

The first international fungal / plant cell wall meeting

**Biarritz, France
March 10 – 14, 2007**

Cell Wall Polysaccharides of fungi and plants



The local organizing Committee

**J.P. Latgé (Institut Pasteur, Paris, France)
H. Höfte (INRA, Versailles, France)**

The International Scientific Committee

**T. Bacic (University of Melbourne, Australia)
R. Calderone (Georgetown University, Washington DC, USA)
N. Gow (University of Aberdeen, UK)
H. Höfte (INRA, Versailles, France)
J.P. Latgé (Institut Pasteur, Paris, France)
Y. Ohya (University of Tokyo, Japan)
M. Pauly (Max Planck Institute, Golm, Germany)
C. Somerville (Carnegie Institution, Stanford, CA, USA)**

CONTENTS

Program	3
Invited Lectures (chronological order) : L 1 to L 48	8
Selected talks of FEMS grantees : F 1 to F 11	59
Posters (alphabetical order) : P 1 to P 44	70
Author index	116

PROGRAM

SATURDAY

2:00 pm – 10:00 pm *Arrival and Check-in*

Introduction

Chairs : J.P. Latgé and H. Höfte

5:00 pm – 5:15 pm **Introductory remarks**

5:15 pm – 6:00 pm **Keynote Lectures**
H. Bussey (Montreal, Canada) L1
The fungal cell wall

6:00 pm – 6:45 pm **K. Keegstra** (East Lansing, USA) L2
The plant cell wall

7:30 PM *Welcome reception*

SUNDAY

7:30 am – 9:00 am *Breakfast*

Structure and organisation of the cell wall

Chairs : N. Gow and A. Bacic

9:00 am – 9:20 am **J.-P. Latgé** (Paris, France) L3
The fungal cell wall polysaccharidome

9:20 am – 9:40 am **M. Hahn** (Athens, USA) L4
Structural and immunological studies of pectins and hemicelluloses in plant cell walls

9:40 am – 10:00 am **F. Klis** (Amsterdam, The Netherlands) L5
Dynamics of the fungal cell wall proteome

10:00 am – 10:20 am **J.P. Knox** (Leeds, UK) L6
Dynamics and diversity of plant cell wall glycans and cell development

10:30 am – 11:00 am *Coffee break*

11:00 am – 11:20 am **M.-C. Ralet** (Nantes, France) L7
Structure-function studies of pectins

- 11:20 am – 11:40 am **J. Arroyo** (Madrid, Spain) L8
Cell wall remodelling in *Saccharomyces cerevisiae* stressed cells
- 11:40 am – 12:00 am **Mami Konomi** (Tokyo, Japan) L9
Ultrastructure of Yeast cell wall revealed by electron microscopy
- 12:00 am – 12:20 am **V. Morris** (Norwich,UK) L10
Atomic force microcopy to study cell wall polymer structures

12:30 am – 2:00 pm Lunch

Cell wall biosynthesis (I)

Chairs : J.M. François and P. Dupree

- 2:00 pm – 2:20 pm **B. Henrissat** (Marseille, France) L11
Carbohydrate-active enzymes in fungal and plant genomes
- 2:20 pm – 2:40 pm **V. Bulone** (Stockholm, Sweden) L12
(1,3)- β -D-glucan and cellulose biosynthesis in plants and in the Oomycete *Saprolegnia monoica*
- 2:40 pm – 3:00 pm **A. Bacic** (Melbourne, Australia) L13
Biosynthesis and regulation of cell wall β -glucans in plants
- 3:00 pm – 3:20 pm **J.C. Ribas** (Salamanca, Spain) L14
Essential functions in septum and cell wall construction of the Bgs family of (1,3)- β -D-glucan synthase catalytic subunits from *Schizosaccharomyces pombe*
- 3:20 pm – 3:50 pm Coffee break**
- 3:50 pm – 4:10 pm **M. Pauly** (Golm, Germany) L15
Identification and characterisation of plant cell wall mutants using a glycosylhydrolase screen
- 4:10 pm – 4:30 pm **C.R. Vazquez de Aldana** (Salamanca, Spain) L16
Glycoside hydrolases and glucanosyl transferases in *S. pombe*
- 4:30 pm – 4:50 pm **S. Persson** (Stanford, USA) L17
Discrete functional roles for cellulose synthase A (CESA) genes during pollen maturation and root development
- 4:50 pm – 5:10 pm **D. Mohnen** (Athens, GA, USA) L18
Pectin synthesis
- 7:00 pm – 9:00 pm Dinner**
- 9:00 pm – 11:00 pm Posters with bubbles

MONDAY

7:30 am – 9:00 am Breakfast

Cell wall biosynthesis (II)

Chairs : C. Boone and D. Mohnen

- 9:00 am – 9:20 am **S. Turner** (Manchester, UK) L19
Carbohydrate synthesis in the *Arabidopsis* secondary cell wall
- 9:20 am – 9:40 am **N.A.R. Gow** (Aberdeen, UK) L20
The chitin skeleton of *Candida albicans*: biosynthesis and regulation under stress
- 9:40 am – 10:00 am **C. Roncero** (Salamanca, Spain) L21
Chitin synthesis regulation in *Saccharomyces cerevisiae*
- 10:00 am – 10:20 am **S. Fry** (Edinburgh, UK) L22
A novel dual-radiolabelling approach to studying the competing pathways of NDP-sugar biosynthesis in vivo

- 10:30 am – 11:00 am** **Coffee break**
- 11:00 am – 11:20 am **H. Horiuchi** (Tokyo, Japan) L23
Functional dissection of the chitin synthase system for the growth and morphogenesis of *Aspergillus nidulans*
- 11:20 am – 11:40 am **M. Azuma** (Osaka, Japan) L24
Characterization of yeast mutants with a defect in cell surface structure and their application to various fields
- 11:40 am – 12:00 am **P. Dupree** (Cambridge, UK) L25
Golgi proteomics to study cell wall polysaccharide synthesis in plants
- 12:00 am – 12:20 am **G. Fincher** (Adelaide, Australia) L26
Structure-function studies of xyloglucan transglycosylases / hydrolases

12:30 am – 2:00 pm **Lunch**

Regulation of cell wall synthesis (I)

Chairs : J. Arroyo and K. Keegstra

- 2:00 pm – 2:20 pm **Y. Ohya** (Tokyo, Japan) L27
Yeast cell cycle checkpoint that monitors cell wall synthesis
- 2:20 pm – 2:40 pm **C. Boone** (Toronto, Canada) L28
A combined computational and experimental approach for a functional interaction map of yeast
- 2:40 pm – 3:00 pm **J.M. François** (Toulouse, France) L29
Tools and identification of cell wall proteins-cell wall polysaccharide interactomics
- 3:00 pm - 3:20 pm **R. Calderone** (Washington DC, USA) L30
The histidine kinase Chk1p and cell wall synthesis in *Candida albicans*

3:20 pm – 3:50 pm **Coffee break**

- 4:00 pm - 6:30 pm **Talks from FEMS grantees**
- C. Bermejo** (Madrid, Spain) F1
E. Danchin (Marseille, France) F2
A. Gastebois (Paris, France) F3
A. Gomez (Salamanca, Spain) F4
R. Hurtado Guerrero (Dundee, Scotland, UK) F5
V. Kumar (Mysore, India) F6
O. Lerouxel (Versailles, France) F7
E. Ragni (Milano, Italy) F8
J. Romano (Tel-Aviv, Israel) F9
V. Seidl (Edinburgh, Scotland, UK) F10
A. Vos (Amsterdam, The Netherlands) F11

8:00 PM **Gala Dinner**

TUESDAY

8:00 am – 9:00 am **Breakfast**

Regulation of cell wall synthesis (II)

Chairs : L. Popolo and S. Turner

- 9:00 am – 9:20 am **H. Höfte** (Versailles, France) F31
Coordination between cell wall synthesis and growth in plants
- 9:20 am – 9:40 am **B.G. Wasteneys** (Vancouver, Canada) F32
The role of the cytoskeleton in cell wall deposition

- 9:40 am – 10:00 am **H. Fukuda** (Tokyo, Japan) F33
Signaling involved in xylogenesis
- 10:00 am – 10:20 am **M. Molina** (Madrid, Spain) F34
Cell wall salvage pathways

10:30 am – 11:00 am *Coffee break*

Cell wall polysaccharide synthesis in other systems

- 11:00 am – 11:20 am **M. Terrak** (Liege, Belgium) F35
Bacterial cell wall biosynthesis
- 11:20 am – 11:40 am **H. Merzendorfer** (Osnabrück, Germany) F36
Chitin synthesis in insects: lessons from the midgut of the tobacco hornworm
- 11:40 am – 12:00 am **V. Eijsink** (As, Norway) F37
Structure-function studies of enzymes and proteins involved in chitin turnover
- 12:00 am – 12:20 am **N. Ohno** (Tokyo, Japan) F38
Contribution of Dectin-1 on Immunomodulating Effect of Soluble beta-Glucan SCG in Mice

12:30 am – 2:00 pm *Lunch*

FREE AFTERNOON

7:30 pm – 9:00 pm *Dinner*

9:00 pm – 11:00 pm Posters with bubbles

WEDNESDAY

7:30 am – 9:00 am *Breakfast*

Biological properties of the cell wall

Chairs : R. Calderone and M.-C. Ralet

- 9:00 am – 9:20 am **D. Poulain** (Lille, France) F39
Structure / biological activity of β 1,2 linked mannoses and their association to a wide range of carrier molecules are under the control of a new family of mannosyl transferases
- 9:20 am – 9:40 am **H. Shimoi** (Higashi-Hiroshima, Japan) F40
A cell wall protein involved in cell surface hydrophobicity and foam formation of sake yeast
- 9:40 am – 10:00 am **D. Perlin** (Newark, USA) F41
Antifungals blocking cell wall polysaccharide biosynthesis
- 10:00 am – 10:20 am **S. McQueen-Mason** (York, UK) F42
Varied functional roles for arabinans in plant cell walls

10:30 am – 11:00 am *Coffee break*

- 11:00 am – 11:20 am **S. Merino** (Copenhagen, Denmark) F43
Biomass conversion and plant cell walls
- 11:20 am – 11:40 am **W. Helbert** (Roscoff, France) F44
Heterogeneous phase biodegradation of cell wall polysaccharides: case of the carrageenan / carrageenase systems
- 11:40 am – 12:00 am **F. Meulewaeter** (Ghent, Belgium) F45
Improved reactivity of cotton fiber through accumulation of cationic polymers in the fiber cell wall
- 12:00 am – 12:20 am **D. van Aalten** (Dundee, UK) F46
Structure and function of chitin deacetylases

12:20 am – 12:40 pm **S. Strahl** (Heidelberg, Germany) F47
O-Mannosylation

12: 40 am – 1:00 pm **W. Steinbach** (Durham, NC, USA) F48
Echinocandin antifungals and other ways to inhibit β 1,3 glucan synthesis

1:00 pm – 2:30 pm **Lunch**

INVITED LECTURES

L 1 to L 48

The Fungal Cell Wall

L 1

While work remains, we have now largely reached the end of the “Age of Identification” for both cell wall structure and the underlying biosynthetic components - at least for a few model ascomycetes! Work is now well advanced at coming to grips with the biochemistry of polysaccharide biosynthesis and its regulated integration with broader cell biology in defining surface morphogenesis. Where do we go from here? A perusal of talk titles at this meeting points the way.

Cell walls are dynamic structures, responding to external cues and stresses as well as to internal growth, cell cycle and morphogenetic cues. We are beginning to know how all this is achieved at a molecular level. Further, attempts are being made to take a systems approach to the building of global cellular networks that capture both cell wall synthesis and cell wall function. Genomics also offers us the prospect of extending cell wall studies in a comparative way to a wider set of organisms within the fungal kingdom. Prominent amongst these are the human pathogens, where the cell wall continues to be a target for specific antifungal drugs.

Howard Bussey

Howard Bussey
Department of Biology, McGill
University
Montreal, QC, Canada
howard.bussey@mcgill.ca

The Plant Cell Wall

L 2

Plant cell walls are a complex composite of polysaccharides, proteins and lignin. Wall composition and organization varies greatly depending upon the plant species as well as the particular tissue and cell type. Growing cells have primary cell walls while differentiated cells normally have more rigid secondary walls. The structure of various primary wall components as well as their organization within the wall matrix will be reviewed. Cellulose is an abundant component found in both primary and secondary walls. Its biosynthesis is a complex process that involves synthesis of microfibrils at the plasma membrane. Other wall polyaccharides are synthesized in the Golgi apparatus and delivered to the cell surface for assembly into the wall matrix. In recent years, our lab has focused on the synthesis of xyloglucan (XyG), a major hemicellulose found in the primary cell walls of many plant species. It forms cross-links between adjacent cellulose microfibrils to form a three dimensional cellulose-xyloglucan network that functions as the principal load-bearing structure of the primary cell wall. Formation of XyG requires the action of a glucan synthase and xylosyltransferases. I will briefly describe our recent studies aimed at identifying these enzymes as well as efforts to disrupt the biosynthesis of XyG via reverse genetics.

David Cavalier
Olivier Lerouxel
Olga Zabolina
Lutz Neumetzler
Markus Pauly
Willie Abasolo
Ingo Burgert
Curt Wilkerson
Natasha Raikhel
Kenneth Keegstra

Kenneth Keegstra
MSU-DOE Plant Research
Laboratory
Michigan State University
East Lansing, MI USA
keegstra@msu.edu

Fungal cell walls are mainly composed of polysaccharides. Two of them, β 1-3 glucan and chitin are common to all species in the fungal kingdom and constitute the skeleton of the cell wall. The three dimensional organisation of the fungal cell wall has been however studied in very few fungal species. A comparison of the chemical composition of cell walls from different fungi demonstrates that a common skeletal core structure exists in almost all fungi. This core skeleton is similar for both yeasts and moulds. It is composed of a branched β 1-3 glucan to which chitin is linked through a β 1-4 linkage to a non reducing end of the lateral β 1-3 glucan chains. This fibrillar core is further decorated with amorphous polysaccharides that are alkali-soluble. In contrast to the structural polysaccharides, the composition of these polysaccharides varies with the species studied and has some taxonomical foundations. Among the most important amorphous polysaccharides, we can cite β 1,6 glucans, α 1,3 glucans and mannans. Proteins of the cell wall do not play a role of linker in the structural organisation of the cell wall. Most cell wall proteins are in transit towards the external milieu. Some of the proteins that were originally anchored to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor play a role in remodeling cell wall polysaccharides whereas other GPI-proteins can become covalently bound to the cell wall polysaccharides; this latter class of proteins remaining at the surface of the cell wall to fulfil their biological function (in cell to cell interaction for example). This conclusion was confirmed by a chemogenomic comparative analysis between yeasts and moulds.

In my talk, I will review our current understanding of the structural organisation of the cell wall with special emphasis on the two models that have been extensively studied, viz the yeast *Saccharomyces cerevisiae* and the mold *Aspergillus fumigatus*. This analysis will take into account recent genomic data from the comparison of yeast and mould genome sequences as well as chemical data on the cell wall of these fungi to identify general structural rules in fungal cell wall ontogeny.

Jean-Paul Latgé
Thierry Fontaine
Anne Beauvais
Cécile Clavaud
Isabelle Mouyna
Willy Morelle
Claude Lamarre
Vishu Kumar

Jean-Paul Latgé
Aspergillus unit
Institut Pasteur
75015 Paris, France
jplatge@pasteur.fr

Structural and immunological studies of pectins and hemicelluloses in plant cell walls

L 4

The cell walls of plants play a prominent role in determining the structure and shape of individual cells, and ultimately the morphology of the plant as a whole. The plant cell wall consists of several macromolecular networks that are composed primarily of polysaccharides. Chemical studies have provided an overall picture of the structure and organization of the major wall polymers [1]. A brief overview will be presented of the major structural features of two prominent classes of polysaccharides present in plant cell walls, the pectins and hemicelluloses. Pectins include linear homogalacturonans (consisting of a 1→4αGalA backbone with varying degrees of methyl esterification), rhamnogalacturonan I (consisting of a backbone of disaccharide 2αRha1→4αGalA1→ repeats with diverse Ara and/or Gal containing side-chains attached to the 2-position of Rha), and the highly branched rhamnogalacturonan II. Hemicelluloses include xyloglucans (consisting of a 1→4βGlc backbone carrying a regular pattern of Xyl side-chains attached at O6, some of which are further extended with Gal and Fuc residues) and xylans (consisting of a 1→4βXyl backbone with Ara, GlcA and/or MeGlcA side-chains). These chemical studies, while informative about structural features of the polysaccharides, do not provide complete information about wall structure and dynamics at the cellular and sub-cellular levels.

Antibodies provide highly specific and sensitive tools to monitor the composition and dynamics of cell walls at the cellular level [2]. An overview will be provided of new monoclonal antibodies that have been generated against pectic and hemicellulosic polysaccharides from plant cell walls. These antibodies recognize diverse epitopes present in these polysaccharides, as demonstrated by direct enzyme-linked immunosorbent assays (ELISAs) against a range of plant cell wall polysaccharide preparations from diverse plants and by competitive ELISAs using purified oligo- and polysaccharides. Very few of these antibodies are specific for individual polysaccharides, suggesting sharing of carbohydrate epitope structures amongst different wall polysaccharides. Nonetheless, these antibodies have proven to be useful tools for localizing such epitopes in plant tissues and cell walls, and for following changes in wall polysaccharide structure as plant cells grow and differentiate. Examples of such uses of these antibodies will be presented describing the distribution of fucosylated and non-fucosylated xyloglucans in germinating seeds of tropical legumes [3] and the analysis of the *irx8* mutant of *Arabidopsis thaliana* [4,5].

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1. O'Neill & York (2003), in *The Plant Cell Wall* (JK Rose, ed.), pp. 1-54.
2. Knox (1997) *Int.Rev.Cytol.* **171**: 79-120.
3. Buckeridge *et al.* (2000) *Plant Physiol.Biochem.* **38**: 141-156.
4. Persson *et al.* (2007) *The Plant Cell* (in press).
5. Peña *et al.* (2007) *The Plant Cell* (in press).

Sami Tuomivaara
David Baldwin
Anathea Albert
Glenn Freshour
Zoë Popper
Tracey Bootten
Alton G. Swennes
Ruth Davis
Alan Darvill
Malcolm O'Neill
William York
Michael G. Hahn

Michael G. Hahn
Complex Carbohydrate Research
Center and Department of Plant
Biology
University of Georgia
315 Riverbend Road
Athens, GA 30602-4712
USA
hahn@ccrc.uga.edu

The budding yeast *Saccharomyces cerevisiae* has a highly elastic wall. Consequently, the cell shrinks when challenged with hypertonic conditions and swells when transferred to a hypotonic medium. This process is reversible. Fixed cells, which cannot sustain turgor pressure any more, are generally also significantly smaller than living cells. This elasticity is due to the presence of a continuous inner wall layer, which consists of moderately branched β -1,3-glucan molecules that laterally associate through the formation of multiple hydrogen bonds. The β -1,3-glucan network may be fortified by the covalent attachment of chitin chains to non-reducing ends of the β -1,3-glucan chains. This happens predominantly at the inside of the β -1,3-glucan network. The network seems to be further strengthened by so-called Pir-proteins, which are assumed to cross-link β -1,3-glucan chains through a recently described ester linkage. The skeletal inner layer is surrounded by an external protein layer, which mainly consists of glycosylated, GPI-dependent cell wall proteins (GPI-CWPs) emanating into the environment. GPI-CWPs are covalently linked to a strongly branched and thus water-soluble β -1,6-glucan molecule, which in turn is linked to a β -1,3-glucan chain, forming the protein-polysaccharide complex CWP-GPI_r \rightarrow β -1,6-glucan \rightarrow β -1,3-glucan. This complex has also been identified in the cell wall of the dimorphic fungus *Candida albicans*, an important human pathogen. Evidence is emerging that this protein-polysaccharide complex is further present in the lateral walls of the fission yeast *Schizosaccharomyces pombe*, the mycelial fungus *Aspergillus niger*, and many other ascomycetous species (1).

A powerful tool to analyze the composition and dynamics of the fungal cell wall proteome is mass spectrometry. Intriguingly, many ascomycetous fungi incorporate a wide variety of GPI-CWPs and other CWPs in their walls (2). For example, wild-type cells of *S. cerevisiae* and *C. albicans* growing in rich medium express at least 20 different CWPs. The composition of the cell wall proteome depends on environmental conditions and even depends on the phase of the cell cycle. This leads to the question what the functions of CWPs are. To illustrate this, we will discuss the changes in the cell wall proteome of *C. albicans* when grown in a vagina-simulative medium under micro-aerobic conditions. As the high-affinity iron uptake system of *C. albicans* requires molecular oxygen, low oxygen levels will probably cause iron deficiency. This is consistent with our observation that the cell wall becomes enriched with two GPI-proteins (Rbt5p and presumably also Pga10p) that are involved in binding heme/hemoglobin, which are known to function as iron sources. We propose that our approach is widely valid for studying the cell wall proteome of fungi, algae, and higher plants.

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Literature

1. Klis FM, Boorsma A, De Groot PWJ 2006. Cell wall construction in *Saccharomyces cerevisiae*. *Yeast* 23: 185-202
2. De Groot PWJ, Ram AF, Klis FM 2005. Features and functions of covalently linked proteins in fungal cell walls. *Fungal Gen Biol* 42: 657-675

Frans M. Klis
Piet W.J. de Groot
Grazyna Sosinska
Qing Yuan Yin
Stanley Brul
Chris de Koster

Frans M. Klis
Swammerdam Institute for Life
Sciences, University of
Amsterdam, Nieuwe Achtergracht
166, 1018 WV Amsterdam, The
Netherlands
F.M.Klis@uva.nl

Cell walls form the structural component of plant organs imparting mechanical properties, tissue cohesion and capacities for extension growth. Plant cell walls can develop into a range of forms and shapes that underpin the diversities of organ anatomies and cell functions. The major macromolecular and functional components of plant cell walls are glycans and a cell wall is viewed as a composite of cellulose microfibrils, hemicelluloses or cross-linking glycans and pectic polysaccharides. These broad groups can contain a great range of structural diversity both in terms of glycosyl linkages, polymer association, domain arrangements and also sugar substitutions and modifications. Understanding the occurrence and organization of cell wall polymers in relation to cell wall construction and function requires appropriate means of detecting glycan structural features *in situ*. Currently, one of the best methods for glycan detection and the molecular imaging of cell wall architectures involves the use of defined monoclonal antibodies and carbohydrate-binding modules capable of the specific recognition of glycan structural features. Sets of probes are now available for the detection of structural features of all three major groups of glycans. The use of these sets of probes are indicating a considerable diversity of cell wall architectures and glycans, in short – that plant cell walls are highly diverse and dynamic both within organs and between species. The talk will focus on recent work on the development of probes for xylan polysaccharides and their use indicating that xylans may have distinct roles in primary and secondary cell walls.

Anthony Blake
José Ordaz-Ortiz
Susan Marcus
J. Paul Knox

J. Paul Knox
Centre for Plant Sciences
University of Leeds
Leeds LS2 9JT
United Kingdom
j.p.knox@leeds.ac.uk

All plant cells are encapsulated in a cell wall, whose most prominent components are polysaccharides: cellulose, hemicelluloses and pectins. When soluble polysaccharides are secreted into the wall, they are associated with newly synthesised cellulose microfibrils to form a strong and extensible network, so that polymers collectively determine cell wall shape and mechanical properties. Network formation implies the setting up of interactions between the wall polysaccharides.

Pectin is an extremely complex polysaccharide that can be viewed as a multiblock biopolymer. The simplest, and the most abundant of these blocks is homogalacturonan (HG), an unbranched polymer of (1,4)-linked galacturonic acid residues that are partly methyl-esterified and sometimes partly acetyl-esterified. A second major block, rhamnogalacturonan I (RG-I) is composed of a repeating disaccharide unit [2)-rhamnose-(1,4)-galacturonic acid-(1)]_n decorated primarily with arabinan and galactan side-chains. Finally, substituted galacturonans such as rhamnogalacturonan II (RG-II), apiogalacturonan, xylogalacturonan, are also present. All those blocks exhibit various interaction properties: HG/HG, HG/extensin, RG-I side-chains/RG-I side-chains, RG-I side-chains/cellulose, RG-II/RG-II.

Pectic blocks can be specifically isolated by chemical or enzymatic means. For example, HG blocks were isolated by exploiting the differences in the susceptibility to acid hydrolysis of the glycosidic linkages. Indeed, under mild acid conditions, the linkages between galacturonic acid and rhamnose in RG-I blocks are much more labile than the linkages between adjacent non-methylated galacturonic acid residues in HG blocks. Isolated HG were then chemically highly remethylated prior to partial random or blockwise demethylation by chemical or enzymatic means, respectively. Series of partly methyl-esterified HG were thereby generated and studied for their interaction properties (HG/HG, HG/extensin). RG-I side-chains can be specifically isolated by enzymatic means. The ability of isolated arabinan and galactan chains to bind to cellulose was studied.

The impact of fine structural variability onto interaction properties will be discussed.

Marie-Christine Ralet
Agata Zykwska
Marie-Jeanne Crépeau
Romain Valentin
Bernard Cathala
Jean-François Thibault

Marie-Christine Ralet
INRA, UR1268 Biopolymères
Interactions Assemblages
Rue de la Géraudière BP 71627
44316 Nantes Cedex 03, France
ralet@nantes.inra.fr

Cell wall remodelling in *Saccharomyces cerevisiae* stressed cells

L 8

The *Saccharomyces cerevisiae* cell wall is a very dynamic structure that needs to be remodelled during cell growth as well as in those conditions in which cells are challenged with cell wall interfering compounds. Under these circumstances, up-regulation of a particular set of genes is triggered, leading to the remodelling of the cell wall structure, a process necessary to ensure cell integrity. This response is mainly regulated by the MAPK Slt2p/Mpk1p, although we have evidences that other MAPK, like the HOG pathway are also involved, particularly under specific cell wall damage conditions.

One of the genes up-regulated in most of the cell wall stress conditions so far studied is *CRH1*. This gene encodes for a glycosyltransferase involved, together with its homolog *CRH2* in the crosslinking between chitin and β -1,6 glucan as deduced from the fact that chitin bound to β -1,6 glucan is completely absent in a double mutant *crh1 crh2*. Interestingly, stress conditions like a temperature shift from 30°C to 38°C lead to an increase of the proportion of chitin attached β -1,6 glucan. This process is dependent both on an increase in the amount of Crh1 synthesized by the cell and also on an enhancement of the localization of these proteins to the cell surface.

Clara Bermejo
E. Cabib*
N. Blanco
J.M. Rodríguez-Peña
R.García
P. Arias
A.B. Sanz
N. Blanco
S. Díez
C. Nombela
J. Arroyo

Javier Arroyo
Dpto. Microbiología II.
Facultad de Farmacia.
Universidad Complutense
Madrid, 28040. Spain
jarroyo@farm.ucm.es

*National Institutes of Diabetes and Digestive and Kidney Diseases. Laboratory of Biochemistry and Genetics, Bethesda, MD 20892, USA.

Ultrastructure of the yeast cell wall revealed by electron microscopy

Electron microscope studies have revealed the structure of the yeast cell wall and the processes involved in its formation. Thin sections of the yeast cell wall show a three-layer structure with each layer having a different electron density. The structure is composed of an inner network of glucans and an outer fibrillar layer composed of mannoproteins. Ultra-high resolution and low-voltage scanning electron microscopy (LVSEM) show that α -galactomannan particles, which entirely cover the cell surface, prevent visualization of the glucan network structure.

