory pathway (0.990) | 1-21 | |
| | At2g28790 | outside (0.705) | secretory pathway (0.994) | 1-24 or 1-21 | |
| | At2g34700 | outside (0.432) | secretory pathway (0.790) | 1-23 or 1-28 | |
| | At3g14310 | plasma membrane (0.460) | secretory pathway (0.227) | 1-40 or 1-37 | no |
| | At3g16850 | outside (0.820) | secretory pathway (0.965) | 1-19 or 1-20 | |
| | At3g21770 | outside (0.820) | secretory pathway (0.967) | 1-27 | |
| | At3g49120 | outside (0.609) | secretory pathway (0.604) | 1-28 or 1-29 | |
| | At3g54400 | outside (0.595) | secretory pathway (0.409) | 1-19 | |
| | At4g30170 | outside (0.695) | secretory pathway (0.980) | 1-25 | |
| | At4g34980 | outside (0.820) | secretory pathway (0.980) | 1-20 | |
| | At5g06860 | outside (0.733) | secretory pathway (0.964) | 1-21 | |
| | At5g07030 | outside (0.820) | secretory pathway (0.559) | 1-19 | |
| | At5g09440 | outside (0.820) | secretory pathway (0.812) | 1-19 or 1-23 | |
| | At5g11420 | outside (0.820) | secretory pathway (0.989) | 1-22 | |
| | At5g25460 | outside (0.820) | secretory pathway (0.979) | 1-19 | |
| | At5g26280 | outside (0.528) | secretory pathway (0.963) | 1-22 or 1-23 | |
| | At5g51260 | outside (0.820) | secretory pathway (0.938) | 1-19 | |
| At5g59090 | outside (0.820) | secretory pathway (0.978) | 1-24 | | |
| At5g66390 | outside (0.820) | secretory pathway (0.973) | 1-23 | | |
| intracellular | At5g20830 | microbody (0.475) | other (0.669) | 1-20 | |
| not clear | At3g42160 | plasma membrane (0.650) | other (0.925) | | no |

colour code:

proteins found at both steps 1 and 2
 proteins found at both steps 1 and 2



* small subunit of RUBISCO

° homolog to storage proteins, most probably vacuolar localization

(a) PSORT : <http://psort.nibb.ac.jp/form.html> [29]

(b) TargetP: <http://www.cbs.dtu.dk/services/TargetP/> [30]

(c) Two sizes are indicated when different signal peptides are predicted by PSORT and TargetP. The first one is predicted with PSORT.

(d) Aramemnon: <http://aramemnon.botanik.uni-koeln.de/> [31]

Additional file 5: Bioinformatic analysis of proteins extracted from cell walls of *A. thaliana* etiolated hypocotyls with SDS and DTT.

Proteins were extracted from *A. thaliana* cell walls of etiolated hypocotyls as described in Figure 6. One extraction was performed using SDS and DTT. All proteins sequences were analyzed with bioinformatic programs to predict their sub-cellular localization. Proteins for which predictions by different bioinformatic programs are in conflict are classified as “not clear”.

| step 3: SDS-DTT | Predicted subcellular localization | Gene (<i>A. thaliana</i>) | PSORT (a) | TargetP (b) | Predicted signal peptide (c) | Aramemnon (d) | |
|-----------------------------|------------------------------------|-----------------------------|---|------------------------------------|------------------------------|---------------|--|
| transmembrane domain | | At2g25300 | chloroplast thylakoid membrane (0.765), plasma membrane (0.650) | chloroplast (0.573) | | yes | |
| | | At4g33230 | chloroplast thylakoid membrane (0.615), plasma membrane (0.600) | other (0.932) | | yes | |
| | | At4g25080 | endoplasmic reticulum (0.600), mitochondry (0.453), chloroplast (0.376) | chloroplast (0.866) | | yes | |
| | | At2g24820 | mitochondry (0.791), microbody (0.635), chloroplast (0.500) | chloroplast (0.897) | | yes | |
| | | At4g15820 | mitochondry (0.861), chloroplast (0.660), plasma membrane (0.650) | mitochondry (0.537) | | yes | |
| | | At1g72990 | outside (0.820) | mitochondry (0.957) | 1-29 | yes | |
| | | At1g27200 | plasma membrane (0.790) | other (0.521) | | yes | |
| | | At3g05610 | plasma membrane (0.790) | mitochondry (0.537) | | yes | |
| | | At3g61270 | plasma membrane (0.790) | other (0.843) | | yes | |
| | | At4g15320 | plasma membrane (0.600) | other (0.879) | | yes | |
| | | At4g16590 | plasma membrane (0.600) | other (0.879) | | yes | |
| | | At5g41390 | plasma membrane (0.600) | mitochondry (0.750), other (0.544) | | yes | |
| | outside | | At1g66270 | outside (0.820) | secretory pathway (0.976) | 1-24 | |
| | | | At1g71695 | outside (0.820) | secretory pathway (0.829) | 1-22 or 1-31 | |
| | | | At2g46570 | outside (0.370) | secretory pathway (0.868) | 1-20 or 1-29 | |
| | | At3g14040 | outside (0.820) | secretory pathway (0.984) | 1-23 or 1-25 | | |
| | | At4g11310 | outside (0.820) | secretory pathway (0.995) | 1-18 or 1-23 | | |
| | | At4g12880 | outside (0.820) | secretory pathway (0.952) | 1-18 or 1-26 | | |
| | | At4g28790 | outside (0.820) | secretory pathway (0.726) | 1-17 or 1-24 | | |
| | | At4g33810 | outside (0.820) | secretory pathway (0.910) | 1-24 | | |
| | | At5g07030 | outside (0.820) | secretory pathway (0.559) | 1-19 | | |
| | | At5g25460 | outside (0.820) | secretory pathway (0.979) | 1-19 | | |
| | | At5g44020 | mitochondry (0.850), outside (0.820) | secretory pathway (0.845) | 1-16 or 1-21 | | |

| | | | | |
|----------------------|------------|---|------------------------------------|--------------|
| intracellular | ArthCp029 | chloroplast | | |
| | ArthCp030 | chloroplast | | |
| | At1g55490 | chloroplast (0.864) | chloroplast (0.979) | |
| | At1g67090* | chloroplast (0.923) | chloroplast (0.769) | |
| | At2g21170 | chloroplast (0.888) | chloroplast (0.950) | |
| | At3g04350 | chloroplast (0.842) | other (0.635), chloroplast (0.451) | |
| | At3g13470 | chloroplast (0.950) | chloroplast (0.924) | |
| | At3g53460 | chloroplast (0.903) | chloroplast (0.903) | |
| | At4g15040 | chloroplast (0.496) | other (0.625), chloroplast (0.289) | |
| | At4g20360 | chloroplast (0.950) | chloroplast (0.975) | |
| | At5g20720 | chloroplast (0.895) | chloroplast (0.901) | |
| | At5g54190 | chloroplast (0.924) | chloroplast (0.957) | |
| | | endoplasmic reticulum (0.910) (C-term KDEL) | secretory pathway (0.952) | 1-24 |
| | At3g09260 | | secretory pathway (0.962) | 1-23 |
| | At4g28520° | outside (0.820) | secretory pathway (0.975) | 1-24 or 1-23 |
| | At5g44120° | outside (0.700) | | |
| | At1g49240 | cytoplasm (0.450) | other (0.891) | |
| | At3g18780 | cytoplasm (0.450) | other (0.883) | |
| | At4g11850 | cytoplasm (0.450) | other (0.806) | |
| | At5g17920 | cytoplasm (0.450) | other (0.713) | |
| | At4g02520 | microbody (0.520) | other (0.556) | |
| | At2g13890 | endoplasmic reticulum (0.850) | other (0.609) | |
| | At1g13440 | microbody (0.539) | other (0.610) | |
| | At1g60120 | microbody (0.704) | other (0.753) | |
| | At1g78380 | microbody (0.640) | other (0.523) | |
| | At3g02230 | microbody (0.603) | other (0.765) | |
| | At3g04120 | microbody (0.472) | other (0.619) | |
| | At3g60120 | microbody (0.640) | other (0.712) | |
| | At5g58690 | microbody (0.499) | other (0.598) | |
| not clear | At3g49400 | plasma membrane (0.650) | other (0.338) | no |
| | At5g37470 | plasma membrane (0.600) | other (0.973) | no |

colour code:

proteins also extracted by CaCl₂ or LiCl

proteins also extracted by CaCl₂ or LiCl

proteins also extracted by CaCl₂ or LiCl



* small subunit of RUBISCO

° homolog to storage proteins, most probably vacuolar localization

(a) PSORT : <http://psort.nibb.ac.jp/form.html> [29]

(b) TargetP: <http://www.cbs.dtu.dk/services/TargetP/> [30]

(c) Two sizes are indicated when different signal peptides are predicted by PSORT and TargetP. The first one is predicted with PSORT.

(d) Aramemnon: <http://aramemnon.botanik.uni-koeln.de/> [31]

SUPPLEMENTARY DATA CHAPTER 4

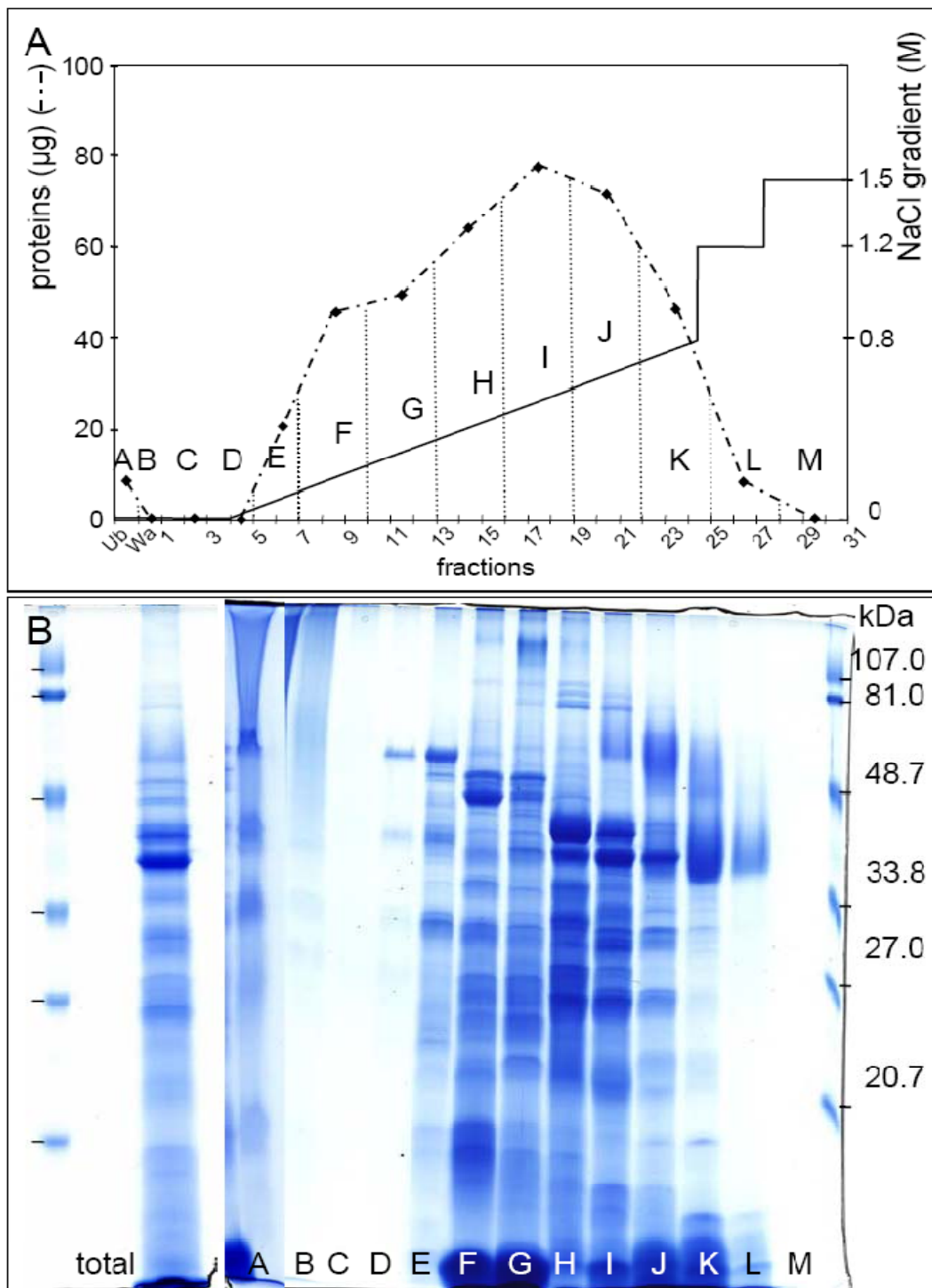


Figure S1, supplementary material. Separation of proteins extracted with CaCl₂ and LiCl from purified cell walls of 5-day-old etiolated hypocotyls by cation exchange chromatography followed by 1D-E.

a. Graph showing amounts of proteins (in µg) in each fraction eluted by a NaCl gradient (from 0 to 0.8 M) followed by two steps at 1.2 and 1.5 M NaCl. Dotted vertical lines show the grouping of chromatography fractions whereas the letters (from A to M) correspond to those analyzed by 1D-E.

b. 1D-E of total CWP (total) and chromatography fractions (Ub stands for unbound proteins, Wa for proteins eluted with loading buffer). Molecular mass markers are in kDa.

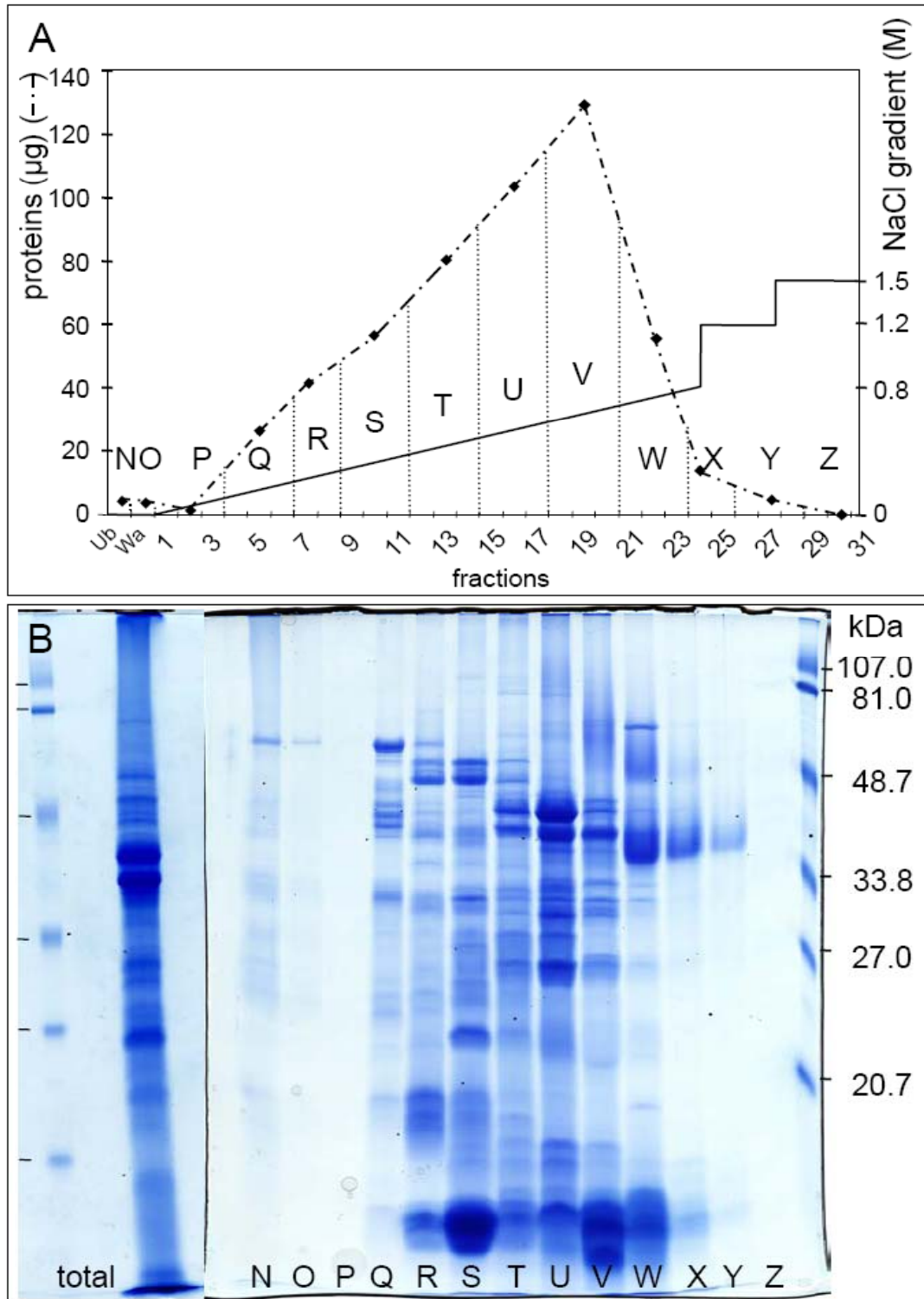


Figure S2, supplementary material. Separation of proteins extracted with CaCl_2 and LiCl from purified cell walls of 11-day-old etiolated hypocotyls by cation exchange chromatography followed by 1D-E.

a. Graph showing amounts of proteins (in μg) in each fraction eluted by a NaCl gradient (from 0 to 0.8 M) followed by two steps at 1.2 and 1.5 M NaCl. Dotted vertical lines show the grouping up of chromatography fractions whereas the letters (from N to Z) correspond to those analyzed by 1D-E.

b. 1D-E of total CWP (total) and chromatography fractions (Ub stands for unbound proteins, Wa for proteins eluted with loading buffer). Molecular mass markers are in kDa.

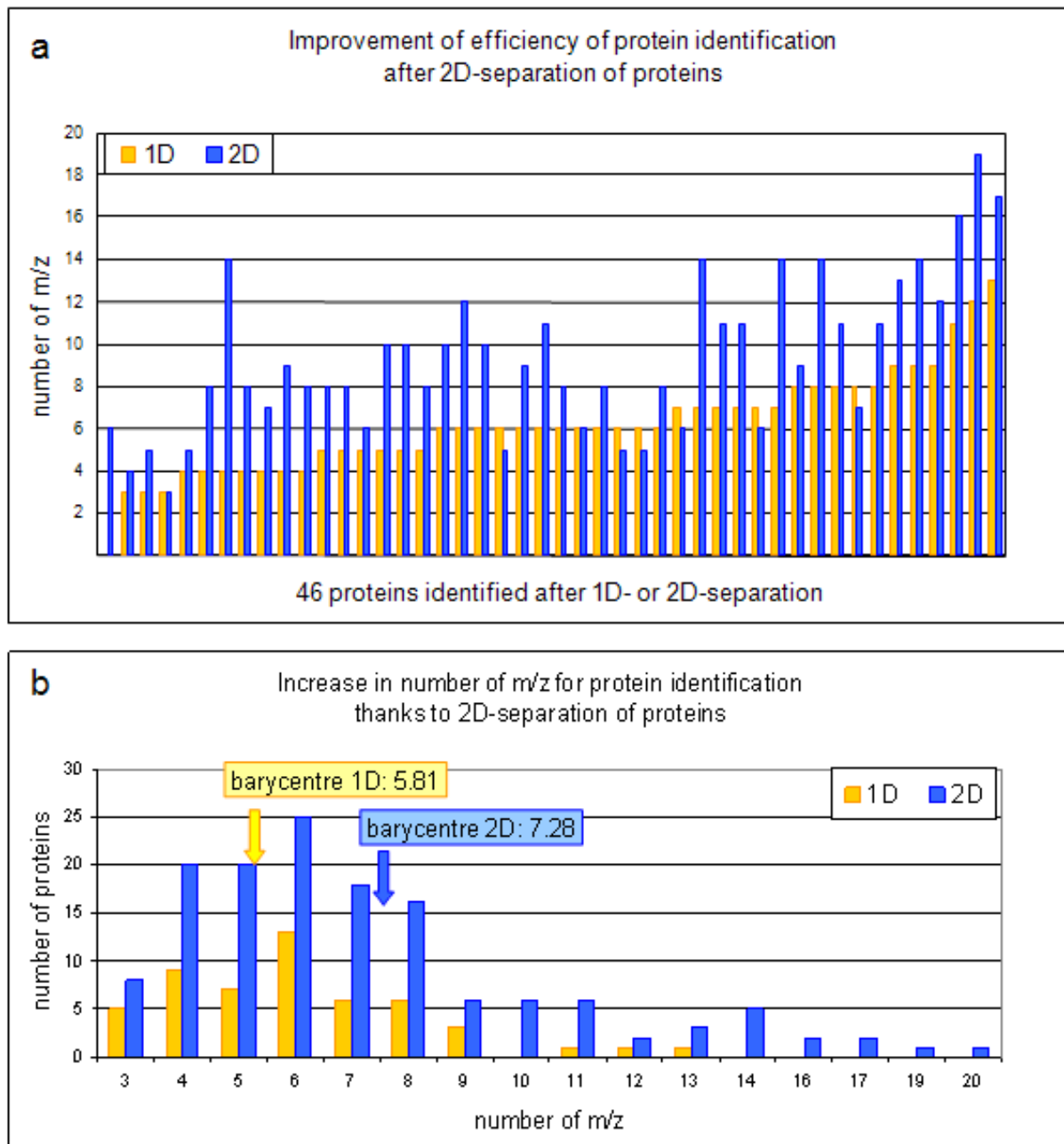


Figure S3, supplementary material. Identification of proteins extracted by CaCl₂ and LiCl from purified cell walls of 5-day-old *Arabidopsis* etiolated hypocotyls by MALDI-TOF MS. **a.** Improvement of efficiency of protein identification after 2D-separation of proteins. Numbers of m/z found for identification of proteins after 1D- (yellow bars) and 2D- (blue bars) separation are shown. Note that only 6 out of 46 proteins are identified with less m/z after 1D- than after 2D-separation. **b.** Increase in number of m/z for protein identification thanks to 2D-separation of proteins. Numbers of proteins identified with 3 to 20 m/z are shown after 1D- (yellow bars) and 2D- (blue bars) separation. Note that the barycentre of this number of m/z used for successful identification is higher after 2D- than after 1D-separation.

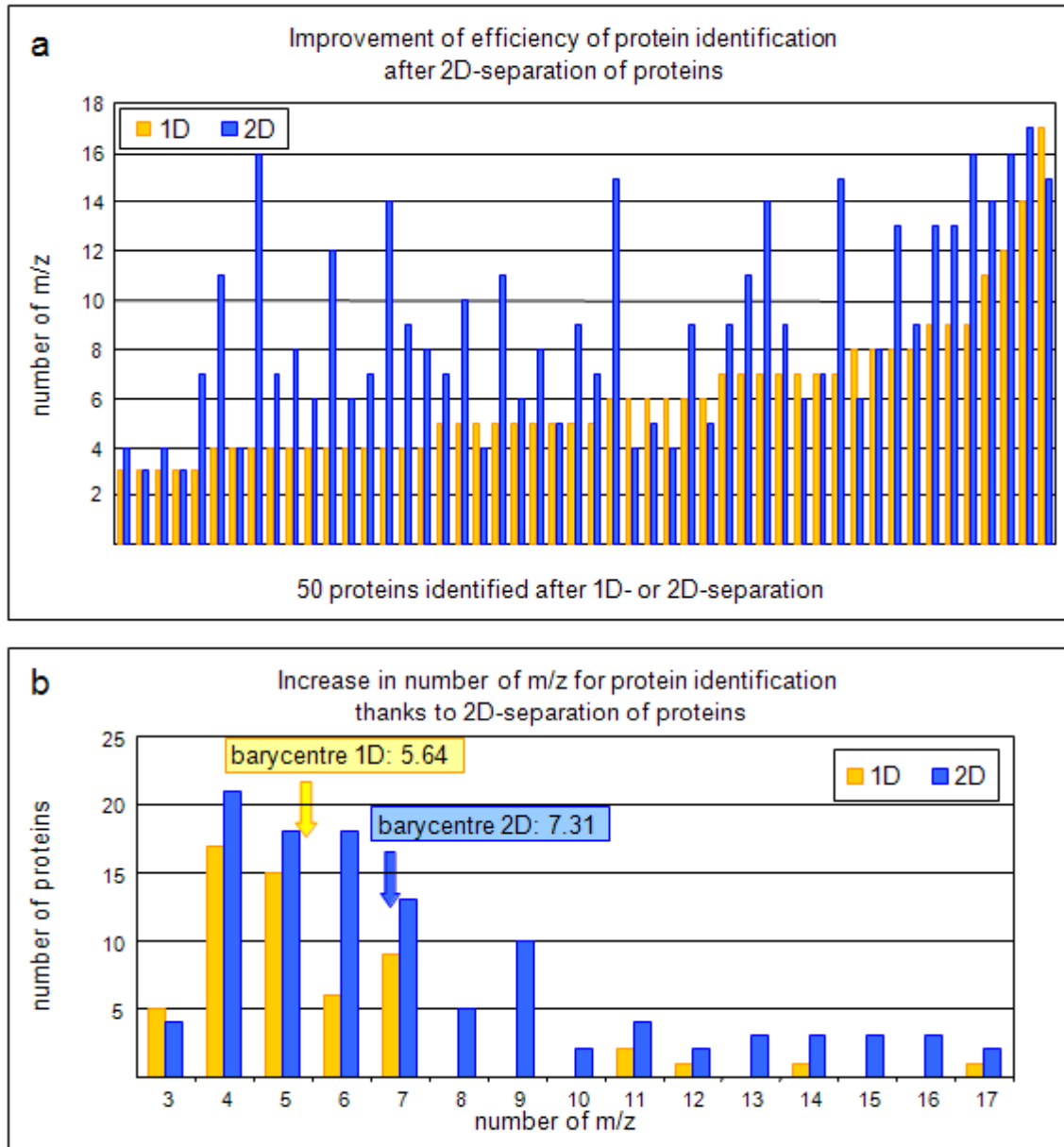







Figure S4, supplementary material. Identification of proteins extracted by CaCl₂ and LiCl from purified cell walls of 11-day-old *Arabidopsis* etiolated hypocotyls by MALDI-TOF MS.

a. Improvement of efficiency of protein identification after 2D-separation of proteins. Numbers of m/z found for identification of proteins after 1D- (yellow bars) and 2D-(blue bars) separation are shown. Note that only 7 out of 50 proteins are identified with less m/z after 1D- than after 2D-separation. **b.** Increase in number of m/z for protein identification thanks to 2D-separation of proteins. Numbers of proteins identified with 3 to 20 m/z are shown after 1D-(yellow bars) and 2D-(blue bars) separation. Note that the barycentre of this number of m/z used for successful identification is higher after 2D- than after 1D-separation.

Table S1, supplementary data

Table S1, supplementary material. Identification of proteins extracted from purified cell walls of 5-day-old *Arabidopsis* hypocotyls by CaCl₂ and LiCl solutions.

* or # proteins that could not be distinguished

Colour code
 secretory proteins (Presence of predicted signal peptide was checked using PSORT: <http://psort.ims.u-tokyo.ac.jp/form.html>, and TargetP: <http://www.cbs.dtu.dk/services/TargetP/>)
 transmembrane domains (Presence of predicted transmembrane domains was checked using Aramemnon: <http://aramemnon.botanik.uni-koeln.de/>)
 GPI anchors (presence or GPI anchors was checked using Aramemnon: <http://aramemnon.botanik.uni-koeln.de/>)
 intracellular proteins
 proteins identified after 1D-separation (1-DE), but not identified after 2D-separation (cationic exchange chromatography followed by 1-DE)





| total protein extract | band number | accession AGI | annotation | MALDI-TOF identification % sequence coverage | number of peptides (m/z) ^a | mature protein MM (kDa) | pI | signal peptide | transmembrane domains GPI anchors | functional domains ^b |
|---|-------------------|-------------------|--|--|---------------------------------------|-------------------------|-------|----------------|-----------------------------------|--|
|  | 1 | At5g17820 | peroxidase (AtPrx57) | 23,0 | 4 | 31636,96 | 11,42 | 1-22 | | peroxidase active site (PS00436) |
| | 2, 3 | At1g68560 | glycoside hydrolase family 31 (alpha-xylosidase) (AtXYL1) | 16,4 | 12 | 99641,37 | 6,26 | 1-27 | | glycoside hydrolases family 31 active site (PS00129) |
| | 4, 6 | At1g28290 | proline-rich protein | 9,7 | 4 | 35913,36 | 10,77 | 1-24 | | histidine-rich region profile (PS50316), proline-rich region profile (PS50099), pollen proteins Ole e 1 family (PF01190) |
| | 5-7 | At3g16850 | glycoside hydrolase family 28 (polygalacturonase) | 27,9 | 8 | 46982,42 | 5,25 | 1-19 | | glycoside hydrolase family 28 (PF00295) |
|  | 7 | At2g27190 | homologous to purple acid phosphatase (PAP1) | 9,8 | 4 | 50962,71 | 5,75 | 1-28 | | metallo-phosphoesterase motif (PS50185), calcineurin-like phosphoesterase (PF00149) |
| | 8, 9, 36-38 | At1g78850* | homologous to lectin (curculin-like) | 14,5 | 6 | 46664,12 | 8,90 | 1-22 | | PAN domain (PF00024), lectin (probable mannose binding) (PF01453) |
| | 8, 9 | At1g78860* | homologous to lectin (curculin-like) | 14,4 | 6 | 46804,29 | 6,00 | 1-22 | | PAN domain (PF00024), lectin (probable mannose binding) (PF01453) |
| | 9-11 | At1g03220 | homologous to carrot EDGP and tomato XEGIP | 17,3 | 6 | 43394,37 | 9,99 | 1-22 | | |
| | 9 | At5g34940 | glycoside hydrolase family 79 (endo beta-glucuronidase/heparanase) | 10,6 | 4 | 57219,88 | 9,13 | 1-21 | | glycoside hydrolase family 79, N-terminal domain (PF03662) |
| | 10, 11 | At1g09750 | homologous to aspartic protease (pepsin family) (Peptidase family A1, subfamily A1B unassigned peptidases, MEROPS) | 16,9 | 8 | 45038,78 | 9,09 | 1-23 | | lipase Ser active site (PS00120), Ser-rich region (PS050324), eukaryotic aspartic protease (PF00026) |
| | 10-12 | At1g03230 | homologous to carrot EDGP and tomato XEGIP | 15,9 | 6 | 43723,02 | 10,27 | 1-23 | | |
| | 12 | At3g54400 | homologous to aspartic protease (pepsin family) (Peptidase family A1, subfamily A1B unassigned peptidases, MEROPS) | 9,9 | 4 | 43313,31 | 10,26 | 1-19 | | eukaryotic aspartyl protease (PF00026) |
| | 13 | At3g43270 | carbohydrate esterase family 8 (pectin methyl-esterase) | 17,5 | 9 | 54929,20 | 9,55 | 1-24 | | pectinesterase (PF01095), plant invertase/pectin methyl-esterase inhibitor (PMEI) (PF04043) |
| | 13-15, 18, 32-34 | At5g11420 | expressed protein (DUF642) | 28,4 | 11 | 37344,57 | 7,70 | 1-22 | | domain of unknown function DUF 642 (PF04862) |
|  | 13 | At5g07030 | homologous to aspartic protease (pepsin family) (Peptidase family A1, subfamily A1B unassigned peptidases, MEROPS) | 11,8 | 5 | 44860,54 | 10,82 | 1-19 | | eukaryotic aspartyl protease (PF00026) |
| | 13, 15 | At1g19170 | glycoside hydrolase family 28 (polygalacturonase) | 11,8 | 6 | 49948,02 | 9,65 | 1-47 | | glycosyl hydrolase family 28 (PF00295) |
| | 13 | At4g33220 | carbohydrate esterase family 8 (pectin methyl-esterase) | 15,6 | 7 | 56832,32 | 6,24 | 1-19 | | pectinesterase (PF01095) |
| | 15 | At3g49120 | peroxidase (AtPrx34) | 17,8 | 6 | 35695,45 | 9,41 | 1-30 | | peroxidase active site signature (PS00436), peroxidases proximal heme-ligand signature (PS00435) |
| | 15, 23, 40, 43-46 | At4g32460 | expressed protein (DUF642) | 22,7 | 8 | 37375,51 | 9,25 | 1-21 | | domain of unknown function DUF 642 (PF04862) |
| | 16, 17 | At3g20820 | expressed protein (LRR domains) | 26,0 | 8 | 37850,70 | 9,90 | 1-19 | | typical LRR profile (PS50506), plant specific LRR profile (PS50502), SDS22-like LRR profile (PS50504) |
| | 16-21, 36-38, 40 | At5g25460 | expressed protein (DUF642) | 35,0 | 13 | 37948,52 | 9,03 | 1-19 | | domain of unknown function DUF 642 (PF04862) |
| | 17, 18, 25, 30 | At3g08030 | expressed protein (DUF642) | 23,6 | 8 | 36993,02 | 6,87 | 1-21 | | domain of unknown function DUF 642 (PF04862) |
| | 18, 36, 38, 39 | At1g70850 | homologous to Bet v I allergen family | 20,9 | 6 | 35569,93 | 4,96 | | | pathogenesis-related protein Bet v I family (PF00407) |
| | 18-20 | At5g12940 | homologous to <i>Phaseolus vulgaris</i> PGIP2 (LRR domains) | 21,6 | 7 | 36784,96 | 10,45 | 1-29 | | plant specific LRR profile (PS50502), Leucine Rich Repeat (PF00560) |
|  | 18 | At1g78830 | homologous to lectin (curculin-like) | 11,2 | 4 | 48101,66 | 9,45 | 1-22 | | PAN domain (PF00024), lectin (probable mannose binding) (PF01453) |
| | 20, 21 | At1g29670 | homologous to lipase/acylhydrolase (GDSL family) | 23,1 | 6 | 37153,43 | 9,65 | 1-24 | | GDLS-like Lipase/Acyhydrolase (PF00657) |
| | 20 | At5g64100 | peroxidase (AtPrx69) | 11,2 | 5 | 33228,93 | 10,72 | 1-23 | | peroxidases active site signature (PS00436) |
| | 22 | At1g53240* | homologous to mitochondrial NAD-dependent malate dehydrogenase | 16,7 | 5 | 35804,43 | 9,49 | | | malate dehydrogenase active site signature (PS00068), lactate/malate dehydrogenase, NAD binding domain (PF00056), lactate/malate dehydrogenase, alpha/beta C-terminal domain (PF02866) |
| | | At3g15020* | homologous to mitochondrial NAD-dependent malate dehydrogenase | 16,7 | 5 | 35875,41 | 9,34 | | | malate dehydrogenase active site signature (PS00068), lactate/malate dehydrogenase, NAD binding domain (PF00056), lactate/malate dehydrogenase, alpha/beta C-terminal domain (PF02866) |

Table S1, supplementary data

| | | | | | | | | | | |
|----------|----------------|-------------------|---|------|----|----------|-------|------|-----------------------|--|
| | 23 | At3g14310 | carbohydrate esterase family 8 (pectin methyltransferase) (AIPME3) | 18,9 | 9 | 60050.61 | 9,59 | 1-40 | transmembrane domains | pectinesterase signature 1 (PS00800), pectinesterase signature 2 (PS00503), pectinesterase (PF01095, plant invertase/pectin methyltransferase inhibitor (PF04043) |
| | 24, 25 | At4g30170 | peroxidase (AtPrx45) | 31,7 | 9 | 33052.57 | 10,43 | 1-25 | | haem peroxidase, plant/fungal/bacterial (PS00435, PS00436, PS50873) |
| | 24 | At2g06850 | glycoside hydrolase family 16 (endoxyloglucan transferase) (AtXTH4) | 20,3 | 7 | 31716.77 | 9,65 | 1-24 | | glycoside hydrolases family 16 (PF00722) |
| | 25 | At3g50990 | peroxidase (AtPrx36) | 10,4 | 3 | 35273.36 | 4,57 | 1-20 | | peroxidases proximal heme-ligand signature (PS00435), peroxidases active site signature (PS00436), plant heme peroxidase family profile (PS50873), peroxidase (PF00141) |
| | 25-27 | At3g45970 | expansin-like A (AtEXLA1) | 18,9 | 4 | 26258.12 | 9,46 | 1-20 | | pollen allergen (PF01357), barwin (PF00967) |
| | 26-28 | At5g43060 | homologous to cysteine proteinase (papain family) (RD21 peptidase, Peptidase family C1, C01.064 MEROPS) | 13,4 | 6 | 36995.63 | 5,79 | 1-22 | | eukaryotic thiol (cysteine) proteases asparagine active site (PS00640), papain family cysteine protease (PF00112), granulin (PF00396) |
| | 26-28 | At5g09440 | homologous to <i>Nicotiana tabacum</i> phi-I | 20,5 | 7 | 27366.44 | 10,15 | 1-23 | | phosphate-induced protein 1 conserved region (PF04674) |
| | 26 | At5g64260 | homologous to <i>Nicotiana tabacum</i> phi-I | 14,1 | 8 | 30550.86 | 9,98 | 1-19 | | phosphate-induced protein 1 conserved region (PF04674) |
| | 27 | At4g38400 | expansin-like A (AtEXLA2) | 16,6 | 5 | 26516.93 | 9,60 | 1-20 | | expansin, cellulose-binding-like domain profile (PS50843), expansin, family-45 endoglucanase-like domain profile (PS50842), rare lipoprotein A (RlpA)-like double-psi beta-barrel (PF03330), pollen allergen (PF01357) |
| | 29 | At3g22640 | expressed protein (cupin domain) | 12,3 | 7 | 52687.99 | 6,45 | 1-22 | | cupin domain profile (PS50849), cupin (PF00190), cupin domain |
| | 29-31 | At2g28790 | homologous to <i>Lycopersicon esculentum</i> osmotin | 12,9 | 3 | 24551.60 | 9,53 | 1-24 | | thaumatin family (PF00314) |
| | 31-34 | At2g28490 | expressed protein (cupin domain) | 12,3 | 6 | 53594.59 | 5,45 | 1-19 | | cupin domain profile (PS50849), cupin (PF00190) |
| | 31 | At4g29270 | homologous to acid phosphatase | 18,0 | 4 | 25900.84 | 9,15 | 1-26 | | HAD superfamily, subfamily IIIB (acid phosphatase) (PF03767) |
| | 33 | At2g40880 | inhibitor family I25 (phytostatin) (cystatin family, I25.014, MEROPS) | 31,2 | 3 | 11955.61 | 9,71 | 1-22 | | cysteine proteases inhibitors signature (PS00287), cystatin domain (PF00031) |
| | 33-35 | At1g20190 | alpha-expansin (AtEXPA11) | 19,8 | 7 | 24817.74 | 9,84 | 1-20 | | expansin, family-45 endoglucanase-like domain profile (PS50842), expansin, cellulose-binding-like domain profile (PS50843), pollen allergen (PF01357) |
| | 33-37 | At4g12880 | early nodulin (AtEN20) (phytoeyanin) | 24,8 | 5 | 14273.34 | 9,64 | 1-18 | | plastocyanin-like domain (PF02298) (copper binding proteins) |
| | 36-38 | At3g02110 | homologous to serine carboxypeptidase D (SCPL25) (Peptidase family S10, S10.005, MEROPS) | 14,4 | 5 | 51075.46 | 9,35 | 1-22 | | serine carboxypeptidases, histidine active site (PS00560), serine carboxypeptidases, serine active site (PS00131), serine carboxypeptidase (PF00450) |
| | 37 | At3g21770 | peroxidase (AtPrx30) | 15,2 | 4 | 32901.55 | 10,54 | 1-27 | | peroxidases active site signature (PS00436) |
| | 41 | At2g05580 | glycine-rich protein | 21,8 | 3 | 21517.58 | 10,21 | 1-20 | | glycine-rich region profile (PS50315) |
| | 43-47 | At1g67090 | RUBISCO small subunit A1 | 26,1 | 6 | 20216.04 | 9,26 | | | ribulose biphosphate carboxylase, small chain (PF00101) |
| | 42-44 | At5g38410* | RUBISCO small subunit B3 | 30,9 | 6 | 20284.25 | 9,40 | | | ribulose biphosphate carboxylase, small chain (PF00101) |
| | 43 | At5g38420* | RUBISCO small subunit B2 | 26,0 | 6 | 20350.22 | 9,24 | | | ribulose biphosphate carboxylase, small chain (PF00101) |
| | 48 | At2g38530 | non-specific lipid transfer protein type 1 (LTP2) | 25,4 | 3 | 9475.92 | 11,90 | 1-25 | | protease inhibitor/seed storage/LTP family (PF00234), plant lipid transfer proteins signature (PS00597) |
| A | 1-3, 9, 10, 11 | At3g16850 | glycoside hydrolase family 28 (polygalacturonase) | 33,8 | 14 | 46982.42 | 5,25 | 1-19 | | glycoside hydrolase family 28 (PF00295) |
| A | 4, 5 | At1g79720 | homologous to aspartic protease (CND41 peptidase) (Peptidase family A1, subfamily A1.050, MEROPS) | 10,1 | 5 | 49324.89 | 8,88 | 1-28 | | eukaryotic and viral aspartyl proteases active site (PS00141), eukaryotic aspartyl protease (PF00026) |
| A | 5-8 | At3g32980 | peroxidase (AtPrx32) | 28,7 | 13 | 35712.41 | 6,00 | 1-29 | | peroxidases active site signature (PS00436) |
| A | 7, 8 | At1g47710 | homologous to serpin (serine protease inhibitor) | 50,6 | 17 | 42639.41 | 4,97 | | transmembrane domains | serpins signature (PS00284), serpin (serine protease inhibitor) (PF00079) |
| A | 10, 11 | At5g22140 | expressed protein (oxido-reductase domain) | 14,2 | 6 | 46982.42 | 5,25 | 1-19 | | glycoside hydrolase family 28 (PF00295) |
| A | 10-13 | At5g43060 | homologous to cysteine proteinase (papain family) (RD21 peptidase, Peptidase family C1, C01.064 MEROPS) | 14,2 | 5 | 36995.63 | 5,79 | 1-22 | | eukaryotic thiol (cysteine) proteases asparagine active site (PS00640), papain family cysteine protease (PF00112), granulin (PF00396) |
| A | 11, 12 | At1g11840 | glyoxalase I homologue (lactoylglutathione lyase) (ATGLX1) | 27,2 | 7 | 31928.38 | 5,19 | | | glyoxalase I signature 1 (PS00934), glyoxalase I signature 2 (PS00935), glyoxalase/bleomycin resistance protein/dioxigenase superfamily (PF00903) |
| A | 11, 12 | At4g01610 | homologous to cysteine proteinase (papain family) (Cathepsin B, Peptidase family C1, C01.049, MEROPS) | 13,6 | 5 | 36995.63 | 5,79 | 1-22 | | eukaryotic thiol (cysteine) proteases histidine active site (PS00639), papain family cysteine protease (PF00112), propeptide_C1 (PF08127) |
| A | 13 | At2g33160 | glycoside hydrolase family 28 (polygalacturonase) | 8,0 | 4 | 72778.41 | 9,68 | 1-20 | | polygalacturonase active site (PS00502), glycoside hydrolases family 28 (PF00295) |

Table S1, supplementary data

| | | | | | | | | | | |
|----------|-----------|------------------|---|------|----|----------|-------|------|---------------------------------|--|
| A | 14,15 | At3g11630 | homologous to 2-Cys peroxiredoxin | 24,8 | 4 | 29091,87 | 7,91 | 1-24 | | AhpC/TSA family (PF00578) |
| A | 16-18 | At5g23820 | expressed protein (ML domain - MD-2-related lipid recognition domain) | 20,1 | 4 | 15240,10 | 4,17 | | | ML domain (PF02221) |
| A | 16,17 | At5g46960 | homologous to plant invertase/pectin methyltransferase inhibitor (PMEI) | 22,4 | 4 | 16869,03 | 4,52 | 1-20 | | plant invertase/pectin methyltransferase inhibitor (PF04043) |
| A | 19 | At3g20390 | homologous to endoribonuclease | 31,0 | 5 | 19815,74 | 9,57 | | | uncharacterized protein family UPF0076 signature (PS01094), endoribonuclease L-PSP (PF01042) |
| B | 1,3 | At2g38530 | non-specific lipid transfer protein type 1 (LTP2) | 16,1 | 2 | 9475,92 | 11,90 | 1-25 | | protease inhibitor/seed storage/LTP family (PF00234), plant lipid transfer proteins signature (PS00697) |
| B | 2 | At1g47710 | homologous to serpin (serine protease inhibitor) | 17,6 | 6 | 42639,41 | 4,97 | | transmembrane domains | serpins signature (PS00284), serpin (serine protease inhibitor) (PF00079) |
| D | 1,2 | At3g16850 | glycoside hydrolase family 28 (polygalacturonase) | 29,6 | 12 | 46982,42 | 5,25 | 1-19 | | glycoside hydrolase family 28 (PF00295) |
| D | 3,4 | At1g54010 | homologous to lipase/acylhydrolase (GDGL family) | 21,1 | 5 | 40021,24 | 9,05 | 1-29 | | GDGL-like Lipase/Acylhydrolase (PF00657) |
| D | 5 | At3g32980 | peroxidase (AtPrx32) | 29,0 | 11 | 35712,41 | 6,00 | 1-29 | | peroxidases active site signature (PS00436) |
| D | 6 | At4g01610 | homologous to cysteine proteinase (papain family) (Cathepsin B, Peptidase family C1, C01.049, MEROPS) | 15,0 | 5 | 36995,63 | 5,79 | 1-22 | | eukaryotic thiol (cysteine) proteases histidine active site (PS00639), papain family cysteine protease (PF00112), propeptide_C1 (PF08127) |
| E | 1, 5-7, 9 | At5g34940 | glycoside hydrolase family 79 (endo beta-glucuronidase/heparanase) | 19,6 | 8 | 57219,88 | 9,13 | 1-21 | | glycoside hydrolase family 79, N-terminal domain (PF03662) |
| E | 2-5, 9 | At3g16850 | glycoside hydrolase family 28 (polygalacturonase) | 28,5 | 12 | 46982,42 | 5,25 | 1-19 | | glycoside hydrolase family 28 (PF00295) |
| E | 5 | At3g55260 | glycoside hydrolase family 20 (beta-hexosaminidase) | 19,2 | 8 | 58810,86 | 5,66 | 1-22 | | glycoside hydrolase family 20 (PF00295), glycoside hydrolase family 20, catalytic domain (PF00728), glycoside hydrolase family 20, domain 2 (PF02838) |
| E | 8 | At5g34850 | homologous to purple acid phosphatase | 22,1 | 8 | 53242,23 | 6,79 | 1-16 | | calcineurin-like phosphoesterase (PS00149), phosphoesterase (PF50185) |
| E | 10 | At2g17120 | expressed protein (LysM domain) | 17,7 | 5 | 35262,82 | 6,24 | 1-23 | GPI anchor | LysM domain (PF01476) |
| E | 11-13, 17 | At3g49120 | peroxidase (AtPrx34) | 32,3 | 8 | 35695,45 | 9,41 | 1-30 | | peroxidase active site signature (PS00436), peroxidases proximal heme-ligand signature (PS00435) |
| E | 12,13 | At1g54010 | homologous to lipase/acylhydrolase (GDGL family) | 43,0 | 13 | 40021,24 | 9,05 | 1-29 | N-terminal transmembrane domain | lipase/acylhydrolase with GDGL-like motif (PS50241), GDGL-like lipase/acylhydrolase (PF00567) |
| E | 12-14 | At3g32980 | peroxidase (AtPrx32) | 29,0 | 11 | 35712,41 | 6,00 | 1-29 | | peroxidases active site signature (PS00436) |
| E | 14 | At1g70850 | homologous to Bet v I allergen family | 37,0 | 8 | 35569,93 | 4,96 | | | pathogenesis-related protein Bet v I family (PF00407) |
| E | 14,15 | At4g19810 | glycoside hydrolase family 18 (chitinase) | 32,2 | 9 | 38510,49 | 9,37 | 1-24 | | glycoside hydrolases family 18 (PF00704) |
| E | 14 | At1g54030 | homologous to lipase/acylhydrolase (GDGL family) | 11,6 | 3 | 46082,76 | 6,20 | | transmembrane domains | lipase/acylhydrolase with GDGL-like motif (PS50241), GDGL-like Lipase/Acylhydrolase (PF00657) |
| E | 14 | At5g06860 | PGIP1 (LRR domains) | 19,4 | 5 | 34324,36 | 9,56 | 1-21 | | LRR (PF00560, PS50502, PS50506) |
| E | 14 | At5g39270 | alpha expansin (AtEXPA22) | 11,5 | 3 | 27348,10 | 9,74 | 1-13 | | expansin, family-45 endoglucanase-like domain profile (PS50842), expansin, cellulose-binding-like domain profile (PS50843), rare lipoprotein A (RlpA)-like double-psi beta-barrel (PF03330), pollen allergen (PF01357) |
| E | 15,16 | At4g36700 | expressed protein (cupin domain) | 16,4 | 7 | 53703,31 | 5,21 | 1-25 | | glutamic acid-rich region profile (PS50313), cupin (PF00190) |
| E | 15-17 | At3g20370 | expressed protein (MATH domain) | 24,3 | 8 | 40734,84 | 5,56 | 1-25 | | 2 MATH domains (PF00917) |
| E | 16,17 | At1g53240 | homologous to mitochondrial NAD-dependent malate dehydrogenase | 16,7 | 4 | 35804,43 | 9,49 | | | malate dehydrogenase active site signature (PS00068), lactate/malate dehydrogenase, NAD binding domain (PF00056), lactate/malate dehydrogenase, alpha/beta C-terminal domain (PF02866) |
| E | 18,19 | At3g12500 | glycoside hydrolase family 19 (basic endochitinase) | 10,2 | 3 | 32367,86 | 6,24 | 1-20 | | chitinase family 19 signature 1 (PS00773), signature 2 (PS00774), chitin recognition or binding domain signature (PS00026), chitinase class I (PF00182), chitin recognition protein (PF00187) |
| E | 19 | At3g02870 | homologous to myo-inositol monophosphatase | 33,6 | 9 | 29121,33 | 5,03 | | transmembrane domains | inositol monophosphatase family signature 1 (PS00629), inositol monophosphatase family signature 2 (PS00630), inositol monophosphatase family (PF00459) |
| E | 19-21 | At4g01610 | homologous to cysteine proteinase (papain family) (Cathepsin B, Peptidase family C1, C01.049, MEROPS) | 20,3 | 7 | 36995,63 | 5,79 | 1-22 | | eukaryotic thiol (cysteine) proteases histidine active site (PS00639), papain family cysteine protease (PF00112), propeptide_C1 (PF08127) |
| E | 20-22 | At1g11840 | glyoxalase I homologue (lactoylglutathione lyase) (ATGLX1) | 27,2 | 7 | 31928,38 | 5,19 | | | glyoxalase I signature 1 (PS00934), glyoxalase I signature 2 (PS00935), glyoxalase/bleomycin resistance protein/dioxygenase superfamily (PF00903) |
| E | 20,21 | At1g67280 | glyoxalase I homologue (lactoylglutathione lyase) (ATGLX1) | 20,9 | 6 | 39167,11 | 7,73 | | | glyoxalase I signature 1 (PS00934), glyoxalase/bleomycin resistance protein/dioxygenase superfamily (PF00903) |
| E | 21-23 | At5g43060 | homologous to cysteine proteinase (papain family) (RD21 peptidase, Peptidase family C1, C01.064 MEROPS) | 15,8 | 8 | 36995,63 | 5,79 | 1-22 | | eukaryotic thiol (cysteine) proteases asparagine active site (PS00640), papain family cysteine protease (PF00112), granulin (PF00396) |

Table S1, supplementary data

| | | | | | | | | | | |
|---|-------------|------------------|--|------|----|----------|-------|------|-----------------------|--|
| E | 21-23 | At1g47128 | homologous to cysteine proteinase (papain family) (RD21A, Peptidase family C1, C01.064 MEROPS) | 11,0 | 5 | 48785,27 | 5,01 | 1-21 | | eukaryotic thiol (cysteine) proteases histidine active site (PS00639), cysteine active site (PS00640), asparagine active site (PS00139), Cys-rich region (PS50311), papaine family cysteine protease (PF00112) |
| E | 22 | At1g30710 | homologous to berberine-bridge enzyme (S)-reticuloin:oxygen oxidoreductase | 9,4 | 5 | 56821,30 | 9,63 | 1-23 | | berberine and berberine like (PF08031), FAD binding domain (PF01565) |
| E | 24, 25 | At3g55440 | triose phosphate isomerase | 23,2 | 7 | 27168,94 | 5,10 | | | triose phosphate isomerase active site (PS00171) |
| E | 25, 26 | At2g28490 | expressed protein (cupin domain) | 11,0 | 4 | 53594,59 | 5,45 | 1-19 | | cupin domain profile (PS50849), cupin (PF00190) |
| E | 27 | At2g15220 | expressed protein (Plant Basic Secreted Protein domain) | 30,2 | 7 | 22772,21 | 9,09 | 1-21 | | neutral zinc metalloproteinases, zinc-binding region signature (PS00142), plant basic secretory protein (PF04450) |
| E | 28 | At1g73260 | inhibitor family I3 (Kunitz-P family) (subfamily I3A unassigned peptidase inhibitor homologues, MEROPS) | 13,0 | 3 | 20982,78 | 5,20 | 1-26 | | trypsin and protease inhibitor Kunitz legume (PF00197) |
| E | 28 | At5g44360 | homologous to berberine-bridge enzyme (S)-reticuloin:oxygen oxidoreductase | 8,1 | 4 | 57931,72 | 9,83 | 1-22 | | oxygen oxido-reductases covalent FAD binding site (PS00862), FAD binding domain (PF01565), berberine and berberine like (PF08031) |
| E | 29 | At5g55730 | fasciolicin-like arabinogalactan protein (AtFLA1) | 10,1 | 3 | 42165,78 | 5,65 | 1-24 | GPI anchor | Beta-Ig-H3/fasciolicin domain (PS50213) |
| E | 30, 31 | At2g04690 | expressed protein (homologous to a human brain CREG protein) | 31,9 | 6 | 20214,11 | 8,00 | 1-28 | | |
| E | 32 | At5g66090 | expressed protein | 37,1 | 6 | 22539,76 | 6,15 | | | UspA (universal stress protein) IPR00616 |
| E | 33-35 | At4g16500 | inhibitor family I25 (cystatin family) (subfamily I25B unassigned peptidase inhibitor homologues, MEROPS) | 33,3 | 5 | 10183,74 | 9,78 | 1-22 | | cystatin domain (PF00031) |
| F | 1, 8-10 | At1g03220 | homologous to carrot EDGP and tomato XEGIP | 31,6 | 10 | 43394,37 | 9,99 | 1-22 | | |
| F | 2, 4, 46-48 | At2g38530 | non-specific lipid transfer protein type 1 (LTP2) | 33,1 | 4 | 9475,92 | 11,90 | 1-25 | | protease inhibitor/seed storage/LTP family (PF00234), plant lipid transfer proteins signature (PS00597) |
| F | 3 | At2g34930 | expressed protein (LRR domains) | 22,9 | 20 | 97059,60 | 9,59 | 1-28 | | leucine-rich region profile (PS50319), serine-rich region profile (PS50324), typical LRR profile (PS50506), SDS22+-like LRR profile (PS50504), plant-specific LRR profile (PS50502) |
| F | 5 | At3g16850 | glycoside hydrolase family 28 (polygalacturonase) | 24,6 | 7 | 46982,42 | 5,25 | 1-19 | | glycoside hydrolase family 28 (PF00295) |
| F | 6, 9, 11 | At1g78860 | homologous to lectin (curculin-like) | 21,0 | 8 | 46804,29 | 6,00 | 1-22 | | PAN domain (PF00024), lectin (probable mannose binding) (PF01453) |
| F | 7-9, 11 | At1g78850 | homologous to lectin (curculin-like) | 26,8 | 11 | 46664,12 | 8,90 | 1-22 | | PAN domain (PF00024), lectin (probable mannose binding) (PF01453) |
| F | 8-12, 18-23 | At1g09750 | homologous to aspartic protease (pepsin family) (Peptidase family A1, subfamily A1B unassigned peptidases, MEROPS) | 16,9 | 9 | 45038,78 | 9,09 | 1-23 | | lipase Ser active site (PS00120), Ser-rich region (PS050324), eukaryotic aspartic protease (PF00026) |
| F | 11-13 | At3g43270 | carbohydrate esterase family 8 (pectin methyltransferase) | 17,8 | 14 | 54929,20 | 9,55 | 1-24 | | pectinesterase (PF01095), plant invertase/pectin methyltransferase inhibitor (PMEI) (PF04043) |
| F | 12-14 | At3g49120 | peroxidase (AtPrx34) | 16,1 | 6 | 35695,45 | 9,41 | 1-30 | | peroxidase active site signature (PS00436), peroxidases proximal heme-ligand signature (PS00435) |
| F | 13, 14 | At3g48460 | homologous to lipase/acylhydrolase (GDSSL family) | 22,8 | 6 | 39627,85 | 8,91 | 1-26 | | lipase/acylhydrolase with GDSSL-like motif (PS50241), GDSSL-like lipase/acylhydrolase (PF00657) |
| F | 13-17 | At1g78830 | homologous to lectin (curculin-like) | 21,5 | 8 | 48101,66 | 9,45 | 1-22 | | PAN domain (PF00024), lectin (probable mannose binding) (PF01453) |
| F | 14-16 | At5g06860 | PGIP1 (LRR domains) | 27,6 | 8 | 34324,36 | 9,56 | 1-21 | | LRR (PF00560, PS50502, PS50506) |
| F | 14-21 | At4g36700 | expressed protein (cupin domain) | 15,2 | 8 | 53703,31 | 5,21 | 1-25 | | glutamic acid-rich region profile (PS50313), cupin (PF00190) |
| F | 14 | At3g32980 | peroxidase (AtPrx32) | 15,6 | 7 | 35712,41 | 6,00 | 1-29 | | peroxidases active site signature (PS00436) |
| F | 15, 16 | At1g70850 | homologous to Bet v I allergen family | 39,6 | 9 | 35569,93 | 4,96 | | | pathogenesis-related protein Bet v I family (PF00407) |
| F | 15 | At1g70830 | homologous to Bet v I allergen family | 26,0 | 7 | 37614,51 | 5,03 | | | pathogenesis-related protein Bet v I family (PF00407) |
| F | 17-19 | At1g29670 | homologous to lipase/acylhydrolase (GDSSL family) | 38,6 | 10 | 37153,43 | 9,65 | 1-24 | | GDSSL-like Lipase/Acylhydrolase (PF00657) |
| F | 17 | At3g01590 | homologous to aldose 1-epimerase | 19,3 | 5 | 34738,64 | 5,72 | | | aldose 1-epimerase (PF01263) |
| F | 18, 19 | At1g53240 | homologous to mitochondrial NAD-dependent malate dehydrogenase | 24,9 | 8 | 35804,43 | 9,49 | | | malate dehydrogenase active site signature (PS00068), lactate/malate dehydrogenase, NAD binding domain (PF00056), lactate/malate dehydrogenase, alpha/beta C-terminal domain (PF02866) |
| F | 18, 19 | At4g18180 | glycoside hydrolase family 28 (polygalacturonase) | 13,0 | 4 | 41118,43 | 10,42 | 1-23 | | polygalacturonase active site (PS00502), glycoside hydrolase family 28 (PF00295) |
| F | 20 | At3g14310 | carbohydrate esterase family 8 (pectin methyltransferase) (A1PME3) | 14,4 | 7 | 60050,61 | 9,59 | 1-40 | transmembrane domains | pectinesterase signature 1 (PS00800), pectinesterase signature 2 (PS00503), pectinesterase (PF01095), plant invertase/pectin methyltransferase inhibitor (PF04043) |
| F | 21 | At5g53370 | carbohydrate esterase family 8 (pectin methyltransferase) | 9,7 | 5 | 64240,96 | 8,80 | | GPI anchor | plant invertase/pectin methyltransferase inhibitor (PF04043), pectinesterase (PF01095) |
| F | 21 | At4g30170 | peroxidase (AtPrx45) | 14,8 | 4 | 33052,57 | 10,43 | 1-25 | | haem peroxidase, plant/fungal/bacterial (PS00435, PS00436, PS50873) |