The fission yeast, *Schizosacharomyces pombe*, has β -1,3-, β -1,6-, and α -1,3-glucans. An analysis of glucan synthase mutants has indicated that the β -1,3- and α -1,3-glucans are essential for the formation of the cell wall layered structure and that each glucan plays a different role in this process. α -glucan possibly acts in packing the inner network [1]. Immunoelectron microscopic studies revealed that glucan localization differs in the septum [2-3]. During septum formation, an electron-transparent, primary septum develops into the cell membrane invagination, which is concentrically formed through the construction of a contractile ring. After about two-thirds of the way through this process, a secondary septum appears adjacent to the cell membrane on both sides of the primary septum. Both β -1,3- and α -1,3-glucans affected secondary septum formation; however, only the β -1,3-glucan synthase mutant was defective in the early stage of the septum formation. The materials for secondary septum formation were found to have already accumulated before two-thirds of the process was complete. The shut-off experiments with the β -1,3-glucan synthase encoded gene, *bgs1*⁺, showed that the shut-off cell had a delay in primary septum formation and no detectable levels of linear β -1,3-glucan, which is located exclusively in the primary septum. These results indicate that only β -1,3-glucan plays a vital role in primary septum formation, and suggest that its synthesis is essential for the progression of septum formation.

We succeeded in applying high-pressure freezing fixation and in developing ultra-low temperature LVSEM and immunoelectron microscopy for the molecular anatomic analysis of yeast [4-5]. Ultra-low temperature LVSEM provided a three-dimensional view of the process of septum formation. SDS-digested freeze fracture replica labeling showed that α -glucan synthase is located on the cell membrane of the septum region during septum formation. These results indicate that the cell membrane during new cell wall synthesis is smooth and excludes the long invagination peculiar to fission yeast. The novel immunoelectron microscopy method makes it possible to visualize the ultrastructure of the cell and simultaneously detect an antigen in one thin section. This method confirmed that Bgs1 is in fact located on the cell membrane of the septum and that it accumulates in the electron dense area beneath the cell membrane, where the future site of invagination first appears. These findings strongly suggest that β -1,3-glucan is required for the initiation and progression of the septum. These analyses allow us to propose an ultrastructure for the fission yeast cell wall during septum formation.

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Mami Konomi
Juan Carlos Cortés
Juan Carlos Ribas
Angel Durán
Masako Osumi

Mami Konomi
 Laboratory of Electron microscopy
 / Open Research Center, and
 Department of Chemical and
 Biological Sciences, Faculty of
 Science,
 Japan Women's University,
 2-8-1 Mejirodai, Bunkyo-ku,
 Tokyo 112-8681, Japan
mkonomi@fc.jwu.ac.jp
em-lab@fc.jwu.ac.jp

Atomic force microscopy of the structure of plant cell walls and plant cell wall polysaccharides.

L 10

Atomic Force Microscopy (AFM) provides a method for visualising biopolymers and their interactions [<http://www.ifr.ac.uk/spm/>]. The technique can be used to image wet plant cell walls and isolated plant cell wall polysaccharides. The use of AFM allows visualisation of the cellulose networks within the plant cell wall. The dynamic motion of the non-cellulose components make them difficult to visualise directly and it is only possible to infer their role from their effects on the cellulose network. However, after extraction from the cell wall it is possible to visualise and characterise cell wall components such as carrageenans, arabinoxylans and pectin. Direct visualisation of molecules allows characterisation of the heterogeneity of the polysaccharides. For extracts such as arabinoxylans and pectin the use of AFM has revealed unexpected branching of the polysaccharides. AFM also provides a means of visualising and characterising protein-carbohydrate interactions. This approach can be adapted to probe the chemical heterogeneity of polysaccharides or, through characterising the nature of protein-carbohydrate interactions, to explain functional behaviour of the polysaccharides.

Victor J Morris

Victor J Morris
Institute of Food Research,
Norwich Research Park,
Coney,
Norwich, NR4 7UA, UK.
vic.morris@bbsrc.ac.uk

Carbohydrate-active enzymes in fungal and plant genomes

L 11

We have named Carbohydrate-active enzymes (CAZymes) the enzymes that built (glycosyltransferases) and cleave (glycoside hydrolases, polysaccharide lyases) glycosidic bonds. As such these enzymes play a pivotal role in the biosynthesis and expansion of the plant and fungal cell walls and the degradation of plant polysaccharides by fungal enzymes represents a promising route for the production of second generation biofuels.

The functional annotation of CAZymes in genomes is challenging for non-specialist, due to the varying modularity of these enzymes and the grouping of enzymes with different substrate specificity in the same sequence-based families. Many errors are therefore created by automated functional annotation pipelines with their consequent accumulation and propagation in public databases. Over the last 15 years we have developed the Carbohydrate-active enzymes database (CAZy; <http://www.cazy.org/CAZY/>), a dedicated family classification system that correlate with the structure and molecular mechanism of CAZymes. Over 280 families of catalytic and ancillary modules are presented online and correspond to more than 60,000 non-redundant entries (February 2007). Although the CAZy classification is widely used by the scientific community, it is more rarely by genome consortia. To improve the situation, we develop tools for unambiguous and high-throughput modular and functional annotation of CAZymes in sequences issued from genomic efforts. We then use these improved and uniformly annotated data sets for comparative genomics. We will present our recent results during our search for CAZyme-encoding genes in the genomes of plants and in fungi.

Bernard Henrissat

Bernard Henrissat

Architecture et Fonction des
Macromolécules Biologiques,
UMR6098, Centre National de la
Recherche Scientifique, Université
de la Méditerranée, Université de
Provence, Case 932, 163 Avenue
de Luminy, 13288 Marseille,
France
[bernard.henrissat@afmb.univ-
mrs.fr](mailto:bernard.henrissat@afmb.univ-mrs.fr)

Cellulose and (1→3)-β-D-Glucan Biosynthesis in Plants and Oomycetes

L 12

Cellulose biosynthesis is one of the most important biochemical processes in plant biology. Callose ((1→3)-β-D-glucan) synthesis is also of great importance as it is essential for normal plant development and plays a major role in the plant defence response to various stresses. These processes are however not well understood despite the identification in several plants of genes that code for the catalytic subunits of the cellulose and callose synthases and for other proteins potentially associated to the enzyme complexes. Cellulose and (1→3)-β-D-glucan synthases also play a central role in such vital processes as the morphogenesis and growth of Oomycetes, which comprise pathogenic species responsible for severe environmental and economical damages. As for the plant enzymes, the proteins associated to the Oomycete glucan synthase complexes and their corresponding mechanisms are not well characterized.

This presentation will compare our latest results on the cellulose and (1→3)-β-D-glucan synthases from poplar cell suspension cultures and the Oomycete *Saprolegnia monoica*. In particular, the significance of our recent discovery that cellulose and (1→3)-β-D-glucan synthases of both organisms are located in so-called plasma membrane microdomains or lipid rafts will be discussed.

Anne Briolay
Laurence Bessueille
Nicolas Sindt
Jamel Bouzenzana
Michel Guichardant
Vincent Bulone

Vincent Bulone
Royal Institute of Technology
School of Biotechnology
AlbaNova University Centre
SE-106 91 Stockholm, Sweden
vincent.bulone@biotech.kth.se

Biosynthesis and regulation of cell wall β -glucans in plants

L 13

Plant cell walls are of fundamental importance in both plant growth and development and in biotechnology. Understanding the biosynthesis, interactions and regulation of the major wall components, the polysaccharides (>90% w/w), has been a major goal of plant biologists. β -glucan homopolymers are a major group of wall polysaccharides and include (1,3)- β -glucans (callose), (1,4)- β -glucans (cellulose) and (1,3;1,4)- β -glucans (mixed linkage glucans; MLGs). Cellulose is the ubiquitous microfibrillar component of all higher plant walls; callose is only a component of walls of certain specialised tissues and of plasmodesmatal plugs but is most often associated with wounding responses; the MLGs are a characteristic feature of the commelinoid monocotyledon group of land plants (eg. grasses).

A number of genes putatively encoding processive polysaccharide synthases have been identified based on their similarity to microbial and fungal genes. These include the *CesA* (*CESA*) genes known to encode cellulose synthases, the *cellulose synthase-like* (*CSL*) genes suggested to encode both cellulose synthases and the synthases that make the backbones of various non-cellulosic polysaccharides and the *glucan synthase-like* (*GSL*) genes proposed to be involved in callose biosynthesis (for a review see Farrokhi *et al.*, 2006, *PBJ* 4, 145-167). The latter group, the *GSL* genes, were identified on the basis of similarities to the fungal *FKS* genes. We will report on (1) the molecular and biochemical characterisation of a callose synthase from pollen tubes and its relatedness to the fungal *FKS* genes, and (2) the molecular evidence for participation of the *CsIF* genes in MLG synthesis in the grasses (Burton *et al.*, 2006, *Science* 311, 1940-1942).

Antony Bacic
Lynette Brownfield
Rachel A. Burton
Monika S. Doblin
Andrew J. Harvey
Sarah M. Wilson
Anne Medhurst
Bruce A. Stone
Edward J. Newbiggin
Steve M. Read
Geoffrey B. Fincher

Antony Bacic
Australian Centre for Plant
Functional Genomics, School of
Botany, University of Melbourne,
Victoria 3010, Australia
abacic@unimelb.edu.au

Essential functions in septum and cell wall construction of the Bgs family of (1,3) β -D-glucan synthase catalytic subunits from *Schizosaccharomyces pombe*.

L 14

In fungal cells, cytokinesis requires coordinated contraction of an actomyosin ring and synthesis of plasmatic membrane, both processes common to animal cells, and synthesis of a septum structure that will constitute the cell wall of the new end. The (1,3) β -D-glucan is the main structural polysaccharide of the fission yeast cell wall. *Schizosaccharomyces pombe* contains four putative (1,3) β -D-glucan synthase (GS) catalytic subunits, Bgs1p-4p that share high identity among them and with fungi and plant Fks homologues. All four Bgs proteins are essential for the cell; Bgs2p for a sporulation-specific GS activity whilst Bgs1p, Bgs3p and Bgs4p are needed for vegetative cells. Our group is interested in the study of Bgs1p and Bgs4p, the two main subunits involved in the synthesis of the different cell wall (1,3) β -D-glucans.

Bgs1p and Bgs4p localize to every place of cell wall synthesis and remodelling, either during the vegetative cycle or during sexual differentiation. *bgs4⁺* shut-off produces a dramatic GS reduction, which is accompanied by cell lysis at poles and mainly at the septum. TEM analysis of *bgs4⁺* shut-off cells shows absence of secondary septum and an excess of lateral cell wall degradation before cell separation, which will eventually lead to cell lysis. These data show that Bgs4p is the main subunit involved in GS activity, in secondary septum synthesis and in cell integrity maintenance during both cytokinesis and polarized growth.

bgs1⁺ was identified from mutants defective in cytokinesis, suggesting its involvement in septum synthesis. We have found the Bgs1p function by analyzing the lethal phenotype of *bgs1⁺* shut-off and *bgs1 Δ* cells. *bgs1⁺* shut-off produces multiseptated and branched cells that ultimately will die. TEM and IEM analyses show strong defects on primary septum and linear (1,3) β -D-glucan (LBG) synthesis. Bgs1p is essential but the *bgs1 Δ* spores can germinate, and the *bgs1 Δ* cells are able to make complete septa in which Calcofluor white staining, primary septum and LBG are absent. These data demonstrate that Bgs1p and primary septum are not necessary for medial ring constriction and septum formation but are needed for cell separation and viability. We show that Bgs1p is the only subunit responsible for the LBG synthesis which in turn is the polysaccharide responsible for the primary septum structure. This presents strong similarities with plant cytokinesis, both using a Bgs homologue (callose synthase) to synthesize a similar primary septum (cell plate) of LBG (callose) by two different septation mechanisms. In addition, these results reveal an evolutionarily convergent strategy in yeast cells by which a different single synthase has specialized in the primary septum chitin (Chs2p, budding yeast) or LBG (Bgs1p, fission yeast) synthesis, thus separating it from the rest of cell wall synthesis and ensuring that cell integrity will be preserved during cell separation.

Juan C. Cortés¹
Mami Konomi²
Javier Muñoz¹
Matthias Sipiczki³
Belén Moreno¹
Ivone M. Martins¹
José A. Clemente¹
Mariona Ramos¹
Masako Osumi²
Angel Durán¹
Juan C. Ribas¹

Juan C. Ribas¹
Instituto de Microbiología
Bioquímica
CSIC/Universidad de Salamanca
37007 Salamanca, Spain
ribas@usal.es

²Laboratory of Electron
Microscopy
Japan Women's University
Tokyo 112-8681, Japan

³Department of Genetics
University of Debrecen
H-4010 Debrecen, Hungary

Identification and characterization of plant cell wall mutants using a hydrolase screen

L 15

In the cell walls of higher plants crystalline cellulose microfibrils are linked non-covalently by hemicelluloses forming a network that is responsible for the structural integrity of the cell and whose metabolism plays a major role in cell elongation. The major hemicellulose in those walls is xyloglucan, a beta-1,4 linked glucan that is substituted with xylosyl-residues and dependent on the plant species and tissue contains additional galactosyl-, fucosyl- and/or arabinosyl substituents.

Forward and reverse genetic approaches have been instrumental in the discovery of numerous enzymes involved in the biosynthesis of xyloglucan. However, many more factors influencing the metabolism of xyloglucan remain to be discovered. Therefore, a mutagenized Arabidopsis population was screened for mutants with altered xyloglucan structures. For this purpose two methods involving xyloglucanases were employed.

For the first method, wall material was subjected to oligosaccharide mass profiling (OLIMP), a method based on enzymatic release of oligosaccharides facilitating the xyloglucanase and subsequent analysis of resulting solubilized oligosaccharide mixture with MALDI-TOF mass spectrometry. OLIMP attributes include high sensitivity and short analysis time. Analysis of ~16,000 Arabidopsis mutants with OLIMP yielded 62 altered xyloglucan (*axy*) mutants that could be classified into 11 groups (*axy1-axy11*) based on distinct structural features.

For the second method Arabidopsis mutant seedlings were grown in liquid culture in the presence of exogenously added recombinant xyloglucanase. Wildtype plants exhibit a distinct morphological growth phenotype, i.e. dwarfed plants with crippled cotyledons. However, the identified (*xeg*) mutants were selected due to their alteration in the visual growth phenotype. Wall analysis of these mutants confirmed that they are indeed xyloglucan mutants.

Taken together *axy* and *xeg* mutants exhibit a whole range of defined structural xyloglucan alterations presenting the unique opportunity to connect metabolic pathways to structural features to functional properties.

Lutz Neumetzler
Florian Kraemer
Nicolai Obel
Sascha Gille
Ulrike Haensel
Mark Ziemann
Markus Pauly

Markus Pauly
DOE-Plant Research Laboratory
Michigan State University
178 Wilson Rd.
East Lansing, MI, 48824, USA
paulymar@msu.edu

Glycoside hydrolases and glucanosyl transferases in *Schizosaccharomyces pombe*

L 16

The *S. pombe* cell wall is composed of α -1,3-glucan, β -glucan (linear β -1,3-glucan, β -1,6-glucan and β -1,6-glucan-branched β -1,3-glucan) and α -galactomannans. The nascent β -1,3-glucan chains remain soluble until covalent linkages to other components of the cell wall are created by different transglycosidase enzymes. We have analyzed the role of proteins from GH families 5 and 72 during the life cycle of fission yeasts.

In *S. cerevisiae*, *C. albicans* and *Aspergillus* proteins from family GH72 (Gas, Phr and Gel) have been shown to have β -1,3-glucanosyl transferase activity and all of them are attached to the membrane through a glycosylphosphatidylinositol (GPI). A search of the *S. pombe* genome for proteins with sequence similarity to *S. cerevisiae* Gas1 indicated the existence of four proteins from this family. Three of them (*gas1*, *gas2* and *gas5*) are periodically expressed during the cell cycle, while expression of the fourth (*gas4*) is strongly induced during sporulation. *gas4* Δ mutants undergo normal meiosis but they fail to form mature spores. Gas4 localizes to the forespore membrane and it is essential for the formation of the sporulation-specific cell wall and for germination of the spores. During vegetative growth *gas1* seems to be an essential gene, while mutants lacking *gas2* had no apparent phenotype and *gas5* Δ mutants showed thicker cell walls during growth at high temperatures. Using GFP fusions, the proteins have localized to the plasma membrane. The proteins have been expressed in *Pichia* and the activity has been analyzed in vitro. They also have β -1,3-glucanosyl transferase activity, but the main products of the reaction are different.

S. pombe contains also three proteins from family GH5, named Exg1, Exg2 and Exg3. These genes are differentially expressed during the vegetative cycle, since *exg1* expression periodically oscillates while *exg2* and *exg3* do not show any variations. In addition, *exg2* is induced during the sporulation process. We have found that these proteins have a different fate in the cell, since Exg1 is secreted to the cell wall and culture medium, Exg2 is a transmembrane protein and Exg3 remains in the cytoplasm. In contrast to other members of this family in *S. cerevisiae*, the *S. pombe* Exg proteins are only active against β -1,6-glucans. Detailed characterization of the activity of Exg1 expressed and purified in *Pichia* indicated that this protein is an endo- β -1,6-glucanase.

Maria de Median Redondo
Encarnación Dueñas Santero
Thierry Fontaine
Evangelina Pablo-Hernando
M. Luisa Alonso Núñez
Javier Encinar del Dedo
Jean Paul Latgé
Francisco del Rey Iglesias
Carlos R. Vázquez de Aldana

Carlos R. Vázquez de Aldana
Inst. Microbiología Bioquímica
Dpto Microbiología y Genética
CSIC/Univ. Salamanca
Edificio Departamental
Plaza Doctores de la Reina
37007 Salamanca, Spain
cvazquez@usal.es

Discrete functional roles for cellulose synthase A (CESA) genes during pollen maturation and root development

L 17

Cellulose production during primary cell wall formation in plants is mediated through a cellulose synthase rosette complex, localized to the plasma membrane. This complex is constituted by three individual cellulose synthase subunits which correspond to CESA1, 3 and 6 in *Arabidopsis thaliana*. Mutations in genes corresponding to two cellulose synthase subunits, CESA1 and 6, result in embryo lethality and in aberrant cell shapes and tissue swelling, respectively. We show that mutation in the third CESA gene, CESA3, cause pollen deformation and sterility. Expression and phylogenetic analyses further reveal that CESA2 is likely to execute CESA6-like functions in the *cesa6* mutants. Double mutant analyses between *cesa2-1* and *prc1-1* show that CESA2 and 6 are indeed functionally redundant.

In addition, disruption of CESA6-like functions by *cesa2 cesa6 cesa9* triple mutant analyses result in pollen deformation and male sterility, suggesting that the primary cellulose synthase complex is necessary for pollen maturation. Complementation of the *prc1-1* mutant using a CESA2 cDNA under the control of the CESA6 promoter further reveal that the functional redundancy between the two subunits is not absolute.

Staffan Persson

Staffan Persson
Carnegie Institution
Stanford, USA
shpersso@stanford.edu

The complex family of plant polysaccharides known as pectin comprises between 10-30% of plant primary walls and roughly 2-5% of secondary walls. The pectic polysaccharides, identified as polymers containing 1,4-linked alpha-D-galacturonic acid (GalA), consist largely of homogalacturonan (HG), rhamnogalacturonan I (RG-I) and the substituted galacturonan RG-II. *Arabidopsis* galacturonosyltransferase 1 (GAUT1) is a Golgi-localized pectin biosynthetic alpha-1,4-galacturonosyltransferase (GalAT) that catalyzes the transfer of GalA from UDP-GalA onto HG (1). It is the only functionally proven HG:GalAT identified to date. GAUT1 is part of a 25 member gene superfamily, the GAUT1-related gene family, which consists of three evolutionarily-related clades of 15 GAUT genes and 10 GAUT-like (GATL) genes. Although some family members show preferential transcript expression in specific tissues, suggesting developmental regulation of particular family members, most family members are expressed in most tissues, suggesting either redundant function within a given tissue or specific function in the synthesis of unique wall polymers. Multiple family members have been localized to the Golgi, consistent with sequence analyses indicating that 11 GAUT genes encode proteins with single predicted transmembrane domains near their N-termini and with predicted type II membrane protein topology. However, the GATL genes and 3 GAUT genes have only signal peptides, leading us to propose that at least some GAUT/GATLs without transmembrane domains may reside in the Golgi by association with Golgi-localized protein complexes that contain at least one membrane-bound subunit. Our recent biochemical studies indicate that GAUT1 can associate in a protein complex with at least one other GAUT in *Arabidopsis* detergent-solubilized protein extracts (2). Analyses of cell walls from multiple GAUT and GATL mutants reveals changes in the amounts of diverse wall polymers including pectin and xylan (3,4). In summary, based on the proven function of GAUT1, the highly conserved sequence similarity of the proposed catalytic domains of the *Arabidopsis* GAUT1-related gene family members, the available cell wall composition data on diverse family mutants, and knowledge of fine scale wall polymer structure (5), we propose that the GAUT1-related gene family encodes GalATs involved in the synthesis of pectin, xylan and other GalA-containing wall polymers.

Melani A. Atmodjo
Kerry H. Caffall
Clint Baugham
Sarah E. Eichler Inwood
Michael G. Hahn
Debra Mohnen

Debra Mohnen
Complex Carbohydrate Research
Center and Department of
Biochemistry & Molecular
Biology
315 Riverbend Rd.
Athens, GA 30602, USA
dmohnen@ccrc.uga.edu

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Carbohydrate synthesis in the Arabidopsis secondary cell wall

L 19

The secondary cell wall is deposited once plant cells have commenced growing and usually involves the synthesis of a thick wall composed of a high proportion of cellulose, lignin and other matrix polysaccharides. It is responsible for the mechanical properties associated with woody material. Mutants in the secondary cell wall are characterised by a collapse of the water conducting, xylem vessels. These mutants, named *irx* (*irregular xylem*) have allowed use to identify several genes involved in cellulose synthesis. This is included three putative catalytic subunits IRX1, IRX3 and IRX5 (AtCesA4, 7, 8). These genes have been used in epitope tagging experiments to probe the structure and organisation of the cellulose synthase complex and also in microarray experiments to identify new genes involved in secondary cell wall biosynthesis. This approach has yielded an additional 7 *irx* mutants. At least five of these mutants appear to be involved in synthesising xylan, the major matrix polysaccharide of the secondary cell wall. The talk will present progress in understanding the structure of the cellulose synthase complex and identifying function of genes expressed during secondary cell wall biosynthesis.

Simon Turner
David Brown
Raymond Wightman

Simon Turner
University of Manchester, Faculty
of Life Science, 3.614 Stopford
Building, Oxford Road,
Manchester, UK
Simon.turner@manchester.ac.uk

Molecular analysis of chitin metabolism in *Candida albicans*

L 20

Chitin represents a minor component of the fungal cell wall but is essential for viability especially under conditions of cell wall damage. In the human fungal pathogen *C. albicans* chitin is synthesised by four chitin synthases Chs1, 2, 3 & 8. We have studied the role and the regulation of each Chs. We have shown using promoter-LacZ fusions that each chitin synthase gene has a distinct pattern of transcriptional regulation and that each *CHS* promoter responds to exogenous calcium and treatment with the echinocandin class of antifungal drugs. Increased expression under these conditions resulted in increased chitin synthase activity *in vitro*, elevated cell wall chitin and consequently some level of protection from echinocandins. The spatial regulation of Chs has been examined by generating YFP-tagged versions. Chs3p and Chs8p are localised at the mother-bud neck prior to septation and cytokinesis and Chs3p is targeted to growing tips of hyphae and sites of bud growth. In agreement with distinct localisation patterns for the Chs we have demonstrated by shadow-cast TEM that chitin synthase isoenzymes generate fibrils that differ in appearance. Chs3p synthesises short stubby rodlets in the yeast and hyphal lateral wall, and Chs8p generates longer interlaced fibrils in the cell wall. Although there have been advances in our understanding of chitin synthesis at the molecular level little is known about how chitin is assembled in the wall and how it is modified after synthesis. For example, chitin chains may be modulated by the controlled action of chitinases and by deacetylation to chitosan. A potential chitin deacetylase gene, *CDA2*, has been identified in the *C. albicans* genome and is expressed under laboratory conditions. The role of *CDA2* in the cell wall structure, morphology and virulence of *Candida albicans* is under investigation. The transcriptome of chitin synthase mutants has been analysed using DNA microarrays. In contrast to inhibition of glucan synthesis or deletion of a number of other important cell wall synthesising genes disruption of chitin synthase genes does not activate the cell wall salvage signalling pathways. Therefore chitin is essential in *C. albicans* and this fungus apparently cannot compensate for loss of chitin in the cell wall. Therefore chitin synthesis remains an attractive yet undeveloped antifungal target.

Sarah A. Milne
Carol A. Munro
Megan D. Lenardon
Louise Walker
Rhian K. Whitton
Neil A.R. Gow

Neil A.R. Gow
School of Medical
Sciences University of
Aberdeen Aberdeen AB25
2ZD Scotlands.
n.gow@abdn.ac.uk

Chitin synthase III requires Chs4p-dependent translocation of Chs3p into the plasma membrane

L 21

Chitin is a minor but essential polymer in the cell wall of most fungi. It acts as a sort of scaffold for the rest of components of this structure. Chitin is made by a family of enzymes called generically Chitin synthases, whose members varied significantly between fungal species. In yeast, most chitin is formed by Chitin synthase III (CSIII), an enzymatic activity whose catalytic subunit is Chs3p, a Class IV enzyme.

Chs3p is a polytopic protein that becomes active at the Plasma membrane (PM). During vegetative growth it accumulates at the neck region synthesizing the chitin ring that support septum formation during cell division. The characterization of mutants lacking the chitin ring allowed the identification of several proteins required for CSIII activity in yeast. Most of these proteins are required for proper intracellular traffic of Chs3p to the PM, although the final steps of this traffic are poorly unknown.

In this work we will present the characterization of Chs4p, a protein originally described as an activator of CSIII, but whose molecular function is unknown. We will show that in the absence of Chs4p, Chs3p fails to localize in the PM, accumulating in intracellular vesicles. Such accumulation is dependent on endocytosis since a blockade in endocytosis maintains Chs3p in the PM independently of Chs4p. Clearly, Chs4p is required for Chs3p stabilization at the PM, rather than for its delivery. Interestingly, CSIII is not functional in the *chs4Δ* mutant even after the endocytosis blockade, indicating that in the absence of Chs4p, Chs3p does not achieve a functional state albeit its insertion in the PM.

Endocytosis appears to be a major way of regulating CSIII activity in yeast. A blockade in this process triggers a significant increase in CSIII and chitin levels. This increase is linked to a redistribution of Chs3p along the PM that promotes chitin synthesis along the cell surface. Immediately after endocytosis blockade, Chs3p redistribute from the neck, a clear indication that the endocytic turnover of Chs3p is a key element in maintaining Chs3p localization at the neck for the formation of the chitin ring. The characterization of several Chs4p mutants during the endocytic blockade suggests that once CSIII becomes activated upon Chs3p/Chs4p interaction, it remains active independently of Chs4p.

These data, taken together, support a model in which Chs4p interaction to Chs3p promotes a posttranslational modification of the later that activates its catalytic site, stabilizing also the protein at the PM. We will analyze the most plausible model for this modification while we will also discuss its hypothetical role in the regulation of other related Chitin synthases.