Table S1, supplementary data

| | | | | | | | | | |
|---|-------------------------|-------------------|--|------|----|-----------|-------|------|--|
| F | 21, 22 | At4g01610 | homologous to cysteine proteinase (papain family) (Cathepsin B, Peptidase family C1, C01.049, MEROPS) | 15,3 | 5 | 36995,63 | 5,79 | 1-22 | eukaryotic thiol (cysteine) proteases histidine active site (PS00639), papain family cysteine protease (PF00112), propeptide_C1 (PF08127) |
| F | 22-24 | At3g45970 | expansin-like A (AtEXLA1) | 29,4 | 8 | 26258,12 | 9,46 | 1-20 | pollen allergen (PF01357), barwin (PF00967) |
| F | 22, 23 | At1g47128 | homologous to cysteine proteinase (papain family) (RD21A, Peptidase family C1, C01.064 MEROPS) | 16,2 | 6 | 48785,27 | 5,01 | 1-21 | eukaryotic thiol (cysteine) proteases histidine active site (PS00639), cysteine active site (PS00640), asparagine active site (PS00139), Cys-rich region (PS50311), papaine family cysteine protease (PF00112) |
| F | 22-26 | At5g43060 | homologous to cysteine proteinase (papain family) (Peptidase family C1, RD21 peptidase, C01.064 MEROPS) | 16,0 | 7 | 36995,63 | 5,79 | 1-22 | eukaryotic thiol (cysteine) proteases asparagine active site (PS00640), papain family cysteine protease (PF00112), granulin (PF00396) |
| F | 26, 27 | At3g55440 | triose phosphate isomerase | 27,5 | 6 | 27168,94 | 5,10 | | triose phosphate isomerase active site (PS00171) |
| F | 27-29 | At4g29270 | homologous to acid phosphatase | 29,3 | 8 | 25900,84 | 9,15 | 1-26 | HAD superfamily, subfamily IIIB (acid phosphatase) (PF03767) |
| F | 27-31 | At2g28490 | expressed protein (cupin domain) | 13,7 | 6 | 53594,59 | 5,45 | 1-19 | cupin domain profile (PS50849), cupin (PF00190) |
| F | 27-29 | At3g63190 | homologous to ribosome recycling factor | 25,8 | 7 | 30422,14 | 6,50 | | ribosome recycling factor (PF01765) |
| F | 27 | At4g36880 | homologous to cysteine protease (papain family) (Peptidase family C1, Brassicain, C01.021 MEROPS) | 16,5 | 5 | 39395,74 | 6,50 | 1-22 | eukaryotic thiol (cysteine) proteases cysteine active site (PS00139), eukaryotic thiol (cysteine) proteases histidine active site (PS00639), eukaryotic thiol (cysteine) proteases asparagine active site (PS00640), papain family cysteine protease (PF00112) |
| F | 28 | At3g57140 | homologous to patatin (phospholipase domain) | 9,7 | 6 | 89933,66 | 6,31 | | patatin-like phospholipase (PF01734) |
| F | 28 | At4g27110 | homologous to COBRA (AICOB110) | 8,4 | 5 | 71335,23 | 9,86 | 1-21 | GPI anchor phytochelatin synthetase-like conserved region (PF04833), carbohydrate binding domain (SSF49394) |
| F | 28-32, 35, 36, 38 | At4g12880 | early nodulin (AtEN20) (phytocyanin) | 24,8 | 5 | 14273,34 | 9,64 | 1-18 | plastocyanin-like domain (PF02298) (copper binding proteins) |
| F | 29 | At5g05110 | inhibitor family I25 (phytostatin) (cystatin family, I25.014, MEROPS) | 10,3 | 3 | 23934,58 | 8,48 | 1-24 | cysteine proteases inhibitors signature (PS00287), cystatin domain (PF00031) |
| F | 33, 34 | At1g17860 | inhibitor family I3 (Kunitz-P family) (subfamily I3A unassigned peptidase inhibitor homologues, MEROPS) | 25,5 | 7 | 19718,68 | 9,76 | 1-21 | soybean trypsin inhibitor (Kunitz) protease inhibitors family signature (PS00283), trypsin and protease inhibitor (PF00197) |
| F | 34-36 | At5g01300 | homologous to phosphatidylethanolamine-binding protein | 50,0 | 7 | 71823,40 | 5,15 | | phosphatidylethanolamine-binding protein (PF01161), |
| F | 35, 36 | At2g04690 | expressed protein (homologous to a human brain CREG protein) | 28,1 | 6 | 20214,11 | 8,00 | 1-28 | |
| F | 35, 36 | At4g25260 | homologous to plant invertase/ pectin methyltransferase inhibitor (PMEI) | 16,9 | 4 | 15737,05 | 5,50 | 1-19 | plant invertase/pectin methyltransferase inhibitor (PF04043) |
| F | 37, 39-42 | At3g15670 | homologous to late embryogenesis abundant protein (LEA) | 36,4 | 6 | 24186,00 | 9,50 | | late embryogenesis abundant protein (LEA) (PF02987) |
| F | 39-43 | At4g39260 | homologous to RNA binding protein (AtGRP8) | 41,4 | 6 | 16578,63 | 5,12 | | glycine-rich region profile (PS50315), eukaryotic RNA Recognition Motif (RRM) profile (PS50102), RNA recognition motif. (a.k.a. RRM, RBD, or RNP domain) (PF00076) |
| F | 39-44 | At1g09310 | expressed protein (DUF538) | 38,5 | 7 | 19947,00 | 5,12 | | domain of unknown function DUF538 (PF04398) |
| F | 40 | At4g23680 | homologous to Bet v 1 allergen family | 45,0 | 7 | 17473,54 | 5,80 | | pathogenesis-related protein Bet v 1 family (PF00407) |
| F | 42-45 | At4g01900 | P-II nitrogen sensing protein (GLB 1) | 34,7 | 7 | 21275,40 | 5,12 | | P-II protein C-terminal region signature (PS00638), nitrogen regulatory protein P-II (PS00543) |
| F | 43-45 | At1g67090 | RUBISCO small subunit A1 | 19,4 | 4 | 20216,04 | 9,26 | | ribulose biphosphate carboxylase, small chain (PF00101) |
| F | 43 | At5g38410* | RUBISCO small subunit B3 | 24,3 | 4 | 20284,25 | 9,40 | | ribulose biphosphate carboxylase, small chain (PF00101) |
| F | 43 | At5g38430* | RUBISCO small subunit B1 | 19,3 | 4 | 20286,18 | 9,24 | | ribulose biphosphate carboxylase, small chain (PF00101) |
| F | 48 | At1g47540 | inhibitor family I18 (mustard trypsin inhibitor-2 family) (family I18 unassigned peptidase inhibitor homologues, MEROPS) | 45,9 | 4 | 9051,17 | 4,97 | 1-18 | scorpion toxin-like domain (PF00537) |
| G | 1 | At3g15670 | homologous to late embryogenesis abundant protein (LEA) | 24,0 | 4 | 24186,00 | 9,50 | | late embryogenesis abundant protein (LEA) (PF02987) |
| G | 2-5, 7, 9-11, 45, 55-59 | At2g38530 | non-specific lipid transfer protein type 1 (LTP2) | 46,6 | 5 | 9475,92 | 11,90 | 1-25 | protease inhibitor/seed storage/LTP family (PF00234), plant lipid transfer proteins signature (PS00597) |
| G | 3, 7 | At1g05570 | glycosyl transferase family 48 (callose synthase) (AtCalS1) | 2,8 | 5 | 225730,52 | 9,51 | | 1,3-beta-glucan synthase component (PF02364) |
| G | 5 | At2g34930 | expressed protein (LRR domains) | 10,9 | 11 | 97059,60 | 9,59 | 1-28 | leucine-rich region profile (PS50319), serine-rich region profile (PS50324), typical LRR profile (PS50506), SDS22+like LRR profile (PS50504), plant-specific LRR profile (PS50502) |
| G | 6-8 | At1g68560 | glycoside hydrolase family 31 (alpha-xylosidase) (AtXYL1) | 8,2 | 7 | 99641,37 | 6,26 | 1-27 | glycoside hydrolases family 31 active site (PS00129) |
| G | 10 | At3g07130 | homologous to purple acid phosphatase | 9,6 | 5 | 58523,64 | 5,71 | 1-17 | metallo-phosphoesterase motif (PS50185), calcineurin-like phosphoesterase (PF00149) |
| G | 11, 12 | At3g61490 | glycoside hydrolase family 28 (polygalacturonase) | 10,9 | 4 | 49497,06 | 5,76 | 1-23 | glycoside hydrolase family 28 (PF00295) |
| G | 12, 13 | At2g27190 | homologous to purple acid phosphatase (PAP1) | 19,0 | 10 | 50962,71 | 5,75 | 1-28 | metallo-phosphoesterase motif (PS50185), calcineurin-like phosphoesterase (PF00149) |
| G | 13-15 | At1g33590 | expressed protein (LRR domains) | 40,6 | 16 | 48103,60 | 9,90 | 1-24 | LRR (PF00560), leucine zipper pattern (PS00029) |

Table S1, supplementary data

| | | | | | | | | | |
|----------|--|-------------------|---|------|----|----------|-------|------|--|
| G | 51, 52 | At1g09310 | expressed protein (DUF538) | 27,9 | 5 | 19947,00 | 5,12 | | domain of unknown function DUF538 (PF04398) |
| G | 52, 53, 54 | At5g38410* | RUBISCO small subunit B3 | 27,6 | 5 | 20284,25 | 9,40 | | ribulose biphosphate carboxylase, small chain (PF00101) |
| G | 52, 53, 57 | At5g38430* | RUBISCO small subunit B1 | 22,7 | 5 | 20286,18 | 9,24 | | ribulose biphosphate carboxylase, small chain (PF00101) |
| G | 52, 53 | At5g38420* | RUBISCO small subunit B2 | 22,7 | 5 | 20350,22 | 9,24 | | ribulose biphosphate carboxylase, small chain (PF00101) |
| G | 53-56 | At1g67090 | RUBISCO small subunit A1 | 22,8 | 5 | 20216,04 | 9,26 | | ribulose biphosphate carboxylase, small chain (PF00101) |
| G | 57, 58 | At4g16500 | inhibitor family I25 (cystatin family) (subfamily I25B unassigned peptidase inhibitor homologues, MEROPS) | 43,6 | 6 | 10183,74 | 9,78 | 1-22 | cystatin domain (PF00031) |
| H | 1, 3, 4, 10, 12-14, 16-32, 35-42, 48-54, 56-59 | At5g11420 | expressed protein (DUF642) | 32,0 | 16 | 37344,57 | 7,70 | 1-22 | domain of unknown function DUF 642 (PF04862) |
| H | 2 | At3g24480 | LRR-extensin (AtLRX4) | 10,5 | 6 | 51682,00 | 6,39 | 1-25 | plant specific LRR profile (PS50502), Leucine Rich Repeat (PF00560), Pro-rich profile (PS50099) |
| H | 3 | At4g13340 | LRR-extensin (AtLRX3) | 5,9 | 4 | 79847,66 | 6,29 | 1-20 | proline-rich region profile (PS50099), leucine-rich repeat (PF00560), extensin-like region (PF04554), leucine-rich repeat, plant specific (PS50502) |
| H | 3 | At4g18670 | LRR-extensin (AtLRX5) | 6,3 | 4 | 87040,18 | 9,02 | 1-27 | histidine-rich region profile (PS50316), plant specific LRR profile (PS50502), serine-rich region profile (PS50324), Leucine Rich Repeat (PF00560) |
| H | 3 | At5g53370 | carbohydrate esterase family 8 (pectin methyltransferase) | 8,7 | 4 | 64240,96 | 8,80 | | GPI anchor plant invertase/pectin methyltransferase inhibitor (PF04043), pectinesterase (PF01095) |
| H | 4 | At1g28290 | proline-rich protein | 6,4 | 3 | 35913,36 | 10,77 | 1-24 | histidine-rich region profile (PS50316), proline-rich region profile (PS50099), pollen proteins Ole e 1 family (PF01190) |
| H | 5-9 | At1g68560 | glycoside hydrolase family 31 (alpha-xylosidase) (XYL1) | 19,3 | 19 | 99641,37 | 6,26 | 1-27 | glycoside hydrolases family 31 active site (PS00129) |
| H | 10 | At3g13790 | glycoside hydrolase family 32 (beta-fructofuranosidase) | 8,9 | 5 | 63490,72 | 9,68 | 1-25 | glycoside hydrolases family 32 active site (PS00609), glycosyl hydrolases family 32 (PF00251) |
| H | 11 | At3g07130 | homologous to purple acid phosphatase | 12,2 | 6 | 58523,64 | 5,71 | 1-17 | metallo-phosphoesterase motif (PS50185), calcineurin-like phosphoesterase (PF00149) |
| H | 12 | At2g27190 | homologous to purple acid phosphatase (PAP12) | 27,9 | 14 | 50962,71 | 5,75 | 1-28 | metallo-phosphoesterase motif (PS50185), calcineurin-like phosphoesterase (PF00149) |
| H | 12 | At3g61490 | glycoside hydrolase family 28 (polygalacturonase) | 12,2 | 4 | 49497,06 | 5,76 | 1-23 | glycoside hydrolase family 28 (PF00295) |
| H | 13 | At1g78850 | homologous to lectin (curcumin-like) | 7,7 | 4 | 46664,12 | 8,90 | 1-22 | PAN domain (PF00024), lectin (probable mannose binding) (PF01453) |
| H | 13 | At1g33590 | expressed protein (LRR domains) | 9,0 | 5 | 48103,60 | 9,90 | 1-24 | LRR (PF00560), leucine zipper pattern (PS00029) |
| H | 14-17 | At1g03230 | homologous to carrot EDGP and tomato XEGIP | 33,2 | 12 | 43723,02 | 10,27 | 1-23 | |
| H | 17 | At5g19110 | homologous to carrot EDGP and tomato XEGIP | 23,5 | 8 | 41369,63 | 10,01 | 1-19 | |
| H | 18 | At4g05200 | homologous to receptor kinase (RLK, DUF26-1b subfamily) | 9,2 | 6 | 72576,6 | 6,09 | 1-25 | trans-membrane domains protein kinases ATP-binding region signature (PS00107), Serine/Threonine protein kinases active-site signature (PS00108), protein kinase domain profile (PF50011), domain of unknown function DUF 26 (PF01657), Protein kinase domain (PF00069), protein tyrosine kinase (PF07714) |
| H | 20-23, 32 | At3g20820 | expressed protein (LRR domains) | 37,5 | 11 | 37850,70 | 9,90 | 1-19 | typical LRR profile (PS50506), plant specific LRR profile (PS50502), SDS22-like LRR profile (PS50504) |
| H | 20 | At1g71695 | peroxidase (AtPrx12) | 26,5 | 7 | 37104,36 | 9,46 | 1-22 | peroxidase (PF00141) |
| H | 21, 22 | At5g12940 | homologous to <i>Phaseolus vulgaris</i> PGIP2 (LRR domains) | 18,6 | 6 | 36784,96 | 10,45 | 1-29 | plant specific LRR profile (PS50502), Leucine Rich Repeat (PF00560) |
| H | 22, 24, 26, 27, 32, 41, 50, 52-53 | At5g25460 | expressed protein (DUF642) | 18,7 | 8 | 37948,52 | 9,03 | 1-19 | domain of unknown function DUF 642 (PF04862) |
| H | 23-29, 32 | At2g06850 | glycoside hydrolase family 16 (endoxylglucan transferase) (AtXTH4) | 42,2 | 14 | 31716,77 | 9,65 | 1-24 | glycoside hydrolases family 16 (PF00722) |
| H | 24, 25 | At5g64100 | peroxidase (AtPrx69) | 19,6 | 8 | 33228,93 | 10,72 | 1-23 | peroxidases active site signature (PS00436) |
| H | 25, 26 | T23B7.10 | homologous to PGIP1 (LRR protein FLR1) | 24,0 | 6 | 33134,88 | 9,62 | 1-23 | typical LRR profile (PS50506), plant specific LRR profile (PS50502), plant disease resistance response protein (PF03018) |
| H | 28 | At5g13870 | glycoside hydrolase family 16 (endoxylglucan transferase) (AtXTH5) | 14,3 | 6 | 31752,61 | 9,53 | 1-21 | xyloglucan endo-transglycosylase (XET) C-terminus (PF06955), glycoside hydrolases family 16 (PF00722) |
| H | 29 | At5g64260 | homologous to <i>Nicotiana tabacum</i> phi-1 | 20,7 | 6 | 30550,86 | 9,98 | 1-19 | phosphate-induced protein 1 conserved region (PF04674) |
| H | 29-34 | At5g09440 | homologous to <i>Nicotiana tabacum</i> phi-1 | 20,5 | 6 | 27366,44 | 10,15 | 1-23 | phosphate-induced protein 1 conserved region (PF04674) |
| H | 30 | At4g38400 | expansin-like A (AtEXLA2) | 13,2 | 4 | 26516,93 | 9,60 | 1-20 | expansin, cellulose-binding-like domain profile (PS50843), expansin, family-45 endoglucanase-like domain profile (PS50842), rare lipoprotein A (RlpA)-like double-psi beta-barrel (PF03330), pollen allergen (PF01357) |

Table S2, supplementary material. Identification of proteins extracted from purified cell walls of 11-day-old *Arabidopsis* hypocotyls by CaCl₂ and LiCl solutions

* proteins that could not be distinguished

Colour code secretory proteins (Presence of predicted signal peptide was checked using PSORT: <http://psort.ims.u-tokyo.ac.jp/form.html>, and TargetP: <http://www.cbs.dtu.dk/services/TargetP/>)
 secretory proteins (Presence of predicted signal peptide was checked using PSORT: <http://psort.ims.u-tokyo.ac.jp/form.html>, and TargetP: <http://www.cbs.dtu.dk/services/TargetP/>)
 transmembrane domains (Presence of predicted transmembrane domains was checked using Aramemnon: <http://aramemnon.botanik.uni-koeln.de/>)
 GPI anchors (presence or GPI anchors was checked using Aramemnon: <http://aramemnon.botanik.uni-koeln.de/>)
 intracellular proteins
 proteins identified after 1D-separation (1-DE), but not identified after 2D-separation (cationic exchange chromatography followed by 1-DE)

| total protein extract | band number | accession AGI | annotation | MALDI-TOF identification % sequence coverage | number of peptides (m/z) ^a | mature protein MM (kDa) | pI | signal peptide | transmembrane domains GPI anchors | functional domains ^b |
|-----------------------|---|-------------------|---|--|---------------------------------------|-------------------------|-------|----------------|-----------------------------------|---|
| 1 | 1-16, 18-24, 27, 29, 30, 32-34, 41, 42, 44, 45, 47, 53-56 | At5g25460 | expressed protein (DUF642) | 48,5 | 17 | 37948,52 | 9,03 | 1-19 | | domain of unknown function DUF 642 (PF04862) |
| 2 | 1-23, 26, 28, 37-40, 43, 44, 52-56 | At5g11420 | expressed protein (DUF642) | 34,7 | 14 | 37344,57 | 7,70 | 1-22 | | domain of unknown function DUF 642 (PF04862) |
| 3 | 1, 2 | At1g28290 | proline-rich protein | 47 | 4 | 35913,36 | 10,77 | 1-24 | | histidine-rich region profile (PS50316), proline-rich region profile (PS50099), pollen proteins Ole e 1 family (PF01190) |
| 4 | 2, 3, 5, 7, 10, 13-15, 24 | At1g03230 | homologous to carrot EDGP and tomato XEGIP | 16,8 | 6 | 43723,02 | 10,27 | 1-23 | | |
| 5 | 3, 7, 9, 10, 12, 14, 15 | At1g03220 | homologous to carrot EDGP and tomato XEGIP | 13,2 | 4 | 43394,37 | 9,99 | 1-22 | | |
| 6 | 5 | At2g06850 | glycoside hydrolase family 16 (endoxylglucan transferase) (AtXTH4) | 13,2 | 5 | 31716,77 | 9,65 | 1-24 | | glycoside hydrolases family 16 (PF00722) |
| 7 | 5 | At5g07030 | homologous to Asp protease (pepsin family) (Peptidase family A1, subfamily A1B unassigned peptidases, MEROPS) | 8,6 | 4 | 44860,54 | 10,82 | 1-19 | | eukaryotic aspartyl protease (PF00026) |
| 8 | 9, 10 | At3g16850 | glycoside hydrolase family 28 (polygalacturonase) | 31,9 | 12 | 46982,42 | 5,25 | 1-19 | | glycoside hydrolase family 28 (PF00295) |
| 9 | 11 | At5g34940 | glycoside hydrolase family 79 (endo beta-glucuronidase/heparanase) | 9,9 | 4 | 57219,88 | 9,13 | 1-21 | | glycoside hydrolase family 79, N-terminal domain (PF03662) |
| 10 | 11 | At1g33590 | expressed protein (LRR domains) | 7,8 | 4 | 48103,60 | 9,90 | 1-24 | | LRR (PF00560), leucine zipper pattern (PS00029) |
| 11 | 13, 14, 41-43 | At1g78850 | homologous to lectin (curculin-like) | 18,1 | 7 | 46664,12 | 8,90 | 1-22 | | PAN domain (PF00024), lectin (probable mannose binding) (PF01453) |
| 12 | 13, 41 | At1g78860 | homologous to lectin (curculin-like) | 14,9 | 6 | 46804,29 | 6,00 | 1-22 | | PAN domain (PF00024), lectin (probable mannose binding) (PF01453) |
| 13 | 13-15 | At1g09750 | homologous to Asp protease (pepsin family) (Peptidase family A1, subfamily A1B unassigned peptidases, MEROPS) | 12,7 | 7 | 45038,78 | 9,09 | 1-23 | | lipase Ser active site (PS00120), Ser-rich region (PS050324), eukaryotic aspartic protease (PF00026) |
| 14 | 16 | At3g43270 | carbohydrate esterase family 8 (pectin methylesterase) | 6,1 | 4 | 54929,20 | 9,55 | 1-24 | | pectinesterase (PF01095), plant invertase/pectin methylesterase inhibitor PME1 (PF04043) |
| 15 | 17-19, 21 | At3g20820 | expressed protein (LRR domains) | 27,9 | 9 | 37850,70 | 9,90 | 1-19 | | typical LRR profile (PS50506), plant specific LRR profile (PS50502), SDS22-like LRR profile (PS50504) |
| 16 | 17, 19, 32 | At3g49120 | peroxidase (AtPrx34) | 8,5 | 5 | 35695,45 | 9,41 | 1-30 | | peroxidase active site signature (PS00436), peroxidases proximal heme-ligand signature (PS00435) |
| 17 | 19-24 | At5g12940 | homologous to <i>Phaseolus vulgaris</i> PGIP2 (LRR domains) | 21,6 | 7 | 36784,96 | 10,45 | 1-29 | | plant specific LRR profile (PS50502), Leucine Rich Repeat (PF00560) |
| 18 | 19 | At3g32980 | peroxidase (AtPrx32) | 15,6 | 7 | 35712,41 | 6,00 | 1-29 | | peroxidases active site signature (PS00436) |
| 19 | 20, 22, 23, 47 | At1g70850* | homologous to Bet v I allergen family | 35,4 | 9 | 35569,93 | 4,96 | | | pathogenesis-related protein Bet v I family (PF00407) |
| 20 | 47 | At1g70830* | homologous to Bet v I allergen family | 17,6 | 4 | 37614,51 | 5,03 | | | pathogenesis-related protein Bet v I family (PF00407) |
| 21 | 21-23, 30, 31 | At3g08030 | expressed protein (DUF642) | 23,6 | 7 | 36993,02 | 6,87 | 1-21 | | domain of unknown function DUF 642 (PF04862) |
| 22 | 22 | At4g37950 | polysaccharide lyase family 4 (rhamnogalacturonate lyase) | 8,8 | 5 | 74062,32 | 9,65 | 1-30 | | rhamnogalacturonate lyase family (PF06045) |
| 23 | 23, 24 | At1g78830 | homologous to lectin (curculin-like) | 13,0 | 8 | 48101,66 | 9,45 | 1-22 | | PAN domain (PF00024), lectin (probable mannose binding) (PF01453) |
| 24 | 23 | At1g80240 | expressed protein (DUF642) | 18,6 | 5 | 37817,29 | 9,57 | 1-22 | | domain of unknown function DUF 642 (PF04862) |
| 25 | 24 | T23B7.10 | homologous to PGIP1 (LRR protein FLR1) | 18,8 | 4 | 33134,88 | 9,62 | 1-23 | | typical LRR profile (PS50506), plant specific LRR profile (PS50502), plant disease resistance response protein (PF03018) |
| 26 | 24 | At5g64100 | peroxidase (AtPrx69) | 18,7 | 6 | 33228,93 | 10,72 | 1-23 | | peroxidases active site signature (PS00436) |
| 27 | 25 | ArthCp030 | RUBISCO large subunit (RBCL) | 21,5 | 11 | 52954,98 | 5,79 | | | ribulose biphosphate carboxylase large chain active site (PS00157), ribulose biphosphate carboxylase large chain, catalytic domain (PF00016), ribulose biphosphate carboxylase large chain, N-terminal domain (PF02788) |
| 28 | 25, 53-58 | At1g67090 | RUBISCO small subunit A1 | 28,9 | 5 | 20216,04 | 9,26 | | | ribulose biphosphate carboxylase, small chain (PF00101) |
| 29 | 28, 29 | At4g30170 | peroxidase (AtPrx45) | 38,8 | 11 | 33052,57 | 10,43 | 1-25 | | haem peroxidase, plant/fungal/bacterial (PS00435, PS00436, PS50873) |

Table S2, supplementary data

| | | | | | | | | | | |
|---|----------|-----------|---|------|----|----------|------|------|-----------------------|---|
| N | 2 | At1g47710 | homologous to serpin (serine protease inhibitor) | 20,2 | 7 | 42639,41 | 4,97 | | transmembrane domains | serpins signature (PS00284), serpin (serine protease inhibitor) (PF00079) |
| N | 3 | At4g16260 | glycoside hydrolase family 17 | 12,8 | 6 | 35905,19 | 6,64 | 1-16 | | glycoside hydrolases family 17 signature (PS00587), glycoside hydrolases family 17 (PF00332) |
| N | 4, 5 | At4g01610 | homologous to cysteine proteinase (papain family) (Cathepsin B, Peptidase family C1, C01.049, MEROPS) | 14,4 | 6 | 36995,63 | 5,79 | 1-22 | | eukaryotic thiol (cysteine) proteases histidine active site (PS00639), papain family cysteine protease (PF00112), propeptide_C1 (PF08127) |
| N | 4-6 | At1g47128 | homologous to cysteine proteinase (papain family) (RD21A, Peptidase family C1, C01.064 MEROPS) | 16,7 | 7 | 48785,27 | 5,01 | 1-21 | | eukaryotic thiol (cysteine) proteases histidine active site (PS00639), cysteine active site (PS00640), asparagine active site (PS00139), Cys-rich region (PS50311), papaine family cysteine protease (PF00112) |
| N | 4, 5 | At3g12500 | glycoside hydrolase family 19 (chitinase) | 9,8 | 3 | 32367,86 | 6,24 | 1-20 | | chitinase family 19 signature 1 (PS00773), signature 2 (PS00774), chitin recognition or binding domain signature (PS00026), chitinase class I (PF00182), chitin recognition protein (PF00187) |
| N | 5, 6 | At5g43060 | homologous to cysteine proteinase (papain family) (RD21 peptidase, Peptidase family C1, C01.064 MEROPS) | 13,6 | 6 | 36995,63 | 5,79 | 1-22 | | eukaryotic thiol (cysteine) proteases asparagine active site (PS00640), papain family cysteine protease (PF00112), granulin (PF00396) |
| N | 5, 6 | At1g11840 | glyoxalase I homologue (lactoylglutathione lyase) (ATGLX1) | 17,6 | 5 | 31928,38 | 5,19 | | | glyoxalase I signature 1 (PS00934), glyoxalase I signature 2 (PS00935), glyoxalase/bleomycin resistance protein/dioxygenase superfamily (PF00903) |
| N | 8 | At1g73260 | inhibitor family I3 (Kunitz-P family) (subfamily I3A unassigned peptidase inhibitor homologues, MEROPS) | 19,1 | 4 | 20982,78 | 5,20 | 1-26 | | trypsin and protease inhibitor Kunitz legume (PF00197) |
| O | 1 | At3g16850 | glycoside hydrolase family 28 (polygalacturonase) | 25,5 | 8 | 46982,42 | 5,25 | 1-19 | | glycoside hydrolase family 28 (PF00295) |
| O | 2 | At4g16590 | glycoside transferase family 2 (cellulose synthase like) (CslA1) | 8,8 | 4 | 60927,18 | 9,80 | 1-27 | transmembrane domains | glycoside hydrolase family 2 (PF00535) |
| O | 3 | At1g23190 | homologous to phosphoglycomutase | 12,3 | 5 | 63170,37 | 5,79 | | | phosphoglucomutase/phosphomannomutase, alpha/beta/alpha domain I (PF02878), phosphoglucomutase/phosphomannomutase, alpha/beta/alpha domain II (PF02879), phosphoglucomutase/phosphomannomutase, alpha/beta/alpha domain III (PF02880), phosphoglucomutase/phosphomannomutase, C-terminal domain (PF00408) |
| O | 4 | At1g47128 | homologous to cysteine proteinase (papain family) (RD21A, Peptidase family C1, C01.064 MEROPS) | 16,7 | 4 | 48785,27 | 5,01 | 1-21 | | eukaryotic thiol (cysteine) proteases histidine active site (PS00639), cysteine active site (PS00640), asparagine active site (PS00139), Cys-rich region (PS50311), papaine family cysteine protease (PF00112) |
| O | 5 | At5g43060 | homologous to cysteine proteinase (papain family) (RD21 peptidase, Peptidase family C1, C01.064 MEROPS) | 13,6 | 3 | 36995,63 | 5,79 | 1-22 | | eukaryotic thiol (cysteine) proteases asparagine active site (PS00640), papain family cysteine protease (PF00112), granulin (PF00396) |
| O | 6 | At5g44360 | homologous to berberine-bridge enzyme (S)-reticulil:oxygen oxidoreductase | 7,9 | 4 | 57931,72 | 9,83 | 1-22 | | oxygen oxido-reductases covalent FAD binding site (PS00862), FAD binding domain (PF01565), berberine and berberine like (PF08031) |
| Q | 1-6 | At3g16850 | glycoside hydrolase family 28 (polygalacturonase) | 36,0 | 16 | 46982,42 | 5,25 | 1-19 | | glycoside hydrolase family 28 (PF00295) |
| Q | 2, 3 | At5g34940 | glycoside hydrolase family 79 (endo beta-glucuronidase/heparanase) | 18,1 | 9 | 57219,88 | 9,13 | 1-21 | | glycoside hydrolase family 79, N-terminal domain (PF03662) |
| Q | 4-6 | At2g17120 | expressed protein (LysM domain, GPI anchor) | 12,3 | 6 | 35262,82 | 6,24 | 1-23 | GPI anchor | LysM domain (PF01476) |
| Q | 7, 8, 10 | At3g49120 | peroxidase (AtPrx34) | 30,0 | 11 | 35695,45 | 9,41 | 1-30 | | peroxidase active site signature (PS00436), peroxidases proximal heme-ligand signature (PS00435) |
| Q | 6 | At3g43270 | carbohydrate esterase family 8 (pectin methyltransferase) | 13,3 | 7 | 54929,20 | 9,55 | 1-24 | | pectinesterase (PF01095), plant invertase/pectin methyltransferase inhibitor PME1 (PF04043) |
| Q | 7 | At2g36870 | glycoside hydrolase family 16 (endoxylglucan transferase) (AtXTH32) | 16,4 | 6 | 31928,02 | 9,92 | 1-24 | | glycoside hydrolase family 16 (PF00722), xyloglucan endo-transglycosidase (XET) C-terminus (PF06955) |
| Q | 8-10 | At3g32980 | peroxidase (AtPrx32) | 31,8 | 14 | 35712,41 | 6,00 | 1-29 | | peroxidases active site signature (PS00436) |
| Q | 10, 11 | At5g06860 | PGIP1 (LRR domains) | 21,5 | 7 | 34324,36 | 9,56 | 1-21 | | LRR (PF00560, PS50502, PS50506) |
| Q | 10, 11 | At1g70850 | homologous to Bet v I allergen family | 41,1 | 13 | 35569,93 | 4,96 | | | pathogenesis-related protein Bet v I family (PF00407) |
| Q | 10, 11 | At1g54030 | homologous to lipase/acylhydrolase (GDSL family) | 14,5 | 5 | 46082,76 | 6,20 | | transmembrane domains | lipase/acylhydrolase with GDSL-like motif (PS50241), GDSL-like Lipase/Acylhydrolase (PF00657) |
| Q | 12, 13 | At3g12500 | glycoside hydrolase family 19 (chitinase) | 10,5 | 4 | 32367,86 | 6,24 | 1-20 | | chitinase family 19 signature 1 (PS00773), signature 2 (PS00774), chitin recognition or binding domain signature (PS00026), chitinase class I (PF00182), chitin recognition protein (PF00187) |
| Q | 14, 15 | At1g47128 | homologous to cysteine proteinase (papain family) (RD21A, Peptidase family C1, C01.064 MEROPS) | 13,8 | 6 | 48785,27 | 5,01 | 1-21 | | eukaryotic thiol (cysteine) proteases histidine active site (PS00639), cysteine active site (PS00640), asparagine active site (PS00139), Cys-rich region (PS50311), papaine family cysteine protease (PF00112) |

Table S2, supplementary data

| | | | | | | | | | |
|---|---------------|-------------------|---|------|----|----------|-------|------|--|
| T | 26-28 | At5g64260 | homologous to <i>Nicotiana tabacum</i> phi-I | 20,0 | 6 | 30550,86 | 9,98 | 1-19 | phosphate-induced protein 1 conserved region (PF04674) |
| T | 27-29 | At5g09440 | homologous to <i>Nicotiana tabacum</i> phi-I | 20,0 | 8 | 27366,44 | 10,15 | 1-23 | phosphate-induced protein 1 conserved region (PF04674) |
| T | 28 | At4g38400 | expansin-like A (AEXLA2) | 20,0 | 7 | 26516,93 | 9,60 | 1-20 | expansin, cellulose-binding-like domain profile (PS50843), expansin, family-45 endoglucanase-like domain profile (PS50842), rare lipoprotein A (RlpA)-like double-psi beta-barrel (PF03330), pollen allergen (PF01357) |
| T | 30, 31 | At2g28790 | homologous to <i>Lycopersicon esculentum</i> osmotin | 10,0 | 4 | 24551,60 | 9,53 | 1-24 | thaumatin family (PF00314) |
| T | 31 | At4g29270 | homologous to acid phosphatase | 25,4 | 4 | 25900,84 | 9,15 | 1-26 | HAD superfamily, subfamily IIIB (acid phosphatase) (PF03767) |
| T | 31-35, 37, 38 | At4g12880 | early nodulin (AtEN20) (phytoecyanin) | 44,7 | 8 | 14273,34 | 9,64 | 1-18 | plastocyanin-like domain (PF02298) (copper binding proteins) |
| T | 32-33 | At1g20190 | alpha-expansin (AtEXPA11) | 19,8 | 5 | 24817,74 | 9,84 | 1-20 | expansin, family-45 endoglucanase-like domain profile (PS50842), expansin, cellulose-binding-like domain profile (PS50843), pollen allergen (PF01357) |
| T | 32 | At1g09560 | germin (subfamily 2, member 1, GLP5) | 14,6 | 4 | 20472,37 | 9,71 | 1-23 | germin (PS00725), cupin (PF00190) |
| T | 34-37 | At5g66590 | homologous to <i>Nicotiana tabacum</i> pathogenesis-related protein PR1 | 38,9 | 5 | 18040,82 | 9,65 | 1-19 | SCP-like extracellular protein (PF00188) (sperm coating glycoprotein) |
| T | 34 | At1g73260 | inhibitor family I3 (Kunitz-P family) (subfamily I3A unassigned peptidase inhibitor homologues, MEROPS) | 19,1 | 3 | 20982,78 | 5,20 | 1-26 | trypsin and protease inhibitor Kunitz legume (PF00197) |
| T | 35 | At3g02110 | homologous to serine carboxypeptidase D (SCPL25) (Peptidase family S10, S10.005, MEROPS) | 20,0 | 4 | 51075,46 | 9,35 | 1-22 | serine carboxypeptidases, histidine active site (PS00560), serine carboxypeptidases, serine active site (PS00131), serine carboxypeptidase (PF00450) |
| T | 36, 37 | At1g17860 | inhibitor family I3 (Kunitz-P family) (subfamily I3A unassigned peptidase inhibitor homologues, MEROPS) | 25,5 | 4 | 19718,68 | 9,76 | 1-21 | soybean trypsin inhibitor (Kunitz) protease inhibitors family signature (PS00283), trypsin and protease inhibitor (PF00197) |
| T | 44-46 | At2g38530 | non-specific lipid transfer protein type 1 (LTP2) | 40,0 | 5 | 9475,92 | 11,90 | 1-25 | protease inhibitor/seed storage/LTP family (PF00234), plant lipid transfer proteins signature (PS00597) |
| T | 44-46 | At4g16500 | inhibitor family I25 (cystatin family) (subfamily I25B unassigned peptidase inhibitor homologues, MEROPS) | 50,0 | 8 | 10183,74 | 9,78 | 1-22 | cystatin domain (PF00031) |
| T | 45 | At5g05110 | inhibitor family I25 (phytoecyanin) (cystatin family, I25.014, MEROPS) | 16,8 | 4 | 23934,58 | 8,48 | 1-24 | cysteine proteases inhibitors signature (PS00287), cystatin domain (PF00031) |
| U | 1-40 | At5g11420 | expressed protein (DUF642) | 30,0 | 13 | 37344,57 | 7,70 | 1-22 | domain of unknown function DUF 642 (PF04862) |
| U | 1-14, 16-40 | At5g25460 | expressed protein (DUF642) | 30,0 | 12 | 37948,52 | 9,03 | 1-19 | domain of unknown function DUF 642 (PF04862) |
| U | 2-7 | At1g28290 | proline-rich protein | 6,4 | 5 | 35913,36 | 10,77 | 1-24 | histidine-rich region profile (PS50316), proline-rich region profile (PS50099), pollen proteins Ole e 1 family (PF01190) |
| U | 3 | At5g59370* | actin (ACT4) | 14,1 | 5 | 41778,87 | 5,80 | | actins and actin-related proteins signature (PS01132), actins signature 1 (PS00406), actins signature 2 (PS00432), actin (PF00022) |
| U | 3 | At3g46520* | actin (ACT12) | 14,1 | 5 | 41794,87 | 5,20 | | actins and actin-related proteins signature (PS01132), actins signature 1 (PS00406), actins signature 2 (PS00432), actin (PF00022) |
| U | 3 | At2g37620# | actin (ACT1) | 13,5 | 5 | 41797,89 | 5,12 | | actins and actin-related proteins signature (PS01132), actins signature 1 (PS00406), actins signature 2 (PS00432), actin (PF00022) |
| U | 3 | At3g53750# | actin (ACT3) | 13,5 | 5 | 41797,89 | 5,12 | | actins and actin-related proteins signature (PS01132), actins signature 1 (PS00406), actins signature 2 (PS00432), actin (PF00022) |
| U | 8 | At3g13790 | glycoside hydrolase family 32 (beta-fructofuranosidase) | 16,4 | 9 | 63490,72 | 9,68 | 1-25 | glycoside hydrolases family 32 active site (PS00609), glycoside hydrolases family 32 (PF00251) |
| U | 11, 12, 20 | At1g03230 | homologous to carrot EDGP and tomato XEGIP | 35,9 | 11 | 43723,02 | 10,27 | 1-23 | |
| U | 11 | At1g03220 | homologous to carrot EDGP and tomato XEGIP | 12,5 | 4 | 43394,37 | 9,99 | 1-22 | |
| U | 11 | At4g16260 | glycoside hydrolase family 17 | 12,4 | 4 | 35905,19 | 6,64 | 1-16 | glycoside hydrolases family 17 signature (PS00587), glycoside hydrolases family 17 (PF00332) |
| U | 13, 14 | At5g19110 | homologous to carrot EDGP and tomato XEGIP | 23,9 | 9 | 41369,63 | 10,01 | 1-19 | |
| U | 13, 14 | At3g43270 | carbohydrate esterase family 8 (pectin methylesterase) | 20,0 | 8 | 54929,20 | 9,55 | 1-24 | pectinesterase (PF01095), plant invertase/pectin methylesterase inhibitor (PMEI) (PF04043) |
| U | 14 | At5g07030 | homologous to Asp protease (pepsin family) (Peptidase family A1, subfamily A1B unassigned peptidases, MEROPS) | 10,0 | 6 | 44860,54 | 10,82 | 1-19 | eukaryotic aspartyl protease (PF00026) |
| U | 16, 17 | At3g20820 | expressed protein (LRR domains) | 35,9 | 10 | 37850,70 | 9,90 | 1-19 | typical LRR profile (PS50506), plant specific LRR profile (PS50502), SDS22+like LRR profile (PS50504) |
| U | 16-19, 21 | At5g12940 | homologous to <i>Phaseolus vulgaris</i> PGIP2 (LRR domains) | 40,0 | 15 | 36784,96 | 10,45 | 1-29 | plant specific LRR profile (PS50502), Leucine Rich Repeat (PF00560) |
| U | 16 | At1g10550 | glycoside hydrolase family 16 (endoxyloglucan transferase) (AtXTH33) | 19,7 | 6 | 32314,04 | 9,73 | 1-21 | glycoside hydrolases family 16 (PF00722), xyloglucan endo-transglycosidase (XET) C-terminus (PF06955) |
| U | 21, 22, 24 | At2g06850 | glycoside hydrolase family 16 (endoxyloglucan transferase) (AtXTH4) | 22,3 | 8 | 31716,77 | 9,65 | 1-24 | glycoside hydrolases family 16 (PF00722) |
| U | 22 | At4g30170 | peroxidase (AtPrx45) | 24,3 | 6 | 33052,57 | 10,43 | 1-25 | haem peroxidase, plant/fungal/bacterial (PS00435, PS00436, PS50873) |

Table S2, supplementary data

| | | | | | | | | | | | |
|---|-----------------|------------|---|------|--|----------|----------|-------|------------------------|--|---|
| W | 1 | At2g30210 | homologous to laccase (AtLAC3) | 14,2 | 7 | 61172,59 | 10,11 | 1-25 | | multicopper oxidases signature 2 (PS00080), multicopper oxidase (PF00394) | |
| W | 2, 5 | At1g28290 | proline-rich protein | 9,2 | 4 | 35913,36 | 10,77 | 1-24 | | histidine-rich region profile (PS50316), proline-rich region profile (PS50099), pollen proteins Ole e 1 family (PF01190) | |
| W | 6 | At3g43270 | carbohydrate esterase family 8 (pectin methyltransferase) | 20,0 | 10 | 54929,20 | 9,55 | 1-24 | | pectinesterase (PF01095), plant invertase/pectin methyltransferase inhibitor (PF04043) | |
| W | 6 | At5g07030 | homologous to Asp protease (pepsin family) (Peptidase family A1, subfamily A1B unassigned peptidases, MEROPS) | 20,0 | 8 | 44860,54 | 10,82 | 1-19 | | eukaryotic aspartyl protease (PF00026) | |
| W | 6, 7, 18 | At5g11420 | expressed protein (DUF642) | 30,0 | 11 | 37344,57 | 7,70 | 1-22 | | domain of unknown function DUF 642 (PF04862) | |
| W | 7-9, 12, 17, 18 | At5g25460 | expressed protein (DUF642) | 20,0 | 7 | 37948,52 | 9,03 | 1-19 | | domain of unknown function DUF 642 (PF04862) | |
| W | 8 | At3g20820 | expressed protein (LRR domains) | 10,0 | 4 | 37850,70 | 9,90 | 1-19 | | typical LRR profile (PS50506), plant specific LRR profile (PS50502), SDS22+like LRR profile (PS50504) | |
| W | 9, 11 | At5g12940 | homologous to <i>Phaseolus vulgaris</i> PGIP2 (LRR domains) | 20,0 | 6 | 36784,96 | 10,45 | 1-29 | | plant specific LRR profile (PS50502), Leucine Rich Repeat (PF00560) | |
| W | 10, 11 | At5g14920 | proline-rich protein | | Edman sequencing + MALDI-TOF-TOF | 3 | 26753,23 | 10,98 | 1-21 | | gibberellin regulated protein (PF02704) |
| W | 12 | At3g14310 | carbohydrate esterase family 8 (pectin methyltransferase) (PME3) | 19,7 | 7 | 60050,61 | 9,59 | 1-40 | trans-membrane domains | pectinesterase signature 1 (PS00800), pectinesterase signature 2 (PS00503), pectinesterase (PF01095), plant invertase/pectin methyltransferase inhibitor (PF04043) | |
| W | 13 | At5g64260 | homologous to <i>Nicotiana tabacum</i> phi-I | 11,0 | 4 | 30550,86 | 9,98 | 1-19 | | phosphate-induced protein 1 conserved region (PF04674) | |
| W | 14 | At5g09440 | homologous to <i>Nicotiana tabacum</i> phi-I | 17,3 | 5 | 27366,44 | 10,15 | 1-23 | | phosphate-induced protein 1 conserved region (PF04674) | |
| W | 15, 16 | At1g20190 | alpha-expansin (AtEXPA11) | 19,8 | 5 | 24817,74 | 9,84 | 1-20 | | expansin, family-45 endoglucanase-like domain profile (PS50842), expansin, cellulose-binding-like domain profile (PS50843), pollen allergen (PF01357) | |
| W | 16 | At4g12880 | early nodulin (AtEN20) (phytolectin) | 33,6 | 4 | 14273,34 | 9,64 | 1-18 | | plastocyanin-like domain (PF02298) (copper binding proteins) | |
| W | 19, 20 | At4g27150* | 2S albumin isoform 2 (protease inhibitor/seed storage/LTP family) | 13,5 | 2 | 17473,78 | 6,34 | 1-17 | | protease inhibitor/seed storage/LTP family (PF00234) | |
| W | 19-21 | At4g27160* | 2S albumin isoform 3 (protease inhibitor/seed storage/LTP family) | 14,0 | 2 | 16909,07 | 7,69 | 1-17 | | protease inhibitor/seed storage/LTP family (PF00234) | |
| X | 1 | At1g28290 | proline-rich protein | 6,4 | 3 | 35913,36 | 10,77 | 1-24 | | histidine-rich region profile (PS50316), proline-rich region profile (PS50099), pollen proteins Ole e 1 family (PF01190) | |
| X | 2, 3 | At5g11420 | expressed protein (DUF642) | 11,7 | 5 | 37344,57 | 7,70 | 1-22 | | domain of unknown function DUF 642 (PF04862) | |
| X | 4 | At3g20820 | expressed protein (LRR domains) | 7,7 | 3 | 37850,70 | 9,90 | 1-19 | | typical LRR profile (PS50506), plant specific LRR profile (PS50502), SDS22+like LRR profile (PS50504) | |
| X | 4 | At5g25460 | expressed protein (DUF642) | 12,5 | 4 | 37948,52 | 9,03 | 1-19 | | domain of unknown function DUF 642 (PF04862) | |
| X | 5 | At5g14920 | proline-rich protein | | Edman sequencing + MALDI-TOF-TOF | 3 | 26753,23 | 10,98 | 1-21 | | gibberellin regulated protein (PF02704) |
| X | 6 | At4g27170 | 2S albumin isoform 4 (protease inhibitor/seed storage/LTP family) | 15,1 | 3 | 16946,26 | 7,80 | 1-21 | | protease inhibitor/seed storage/LTP family (PF00234) | |
| Y | 1 | At5g11420 | expressed protein (DUF642) | 10,0 | 5 | 37344,57 | 7,70 | 1-22 | | domain of unknown function DUF 642 (PF04862) | |
| Y | 2 | At5g25460 | expressed protein (DUF642) | 10,0 | 4 | 37948,52 | 9,03 | 1-19 | | domain of unknown function DUF 642 (PF04862) | |
| Y | 3 | At5g14920 | proline-rich protein | | Edman sequencing + MALDI-TOF-TOF MS ^c | 3 | 26753,23 | 10,98 | 1-21 | | gibberellin regulated protein (PF02704) |

a: The following criteria were retained for protein identification: at least 4 peptides are required for proteins larger than 15 kDa, only 3 peptides can be used for proteins smaller than 15 kDa. Allowed modifications are 1 miscleavage and Met oxidation. In each series of samples (i.e., total and M to Z), the best score of identification is shown.

b: Functional domains were predicted as described in Material and methods. PF stands for PFAM, PS for PROSITE and IPR for InterPro.

c: N-terminal sequencing, XXTLPS; LC-MS/MS sequencing, CGQHSR.

Table S3, supplementary data

| Gene ID | Gene Name | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|--|-----------|--|---|---|---|---|---|---|---|---|----|----|----|
| oxido-reductases | | | | | | | | | | | | | |
| 33 | At1g71695 | peroxidase (AtPrx12) | | | | | | | | 1 | | | |
| 34 | At3g21770 | peroxidase (AtPrx30) | 1 | | | | | | | 1 | | | |
| 35 | At3g32980 | peroxidase (AtPrx32) | | 1 | 1 | 1 | 1 | | | | | | |
| 36 | At3g49110 | peroxidase (AtPrx33) | | | | | | | | | 1 | 1 | |
| 37 | At3g49120 | peroxidase (AtPrx34) | | | | | | | | | 1 | 1 | |
| 38 | At3g50990 | peroxidase (AtPrx36) | | | | | | | | | | | |
| 39 | At5g25980 | peroxidase (AtPrx43) | | | | | | | | | 1 | 1 | |
| 40 | At4g30170 | peroxidase (AtPrx45) | | | | | | | | 1 | | | |
| 41 | At5g17820 | peroxidase (AtPrx57) | | | | | | | | | | 1 | 1 |
| 42 | At5g64100 | peroxidase (AtPrx69) | | | | | | | | | | | 1 |
| 43 | At5g66390 | peroxidase (AtPrx72) | | | | | | | | | | | 1 |
| 44 | At2g30210 | homologous to laccase (ATLAC3) | | | | | | | | | | | 1 |
| 45 | At1g30710 | homologous to berberine-bridge enzyme (S)-reticulin:oxygen oxidoreductase | | | | | | | | | | | 1 |
| 46 | At4g20860 | homologous to berberine-bridge enzyme (S)-reticulin:oxygen oxidoreductase | | | | | | | | | | | 1 |
| 47 | At5g44360 | homologous to berberine-bridge enzyme (S)-reticulin:oxygen oxidoreductase | | | | | | | | | | | 1 |
| 48 | At5g44410 | homologous to berberine-bridge enzyme (S)-reticulin:oxygen oxidoreductase | | | | | | | | | | | 1 |
| 49 | At1g01980 | homologous to berberine-bridge enzyme (S)-reticulin:oxygen oxidoreductase | | | | | | | | | | | 1 |
| 50 | At2g02850 | plantacyanin (AtPNC) (phytoeyanin) | | | | | | | | | | | 1 |
| 51 | At4g12880 | early nodulin (AtEN20) (phytoeyanin) | | | | | | | | | | | 1 |
| 52 | At5g22140 | expressed protein (oxido-reductase domain) | | | | | | | | | | | 1 |
| 53 | At5g56490 | expressed protein (FAD binding domain) | | | | | | | | | | | 1 |
| proteins with interacting domains | | | | | | | | | | | | | |
| 54 | At1g53070 | homologous to lectin (legume lectin domains) | | | | | | | | | | | 1 |
| 55 | At1g78820 | homologous to lectin (curculin-like) | | | | | | | | | | | 1 |
| 56 | At1g78830 | homologous to lectin (curculin-like) | | | | | | | | | | | 1 |
| 57 | At1g78850 | homologous to lectin (curculin-like) | | | | | | | | | | | 1 |
| 58 | At1g78860 | homologous to lectin (curculin-like) | | | | | | | | | | | 1 |
| 59 | At5g06860 | PGIP1 (LRR domains) | | | | | | | | | | | 1 |
| 60 | T23B7.10 | homologous to PGIP1 (LRR protein FLR1) | | | | | | | | | | | 1 |
| 61 | At5g12940 | homologous to <i>Phaseolus vulgaris</i> PGIP2 (LRR domains) | | | | | | | | | | | 1 |
| 62 | At1g33590 | expressed protein (LRR domains) | | | | | | | | | | | 1 |
| 63 | At2g34930 | expressed protein (LRR domains) | | | | | | | | | | | 1 |
| 64 | At3g20820 | expressed protein (LRR domains) | | | | | | | | | | | 1 |
| 65 | At2g17120 | expressed protein (LysM domain) | | | | | | | | | | | 1 |
| 66 | At1g03220 | homologous to carrot EDGP and tomato XEGIP | | | | | | | | | | | 1 |
| 67 | At1g03230 | homologous to carrot EDGP and tomato XEGIP | | | | | | | | | | | 1 |
| 68 | At5g19110 | homologous to carrot EDGP and tomato XEGIP | | | | | | | | | | | 1 |
| 69 | At1g47710 | homologous to serpin (serine protease inhibitor) | | | | | | | | | | | 1 |
| 70 | At1g17860 | inhibitor family I3 (Kunitz-P family) (subfamily I3A unassigned peptidase inhibitor homologues, MEROPS) | | | | | | | | | | | 1 |
| 71 | At1g73260 | inhibitor family I3 (Kunitz-P family) (subfamily I3A unassigned peptidase inhibitor homologues, MEROPS) | | | | | | | | | | | 1 |
| 72 | At1g47540 | inhibitor family I18 (mustard trypsin inhibitor-2 family) (family I18 unassigned peptidase inhibitor homologues, MEROPS) | | | | | | | | | | | 1 |
| 73 | At2g40880 | inhibitor family I25 (phytostatin) (cystatin family, I25.014, MEROPS) | | | | | | | | | | | 1 |
| 74 | At5g05110 | inhibitor family I25 (phytostatin) (cystatin family, I25.014, MEROPS) | | | | | | | | | | | 1 |
| 75 | At4g16500 | inhibitor family I25 (cystatin family) (subfamily I25B unassigned peptidase inhibitor homologues, MEROPS) | | | | | | | | | | | 1 |
| 76 | At4g25260 | homologous to plant invertase/pectin methylesterase inhibitor (PMEI) | | | | | | | | | | | 1 |
| 77 | At5g46940 | homologous to plant invertase/pectin methylesterase inhibitor (PMEI) | | | | | | | | | | | 1 |
| 78 | At5g46960 | homologous to plant invertase/pectin methylesterase inhibitor (PMEI) | | | | | | | | | | | 1 |

Table S4, supplementary material. Semi-quantification of CWP identified by MALDI-TOF MS from 5- and 11-day-old etiolated *Arabidopsis* hypocotyls.