Abigail Reyes
Cristina Jimenez
Angel Duran
Cesar Roncero

Cesar Roncero
Instituto de Microbiología Bioquímica
and Departamento de Microbiología y
Genética. CSIC/Univ. Salamanca.
37007-Salamanca, Spain.
crm@usal.es

A novel dual-radiolabelling approach to studying the competing pathways of NDP-sugar biosynthesis *in vivo*

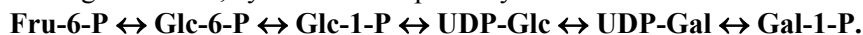
Sandra C. Sharples
Stephen C. Fry

Stephen C. Fry
The Edinburgh Cell Wall Group,
Inst. of Molecular Plant Sciences,
School of Biological Sciences,
The King's Buildings,
Edinburgh EH9 3JH,
United Kingdom
S.C.Fry@ed.ac.uk

Cell wall polysaccharide biosynthesis often represents the plant's principal anabolic activity. Astonishingly, however, the metabolic pathways involved remain controversial. Wall polysaccharide biosynthesis is catalysed by membrane-bound glycosyltransferases using cytosolic sugar-nucleotides such as UDP-glucose — where necessary transported into, and further metabolised within, the Golgi lumen. Proposed routes of sugar-nucleotide production include several 'competing' pathways. For example, UDP-GlcA could be produced either by UDP-Glc dehydrogenation or from Glc-6-P via the *myo*-inositol pathway. Patterns of gene expression suggest when and where the enzymes of these pathways may be present; and immunological or enzymic assays sometimes support such suggestions. However, an enzyme's mere presence (let alone its mRNA's presence) does not guarantee that it actually fulfils any metabolic role *in vivo*.

Mutational studies may fail to distinguish between competing pathways because plants often circumvent genetic blocks by shunting substrates through 'reserve' pathways. Likewise, *in-vivo* feeding of pathway-specific radiolabelled intermediates does not reliably identify the predominating pathway. For example, feeding of exogenous *myo*-[³H]inositol causes radiolabelling of GalA, Ara and Xyl residues. However, this establishes only that the *myo*-inositol pathway can operate (perhaps opportunistically, scavenging any occasionally surplus inositol), not that it normally does operate.

We are exploring a dual-radiolabelling methodology with cultured *Arabidopsis* cells to which two pathway-nonspecific radiolabelled intermediates are simultaneously fed: [1-³H]galactose and [U-¹⁴C]fructose. The two radioisotopes thereby infiltrate the metabolic landscape from opposite ends of the following reversible, cytosolic 'core pathway' of intermediates:



Each cytosolic 'core metabolite' gets a unique ³H:¹⁴C ratio. Products stemming from a given core metabolite will inherit the same ³H:¹⁴C ratio as that core metabolite (if downstream reactions do not cause loss of ³H or ¹⁴C).

By this method, we found that the 'core metabolite' giving rise to polymer-bound GalA residues was UDP-Glc, not Glc-6-P or UDP-Gal. Therefore, postulated pathways involving *myo*-inositol oxidation or UDP-Gal dehydrogenation were not predominant in these cells. The *in-vivo* favoured pathway is thus UDP-Glc → UDP-GlcA → UDP-GalA.

The core metabolite from which polymer-bound Man and Fuc residues arose was Fru-6-P, not Glc-1-P. Therefore, a proposed pathway involving GDP-Glc → GDP-Man epimerisation was not predominant in these cells. The *in-vivo* favoured pathway is thus Fru-6-P → Man-6-P → Man-1-P → GDP-Man → GDP-Fuc.

Our approach should be generally applicable to diverse cell-types and to additional pathways.

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Functional Dissection of the Chitin Synthase System for the Growth and Morphogenesis of *Aspergillus nidulans*

Hiroyuki Horiuchi

Hiroyuki Horiuchi;
Department of Biotechnology
The University of Tokyo
1-1-1 Yayoi, Bunkyo-ku,
Tokyo 113-8657, Japan
ahhoriu@mail.ecc.u-tokyo.ac.jp

Chitin is one of the major cell wall components in most filamentous fungi. Chitin synthases catalyze the polymerization of *N*-acetyl-D-glucosamine (GlcNAc) using UDP-GlcNAc as a substrate. Recent analyses of genome sequences in many filamentous fungi demonstrate that there are several genes that encode chitin synthases (*chs* genes) in each fungus.

Aspergillus nidulans is ascomycete filamentous fungus and is used as one of the model organism in many filamentous fungi. We have cloned six *chs* genes from *A. nidulans* and analyzed their roles in hyphal tip growth and morphogenesis (reviewed in references 1 and 2). These are, *chsA*, *chsB*, *chsC*, *chsD*, *csmA*, and *csmB*, and their gene products belong to classes II, III, I, IV, V, and VI, respectively.

chsB-deletion mutants formed very small colonies with many hyphal branches, suggesting that *chsB* plays an important role in hyphal tip growth. Deletions of *chsC* and/or *chsD* did not cause any defects. Deletion of *chsA* caused slight reduction of conidiation efficiency. In contrast, *chsA* and *chsC* double deletion mutants showed pleiotropic defects in asexual and sexual developments and growth sensitivity to various reagents. These results suggest that *chsA* and *chsC* have overlapping functions in these processes.

csmA and *csmB* encode the proteins consisting of a myosin-motor like domain at their N-termini and a chitin synthase domain at their C-termini.

csmA deletion mutants showed growth sensitivity to hypo-osmotic stress and formed swollen hyphae and intrahyphal hyphae. These phenotypic defects were also observed in *csmB* deletion mutants. Deletions of both *csmA* and *csmB* caused synthetic lethality. Overexpression of *csmA* could not suppress the defects of the *csmB* deletion mutant and overexpression of *csmB* could not suppress those of the *csmA* deletion mutant. These results suggest that although *csmA* and *csmB* have some overlapping functions, they have different functions in hyphal growth.

The localizations of *chs* gene products were investigated by using epitope-tagged chitin synthases. HA-ChsA mainly localized at forming septa, while FLAG-ChsB, FLAG-ChsC, CsmA-HA, and CsmB-FLAG localized primarily at hyphal tips and forming septa. To investigate the movement of chitin synthase in living hyphae, we analyzed it using EGFP-ChsB and found that it moved from the outer rim to the center of septation sites during septum development.

These results suggest that chitin synthesis in hyphal growth and septum formation is a complex process and several chitin synthases function in it

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Characterization of yeast mutants with a defect in cell surface structure and their application to various fields - *Saccharomyces cerevisiae* Mutant Displaying β -Glucans on Cell Surface

Masayuki Azuma

Masayuki Azuma
Dept. of Applied Chemistry and Bioengineering,
Graduate School of Engineering,
Osaka City University,
Sugimoto 3-3-138, Sumiyoshi-ku,
Osaka 558-8585, Japan
azuma@bioa.eng.osaka-cu.ac.jp

Understanding the structure and the biosynthesis of the fungal cell wall should contribute to developments in various fields, such as development of medicine and food, environmental improvement. To be concrete, the cell wall of *Saccharomyces cerevisiae* attracts attention at next points.

1. Screening of new antifungal agents that target the cell wall
2. Application of the cell wall β -glucans that have the ability to stimulate the human immune system
3. Cell-surface engineering (cell-surface display of a new catalytic function for the use as a biocatalyst)
4. Separation of cells from culture broth using cell flocculation in the brewing industry
5. Application of the cell wall components as a food coating agent

It is thought that there are suitable cell wall mutants in each field mentioned above, and our group pushes forward examination to apply *S. cerevisiae* cell wall mutants to those fields. Here I will introduce a mutant displaying β -glucans on cell surface.

The *S. cerevisiae* cell wall is composed of mannoproteins, β -1,3-glucan, β -1,6-glucan, and chitin. Normally cells have the most outer layer consisting of mannoproteins, and an exposure of β -glucans on cell surface is not observed. Glycosylphosphatidylinositol (GPI) anchor connects mannoproteins to β -glucans and chitin. Most proteins involved in the synthesis of the GPI core structure are essential for growth. Before we screened deletion mutants in genes involved in GPI synthesis for osmotic remedial growth to explore the relationship between the GPI anchor structure and β -1,6-glucan synthesis. Heterozygous diploid strains were dissected on medium with osmotic support, and slow growth of the *mcd4* deletion mutant (*mcd4* Δ) was observed (1). It is known that Mcd4p (morphogenesis checkpoint dependent) are involved in adding phosphoethanolamine (EtN-P) to the mannose chain of the GPI core structure, and recently Zhu *et al.* reported that the EtN-P is required for the transport of GPI proteins from ER to Golgi. We examined characterization of *mcd4* Δ . *mcd4* Δ showed abnormal morphology and cell aggregation, and was hypersensitive to SDS, hygromycin B and K1 killer toxin. The mutation also caused a decrease in mannan levels and an increase in alkali-insoluble β -1,6-glucan and chitin levels in the cell wall. Cell surface of *mcd4* Δ was clearly different from that of WT from SEM images. These results suggested that *mcd4* Δ displays β -glucans on cell surface without a mannan cover.

There have been several reports on the physiological function (immunomodulating effects) of β -glucans extracted from *S. cerevisiae*. Zymosan, which is a cell wall preparation containing β -glucans and mannan, activates macrophages and stimulates the secretion of inflammatory products, such as tumor necrosis factor- α (TNF- α). β -Glucan particles also induce the production of TNF- α and interleukin-1 β via the stimulation of human monocyte β -glucan receptors. Therefore, we expected that *mcd4* Δ cells displaying β -glucans on cell surface activate macrophage and examined the effect of contact *in vitro* between *mcd4* Δ cells and mouse macrophages.

As a result *mcd4Δ* cells strongly activated macrophages and stimulated the TNF- α secretion compared with WT cells. We further examined the effect of intraperitoneal ethanol-fixed *mcd4Δ* cells on the survival period of mice infected with *Candida albicans*. *mcd4Δ* cells tended to prolong the survival period compared with WT cells, implying that *mcd4Δ* cells may enhance the immune function of mice via macrophage activation (2). Moreover, we examined the structures of β -glucans extracted from *mcd4Δ* with NMR. The structures of the β -glucans from *mcd4Δ* differed from those of WT; however, there was no difference in TNF- α secretion level from macrophages between β -glucans from *mcd4Δ* and those from WT. *mcd4Δ* may be a superior strain for the preparation of β -glucans because the yield of purified β -glucans obtained from dry cells of *mcd4Δ* was higher than that obtained from dry cells of WT (2). However, the growth rate of *mcd4Δ* cells is very slow. By solving the problem of the growth rate, in the future, the day intact yeasts displaying β -glucans on cell surface are available in the food industry could come.

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Golgi apparatus proteomics to study enzymes of polysaccharide synthesis.

The plant Golgi apparatus is specialized for glycosylation reactions. Many plant cell wall polysaccharides and proteoglycans are synthesized here by a largely unknown set of enzymes. These cell wall components are then trafficked in vesicles to the cell surface. To discover proteins involved in the synthesis of these glycans and in their sorting to the plasma membrane, we are analyzing the protein composition of the Golgi apparatus. The premise is that the major Golgi proteins will carry out the synthesis of the most abundant cell wall polysaccharides. We have developed LOPIT (Localization of Organelle Proteins by Isotope Tagging), a new proteomic tool for high-throughput membrane protein localization. Organelles are partially separated using centrifugation. Proteins from the same organelle co-fractionate and therefore exhibit similar distributions in density gradients. Protein distributions can be determined through use of isotope-coded affinity tags to enable relative quantitation of protein levels by mass spectrometry. Novel proteins are then localized by using multivariate data analysis techniques to match their distributions to those of proteins that are known to reside in specific organelles. Using LOPIT we have identified putative glycosyltransferases, transporters and other novel proteins in the Golgi apparatus in Arabidopsis. We are studying mutants in these enzymes using the enzymatic polysaccharide profiling technique PACE, which reveals structure and quantity of oligosaccharides released by cell wall digestion. To gain further knowledge on the pathways of synthesis, we are developing further proteomic approaches to identify protein complexes, called ProCoDeS. We believe that understanding both localization and complex formation will help in prioritization of experiments to study enzyme function.

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**Godfrey P Miles¹,
Nicholas T Hartman¹,
Tom P Dunkley²,
Pawel Sadowski²,
Thilo Weimar¹,
Marcelo P Segura¹,
Jennifer Mortimer¹,
Chris J Barton¹,
H-C. Liang¹,
Florence Goubet¹,
Elaine Stephens³,
Kathryn S Lilley²
Paul Dupree¹**

Paul Dupree

¹Department of Biochemistry,
University of Cambridge, Building
O, Downing Site, Cambridge, CB2
1QW, UK

²Cambridge Centre for Proteomics,
Cambridge Systems Biology
Institute, Tennis Court Road,
University of Cambridge,
Cambridge, CB2 1QR, UK,

³Department of Chemistry,
University of Cambridge,
Lensfield Road, Cambridge CB2
1EW, UK.

p.dupree@bioc.cam.ac.uk

Structure-Function Studies of Xyloglucan Transglycosylases/Hydrolases

In higher plants, molecular interactions between wall polysaccharides, including cellulose and non-cellulosic polysaccharides such as xyloglucans and (1,3;1,4) β -D-glucans, are fundamental to wall properties. The interactions have been generally assumed to be non-covalent in nature. We have purified a barley xyloglucan xyloglucosyl transferase HvXET5 (EC 2.4.1.207), a member of the GH16 group of glycoside hydrolases, which catalyses the formation of covalent linkages between xyloglucans and cellulosic substrates, and between xyloglucans and (1,3;1,4) β -D-glucans. The rate of covalent bond formation catalyzed by HvXET5 with hydroxyethylcellulose (HEC)-D-glucan is slower. Mass spectrometric analyses show that oligosaccharides released from a fluorescent HEC:xyloglucan conjugate -D-glucan endohydrolase consist of xyloglucan substrate with glucosyl residues attached. Ancillary peaks contain hydroxyethyl substituents and confirm that the parent material consisted of HEC covalently linked with xyloglucan. Similarly, pD-glucan:xyloglucan conjugate -D-glucan endohydrolase reveals the presence of a series of fluorescent oligosaccharides that consist of the fluorescent xyloglucan acceptor substrate linked covalently with glucosyl residues. These findings raise the possibility that XETs might link different polysaccharides *in vivo*, and hence influence cell wall strength, flexibility and porosity.

Maria Hrmova
Vladimir Farkas
Jelle Lahnstein
Geoffrey B. Fincher

Geoffrey B. Fincher
Australian Centre for Plant
Functional Genomics, University
of Adelaide, Waite Campus, Glen
Osmond, SA 5064, Australia
geoff.fischer@adelaide.edu.au

Cell wall integrity checkpoint that monitors cell wall remodeling in *Saccharomyces cerevisiae*

L 27

Cell wall remodeling and cell morphogenesis are tightly coordinated with progression of the cell cycle in many organisms. In the budding yeast, *Saccharomyces cerevisiae*, a major cell wall component, 1,3 β -glucan is synthesized at the budding site in the early stage of the cell cycle, and constructed in the primary septum before cytokinesis. Yeast possesses both forward and feedback system to achieve temporal and spatial regulation of cell wall construction. Forward system is composed of a cell cycle dependent morphological pathway that is regulated by small GTPase, Rho1p (1,2). In addition, the feedback regulatory system, called cell wall integrity checkpoint was recently studied and characterized in our laboratory (3).

We found that yeast cells stop growing at the specific stage of the cell cycle after perturbation of 1,3 β -glucan synthesis. These cells arrest with post-replicative DNA but quite low level of Clb2p, and without forming bipolar spindles. *wac1* (wall-checkpoint defective) mutation that abolishes this arrest causes the accumulation of Clb2p and *CLB2* mRNA and leads to formation of bipolar spindles despite 1,3--glucan-synthesis perturbation. Cell wall defects caused by the temperature-sensitive mutation of a putative mannosidase (*dfg5*) also cause the cells to arrest in the cell cycle before the separation of the spindle pole bodies and the formation of a spindle (4). These results indicate the existence of a novel cell cycle checkpoint to coordinate entry into mitosis, and suggest that Wac1p is required to achieve this checkpoint function through a transcriptional regulation of *CLB2*. We revealed that *WAC1* is identical with *ARPI* (actin-related protein). Arp1p, Nip100p and Jnm1p, which are components of the dynactin complex, are all required to achieve the G2 arrest while keeping cells highly viable. These results imply that the dynactin complex has a regulatory role in the novel checkpoint to monitor cell wall remodeling. In this meeting we report our recent findings on the mechanism of the cell wall integrity checkpoint and discuss about implication of cell wall integrity checkpoint in yeast cell cycle regulation.

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Yoshikazu Ohya
Masaya Suzuki
Ryoji Igarashi
Yo Kikuchi
Takahiro Negishi

Yoshikazu Ohya, PhD
Professor of Laboratory of Signal Transduction
Department of Integrated Biosciences
Graduate School of Frontier Sciences
University of Tokyo
Bldg. FSB-101, 5-1-5
Kashiwanoha
Kashiwa, Chiba Prefecture 277-8562 Japan
Tel. +81 (0)4-7136-3650
Fax. +81 (0)4-7136-3651
ohya@k.u-tokyo.ac.jp

Global Mapping of Synthetic Genetic Interactions in Yeast

L 28

We are applying synthetic genetic array (SGA) analysis to the large-scale mapping of genetic interaction networks in yeast. A network containing ~1000 genes and ~4000 interactions was mapped by crossing mutations in 132 different query genes into a set of ~5000 viable gene deletion mutants and scoring the resultant double mutant progeny for a fitness defect. The average query gene showed ~30 synthetic genetic interactions, indicating that the resulting genetic network is complex and may contain as many as ~100,000 interactions. Connectivity of a gene in the network is predictive of function because query genes tend to interact with genes of related function. Moreover, subsets of genes displaying similar patterns of genetic interactions often encode components of the same pathway or complex. To investigate networks of essential genes, we have taken advantage to temperature sensitive and promoter shut-off alleles for essential yeast genes. In an application of the genetic network analysis, we showed that clustering chemical-genetic profiles and genetic interaction profiles identifies target pathways or proteins, providing a powerful means of inferring mechanism of drug action.

Charles Boone

Charles Boone
Banting & Best Dept. of Medical
Research, University of Toronto,
Toronto, ON, Canada, M5G 1L6.
charlie.boone@utoronto.ca

Tools and identification of cell wall proteins - cell wall polysaccharides interactomics

L 29

The assembly of yeast cells wall components, namely β -1, 3 and β -1,6 glucan, mannans and chitin is a highly complex and regulated process involving structural enzymes under the control of several regulatory pathways, among which the PKC1-MAPK kinase module seems to be the most important. Our major goal is at two levels (1) within the European project “Fungwall”, we wish to characterize how and what the so-called ‘carbohydrate modifying enzymes (e.g. transglycosidase, glucanosyltransferase, glycosidases , etc) recognize the polysaccharides moieties at the cell surface to catalyse the remodelling of the cell wall structure and (2) to identify regulatory proteins involved in cell wall remodelling that might be potential antifungal targets.

With respect to the first aim, we considered the ‘protein chips’ and other analytical tools for measuring the binding/ interaction between purified cell wall proteins and specific cell wall polysaccharide . As a proof of principle, we expressed and purified from *E. coli*, 5 yeast cell wall proteins, namely Bgl2, Cts1, Eng1, Crr1 and Gas2 and one protein, ChiB, from *Aspergillus niger*. These proteins were bound on CM5 sensor chips from BIAcore. Using the Surface plasmon Resonance technique, we were able to detect relative specific interaction of these proteins with either short β -1,3 glucan, laminarine, chitopentaose and mannans. Meanwhile, the same proteins were expressed at the cell surface of yeast cells using the Yeast Surface Display method from *In vitro*gen (construction were made by Dr. E. Ragni in Dr. Strahl’ lab and kindly given to us). Yeast expressing these proteins as well as DTT-released proteins were spotted on epoxy-activated glass slides to produce cell wall protein chips. In contrast to the first technique, these protein chips required labelled oligosaccharides to detect potential interaction. We will report on the first results using these chips.

With respect to the second aspect of our project, we will summarize the work on a very curious regulatory protein, encoded by *KNR4/ SMII* that is implicated in cell wall synthesis and seems to connect this process to cell growth. To quickly identify genes and proteins interactions on a global scale, we have developed a WEB-interface navigation and visualization tool called “MAPyeast”. This system allows us to easily visualize the Knr4 interactomics from all publicly available data. In addition to our work showing a physical interaction of Knr4 with components of the cell wall integrity PKC1-MAP kinase signalling cascade, this tool illustrates that *KNR4* is synthetic lethal with > 80 genes, and in particular with genes implicated in cell wall, polarisome and cell division. Quite surprisingly, search for suppressors of synthetic lethal between *knr4* mutant and mutant in genes of the MAP kinase pathway (*BCK1*, *SLT2*, *RLM1*) has failed, suggesting that there is no compensatory mechanism when this signalling pathway together with the Knr4 pathway were defective. These results together with the fact that Knr4 is specific to the fungal kingdom may pose this protein as a potential antifungal target.

Jean Marie François
Adilia Dagkessamanskaia
Jean Ph Siguier
Emmanuelle Trévisiol
Khalid Fakir,
Fabien Durand
Helene Martin-Yken.

Jean Marie François
Molecular Microbial Physiology
Team, Laboratory Engineering of
Biological Systems and
Bioprocess ;
UMR-CNRS 5504 & INRA-792,
Av. de Rangueil,
F-31077 Toulouse Cedex 04,
ph: 33 5 61 55 9492 ; m
fran_jm@insa-toulouse.fr

The Chk1p histidine kinase of *Candida albicans* and cell wall biosynthesis

Virulence of *Candida albicans* is associated with several factors including secreted proteases, the ability to undergo a reversible morphogenic shift from yeast to hypha, and cell wall adherence glycoproteins. Previously, we have investigated the functional role of two-component signal proteins in growth, cell wall biosynthesis, oxidant adaptation, and virulence of this yeast. Two-component signal proteins are unique to bacteria as well as fungi such as *C. albicans* and are not found in human cells which make them ideal targets for antifungal development. We have focused much of our work on the hybrid-histidine kinase Chk1p. A strain lacking this gene has been shown to have a number of defects in the cell wall, including alterations of glucan structure and *N*-linked mannan which may contribute to its reduced ability to adhere to human esophageal cells. In this current investigation, we show that the *chk1* mutant is highly resistant to the cell wall perturbing compound, Congo red. A series of other deletion mutants has been constructed to determine the role of specific protein domains in Congo red sensitivity. We found that deletion of a partial MAP kinase domain resulted in increased sensitivity to Congo red. Phenotypes of other domain mutants will be discussed. We have also completed an analysis of the mannan structure among a matched set of strains. For these experiments, all strains were grown overnight in YPD, washed, and cells bead-disrupted. Subsequently, mannan was isolated by precipitation with Fehling solution, washed with methanol, and the resulting precipitate was subjected to GPC/MALLS and GC/MS analysis. Our data indicate that the mannan in both CAF2-1 and CHK23 (the reconstituted *CHK1* heterozygote) assumed a trimodal polymer distribution suggesting a more heterogeneous mannan structure, representing a mixture of high, moderate, and lower molecular weight polymers. While the *chk1* null strain does appear to have some high and moderately sized mannan, the majority of the polymer (90%) was present in a lower molecular weight form. These data support our observation that Chk1p provides important functions in the assembly and composition of the *C. albicans* cell wall that are reflected in the virulence of this organism.

Dongmei Li
Michael Kruppa
David Williams
Douglas Lowman
Richard Calderone

Richard Calderone
Department of Microbiology &
Immunology
Georgetown University Medical
Center
3900 Reservoir Rd, NW
Washington, DC 20057
calderor@georgetown.edu

Wall integrity sensing in growing cells in Arabidopsis

L 31

Plant cells are surrounded by a strong polysaccharide-rich wall. A major challenge is to understand how cell walls are correctly assembled and remodeled at the surface of expanding cells often while being challenged by wall-degrading pathogens. Plant cells, like yeast, can react to cell wall perturbations as shown by changes in gene expression, accumulation of ectopic lignin and growth arrest caused by the inhibition of cellulose synthesis. We have identified a plasma membrane-bound receptor-like kinase (THESEUS1), which is expressed in elongating cells and vascular tissue. Mutations in THE1 and overexpression of a functional THE1-GFP fusion protein did not cause a phenotype in a wild type background but respectively attenuated and enhanced growth inhibition and ectopic lignification in seedlings mutated in cellulose synthase CESA6 without rescuing the cellulose deficiency. In addition, a T-DNA insertion mutant for *THE1* attenuated the growth defect and ectopic lignin production in other but not all cellulose-deficient mutants and not in a dwarf cytoskeleton mutant. A subset of the genes that were deregulated in *cesA6* mutants were shown to be regulated by THE1. Some of these genes potentially play a role in the defense against pathogens, in cross-linking the cell wall and in protecting the cell against reactive oxygen species. Together, these results demonstrate a role for THE1 in the surveillance of the wall integrity of growing plant cells.

Kian Hématy
Pierre-Etienne Sado
Ageeth Van Tuinen,
Soizic Rochange
Sandra Pelletier
Jean-Pierre Renou
Herman Höfte

Herman Höfte
Laboratoire de Biologie Cellulaire,
UR501, Institut Jean-Pierre
Bourgin, INRA, Rte de Saint Cyr,
78026 Versailles cedex
herman.hofte@versailles.inra.fr

Microtubule organization and the mechanical properties of cellulose

Cortical microtubules have long been recognized as important regulators of the mechanical properties of the plant cell wall. Until recently, this function has been thought to be mediated through microtubules regulating the orientation of cellulose microfibrils, and thereby restricting expansion in the direction in which they are aligned. Studies in my lab, however, have shown that disrupting the cortical microtubule array, through mutation or drug treatment, has surprisingly little effect on microfibril orientation. To account for this, I recently postulated that microtubules interact with the cellulose synthase machinery to ensure that long and mechanically sound cellulose microfibrils are generated. In this talk, I will present work that investigates the relationship between microtubule organization and cellulose crystallinity. For this work, we measured the degree of cellulose crystallinity by X-ray diffraction, comparing wall material from wild-type and the temperature-sensitive mutant *mor1-1*, whose microtubule arrays become disorganized at 29°C. To obtain enough primary wall material, we harvested the growing regions of the inflorescence stem. Just as previously described for other tissues of the *mor1-1* mutant, microtubules became disrupted and short, while microfibril orientation remained highly ordered in spite of the loss of growth anisotropy at the restrictive temperature. X-ray diffraction analysis demonstrated that the degree of cellulose crystallinity increased significantly in the mutant at restrictive temperature. This result indicates that microtubule organization helps to regulate cellulose crystallinity, and that cellulose crystallinity helps to define the mechanical properties of the primary cell wall.

Geoffrey O. Wasteneys¹,
Miki Fujita¹
Eiko Kawamura¹
Shawn D. Mansfield²

Geoffrey O. Wasteneys¹,
¹Department of Botany,
²Department of Forestry,
University of British Columbia,
Vancouver, BC, Canada, V6T 1Z4
geoffwas@interchange.ubc.ca

Xylogens; Arabinogalactan proteins that act as intercellular signaling molecules in cell walls

L 33

The plasma membranes of two adjacent plant cells are separated by thick cell walls. Therefore the cell walls play a crucial role as the place of intercellular signaling. To understand intercellular signaling machinery through cell walls, we have tried to find out intercellular signaling molecules resident in cell walls, in particular, in plant vascular tissues. For this purpose we have used a *Zinnia elegans* xylogenic culture in which isolated single mesophyll cells transdifferentiate into xylem cells.

Using the culture, we isolated an extracellular protein, designated xylogen, which promotes xylem cell differentiation. Interestingly, xylogen derived from the dicot *Zinnia* could promote TE differentiation from mesophyll cells of the monocot *Asparagus officinalis* L., suggesting that xylogen is not species-specific but common to diverse angiosperms. Isolation of xylogen gene and its structural analysis revealed that xylogen is a non-classical arabinogalactan protein with non-specific lipid transfer protein-like sequence and a putative GPI anchor domain. Xylogen transcript and protein predominantly accumulated in the meristem, procambium, and xylem. Interestingly, in the xylem, xylogen was polarly localized in the cell walls of very immature tracheary elements. Together with the fact that double-knockouts of *Arabidopsis* lacking both *AtXYP1* and *AtXYP2*, which are two *Arabidopsis* homologous genes and expressed in vascular tissues, exhibited discontinuous veins, we present a hypothesis that polarly secreted xylogen draws neighboring cells into the pathway of vascular differentiation to direct continuous vascular development.