A quantification index (QI) is attributed to each protein in each sample (5- and 11-day-old etiolated hypocotyls). It was calculated by adding the percentages of coverage by peptide mass mapping in all the bands in which the protein was identified. When the protein was only identified in the total extracts after 1D-E (1D-separation), the values obtained for this identification were retained. When the protein was identified only after cation exchange chromatography and 1D-E of the eluted fractions (2D-separation), the values obtained for this identification were retained. When the protein was identified in both cases, only the values corresponding to the latter were taken into account.

Only differences higher than 2-fold were considered as significant and are indicated in bold red.

CWPs identified only in 5-day-old etiolated hypocotyls
 CWPs identified only in 11-day-old etiolated hypocotyls
 nd QI was not calculated since the protein was identified in the same separation conditions in the two samples.

| accession AGI | annotation | 5 day-old hypocotyls QI | 11day-old hypocotyls QI | 1D- and 2D-separations | 1D-separation only | 2D-separation only |
|---|--|-------------------------|-------------------------|------------------------|--------------------|--------------------|
| proteins acting on carbohydrates | | | | | | |
| At2g06850 | glycoside hydrolase family 16 (endoxyloglucan transferase) (AtXTH4) | 4,59 | 1,35 | 1 | | |
| At5g13870 | glycoside hydrolase family 16 (endoxyloglucan transferase) (AtXTH5) | 0,40 | | | | 1 |
| At3g44990 | glycoside hydrolase family 16 (endoxyloglucan transferase) (AtXTH31) | 0,41 | | | | 1 |
| At2g36870 | glycoside hydrolase family 16 (endoxyloglucan transferase) (AtXTH32) | | 0,16 | | | 1 |
| At1g10550 | glycoside hydrolase family 16 (endoxyloglucan transferase) (AtXTH33) | 0,56 | 0,20 | | | 1 |
| At4g16260 | glycoside hydrolase family 17 | | 0,32 | | | 1 |
| At4g19810 | glycoside hydrolase family 18 (chitinase) | 0,74 | 0,18 | | | 1 |
| At3g12500 | glycoside hydrolase family 19 (chitinase) | 0,34 | 0,41 | | | 1 |
| At3g55260 | glycoside hydrolase family 20 (beta-hexosaminidase) | 0,17 | | | | 1 |
| At1g19170 | glycoside hydrolase family 28 (polygalacturonase) | 0,21 | | | 1 | |
| At4g18180 | glycoside hydrolase family 28 (polygalacturonase) | 0,25 | | | | 1 |
| At3g16850 | glycoside hydrolase family 28 (polygalacturonase) | 3,77 | 2,38 | 1 | | |
| At3g06770 | glycoside hydrolase family 28 (polygalacturonase) | | 0,28 | | | 1 |
| At3g61490 | glycoside hydrolase family 28 (polygalacturonase) | 0,39 | 0,33 | | | 1 |
| At2g33160 | glycoside hydrolase family 28 (polygalacturonase) | 0,08 | | | | 1 |
| At1g68560 | glycoside hydrolase family 31 (alpha-xylosidase) (AtXYL1) | 1,62 | 0,82 | 1 | | |
| At3g13790 | glycosyl hydrolase family 32 (beta-fructofuranosidase) | 0,09 | 0,16 | | | 1 |
| At5g34940 | glycosyl hydrolase family 79 (endo-beta-glucuronidase/heparanase) | 0,94 | 0,11 | 1 | | |
| At1g53830 | carbohydrate esterase family 8 (pectin methylesterase) (AtPME2) | 0,78 | 0,10 | | | 1 |
| At3g14310 | carbohydrate esterase family 8 (pectin methylesterase) (AtPME3) | 1,08 | 0,65 | 1 | | |
| At3g43270 | carbohydrate esterase family 8 (pectin methylesterase) | 2,24 | 1,23 | 1 | | |
| At4g33220 | carbohydrate esterase family 8 (pectin methylesterase) | 0,42 | 0,77 | 1 | | |
| At1g11580 | carbohydrate esterase family 8 (pectin methylesterase) | 0,96 | | | | 1 |
| At5g53370 | carbohydrate esterase family 8 (pectin methylesterase) | 0,10 | | | | 1 |
| At4g37950 | polysaccharide lyase family 4 (rhamnogalacturonate lyase) | | 0,90 | 1 | | |
| At1g05570 | glycoside transferase family 48 (callose synthase 1) (AtCalS1) | 0,04 | 0,03 | | | 1 |
| At5g02260 | alpha-expansin (ATEXP9) | 1,22 | 0,43 | | | 1 |
| At1g20190 | alpha-expansin (ATEXPA11) | 2,22 | 2,59 | 1 | | |
| At5g39270 | alpha-expansin (ATEXPA22) | 0,11 | | | | 1 |
| At3g45970 | expansin-like A (ATEXLA1) | 3,45 | 2,04 | 1 | | |
| At4g38400 | expansin-like A (ATEXLA2) | 1,48 | 0,67 | 1 | | |

Table S4, supplementary data

| | | | | | | |
|-------------------------|--|-------|------|---|---|---|
| At3g45960 | expansin-like A (ATEXLA3) | 0,14 | | | 1 | |
| oxido-reductases | | | | | | |
| At1g71695 | peroxidase (AtPrx12) | 0,27 | 0,87 | | | 1 |
| At3g21770 | peroxidase (AtPrx30) | 0,15 | 0,15 | | 1 | |
| At3g32980 | peroxidase (AtPrx32) | 3,18 | 0,87 | | | 1 |
| At3g49110 | peroxidase (AtPrx33) | | 0,16 | | | 1 |
| At3g49120 | peroxidase (AtPrx34) | 1,53 | 2,04 | 1 | | |
| At3g50990 | peroxidase (AtPrx36) | 0,10 | | | 1 | |
| At4g25980 | peroxidase (AtPrx43) | | 0,19 | | 1 | |
| At4g30170 | peroxidase (AtPrx45) | 1,92 | 2,57 | 1 | | |
| At5g17820 | peroxidase (AtPrx57) | 0,23 | | | 1 | |
| At5g64100 | peroxidase (AtPrx69) | 0,35 | 0,26 | 1 | | |
| At5g66390 | peroxidase (AtPrx72) | | 0,19 | | | 1 |
| At2g30210 | homologous to laccase (AtLAC3) | 0,40 | 0,56 | | | 1 |
| At4g20860 | homologous to berberine-bridge enzyme (S)-reticul: oxygen oxidoreductase | | 0,16 | 1 | | |
| At5g44360 | homologous to berberine-bridge enzyme (S)-reticul: oxygen oxidoreductase | 0,08 | 0,16 | | | 1 |
| At5g44410 | homologous to berberine-bridge enzyme (S)-reticul: oxygen oxidoreductase | | 0,12 | | | 1 |
| At1g30710 | homologous to berberine-bridge enzyme (S)-reticul: oxygen oxidoreductase | 0,16 | | | | 1 |
| At1g09610 | homologous to berberine-bridge enzyme (S)-reticul: oxygen oxidoreductase | | 0,12 | | | |
| At4g12880 | early nodulin (AtEN20) (phytyocyanin) | 11,84 | 5,66 | 1 | | |
| At2g02850 | plantacyanin (AtPNC) (phytyocyanin) | | 0,20 | | | 1 |
| At5g22140 | expressed protein (oxidoreductase domain) | 0,28 | | | | 1 |
| At5g56490 | expressed protein (FAD binding domain) | 0,15 | | | | 1 |
| proteases | | | | | | |
| At5g07030 | homologous to aspartic protease (pepsin family) (Peptidase family A1, subfamily A1B unassigned peptidases, MEROPS) | 1,43 | 0,86 | 1 | | |
| At1g09750 | homologous to aspartic protease (pepsin family) (Peptidase family A1, subfamily A1B unassigned peptidases, MEROPS) | 1,94 | 0,97 | 1 | | |
| At3g54400 | homologous to aspartic protease (pepsin family) (Peptidase family A1, subfamily A1B unassigned peptidases, MEROPS) | 0,59 | 0,78 | 1 | | |
| At5g10770 | homologous to aspartic protease (CND41 peptidase) (Peptidase family A1, A01.050, MEROPS) | 0,34 | 0,42 | | | 1 |
| At3g02740 | homologous to aspartic protease (pepsin family) (Peptidase family A1, subfamily A1B unassigned peptidases, MEROPS) | 0,14 | | | | 1 |
| At1g79720 | homologous to aspartic protease (CND41 peptidase) (Peptidase family A1, subfamily A1.050, MEROPS) | 0,20 | | | | 1 |
| At3g52500 | homologous to aspartic protease (pepsin family) (Peptidase family A1, subfamily A1B unassigned peptidases, MEROPS) | 0,28 | | | | 1 |
| At5g43060 | homologous to cysteine proteinase (papain family) (RD21 peptidase, Peptidase family C1, C01.064 MEROPS) | 1,78 | 1,65 | 1 | | |
| At1g47128 | homologous to cysteine proteinase (papain family) (RD21A, Peptidase family C1, C01.064 MEROPS) | 0,73 | 1,30 | 1 | | |
| At4g01610 | homologous to cysteine proteinase (papain family) (Cathepsin B, Peptidase family C1, C01.049, MEROPS) | 1,43 | 1,50 | 1 | | |
| At4g36880 | homologous to cysteine proteinase (papain family) (Peptidase family C1, Brassicain, C01.021 MEROPS) | 0,16 | | | | 1 |
| At4g30610 | homologous to serine carboxypeptidase (BRS1 - Brassinosteroid-Insensitive BRI suppressor 1) (Peptidase family S10, S10.015 MEROPS) | 0,45 | | | | 1 |
| At3g02110 | homologous to serine carboxypeptidase D (SCPL25) (Peptidase family S10, S10.005, MEROPS) | 1,69 | 0,71 | 1 | | |
| At5g23210 | homologous to serine carboxypeptidase (SCPL34) (Peptidase family S10, S10.005, MEROPS) | | nd | 1 | | |

Table S4, supplementary data

| proteins with interacting domains | | | | | | |
|-----------------------------------|--|------|------|---|--|---|
| At1g53070 | homologous to lectin (legume lectin domains) | 0,71 | 0,16 | | | 1 |
| At1g78820 | homologous to lectin (curculin-like) | 0,39 | | | | 1 |
| At1g78830 | homologous to lectin (curculin-like) | 2,38 | 1,48 | | | |
| At1g78850 | homologous to lectin (curculin-like) | 1,93 | 1,98 | 1 | | |
| At1g78860 | homologous to lectin (curculin-like) | 0,84 | 0,24 | 1 | | |
| At5g06860 | PGIP1 | 1,16 | 1,91 | | | 1 |
| T23B7.10 | homologous to PGIP1 (LRR protein FLR1) | 0,61 | 1,55 | 1 | | |
| At5g12940 | homologous to <i>Phaseolus vulgaris</i> PGIP2 (LRR domains) | 3,85 | 4,44 | 1 | | |
| At1g33590 | expressed protein (LRR domains) | 1,19 | 1,11 | 1 | | |
| At2g34930 | expressed protein (LRR domains) | 0,46 | 0,24 | | | 1 |
| At3g20820 | expressed protein (LRR domains) | 2,15 | 5,12 | 1 | | |
| At2g17120 | expressed protein (LysM domain) | 1,18 | 0,35 | | | 1 |
| At5g19110 | homologous to carrot EDGP and to tomato XEGIP | 0,84 | 0,39 | | | 1 |
| At1g03220 | homologous to carrot EDGP and to tomato XEGIP | 2,37 | 4,02 | 1 | | |
| At1g03230 | homologous to carrot EDGP and to tomato XEGIP | 1,65 | 1,55 | 1 | | |
| At1g47710 | homolog to serpin (serine protease inhibitor) | 1,33 | 0,20 | | | 1 |
| At1g17860 | inhibitor family I3 (Kunitz-P family) (subfamily I3A unassigned peptidase inhibitor homologues, MEROPS) | 1,78 | 1,81 | 1 | | |
| At1g73260 | inhibitor family I3 (Kunitz-P family) (subfamily I3A unassigned peptidase inhibitor homologues, MEROPS) | 0,30 | 5,13 | 1 | | |
| At1g47540 | inhibitor family I18 (mustard trypsin inhibitor-2 family) (family I18 unassigned peptidase inhibitor homologues, MEROPS) | nd | nd | 1 | | |
| At2g40880 | inhibitor family I25 (phytolectin) (cystatin family, I25.014, MEROPS) | 0,22 | 0,31 | 1 | | |
| At5g05110 | inhibitor family I25 (phytolectin) (cystatin family, I25.014, MEROPS) | 0,10 | 0,17 | | | 1 |
| At4g16500 | inhibitor family I25 (cystatin family) (subfamily I25B unassigned peptidase inhibitor homologues, MEROPS) | 2,12 | 5,61 | | | 1 |
| At4g25260 | homologous to plant invertase/ pectin methylesterase inhibitor (PMEI) | 0,34 | 0,17 | | | 1 |
| At5g46940 | homologous to plant invertase/pectine methylesterase inhibitor (PMEI) | 0,19 | | | | 1 |
| At5g46960 | homologous to plant invertase/pectine methylesterase inhibitor (PMEI) | 0,44 | | | | 1 |
| structural proteins | | | | | | |
| At1g28290 | proline-rich protein | 2,61 | 2,14 | 1 | | |
| At5g14920 | proline-rich protein | 0,17 | 0,29 | | | 1 |
| At2g05580 | glycine-rich protein | 0,74 | 1,61 | 1 | | |
| At4g13340 | LRR-extensin (AtLRX3) | 0,08 | | | | 1 |
| At3g24480 | LRR-extensin (AtLRX4) | 0,12 | 0,33 | | | 1 |
| At4g18670 | LRR-extensin (AtLRX5) | 0,07 | 0,11 | | | 1 |
| signaling | | | | | | |
| At4g05200 | homologous to receptor-kinase (RLK, DUF26-1b subfamily) | 0,09 | | | | 1 |
| At5g55730 | fasciclin-like arabinogalactan protein (AtFLA1) | 0,19 | 0,58 | | | 1 |

Table S4, supplementary data

| proteins related to lipid metabolism | | | | | | |
|--------------------------------------|---|-------|-------|---|---|---|
| At1g29670 | homologous to lipase/acylhydrolase (GDSL family) | 2,27 | 0,75 | 1 | | |
| At1g54010 | homologous to lipase/acylhydrolase (GDSL family) | 1,67 | | | | 1 |
| At1g54030 | homologous to lipase/acylhydrolase (GDSL family) | 0,12 | 0,23 | | 1 | |
| At5g15720 | homologous to lipase/acylhydrolase (GDSL family) | | 0,13 | | | 1 |
| At3g48460 | homologous to lipase/acylhydrolase (GDSL family) | 0,98 | 0,21 | | | 1 |
| At2g38530 | non-specific lipid transfer protein type 1 (LTP2) | 5,26 | 9,07 | 1 | | |
| At5g23820 | expressed protein (ML domain - MD-2-related lipid recognition domain) | 0,80 | | | | 1 |
| At2g16001 | expressed protein (lipid recognition domain) | 0,61 | | | | 1 |
| miscellaneous functions | | | | | | |
| At2g28790 | homologous to <i>Lycopersicon esculentum</i> osmotin | 1,06 | 0,96 | 1 | | |
| At5g15230 | gibberellin-regulated protein (GASA4) | 0,47 | | | | 1 |
| At3g07130 | homologous to <i>Glycine max</i> phytase and to purple acid phosphatase | 0,25 | 0,11 | | | 1 |
| At2g27190 | homologous to purple acid phosphatase (PAP1) | 0,60 | 0,24 | 1 | | |
| At5g34850 | homologous to purple acid phosphatase | 0,38 | | | | 1 |
| At4g29270 | homologous to acid phosphatase | 2,53 | 0,79 | 1 | | |
| At4g24340 | homologous to phosphorylase | 0,33 | | | | 1 |
| At3g02870 | homologous to myo-inositol monophosphatase | 0,34 | | | | 1 |
| At5g09440 | homologous to <i>N. tabacum</i> phi-I | 3,35 | 2,52 | 1 | | |
| At5g64260 | homologous to <i>N. tabacum</i> phi-I | 2,37 | 1,67 | 1 | | |
| At4g27110 | homologous to COBRA (AtCOBL10) | 0,08 | | | | 1 |
| At5g66590 | homologous to <i>Nicotiana tabacum</i> pathogenesis-related protein PR1 | 7,86 | 5,2 | 1 | | |
| At1g09560 | germin (subfamily 2, member 1, GLP5) | | 0,29 | | | 1 |
| unknown function | | | | | | |
| At3g56750 | expressed protein | | 0,09 | | 1 | |
| At3g22000 | expressed protein (DUF26) | | 0,15 | | 1 | |
| At1g26850 | expressed protein (DUF248) | | 0,07 | | | 1 |
| At1g80240 | expressed protein (DUF642) | nd | nd | 1 | | |
| At3g08030 | expressed protein (DUF642) | 0,73 | 0,76 | | 1 | |
| At4g32460 | expressed protein (DUF642) | 1,21 | 0,50 | 1 | | |
| At5g11420 | expressed protein (DUF642) | 20,05 | 24,30 | 1 | | |
| At5g25460 | expressed protein (DUF642) | 20,12 | 23,77 | 1 | | |
| At3g20370 | expressed protein (MATH domain) | 0,57 | | | | 1 |
| At2g04690 | expressed protein (homologous to a human CREG protein) | 1,07 | 1,11 | 1 | | |
| At1g78460 | expressed protein (SOUL heme binding domain) | | 0,17 | | | 1 |
| At2g34700 | expressed protein (Ole e1 allergen domain) | 1,51 | 1,00 | | | 1 |
| At2g15220 | expressed protein (Plant Basic Secreted Protein domain) | 0,30 | 0,85 | | | 1 |
| At2g28490 | expressed protein (cupin domain) | 1,23 | 0,09 | 1 | | |
| At3g22640 | expressed protein (cupin domain) | 0,12 | 0,09 | 1 | | |
| At4g36700 | expressed protein (cupin domain) | 1,96 | | | | 1 |

SUPPLEMENTARY DATA CHAPTER 5

Additional data file 1

Table 1. Cell Wall Genes (CWGs) with detectable levels of transcripts in 5- and 11-day-old hypocotyls. CWGs were selected according to several papers and databases as mentioned at the bottom of the Table. The intensity of the signal is expressed as \log_2 , the ratio between the levels of transcripts at 11 days and 5 days as well as the p-value are indicated. CWGs are classified by gene family and level of transcripts: below background in black, low level in blue (between background and 9), moderate level in green (between 9 and 10) and high level in red (higher than 10). Significant Bonferroni p-values are highlighted in green ($p < 5\%$)

| Gene family | AGI number | Predicted or known gene function | CATMA probe | 5 day-old hypocotyls | 11 day-old hypocotyls | ratio 11 days / 5 days | p-value |
|---|-------------|----------------------------------|--------------|----------------------|-----------------------|------------------------|----------|
| prolyl-4-hydroxylases ^a | AT5G18900 | | CATMA5A17270 | 9,67 | 9,60 | -0,07 | 1,00 |
| | AT2G43080 | AtP4H1 | CATMA2A41480 | 8,77 | 8,77 | -0,01 | 1,00 |
| | AT4G33910 | | CATMA4A35710 | 8,70 | 7,96 | -0,74 | 1,85E-02 |
| | AT1G20270 | | CATMA1A19270 | 7,54 | 7,62 | 0,08 | 1,00 |
| | AT3G28480 | | CATMA3A28370 | 7,23 | 7,07 | -0,16 | 1,00 |
| | AT4G25600 | | CATMA4A27290 | 6,87 | 7,00 | 0,13 | 1,00 |
| nucleotide-sugar interconversion pathway ^b | AT5G39320 | UGD1 | CATMA5A34920 | 11,43 | 11,03 | -0,40 | 1,00 |
| | AT1G08200 | AXS2 | CATMA1A07190 | 10,34 | 9,98 | -0,36 | 1,00 |
| | AT5G15490 | UGD3 | CATMA5A13740 | 9,73 | 9,46 | -0,27 | 1,00 |
| | AT5G28840 | GME1 | CATMA5A26920 | 9,53 | 9,49 | -0,04 | 1,00 |
| | AT1G53500 | RHM2 | CATMA1A44530 | 9,11 | 8,83 | -0,28 | 1,00 |
| | AT1G17890 | GER2 | CATMA1A16900 | 9,00 | 8,83 | -0,18 | 1,00 |
| | AT4G30440 | GAE1 | CATMA4A32050 | 8,86 | 8,69 | -0,17 | 1,00 |
| | AT2G27860 | AXS1 | CATMA2A26260 | 8,75 | 8,68 | -0,07 | 1,00 |
| | AT5G66280 | GMD1 | CATMA5A61645 | 8,59 | 8,17 | -0,42 | 1,00 |
| | AT3G23820 | GAE6 | CATMA3A23780 | 8,54 | 8,51 | -0,02 | 1,00 |
| | AT3G46440 | SUD1 | CATMA3A39510 | 8,49 | 8,36 | -0,13 | 1,00 |
| | AT4G00110 | GAE3 | CATMA4A00110 | 7,89 | 7,81 | -0,08 | 1,00 |
| | AT1G73250 | GER1 | CATMA1A62530 | 7,82 | 8,02 | 0,20 | 1,00 |
| | AT1G30620 | UXE1 (MUR4) | CATMA1A28670 | 7,78 | 7,82 | 0,03 | 1,00 |
| | AT2G47650 | AUD2 | CATMA2A46090 | 7,75 | 7,61 | -0,13 | 1,00 |
| | AT1G02000 | GAE2 | CATMA1A00990 | 7,70 | 7,58 | -0,12 | 1,00 |
| | AT1G26570 | UGD4 | CATMA1A24800 | 7,58 | 7,31 | -0,27 | 1,00 |
| | AT2G34850 | UXE2 | CATMA2A32970 | 7,51 | 7,49 | -0,02 | 1,00 |
| | AT2G45310 | GAE4 | CATMA2A43710 | 7,46 | 7,71 | 0,25 | 1,00 |
| | AT1G12780 | UGE1 | CATMA1A11765 | 7,29 | 7,78 | 0,49 | 1,00 |
| AT1G64440 | UGE4 | CATMA1A53740 | 7,25 | 7,04 | -0,21 | 1,00 | |
| AT3G62830 | AUD | CATMA3A55995 | 7,21 | 7,06 | -0,15 | 1,00 | |
| AT1G63000 | UER1 | CATMA1A52170 | 7,18 | 7,08 | -0,10 | 1,00 | |
| AT3G51160 | GMD2 (MUR1) | CATMA3A44165 | 6,91 | 6,87 | -0,05 | 1,00 | |
| glycosyl transferases (GTs) | | | | | | | |
| GT8 ^c | AT5G18480 | Group E | CATMA5A16780 | 9,66 | 9,36 | -0,30 | 1,00 |
| | AT1G77130 | Group A | CATMA1A66340 | 9,40 | 8,95 | -0,45 | 1,00 |
| | AT2G20810 | Group D (GAUT10) | CATMA2A19380 | 9,11 | 8,64 | -0,47 | 1,00 |
| | AT3G62660 | Group C (GATL7) | CATMA3A55830 | 8,70 | 8,52 | -0,18 | 1,00 |
| | AT3G02350 | Group D (GAUT9) | CATMA3A01330 | 8,69 | 8,47 | -0,22 | 1,00 |
| | AT5G15470 | Group D (GAUT14) | CATMA5A13720 | 8,26 | 8,01 | -0,25 | 1,00 |
| | AT1G18580 | Group D (GAUT11) | CATMA1A17620 | 8,09 | 7,88 | -0,21 | 1,00 |
| | AT1G19300 | Group C (GATL1=Parvus) | CATMA1A18330 | 8,06 | 7,93 | -0,14 | 1,00 |
| | AT3G01040 | Group D (GAUT13) | CATMA3A00060 | 8,06 | 7,82 | -0,24 | 1,00 |
| | AT2G38650 | Group D (GAUT7) | CATMA2A36930 | 8,03 | 8,02 | -0,01 | 1,00 |
| | AT3G61130 | Group D (GAUT1) | CATMA3A54300 | 7,99 | 7,87 | -0,12 | 1,00 |
| | AT1G24170 | Group C (GATL8) | CATMA1A23060 | 7,89 | 7,94 | 0,05 | 1,00 |