We will also present results about the secretion of xylogen with a GFP-fusion protein and the expression of other xylogen-like genes.

Hiroo Fukuda
Yuki, Kobayashi
Hiroyasu Motose

Hiroo Fukuda
Department of Biological
Sciences,
Graduate School of Science,
The University of Tokyo
7-3-1, Hongo, Tokyo 113-0033,
JAPAN
fukuda@biol.s.u-tokyo.ac.jp

Cell wall salvage pathway: novel regulatory mechanisms of the cell integrity MAPK Slt2/Mpk1

L 34

Environmental factors, such as high temperature or cell wall-perturbing agents can impair the stability of the yeast cell wall. Yeast cells respond to cell wall stress by activating salvage mechanisms that guarantee cell survival through remodelling of this essential extracellular structure (Popolo,). In *Saccharomyces cerevisiae*, cell wall construction and remodelling is monitored and regulated by the cell wall integrity (CWI) signalling pathway (Levin, 2005). Stimulation of signalling through this pathway results in activation of the Slt2/Mpk1 MAP kinase, which triggers a transcriptional response by phosphorylating different transcription factors, being Rlm1 the one mainly involved in the compensatory mechanism against cell wall damage. However, recent findings revealed that MAPKs may play a structural role by binding to transcription complexes at target genes in addition to phosphorylating transcription factors and co-regulatory proteins (Edmunds and Mahadevan, 2006). Therefore, regulation of gene expression by MAPKs seems to have several alternative mechanisms that are only starting to be understood. Interestingly, Slt2 and its mammalian ortholog ERK5 are unusual among MAP kinases in that they possess the ability to activate transcription of a *GAL1-lacZ* reporter when fused to the DNA-binding domain of the Gal4 transcription factor. The transcriptional activation domain of ERK5 has been shown to be required for coactivating the MEF2 family of transcription factors (Kasler *et al*, 2000), to which the Slt2-target Rlm1 belongs.

We have demonstrated that transcriptional activation of a Gal4-Slt2 fusion is responsive to cell wall stress and requires phosphorylation of Slt2. We have identified two neighboring, but separable transcription activation domains within the C-terminal half of Slt2. Our data suggest that intramolecular interactions controlled by phosphorylation of Slt2 regulate the function of these domains, which are masked by the N-terminal catalytic domain under inactive conditions. Finally, we have demonstrated that Slt2 self-associates, likely through a glutamine-rich region within the C-terminal half of the protein. Therefore, we propose that, like ERK5, both intra- and inter-molecular interactions are operating in this yeast MAPK.

Inmaculada C. Cosano
Ki-Young Kim*
David E. Levin*
Humberto Martín
María Molina

María Molina
Dpto. Microbiología II
Fac. Farmacia. Univ. Complutense.
Plaza Ramon y Cajal s/n
28040- Madrid. Spain
molmifa@farm.ucm.es

*Dept. Biochemistry and Molecular
Biology. Johns Hopkins Bloomberg
School of Public Health.
Baltimore, MD 21205, USA

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Most bacteria are surrounded by peptidoglycan (PG) cell wall, a net-like polymer consisting of glycan strands made of alternating β -1,4-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues cross-linked by peptides. PG metabolism is linked to cell cycle and function to confer shape and osmotic stability to the cell. Its assembly is complex and requires the contribution of several proteins (polymerases, hydrolases and scaffolding proteins) which are believed to form a multienzyme complex machinery specialized in cell elongation or division.

Multimodular penicillin-binding proteins (PBPs) are the main enzymes responsible for PG polymerization. They are essentially two-domains proteins that belong either to class A or class B, depending on the structure and the catalytic activity of their N-terminal domain. The C-terminal penicillin-binding domain of both classes has a transpeptidase (TP) activity catalyzing peptide cross-linking between two adjacent glycan chains. In class A, the N-terminal domain is responsible for their glycosyltransferase (GT) activity, catalyzing the polymerization of uncross linked glycan chains of the PG. In class B the N-terminal domain play role in cell morphogenesis. Monofunctional enzymes similar to the GT domain of class A PBPs also exist in some bacteria but their exact role is still unknown. They both belong to GT51 family, characterized by five conserved motifs and uses lipid II (undecaprenyl-P-P-GlcNAc-MurNAc-pentapeptide) as substrate.

Amino acid sequences alignment of the GTs reveals four highly conserved acidic residues (E233, D234, E290 and D298, in *E. coli* PBP1b). Their analysis by site directed mutagenesis and biochemical characterization showed that Glu233 of the first motif ($E^{233}DxxFxxHxG$) is the essential element of the active site. From substrate specificity study of the PBP1b it seems that the lipid chain is required on the acceptor but not on the donor, that the peptide moiety play role in the recognition of the substrate and that growing chain could serve as donor. Metal ions stimulate the activity of most characterized proteins but, their exact role still need to be determined. A single basic residue was titrated, with a pKa of 7.0. Taken together, these data suggest a mechanism for PBP1b where direct attack of the growing polysaccharide chain by Lipid II is catalyzed by deprotonation of the 4-OH hydroxylic nucleophile of GlcNAc by an active site base (Glu233), concomitant with stabilization of the leaving group by a divalent metal.

The biosynthesis of the bacterial PG wall has been and remains an attractive target for antibacterial interventions. Penicillins, glycopeptides and moenomycin (not used in human therapy) are the best studied antibiotics known to interfere with PG polymerization. However, bacterial resistance limits their effectiveness causing a major public health problem. The first step of PG polymerization catalyzed by the GTs is validated target which is still underexplored. Recently, glycopeptides and moenomycin derivatives were found to be effective against resistant pathogens such as MRSA.

Mohammed Terrak
Martine Nguyen-Distèche
Jean-Marie Frère

Mohammed Terrak
Centre d'Ingenierie des Proteines,
University of Liege, Institut de
Chimie, B6a, B-4000 Liege (Sart-
Tilman), Belgium.
mterrak@ulg.ac.be

Chitin synthesis in insects: lessons from the midgut of the tobacco hornworm

L 36

The ability to form chitin is strictly tied to the presence of chitin synthase, a family II glycosyltransferase, whose biochemical properties are largely unknown due to the lack of heterogeneous expression systems and sufficient purification procedures (1). Previously, we have demonstrated that the chitin portion of the peritrophic matrix in the midgut of the tobacco hornworm, *Manduca sexta*, is produced by chitin synthase 2 (CHS2), one of two isoforms encoded by different *CHS* genes (2). Immunological studies revealed that CHS2 is located at the apical tips of the brush border microvilli formed by columnar cells (3). Here we report the purification of the chitin synthase from the *Manduca* midgut. Several lines of experiments suggest that the chitin synthase exists as a trimeric complex of which the monomers are proteolytically processed. Moreover, screening for midgut proteins interacting with different soluble CHS2 domains in the yeast two-hybrid system yielded a novel chymotrypsin-like protease (CTLP1), which binds to the extracellular C-terminal domain of CHS2. This binding was independently confirmed by co-immunoprecipitation. CTLP1 is a secretory protease, which is expressed in the same midgut region as CHS2 and activated by trypsin. As we could previously show that trypsin stimulates chitin synthase activity without affecting chitin synthase directly, CTLP1 might be required to process and activate CHS2.

Lars Zimoch
Gunnar Broehan
Anton Wessels
Beyhan Ertas
Judith Krysiak
Hans Merzendorfer

Hans Merzendorfer
University of Osnabrueck FB05
Barbarastrasse 11
49076 Osnabrueck, Germany
merzendorfer@biologie.uni-osnabrueck.de

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Structure-function studies of enzymes and accessory proteins involved in chitin turnover

L 37

Serratia marcescens produces three family 18 chitinases and a chitin-binding protein, CBP21, that convert the recalcitrant insoluble polysaccharide chitin to chitobiose. Using the unique experimental possibilities provided by the soluble chitin-derivative chitosan (partially deacetylated chitin), we have studied the properties of the three chitinases. ChiC is a non-processive endo-acting enzyme, whereas ChiA and ChiB act in a processive fashion after initial endo-binding, presumably in opposite directions [1,2]. Mutation of aromatic residues in sugar-binding sites close to the catalytic centre of ChiB revealed that some of these residues are essential for processivity [3]. ChiB variants displaying reduced processivity were less effective in degrading solid chitin, presumably because detached single polymer chains are no longer kept from re-associating with the solid material in between catalytic events. Most remarkably, these same mutants showed a large increase in the degradation rate for non-solid substrates, such as the single soluble polymer chains of chitosan. Thus, processivity comes at a cost in terms of enzyme speed [3]. The non-catalytic CBP21 adds to chitin degradation by increasing the accessibility of the substrate for chitinases [4,5]. Whereas many carbohydrate-binding modules have aromatic surfaces, binding of CBP21 to chitin involves a patch of conserved, mostly polar surface residues [4]. Individual mutations of these surface residues only moderately reduced chitin-binding but abolished the accessory function in chitin-degradation [5]. Accessory proteins such as CBP21 are a potentially valuable tool for biomass turnover and may provide an alternative for processivity as a mechanism for improving substrate disruption and accessibility.

Svein J. Horn
Gustav Vaaje-Kolstad1
Pawel Sikorski
Bjørnar Synstad
Morten Sørli
Daan M.F. van Aalten
Kjell M. Vårum
Vincent Eijsink

Vincent Eijsink
Norwegian University of Life
Sciences
Department of Chemistry,
Biotechnology and Food Science
P.O. Box 5003
N-1432 Ås, Norway
Vincent.eijsink@umb.no

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Contribution of Dectin-1 on Immunomodulating Effect of Soluble β -Glucan SCG in Mice

L 38

SCG is a major 6-branched 1,3- β -D-glucan in *Sparassis crispa* Fr. SCG shows antitumor activity and also enhances the hematopoietic response in cyclophosphamide (CY)-treated mice. Splenocytes from naive DBA/1 and DBA/2 mice are potently induced by SCG to produce IFN- γ , TNF- α , GM-CSF, and IL-12p70, and that GM-CSF plays a key biologic role among these cytokines. Cell-cell contact involving ICAM-1 and LFA-1 was an essential step for the induction of GM-CSF and IFN- γ by SCG but not for the induction of TNF- α or IL-12p70 by SCG. SCG directly induced adherent splenocytes to produce TNF- α and IL-12p70. GM-CSF was required for the induction of TNF- α by SCG, and in turn, TNF- α enhanced the release of GM-CSF and thereby augmented the induction of IL-12p70 and IFN- γ by SCG. Neutralization of IL-12 significantly inhibited the induction of IFN- γ by SCG. We concluded that induction of GM-CSF production by SCG was mediated through ICAM-1 and LFA-1 interaction, GM-CSF subsequently contributed to further cytokine induction by SCG, and reciprocal actions of the cytokines were essential for enhancement of the overall response to SCG in DBA/2 mice. In bone marrow derived dendritic cells from dectin-1 KO mice, almost all of the cytokine productivity and induction of the co-stimulatory molecules were disappeared, strongly indicated contribution of dectin-1 on major SCG mediated signalling.

In CY-treated mice, the levels of IFN- γ , TNF- α , GM-CSF, IL-6 and IL-12p70 were significantly increased by SCG. GM-CSF production in the splenocytes from the CY-treated mice was higher than that in normal mice regardless of SCG stimulation. Neutralizing GM-CSF significantly inhibited the induction of IFN- γ , TNF- α and IL-12p70 by SCG. The level of cytokine induction by SCG was regulated by the amount of endogenous GM-CSF produced in response to CY treatment in a dose-dependent manner. The expression of β -glucan receptors, such as CR3 and dectin-1, was up-regulated by CY treatment. Blocking dectin-1 significantly inhibited the induction of TNF- α and IL-12p70 production by SCG. Taken together, these results suggest that the key factors in the cytokine induction in CY-treated mice were the enhanced levels of both endogenous GM-CSF production and dectin-1 expression.

**Toshie Harada,
Hiromi Kawaminami,
Noriko N. Miura,
Yoshiyuki Adachi,
Mitsuhiko Nakajima,
Toshiro Yadomae,
Naohito Ohno**

Naohito Ohno
Lab Immunopharmac Microbial
Products, School of Pharmacy,
Tokyo University of Pharmacy and
Life Science, 1432-1 Horinouchi,
Hachioji, Tokyo 192-0392, Japan.
ohnonao@ps.toyaku.ac.jp

Structure/biological activity of β -1,2 mannoses, their association to different carrier molecules are under the control of a new family of mannosyl transferases.

L 39

The pathogenic yeast *C. albicans* shares with some other pathogenic and non pathogenic microbes the ability to build mannose blocks bound through an unusual type of linkage: the β -1,2 oligomannosides (β -Mans). In *C. albicans* β -Mans are prominently expressed at the cell wall surface. An important body of clinical and experimental evidences suggests that this phenotypic character contributes to *C. albicans* virulence. Indeed, the unique spacial conformation of β -Mans is specifically detected by mammals innate and adaptative immune systems. β -Mans act as adhesins for host cells, induce specific response through binding to galectin-3, and TLR2 dependent pathways; generation of anti- β -Man antibodies are important factors contributing to animal protection against both systemic and mucosal candidosis. These evidences were gained by using β -Mans either chemically released from the cell wall or chemically synthesized.

With regard to these advances concerning the biological activities of β -Mans, present knowledge concerning their biogenesis and their mode of expression at the *C. albicans* cell wall is limited. Definitive chemical demonstration (NMR) for the presence of β -Mans in *C. albicans* cell wall molecules was only gained for two of them. Both are non-covalently linked to the cell wall and consist in the phosphopeptidomannan (PPM) acid labile and acid stable fractions (serotypes A and B and serotype A respectively) and the phospholipomannan (differing between serotypes A and B). By contrast two important gaps remain in our knowledge: i) a large number of immunological observations consistently suggest the presence of β -Man epitopes in the glycan moiety of other cell wall molecules among which are mannoproteins ii) identification of the genes responsible for β -Mans biogenesis which could help to dissect this process are unknown.

These two questions have been addressed jointly. First a proteomic-glycomic analysis of *C. albicans* cell wall mannoproteins demonstrated that β -mannosylation was part of the O-mannosylation process for representative members of the so called PIR proteins, linked to β -1,3 glucans and GPI anchored proteins, linked to β -1,6 glucans. Second we have identified the family of genes involved in beta mannose transfer (*BMT*) in *C. albicans*. The construction of individual deletion mutants combined with immunochemical and structural analysis showed that each of the 9 *C. albicans* genes encodes for Bmtps that are involved in the sequential steps of β -mannosylation of structurally diverse glycoconjugates which are PPM, PLM and mannoproteins. Interestingly individual deletions had only limited impact on global surface expression and virulence. This is coherent with previous studies having evidenced a complex expression mechanism and suggested a possible global regulation. As far as all the genes and all the potential carrier molecules have been identified, it can be anticipated that the questions of β -Mans functions in *C. albicans* and its hosts can now be more rationally addressed.

Céline Mille,
Chantal Fradin
Thierry Jouault
Pierre André Trinel
Yann Guerardel
Guilhem Janbon
Stefan Wildt
Daniel Poulain

Daniel Poulain
Unité Inserm 799, Faculté de
Médecine, Centre Hospitalo-
Universitaire, Place Verdun,
59045 Lille Cedex.
dpoulain@univ-lille2.fr

A cell wall protein involved in cell surface hydrophobicity and foam formation of sake yeast

L 40

Hitoshi Shimoi

Hitoshi Shimoi
National Research Institute of
Brewing
3-7-1, Kagamiyama
Higashihiroshima
739-0046 JAPAN
simoi@nrib.go.jp

Sake is a Japanese traditional alcohol beverage made from steamed rice, using *Aspergillus oryzae*, which is a source of saccharification enzymes, and sake yeast classified as *Saccharomyces cerevisiae*, which produces ethanol from glucose. Almost all sake yeasts form a thick foam layer on sake mash during vigorous fermentation. This foam formation has been used as an indicator of fermentation progress: the foam rises when fermentation becomes strong and it disappears when fermentation becomes weak. However, this characteristic sometimes reduces the efficiency of sake fermentation because a large part of the fermentation tank is occupied by a thick foam layer. Thus, an absence of foam formation during fermentation is a preferable property. Nonfoaming mutants were screened from foaming industrial sake yeast strains using cell affinity for bubbles, and they are now widely used in commercial sake brewing. Comparison of the nonfoaming mutant with its parent revealed that the cell surface of the former is less hydrophobic than that of the latter, suggesting that cell surface hydrophobicity is related to foaming ability. However, the detailed molecular mechanism of foaming ability of sake yeast was unknown.

We have cloned a gene from a foam-forming sake yeast that confers foaming ability to a nonfoaming mutant. This gene was named *AWAI* and structures of the gene and its product were analyzed. The N- and C-terminal regions of Awa1p have the characteristic sequences of a glycosyl-phosphatidylinositol anchor protein. The entire protein is rich in serine and threonine residues and has a lot of repetitive sequences. These results suggest that Awa1p is localized in the cell wall. This was confirmed by immunofluorescence microscopy and Western blotting analysis using hemagglutinin-tagged Awa1p. Moreover, an *awa1* disruptant of sake yeast was hydrophilic and showed a nonfoaming phenotype in sake mash. We conclude that Awa1p is a cell wall protein and is required for the foam-forming phenotype and the cell surface hydrophobicity of sake yeast.

Although Awa1p is homologous to YOL155C of *S. cerevisiae* S288C, the N-terminal region of Awa1p is also similar to a part of YJR151C of S288C. To elucidate the relationship between structure and function of Awa1p, we constructed four kinds of deletion mutants of *AWAI* and analyzed their phenotypes. The mutants lacking the N-terminal region that is homologous to YJR151C and a GPI-anchor signal had less hydrophobic cell surfaces and did not make foam in sake mash, suggesting that these regions are important for cell surface hydrophobicity and foam formation.

We next analyzed the *AWAI* gene of a nonfoaming mutant strain K701 derived from a parental foam-forming strain K7. K701-*AWAI* was cloned in a cosmid and its sequence was compared with that of K7-*AWAI*. Although the 5' half of K701-*AWAI* was identical to that of K7-*AWAI*, the 3' half of K701-*AWAI* was different from that of K7-*AWAI*, resulting in a loss of the C-terminal hydrophobic sequence of Awa1p. Since this sequence is required for the anchoring of Awa1p to the cell wall, K7-Awa1p can not confer both cell surface hydrophobicity and foam-forming ability to strain K701 cells. We further analyzed K701-*AWAI* by pulsed-field gel electrophoresis Southern blot analyses. The results suggest that the left subtelomeric region of chromosome IX in strain K7 was translocated to the *AWAI* gene in chromosome XV by a nonreciprocal recombination, resulting in the loss of 3' half of K701-*AWAI* in chromosome XV.

Antifungals blocking cell wall polysaccharide biosynthesis

L 41

The echinocandin antifungal drugs are the first new class compounds that target the fungal cell wall by blocking β -1,3-D-glucan synthase (GS). These compounds act largely with non-competitive inhibition kinetics. Membrane-bound GS is 10-fold less sensitive to drug relative to detergent-extracted and highly enriched product entrapped enzyme. Resistance to echinocandin drugs among clinical isolates is associated with amino acid substitutions in two “hot-spot” regions of Fks1, the major subunit of glucan synthase. The mutations, yielding highly elevated MIC values, are genetically dominant and confer cross-resistance to all echinocandin drugs. Prominent Fks1 mutations decrease the sensitivity of glucan synthase for drug by one thousand-fold or more. Resistant glucan synthase enzymes show normal K_m (0.16 mM) values further supporting the notion that the site of action of echinocandins is independent of substrate binding. The Fks1-mediated resistance mechanism is conserved in a wide variety of *Candida* spp. and can account for intrinsic reduced susceptibility of certain species. Fks1 mutations confer resistance in both yeasts and moulds suggesting that this mechanism is pervasive in the fungal kingdom.

Steven Park
Guillermo Garcia-Effron
David S. Perlin

David S. Perlin
Public Health Research Institute,
New Jersey medical School-
UMDNJ, 225Warren Street,
Newark, NJ 07103 USA
perlin@phri.org

Varied functional roles for arabinans in plant cell walls

L 42

Arabinans are believed to form part of the pectic network in plant cells, most notably as side chains on rhamnogalacturonan I. Using specific hydrolytic enzymes, we have shown that arabinans play an essential role in the opening and closing of plant stomatal pores and may help endow the cell walls of stomatal guard cells with their unusual elastic properties. To investigate the role of arabinans in living plants we have adopted a reverse genetic approach in *Arabidopsis thaliana* targeting genes potentially involved in arabinan metabolism. T-DNA insertion mutations in two putative endo arabinanase genes both gave a similar phenotype of very poor seedling establishment. This observation and subsequent studies strongly suggest that cell wall arabinan may serve as a storage reserve in *Arabidopsis* embryos.

**Simon McQueen-Mason,
Leonardo Gomez,
Louise Jones,
Jonathan Foster**

Simon McQueen-Mason
CNAP, Biology
department, University of York, PO
Box 373, York, YO10 5YW, UK
sjmm1@york.ac.uk

The fungus, *Trichoderma reesei*, secretes numerous enzymes involved in the metabolism of plant polysaccharides such as xylan, starch, pectin, and cellulose. These enzymes play a critical role in the conversion of cellulosic waste into fuels, by breaking down polymeric sugars to fermentable monomers. Recently, there has been a significant effort to reduce the cost of enzymes required for biofuels production. New enzymes have been identified, the efficiency of enzymes has been improved, and new enzyme mixes are being created based on substrate type. This presentation will discuss recent advances in enzyme technology for conversion of biomass to fermentable sugars.

Sandy Merino
Joel Cherry

Sandy Merino
Novozymes Inc.
1445 Drew Ave
Davis, CA 95618 USA
SaMe@novozymes.com

Heterogeneous phase biodegradation of cell wall polysaccharides: Example of the carrageenan / carrageenase systems

L 44

Carrageenans are the main components of the red algae (Rhodophyta) cell wall wherein they are laid out as a tri-dimensional network of semi-crystalline fibres. Carrageenans are made up of linear chains of D-galactose (G), with alternating α -(1 \rightarrow 3) and β -(1 \rightarrow 4) linkages. They are classified according to the number and the position of sulphated ester (S) and by the occurrence of 3,6 anhydro-bridges in the α -linked residues (DA unit) found in gelling carrageenans. The three most industrially exploited carrageenans, namely, kappa- (κ , DA-G4S), iota- (ι , DA2S-G4S), and lambda- (λ , D2S6S-G2S) carrageenans, are distinguished by the presence of one, two, and three ester-sulphate groups per repeating disaccharide unit, respectively. At the laboratory, these polysaccharides can be prepared under various physico-chemical states (coil, helix and gel) simply by adjusting the nature and the concentration of the salt in the medium. Carrageenases are enzymes produced by marine bacterium involved in the bioconversion of algal biomass. *Pseudoalteromonas carageenovora* κ -carrageenase and *Alteromonas fortis* ι -carrageenase of have been cloned, overexpressed and crystallised. We have undertaken the characterisation of the mode of action of these two enzymes using chromatography and transmission electron microscopy. These experiments were performed using carrageenan substrates under various physico-chemical states (i.e. gel, amorphous powder, coil and helix). We have found that when enzymatic degradation is achieved in heterogeneous phase conditions (solid substrate: gel, powder), the κ - and ι -carrageenase feature an endo-processive mode of action which are corroborated by the tunnel topology of their actives sites. Results recorded when degradation occurred in homogenous phase (soluble substrate : coil, helix) suggest the random degradation of the polysaccharides by endo-acting enzymes. In conclusion, the mode of action of the carrageenases is modulated by the physical state of the substrate: solid or soluble, but don't seems to be affected by their conformational state coil or helix.

William Helbert
Maud Lemoine
Pi Nyvall Collen
William Helbert

William Helbert
Marine Plants and
biomoleculairesCNRS-University
Paris VIStation BiologiquePl. G.
TeissierF-29680 RoscoffFRANCE
helbert@sb-roscoff.fr

Improved reactivity of cotton fiber through accumulation of cationic polymers in the fiber cell wall

L 45

The cotton fiber is globally the most commonly used natural fiber in the textile industry. So far, cotton seed companies have focused on improving the yield, length and strength of the cotton fiber but have spent little effort on improving the value of the fiber for the downstream processing. The hydroxyl groups of the cotton fiber cellulose have only limited reactivity and most of them are involved in hydrogen bonds that are important for the fiber structure. These attributes lead to an inefficient application of chemicals during the cotton processing in the mill. One approach that has been described to overcome this limitation is to graft the cotton fiber with a cationic polymer, such as chitin. This has resulted in a fiber that reacts more easily with commercial dyes. Using a biotechnological approach we have now produced a cationic fiber on the cotton plant. We show that expression of enzymes that produce β -1,4 linked N-acetyl glucosamine polymers, such as NodC or chitin synthase, in the Golgi results in the formation of cationic cell walls. Production of the chitin-like polymer in the cotton fiber allows a more efficient application of dyes to the fiber

**Marc De Block
Greet Vanderkimpen,
Stephan Soyka,
Frank Meulewaeter**

Frank Meulewaeter
Bayer BioScience N.V.,
Technologiepark 38, 9052 Gent,
Belgium.
frank.meulewaeter@bayercropscience.com

Chitinases are enzymes that catalyze the hydrolysis of the $\beta(1,4)$ -glycosidic bonds between the *N*-acetyl-D-glucosamine (GlcNAc) monomers of chitin. Within the family 18 chitinases two subfamilies exist, the extensively studied bacterial-type chitinases and the less well studied plant-type chitinases with as prototype hevamine from *Hevea brasiliensis*. Both classes of enzymes are found in the genomes of yeast and fungi. Whereas the bacterial-type family 18 chitinases are non-essential, genetic data on the plant-type family 18 chitinases points to a role in cell wall morphology. For example, disruption of *Aspergillus nidulans* *chiA* leads to a defect in germination and hyphal growth, whereas the *Saccharomyces cerevisiae* chitinase 1 CTS1 plays a key role in separation of mother and daughter cells at the end of cell division. Specific inhibitors of these enzymes would be useful as tools to study their role in cell wall morphogenesis and could possess anti-fungal properties. Progress towards understanding the structure/mechanism of these chitinases will be described, together with screening-based discovery of inhibitors.

Daan van Aalten

Daan van Aalten

Wellcome Trust Biocentre, School
of Life Sciences
Univ. of Dundee, Dundee DD1
5EH, UK
dava@davapc1.bioch.dundee.ac.uk

Protein O-Mannosylation is Crucial for Cell Wall Integrity, Septation and Viability of the Fission Yeast *Schizosaccharomyces pombe*

L 47

Protein O-mannosyltransferases (PMTs) initiate the assembly of O-mannosyl glycans. The evolutionarily conserved PMT family is classified into PMT1, PMT2 and PMT4 subfamilies. In higher eukaryotes only one member of the PMT2 and the PMT4 subfamily, respectively, is present, whereas in budding yeast the PMT family is highly redundant. In *Saccharomyces cerevisiae* the simultaneous deletion of one member of each PMT subfamily causes lethality. In humans, mutations of the PMT4 subfamily member cause congenital muscular dystrophies that are associated with brain abnormalities, proving that O-mannosylation is of fundamental importance for lower and higher eukaryotes.

We identified and characterized the PMT family of the archiascomycete *Schizosaccharomyces pombe* which is in many respects more similar to higher eukaryotes than to budding yeast. Different *S. cerevisiae*, in *S. pombe* only one member of each PMT subfamily is present namely *pmt1*⁺, *pmt2*⁺ and *pmt4*⁺. They all act as protein O-mannosyltransferases *in vivo*. *pmt1*⁺ and *pmt2*⁺ form heteromeric protein complexes and recognize different protein substrates compared to *pmt4*⁺, suggesting that similar principles underlie mannosyltransfer reaction of *S. pombe* and budding yeast. Characterization of the viable *pmt1*Δ and *pmt4*Δ single mutants showed in that protein O-mannosylation is of special importance for cell wall structure and septation. Deletion of *pmt2*⁺ is lethal. *S. pombe pmt2*⁺ is the first single fungal PMT family member which is essential for vegetative cell growth.