Table S1, supplementary data

| | | | | | | | |
|-------------------|-----------|-------------------|--------------|-------|-------|-------|----------|
| | AT4G38270 | Group D (GAUT3) | CATMA4A39780 | 7,75 | 7,92 | 0,17 | 1,00 |
| | AT3G18660 | Group A (PGS1P1) | CATMA3A18270 | 7,64 | 7,18 | -0,46 | 1,00 |
| | AT1G13250 | Group C (GATL3) | CATMA1A12260 | 7,58 | 7,33 | -0,25 | 1,00 |
| | AT1G70090 | Group C (GATL9) | CATMA1A59370 | 7,38 | 7,28 | -0,10 | 1,00 |
| | AT5G47780 | Group D (GAUT4) | CATMA5A43750 | 7,37 | 7,49 | 0,12 | 1,00 |
| | AT3G28340 | Group C (GATL10) | CATMA3A28190 | 7,11 | 6,99 | -0,13 | 1,00 |
| | AT1G02720 | Group C (GATL5) | CATMA1A01680 | 6,91 | 6,84 | -0,07 | 1,00 |
| | AT3G58790 | Group D (GAUT15) | CATMA3A51790 | 6,78 | 6,72 | -0,06 | 1,00 |
| | AT2G35710 | Group E | CATMA2A33890 | 6,77 | 6,80 | 0,03 | 1,00 |
| | AT1G56600 | Group B (AtGoIS2) | CATMA1A47690 | 6,73 | 7,26 | 0,53 | 1,00 |
| | AT5G30500 | Group B | CATMA5A27960 | 6,68 | 6,77 | 0,10 | 1,00 |
| GT31 ^d | AT4G00300 | Group A | CATMA4A00350 | 9,68 | 8,84 | -0,84 | 4,22E-04 |
| | AT1G05170 | Group E | CATMA1A04030 | 9,55 | 9,00 | -0,55 | 1,00 |
| | AT1G77810 | Group E | CATMA1A66970 | 8,26 | 8,19 | -0,07 | 1,00 |
| | AT3G14960 | Group C | CATMA3A14310 | 8,22 | 8,38 | 0,16 | 1,00 |
| | AT2G32430 | Group E | CATMA2A30720 | 8,19 | 7,96 | -0,23 | 1,00 |
| | AT3G11420 | Group A | CATMA3A10410 | 7,86 | 8,03 | 0,17 | 1,00 |
| | AT2G26100 | Group C | CATMA2A24460 | 7,68 | 7,71 | 0,04 | 1,00 |
| | AT5G41460 | Group A | CATMA5A37060 | 7,00 | 6,72 | -0,28 | 1,00 |
| | AT1G33250 | Group A | CATMA1A31530 | 6,95 | 7,11 | 0,16 | 1,00 |
| | AT5G53340 | Group D | CATMA5A49250 | 6,93 | 6,95 | 0,02 | 1,00 |
| | AT1G53290 | Group C | CATMA1A44310 | 6,88 | 6,86 | -0,02 | 1,00 |
| GT34 ^e | AT2G22900 | | CATMA2A21380 | 10,09 | 9,72 | -0,36 | 1,00 |
| | AT4G02500 | | CATMA4A02820 | 9,31 | 8,99 | -0,31 | 1,00 |
| | AT3G62720 | XT1 | CATMA3A55880 | 7,63 | 7,47 | -0,17 | 1,00 |
| | AT1G74380 | | CATMA1A63770 | 7,60 | 7,37 | -0,23 | 1,00 |
| | AT2G22900 | | CATMA2B21380 | 7,35 | 7,21 | -0,14 | 1,00 |
| | AT1G18690 | | CATMA1A17720 | 6,68 | 6,77 | 0,09 | 1,00 |
| GT37 ^f | AT2G03220 | FUT1 (MUR2) | CATMA2A02125 | 6,74 | 6,79 | 0,05 | 1,00 |
| | AT1G14070 | FUT7 | CATMA1A13020 | 6,77 | 6,69 | -0,08 | 1,00 |
| GT47 ^g | AT2G28110 | Group E (FRA8) | CATMA2A26510 | 11,39 | 10,73 | -0,67 | 1,86E-01 |
| | AT5G22940 | Group E | CATMA5A20440 | 9,40 | 8,74 | -0,66 | 2,06E-01 |
| | AT5G61840 | Group E (AtGUT2) | CATMA5A57440 | 8,58 | 8,34 | -0,24 | 1,00 |
| | AT4G16745 | Group D | CATMA4A17710 | 8,23 | 7,85 | -0,38 | 1,00 |
| | AT2G20370 | Group A (MUR3) | CATMA2A18850 | 8,06 | 7,78 | -0,27 | 1,00 |
| | AT4G22580 | Group A (AtGT19) | CATMA4A24310 | 8,04 | 8,05 | 0,01 | 1,00 |
| | AT1G27440 | Group E (AtGUT1) | CATMA1A25670 | 7,83 | 7,82 | 0,00 | 1,00 |
| | AT4G38040 | Group D | CATMA4A39540 | 7,68 | 7,72 | 0,03 | 1,00 |
| | AT3G57630 | Group E | CATMA3A50640 | 7,61 | 7,28 | -0,33 | 1,00 |
| | AT5G62220 | Group A (AtGT18) | CATMA5A57810 | 7,60 | 7,53 | -0,06 | 1,00 |
| | AT5G33290 | Group C | CATMA5A28910 | 7,57 | 7,32 | -0,26 | 1,00 |
| | AT2G31990 | Group A (AtGT15) | CATMA2A30260 | 7,56 | 7,55 | -0,01 | 1,00 |
| | AT1G21480 | Group D | CATMA1A20530 | 7,43 | 7,38 | -0,05 | 1,00 |
| | AT2G35100 | Group B (ARAD1) | CATMA2A33240 | 7,34 | 7,17 | -0,17 | 1,00 |
| | AT1G34270 | Group B | CATMA1A32570 | 7,26 | 7,15 | -0,11 | 1,00 |
| | AT1G67410 | Group B | CATMA1A56730 | 7,12 | 6,89 | -0,23 | 1,00 |
| | AT5G16890 | Group B | CATMA5A15200 | 7,10 | 6,95 | -0,15 | 1,00 |
| | AT4G16745 | Group D | CATMA4A17700 | 7,09 | 7,20 | 0,11 | 1,00 |
| | AT1G74680 | Group B | CATMA1A64050 | 7,09 | 6,87 | -0,22 | 1,00 |
| | AT5G33290 | Group C | CATMA5A28900 | 6,98 | 6,93 | -0,04 | 1,00 |
| | AT5G44930 | Group B | CATMA5A40770 | 6,91 | 7,11 | 0,20 | 1,00 |
| | AT5G11610 | Group D | CATMA5A10380 | 6,90 | 6,90 | 0,00 | 1,00 |

Table S1, supplementary data

| | | | | | | | |
|---|---|---|--|--|--|--|--|
| | AT4G13990 AT5G03800 AT5G19670 AT5G25310 AT5G03800 | Group A (AtGT14) Group C Group D Group C Group C | CATMA4A14190 CATMA5A02990 CATMA5A18100 CATMA5A23020 CATMA5A03000 | 6,88 6,84 6,80 6,80 6,79 | 6,83 6,80 6,83 6,63 6,77 | -0,05 -0,04 0,03 -0,17 -0,02 | 1,00 1,00 1,00 1,00 1,00 |
| GT77 ^h | AT1G19360 AT2G35610 AT2G35610 AT5G44820 AT1G75110 AT1G14590 | RRA2 | CATMA1A18380 CATMA2A33740 CATMA2A33750 CATMA5A40660 CATMA1A64440 CATMA1A13610 | 9,97 7,60 7,33 7,23 7,01 6,94 | 9,52 7,41 7,17 7,01 6,90 6,95 | -0,45 -0,20 -0,16 -0,22 -0,12 0,01 | 1,00 1,00 1,00 1,00 1,00 1,00 |
| GT2 (cellulose synthases) ⁱ | AT5G05170 AT5G09870 AT4G39350 AT4G32410 AT2G25540 AT5G44030 AT5G17420 | AtCESA3 (primary wall) AtCESA5 (unknown function) AtCESA2 (unknown function) AtCESA1 (primary wall) AtCesA10 (unknown function) AtCESA4 (secondary wall) AtCesA7 (secondary wall) | CATMA5A04375 CATMA5A08637 CATMA4A40743 CATMA4A34150 CATMA2A23880 CATMA5A39800 CATMA5A15680 | 10,36 10,01 9,80 9,80 7,63 7,60 6,94 | 9,89 9,11 8,99 9,53 7,25 7,28 6,87 | -0,47 -0,90 -0,82 -0,27 -0,38 -0,31 -0,08 | 1,00 3,26E-05 9,50E-04 1,00 1,00 1,00 1,00 |
| GT2 (cellulose synthases-like) ⁱ | AT5G03760 AT3G28180 AT5G22740 AT2G35650 AT3G07330 AT2G33100 AT1G55850 AT1G23480 | AtCSLA9 AtCSLC4 AtCSLA2 AtCSIA7 AtCSLC6 AtCslD1 AtCSLE1 AtCslA3 | CATMA5A02940 CATMA3A06563 CATMA5A20200 CATMA2A33860 CATMA3A28010 CATMA2A31270 CATMA1A47044 CATMA1A22512 | 8,94 8,93 8,63 8,60 8,57 7,32 7,19 7,04 | 8,47 8,66 8,45 8,21 9,03 6,99 7,92 6,99 | -0,47 -0,26 -0,18 -0,39 0,46 -0,33 0,73 -0,05 | 1,00 1,00 1,00 1,00 1,00 1,00 1,98E-02 1,00 |
| GT48 (callose synthases) ^j | AT1G05570 AT2G31960 AT5G13000 AT2G36850 AT3G07160 | AtGSL6 (CAL51) AtGSL3 AtGSL12 AtGSL8 AtGSL10 | CATMA1A04500 CATMA2A30240 CATMA5A11220 CATMA2A35110 CATMA3A06350 | 9,17 9,15 8,68 7,90 7,87 | 8,14 8,71 8,64 7,92 7,77 | -1,03 -0,44 -0,04 0,02 -0,09 | 8,89E-08 1,00 1,00 1,00 1,00 |
| vesicle trafficking (emp24/gp25L/p24 family protein) ^k | AT1G69460 AT1G57620 AT3G07680 AT1G14010 AT1G21900 AT1G09580 | | CATMA1A58750 CATMA1A47920 CATMA3A06940 CATMA1A12960 CATMA1A21000 CATMA1A08430 | 10,17 8,70 8,26 8,03 7,69 7,05 | 10,55 9,08 8,69 8,03 7,75 7,09 | 0,37 0,37 0,43 0,00 0,06 0,03 | 1,00 1,00 1,00 1,00 1,00 1,00 |
| glycosyl hydrolases (GHs) | | | | | | | |
| GH9 (endoglucanases) ^l | AT4G02290 AT1G64390 AT5G49720 AT1G70710 AT1G75680 AT4G24260 AT1G71380 AT2G32990 AT4G39000 | AtKOR AtCEL1 | CATMA4A02560 CATMA1A53700 CATMA5A45658 CATMA1A59990 CATMA1A64980 CATMA4A25995 CATMA1A60700 CATMA2A31180 CATMA4A40450 | 11,10 10,68 9,64 9,32 8,37 7,70 7,70 7,35 6,68 | 10,08 10,19 9,94 8,93 8,20 7,88 7,68 7,23 6,83 | -1,02 -0,49 0,30 -0,39 -0,17 0,18 -0,02 -0,12 0,15 | 1,57E-07 1,00 1,00 1,00 1,00 1,00 1,00 1,00 1,00 |
| GH16 (xyloglucan endotransglycosylases/hydrolases) (XTHs) | AT2G06850 AT4G14130 AT4G30290 | At-XTH4 At-XTH15 At-XTH19 | CATMA2A05540 CATMA4A14375 CATMA4A31900 | 12,98 12,72 11,37 | 12,15 11,98 11,40 | -0,83 -0,74 0,03 | 6,40E-04 1,62E-02 1,00 |

Table S1, supplementary data

| | | | | | | | |
|--|-----------|-------------------|--------------|-------|-------|-------|----------|
| | AT4G30270 | At-XTH24 (MERI-5) | CATMA4A31885 | 10,84 | 11,64 | 0,80 | 1,63E-03 |
| | AT1G32170 | At-XTH30 | CATMA1A30510 | 10,06 | 9,85 | -0,21 | 1,00 |
| | AT2G01850 | At-XTH27 | CATMA2A00905 | 9,92 | 9,56 | -0,36 | 1,00 |
| | AT1G11545 | At-XTH8 | CATMA1A10510 | 9,18 | 9,03 | -0,15 | 1,00 |
| | AT1G14720 | At-XTH28 | CATMA1A13765 | 8,78 | 8,92 | 0,14 | 1,00 |
| | AT4G37800 | At-XTH7 | CATMA4A39300 | 8,70 | 8,17 | -0,52 | 1,00 |
| | AT2G36870 | At-XTH32 | CATMA2A35140 | 8,31 | 7,86 | -0,45 | 1,00 |
| | AT5G13870 | At-XTH5 | CATMA5A12105 | 8,17 | 8,15 | -0,02 | 1,00 |
| | AT3G23730 | At-XTH16 | CATMA3A23670 | 7,79 | 7,81 | 0,02 | 1,00 |
| | AT5G48070 | At-XTH20 | CATMA5A44050 | 7,60 | 7,56 | -0,05 | 1,00 |
| | AT4G25820 | At-XTH14 | CATMA4A27496 | 6,87 | 6,76 | -0,11 | 1,00 |
| | AT2G14620 | At-XTH10 | CATMA2A13330 | 6,73 | 6,78 | 0,05 | 1,00 |
| | AT5G65730 | At-XTH6 | CATMA5A61080 | 6,69 | 6,78 | 0,10 | 1,00 |
| GH17 ⁿ | AT5G42100 | Group C | CATMA5A37820 | 9,93 | 9,88 | -0,05 | 1,00 |
| | AT3G04010 | Group B | CATMA3A02980 | 9,85 | 8,30 | -1,55 | 0,00E+00 |
| | AT5G58090 | Group B | CATMA5A53850 | 9,33 | 9,56 | 0,23 | 1,00 |
| | AT3G07320 | Group E | CATMA3A06560 | 8,55 | 8,29 | -0,25 | 1,00 |
| | AT5G42720 | Group C | CATMA5A38510 | 8,54 | 8,49 | -0,05 | 1,00 |
| | AT4G31140 | Group B | CATMA4A32830 | 8,47 | 8,22 | -0,24 | 1,00 |
| | AT2G26600 | Group C | CATMA2A24920 | 8,41 | 8,09 | -0,32 | 1,00 |
| | AT2G01630 | Group D | CATMA2A00680 | 8,40 | 8,37 | -0,03 | 1,00 |
| | AT5G20330 | Group A (AtBG4) | CATMA5A18800 | 8,26 | 8,04 | -0,22 | 1,00 |
| | AT1G64760 | Group B | CATMA1A54070 | 8,13 | 8,09 | -0,04 | 1,00 |
| | AT5G58480 | Group B | CATMA5A54240 | 7,81 | 7,61 | -0,20 | 1,00 |
| | AT3G55430 | Group C | CATMA3A48400 | 7,78 | 7,75 | -0,03 | 1,00 |
| | AT2G27500 | Group C | CATMA2A25930 | 7,73 | 8,01 | 0,28 | 1,00 |
| | AT4G29360 | Group D | CATMA4A30990 | 7,64 | 7,60 | -0,04 | 1,00 |
| | AT5G20870 | Group B | CATMA5A19400 | 7,36 | 7,20 | -0,16 | 1,00 |
| | AT5G56590 | Group D | CATMA5A52400 | 7,29 | 7,03 | -0,26 | 1,00 |
| | AT5G18220 | Group B | CATMA5A16490 | 7,25 | 7,14 | -0,11 | 1,00 |
| | AT1G30080 | Group C | CATMA1A28100 | 7,08 | 7,02 | -0,06 | 1,00 |
| | AT1G66250 | Group D | CATMA1A55520 | 6,99 | 6,89 | -0,10 | 1,00 |
| | AT4G17180 | Group B | CATMA4A18200 | 6,92 | 6,88 | -0,04 | 1,00 |
| | AT5G20560 | Group A | CATMA5A19060 | 6,87 | 6,87 | -0,01 | 1,00 |
| | AT1G32860 | Group C | CATMA1A31160 | 6,84 | 7,01 | 0,17 | 1,00 |
| | AT3G15800 | Group C | CATMA3A15190 | 6,82 | 6,73 | -0,09 | 1,00 |
| GH18 (yieldins) ^o | AT5G24090 | | CATMA5A21620 | 7,22 | 7,30 | 0,09 | 1,00 |
| | AT4G19730 | | CATMA4A20960 | 6,98 | 6,91 | -0,08 | 1,00 |
| | AT4G19810 | | CATMA4A21020 | 6,88 | 6,89 | 0,00 | 1,00 |
| GH28 (polygalacturonases) ^p | AT4G23820 | Group A | CATMA4A25640 | 9,36 | 8,91 | -0,45 | 1,00 |
| | AT3G06770 | Group A | CATMA3A05990 | 9,01 | 8,78 | -0,23 | 1,00 |
| | AT5G41870 | Group A | CATMA5A37590 | 8,64 | 8,35 | -0,29 | 1,00 |
| | AT3G55790 | Group A | CATMA3A48760 | 8,13 | 8,58 | 0,45 | 1,00 |
| | AT3G61490 | Group A | CATMA3A54630 | 8,09 | 8,55 | 0,45 | 1,00 |
| | AT3G62110 | Group A | CATMA3A55250 | 7,93 | 7,77 | -0,16 | 1,00 |
| | AT1G80170 | Group D | CATMA1A69350 | 7,64 | 7,64 | 0,00 | 1,00 |
| | AT5G49215 | Group A | CATMA5A45170 | 7,36 | 7,19 | -0,16 | 1,00 |
| | AT3G42950 | Group A | CATMA3A35240 | 7,32 | 7,30 | -0,02 | 1,00 |
| | AT3G15720 | Group B | CATMA3A15120 | 7,27 | 7,42 | 0,15 | 1,00 |
| | AT3G16850 | Group A | CATMA3A16240 | 7,08 | 6,95 | -0,13 | 1,00 |
| | AT4G32375 | Group B | CATMA4A34110 | 6,97 | 6,84 | -0,13 | 1,00 |

Table S1, supplementary data

| | | | | | | | |
|---|-------------------------------------|-------------------------------|--|----------------------|----------------------|------------------------|----------------------|
| | AT5G14650 AT4G23500 AT1G70500 | Group C Group A Group D | CATMA5A12860 CATMA4A25330 CATMA1A59770 | 6,90 6,79 6,77 | 6,91 6,75 6,75 | 0,01 -0,04 -0,02 | 1,00 1,00 1,00 |
| GH35 (beta-galactosidases) ^q | AT5G56870 | AtBGAL4 | CATMA5A52625 | 13,18 | 11,97 | -1,22 | 5,60E-12 |
| | AT1G45130 | AtBGAL5 | CATMA1A37985 | 11,21 | 10,42 | -0,78 | 3,24E-03 |
| | AT5G20710 | AtBGAL7 | CATMA5A19240 | 8,89 | 7,98 | -0,92 | 1,72E-05 |
| | AT5G63810 | AtBGAL10 | CATMA5A59336 | 8,51 | 8,25 | -0,26 | 1,00 |
| | AT3G52840 | AtBGAL2 | CATMA3A45773 | 8,44 | 7,71 | -0,73 | 2,32E-02 |
| | AT2G28470 | AtBGAL8 | CATMA2A26900 | 8,12 | 7,77 | -0,35 | 1,00 |
| | AT5G63800 | AtBGAL6 | CATMA5A59333 | 7,59 | 7,34 | -0,25 | 1,00 |
| | AT1G72990 | AtBGAL17 | CATMA1A62200 | 7,16 | 7,03 | -0,12 | 1,00 |
| | | | | | | | |
| carbohydrate esterases (CEs) | | | | | | | |
| CE8 (pectin methylesterases) ^r | AT2G43050 | | CATMA2A41450 | 12,56 | 10,76 | -1,80 | 0,00E+00 |
| | AT3G49220 | | CATMA3A42250 | 11,81 | 10,99 | -0,82 | 8,65E-04 |
| | AT3G14310 | AtPME3 | CATMA3A13580 | 10,79 | 9,90 | -0,90 | 4,05E-05 |
| | AT1G53840 | AtPME1 | CATMA1A44933 | 9,39 | 9,04 | -0,35 | 1,00 |
| | AT3G43270 | | CATMA3A35650 | 8,75 | 8,37 | -0,38 | 1,00 |
| | AT1G53830 | AtPME2 | CATMA1A44930 | 8,35 | 7,61 | -0,74 | 1,61E-02 |
| | AT3G49220 | | CATMA3A42240 | 8,20 | 8,14 | -0,06 | 1,00 |
| | AT4G33220 | | CATMA4A34970 | 8,08 | 7,75 | -0,33 | 1,00 |
| | AT5G09760 | | CATMA5A08570 | 7,80 | 7,64 | -0,16 | 1,00 |
| | AT3G10720 | | CATMA3A09760 | 7,54 | 7,26 | -0,28 | 1,00 |
| | AT3G29090 | | CATMA3A29170 | 7,50 | 7,78 | 0,28 | 1,00 |
| | AT5G64640 | | CATMA5A60070 | 7,44 | 7,93 | 0,49 | 1,00 |
| | AT5G53370 | | CATMA5A49270 | 7,41 | 7,30 | -0,11 | 1,00 |
| | AT5G64640 | | CATMA5A60070 | 7,35 | 7,17 | -0,18 | 1,00 |
| | AT3G59010 | | CATMA3A52040 | 7,31 | 6,81 | -0,50 | 1,00 |
| | AT4G02330 | | CATMA4A02630 | 7,05 | 7,00 | -0,05 | 1,00 |
| | AT1G05310 | | CATMA1A04190 | 7,03 | 6,81 | -0,22 | 1,00 |
| | AT5G47500 | | CATMA5A43480 | 6,90 | 7,06 | 0,17 | 1,00 |
| | AT5G04970 | | CATMA5A04150 | 6,89 | 6,86 | -0,03 | 1,00 |
| | AT3G05610 | | CATMA3A04630 | 6,83 | 6,91 | 0,08 | 1,00 |
| | AT3G47400 | | CATMA3A40400 | 6,74 | 6,76 | 0,03 | 1,00 |
| | | | | | | | |
| CE13 (pectin acylesterases) ^s | AT3G05910 | | CATMA3A04940 | 10,44 | 10,14 | -0,30 | 1,00 |
| | AT4G19410 | | CATMA4A20590 | 8,25 | 8,09 | -0,17 | 1,00 |
| | AT2G46930 | | CATMA2A45390 | 7,97 | 8,07 | 0,10 | 1,00 |
| | AT3G09410 | | CATMA3A08300 | 7,25 | 7,31 | 0,06 | 1,00 |
| | AT3G62060 | | CATMA3A55190 | 7,15 | 7,01 | -0,14 | 1,00 |
| | AT5G23870 | | CATMA5A21340 | 6,94 | 6,87 | -0,06 | 1,00 |
| | | | | | | | |
| carbohydrate lyases (CLs) | | | | | | | |
| PL1 (pectate lyases) ^t | AT5G48900 | Group A | CATMA5A44810 | 10,92 | 10,23 | -0,70 | 6,69E-02 |
| | AT1G04680 | Group A | CATMA1A03520 | 10,73 | 10,22 | -0,52 | 1,00 |
| | AT1G67750 | Group A | CATMA1A57110 | 10,66 | 10,42 | -0,24 | 1,00 |
| | AT3G07010 | Group A | CATMA3A06210 | 10,26 | 9,56 | -0,70 | 6,16E-02 |
| | AT4G24780 | Group A | CATMA4A26470 | 8,40 | 9,43 | 1,03 | 1,03E-07 |
| | AT5G63180 | Group A | CATMA5A58780 | 8,03 | 7,52 | -0,52 | 1,00 |
| | AT3G55250 | Group B | CATMA3A48250 | 7,95 | 8,14 | 0,18 | 1,00 |
| | AT3G09540 | Group B | CATMA3A08400 | 7,76 | 7,56 | -0,20 | 1,00 |
| | AT5G04310 | Group C | CATMA5A03510 | 7,42 | 7,17 | -0,26 | 1,00 |
| | AT3G55140 | Group B | CATMA3A48150 | 7,37 | 7,27 | -0,11 | 1,00 |
| | | | | | | | |

Table S1, supplementary data

| | | | | | | | |
|--|-----------|----------------------------------|--------------|-------|-------|-------|----------|
| | AT3G24670 | Group A | CATMA3A24620 | 7,37 | 7,22 | -0,15 | 1,00 |
| | AT4G13710 | Group A | CATMA4A13890 | 7,29 | 7,03 | -0,27 | 1,00 |
| | AT5G09280 | Group B | CATMA5A08090 | 7,14 | 7,37 | 0,23 | 1,00 |
| | AT5G15110 | Group B | CATMA5A13390 | 6,89 | 6,92 | 0,03 | 1,00 |
| | AT1G30350 | Group B | CATMA1A28360 | 6,80 | 6,78 | -0,03 | 1,00 |
| | AT1G14420 | Group B | CATMA1A13420 | 6,77 | 6,73 | -0,04 | 1,00 |
| | AT3G27400 | Group A | CATMA3A27260 | 6,59 | 6,94 | 0,35 | 1,00 |
| PL4 (rhamnolacturonan lyases) ^u | AT1G09910 | | CATMA1A08780 | 8,04 | 7,87 | -0,17 | 1,00 |
| | AT1G09890 | | CATMA1A08750 | 6,80 | 6,73 | -0,07 | 1,00 |
| expansins ^v | AT5G02260 | AtEXPA9 (alpha expansin) | CATMA5A01340 | 12,71 | 12,44 | -0,28 | 1,00 |
| | AT1G69530 | AtEXPA1 (alpha expansin) | CATMA1A58850 | 10,62 | 10,86 | 0,24 | 1,00 |
| | AT2G37640 | AtEXPA3 (alpha expansin) | CATMA2A35920 | 10,34 | 10,52 | 0,18 | 1,00 |
| | AT2G40610 | AtEXPA8 (alpha expansin) | CATMA2A38930 | 9,95 | 9,85 | -0,10 | 1,00 |
| | AT2G28950 | AtEXPA6 (alpha expansin) | CATMA2B27340 | 9,26 | 9,80 | 0,54 | 1,00 |
| | AT4G28250 | AtEXPB3 (beta expansin) | CATMA4A29890 | 9,07 | 10,38 | 1,31 | 0,00E+00 |
| | AT1G20190 | AtEXPA11 (alpha expansin) | CATMA1A19190 | 8,76 | 8,72 | -0,03 | 1,00 |
| | AT2G03090 | AtEXPA15 (alpha expansin) | CATMA2A02000 | 8,01 | 8,56 | 0,55 | 1,00 |
| | AT4G38400 | AtEXLA2 (expansin like family A) | CATMA4A39920 | 7,98 | 7,74 | -0,24 | 1,00 |
| | AT4G38210 | AtEXPA20 (alpha expansin) | CATMA4A39720 | 7,91 | 8,42 | 0,51 | 1,00 |
| | AT2G28950 | AtEXPA6 (alpha expansin) | CATMA2A27340 | 7,88 | 8,09 | 0,21 | 1,00 |
| | AT1G26770 | AtEXPA10 (alpha expansin) | CATMA1A24995 | 7,87 | 7,97 | 0,10 | 1,00 |
| | AT2G20750 | AtEXPB1 (beta expansin) | CATMA2A19310 | 7,47 | 7,31 | -0,16 | 1,00 |
| | AT3G55500 | AtEXPA16 (alpha expansin) | CATMA3A48450 | 7,43 | 7,30 | -0,13 | 1,00 |
| | AT5G05290 | AtEXPA2 (alpha expansin) | CATMA5A04475 | 7,27 | 7,64 | 0,37 | 1,00 |
| | AT5G56320 | AtEXPA14 (alpha expansin) | CATMA5A52120 | 7,25 | 6,99 | -0,26 | 1,00 |
| | AT3G45960 | AtEXLA3 (expansin like family A) | CATMA3A38990 | 6,97 | 6,91 | -0,06 | 1,00 |
| | AT3G29030 | AtEXPA15 (alpha expansin) | CATMA3A29035 | 6,71 | 6,80 | 0,09 | 1,00 |
| arabinogalactan proteins (AGPs) ^w | AT2G14890 | AtAGP9 | CATMA2A13650 | 11,71 | 11,82 | 0,12 | 1,00 |
| | AT5G11740 | AtAGP15 (AG peptide) | CATMA5A10540 | 11,03 | 11,71 | 0,68 | 1,10E-01 |
| | AT5G10430 | AtAGP4 | CATMA5A09183 | 10,84 | 10,65 | -0,19 | 1,00 |
| | AT1G28290 | AtAGP31 | CATMA1A26460 | 10,97 | 11,42 | 0,46 | 1,00 |
| | AT3G13520 | AtAGP12 (AG peptide) | CATMA3A12660 | 10,77 | 11,51 | 0,74 | 1,50E-02 |
| | AT5G53250 | AtAGP22 (AG peptide) | CATMA5A49170 | 9,48 | 9,98 | 0,50 | 1,00 |
| | AT3G61640 | AtAGP20 (AG peptide) | CATMA3A54770 | 8,69 | 9,53 | 0,85 | 3,14E-04 |
| | AT2G47930 | AtAGP26 | CATMA2A46360 | 8,46 | 9,10 | 0,63 | 5,19E-01 |
| | AT5G18690 | AtAGP25 | CATMA5A17040 | 7,90 | 8,07 | 0,17 | 1,00 |
| | AT5G64310 | AtAGP1 | CATMA5A59763 | 7,85 | 8,47 | 0,62 | 7,55E-01 |
| | AT4G09030 | AtAGP10 | CATMA4A09010 | 7,75 | 8,34 | 0,58 | 1,00 |
| | AT4G37450 | AtAGP18 (Lys-rich AGP) | CATMA4A39020 | 7,68 | 7,73 | 0,04 | 1,00 |
| | AT1G68725 | AtAGP19 (Lys-rich AGP) | CATMA1A58110 | 7,64 | 7,57 | -0,08 | 1,00 |
| fasciclin AGPs (FLAs) ^x | AT1G03870 | AtFLA9 | CATMA1A02720 | 12,49 | 12,25 | -0,24 | 1,00 |
| | AT2G45470 | AtFLA8 | CATMA2A43870 | 11,96 | 11,53 | -0,43 | 1,00 |
| | AT4G12730 | AtFLA2 | CATMA4A12870 | 10,85 | 10,44 | -0,41 | 1,00 |
| | AT2G04780 | AtFLA7 | CATMA2A03690 | 9,27 | 9,69 | 0,42 | 1,00 |
| | AT3G12660 | AtFLA14 | CATMA3A11630 | 8,54 | 8,09 | -0,46 | 1,00 |
| | AT5G55730 | AtFLA1 | CATMA5A51470 | 8,18 | 7,96 | -0,22 | 1,00 |
| | AT3G11700 | AtFLA18 | CATMA3A10640 | 8,01 | 8,28 | 0,27 | 1,00 |
| | AT5G44130 | AtFLA13 | CATMA5A39880 | 7,76 | 7,48 | -0,28 | 1,00 |
| | AT5G03170 | AtFLA11 | CATMA5A02280 | 7,75 | 7,66 | -0,09 | 1,00 |