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Tobias Willer
Martin Brandl
Matthias Sipiczki
Sabine Strahl

Sabine Strahl
Heidelberg Institute of Plant
Science, Ruprecht-Karls-University
Heidelberg
Im Neuenheimer Feld 360
D-69120 Heidelberg, Germany
sstrahl@hip.uni-heidelberg.de

Echinocandin antifungals and other ways to inhibit β 1,3 glucan synthesis

L 48

The echinocandin class of antifungals (caspofungin, micafungin, and anidulafungin) are the first completely novel approved class of antifungals in years. They have unique antifungal properties as they display fungicidal activity against *Candida* species, yet fungistatic activity against *Aspergillus* species. Against *Aspergillus* species, the echinocandins selectively inhibit the growing hyphal tip as shown through cellular staining. The class of agents may have a role in combination antifungal therapy against invasive aspergillosis, whereby the cell wall mechanism could be paired with cell membrane inhibition to exert synergistic effects. The echinocandins also stand as a powerful laboratory tool where the cell wall can be pharmacologically inhibited more easily to mimic genetic inhibition and therefore allow further characterization. Our laboratory has also explored additional methodologies of cell wall activity by inhibiting the calcineurin pathway, both pharmacologically as well as genetically. Inhibition of calcineurin yields effects on hyphal elongation, akin to the echinocandin activity on hyphal tips, and the combination of calcineurin inhibition and echinocandin use shows additive effects with real clinical implications.

William J. Steinbach

William J. Steinbach
Durham, NC, USA
Stein022@mc.duke.edu

Selected talks of FEMS grantees

F 1 to F 11

Transcriptional response to different cell wall damage is regulated by different membrane proteins

F 1

The cell wall of *S.cerevisiae* is a dynamic structure responsible of morphology and cell integrity. It is basically composed of manoproteins, β -1,3-glucan, β -1,6-glucan and chitin. Stress situations that damage the cell wall activate a survival response. This response has been called compensatory mechanism which includes an increase in chitin synthesis and manoproteins production together with certain changes in crosstalk of different polymers to strengthen this structure.

Using DNA microarrays, characterization of the transcriptional response to different cell wall damage stimulation (congo red that binds chitin, zymolyase that degrades the β -1,3-glucan network and pneumocandin that inhibits synthesis of β -1,3-glucan) was carried out in wild type strains of *S.cerevisiae*. Then, using bioinformatics tools we compared these responses in detail. Although there is a common response to those different damages involving the induction of genes related to cell wall construction and its repair, there is also a specific response in each treatment.

In order to analyze if these responses are regulated in a similar manner, the transcriptional response to these three different drugs was studied in strains deleted in Mid2p, Wsc1 (sensors of integrity pathway controlled by Slr2p MAPK) and Sho1p (transmembrane protein from HOG pathway mediated by Hog1p MAPK). While the response to zymolyase is dependent on Sho1p, the response to pneumocandin is completely dependent on Wsc1p and the response to congo red is mainly regulated by Mid2p. These results indicate that responses are differentially regulated depending on the nature of damage.

In addition, cells deleted in Mid2p and treated with congo red unleash a new transcriptional response, different from the wild type, involving transcription and metabolism genes. This kind of mutant specific response to the drug is reduced in *SHO1Δ* mutants treated with zymolyase and it does not exist in *WSC1Δ* mutants treated with pneumocandin.

Clara Bermejo
Raúl García
Sonia Díez
Noelia Blanco
Ana Belén Sanz
Patricia Arias
JM Rodríguez-Peña
Javier Arroyo

Bermejo Clara
Dep. Microbiología II Fcc
Farmacia
Pza. Ramón y Cajal s/n
28040 Madrid-Spain
cbermejo@farm.ucm.es

Comparative and Evolutionary Analysis of Fungal repertoires of Carbohydrate-Active enZymes (CAZymes).

F 2

Carbohydrate-Active enZymes (CAZymes) are crucial in many aspects of the biology of fungal and other species having close relationships with carbohydrates (like plants). In fungi, CAZymes are, for example, involved in key aspects such as biosynthesis, plasticity and remodelling of fungal cell wall, N/O-glycosylation and interaction with the environment. The fungal kingdom features many lifestyles lifeforms and ecological niches. Most fungi studied thus far are saprophytes and / or plant pathogens. Fungi directly secrete enzymes in the environment to degrade bio-polymers. An important proportion of these secreted enzymes are CAZymes which degrade plant cell wall polysaccharides. Here we studied variations in the distribution, composition and relative abundance of fungal CAZymes repertoires as a function of the species' evolutionary and adaptive history. We analyzed whether correlations can be found between a species' CAZyme repertoire's composition and its ecology. We studied a dataset composed of 16 fungal genomes (including saprobies, parasites, pathogens and symbionts) from which we extracted and annotated the encoded CAZymes' sets. The results of our analysis showed that composition of CAZyme sets in fungi follows both the phylogeny and the lifestyle. For example, in the phylum Sordariomycotina the two plant pathogens included in our analysis (*M.grisea* and *G.zea*) appeared to have the closest relative sets of CAZymes while they are in fact the most evolutionary distant species from this lineage. In this case similarities in the repertoires of CAZymes seem to reflect a convergent adaptation to plant pathogenicity. In contrast, in *Aspergilli*, variations in CAZymes repertoires exactly follow the phylogeny and seem to only reveal divergence due to evolutionary distance.

When classifying CAZyme families according to their phylogenetic pattern (pattern of relative abundance between species), we showed that resulting clusters led to groups of enzymes involved in a common process (ie degradation of cellulose, degradation of pectins...). Interestingly, several families of yet unknown function clustered inside those groups. Those families may represent good candidate for the discovery of new enzymes important in biological processes of biological or industrial interest.

Analysis of the evolutionary behaviour of CAZyme families also revealed that sets of secreted CAZymes presented significantly higher rates of evolution (in terms of expansions / reductions of family size) throughout the fungal biodiversity compared to intracellular CAZymes. This is consistent with the idea that repertoires of secreted CAZymes reflect the ecology and lifestyles of fungal species.

Etienne G.J. Danchin
Pedro M. Coutinho
Corinne Rancurel
Bernard Henrissat

Danchin Etienne G.J.
Architecture et Fonction des
Macromolécules Biologiques
(AFMB) UMR6098 CNRS,
Universités d'Aix-Marseille I & II.
163 Av. de Luminy, Case 932,
13288 Marseille Cedex 09.
etienne.danchin@afmb.univ-mrs.fr

Molecular characterisation of Gel4p, a member of the Gelp family, involved in cell wall morphogenesis of *Aspergillus fumigatus*

F 3

The cell wall of the human opportunistic pathogen is a complex structure mainly composed of polysaccharides, $\beta(1-3)$ glucan being the most abundant. In a way similar to other fungi, $\beta(1-3)$ glucan of *A. fumigatus* serve as a skeleton on which the other polysaccharides of the cell wall (chitin and galactomannan) become anchored.

A new $\beta(1-3)$ glucanotransferase (Gel1p) isolated from the cell wall of *A. fumigatus* has been discovered and characterised. This enzyme splits internally a $\beta(1-3)$ glucan molecule and transfers the newly generated reducing end to the non reducing end of another $\beta(1-3)$ glucan molecule. The creation of a new $\beta(1-3)$ glucan resulted in the elongation of $\beta(1-3)$ glucan chains. Gel1p belongs to a family of seven members, which are GPI anchored protein. Only Gel1p, Gel2p and Gel4p are expressed in normal growth conditions. An analysis of these 3 proteins using biochemical and molecular method is presented here. *GEL1* deletion has no phenotype but disruption of *GEL2* gene results in alteration of polar growth, reduced growth, abnormal conidiophores and a decrease in virulence.

All attempt to disrupt *GEL4* have been unsuccessful to date this results suggest that *GEL4* is essential for *A. fumigatus*. The enzymatic activity of Gel4p is being investigated using a recombinant protein produced in *Pichia pastoris*.

**Amandine Gastebois¹,
Isabelle Mouyna¹,
Jean-Paul Latgé¹**

Gastebois Amandine¹
Unité des *Aspergillus*
Institut Pasteur
25, rue du Docteur Roux
75015 Paris
agastebo@pasteur.fr

Cell wall construction and the interaction of RIM101 and PKC signal transduction pathways.

F 4

Alberto Gomez
Cesar Roncero

Gomez Alberto
Instituto de Microbiología
Bioquímica and Departamento de
Microbiología y Genética.
CSIC/Univ. Salamanca. 37007-
Salamanca, Spain.
agsanz@usal.es

The fungal cell wall is a structure that confers cell shape as well as protection from the extracellular environment. There are several signal transduction pathways that allow the cells to respond to cell wall damage.

One of them is the cell integrity or protein kinase C (PKC) pathway, that coordinates the morphogenesis and the cell cycle. In addition, it controls directly the compensatory response through the transcription factor Rlm1p. There are several plasma membrane proteins that can act as sensors for this route, but the most important members are Wsc1p and Mid2p. When they sense a damage, the signal goes through a MAP kinase cascade, and the MAPK Slt2p activates the transcription factor Rlm1p by phosphorylation. It can occur under several stressful conditions including direct damage of the cell wall.

Recent studies in our group show how another route, RIM101, is involved in yeast cell wall assembly. It responds to alkaline pH and differs from PKC pathway in its activation form, because its transcription factor, Rim101p, suffers a proteolytic process. The signalling machinery seems to be conserved in most fungi, although it is not well understood.

These two routes do not seem to share components, but there are some evidences that have encourage us to study the possible relation between them and their rule in the cell wall formation. The *rim21Δ* mutant, defective in the putative sensor of the RIM101 pathway, was identified in our laboratory as moderately resistant to calcofluor. It also shows a low sensibility to SDS, caffeine and zymoliase. Moreover, this mutant presents a reduced compensatory response after a direct damage on the cell wall. Both, *rim21Δ* and *rim101Δ*, are synthetic lethal with *slt2Δ*. This defect is partially restored when the media was supplemented with an osmotic stabilizer. The double mutants *slt2Δrim101Δ* have a thicker cell wall and show extremely hypersensitive to zymoliase. Taken together, these results suggest that the RIM101 pathway acts in parallel with the PKC cascade in the assembly of the yeast cell wall. This relation is independent of the compensatory response mediated by Rlm1p.

We are trying to obtain suppressors of *slt2Δrim101Δ*. That could indicate us intracellular targets of Slt2p to explain the role of the RIM101 pathway in the *Saccharomyces cerevisiae* cell wall construction.

We have isolated multicopy suppressors of the synthetic *slt2Δrim101Δ* lethality. They include genes involved in metabolism or cell cycle and their function in cell wall assembly is currently under scrutiny.

Enzymes of the UDP-GlcNAc biosynthetic pathway as potential targets for drug discovery against *Aspergillus fumigatus*

F 5

Aspergillus fumigatus is the most common mould causing infection worldwide in immunocompromised patients. Because of the increased number of infections caused by this filamentous fungi and the inefficacy of the available drugs, there is an urgent need for better drugs. The fungal cell wall is essential for the viability of *Aspergillus fumigatus* and is composed mainly of a fibrillar branched β 1,3-glucan core bound to chitin, galactomannan and β 1,3-1,4-glucan, embedded in an amorphous cement composed of α 1,3-glucan, galactomannan and polygalactosamine. Chitin is a 1,4 β linked polymer of N-acetyl-D-glucosamine (GlcNAc) synthesised by chitin synthase and it is essential for cell viability and mother-daughter cell separation. Thus, the synthesis of UDP-GlcNAc, which is the substrate of chitin synthase, is also essential. Four different enzymes carry out the biosynthesis of UDP-GlcNAc. We describe the crystal structures of two of them: the *Aspergillus fumigatus* glucosamine-6-phosphate N-acetyltransferase (*AfGNA1*), in complex with glucose-6P, and the phosphorylated form of GlcNAc-phosphomutase (*AfAGM1*), in complex with GlcNAc-6P and magnesium. In addition we also show the native human GNA1 crystal structure in complex with GlcNAc-6P and CoA, in order to compare the active sites. Inferred by mutagenesis studies and the different environment around the sugar compared to the human crystal structure, we suggest *AfGNA1* as an attractive drug target. We also discuss the catalytic mechanism based on kinetic and structural studies of Tyr174Phe from *AfGNA1*. With regard to the *AfAGM1* crystal structure, we discuss the trapping of a catalytic intermediate.

Ramon Hurtado-Guerrero, Wale Raimi, Laura Vallius, Sharon Shepard, Adel Ibrahim, Structural Genomics Consortium (Toronto, Ca) and Daan MF van Aalten.

Hurtado-Guerrero Ramon
School of Chemistry & Molecular Microbiology,
School of Life Sciences,
University of
Dundee, Dundee DD1 5EH,
Scotland, UK
r.hurtadoguerrero@dundee.ac.uk

Chitosanalytic activity of hemicellulase from *Aspergillus niger* – A non-specific activity

F 6

Hemicellulase from *Aspergillus niger* caused depolymerization of chitosan obtained after partial de-N-acetylation of shrimp chitin, a polysaccharide made up of N-acetylglucosamine residues linked by 1-4 glycosidic linkage. Optimum depolymerisation occurred at pH 3.0 and ambient temperature with K_m and V_{max} values of 7.86 mg mL^{-1} and $1270 \text{ nmoles min}^{-1} \text{ mg}^{-1} \text{ protein}$, respectively. Chitosanalysis by hemicellulase resulted in low molecular weight chitosans (LMWC) of M_w in the range of 18-22 kDA (as determined by HPSEC) and GlcNAc/GlcN (HPLC analysis), indicating the exo-action of the enzyme that was further confirmed by a slow decrease in the viscosity of chitosan solution. The LMWC obtained showed a degree of acetylation (DA) of 15-19% in comparison with 26% for native chitosan, as determined by FTIR and solid state ^{13}C -NMR analyses. The LMWCs also showed better antibacterial activity towards *B cereus* and *E coli* compared to native chitosan. Use of hemicellulase in place of expensive chitosanase is of commercial importance as the products could be tailor-made that could be used as antibacterial agent.

Vishu Kumar A.B.
Tharanathan R.N.

Kumar Vishu A.B.
Department of Biochemistry and
Nutrition
Central Food Technological
Research Institute, Mysore
570020, India
vkumar@pasteur.fr

***CsIC* Genes from *Arabidopsis* and *Nasturtium* Encode a Glucan Synthase Activity Involved in Xyloglucan Biosynthesis.**

F 7

The hemicellulosic polysaccharide xyloglucan (XyG) is a key polymer in primary plant cell walls, imparting both strength and dynamic features by cross-linking cellulose microfibrils and by being modified during cell expansion. Despite its central role in plant cell wall dynamics XyG biosynthesis is not fully understood. The major gap, as for almost all plant polysaccharides, is the identification of the enzymes that synthesize the polysaccharide backbone. In the case of XyG example, with a β ,1-4 Glc backbone this requires the activity of a glucan synthase (GS) activity.

In an effort to identify XyG GS, we have taken a genomic approach by exploiting the ability of *Nasturtium* (*Tropaeolum majus*) to synthesize huge quantities of storage XyG during seed development. A *Nasturtium* cDNA library was produced, and the sequence of 10,000 ESTs was determined. This analysis revealed several ESTs with sequence similarity to *Arabidopsis* genes involved in XyG biosynthesis. Interestingly, a *Nasturtium* homolog to *Cellulose synthase-like (Csl) C4* from *Arabidopsis thaliana* was identified several times in this *Nasturtium* cDNA library.

Therefore heterologous expression of *AtCslC4*, and the *TmCslC* gene identified in the *Nasturtium* cDNA library, were carried out in *Pichia pastoris*. Despite several attempts, we were unable to identify an in-vitro GS activity using a radioactive assay and microsomal membranes. However, we successfully overcame this difficulty by using an alternative strategy that looked for a β -1,4- glucan product specifically synthesized in the cells expressing the *CsIC* genes. In short, *Pichia* cells were broken and separated into two fractions: a fraction called “soluble” corresponding to the cellular content (cytosol and intracellular membranes) and an “insoluble” fraction mainly corresponding to the *Pichia* cell wall. Using a combination of methods, including Biogel-P2, HPAEC-PAD, GC/MS, enzymatic degradation, IT-MALDI MS and $^1\text{H-NMR}$, we have been able to characterize the presence of β -1,4-glucan oligosaccharides (DP = 4 to 7) in the cells expressing the *CsIC* genes.

Finally, a *Pichia* line co-expressing *AtCslC4* and *AtXT1* (XyG xylosyltransferase) was created in an effort to enhance the XyG GS activity by expressing its putative partner. Remarkably, this line accumulates significant quantities of β -1,4- glucan in the insoluble fraction, whereas lines containing only *CsIC* genes do not accumulate this polymer. Taken together, these results support our conclusion that *CsIC* genes encode a β -1,4-glucan synthase activity that is involved in creating the backbone of XyG.

Olivier Lerouxel^{1,2}
Jean-Christophe Cocuron¹
Natasha Raikhel³
Curtis Wilkerson¹
Kenneth Keegstra¹

Lerouxel Olivier

¹MSU-DOE Plant Research Lab,
Michigan State University, East
Lansing MI 48824

²Laboratoire de Biologie
Cellulaire, IJPB, INRA
78026 Versailles, France

³CEPCEB, Botany and Plant
Sciences, University of California
Riverside

lerouxel@msu.edu

A cysteine-rich domain related to the plant CBM43 is essential for the $\beta(1,3)$ -glucanotransferase activity of Gas family of proteins of *Saccharomyces cerevisiae*

F 8

The *GAS* multigene family is constituted by 5 genes (*GAS1* to *GAS5*). *GAS1* is the best characterized gene to date. It encodes the major GPI-anchored plasma membrane protein in the yeast *Saccharomyces cerevisiae*. Gas1p is endowed of a $\beta(1,3)$ -glucanotransferase activity that is essential for the proper assembly of the glucan network of the cell wall during vegetative growth. The absence of this activity causes a weakening of the cell wall that activates a salvage pathway. *GAS2* and *GAS4* are expressed during sporulation and are essential for the assembly of the spore wall. Gas proteins belong to a broader family of extracellular enzymes from fungal and yeast species that includes also Phr1 and Phr2 from *Candida albicans* and Gel proteins from *Aspergillus fumigatus*. At the moment 70 protein sequences similar to Gas1p were identified and constitute Family 72 of the Glycoside Hydrolase database. In this work we focused on the Gas family of proteins as representative of the GH72 family. Gas proteins share an N-terminal domain of about 330-350 amino acids, where two catalytic residues are located, whereas they are dissimilar in the C-terminal portion. Out of the five Gas proteins, only Gas1 and Gas2 proteins share a cysteine-enriched domain of about 100 amino acids in their C-terminal region. This module, named Cys-box is similar to a novel Carbohydrate Binding Module (CBM), namely CBM43, an independent module that tightly binds laminarin in some plant $\beta(1,3)$ -glucanases. Family GH72 appears to be divided into two subfamilies: one comprehends proteins with the Cys-box (GH72⁺) and the other one includes proteins without the Cys-box (GH72⁻). First we tested the activity of all the Gas proteins. Recombinant forms were produced in soluble form and purified from *P. pastoris* medium. Gas2 protein exhibited *in vitro* a $\beta(1,3)$ -glucan-transferase activity identical to that of Gas1p whereas Gas4 and Gas5 proteins an activity similar to Gas1p. In order to study the role of the Cys-box we carried out a truncation analysis from the C-terminal end of Gas1 and Gas2 proteins. The removal of the Cys-box did not affect the folding of the proteins, as assessed by different spectroscopic analysis, but totally abolished the activity and also slightly reduced the thermal stability of the proteins. More extensive truncations greatly affected folding of the recombinant proteins and the putative catalytic N-terminal domain could not be produced in a proper conformation indicating that it does not constitute a structurally and functionally independent module. The results obtained suggest a possible interdependent relation of the N-terminal and C-terminal region in the GH72⁺ enzymes indicating a different role of the Cys-box in the fungal glucan transferases with respect to the CBM43 of plant glucanases. The analysis of a phylogenetic tree of the N-terminal domains of Family GH72 revealed a distinct molecular evolution of the GH72⁻ and GH72⁺ subfamilies providing support to the hypothesis that the type of C-terminal region imposed constraints to the evolution of the N-terminal portion.

Enrico Ragni
Oscar Palomares
Eleonora Rolli
Julia Calderon
Laura Popolo

Ragni Enrico
Università degli Studi di Milano
Dipartimento di Scienze
Biomolecolari e Biotecnologie
Via Celoria 26
20133 Milano, Italy
enrico.ragni@unimi.it

Tandem Repeat Containing Genes Generate Genetic Diversity in *Aspergillus fumigatus*

F 9

Genes containing multiple coding tandem repeats are highly dynamic components of genomes. In humans, expansion of coding repeats is associated with various diseases, including Huntington's Disease and Fragile X Syndrome. In fungi, many repeat-containing genes encode for cell-wall proteins containing a leader sequence and glycosylphosphatidylinositol (GPI)-anchor. Several encode for known adhesins (ie. *Saccharomyces cerevisiae* FLO11, *Candida albicans* ALS1, ALS5, ALS7 and ALS9, *Candida glabrata* EPA1). They undergo frequent recombination-dependent expansion or contraction in size, creating size-variability between different yeast isolates and alterations in adhesion. This variation provides the functional diversity in cell-surface antigens which allows rapid adaptation to the environment.

Aspergillus fumigatus is the most common mold pathogen in man, causing both deadly invasive diseases in immunocompromised patients and allergic diseases in patients with atopic immune systems. We analyzed the entire genome of *A. fumigatus* for open-reading frames (ORFs) containing putative repeats, using the ETANDEM software program, a numerical score for tandem repeats in a nucleotide sequence. Fourteen of 30 top-ETANDEM-scoring genes analyzed showed strain-specific size variation of repeat containing-regions. Four of these, (*Afu3g08990*, *Afu2g05150* (MP-2), *Afu4g09600*, and *Afu6g14090*) are putative cell-wall proteins containing a leader sequence and GPI-anchor motif. All four are expressed genes and produce size-variable mRNA encoding a discrete number of repeat amino-acid units. Their expression during development and in response to cell-wall disrupting agents was analyzed.

Our findings suggest that a subset of the *A. fumigatus* cell-surface proteins may be hyper-variable due to recombination events in their internal tandem repeats. We have created knock out strains in *A. fumigatus* 293 for two putative cell-wall coding genes (*Afu3g08990* and *Afu6g14090*). We are currently studying the effect of the deletion of these genes on the phenotype. Preliminary results indicate that compared to the wild type strain, *Afu3g08990*-deleted strain exhibits early germination rates and reduced adhesion to ECM (extra cellular matrix) of alveolar lung cells (A549 cells). In addition, we are analyzing *Afu3g08990* and *Afu6g14090* cellular location by tagging the genes with a myc epitope.

Jacob Romano
Emma Levdansky
Kevin J. Verstrepen
Gerald R. Fink
Nir Osherov

Romano Jacob
Department of Human
Microbiology, Sackler School of
Medicine, Tel-Aviv University,
Tel-Aviv, Israel. E-mail:
cobby@post.tau.ac.il

Verena Seidl
Nick D Read
Christian P Kubicek

Seidl Verena
Fungal Cell Biology Group
Institute of Cell Biology
University of Edinburgh
Edinburgh EH9 3JH, UK.
Verena@fungalculture.org

The recent advances in the sequencing and analysis of fungal genomes showed that fungi have a large number of different chitinolytic enzymes. In a phylogenetic analysis of GH 18 chitinases we could identify three different subgroups of fungal chitinases. Proteins in group A do not have any carbohydrate binding domains and contain the chitinases that have been described as class V (fungal/bacterial) chitinases. Most group B chitinases (class III, fungal/plant chitinases) have a cellulose binding domain. Group C is a novel group of fungal chitinases that have not been characterized so far. They are predicted to be large proteins (140 – 170 kDa), are targeted to the secretory pathway, have a chitin binding domain and two LysM domains and show similarity to yeast killer toxins. We cloned recently the first of those chitinases (*chi18-10*) from the mycoparasitic fungus *Hypocrea atroviridis* and could show that its transcription is not induced by chitin, but is specifically triggered upon growth on cell walls of the plant pathogen *Rhizoctonia solani* which can be antagonized by *H. atroviridis*.

Selected species of *Hypocrea/Trichoderma*, e.g. *H. atroviridis* (anamorph: *Trichoderma atroviride*) and *H. lixii* (*T. harzianum*) are potent producers of chitinases, which has been related to their mycoparasitic abilities since lysis of the host cell wall has been shown to be an important step during mycoparasitism. The genome of *H. atroviridis*, which is used in agriculture as a biocontrol agent, is currently being sequenced. A preliminary analysis of group C chitinases showed that out of the 4 proteins of this group that can be found in the *H. jecorina* genome, only one is strongly conserved in the *H. atroviridis* genome and other group C members seem to have strongly different aa-sequences. This suggests that some of these chitinases might have specialized functions such as involvement in the mycoparasitic attack.

Many fungal chitinases that can be identified in the *Hypocrea*-genomes have homologues in non-pathogenic species such as *Neurospora crassa* and might therefore be relevant for morphological processes such as localized cell wall breakdown during hyphal fusion events and hyphal branching or autolysis. One of the aims of the *Neurospora* genome project (<http://www.dartmouth.edu/~neurosporagenome/index.html>) is the generation of knockout mutants of all annotated genes, which can be obtained from the FGSC. We are using this strain collection to compare *N. crassa* chitinase knockouts with respect to their phenotype and autolysis behaviour to obtain information about the possible roles of some of those chitinases.

Besides chitinases *N*-acetylglucosaminidases (NAGases, GH 20) are involved in chitin degradation. Interestingly, fungal genomes contain ca. between 11-20 chitinases, but only 2 NAGases. We could recently show that the Nag1 enzyme of *H. atroviridis* is expressed under all growth conditions but that its expression level is dependent on the carbon source and we could identify carbohydrates with certain features such as alpha-glucans and galactose-containing oligosaccharides that especially enhanced Nag1 expression. We are investigating the inducibility of those enzymes by *N*-acetylglucosamine among different fungal species and we are studying the localization of NAGases with fluorescence-imaging techniques.

Role of the SYN Domain of Ags1p in Cell Wall alpha-Glucan Biosynthesis in Fission Yeast

F11

The cell wall is important for the maintenance of structural integrity and morphology of fungal cells. Besides beta-glucan and chitin, alpha-glucan is a major polysaccharide in the cell wall of many fungi. In the fission yeast *Schizosaccharomyces pombe*, the alpha-glucan synthase Ags1p was identified using a temperature-sensitive (*ts*) mutant strain, *ags1-1^{ts}*, whose cells lyse at the restrictive temperature due to a weakened cell wall unable to withstand internal osmotic pressure. These observations showed that cell wall alpha-glucan is an essential component of the *S. pombe* cell wall. Recent observations showed that this cell wall alpha-glucan consists mainly of (1,3)-alpha-glucan with some ten percent (1,4)-linked alpha-glucose residues. Ags1p is a multidomain protein with two probable catalytic domains predicted to reside at opposite sides of the plasma membrane, as well as a multipass transmembrane domain. This overall domain structure of Ags1p is conserved among the four Ags1p homologs of *S. pombe*, Mok11p, Mok12p, Mok13p, and (partly) Mok14p, whose genes are expressed during sporulation. Importantly, this domain structure is also well conserved among Ags1p homologs in other cell wall alpha-glucan-containing fungi, such as several human fungal pathogens like *C. neoformans* and *A. fumigatus*. In the present study, we focus on the role of the putative intracellular domain of the *S. pombe* Ags1p, denoted here as the SYN domain. This domain shares amino acid sequence similarities with (1,4)-alpha-glucan synthases such as bacterial glycogen synthases and plant starch synthases, suggesting that Ags1p may be involved in the synthesis of the (1,4)-alpha-glucan constituent of cell wall alpha-glucan. Our data indicate that the Ags1-SYN domain is involved in the production of (1,4)-alpha-glucan for the biosynthesis of cell wall alpha-glucan.