Table S1, supplementary data

| | | | | | | | |
|--|--|---|--|--|---|---|--|
| | AT5G60490 AT3G60900 AT2G24450 | AtFLA12 AtFLA10 AtFLA3 | CATMA5A56200 CATMA3A53870 CATMA2A22780 | 7,57 7,28 7,19 | 7,42 7,12 7,12 | -0,15 -0,16 -0,07 | 1,00 1,00 1,00 |
| GPI-anchored peptide (GAPEP) family ^y | AT3G01950 AT5G40970 AT5G40960 AT3G01940 AT5G50660 | | CATMA3A00940 CATMA5A36640 CATMA5A36630 CATMA3A00930 CATMA5A46550 | 7,46 7,29 6,96 6,85 6,83 | 7,94 7,74 8,07 7,15 6,84 | 0,48 0,44 1,11 0,31 0,01 | 1,00 1,00 1,58E-09 1,00 1,00 |
| COBRA-like proteins ^z | AT5G15630 AT4G16120 AT3G02210 AT3G29810 AT5G60920 AT5G49270 | AtCOBL4 AtCOBL7 AtCOBL1 AtCOBL2 COB COBL9 | CATMA5A13880 CATMA4A16865 CATMA3A01200 CATMA3A30250 CATMA5CTRL29 CATMA5A45240 | 7,65 7,51 7,44 7,41 6,83 6,78 | 7,23 7,38 7,50 7,53 6,79 6,76 | -0,43 -0,12 0,06 0,11 -0,04 -0,02 | 1,00 1,00 1,00 1,00 1,00 1,00 |
| leucine-rich repeat extensins (LRXs) ^{aa} | AT3G24480 AT2G15890 AT4G33980 AT1G62440 AT4G13340 AT3G22800 AT4G18670 | AtLRX4 AtPEX3 AtPEX4 AtLRX2 AtLRX3 AtLRX6 AtLRX5 | CATMA3A24360 CATMA2A14770 CATMA4A35800 CATMA1A51580 CATMA4A13500 CATMA3A22740 CATMA4A19790 | 9,68 9,07 8,52 7,57 6,89 6,78 6,70 | 9,56 9,38 8,89 7,44 6,83 6,84 6,79 | -0,11 0,31 0,37 -0,12 -0,06 0,06 0,09 | 1,00 1,00 1,00 1,00 1,00 1,00 1,00 |
| Hyp/Pro-rich proteins (H/PRP) ^{ab} | AT2G21140 AT4G38770 | AtPRP2 AtPRP4 | CATMA2A19790 CATMA4A40195 | 11,67 8,94 | 11,15 8,78 | -0,51 -0,16 | 1,00 1,00 |
| extensins ^{ac} | AT1G23720 AT3G28550 AT3G54580 | | CATMA1A22610 CATMA3A28430 CATMA3A47520 | 7,01 6,91 7,01 | 6,92 6,96 6,94 | -0,08 0,05 -0,07 | 1,00 1,00 1,00 |
| lignin toolbox ^{ad} | AT4G39330 AT2G30490 AT1G76790 AT5G48930 AT2G40890 AT1G20480 AT5G54160 AT5G58490 AT1G15950 AT3G21240 AT3G10340 AT1G72680 AT3G19450 AT3G61990 | Arath;CAD1(cinnamyl alcohol dehydrogenase) Arath;C4H (CYP73A5) (trans-cinnamate 4-hydroxylase) Arath;COMT-like8 (caffeic acid O-methyltransferase) Arath;HCT (hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyl transferase) Arath;C3H2 (CYP98A3) (p-coumarate 3-hydroxylase) Arath;4CL-like4 (4-coumarate CoA ligase) Arath;COMT (caffeic acid O-methyltransferase) Arath;CCR-like5 (cinnamoyl-CoA reductase) Arath;CCR1 (cinnamoyl-CoA reductase) Arath;4CL2 (4-coumarate CoA ligase) Arath;PAL4 (Phe ammonia lyase) Arath;CAD9 (cinnamyl alcohol dehydrogenase) Arath;CAD2 (cinnamyl alcohol dehydrogenase) Arath;CCoAOMT3 (caffeoyl-CoA O-methyltransferase) | CATMA4A40735 CATMA2A28746 CATMA1A66010 CATMA5A44840 CATMA2A39220 CATMA1A19480 CATMA5A50073 CATMA5A54250 CATMA1A14998 CATMA3A21086 CATMA3A09340 CATMA1A61900 CATMA3A19045 CATMA3A55120 | 11,03 10,84 10,37 10,24 9,83 9,57 9,55 8,96 8,74 8,62 8,46 8,40 8,40 8,27 | 10,33 10,43 9,48 10,28 9,16 9,02 9,02 9,14 8,79 8,40 8,52 8,53 7,96 8,06 | -0,70 -0,41 -0,89 0,04 -0,67 -0,55 -0,53 0,18 0,05 -0,22 0,06 0,13 -0,44 -0,21 | 7,14E-02 1,00 4,45E-05 1,00 1,45E-01 1,00 1,00 1,00 1,00 1,00 1,00 1,00 1,00 1,00 |

Table S1, supplementary data

| | | | | | | | |
|---------------------------|-----------|---|--------------|-------|-------|-------|----------|
| | AT2G02400 | Arath;CCR-like2 (cinnamoyl-CoA reductase) | CATMA2A01315 | 8,09 | 8,02 | -0,08 | 1,00 |
| | AT5G04230 | Arath;PAL3 (Phe ammonia lyase) | CATMA5A03420 | 8,06 | 7,83 | -0,23 | 1,00 |
| | AT4G34230 | Arath;CAD6 (cinnamyl alcohol dehydrogenase) | CATMA4A36050 | 7,83 | 7,53 | -0,30 | 1,00 |
| | AT1G20510 | Arath;4CL-like1 (4-coumarate CoA ligase) | CATMA1A19510 | 7,78 | 7,40 | -0,38 | 1,00 |
| | AT2G21890 | Arath;CAD8 (cinnamyl alcohol dehydrogenase) | CATMA2A20490 | 7,66 | 7,41 | -0,25 | 1,00 |
| | AT1G51680 | Arath;4CL1 (4-coumarate CoA ligase) | CATMA1A42785 | 7,64 | 8,15 | 0,51 | 1,00 |
| | AT5G63380 | Arath;4CL-like8 (4-coumarate CoA ligase) | CATMA5A58930 | 7,60 | 7,42 | -0,18 | 1,00 |
| | AT2G21730 | Arath;CAD7 (cinnamyl alcohol dehydrogenase) | CATMA2A20370 | 7,44 | 7,31 | -0,13 | 1,00 |
| | AT4G26220 | Arath;CCoAOMT7 (caffeoyl-CoA O-methyltransferase) | CATMA4A27740 | 7,23 | 7,48 | 0,24 | 1,00 |
| | AT5G04330 | Arath;F5H2 (CYP84A4) | CATMA5A03540 | 7,15 | 6,89 | -0,27 | 1,00 |
| | AT2G37040 | Arath;PAL1 (Phe ammonia lyase) | CATMA2A35330 | 7,01 | 7,94 | 0,93 | 8,18E-06 |
| | AT3G21230 | Arath;4CL4 (4-coumarate CoA ligase) | CATMA3A21070 | 6,99 | 6,91 | -0,07 | 1,00 |
| | AT1G80820 | Arath;CCR2 (cinnamoyl-CoA reductase) | CATMA1A70050 | 6,90 | 6,81 | -0,09 | 1,00 |
| | AT4G19010 | Arath;4CL-like6 (4-coumarate CoA ligase) | CATMA4A20160 | 6,89 | 6,82 | -0,07 | 1,00 |
| | AT5G53810 | Arath;COMT-like13 (caffeic acid O- | CATMA5A49700 | 6,82 | 6,72 | -0,10 | 1,00 |
| | AT4G36220 | Arath;F5H1 (CYP84A1) | CATMA4A37873 | 6,68 | 6,84 | 0,15 | 1,00 |
| peroxidases ^{ae} | AT2G37130 | AtPrx21 | CATMA2A35430 | 14,28 | 12,86 | -1,42 | 0,00 |
| | AT4G21960 | AtPrx42 | CATMA4A23655 | 13,17 | 12,40 | -0,77 | 5,29E-03 |
| | AT1G05260 | AtPrx03 | CATMA1A04113 | 10,68 | 10,18 | -0,51 | 1,00 |
| | AT4G33870 | AtPrx48 | CATMA5A59590 | 10,52 | 9,25 | -1,27 | 0,00 |
| | AT1G71695 | AtPrx12 | CATMA1A60876 | 10,17 | 9,54 | -0,63 | 4,86E-01 |
| | AT5G64120 | AtPrx71 | CATMA4A35650 | 10,03 | 9,85 | -0,18 | 1,00 |
| | AT3G21770 | AtPrx30 | CATMA3B21635 | 9,54 | 9,77 | 0,23 | 1,00 |
| | AT3G21770 | AtPrx30 | CATMA3A21635 | 9,29 | 9,52 | 0,23 | 1,00 |
| | AT5G51890 | AtPrx66 | CATMA5A47820 | 8,92 | 9,12 | 0,21 | 1,00 |
| | AT5G66390 | AtPrx72 | CATMA5A61725 | 8,70 | 8,57 | -0,13 | 1,00 |
| | AT4G37520 | AtPrx50 | CATMA4A39085 | 8,28 | 8,32 | 0,04 | 1,00 |
| | AT5G64110 | AtPrx70 | CATMA5A59580 | 8,24 | 8,37 | 0,13 | 1,00 |
| | AT5G39580 | AtPrx62 | CATMA5A35160 | 7,83 | 7,95 | 0,11 | 1,00 |
| | AT2G38390 | AtPrx23 | CATMA2A36690 | 7,80 | 8,96 | 1,15 | 1,96E-10 |
| | AT3G17070 | AtPrx29 | CATMA3A16470 | 7,73 | 7,62 | -0,11 | 1,00 |
| | AT1G44970 | AtPrx09 | CATMA1A37915 | 7,72 | 7,56 | -0,16 | 1,00 |
| | AT2G41480 | AtPrx25 | CATMA2A39890 | 7,65 | 8,09 | 0,44 | 1,00 |
| | AT4G30170 | AtPrx45 | CATMA4A31806 | 7,52 | 7,65 | 0,13 | 1,00 |
| | AT2G22420 | AtPrx17 | CATMA2A20940 | 7,34 | 7,48 | 0,14 | 1,00 |
| | AT4G37530 | AtPrx51 | CATMA4A39090 | 7,24 | 7,29 | 0,05 | 1,00 |
| | AT5G05340 | AtPrx52 | CATMA5A04520 | 7,04 | 7,24 | 0,20 | 1,00 |
| | AT5G19890 | AtPrx59 | CATMA5A18330 | 7,13 | 7,20 | 0,08 | 1,00 |
| | AT5G40150 | AtPrx63 | CATMA5A35810 | 7,32 | 7,19 | -0,13 | 1,00 |
| | AT5G19880 | AtPrx58 | CATMA5A18320 | 6,98 | 6,99 | 0,01 | 1,00 |
| | AT2G24800 | AtPrx18 | CATMA2A23170 | 7,03 | 6,95 | -0,08 | 1,00 |
| | AT2G18980 | AtPrx16 | CATMA2A17560 | 7,11 | 6,93 | -0,18 | 1,00 |
| | At4g36430 | AtPrx49 | CATMA4A38005 | 6,77 | 6,90 | 0,13 | 1,00 |
| | AT2G18150 | AtPrx15 | CATMA2A16820 | 6,63 | 6,88 | 0,25 | 1,00 |
| | AT5G22410 | AtPrx60 | CATMA5A19865 | 6,86 | 6,81 | -0,05 | 1,00 |
| | AT5G17820 | AtPrx57 | CATMA5A16105 | 6,99 | 6,76 | -0,23 | 1,00 |
| | AT3G01190 | AtPrx27 | CATMA3A00185 | 6,78 | 6,73 | -0,05 | 1,00 |

Table S1, supplementary data

| | | | | | | | |
|------------------------------|---|------------------------|--------------|--------------|-------|-------|----------|
| laccases ^{af} | AT5G60020 | AtLAC17 | CATMA5A55770 | 9,53 | 9,23 | -0,30 | 1,00 |
| | AT5G03260 | AtLAC11 | CATMA5A02370 | 9,23 | 9,39 | 0,17 | 1,00 |
| | AT2G38080 | AtLAC4 | CATMA2A36370 | 8,91 | 8,33 | -0,58 | 1,00 |
| | AT2G30210 | AtLAC3 | CATMA2A28490 | 8,23 | 8,14 | -0,09 | 1,00 |
| | AT2G29130 | AtLAC2 | CATMA2A27560 | 7,29 | 7,01 | -0,28 | 1,00 |
| | AT5G05390 | AtLAC12 | CATMA5A04570 | 7,01 | 7,04 | 0,03 | 1,00 |
| | AT2G40370 | AtLAC5 | CATMA2A38650 | 6,93 | 6,86 | -0,07 | 1,00 |
| | AT5G01190 | AtLAC10 | CATMA5A00220 | 6,88 | 6,77 | -0,12 | 1,00 |
| | AT5G48100 | AtLAC15 | CATMA5A44080 | 6,77 | 6,68 | -0,09 | 1,00 |
| | SKU-like proteins (multi-copper oxidases) ^{ag} | AT1G76160 | SKS5 | CATMA1A65380 | 10,33 | 9,91 | -0,43 |
| AT1G41830 | | SKS6 | CATMA1A35650 | 9,76 | 9,27 | -0,49 | 1,00 |
| AT4G12420 | | SKU5 | CATMA4A12530 | 9,28 | 8,97 | -0,31 | 1,00 |
| AT5G48450 | | SKS3 | CATMA5A44430 | 9,11 | 8,93 | -0,18 | 1,00 |
| AT4G22010 | | SKS4 | CATMA4A23690 | 8,47 | 8,11 | -0,36 | 1,00 |
| AT3G13990 | | SKS11 | CATMA3A13220 | 7,66 | 7,51 | -0,15 | 1,00 |
| AT1G41830 | | SKS6 | CATMA1A35660 | 7,11 | 6,96 | -0,14 | 1,00 |
| AT4G28090 | | SKS10 | CATMA4A29740 | 6,82 | 6,72 | -0,09 | 1,00 |
| phytochemicals ^{ah} | AT4G12880 | AtEN20 (early nodulin) | CATMA4A13020 | 10,84 | 11,19 | 0,36 | 1,00 |
| | AT2G32300 | AtUCC1 (uclacyanin) | CATMA2A30615 | 10,61 | 10,60 | -0,02 | 1,00 |
| | AT5G15350 | AtEN22 (early nodulin) | CATMA5A13625 | 9,07 | 9,35 | 0,28 | 1,00 |
| | AT1G08500 | AtEN21 (early nodulin) | CATMA1A07465 | 8,78 | 9,08 | 0,30 | 1,00 |
| | AT5G53870 | AtEN1 (early nodulin) | CATMA5A49760 | 8,24 | 8,22 | -0,02 | 1,00 |
| | AT1G72230 | AtUCC8 (uclacyanin) | CATMA1A61460 | 8,10 | 8,34 | 0,24 | 1,00 |
| | AT4G28360 | AtEN2 (early nodulin) | CATMA4A30000 | 8,07 | 8,52 | 0,46 | 1,00 |
| | AT3G20570 | AtEN14 (early nodulin) | CATMA3A20300 | 7,83 | 8,16 | 0,33 | 1,00 |
| | AT3G60280 | AtUCC3 (uclacyanin) | CATMA3A53305 | 7,78 | 7,81 | 0,03 | 1,00 |
| | AT4G31840 | AtEN13 (early nodulin) | CATMA4A33490 | 7,53 | 8,19 | 0,66 | 2,47E-01 |
| | AT2G27035 | AtEN17 (early nodulin) | CATMA2A25400 | 7,33 | 7,57 | 0,24 | 1,00 |
| | AT4G27520 | AtEN12 (early nodulin) | CATMA4A29090 | 7,33 | 7,49 | 0,16 | 1,00 |
| | AT5G26330 | AtSTC3 (stellacyanin) | CATMA5A24030 | 7,23 | 7,32 | 0,09 | 1,00 |
| | AT3G27200 | AtUCC6 (uclacyanin) | CATMA3A27010 | 7,20 | 7,63 | 0,43 | 1,00 |
| | AT5G25090 | AtEN6 (early nodulin) | CATMA5A22800 | 6,98 | 7,12 | 0,15 | 1,00 |
| | AT2G25060 | AtEN7 (early nodulin) | CATMA2A23390 | 7,06 | 7,40 | 0,34 | 1,00 |
| | AT4G33930 | AtEN26 (early nodulin) | CATMA4A35740 | 6,91 | 6,87 | -0,03 | 1,00 |
| | AT2G02850 | AtPNC (plantacyanin) | CATMA2A01770 | 6,84 | 6,90 | 0,06 | 1,00 |
| | AT5G07475 | AtUCC5 (uclacyanin) | CATMA5A06680 | 6,81 | 6,84 | 0,03 | 1,00 |
| | AT5G20230 | AtSTC1 (stellacyanin) | CATMA5A18670 | 6,79 | 7,42 | 0,62 | 0,67 |
| AT5G57920 | AtEN3 (early nodulin) | CATMA5A53680 | 6,69 | 6,82 | 0,13 | 1,00 | |
| subtilases ^{ai} | AT3G14067 | AtSBT1.4 | CATMA3A13290 | 11,85 | 10,71 | -1,15 | 2,80E-10 |
| | AT2G05920 | AtSBT1.8 | CATMA2A04690 | 11,54 | 10,90 | -0,64 | 4,50E-01 |
| | AT5G67360 | AtSBT1.7 (ARA12) | CATMA5A62810 | 11,45 | 11,12 | -0,33 | 1,00 |
| | AT5G51750 | AtSBT1.3 | CATMA5A47680 | 11,04 | 10,80 | -0,24 | 1,00 |
| | AT5G59090 | AtSBT4.12 | CATMA5A54840 | 10,47 | 9,49 | -0,99 | 7,99E-07 |
| | AT3G14240 | AtSBT1.5 | CATMA3A13520 | 9,92 | 9,76 | -0,16 | 1,00 |
| | AT1G20160 | AtSBT5.2 | CATMA1A19170 | 8,84 | 8,90 | 0,06 | 1,00 |
| | AT4G34980 | AtSBT1.6 | CATMA4A36760 | 8,49 | 8,32 | -0,17 | 1,00 |
| | AT4G00230 | AtSBT4.14 (XSP11) | CATMA4A00245 | 8,20 | 8,20 | 0,00 | 1,00 |
| | AT2G04160 | AtSBT5.3 (AIR3) | CATMA2A03130 | 7,94 | 7,37 | -0,57 | 1,00 |
| | AT1G30600 | AtSBT2.1 | CATMA1A28650 | 7,35 | 7,20 | -0,15 | 1,00 |
| | AT5G19660 | AtSBT6.1 | CATMA5A18090 | 7,27 | 7,12 | -0,15 | 1,00 |
| | AT5G44530 | AtSBT2.3 | CATMA5A40320 | 7,15 | 6,88 | -0,27 | 1,00 |
| | AT5G45650 | AtSBT5.6 | CATMA5A41630 | 7,03 | 6,83 | -0,20 | 1,00 |

Table S1, supplementary data

| | | | | | | | |
|------------|---------------------------|---------------------------|--------------|-------------|-------------|-------|----------|
| | AT2G39850 | AtSBT4.2 | CATMA2A38090 | 6,73 | 6,82 | 0,09 | 1,00 |
| | AT1G32950 | AtSBT3.4 | CATMA1A31250 | 6,79 | 6,80 | 0,01 | 1,00 |
| | AT5G59190 | AtSBT4.3 | CATMA5A54940 | 6,76 | 6,79 | 0,03 | 1,00 |
| | AT5G58840 | AtSBT4.9 | CATMA5A54600 | 6,78 | 6,77 | 0,00 | 1,00 |
| | AT5G67090 | AtSBT1.9 | CATMA5A62540 | 6,81 | 6,77 | -0,04 | 1,00 |
| | AT5G03620 | AtSBT4.15 | CATMA5A02800 | 6,79 | 6,74 | -0,06 | 1,00 |
| | AT2G19170 | AtSBT2.5 | CATMA2A17780 | 6,89 | 6,72 | -0,18 | 1,00 |
| | AT5G59100 | AtSBT4.4 | CATMA5A54850 | 6,77 | 6,65 | -0,11 | 1,00 |
| a) (PMEIs) | AT2G31430 | | CATMA2A29670 | 8,79 | 8,76 | -0,03 | 1,00 |
| | AT3G12880 | | CATMA3A11840 | 8,78 | 8,51 | -0,27 | 1,00 |
| | AT4G25260 | | CATMA4A26950 | 8,67 | 9,10 | 0,43 | 1,00 |
| | AT1G62770 | | CATMA1A51910 | 8,22 | 8,37 | 0,15 | 1,00 |
| | AT5G64620 | | CATMA5A60045 | 8,20 | 8,28 | 0,08 | 1,00 |
| | AT5G62350 | | CATMA5A57950 | 8,09 | 8,47 | 0,38 | 1,00 |
| | AT3G47670 | | CATMA3A40660 | 7,72 | 7,72 | 0,00 | 1,00 |
| | AT3G47380 | | CATMA3A40380 | 7,53 | 7,80 | 0,27 | 1,00 |
| | AT2G01610 | | CATMA2A00660 | 7,52 | 7,88 | 0,36 | 1,00 |
| | AT4G12390 | | CATMA4A12500 | 7,44 | 7,34 | -0,10 | 1,00 |
| | AT1G47960 | | CATMA1A39055 | 7,19 | 7,83 | 0,65 | 3,44E-01 |
| | AT3G17220 | | CATMA3A16630 | 7,18 | 7,37 | 0,19 | 1,00 |
| | AT3G49330 | | CATMA3A42360 | 7,05 | 7,10 | 0,05 | 1,00 |
| | AT5G20740 | | CATMA5A19270 | 7,02 | 6,90 | -0,12 | 1,00 |
| | AT1G70720 | | CATMA1A60000 | 6,97 | 6,91 | -0,06 | 1,00 |
| | AT1G23205 | | CATMA1A22290 | 6,95 | 7,17 | 0,22 | 1,00 |
| | AT5G24370 | | CATMA5A21980 | 6,94 | 6,99 | 0,05 | 1,00 |
| | AT1G14890 | | CATMA1A13920 | 6,94 | 7,35 | 0,41 | 1,00 |
| | AT1G48010 | | CATMA1A39095 | 6,83 | 6,81 | -0,02 | 1,00 |
| | AT3G62820 | | CATMA3A55990 | 6,77 | 6,65 | -0,12 | 1,00 |
| | AT3G55680 | | CATMA3A48660 | 6,75 | 6,80 | 0,05 | 1,00 |

a: <http://cellwall.genomics.purdue.edu/families/4-6-5.html>
b: <http://cellwall.genomics.purdue.edu/families/1-1.html>
c: <http://cellwall.genomics.purdue.edu/families/2-3-1.html>
d: <http://cellwall.genomics.purdue.edu/families/2-3-5.html>
e: <http://cellwall.genomics.purdue.edu/families/2-3-4.html>
f: <http://cellwall.genomics.purdue.edu/families/1-1.html>
g: <http://cellwall.genomics.purdue.edu/families/2-3-2.html>
h: <http://www.cazy.org/fam/GT77.html>, [47]
i: <http://cellwall.genomics.purdue.edu/families/2-2.html>
j: <http://cellwall.genomics.purdue.edu/families/2-4.html>
k: <http://cellwall.genomics.purdue.edu/families/3-1.html>
l: <http://cellwall.genomics.purdue.edu/families/4-3-2-1.html>
m: <http://labs.plantbio.cornell.edu/xth/genes.htm>
n: <http://cellwall.genomics.purdue.edu/families/4-3-2-2.html>
o: <http://cellwall.genomics.purdue.edu/families/4-1-2.html>
p: <http://cellwall.genomics.purdue.edu/families/4-3-3.html>
q: <http://cellwall.genomics.purdue.edu/families/4-3-1-1.html>
r: <http://cellwall.genomics.purdue.edu/families/4-5-1.html>

s: <http://cellwall.genomics.purdue.edu/families/4-5-2.html>
t: <http://cellwall.genomics.purdue.edu/families/4-4-1.html>
u: <http://cellwall.genomics.purdue.edu/families/4-4-2.html>
v: <http://www.bio.psu.edu/expansins/arabidopsis.htm#At-EXLB1>
w: [48-51]
x: [48]
y: <http://cellwall.genomics.purdue.edu/families/6-4-10.html>
z: [14]
aa: [52]
ab: [53]
ac: our own annotation
ad: [54]
ae: <http://peroxidase.isb-sib.ch/listing.php?action=view&id=4362>
af: [56, 57]
ag: [58]
ah: [59]
ai: <http://csbdb.mpimp-golm.mpg.de/csbdb/dbcawp/psdb/pub/sgenes.html>
aj: Pr Jérôme Pelloux (Amiens University, France), personal communication

| | | | | | | | | |
|----|---|--|---|------------------|-------|----------|----------|----------|
| 18 | Proteins related to lipid metabolism | proteins homologous to plant lipid transfer protein/seed storage/trypsin alpha amylase inhibitor | AT3G53980 | 8,99 | 9,99 | 1,00 | 3,81E-07 | |
| 19 | | | AT3G18280 | 9,93 | 11,63 | 1,70 | 0,00E+00 | |
| 20 | | others | AT5G14180 homolog to lipase | 8,90 | 10,00 | 1,10 | 3,79E-09 | |
| 21 | Miscellaneous | proteins homologous to late embryogenesis abundant proteins | AT3G17520 | 9,06 | 10,84 | 1,78 | 0,00E+00 | |
| 22 | | | AT2G41260 M17 | 9,45 | 10,40 | 0,95 | 3,44E-06 | |
| 23 | | hormone-related proteins | AT1G04560 homolog to AWPM (ABA-induced) | 7,92 | 10,17 | 2,25 | 0,00E+00 | |
| 24 | | | AT5G15230 gibberellin regulated protein (GASA4) | 12,58 | 13,42 | 0,84 | 4,08E-04 | |
| 25 | | | AT4G09600 gibberellin regulated protein (GASA3) | 8,11 | 9,53 | 1,43 | 0,00E+00 | |
| 26 | | others | AT2G22860 phytosulfokine (AtPSK2) | 7,61 | 8,49 | 0,88 | 7,46E-05 | |
| 27 | | AT3G49780 phytosulfokine (AtPSK4) | 7,64 | 8,61 | 0,97 | 1,97E-06 | | |
| 28 | Unknown function | expressed proteins | AT1G67785 | 8,98 | 9,74 | 0,76 | 8,15E-03 | |
| 29 | | | | AT1G16850 | 8,12 | 9,01 | 0,90 | 3,70E-05 |
| 30 | | | | AT1G54860 | 7,46 | 8,66 | 1,20 | 1,68E-11 |
| 31 | | | | AT5G40960 | 6,96 | 8,07 | 1,11 | 1,58E-09 |
| 32 | | | | AT4G38080 | 7,16 | 8,39 | 1,23 | 0,00E+00 |
| 33 | | | | AT5G64510 | 7,49 | 8,96 | 1,48 | 0,00E+00 |
| 34 | | | | AT5G53650 | 7,87 | 8,96 | 0,91 | 2,43E-05 |
| 35 | | | expressed proteins with domains of unknown function (DUF) or uncharacterized protein families (UPF) | AT3G54260 DUF231 | 7,38 | 9,00 | 1,62 | 0,00E+00 |
| 36 | | | AT4G30660 UPF0057 | 8,52 | 9,37 | 0,85 | 2,45E-04 | |
| 37 | | expressed proteins with diverse structural domains | AT3G22640 cupin domain | 9,52 | 10,52 | 1,00 | 4,06E-07 | |
| 38 | | | AT1G49320 BURP domain | 7,16 | 8,01 | 0,86 | 2,10E-04 | |

Additional data file 4

Table 4. Proteins extracted and identified by mass spectrometry from purified cell walls of 5- and 11-day-old etiolated hypocotyls of *A. thaliana*. All these proteins were annotated using bioinformatic softwares for sub-cellular localization and functional domains as described in Material and methods. Proteomic data are in Irshad *et al.* (2008, submitted for publication). Presence of proteins at 5-days or 11-days is indicated by a ticked box. For transcriptomics, the intensity of the signal is expressed as log₂, the ratio between the levels of transcripts at 11-days and 5-days as well as the p-value are indicated. Background level for transcriptomics is 6.75 (grey boxes indicate results below background). Genes encoding proteins with predicted GPI anchors or transmembrane domains are in bold, and in pink boxes respectively.