Alina Vos
Nick Dekker
Ben Distel
Jack Leunissen
Frans Hochstenbach

Vos Alina
Dept. of Medical Biochemistry,
Academic Medical Center,
University of Amsterdam
Meibergdreef 15
1105 AZ Amsterdam
The Netherlands
a.vos@amc.uva.nl

POSTERS

P 1 to P 44

Habituation of bean (*Phaseolus vulgaris* L.) cells to dichlobenil is linked to an increase in Xyloglucan endotransglucosylase (XET) activity

P 1

The habituation of cell cultures to the specific inhibitor of cellulose biosynthesis dichlobenil (DCB) constitutes a suitable system to expand our knowledge about xyloglucan endotransglucosylase (XET; EC 2.4.1.207) activity, since these habituated cells possess a modified cell wall (with lower amounts of cellulose and xyloglucan) as well as a growth rate similar to non-habituated cells.

In this work, we cultivated non-habituated callus bean cells in the absence and presence of lethal concentrations of DCB or isoxaben (another cellulose inhibitor) for one week. In addition, we also used bean cells that have been habituated to grow in lethal concentrations of DCB by stepwise subcultures in increasing DCB concentrations up to 2 μ M.

The 2 μ M DCB habituated cells were impoverished in cellulose and xyloglucan, they had a markedly increased XET activity, along with an increased growth rate and a decreased molecular size of xyloglucan. However, the XET activity of non-habituated cells was not affected by the presence of DCB or isoxaben, and little effects on xyloglucan molecular size distribution were noted.

We propose that the weakening of plant cell wall provoked by a decrease in the cellulose content might promote the xyloglucan tethers and increase the ability of xyloglucan to bind to cellulose in order to give rigidity to the wall.

Ana Alonso-Simón
Penélope García-Angulo
Antonio Encina
Jesús M. Álvarez
Takahisa Hayashi
José Luis Acebes

Acebes José Luis
Área de Fisiología Vegetal
Facultad de CC. Biológicas y
Ambientales
Universidad de León
E-24071 León, Spain
jl.acebes@unileon.es

alpha1,3 glucan is a cell wall polysaccharide with multiple functions in *Aspergillus fumigatus*

P 2

A major cell wall component : alpha1,3 glucans represent 40 % of the cell wall polysaccharides in *Aspergillus fumigatus*. They are recovered in the alkali-soluble fraction of the cell wall. Chemical analysis have shown that cell wall alpha1,3 glucans are composed of linear chains with episodic intra-chains of alpha1,4 linked glucose. The cell wall of the mycelium developed in static aerial (StA) or shaken immersed (ShI) conditions, differs in composition and localization of some polysaccharides. alpha1,3 glucans are present in similar amount in StA or ShI cell wall but they are localized exclusively in a thick layer at the surface of the StA cell wall whereas a thin layer of alpha1,3 glucans and an intermediate layer were observed in the ShI cell wall.

A glue sticking together *A. fumigatus* aerial hyphae in a biofilm structure : an hydrophobic strong, non-dissociable network of hyphae is formed in the StA condition whereas the ShI condition allow the development of dispersed hydrophilic hyphae. The aerial network of hyphae (StA) was fused by an extracellular amorphous matrix which also covers the mycelial mat like in bacterial or yeast biofilms. The analysis of the extracellular matrix shows that one major component of this extracellular matrix is the alpha1,3glucans.

An anchor for melanin : alpha1,3glucans are also present in the cell wall of the conidia of *A. fumigatus*. They are mostly localized at the cell wall surface where they colocalize with the melanin layer.

Three putative alpha1,3glucans synthase genes *AGS1*, *AGS2* and *AGS3* have been identified. The gene deletion of *AGS1* only results in a partial reduction of the alpha1,3 glucan concentration in the cell wall. Deletion in *AGS1* or *AGS2* are not defective in virulence. In contrast, *AGS3* can modulate the virulence of *A. fumigatus*. An *AGS3* deletion strain was found to produce conidia more resistant to H₂O₂ than the parental strain. The *ags3* mutant causes more rapid and progressive disease in a mice model than the wild type. The apparent hyper-virulence was correlated with an increased melanin content of the conidial cell wall.

Anne Beauvais
C. Schmidt
W. Morelle
S. Guadagnini
S. Park
D.S. Perlin
J.P. Latgé

Beauvais Anne
Unité des *Aspergillus*
Institut Pasteur
25 rue du Docteur Roux
75015 Paris, France
abeauvai@pasteur.fr

Phytopathogenic fungi can degrade xylan, an abundant hemicellulose in plant cell walls, by the coordinate action of a group of extracellular enzymes. Among these, endo-(1,4)- β -xylanases (xylanases; EC 3.2.1.8) carry out the initial breakdown by cleaving internal bonds in the xylan polymer backbone to produce short xylo-oligosaccharides. In addition microbial xylanases have been studied for their role in several industrial food processes, mainly in baking, wheat processing and as supplements in animal feed production.

In our Laboratory, we have produced and characterized fungal xylanases from *Botrytis cinerea* (XynBc1), *Penicillium griseofulvum* (PgXynA) and *Penicillium funiculosum* (PfXynC), all belonging to family 11 of glycoside hydrolases (<http://afmb.cnrs-mrs.fr/CAZY/>).

The enzymes were heterologously expressed in *Escherichia coli* or *Pichia pastoris*, purified and enzymatically characterised. The recombinant proteins demonstrated high catalytic activity and efficiently degraded low viscosity xylan. Although both *Penicillium* enzymes showed the predominant production of xylotriose and xylobiose as end products, the enzymes differed by their rate and pattern of hydrolysis on wheat soluble arabinoxylan and xylo-oligosaccharides. In addition XynBc1 and PfXynC but not PgXynA were found sensitive to the wheat inhibitor XIP-I, recently identified as a pathogenesis-related (PR) protein. We also showed that *B. cinerea* produced xylanase during plant tissue infection, reinforcing a role for this type of enzyme in the plant attack.

In the light of the recently available PfXynC crystal structure, free and in complex with XIP-I, we have identified potential determinants responsible for the variations in substrate and inhibitor specificity. The importance of the interaction between fungal xylanases and protein inhibitors remains to be investigated *in planta*. The reported differences in xylanase specificities have fundamental biological and industrial implications.

Jean-Guy Berrin
Alexandre Brutus
El Hassan Ajandouz
Jacques Georis
Filip Arnaut
Daniela Bellincampi
Thierry Giardina
Nathalie Juge

Berrin Jean-Guy
Laboratoire Biosciences
Université Paul Cézanne Aix
Marseille III
13397 Marseille Cedex 20
France
jean-guy.berrin@univ-cezanne.fr

Amorphous chitin is a structural component of the cell wall of the phytopathogenic oomycete *Aphanomyces euteiches*

P 4

The oomycetes is a group of diploid eukaryotes that include the most numerous, most important and earliest known water molds. For many years they were classified within the fungi because of their similar ecological and morphological traits, but recent studies showed that they are phylogenically closer to diatoms, chromophyte algae and other heterokont protists. A feature that is often mentioned when distinguishing oomycetes from true fungi is the presence of cellulose and the absence of chitin in their cell walls. However, chitin was detected and chitin synthase genes were recently identified in a few oomycete species. Here we present evidence that chitin is a structural component of the cell wall of *Aphanomyces euteiches*, an oomycete parasite of legume plants belonging to the Saprolegniales. Presence of chitin was shown by the following : (i) biochemical analysis of the cell walls indicated the presence of hexosamines, (ii) treatment of the cell walls with a chitinase released N-acetylglucosamine (NAG), and (iii) a Wheat-Germ-Agglutinin-FITC conjugate stained intensely the cell walls. This data was strengthened by the identification of chitin synthase (CHS) sequences within *A. euteiches* ESTs generated at the laboratory, and the observation that *A. euteiches* growth was inhibited in presence of the CHS inhibitor Nikkomycine Z. When the cell wall amorphous material was extracted by mild hydrochloric acid treatment, most of the NAG-containing material was extracted and X-ray analysis of the residue failed to detect significant amounts of crystalline chitin. This suggests that chitin is involved in cell wall function in *A. euteiches* by acting as an amorphous polymer, possibly crosslinking the other glucans, and not as a crystalline scaffold. Taken together, our data show that chitin synthases could be considered as potential targets in the search for antioomycete compounds.

**Ihham Badreddine
Claude Lafitte
Elodie Gaulin
Bernard Dumas
Arnaud Bottin**

Bottin Arnaud
UMR5546 CNRS-UPS
Surfaces Cellulaires et
Signalisation chez les Végétaux
Pôle de Biotechnologie Végétale
24, Chemin de Borde-Rouge
BP 42617, Auzeville
31326 Castanet-Tolosan
France
bottin@scsv.ups-tlse.fr

Compartmentalization analysis of cell wall-synthesizing enzymes within the endomembrane system

P 5

A major function of the Golgi apparatus (GA) in higher plants is the synthesis of cell wall matrix polysaccharides, such as pectins and xyloglucan. These complex polysaccharides are assembled in the GA and are shuttled by GA-derived secretory vesicles to the cell wall. The synthesis of these glycomolecules requires the action of a set of Golgi glycosyltransferases, in addition to nucleotide sugar transporters and nucleotide sugar interconversion enzymes.

In an attempt to learn more on the functional organization of pectin-synthesizing enzymes, we have undertaken a study aiming at mapping an α -1,5 arabinosyltransferase (ARAD1) and an UDP-D-Xylose-4-epimerase (MUR4) within the endomembrane system. As a first step towards this goal, transgenic tobacco plants expressing fusion proteins (MUR4:GFP or ARAD1:GFP) were produced. These were then subjected to microscopical and immunocytochemical analyses to determine the localization patterns of the enzymes using direct GFP imaging as well as anti-GFP antibody-immunogold labeling. Our preliminary data show that while ARAD1:GFP is localized exclusively within Golgi cisternae, MUR4:GFP is found associated with both Golgi stacks and endoplasmic reticulum.

**Sophie Bernard,
Laurence Chevalier,
Claude Saint Jore-Dupas,
Marie-Laure Follet-Gueye,
Véronique Gomord,
Azeddine Driouich**

Azeddine Driouich
UMR CNRS 6037.
IFRMP23. Université de Rouen.
76821 Mont Saint Aignan, France
azeddine.driouich@univ-rouen.fr

The structure and enzymatic properties of a *Streptomyces* family 19 chitinase reveal differences with plant enzymes

P 6

We describe the cloning, over-expression, purification, characterization and crystal structure of ChiG, a single domain family 19 chitinase from the Gram-positive bacterium *Streptomyces coelicolor* A3(2). Studies of the enzymatic properties of ChiG revealed that the enzyme is active towards a variety of soluble chitinous substrates at rates similar to those observed for other chitinases, but has a very limited ability to degrade crystalline chitin [1]. The crystal structures of ChiG [1] and a related *Streptomyces* chitinase, ChiC [2], showed that bacterial family 19 chitinases lack several loops that extend the substrate-binding grooves in family 19 chitinases from plants. In all cases, the grooves are shallow, suggesting endo-activity and lack of processivity. In accordance with the structurally observed shortening of the substrate-binding groove, detailed analysis of the degradation of chito-oligosaccharides by ChiG showed that the enzyme has only four subsites (as opposed to six for plant enzymes [3,4]). This finding, in combination with the fact that ChiG has a very open active site, may explain the low activity towards crystalline substrates. The most prominent structural difference causing a reduced size of the substrate-binding groove is the deletion of a 13-residue loop in between the two putatively catalytic glutamates. The importance of these latter two residues for catalysis was confirmed by a site-directed mutagenesis study.

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Ingunn A. Hoell¹
Bjørn Dalhus²
Ellinor B. Heggset^{1,3}
Stein Ivar Aspmo¹
Vincent G. H. Eijsink¹

Eijsink Vincent G. H.

¹Department of Chemistry,
Biotechnology and Food Science,
Norwegian University of Life
Sciences, P.O. Box 5003, N-1432
Ås, Norway;
vincent.eijsink@umb.no

²Institute of Medical
Microbiology, Section for
Molecular Biology, National
University Hospital, N-0027 Oslo,
Norway

³NOBIPOL, Department of
Biotechnology, the Norwegian
University of Science and
Technology, NTNU, 7491
Trondheim, Norway

Expression of Plant Cell Wall Glycosyltransferases in *Pichia*, Tobacco and Pea

P 7

A main challenge in the post genomic is the assignment of function to the ORFs with unknown or predicted function. In the Carbohydrate EnZyme (CAZy) database 448 putative Arabidopsis glycosyltransferase (GT) encoding sequences are classified into 40 GT families. The plant cell wall consists of numerous complex carbohydrate polymer structures, many unique to the plant kingdom. Although the fine structure (sugar composition) of the various plant CW components are known to some detail, very little is known with respect to the biosynthetic machinery responsible for their synthesis. Activity from heterologously expressed plant CW GTs have been obtained in very few cases. We have previously presented data on two highly homologous plant-specific CW xylosyltransferases, designated *RGXT1* and *2*, which adopt a typical type II membrane protein structure. We expressed the soluble secreted forms of *RGXT1* & *2* in the Baculo virus system and showed that both proteins were active in "the free sugar assay", transferring xylose from UDP-xylose on to fucose. Partly based on these data, *RGXT1* and *RGXT2* were demonstrated to function in the biosynthesis of the A-chain of pectic rhamnogalacturonan II (RG-II). In the present study, a Flag-Tag (MDYKDDDD) was fused to either the N- or the C-terminus of the soluble part of *RGXT1* and/or *2* and expressed in *Pichia pastoris* as secreted tagged soluble proteins or in *Pisum sativum* (using the Pea Early Browning Virus (PEBV) as expression vector) as tagged soluble intra-cellular protein (*P. sativum*). In addition, full length *RGXT2* with a Flag-Tag fused to the C-terminus, was expressed as a membrane bound golgi localized protein in *Nicotiana tabacum*. The amount of expressed protein were estimated using anti Flag Ab (Invitrogen) and activity were demonstrated using the free sugar assay or modifications hereof. Pros and cons of using the Baculo virus system, *Pichia pastoris*, *N. tabacum* or PEBV mediated expression, are discussed.

**Kirsten Faber¹,
Iben Damager^{1,3},
Jack Egelund¹,
Jacob Krüger Jensen^{2,3}, Merete
Albrechtsen^{1,3}, Henrik Vibe
Scheller^{2,3}, Peter Ulvskov^{1,3},
Bent Larsen Petersen^{1,3}**

Faber Kirsten¹
¹Department of Genetics and
Biotechnology, Faculty of
Agricultural Sciences, University
of Aarhus, ²Dept. of Plant
Biology, Faculty of Life Sciences,
³Center for Molecular Plant
Physiology (PlaCe), Copenhagen
University, ³Thorvaldsensvej 40,
DK-1871 Frederiksberg C,
Denmark
k.faber@dias.kvl.dk

Secondary cell wall formation as a unique rescue mechanism during acid-induced autolysis in the yeast *Cryptococcus neoformans*

P 8

The yeast *Cryptococcus neoformans* is an opportunistic pathogen causing severe diseases in immunocompromised persons. The surface of its cells is covered by a thick capsule composed of polysaccharides and proteins. A number of ecto-glycanases (glycosyl hydrolases) associated with the capsules and/or the cell walls have been identified. The enzyme activities included α -mannosidase, α -, and β -glucosidase, α -, and β -galactosidase, β -xylosidase, β -glucuronidase, endo- β -1,3-glucanase and chitinase. The cell-wall associated endo- β -1,3-glucanases exhibited highest activity in the acidic range between pH 2.5 and 5.0 while the pH optimum for chitinase was at pH 5.0 - 5.5. The main products of cell wall autolysis were glucose and β -1,3-linked glucooligosaccharides. In spite of the presence of the whole array of glycanase activities on the cell surfaces, capsular polysaccharides released from *C. neoformans* cells into the growth medium were essentially metabolically stable. During transition from late exponential to stationary phase of growth, pH of the medium decreased from 6.5 to 2.6 and many cells showed morphological aberrations and signs of autolysis of their walls. Some cells died but in a large population of the cells a new, 'secondary' cell wall was formed underneath the original one in places where the old cell wall was eroded by autolysis. The remnants of the old cell walls remained attached to the cell surfaces as 'caps' and were gradually digested or shed into the medium. Freeze-fracture electron microscopy revealed that the newly formed, secondary cell walls were incomplete, exposing bundles of polysaccharide microfibrils only partially masked by amorphous cell wall matrix. The transition from exponential to early stationary phase of growth was accompanied by an increase in cell-associated autolytic enzyme activities (β -1,3-glucanase and chitinase). The cell wall lysis and the formation of secondary cell walls could be suppressed by buffering the medium to pH 6-7. Cells from the stationary phase of growth contained three to five times higher contents of principal cell wall polysaccharides β -1,3-glucan and chitin than the log-phase cells. The chitin/glucan ratio was about two times higher in the cells having secondary cell walls than in those with normal cell walls indicating that, similarly as in other yeasts, increased synthesis of chitin plays the principal role in the cell wall repair. The formation of secondary cell walls represents a unique rescue mechanism to compensate the cell wall damage in yeast.

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Vladimir Farkas^{1,2}
Danka Macekova¹
Kanji Takeo²
Misako Ohkusu²
Soichi Yoshida²
Masami Ohkubo²

Farkas Vladimir^{1,2}
¹Institute of Chemistry, Slovak Academy of Sciences, Centre of Excellence GLYCOBIOS, Dúbravská cesta 9, 84538 Bratislava, Slovakia, and ²Research Centre for Pathogenic Fungi and Microbial Toxicoses, Chiba University, 1-8-1, Inohana, Chuo-ku, Chiba 260-8673, Japan chemvfar@savba.sk

Structural and functional aspects of glycosyltransferases : the gt8 family as a case study

P 9

Glycosyltransferases (GTs) constitute a large family of enzymes that are involved in the biosynthesis of oligosaccharides, polysaccharides and glycoconjugates. These enzymes transfer a sugar residue from an activated donor substrate, usually a nucleotide sugar donor, to an acceptor that may be a protein, a lipid or a growing oligosaccharide. GTs generally displays exquisite specificity for both the glycosyl donor and the acceptor substrates.

GTs have been classified into families on the basis of amino acid sequence similarities in the CAZy database (<http://afmb.cnrs-mrs.fr/CAZY>). To date, the database comprises more than 20,000 known and putative GT sequences that have been divided into 85 families. Large differences are observed among GT families. Some of them are polyspecific and comprise a huge number of sequences from various sources and whose functions are widespread whereas others are monospecific and contain only a few sequences. For the many putative GT genes, which arose from the systematic sequencing programmes of various genomes, and which fall within these polyspecific families, it is often difficult to assign a precise function to these genes on the basis of sequence similarities. For those families, we are wondering if the knowledge of the 3D-structure could help to decipher the substrate specificities of the many putative GT sequences.

The large polyspecific GT8 family was considered as an interesting case study because (i) it comprises two 3D-representatives catalyzing very different reactions (LgtC, an 4-GalT from *Neisseria meningitidis*, and the rabbit glycogenin, a self-a-glucosylating protein), (ii) it includes sequences from prokaryotic and eukaryotic species which exhibit very different enzyme functions, and (iii) it also comprises a high number (approx. 40) of non-annotated *Arabidopsis thaliana* ORFs. We took advantage of the structural data, to further explore the sequence determinants along the polypeptide chains that confer substrate specificities and to tentatively assign a function for the many *Arabidopsis* hypothetical proteins.

Sara Fasmer Hansen
Cédric Montanier
Anne Imberty
Christelle Breton

Fasmer Hansen Sara
Molecular Glycobiology
CERMAV-CNRS, BP 53
38041 Grenoble cedex 9, France
fasmer@cermav.cnrs.fr

Conditional inactivation of *SUN* genes in *Candida albicans* reveals an essential and conserved cell-wall related function required at a late step of cell separation.

P 10

SUN genes are ascomycete-specific genes. Conserved features of the Sun proteins include an amino-terminal signal peptide and a highly homologous carboxy-terminal domain containing a four cysteines motif predicted to bind iron. The function of *SUN* genes remains unclear. They have been mis-annotated as having a beta-glucosidase activity and analysis of *S. cerevisiae* strains with null mutations in the *SUN* genes uncovered pleiotropic phenotypes linking these genes to several cellular processes : aging, DNA replication, mitochondrial biogenesis and septation. In *C. albicans*, three *SUN* genes have been identified : *SUN41* (orf19.3642), *SUN42* (orf19.5032) and a third member with significant divergence at the protein sequence level (orf19.5896). Here, we report the characterization of the *C. albicans* *SUN41* and *SUN42* genes.

C. albicans strains with null mutations in the *SUN41* or *SUN42* genes were constructed. Phenotypic analysis of these deletion mutants revealed that *SUN41* is necessary for cell separation, hyphal elongation and biofilm formation while inactivation of *SUN42* results in minor phenotypic alterations. Repeated attempts to obtain *C. albicans* *sun41* $\Delta\Delta$ *sun42* $\Delta\Delta$ mutants were unsuccessful. Thus, conditional mutants with one *SUN* gene inactivated and the second placed under the control of the repressible *MET3* promoter were generated. Under repressible conditions, the conditional double mutants showed a osmo-remediable lethal phenotype mainly due to mother cell lysis following septation (both in yeast and hyphal conditions). These data indicate that the *C. albicans* Sun41 and Sun42 proteins share a common essential cell wall related function required at a late step of the cell separation process. The conserved carboxy-terminal cysteine residues were shown to be critical for SUN activity. Additional data suggest that the *C. albicans* *SUN* genes perform identical function but are differentially regulated at the transcriptional level. The essential function of the *C. albicans* *SUN* genes is conserved among yeasts since ectopic integration of *S. cerevisiae* orthologues in *C. albicans* conditional *sun* mutants can suppress the lethal phenotype associated to the inactivation of *C. albicans* *SUN* genes. We are currently investigating a probable enzymatic activity of the Sun proteins towards cell wall polymers that is suggested by our data and those obtained by others in *S. cerevisiae*.

Arnaud Firon
Sylvie Aubert
Ismail Iraqui
Sophie Goyard
Guilhem Janbon
Christophe d'Enfert

Firon Arnaud
Institut Pasteur
Fungal Biology and Pathogenicity
25, rue du Dr Roux
75015 Paris, France
afiron@pasteur.fr

Glycosyl-Inositolphosphatidyl-ceramide-anchored molecules and *Aspergillus fumigatus* cell wall

P 11

Fungal plasma membrane is an active interface between the cell wall and the cytoplasm and is the site of synthesis of most structural polysaccharides. Accordingly, lipid-anchored molecules putatively associated to plasma membrane are expected to be involved in the cell wall organisation and maturation. Several glycosylphosphatidylinositol (GPI)-anchored-proteins with β 1-3glucanoyl-transferase or putative glucanase activities take part in the cell wall remodeling. The galactomannan, a cell wall polysaccharide, is also membrane-bound through a GPI-anchor. Using different specific methodologies, we have found that the GPI structures from 4 proteins and from the lipogalactomannan in *A. fumigatus* mycelium were identical and characterized by the presence of 5 mannose residues linked to a glucosaminyl-inositol-phosphoceramide. Glycosphingolipids have been also characterized. Besides mannosylated inositol-phosphoceramide already described, new galactofuranose containing glycosphingolipids and GPI like structures have been identified. An in vitro assay with mycelium lysate has allowed to observe two mannosylation pathways in GPI biosynthesis in *A. fumigatus*. All these data have indicated that the galactomannan is a unique fungal polysaccharide which it is found under 3 different forms: free in the culture medium; cross-linked to the β 1-3glucan chains into the cell wall; and bound to cellular membrane through a GPI-anchor.

Thierry Fontaine¹
Catherine Simenel
Bernadette Coddeville³
Corina Costachel¹
Muriel Delepierre²
Mike A.J. Ferguson⁴
Jean-Paul Latgé¹

Fontaine Thierry¹,
¹ Unité des Aspergillus,
² Laboratoire de RMN, Institut Pasteur, Paris, France
³ Unité de Glycobiologie structurale et fonctionnelle, Université de Lille I,
⁴ Division of biological chemistry and molecular microbiology, University of Dundee, Wellcome Trust Biocentre, Dundee, UK.
tfontain@pasteur.fr

Identification of the first Oomycete glycosyltransferase gene encoding a putative cellulose synthase catalytic subunit

P 12

Oomycetes are classified in the Stramenopile eukaryotic kingdom, among heterokont algae and water moulds. Many plant and animal pathogens as well as saprophytes belong to the Oomycete family. *Phytophthora infestans*, responsible for potato blight, is the most economically important example of an Oomycete pathogen. The order Saprolegniales includes fish pathogens among which *Saprolegnia parasitica* causes the most severe economical losses. Even though the Oomycete cell walls consist essentially of carbohydrates, so far none of the enzymes responsible for the synthesis of the major cell wall polysaccharides have been identified and characterized. Here we report the identification of the first putative cellulose synthase gene (*CesA*) from the Oomycete *Saprolegnia monoica*.

An EST sequence from *S. parasitica*, containing the conserved processive glycosyltransferase motif QXXRW, was used to design degenerated primers and identify a putative *CesA* fragment from *S. monoica* by rapid elongation of cDNA ends (RACE). Primers corresponding to the fragment identified by RACE were used to amplify the full gene, which was subsequently cloned and sequenced. The gene is approximately 3,500 bp long and the translated product contains the D,D,D, QXXRW motif conserved among processive glycosyltransferases in family 2 in the CAZY-database (<http://afmb.cnrs-mrs.fr/CAZY/>). The gene product is a putative transmembrane protein with two predicted soluble domains. The C-terminal soluble part shows significant similarity to cellulose synthases from the cyanobacteria *Nostoc sp. PCC 7120* and *Anabaena variabilis ATCC 29413*, as well as from the slime mould *Dictyostelium discoideum*. The N-terminal part is less conserved. This is the first Oomycete glycosyltransferase identified and more characterization is needed to firmly demonstrate its role as a cellulose synthase catalytic subunit, and reveal its phylogenetic relationship to the other glycosyltransferases from the same family.

Johanna Fugelstad
Jamel Bouzenzana
Soraya Djerbi
Ines Ezcurra
Tuula Teeri
Vincent Bulone

Fugelstad Johanna
Royal Institute of Technology
(KTH)
School of Biotechnology
Alba Nova University Centre
SE-104 06 Stockholm, Sweden
jfu@kth.se

Characterization of Novel *A. thaliana* Xyloglucan Mutants Identified by a Hydrolase Screen

P 13

Hemicelluloses are complex polysaccharides present in plant cell walls that bind to cellulose microfibrils, thereby forming the loadbearing network of plant cell walls. Xyloglucan (XyG) is the quantitatively most abundant hemicellulosic polysaccharide in the primary wall of dicots and non-graminaceous monocots, consisting of a highly substituted glucan backbone. In an attempt to gain further insights into the function of XyG we tried to identify plant mutants with altered XyG structures. Mutants were identified using a liquid culture mutant screen where mutagenized *Arabidopsis thaliana* seed populations were subjected to growth in an exogenous fungal XyG-specific endoglucanase (XEG) solution. To date we were able to identify about 40 mutants, displaying an altered morphological phenotype, when grown in presence of XEG. Some mutated genes were identified by either TAIL- or I-PCR and include genes encoding for proteins with unknown functions, transcription factors as well as one gene encoding for a putative protein with carbohydrate catabolism activity. For one of these lines (*xeg100*) we were able to detect significant changes in the XyG oligosaccharide composition. In another mutant (*xeg5*) we could observe a significant increase in glycosyl residues of the XyG, when compared to the wild type. These data confirm that the screening method is capable of identifying XyG mutants.

Additional data on the wall structure of many of the mutants will be presented as well as the nature of the identified genes highlighting their involvement in establishing and maintaining the XyG network.

Sascha Gille
Ulrike Haensel
Mark Ziemann
Markus Pauly

Gille Sascha
MSU-DOE Plant Research Lab
Michigan State University
178 Wilson Road
East Lansing, MI 48824
USA
gillesas@msu.edu

Light control of primary cell wall cellulose synthases redundancy

P 14

Cellulose microfibrils are synthesized by large complexes called rosettes located at the plasma membrane and containing several distinct cellulose synthase subunits (CESA). In Arabidopsis, the *CESA* gene family comprises 10 members, some of which are expressed during primary cell wall synthesis whereas others are expressed throughout the secondary cell wall deposition. Mutants *AtCESA4*, 7 and 8 lack the secondary thickening in xylem cells which cause the collapse of the xylem whereas mutants of *AtCESA1*, *AtCESA3* and *AtCESA6* genes show all a reduced cellulose content and a growth defect in roots and dark-grown hypocotyls indicating that these three isoforms are required for normal cellulose synthesis in primary cell walls (for a review, Höfte et al, 2007). The function of the remaining four isoforms still has to be determined. *AtCESA2*, 5 and 9 are encoded by recently duplicated genes and are most closely related to *AtCESA6*. Xie et al, recently have shown that knockout of the *AtCESA2* gene affects microtubule orientation and causes abnormal cell expansion in *A. thaliana* Landsberg ecotype.

In this work, we investigate the roles of *AtCESA2* and *AtCESA5* in cell elongation. Mutants in these genes do not show a clear cell wall or growth phenotype. The double and triple mutant combinations *cesA2/cesA5*, *cesaA2/cesA6* and *cesA5/cesA6* have been realized and reveal the partial redundancy of *CESA2*, 5 and 6.

Interestingly, the hypocotyl growth defect in *AtcesaA6^{prc1-1}* is reverted in the presence of light. *AtCESA5* is expressed in the light and not in dark-grown hypocotyls. We will describe the control by light of *AtCESA* functionality during cell elongation and also the role of the various *AtCESA* isoforms in the resistance to the herbicide isoxaben.

Michel Juraniec
Samantha Vernhettes
Hélène Jouy
Herman Höfte
Martine Gonneau

Gonneau Martine
Laboratoire de Biologie Cellulaire
Institut Jean-Pierre Bourgin
INRA
Route de Saint-Cyr
78026 Versailles cedex
Tel: 33-1-30833046
gonneau@versailles.inra.fr

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Deposition of hemicelluloses in cell walls during wheat endosperm development. FT-IR study

P 15

Cereal grains are an important crop in Europe as cereal food represents a main component of our diet. The cell walls from wheat grain have a strong impact on cereal technology and its derived products. Therefore it is important to control their content and properties.

In wheat grain, arabinoxylans (AX) and (1->3),(1->4)- β -Glucans are the major components of cell walls. Heterogeneity in cell wall composition has been demonstrated on samples recovered from wheat grain fractionation processes. Immunolabelling experiments (Guillon et al. 2004) and FT-IR microspectroscopy have suggested (Barron et al., 2004) that some local changes in cell wall composition occur, as a function of the wheat endosperm regions (aleurone layer, peripheral and central starchy endosperm).

Recently, FTIR assignments of AX, β -(1->3)(1->4) glucans and AGPs spectra were performed (Robert et al. 2005). These assignments are useful to further explore the cell wall composition within the grain endosperm.

Using FT-IR microspectroscopy, local heterogeneity in cell wall composition of wheat grain in development was investigated. Attention was paid to:

- the time course of synthesis and distribution of AX and (1->3),(1->4)- β -Glucans,
- changes in the AX substitution.

While a global (thermal) source was found appropriate to study cell walls of the starchy endosperm, synchrotron radiation, thanks to its intrinsic brightness properties, was used, to explore the aleurone layer, due the layer thickness.

3 stages of development were considered: cell division and differentiation (11-12 day after anthesis (DAA)), accumulation of storage products (26 DAA), mature stage.

AX are detected at the beginning of endosperm cell differentiation, when the grain has reached its final length. Changes of AX structural features are observed depending on either the developmental stage or localization in grain. As a matter of fact, the degree of substitution of AX by arabinose is higher in cell wall of peripheral cells than in central cells at the beginning of cell differentiation. In the mature grain, AX are less substituted. In addition, no difference in AX substitution is observed within the starchy endosperm.

In the walls of aleurone layer, AX appears to be little substituted whatever the stage of development. Changes in cell wall composition seem to occur between periclinal and anticlinal aleurone cell walls.

These results indicate a fine spatial and temporal tuning of the biosynthesis of cell wall polymers. The knowledge of these events gives new research direction to unravel the genes involved in the biosynthesis of cell wall polymers.

Philippe Sully
Jamme Frédéric
Robert Paul
Dumas Paul
Saulnier Luc
Guillon Fabienne

Guillon Fabienne
INRA, BIA
BP 71627
44 000 Nantes, France
fabienne.guillon@nantes.inra.fr

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Determination of glycosyltransferase specificities using a carbochip approach

P 16

Glycosyltransferases (GTs) play an essential role in the synthesis of cell wall polysaccharides of plants. Despite the fact that most of the cell wall polysaccharides are characterised to a high extent, the knowledge about their synthesis at a molecular level is limited. The genome of *Arabidopsis thaliana* contains over 400 GTs of which only a few are biochemically characterized. The complicating factor in the research of GTs is that many acceptor and donor substrates have to be tested.

In our lab a method has been developed that is amenable to high throughput analysis of GT substrate specificities. Acceptor substrates (such as a polysaccharide) and a donor-substrate (such as a nucleotide sugar) are incubated with a recombinant GT. The reaction product is then spotted on a PVDF membrane covered target plate, where it can be washed, fragmented with appropriate hydrolases and analysed by MALDI TOF (matrix assisted laser desorption/ionisation time of flight) mass spectrometry. The activity and specificity of two well characterised plant derived carbohydrate active enzymes, fucosyltransferase and a pectin methyl esterase, has already been successfully tested on the carbochip.

Edouard Leboeuf
Peter Immerzeel
Markus Pauly

Immerzeel Peter
Max-Planck Institute for molecular
Plant Physiology
Am Muehlenberg 1
14476 Golm
GERMANY
Immerzeel@mpimp-golm.mpg.de

Immunomodulating, antioxidant, and antimutagenic properties of yeast cell wall polysaccharides

P 17

Polysaccharides constitute the major part of the dry weight of the yeast cell wall and build the skeletal carcass defining cell wall stability and cell morphology (D-glucans) or D-glucans reveal immunomodulating properties, which allows for their application in antiinfective and antitumor D-glucan isolated from the cell wall of baker's yeast *Saccharomyces cerevisiae*, and isolated glucomannan from the industrial yeast *Candida utilis* D-glucan demonstrated potent inhibitory effect on lipid peroxidation comparable to that of the known antioxidants and exerted DNA protection from oxidative damage. The antioxidant activity of D-glucan derivatives was corroborated by spin-trap ESR technique. Antimutagenic and antigenotoxic activity of the yeast polysaccharides was demonstrated using yeast, bacterial, and algal models as well as in the model of inhibition of DNA oxidative damage using comet assay. The presented results indicate significant protective antioxidant, antimutagenic, and antigenotoxic activities of the yeast polysaccharides and imply their potential application in anticancer prevention/therapy.

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Grigorij Kogan
Eva Miadoková
Viera Vlčková
Darina Slameňová
Peter Rauko
Melánia Babincová

Kogan Grigorij
Institute of Chemistry, Slovak
Academy of Sciences,
Dúbravská cesta 9
SK-84538 Bratislava, Slovakia
chemkogi@savba.sk

Phenotyping of cell wall in relation with tomato fruit texture

P 18

Plant cell wall polysaccharides structure, interactions and organization allow cell to withstand turgor pressure and mechanical stresses and are responsible for tissue cohesion by contributing to cell adhesion. At the tissue level, the wall chemical heterogeneity and distribution seen with regard to cell shape, cell size, and cell distribution are thought to contribute to the overall mechanical properties of plant tissues and organs. In order to define the relationships between the cell wall construction at these different scales with plant mechanical properties, we develop two cell wall phenotyping methods. One is based on enzymatic degradation of wall polysaccharides and analysis of products (enzymatic fingerprinting) and the other is based on video image acquisition and image texture analysis of tissue sections. We applied these methods to the study of cell walls in a collection of texture contrasted tomato lines hosting genetic markers related to texture (QTLs). Fruit cell walls are predominant factors expected to control the mechanical properties perceived as texture. At the molecular level, enzymatic fingerprinting revealed strong correlations between QTLs on chromosomes 1, 2, 4 and 9 related to firmness and the content of heteroxylan and arabinan side chains on pectins in cell wall of pericarp tissue, and between a QTL on chromosome 9 for mealiness with the content in galactan side chains in pectins. At the tissue scale, image texture analysis revealed correlations between firmness and a heterogeneous cell distribution in the pericarp, skin toughness and the presence of small cells under the cuticle, and between mealiness and thick pericarps composed of elongated cells. The results demonstrate that these methods allowed discrimination of genetically close fruits with contrasted texture on the basis of wall construction. The methods will be applied to larger fruit collections to validate these correlations and to test for relationships between histology, wall chemistry and texture.

Marc Lahaye 1
Bernard Quemener 2
Marie Françoise Devaux 3
Jamila Chaïb4
Dominique Bertrand5
Isabelle Marty6
Mathilde Causse7

Lahaye Marc 1
INRA, UR 1268 Biopolymères, Interactions, Assemblages, 44316 Nantes, France
2,3: **INRA, UR 1268** Biopolymères, Interactions, Assemblages, 44316 Nantes, France
4: **INRA, UMR 408** Sécurité et Qualité des Produits d'Origine Végétale, 84143 Avignon/ UR 1052 Génétique et Amélioration des Fruits et Légumes, 84143 Montfavet, France
5: **ENITIAA-INRA, UMR** Sensométrie Chimiométrie, 44316 Nantes, France
6: **INRA, UMR 408** Sécurité et Qualité des Produits d'Origine Végétale, 84143 Avignon, France
7: **INRA, UR 1052** Génétique et Amélioration des Fruits et Légumes, 84143 Montfavet, France
lahaye@nantes.inra.fr

Characterization of null mutants for the *Arabidopsis* 3-deoxy-*d*-manno-2-octulosonic acid-8-phosphate (Kdo-8-P) synthase *AtkdsA1* and *AtkdsA2* genes and a CMP-Kdo synthase gene.

P 19

Despite a very complex structure, the sugar composition of the rhamnogalacturonan II (RG-II) pectic fraction is extremely conserved. Among its constituting monosaccharides is the seldom eight carbon sugar 3-deoxy-*d*-manno-octulosonic acid (Kdo). Kdo is first synthesized by Kdo-8-P synthase (KDSA) as a phosphorylated precursor. After dephosphorylation, Kdo is then activated by coupling to CMP prior to its incorporation into the RG-II in a reaction catalyzed by CMP-Kdo synthetase (KDSB). As an attempt to alter specifically the RG-II structure in its sugar composition and assess the consequences on the function of RG-II in cell wall and its relationship with growth, we looked for *Arabidopsis* null mutants in the genes encoding KDSA and KDSB. We here describe the isolation and characterization of one null mutant for the isoform 1 (*AtkdsA1-S*) and two distinct null mutants for the isoform 2 of *Arabidopsis* Kdo-8-P synthase (*AtkdsA2-V* and *AtkdsA2-S*). We provide evidence that *AtkdsA2* gene expression is preferentially associated with plantlet organs displaying a meristematic activity, and that it accounts for 75% of the mRNAs to be translated into KDSA. Furthermore, quantification of cytosolic Kdo content in the various mutants confirmed this predominant expression of *AtKDSA2*. We could never obtain any homozygous *AtkdsA1*^{-/-} *AtkdsA2*^{-/-} double mutant, suggesting embryo-lethality from the complete knock-out of KDSA function. Interestingly our attempts to obtain a homozygous *AtkdsB* mutant for CMP-Kdo synthase was similarly unsuccessful. Self-crossed plants from the original T-DNA tagged *AtkdsB* mutant gave rise to siliques harbouring 23.9% of empty seeds or seeds containing severely affected embryos. Altogether these data suggest an important role for the Kdo synthesis pathway in embryogenesis.

Frédéric Delmas¹
Martial Séveno²
Patrice Lerouge²
Christian Chevalier¹

Lerouge Patrice²
² UMR CNRS 6037, IFRMP 23,
Université de Rouen 76821 Mont
Saint Aignan France
¹ UMR 619 de Biologie du Fruit,
IFR103, INRA, Université de
Bordeaux, Centre de Recherche
INRA-Bordeaux, BP 81; 33883
Villenave d'Ornon Cedex, France
patrice.lerouge@univ-rouen.fr

Isolation of cDNA sequences encoding the MAP kinase HOG1 and the MAP kinase kinase PBS2 of *Alternaria tenuissima* through a genetic approach

P 20

Alternaria tenuissima is a fungus widely present in the environment and causes diseases in plants and humans in the world. In this study, we constructed an *A. tenuissima* cDNA expression library in a centromeric yeast vector that allows the isolation of functional cDNA sequences from this environmental and pathogenic fungus. Through a genetic approach we have isolated and functionally characterized the cDNA sequences encoding the MAP kinase (MAPK) Hog1p and the MAPK kinase Pbs2p of *A. tenuissima*. AtHOG1 cDNA encodes a protein of 355 amino acids, while AtPBS2 cDNA encodes a protein of 683 amino acids. Further studies on the regulation of cell wall synthesis by AtHOG1 and AtPBS2 are being undertaken.

Fei Feng
Dewen Qiu
Linghuo Jiang

Linghuo Jiang
Institute of Plant Protection
Chinese Academy of Agricultural
Science
Beijing, China 100080
linghuojiang2@yahoo.com.cn

Chemo-enzymatic synthesis of xylogluco-oligosaccharides and study of their affinity with cellulose

P 21

Cellulose microfibrils and xyloglucans (XG) are the main polysaccharides forming the load bearing network of the non commelinoids primary plant cell wall.

Xyloglucan consists of (1,4)- β -linked D-glucopyranosyl backbone, where three out of every four glucose units carry a α -(1,6) linked D-xylosyl substituent, either as a single sugar or as a β -D-Gal-(1,2)- α -D-Xyl or α -L-Fuc-(1,2)- β -D-Gal-(1,2)- α -D-Xyl side chains.

Some studies of their interaction with cellulose were carried out and possible structure property relationships have been put forward^{1,2}. However the apparent statistical distribution of substituents and the impact of molecular weight such as a minimal cooperative³ sequence for efficient binding still need to be clarified.

The first aim of the present work is to synthesize xylogluco-oligosaccharides (XGO) with a controlled structure from tamarin seed xyloglucans, applying a chemo-enzymatic strategy based on the glucoside-hydrolases and glycosynthases activities. For instance, an oligosaccharide containing twelve glucosyl residus is obtained by recoupling of three so called XXLG motif. These XGO chemo-enzymatically synthesized have well defined chain length and side chains composition. The study of their interaction with cellulose enables to evaluate the influence of the substitution.

In a second step, interactions are studied *in vitro* by Isothermal Titration Calorimetry (ITC) and determination of adsorption isotherms. These thermodynamic analyses are made using different XG as native XG or synthesized and purified XGO and several celluloses as bacterial microcrystalline cellulose or cellulose whiskers, a colloidal dispersion of pulp sulphated microcrystals. The main adsorption event appears to be endothermal as shown by the temperature effect on isotherms or the ITC thermograms. However an initial exothermal effect requires further investigation and suggests a dual mode or heterogeneous mechanism.

Marie López^{1,2}
Hévé Bizot²
Gérard Chambat¹
Alain Buléon²
Hugues Driguez¹

López Marie
¹ CERMAV BP53
38041 Grenoble Cedex 9 - France
² INRA BP 71627
44316 Nantes Cedex 3 - France
marie.lopez@cermav.cnrs.fr

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Heterologous expression of Chitin Synthase 2 from *Saccharomyces cerevisiae*

P 22

Chitin is a structural polysaccharide that is an essential component of the fungal cell wall. As chitin synthase is essential for the fungus and is not present in humans, it is a good target for antifungal drugs, and knowledge of its three-dimensional structure could be crucial in designing these drugs. Therefore, we have cloned the gene of the essential chitin synthase from *Saccharomyces cerevisiae*. The enzyme consists of a single polypeptide chain, which forms a 25 kDa N-terminal domain of unknown function, a 48 kDa central catalytic domain and a 37 kDa membrane-bound C-terminal domain. To obtain the amount of protein needed for structural and functional investigations, we are working on developing a suitable expression system for both the entire protein, and a mutant lacking the N-terminal domain. We are testing expression in *E. coli* and in the yeast *Pichia pastoris*, as well as cell-free expression.

Fuensanta W. Martinez-Ruboco
Anke C. Terwisscha van Scheltinga
Luise Eckhardt-Strelau

Martinez-Ruboco Fuensanta W.
Anke C. Terwisscha van Scheltinga

Max Planck Institute of Biophysics
Max-von-Laue Str. 3
60438 Frankfurt am Main
Germany

fuensanta.martinez@mpibp-frankfurt.mpg.de

anke.terwisscha@mpibp-frankfurt.mpg.de

Differential regulation of two β -D-xylosidase genes during tomato fruit development and their relation to firmness.

P 23

Softening that occurs during fruit ripening is known to be associated with characteristic changes of cell wall polysaccharides, mostly mediated by the action of hydrolases that affect selective glycosidic bonds of the different cell wall compounds. Several studies reported on the ripening-specific regulation of genes encoding cell-wall hydrolases (Polygalacturonase, Pectinesterases, Glucanases...) but transgenic experiments leading to the suppression of corresponding enzyme activity did not reveal significant effect on fruit firmness. These data suggested that softening and textural changes in fruits involved coordinated and interdependent activities of a large range of cell-wall modifying proteins. Within the scope of identifying the gene network involved in tomato softening, we screened a large number of cell-wall hydrolase genes (referred in TIGR) in two nearly isogenic lines that differ in firmness : Levovil (Lev) a large fruited line with rapid ripening and softening and LCx, a firm line with Levovil genetic background and several texture-related QTL alleles from a cherry tomato (referred as Cervil). From this screening, two β -D-xylosidases (Xylosidase 1 and 2, homologous to 90%) among seven analyzed, showed a differential gene expression between these two lines. The regulation of the iso-genes was analyzed with respect to genetic control, fruit firmness, fruit and plant development and fruit compartment location. Gene expression profiles were also compared to the corresponding protein data obtained by Western-blot. Results showed that the two xylosidases have drastic spatio-temporal differences in gene expression : (i) Xylo1 is predominantly expressed in fruits compared to Xylo2 that is also strongly expressed in other plant organs as flowers, young leaves and stems (ii) Xylo1 is ripening regulated while Xylo2 is early expressed and associated to cell expansion process, (iii) Xylo1 expression is predominantly localised in radial pericarp while Xylo2 is localised in external and radial pericarp as in skin tissues. These differences in gene expression suggest that the biochemical role of β -xylosidase would be accomplished at different developmental or ripening stage of fruits and also in different plant organs. Although the expression of a ripening-regulated xylosidase gene has been correlated with firmness in strawberry (*Bustamante et al., Plant Sc., 2006*), no relation was established in this study between the tomato lines hosting firmness-related QTLs. Transgenic tomatoes with suppressed xylo1 and xylo2 activities are in progress and would inform about their *in vivo* role on fruit firmness

Marty Isabelle
Adeline Moïse
Guillaume Quinonero
Line Tichit
Fabienne Guillon
Mathilde Causse
Marc Lahaye

Marty Isabelle
INRA SQPOV
Domaine Saint-Paul
Sitae Agroparc
84914 Avignon Cedex 9
marty@avignon.inra.fr

Tomato texture is one of the critical components for the consumer's perception of fruit quality. Texture is a complex character composed of several attributes (firmness, mealiness, skin toughness and juiciness) that are difficult to evaluate and which change during fruit ripening. The objective of this work is to identify candidate genes related to QTL controlling texture variations of tomato fruit using proteomic and transcriptomic approaches.

Five chromosome regions carrying QTLs for quality traits, previously mapped in a RILs population, were transferred following a marked-assisted backcross scheme from a cherry tomato line into two modern lines. Two of these regions located on chromosomes 4 and 9 correspond to QTLs with a strong effect on fruit firmness and mealiness. Two types of genotypes were used: QTL-NILs which differed from the recipient line only for the region of the chromosome 4 or of the chromosome 9 and lines cumulating the five chromosome fragments.

The research of candidate genes with a function or an expression level related to texture variations was performed using proteomic (two-dimensional electrophoresis) and transcriptomic (microarrays) approaches. Expression profiles of the parental lines were respectively compared to those of the corresponding line cumulating the five regions. Several candidate genes were identified essentially related to cell wall-modifying enzymes, such as endo-polygalacturonase, β -D-xylosidase, glucan endo-1,3-beta-D-glucosidase, α -mannosidase. The level of expression of these genes was then assessed by quantitative RT-PCR (i) on a kinetic of fruit development composed of seven stages distributed from early stages to over-ripe (ii) on the same lines firstly used but also on the QTL-NIL described above. The results obtained confirmed the involvement of these genes in fruit texture variations but also suggest that several complementary cell wall modifications contributed to texture change. At the same time, the candidate genes identified were mapped and a few co-localisations with texture related QTL were observed.

**Jamila Chaïb
Stéphane Munoz
Philippe Duffé
Esther Pelpoir
Line Tichit
Mireille Faurobert
Mathilde Causse
Marty Isabelle**

Marty Isabelle
INRA SQPOV
Domaine Saint-Paul
Sitae Agroparc
84914 Avignon Cedex 9
marty@avignon.inra.fr

Analysis of a tomato orthologous QUASIMODO gene predominantly expressed in tomato fruit development

P 25

Pectins are major components of primary cell wall and contribute to the physical and biochemical properties of tissues. An allelic mutant of *Arabidopsis thaliana* (*Quasimodo-1*) has been identified that leads to a dwarf plant phenotype and a reduction in cell adhesion, particularly of epidermal cells in seedlings and young leaves. In addition, it presents a reduced homogalacturonan content in isolated cell walls (*Bouton et al., Plant Cell, 2002*). Such results support that *QUAI* gene encodes a putative glycosyltransferase of the multigenic family 8 implicated in pectin and hemicellulose cell wall synthesis (*Orfila et al., Planta, 2005*). Despite their potentially crucial contribution to fruit texture properties, these glycosyltransferase activities have not yet been identified in fruits. Our work consisted in finding orthologous genes of *QUAI* in tomato. Their expression pattern was investigated with respect to fruit and plant development and to fruit texture. Gene expression profiles were compared to the corresponding protein accumulation followed by Western-blot. The role of these genes on texture elaboration during fruit development and maturation is also investigated by the creation of RNAi silencing and surexpression lines. Results showed that only one tomato gene is strongly homologous to *A.thaliana* *Qua1* (*QUA A*) and that (i) is mainly expressed in early stages of fruit development (ii) its expression is predominantly in the fruit than in other plant tissues like flower, young leaves and stems and that (iii) is more strongly localized in pericarp than in skin, gel and seeds. Western-blot analysis showed a similar accumulation pattern of a 63-kDa protein corresponding to the theoretical molecular weight of the *QUA A* protein. Up to now, gene expression and protein accumulation profiles suggest that *QUA A* is involved in the establishment of the cell wall through pectin synthesis during cell expansion phase of the fruits. The first RNAi transgenic plants were obtained and analysis are in progress in order to better understand their role in fruit firmness.

Adeline Moïse
Guillaume Quinero
Line Tichit
Patrick Rousselle
Michel Hernould
Fabienne Guillon
Mathilde Causse
Marc Lahaye

Moïse Adeline
INRA SQPOV
Domaine Saint-Paul
Sitaé Agroparc
84914 Avignon Cedex 9
adeline.moise@avignon.inra.fr

Molecular characterization of the mannosyltransferase *PMT4* of *Aspergillus fumigatus* involved in cell wall morphogenesis.

P 26

O-glycosylation is a major post-translational modification of proteins. O-glycosylated proteins play major roles in eukaryotic cells from fungi to humans. The initial reaction of mannose transfer to serine and threonine residues is catalysed by protein O-mannosyltransferase in the endoplasmic reticulum. Seven PMT (1-7) has been characterized in *S. cerevisiae*. Phylogenetic analysis showed that protein O-mannosyltransferases can be divided into three subfamilies, the *PMT1*, *PMT2* and *PMT4* subfamilies. Disruption of three different types of PMT genes resulted in death of the yeast cells. In the filamentous fungus *Aspergillus fumigatus*, 3 orthologs are present in the genome and are called *PMT1*, *PMT2* and *PMT4* since every of them belong to a different subfamily. All these proteins showed hydrophathy profiles typical of protein O-mannosyltransferase of the PMT family with several transmembranes domains. The role of *PMT4* has been now investigated in *A. fumigatus* and for the first time in filamentous fungi. This gene encoded for a transmembrane protein of 780 amino acids. Δ PMT4 of *A. fumigatus* has been constructed by gene replacement. The mutant show severe morphological defects : reduced growth, altered mycelium, higher sensitivity to antifungal drugs, reduced conidiation.

**Isabelle Mouyna
Jean-Paul Latgé**

Mouyna Isabelle
Unité des *Aspergillus*
Pasteur Institut
25 rue du Docteur Roux
75015 Paris
imouyna@pasteur.fr

Cell Wall Integrity Checkpoint regulates G2/M phase Cell Cycle Arrest through Dynactin Complex and its Interacting Proteins.

P 27

Cell cycle events in *Saccharomyces cerevisiae* are strictly monitored and regulated by checkpoint mechanisms, which ensure the correct cell division and regulate the necessary arrest or delay in cell cycle in response to cell perturbation. We have established the concept of a novel checkpoint mechanism termed cell wall integrity checkpoint, which couples the cell wall synthesis and mitosis. Temperature sensitive mutant of 1,3-*beta*-glucan synthase, *fks1-1154*, shows cell cycle arrest with post-replicative DNA content in G2 phase exhibiting tiny bud projection with duplicated but unseparated SPBs when incubated at the restrictive temperature. Random mutagenesis analysis lead us to find that Arp1p, the main component of yeast dynactin complex, is required for the proper function of the checkpoint. Mutation in Arp1p (*wac1*, P268L) in *fks1-1154* background elicits defect in the checkpoint function when incubated at the restrictive temperature resulting in the accumulation of small budded cells with elongated nuclear spindle. In search for other components contributing to the cell wall integrity checkpoint, proteins physically interacting with the dynactin complex were systematically examined for their involvements in the checkpoint. Among the proteins examined, we found that mutations in Bzz1p and Srp1p, two-hybrid interacting proteins of dynactin components (Nip100p and Arp1p, respectively), in *fks1-1154* background cells show the accumulation of small budded cells with elongated spindle at the restrictive temperature. The phenotype observed by fluorescence microscopy is considered to characterize at least a partial loss of the checkpoint function and Bzz1p and Srp1p are suggested to have roles in the checkpoint. In addition, dynactin complex shows different localization pattern in checkpoint defective cells compared to the checkpoint intact cells. The proper localization of dynactin complex may be responsible for the function of the cell wall integrity checkpoint. These data imply that dynactin complex potentially has a major role in the checkpoint mechanism. This study will help us farther our understanding of the relationship between cell wall biogenesis and cell cycle in *Saccharomyces cerevisiae*.