| accession AGI | annotation | proteomic data | | transcriptomic data | | ratio 11-days/5-days | p-value |
|---|--|----------------|---------|---------------------|---------|----------------------|----------|
| | | 5-days | 11-days | 5-days | 11-days | | |
| proteins acting on carbohydrates | | | | | | | |
| At2g06850 | glycoside hydrolase family 16 (endoxyloglucan transferase) (AtXTH4) | ✓ | ✓ | 12,98 | 12,15 | -0,83 | 6,40E-04 |
| At5g13870 | glycoside hydrolase family 16 (endoxyloglucan transferase) (AtXTH5) | ✓ | | 8,17 | 8,15 | -0,02 | 1,00E+00 |
| At3g44990 | glycoside hydrolase family 16 (endoxyloglucan transferase) (AtXTH31) | ✓ | | nd | nd | | |
| At2g36870 | glycoside hydrolase family 16 (endoxyloglucan transferase) (AtXTH32) | | ✓ | 8,31 | 7,86 | -0,45 | 1,00E+00 |
| At1g10550 | glycoside hydrolase family 16 (endoxyloglucan transferase) (AtXTH33) | ✓ | ✓ | 6,21 | 6,22 | 0,01 | 1,00E+00 |
| At4g16260 | glycoside hydrolase family 17 | | ✓ | 6,56 | 6,68 | 0,12 | 1,00E+00 |
| At4g19810 | glycoside hydrolase family 18 (chitinase) | ✓ | ✓ | 6,88 | 6,89 | 0,00 | 1,00E+00 |
| At3g12500 | glycoside hydrolase family 19 (chitinase) | ✓ | ✓ | 8,08 | 7,72 | -0,36 | 1,00E+00 |
| At3g55260 | glycoside hydrolase family 20 (beta-hexosaminidase) | ✓ | | 9,64 | 9,13 | -0,51 | 1,00E+00 |
| At1g19170 | glycoside hydrolase family 28 (polygalacturonase) | ✓ | | 6,70 | 6,68 | -0,02 | 1,00E+00 |
| At2g33160 | glycoside hydrolase family 28 (polygalacturonase) | ✓ | | 6,56 | 6,46 | -0,10 | 1,00E+00 |
| At3g06770 | glycoside hydrolase family 28 (polygalacturonase) | | ✓ | 9,01 | 8,78 | -0,23 | 1,00E+00 |
| At3g16850 | glycoside hydrolase family 28 (polygalacturonase) | ✓ | ✓ | 7,08 | 6,95 | -0,13 | 1,00E+00 |
| At3g61490 | glycoside hydrolase family 28 (polygalacturonase) | ✓ | ✓ | 8,09 | 8,55 | 0,45 | 1,00E+00 |
| At4g18180 | glycoside hydrolase family 28 (polygalacturonase) | ✓ | | 6,57 | 6,55 | -0,03 | 1,00E+00 |
| At1g68560 | glycoside hydrolase family 31 (alpha-xylosidase) (AtXYL1) | ✓ | ✓ | 10,6 | 10,13 | -0,47 | 1,00E+00 |
| At3g13790 | glycoside hydrolase family 32 (beta-fructofuranosidase) | ✓ | ✓ | 6,89 | 6,78 | -0,11 | 1,00E+00 |
| At5g34940 | glycoside hydrolase family 79 (endo beta-glucuronidase/heparanase) | ✓ | ✓ | 6,98 | 6,90 | -0,09 | 1,00E+00 |
| At1g11580 | carbohydrate esterase family 8 (pectin methyltransferase) | ✓ | | 7,29 | 7,26 | -0,04 | 1,00E+00 |
| At1g53830 | carbohydrate esterase family 8 (pectin methyltransferase) (AtPME2) | ✓ | ✓ | 8,35 | 7,61 | -0,74 | 1,61E-02 |
| At3g14310 | carbohydrate esterase family 8 (pectin methyltransferase) (AtPME3) | ✓ | ✓ | 10,79 | 9,90 | -0,90 | 4,05E-05 |
| At3g43270 | carbohydrate esterase family 8 (pectin methyltransferase) | ✓ | ✓ | 6,30 | 6,46 | 0,16 | 1,00E+00 |
| At4g33220 | carbohydrate esterase family 8 (pectin methyltransferase) | ✓ | ✓ | 8,08 | 7,75 | -0,33 | 1,00E+00 |
| At5g53370 | carbohydrate esterase family 8 (pectin methyltransferase) | ✓ | | 7,41 | 7,30 | -0,11 | 1,00E+00 |
| At4g37950 | polysaccharide lyase family 4 (rhannogalacturonate lyase) | | ✓ | 6,68 | 6,73 | 0,05 | 1,00E+00 |
| At1g05570 | glycosyl transferase family 48 (callose synthase) (AtCalS1) | ✓ | ✓ | 6,58 | 6,58 | 0,00 | 1,00E+00 |
| At5g02260 | alpha-expansin (AtEXPA9) | ✓ | ✓ | 12,71 | 12,44 | -0,28 | 1,00E+00 |
| At1g20190 | alpha-expansin (AtEXPA11) | ✓ | ✓ | 8,76 | 8,72 | -0,03 | 1,00E+00 |
| At5g39270 | alpha expansin (AtEXP22) | ✓ | | nd | nd | | |
| At3g45970 | expansin-like A (AtEXLA1) | ✓ | ✓ | nd | nd | | |
| At4g38400 | expansin-like A (AtEXLA2) | ✓ | ✓ | 7,98 | 7,74 | -0,24 | 1,00E+00 |
| At3g45960 | expansin-like A (AtEXLA3) | ✓ | | 6,97 | 6,91 | -0,06 | 1,00E+00 |
| oxido-reductases | | | | | | | |
| At1g71695 | peroxidase (AtPrx12) | ✓ | ✓ | 10,17 | 9,54 | -0,63 | 4,86E-01 |
| At3g21770 | peroxidase (AtPrx30) | ✓ | ✓ | 9,54 | 9,77 | 0,23 | 1,00E+00 |
| At3g32980 | peroxidase (AtPrx32) | ✓ | ✓ | nd | nd | | |
| At3g49110 | peroxidase (AtPrx33) | ✓ | ✓ | 6,28 | 6,37 | 0,09 | 1,00E+00 |
| At3g49120 | peroxidase (AtPrx34) | ✓ | ✓ | nd | nd | | |
| At3g50990 | peroxidase (AtPrx36) | ✓ | | 6,47 | 6,58 | 0,11 | 1,00E+00 |
| At5g25980 | peroxidase (AtPrx43) | ✓ | ✓ | 6,52 | 6,54 | 0,02 | 1,00E+00 |
| At4g30170 | peroxidase (AtPrx45) | ✓ | ✓ | 7,52 | 7,65 | 0,13 | 1,00E+00 |
| At5g17820 | peroxidase (AtPrx57) | ✓ | ✓ | 6,99 | 6,76 | -0,23 | 1,00E+00 |
| At5g64100 | peroxidase (AtPrx69) | ✓ | ✓ | 6,72 | 6,68 | -0,05 | 1,00E+00 |
| At5g66390 | peroxidase (AtPrx72) | ✓ | ✓ | 8,70 | 8,57 | -0,13 | 1,00E+00 |
| At2g30210 | homologous to laccase (AtLAC3) | ✓ | ✓ | 8,23 | 8,14 | -0,09 | 1,00E+00 |
| At1g30710 | homologous to berberine-bridge enzyme (S)-reticul: oxygen oxidoreductase | ✓ | | 6,32 | 6,41 | 0,09 | 1,00E+00 |
| At4g20860 | homologous to berberine-bridge enzyme (S)-reticul: oxygen oxidoreductase | | ✓ | 6,80 | 6,78 | -0,01 | 1,00E+00 |
| At5g44360 | homologous to berberine-bridge enzyme (S)-reticul: oxygen oxidoreductase | ✓ | ✓ | 6,56 | 6,64 | 0,07 | 1,00E+00 |
| At5g44410 | homologous to berberine-bridge enzyme (S)-reticul: oxygen oxidoreductase | ✓ | ✓ | 6,78 | 6,91 | 0,12 | 1,00E+00 |
| At1g01980 | homologous to berberine-bridge enzyme (S)-reticul: oxygen oxidoreductase | | ✓ | 6,22 | 6,34 | -0,11 | 1,00E+00 |
| At5g22140 | expressed protein (oxido-reductase domain) | ✓ | | nd | nd | | |
| At5g56490 | expressed protein (FAD binding domain) | ✓ | | nd | nd | | |
| At2g02850 | plantacyanin ARPN (blue copper binding protein) | | ✓ | 6,84 | 6,90 | 0,06 | 1,00E+00 |
| At4g12880 | early nodulin A1EN20 homologous to blue copper binding protein | ✓ | ✓ | 10,84 | 11,19 | 0,36 | 1,00E+00 |

| proteins with interacting domains | | | | | | | | |
|-----------------------------------|--|---|---|----|-------|-------|-------|----------|
| At1g53070 | homologous to lectin (legume lectin domains) | ✓ | ✓ | | 7,92 | 7,74 | -0,18 | 1,00E+00 |
| At1g78820 | homologous to lectin (curculin-like) | ✓ | ✓ | | 8,96 | 8,04 | -0,93 | 1,15E-05 |
| At1g78830 | homologous to lectin (curculin-like) | ✓ | ✓ | | 9,82 | 9,22 | -0,60 | 1,00E+00 |
| At1g78850 | homologous to lectin (curculin-like) | ✓ | ✓ | | 11,95 | 11,42 | -0,53 | 1,00E+00 |
| At1g78860 | homologous to lectin (curculin-like) | ✓ | ✓ | | 7,05 | 7,20 | 0,15 | 1,00E+00 |
| At5g06860 | PGIP1 (LRR domains) | ✓ | ✓ | nd | nd | | | |
| T23B7.10 | homologous to PGIP1 (LRR protein FLR1) | ✓ | ✓ | nd | nd | | | |
| At5g12940 | homologous to <i>Phaseolus vulgaris</i> PGIP2 (LRR domains) | ✓ | ✓ | | 9,68 | 9,05 | -0,63 | 5,72E-01 |
| At1g33590 | expressed protein (LRR domains) | ✓ | ✓ | nd | nd | | | |
| At2g34930 | expressed protein (LRR domains) | ✓ | ✓ | | 7,46 | 7,68 | 0,22 | 1,00E+00 |
| At3g20820 | expressed protein (LRR domains) | ✓ | ✓ | | 10,49 | 9,99 | -0,50 | 1,00E+00 |
| At2g17120 | expressed protein (LysM domain) | ✓ | ✓ | | 9,22 | 9,03 | -0,19 | 1,00E+00 |
| At1g03220 | homologous to carrot EDGP and tomato XEGIP | ✓ | ✓ | nd | nd | | | |
| At1g03230 | homologous to carrot EDGP and tomato XEGIP | ✓ | ✓ | nd | nd | | | |
| At5g19110 | homologous to carrot EDGP and tomato XEGIP | ✓ | ✓ | | 8,10 | 8,09 | -0,01 | 1,00E+00 |
| At1g47710 | homologous to serpin (serine protease inhibitor) | ✓ | ✓ | | 8,01 | 7,97 | -0,04 | 1,00E+00 |
| At1g17860 | inhibitor family I3 (Kunitz-P family) (subfamily I3A unassigned peptidase inhibitor homologues, MEROPS) | ✓ | ✓ | | 8,78 | 8,95 | 0,17 | 1,00E+00 |
| At1g73260 | inhibitor family I3 (Kunitz-P family) (subfamily I3A unassigned peptidase inhibitor homologues, MEROPS) | ✓ | ✓ | | 12,00 | 12,50 | 0,50 | 1,00E+00 |
| At1g47540 | inhibitor family I18 (mustard trypsin inhibitor-2 family) (family I18 unassigned peptidase inhibitor homologues, MEROPS) | ✓ | ✓ | nd | nd | | | |
| At2g40880 | inhibitor family I25 (phytostatin) (cystatin family, I25.014, MEROPS) | ✓ | ✓ | nd | nd | | | |
| At5g05110 | inhibitor family I25 (phytostatin) (cystatin family, I25.014, MEROPS) | ✓ | ✓ | | 7,84 | 7,93 | 0,09 | 1,00E+00 |
| At4g16500 | inhibitor family I25 (cystatin family) (subfamily I25B unassigned peptidase inhibitor homologues, MEROPS) | ✓ | ✓ | | 8,08 | 8,42 | 0,34 | 1,00E+00 |
| At4g25260 | homologous to plant invertase/pectin methyltransferase inhibitor (PMEI) | ✓ | ✓ | | 8,67 | 9,10 | 0,43 | 1,00E+00 |
| At5g46940 | homologous to plant invertase/pectin methyltransferase inhibitor | ✓ | ✓ | | 6,36 | 6,33 | -0,03 | 1,00E+00 |
| At5g46960 | homologous to plant invertase/pectin methyltransferase inhibitor | ✓ | ✓ | nd | nd | | | |
| proteases | | | | | | | | |
| At1g09750 | homologous to aspartic protease (pepsin family) (Peptidase family A1, subfamily A1B unassigned peptidases, MEROPS) | ✓ | ✓ | nd | nd | | | |
| At3g02740 | homologous to aspartic protease (pepsin family) (Peptidase family A1, subfamily A1B unassigned peptidases, MEROPS) | ✓ | ✓ | | 7,22 | 7,32 | 0,10 | 1,00E+00 |
| At3g52500 | homologous to aspartic protease (pepsin family) (Peptidase family A1, subfamily A1B unassigned peptidases, MEROPS) | ✓ | ✓ | | 8,8 | 8,34 | -0,46 | 1,00E+00 |
| At3g54400 | homologous to aspartic protease (pepsin family) (Peptidase family A1, subfamily A1B unassigned peptidases, MEROPS) | ✓ | ✓ | | 9,33 | 8,92 | -0,42 | 1,00E+00 |
| At5g07030 | homologous to aspartic protease (pepsin family) (Peptidase family A1, subfamily A1B unassigned peptidases, MEROPS) | ✓ | ✓ | nd | nd | | | |
| At1g97920 | homologous to aspartic protease (CND41 peptidase) (Peptidase family A1, subfamily A1.050, MEROPS) | ✓ | ✓ | | 8,93 | 8,43 | -0,51 | 1,00E+00 |
| At5g10770 | homologous to aspartic protease (CND41 peptidase) (Peptidase family A1, A01.050, MEROPS) | ✓ | ✓ | | 12,46 | 9,97 | -2,49 | 0,00E+00 |
| At1g47128 | homologous to cysteine proteinase (papain family) (RD21A, Peptidase family C1, C01.064 MEROPS) | ✓ | ✓ | nd | nd | | | |
| At5g43060 | homologous to cysteine proteinase (papain family) (RD21 peptidase, Peptidase family C1, C01.064 MEROPS) | ✓ | ✓ | nd | nd | | | |
| At4g01610 | homologous to cysteine proteinase (papain family) (Cathepsin B, Peptidase family C1, C01.049, MEROPS) | ✓ | ✓ | | 12,4 | 12,38 | -0,02 | 1,00E+00 |
| At4g36880 | homologous to cysteine proteinase (papain family) (Peptidase family C1, Brassicain, C01.021 MEROPS) | ✓ | ✓ | | 8,63 | 8,79 | 0,16 | 1,00E+00 |
| At3g02110 | homologous to serine carboxypeptidase D (SCPL25) (Peptidase family S10, S10.005, MEROPS) | ✓ | ✓ | | 7,92 | 7,71 | -0,20 | 1,00E+00 |
| At5g23210 | homologous to serine carboxypeptidase (SCPL34) (Peptidase family S10, S10.005, MEROPS) | ✓ | ✓ | | 9,49 | 8,82 | -0,67 | 1,49E-01 |
| At4g30610 | homologous to serine carboxypeptidase (BRS1 - Brassinosteroid-Insensitive BRI suppressor 1) (Peptidase family S10, S10.015 MEROPS) | ✓ | ✓ | | 7,42 | 7,21 | -0,21 | 1,00E+00 |
| structural proteins | | | | | | | | |
| At1g28290 | proline-rich protein | ✓ | ✓ | | 10,97 | 11,42 | 0,46 | 1,00E+00 |
| At5g14920 | proline-rich protein | ✓ | ✓ | | 8,11 | 7,79 | -0,31 | 1,00E+00 |
| At2g05580 | glycine-rich protein | ✓ | ✓ | | 8,12 | 8,40 | 0,29 | 1,00E+00 |
| At4g13340 | LRR-extensin (AtLRX3) | ✓ | ✓ | | 6,89 | 6,83 | -0,06 | 1,00E+00 |
| At3g24480 | LRR-extensin (AtLRX4) | ✓ | ✓ | | 9,68 | 9,56 | -0,11 | 1,00E+00 |
| At4g18670 | LRR-extensin (AtLRX5) | ✓ | ✓ | | 6,70 | 6,79 | 0,09 | 1,00E+00 |

| signaling | | | | | | | |
|--------------------------------------|---|---|---|-------|-------|-------|----------|
| At4g05200 | homologous to receptor kinase (RLK, DUF26-1b subfamily) | ✓ | | 6,50 | 6,51 | 0,01 | 1,00E+00 |
| At5g55730 | fasciclin-like arabinogalactan protein (AtFLA1) | ✓ | ✓ | 8,18 | 7,96 | -0,22 | 1,00E+00 |
| proteins related to lipid metabolism | | | | | | | |
| At1g29670 | homologous to lipase acylhydrolase (GDSL family) | ✓ | ✓ | nd | nd | | |
| At1g54010 | homologous to lipase/acylhydrolase (GDSL family) | ✓ | | nd | nd | | |
| At1g54030 | homologous to lipase/acylhydrolase (GDSL family) | ✓ | ✓ | 10,10 | 9,75 | -0,35 | 1,00E+00 |
| At3g48460 | homologous to lipase acylhydrolase (GDSL family) | ✓ | ✓ | 8,05 | 7,79 | -0,26 | 1,00E+00 |
| At5g15720 | homologous to lipase acylhydrolase (GDSL family) | | ✓ | 6,85 | 6,73 | -0,13 | 1,00E+00 |
| At2g38530 | non-specific lipid transfer protein 2 (LTP2) | ✓ | ✓ | nd | nd | | |
| At5g23820 | expressed protein (ML domain - MD-2-related lipid recognition domain) | ✓ | | nd | nd | | |
| At2g16001 | expressed protein (lipid recognition domain) | ✓ | | nd | nd | | |
| miscellaneous functions | | | | | | | |
| At2g27190 | homologous to purple acid phosphatase (PAP1) | ✓ | ✓ | 10,67 | 10,42 | -0,25 | 1,00E+00 |
| At3g07130 | homologous to purple acid phosphatase | ✓ | ✓ | 6,62 | 6,52 | -0,10 | 1,00E+00 |
| At5g34850 | homologous to purple acid phosphatase | ✓ | | 11,84 | 11,39 | -0,45 | 1,00E+00 |
| At4g29270 | homologous to acid phosphatase | ✓ | ✓ | nd | nd | | |
| At4g24340 | homologous to phosphorylase | ✓ | | nd | nd | | |
| At3g02870 | homologous to myo-inositol monophosphatase | ✓ | | 7,17 | 7,38 | 0,21 | 1,00E+00 |
| At5g09440 | homologous to <i>Nicotiana tabacum</i> phi-I | ✓ | ✓ | 11,87 | 10,73 | -1,14 | 3,41E-10 |
| At5g64260 | homologous to <i>Nicotiana tabacum</i> phi-I | ✓ | ✓ | 6,47 | 6,47 | 0,00 | 1,00E+00 |
| At5g66590 | homologous to <i>Nicotiana tabacum</i> pathogenesis-related protein PR1 | ✓ | ✓ | 6,42 | 6,40 | -0,02 | 1,00E+00 |
| At2g28790 | homologous to <i>Lycopersicon esculentum</i> osmotin | ✓ | ✓ | 8,00 | 8,45 | 0,45 | 1,00E+00 |
| At5g15230 | gibberellin-regulated protein (GASA4) | ✓ | | 12,58 | 13,42 | 0,84 | 4,08E-04 |
| At4g27110 | homologous to COBRA (AtCOBL10) | ✓ | | 6,39 | 6,38 | -0,01 | 1,00E+00 |
| At1g09560 | germin (subfamily 2, member 1, GLP5) | | ✓ | 10,49 | 9,99 | -0,50 | 1,00E+00 |
| unknown function | | | | | | | |
| At3g56750 | expressed protein | | ✓ | nd | nd | | |
| At3g22000 | expressed protein (DUF26) | | ✓ | nd | nd | | |
| At1g26850 | expressed protein (DUF248) | | ✓ | nd | nd | | |
| At1g80240 | expressed protein (DUF642) | ✓ | ✓ | 8,11 | 7,73 | -0,38 | 1,00E+00 |
| At3g08030 | expressed protein (DUF642) | ✓ | ✓ | 12,03 | 11,69 | -0,33 | 1,00E+00 |
| At4g32460 | expressed protein (DUF642) | ✓ | ✓ | 9,52 | 9,02 | -0,51 | 1,00E+00 |
| At5g11420 | expressed protein (DUF642) | ✓ | ✓ | 11,97 | 11,68 | -0,29 | 1,00E+00 |
| At5g25460 | expressed protein (DUF642) | ✓ | ✓ | 9,81 | 9,24 | -0,57 | 1,00E+00 |
| At1g78460 | expressed protein (SOUL heme binding domain) | | ✓ | 6,52 | 6,54 | 0,02 | 1,00E+00 |
| At2g04690 | expressed protein (homologous to a human brain CREG protein) | ✓ | ✓ | 9,11 | 9,24 | 0,14 | 1,00E+00 |
| At2g15220 | expressed protein (Plant Basic Secreted Protein domain) | ✓ | ✓ | nd | nd | | |
| At2g34700 | expressed protein (Ole el allergen domain) | ✓ | ✓ | 10,14 | 8,35 | -1,80 | 0,00E+00 |
| At3g20370 | expressed protein (MATH domain) | | ✓ | nd | nd | | |
| At2g28490 | expressed protein (cupin domain) | ✓ | ✓ | 10,09 | 10,69 | 0,59 | 1,00E+00 |
| At3g22640 | expressed protein (cupin domain) | ✓ | ✓ | 9,52 | 10,52 | 1,00 | 4,06E-07 |
| At4g36700 | expressed protein (cupin domain) | ✓ | | 6,72 | 7,11 | 0,39 | 1,00E+00 |

Additional data file 5**Table 5. Nucleotide primers used for PCR amplifications**

| Accession number | Primer sequence (5'→3', sense orientation) | Primer sequence (5'→3', antisense orientation) | Length of expected fragment (bp) |
|-------------------------|---|---|---|
| At1g05570 | CACTTCCTCTGATAACAATCAAAGAC | GCTTACACGCTTGTGCAATG | 96 |
| At1g10550 | CAATTCAGTAAGATCGCCATTG | TTTGACACCAACCCAGCTC | 125 |
| At1g28290 | AACCATAAGACTCAAACCCTTC | TGAGGGTGGTGGTGATGAG | 95 |
| At1g49240 | CACCCGAGAGGAAGTACAGTG | CATACTCTGCCTTAGAGATCCACA | 93 |
| At1g66180 | ACCACAAGCTCAACAAATGGT | GGAGGAAGCTTTTTACGATGAC | 78 |
| At1g68560 | GTCGCCGTAAATGTTGTTG | CCCATCTACATTGATGAAATCCT | 95 |
| At1g69530 | GCATCGCTCAATACAGAGC | GAGTGTCCGTTTATCGTAAACCTT | 97 |
| At2g21140 | TCTCATCCTTGATAAAGATGC | TCTACGTAGAATTCAACAAAGC | 111 |
| At2g28790 | GTGCCACTACAACGGAAAA | TGAGCTAGAGAAGCTGGTGGT | 100 |
| At3g07130 | GGGATCGGCAACCTGATTA | GCCCATGTCTCGTTCTTCAT | 82 |
| At3g16850 | GTAGCCTTCAGAACCACCAGA | TCAACGCTTGTGAGATCGAC | 80 |
| At3g43270 | TTCCGATCTGCTACATTTGCT | GACCGCTTGGTGTTTTTCC | 96 |
| At4g12880 | GGCATGAAGCTAGATGTTTTAGTTG | ACATAAAATTGATTTCTTATTGTGCTG | 91 |
| At4g18670 | CACCACCTCCAATCTACGAAG | GCCCCTTTTGAGAACATTCTG | 112 |
| At5g05850 | TGGTACCACTGGAGGAAAAGC | TTGCAACTCCATAGCCACAG | 95 |
| At5g11420 | GTCTCTTCTCTTTACTTTGGTCGTC | AGTCGCCGTTTGGTAACATC | 126 |
| At5g44360 | AGGAGCTTATTTGAATTACCGAGA | TCGCATCTTCAAAGCTCGTA | 74 |
| At5g64100 | GCAAGACTTCGCTGCTAAAAC | GCCGTTGAAGTTAACGAACC | 118 |
| desmin ^a | CAGCCTCAGTCTCCAAATCACA | TAGGCCTGAGGTCACAGAGGT | |

a : Pig desmin RNA was used as an internal control for reverse transcription.

Muhammad IRSHAD

**Dynamique des protéines pariétales au cours de l'élongation cellulaire
dans des hypocotyles étiolés d'*Arabidopsis thaliana* :
approches protéomique et transcriptomique**

Résumé

La paroi cellulaire des végétaux supérieurs est une structure complexe jouant de nombreux rôles au cours du développement ainsi qu'en réponse aux stress. Les protéines pariétales sont notamment importantes au cours de l'élongation cellulaire. Des hypocotyles étiolés d'*Arabidopsis thaliana* ont été choisis comme modèle d'élongation cellulaire parce qu'ils subissent une élongation polarisée et rapide en l'absence de division cellulaire. Deux stades de développement ont été comparés grâce à des analyses protéomique et transcriptomique : pendant leur élongation *vs* après la fin de leur croissance. Pour rendre l'analyse protéomique efficace, une nouvelle méthode de purification de parois et une stratégie de séparation des protéines pariétales ont été établies. Les protéines ont été identifiées par cartographie peptidique massive en utilisant la spectrométrie de masse de type MALDI-TOF. Cette étude a permis d'identifier 137 protéines parmi lesquelles 51 n'avaient pas été identifiées auparavant. Plusieurs familles de protéines connues pour être impliquées dans l'élongation cellulaire ou son arrêt ont été trouvées par l'une ou l'autre approche (XTH, PG, expansines, peroxydases, laccases). De nouvelles protéines candidates pour jouer des rôles dans l'élongation cellulaire ont été identifiées, telles des protéases, des protéines liées au métabolisme des lipides ou des protéines de fonction inconnue. La comparaison des résultats de protéomique et de transcriptomique ne montre pas de cohérence systématique, suggérant l'existence de mécanismes de régulation post-transcriptionnels des gènes codant les protéines pariétales.

Mots-clefs :

Arabidopsis thaliana, développement, protéines pariétales, paroi cellulaire végétale, protéomique, transcriptomique

**Dynamics of cell wall proteins during cell elongation in etiolated hypocotyls
of *Arabidopsis thaliana* as shown by proteomic and transcriptomic surveys**

Abstract

The cell wall of higher plants is a complex dynamic entity that performs a variety of functions during growth and development as well as in response to environmental stresses. Cell wall proteins play important roles in cell elongation. Etiolated hypocotyls of *Arabidopsis thaliana* were chosen as elongating organs since they undergo rapid and polar elongation without cell division. Two developmental stages were compared through proteomic and transcriptomic surveys: active elongation (5-day-old hypocotyls) *vs* after growth arrest (11 day-old hypocotyls). As a prerequisite to proteomic study, an efficient cell wall purification method was established as well as a powerful strategy for protein separation. Proteins were identified by peptide mass mapping using MALDI-TOF mass spectrometry and bioinformatics. This study resulted in identification of 137 proteins among which 51 were not identified before by proteomics, and 36 (resp. 17) were found only at 5-days (resp. 11-days). Overall, many protein families expected to be involved in cell wall elongation or in elongation arrest were found by either survey such as XTHs, PGs, expansins, peroxidases, and laccases. However, some of them were found all along hypocotyl development which suggests other roles in cell walls. In addition, many proteins not described before to play roles during cell wall elongation were identified, such as proteases, proteins related to lipid metabolism and proteins of yet unknown function. Finally, proteomic and transcriptomic results revealed no systematic consistency between them suggesting post-transcriptional regulation steps for genes encoding cell wall proteins.

Key words:

Arabidopsis thaliana, development, cell wall proteins, plant cell wall, proteomics, transcriptomics