Takahiro Negishi
Ryoji Igarashi
Miyuki ImanariSatoru
NogamiYoshikazu
OhyaTakahiro

Negishi Takahiro
Department of Integrated
Biosciences Graduate School of
Frontier Sciences, University of
TokyoKashiwanoha, Kashiwa,
Chiba 277-8562, Japan.
67514@ib.k.u-tokyo.ac.jp

Homologous subunits of yeast 1,3-beta-glucan synthase are important for spore wall assembly in *Saccharomyces cerevisiae*

P 28

During sporulation in *Saccharomyces cerevisiae*, the four haploid nuclei are encapsulated within multilayered spore walls. Glucan, the major constituent of the spore wall, is synthesized by 1,3-beta-glucan synthase, which is composed of a putative catalytic subunit encoded by *FKS1* and *FKS2*. Although another homolog, encoded by *FKS3*, was identified by homology searching, its function is unknown. In this report, we show that *FKS2* and *FKS3* are required for spore wall assembly. The ascospores of *fks2* and *fks3* mutants were enveloped by an abnormal spore wall with reduced resistance to diethyl ether, elevated temperatures, and ethanol. However, deletion of the *FKS1* gene did not result in a defective spore wall. The construction of fusion genes that expressed Fks1p and Fks2p under the control of the *FKS2* promoter revealed that asci transformed with *FKS2*p-driven Fks1p and Fks2p were resistant to elevated temperatures, which suggests that the expression of *FKS2* plays an important role in spore wall assembly. The expression of *FKS1*p-driven Fks3p during vegetative growth effectively suppressed the growth defect of the temperature-sensitive *fks1* mutant. Quantitative analysis of cell morphology and glucan content of mutant cells revealed that morphological abnormality and reduced glucan content of the temperature-sensitive *fks1* mutant was suppressed. Whereas *FKS1*p-driven Fks3p did not affect 1,3-beta-glucan synthase activity *in vitro*, the amount of active form of Rho1p, which is a regulatory subunit of 1,3-beta-glucan synthase, was increased *in vivo*, suggesting that it suppressed the growth defect of the *fks1* mutant by stabilizing active-Rho1p *in vivo*. Based on these results, we propose that *FKS2* encodes the primary 1,3-beta-glucan synthase in sporulation and that *FKS3* is required for normal spore wall formation because it affects the upstream regulation of 1,3-beta-glucan synthase.

Satoru Ishihara
Satoru Nogami
Aiko Hirata
Anne Beauvais
Jean-Paul Latge
Yoshikazu Ohya

Nogami Satoru
Department of Integrated Biosciences
Graduate School of Frontier Sciences
University of Tokyo
Bldg. FSB-101, 5-1-5 Kashiwanoha,
Kashiwa, Chiba 277-8562, Japan
knogami@mail.ecc.u-tokyo.ac.jp

Carbohydrate microarrays: analysis of enzyme activity by epitope deletion

P 29

As a tool for the high-throughput screening of enzymatic activities carbohydrate microarrays were constructed by high density spotting of a range of plant polysaccharides onto nitrocellulose membranes. Different polysaccharide degrading enzymes were subsequently tested for their activity against samples present on the array. Monoclonal antibodies were used to measure decrease in epitope in each sample thus indicating the action patterns of the enzymes tested. Our analysis indicates that this type of carbohydrate microarray can be used for screening of individual enzyme activities against many different carbohydrates simultaneous. Additionally, a reverse approach was tested where nitrocellulose membranes were coated with a single polysaccharide sample and several enzymes spotted onto these coated membranes using a microarrayer. This second approach provides a rapid screening technique for testing numerous enzymes against a single target carbohydrate.

Jens Øbro
Iben Sørensen
Isabel Møller
Jørn Dalgaard Mikkelsen
Michael Skjøt
William G.T. Willats

Øbro Jens
University of Copenhagen
Department of Molecular Biology
Ole Maaløes Vej 5
2200 København N
Denmark
jensobro@my.molbio.ku.dk

Analysis of Coronary arteritis induced by *C. albicans* extracellular mannoprotein in various strains of mice

P 30

CAWS is a water-soluble extracellular polysaccharide fraction obtained from the culture supernatant of *Candida albicans* NBRC1385. CAWS induced toxic reactions, such as acute anaphylactoid reaction, by intravenous administration and coronary arteritis by intraperitoneal administration. CAWS-vasculitis is similar to that induced in Kawasaki disease.

We analyzed differences in the occurrence of coronary arteritis among mouse strains, inbred strains, hybrids and mutants. CAWS vasculitis was induced in almost all of the inbred strains tested, except for CBA/j mice. CAWS vasculitis was induced also in hybrids, CDF1 and BDF1. In mutant strains of various immunological defects, such as C57BL/6J Ham Slc-bg, Balb/c nu/nu, C.B.17/Icr-scid/scid, WBB6F1-W/Wv mice, they all induced CAWS vasculitis but relatively weak. It is already postulated that CAWS vasculitis was regulated by various genes related to acute as well as chronic inflammation.

To clarify the structure responsible for these toxic reactions, *C. albicans* was cultured in pH- and temperature-controlled conditions and prepared with CAWS with or without the beta-1,2-linked mannosyl segment (BM). The structure of CAWS was assessed by immunochemical and spectroscopic methodologies, and we found that CAWS prepared under the natural culture conditions contained only small amounts of BM and CAWS prepared at neutral conditions at 27 degrees C contained a significantly higher percentage of BM. Both the acute lethal toxicity and coronary arteritis induction was significantly more severe in the absence of BM. Activation of a complement pathway, the lectin pathway, by CAWS was significantly stronger in the absence of BM. These facts strongly suggest that BM linkages in CAWS negatively modulate acute and chronic toxicity of CAWS, and may be strongly related to the lectin pathway of the complement activation. Considering these characteristics, CAWS vasculitis might respond to various types of therapeutic strategies.

Noriko Nagi-Miura,
Hiroyasu Shinohara,
Motohiko Komai,
Yoshiyuki Adachi,
Toshiaki Oharaseki*,
Kei Takahashi*,
Akiko Ishida-Okawara,**
Kazuo Suzuki,**
Naohito Ohno

Tokyo Univ Pharm Life Sci,

* Toho Univ Sch Medicine,

** Nat Inst Infect Dis.

Ohno Naohito

Lab Immunopharmac Microbial
Products, School of Pharmacy,
Tokyo University of Pharmacy and
Life Science, 1432-1 Horinouchi,
Hachioji, Tokyo 192-0392, Japan.
ohnonao@ps.toyaku.ac.jp

Functional analysis of *Arabidopsis thaliana* RHM2/MUM4, a multidomain protein involved in UDP- D-glucose to UDP-L-rhamnose conversion

P 31

UDP-L-rhamnose is required for the biosynthesis of cell wall rhamnogalacturonan-I, rhamnogalacturonan-II and natural compounds in plants. It has been suggested that the *RHM2/MUM4* gene is involved in conversion of UDP-D-glucose to UDP-L-rhamnose on the basis of its effect on rhamnogalacturonan-I-directed development in *Arabidopsis thaliana*. *RHM2/MUM4* related genes, *RHM1* and *RHM3*, can be found in the *A. thaliana* genome. Here we present direct evidence that all three RHM proteins have UDP-D-glucose 4,6-dehydratase, UDP-4-keto-6-deoxy-D-glucose 3,5-epimerase and UDP-4-keto-L-rhamnose 4-keto-reductase activities in the cytoplasm when expressed in the yeast *Saccharomyces cerevisiae*. Functional domain analysis revealed that the N-terminal region of RHM2 (RHM2-N; amino acids 1-370) has the first activity and the C-terminal region of RHM2 (RHM2-C; amino acids 371-667) has the two following activities. This suggests that the RHM2 converts UDP-D-glucose to UDP-L-rhamnose *via* an UDP-4-keto-6-deoxy-D-glucose intermediate. Site-directed mutagenesis of RHM2 revealed that mucilage defects in *MUM4-1* and *MUM4-2* mutant seeds of *A. thaliana* are caused by abolishment of RHM2 enzymatic activity in the mutant strains and further, that the GxxGxx(G/A) and YxxxK motifs are important for enzymatic activity. Moreover, a kinetic analysis of purified 6 x HIS tagged RHM2-N protein revealed 5.9-fold higher affinity of RHM2 for UDP-D-glucose than for dTDP-D-glucose, the preferred substrate for dTDP-D-glucose 4,6-dehydratase from bacteria. RHM2-N activity is strongly inhibited by UDP-L-rhamnose, UDP-D-xylose and UDP but not by other sugar nucleotides, suggesting that RHM2 maintains cytoplasmic levels of UDP-D-glucose and UDP-L-rhamnose *via* feedback inhibition by UDP-L-rhamnose and UDP-D-xylose.

Takuji Oka
Tadashi Nemoto
Yoshifumi Jigami

Oka Takuji
Research Center for Glycoscience
National Institute of Advanced
Industrial Science and Technology
(AIST), Tsukuba, Ibaraki, Japan
oka-takuji@aist.go.jp

Impact of storage conditions on the yield and physiochemical properties of ulvans extracted from the cell wall of *Ulva armoricana* (Chlorophyta)

P 32

The polysaccharides contained in the cell wall of marine green algae belonging to Ulvales (Chlorophyta, *Ulva* and *Enteromorpha* sp.) are currently drawing increasing interest as potential sources of new functional biopolymers. Green algae are very abundant and frequently involved in proliferations such as the “green-tides” that occur seasonally in eutrophicated coastal and lagoon waters. Cell wall polysaccharides in green algae are essentially represented by ulvans, which are mainly composed of rhamnose 3-sulfate and glucuronic acid or iduronic acid. These sugars occur as repeating sulphated aldobiuronic acids named ulvanobiuronic acid 3-sulphate A or B, respectively. Ulvans were shown to have many potential uses in pharmaceutical and agronomical applications due to their biological properties, in food and feed conception as dietary fibers and in applications were metal chelation, gelling ability or nanostructuration of composite materials is required.

In spite of this strong application potential, little is known about quantitative and qualitative consistency of the cell wall polysaccharides among *Ulva* biomass. When “green tides” occur, seaweeds are usually collected upon stranding on beaches and need to be processed rapidly due to their high fermentability. In the work reported here, we have assessed the impact of stabilisation methods of *Ulva armoricana* on the yield and physiochemical characteristics of ulvans. The effect of drying, freezing, brining and dry-salting conditions will be presented. Our results indicate that depending on stabilisation method, different ulvan qualities and properties are obtained. Highest ulvan yield was from brined algae. Purer ulvans were obtained from heat-dried algae. The highest ulvan viscosities were obtained from frozen algae. Stabilisation methods can therefore be selected with respect to cost-effectiveness and in accordance with ulvan characteristics needed to meet specific application criteria.

Audrey Robic^{1,2},
Jean-François Sassi¹,
Yannick Lerat¹
Marc Lahaye²

Robic Audrey¹
¹CEVA, BP3, Pleubian, France
²INRA, BIA, BP 71627, 44316
Nantes, France
algue@ceva.fr

Bio-integrative approach to study the biosynthesis of wheat endosperm cell walls.

P 33

Plant and fungi cell wall are made of a complex network of polysaccharides whose biosynthesis is not fully understood. Even though, most of the glycosyltransferases, the enzyme responsible for the synthesis of glycosidic linkages, are identified in sequenced genomes (<http://afmb.cnrs-mrs.fr/CAZY/>). However their precise biochemical functions have been assigned for only a very small number of these glycosyltransferases.

In our model, the wheat endosperm, the cell wall is made of arabinoxylan (70%), β -glucan mixed (20%), glucomannan (2-7%) and cellulose (2-4%). The biosynthesis of the main polysaccharide is unknown, whereas the synthesis of the other three is known (β -glucan mixed by GT2-CslF, mannan by GT2-CslA and cellulose by GT2-CesA). A bioinformatic approach was set up to identify candidate genes for the biosynthesis of the arabinoxylan, their deposition in the wall, their modifications, and their cross linking by ferulic acid. Based on our knowledge of the cellular localisation and the time course of appearance of arabinoxylan during the grain development (see F. Guillon's abstract or ¹), we used an extensive approach to identify and classified most of the wheat glycosyltransferases present in public library (TGI). A local core data base was constructed with arabidopsis and rice glycosyltransferases (using CAZY, TAIR, IRGSP and TIGR). After validation of the core data base, the wheat contigs were compared individually with the local data base (with BLASTX algorithm). Thus, most of the wheat contigs sharing sequence similarity (at the amino acid level) with a glycosyltransferase from either arabidopsis or rice were identified and grouped by family (according to CAZY).

A second round of validation both automatic and manual, finally led to the following results:

36 GT families were identified in wheat with 524 contigs (41 GT families for 470 genes and 39 GT families for 470 genes, in arabidopsis and rice respectively). The origin of the ESTs composing the contigs, show that 83 of these contigs are expressed in the endosperm (although not exclusively). A selection of candidate genes was made based on the expression in the endosperm tissue, the implication of GT-families in cell wall biosynthesis in other plant model, and sub-family grouping using multiple alignments and guide trees.

Molecular validation of these candidate genes is in progress (using RT-PCR, hybridisation *in situ*). More than 20 genes have already been confirmed to be expressed in the endosperm during the period of active arabinoxylan biosynthesis. A functional validation for some of these genes has been initiated (using RNAi, and ecotype screening). The aim of this project is to understand the whole biosynthesis process in detail in order to exploit and control the genetic bio-diversity.

¹ Philippe, S., L. Saulnier, et al. (2006). "Arabinoxylan and (1-->3),(1-->4)-beta-glucan deposition in cell walls during wheat endosperm development." *Planta*: 1-13.

Pierre-Etienne Sado
Dominique Tessier
Khalil El Morjani
Fabienne Guillon
Luc Saulnier

Sado Pierre-Etienne
INRA, BIA
BP 71627
44 000 Nantes, France
sado@nantes.inra.fr

Molecular Characterization of the PMT Protein O-Mannosyltransferase Family in Yeast

P 34

It is initiated at the endoplasmic reticulum by the transfer of mannose from dolichyl phosphate-activated mannose to serine or threonine residues of secretory proteins. This reaction is catalyzed by a family of protein O-mannosyltransferases (PMTs) that is studied best to date in the yeast *S. cerevisiae*. The PMT family is divided into the PMT1, PMT2 and PMT4 subfamilies. In yeast, members of the PMT1 and PMT2 subfamilies show marked similarities and distinctions from PMT4 subfamily members. For example, the PMT1/PMT2 and PMT4 subfamilies use different acceptor protein substrates *in vivo*. Further, members of the PMT1 subfamily interact in pairs with members of the PMT2 subfamily, whereas the unique representative of the PMT4 subfamily forms homomeric complexes.

We proposed a seven-transmembrane structural model for ScPmt1p. A large, hydrophilic, endoplasmic reticulum-oriented segment is flanked by five aminoterminal and two carboxyl-terminal membrane-spanning domains. Based on this model, structure-function analysis was performed which will be discussed.

Andrea Schott
Verena Girrbach
Sabine Strahl

Schott Andrea
Heidelberg Institute of Plant
Science, Ruprecht-Karls-University
Heidelberg
Im Neuenheimer Feld 360
D-69120 Heidelberg, Germany
aschott@hip.uni-heidelberg.de

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Reduction of Peptidase Activity in the Yeast Cell Wall Fraction as a Carrier of Immobilized Enzymes.

P 35

Large-scale supply of variety of glycosyltransferase is required for producing functional oligosaccharides and glycoproteins. We, therefore, are constructing an immobilized human glycosyltransferase library using Pir cell wall proteins as an anchor to bind recombinant enzymes at the yeast cell wall. Cell wall immobilized enzymes have several merits, such as easy preparation of enzyme source, repeated use of enzymes, increase of enzyme stability, etc. However, yeast cell wall fraction, which was prepared by homogenized with glass-beads, showed significant degradation of substrate Muc1 peptides, when immobilized enzymes were used for the glycopeptide synthesis.

In order to remove peptidase activity that was contaminated in the host cell wall fraction, some protease inhibitors were treated. Inhibitors for serine proteases such as PMSF decreased the peptidase activity, and the cell wall fraction washed with detergents showed the decreased peptidase activity. Peptidase assay of EUROSCARF gene deletants of several protease genes revealed that pep4, kex2, and rbd2 showed the decreased peptidase activity. It is suggested that membrane bound serine proteases, which would be activated by yapsin (YPS1, MKC7, YPS3, etc.) or vacuole proteases (PEP4, PRB1, etc.), were involved. Multi-gene disruptants were constructed and the peptidase activity was measured. The peptidase activity of pep4 gene disruptant was about 50% of wild type, while that of pep4 mkc7 double disruptant showed higher activity than pep4 single disruptant. But pep4 yps1 mkc7 yps3 quadruple disruptant showed the same level of peptidase activity with pep4 single disruptant, suggesting that disruption of mkc7 may induce the YPS1 and YPS3 activity. The pep4 prb1 yps1 mkc7 yps3 rbd2 disruptant cells showed residual peptidase activity against Muc1 peptide, but no activities were detected for casein hydrolysis.

The immobilized glycosyltransferases were successfully expressed at the cell wall of these peptidase deficient cells and the stability of these enzymatic activities were examined.

Yoh-ichi Shimma
Tomoko Ishii
Fumie Saito
Yoshifumi Jigami

Shimma Yoh-ichi
Research Institute for Cell
Engineering (RICE), National
Institute of Advanced and
Industrial Science and Technology
Aist, Japan
yohichi-shimma@aist.go.jp

Cell wall mutants of *Botrytis cinerea* and role of chitin synthases in plant infection.

P 36

Epidemics caused by *Botrytis cinerea* can be severe and economically damaging to many agricultural and horticultural crops and these losses continue to occur despite availability of new botryticides. Indeed, the extensive use of fungicides, including systemic compounds has caused serious problems of resistance. An effective control of *B. cinerea*, a necrotrophic fungus which causes the grey mould disease on grapevine, relies on the development of new strategies. Chitin, an essential ultrastructural constituent of fungal cell walls, could be a suitable target for fungicides.

Chitin, a β -1,4 N-acetylglucosamine polymer, is biosynthesized by a family of chitin synthases. Actually, five chitin synthase genes belonging to class I, III, IV and VI have been sequenced in *Botrytis*. Moreover, genes of class II, V and VII have been identified and the recent sequencing of the fungus (Broad Institute USA and Genoplante France) will allow us to know the full gene sequence (1). Three mutants disrupted in class I, IIIa or IV chitin synthase genes were obtained and characterized (2,3) and class VI and VII mutants are under investigation. Interestingly, the three mutants show different aggressiveness phenotypes on *Vitis vinifera* or *Arabidopsis thaliana*: *DBcchsIV* displays a slight delay in infection whereas *DBcchsI* and *DBcchs3a* show a reduction of aggressiveness of 25 and 70% respectively. The drastically reduced aggressiveness of *DBcchs3a* might be due to the altered cell wall structure causing increased plant defence induction. To examine this hypothesis we tested it on different *Arabidopsis thaliana* mutants. First results obtained will be discussed on the poster. Moreover, the *Bcchs3a* mutant displays a specific phenotype *in vitro*: the radial growth rate is severely reduced, the hyphal tips are surrounded by an extracellular matrix and the mycelium produced numerous branched hyphae (microscopic studies). These results suggested that this enzyme BcCHS3a functions at the apical tips of the hyphae. Altogether, we propose that *chs3a* gene product is a good antifungal target in phytopathogenic fungi as previously demonstrated for human pathogens.

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Claude Périno (a)
Pierrette Malfatti (a)
Mathias Choquer (b)
Anne Vidal-Cros (b)
Marie-Christine Soulié (a)
Caroline Kunz,
Claude Périno

Soulié Marie-Christine
(a) UMR 217 INRA/Université ParisVI/INA-PG, Laboratoire Interactions plantes-pathogènes, 16 rue Claude Bernard, 75231 Paris cedex 05
(b) UMR 7613 CNRS/Université Paris VI, Structure et Fonction de Molécules Bioactives, boîte 182, 4 place Jussieu, 75252 Paris cedex 05.
soulie@ccr.jussieu.fr

Endo- versus exo-mechanism and the role of processivity in chitin degradation by family 18 chitinases

P 37

The Gram-negative soil bacterium *Serratia marcescens* produces three family 18 chitinases and a chitin-binding protein, CBP21 [1], that together effectively convert chitin to chitobiose. Using the unique experimental possibilities provided by the soluble chitin-derivative chitosan (partially deacetylated chitin), we have studied the properties of the three chitinases in detail. ChiC is an endo-acting enzyme with a shallow active site cleft. ChiA and ChiB have deep active site clefts and degrade chitosan in a processive fashion after initial endo-binding, presumably in opposite directions [2-5]. The initial endo-binding suggests that the exo-activity observed with crystalline chitin [6] may be due to the accessibility of the chain ends rather than to intrinsic properties of the enzymes. Studies on the contributions of aromatic residues to the functionality of ChiB revealed that two tryptophans close to the catalytic center are crucial for processivity and for effective degradation of crystalline material. Most remarkably, the loss of processivity in the mutants was accompanied by a large increase in the degradation rate for non-crystalline material such as the single soluble polymer chains of chitosan [7]. Processivity is thought to contribute to the degradation of crystalline polysaccharides because detached single polymer chains are kept from re-associating with the solid material. The results of the mutagenesis study show that this processivity comes at a large cost in terms of enzyme speed.

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S.J. Horn¹
A. Sørbotten²
P. Sikorski³
Synstad¹
M. Sørli¹
G. Vaaje-Kolstad¹
K.M. Vårum²
V.G.H. Eijsink¹

Vaaje-Kolstad G.
¹Department of Chemistry, Biotechnology and Food Science, the Norwegian University of Life Sciences, P.O. Box 5003, 1432 Ås, Norway
gustav.vaaje-kolstad@umb.no

²NOBIPOL, Department of Biotechnology, the Norwegian University of Science and Technology, NTNU, 7491 Trondheim, Norway

³Biophysics and Medical Technology, Department of Physics, NTNU, 7491, Trondheim, Norway

Structure and function of CBP21, a non-catalytic chitin-binding protein promoting chitin degradation

P 38

The Gram-negative soil bacterium *Serratia marcescens* produces three family 18 chitinases [1,2] that enable the bacterium to efficiently degrade chitin, an abundant insoluble carbohydrate polymer composed of $\beta(1,4)$ -linked units of *N*-acetylglucosamine. During chitin degradation, *S. marcescens* also secretes a non-catalytic chitin-binding protein, CBP21, which binds to the crystalline substrate [2,3]. The structure of CBP21 was determined at 1.55 Å resolution and showed a budded fibronectin type-III fold with a patch of conserved, mainly hydrophilic, residues on the surface [3]. Site-directed mutagenesis was used to construct single point mutants of selected residues on the conserved surface patch. Analysis of these mutants showed that CBP21 binds its substrate through specific, mostly polar interactions that disrupt the chitin structure. Chitin degradation assays using combinations of the *S. marcescens* chitinases and CBP21 showed that the chitin-binding protein strongly promoted hydrolysis of crystalline β -chitin by chitinases A and C, while it was essential for the complete degradation of chitin by chitinase B [4]. Interestingly, homologues of CBP21 occur in most chitin-degrading microorganisms, suggesting a general mechanism by which chitin-binding proteins enhance chitinolytic activity. Homologues also occur in chitinase-containing insect viruses, whose infectiousness is known to depend on chitinase efficiency.

G. Vaaje-Kolstad¹
S.J. Horn¹
D.R. Houston²
B. Synstad¹
D.M.F. van Aalten²
V.G.H. Eijsink¹

Vaaje-Kolstad G.
¹Department of Chemistry,
Biotechnology and Food Science,
Norwegian University of Life
Sciences, P.O. Box 5003, 1432 Ås,
Norway
gustav.vaaje-kolstad@umb.no

²Wellcome Trust Biocentre,
University of Dundee, Scotland

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Cytoskeleton and cell wall changes during the biphasic growth of dark-grown *Arabidopsis thaliana* Col-0 hypocotyls

P 39

Previous studies revealed that the dark-grown hypocotyls of *Arabidopsis thaliana* (Col-0) elongate in two phases. At the onset of the first phase (at 24h after imbibition), cell elongation was initiated in all cells of the hypocotyls. Between 24h and 48h, both cell 3 (at the base) and cell 13 (at the top) showed a comparable slow growth rate of approximately 1 $\mu\text{m}/\text{h}$. Both cells reached a similar length of about 38 μm at 48h. In the second phase (between 48 and 50h), the growth of cell 3 accelerated, and at 60h it had reached a length of about 225 μm . By contrast, cell 13 maintained at the slow elongation rate, reaching a length of only 45 μm at 60h (Refrégier *et al.*, 2004). During the next hours the growth acceleration propagated, following an acropetal gradient. By 60h the lower two-thirds of the hypocotyl had entered the rapid elongation phase (Gendreau *et al.*, 1997).

The aim of this project is to study changes in the cytoskeleton and the cell wall during slow elongation and the transition to the fast elongation phase. MAP4-GFP, a transgenic line bearing GFP fused to a microtubule binding domain (MBD), was used to visualize cortical microtubules (CMTs). The cell wall has been studied by Field Emission Scanning Electron Microscopy (FESEM) and by the analysis of transgenic plants bearing functional GFP-fusions with cellulose synthase catalytic subunits CESA3 and CESA6 (Desprez *et al.*, unpublished results). Here we show that the orientation of CMTs and the deposition of cortical microfibrils (CMFs) mirror each other. At the beginning of the slow elongation phase, CMTs and CMFs showed a random orientation. During the slow elongation phase and the transition to the fast elongation phase the CMTs and CMFs remained predominantly perpendicular to the elongation axis. When cell elongation ceased, a reorganization occurred from a transverse to an oblique and eventually to a longitudinal orientation. As shown before (Paredez *et al.*, 2006), CMFs are most likely being deposited according to the orientation of microtubules.

To study the actin cytoskeleton, we used transgenic plants with GFP fused to a fimbrin actin-binding domain (FABD2) (Voigt *et al.*, 2005). Using these we found that the actin cytoskeleton changed in three steps: 1) at the start of the slow elongation, thick actin cables were found, especially around the nucleus; 2) just before the start of the fast elongation, there was a fine network of actin cables throughout the whole cytoplasm; 3) at the beginning of and during fast elongation thick longitudinal cables were detected together with a fine network of actin cables throughout the cell. A similar actin reorientation pattern has been showed before in *Arabidopsis* roots and in maize (Voigt *et al.*, 2005; Baskin *et al.*, 2001).

Jürgen Van Orden
Sandra Pelletier
Thierry Desprez*
Samantha Vernhettes*
Herman Höfte*
Jean-Pierre Verbelen
Kris Vissenberg

*Cell Biology Laboratory, Institut
Jean-Pierre Bourgin, INRA, Route
de Saint Cyr, 78026 Versailles
cedex, France

Van Orden Jürgen
University of Antwerp, Biology
Department, Groenenborgerlaan
171, 2020 Antwerp, BELGIUM
jurgen.vanorden@ua.ac.be

Novel monoclonal antibodies to pectic polysaccharides of plant cell walls

P 40

Plant cell walls are key cellular components underpinning plant growth and development. They are also major components of the terrestrial biomass and are used in fuel, food, paper, textiles, plastics and pharmaceutical industries. It is clear that plant cell walls are highly complex structures with a great diversity of components and architectures across species and methodologies are required to assay and understand this diversity. Plant cell wall polysaccharides occur in three major classes: cellulose, cross-linking glycans (or hemicelluloses) and pectins. Pectins are a highly structurally diverse class with distinct domains such as homogalacturonans (HG), rhamnogalacturonans (RG), xylogalacturonans (XGs), arabinans and arabinogalactans. Although recent studies have indicated roles for pectins in growth, cell adhesion, cell-cell signalling, defence and cell differentiation it is still unclear how these complex cell wall components function *in vivo*. Monoclonal antibodies are powerful tools to determine the regulation of the structural features of pectic polysaccharides and to study how they are integrated into diverse cell wall architectures and modulated in response to genetic and environmental impacts. Monoclonal antibodies can thus provide insight into biological roles. A range of new monoclonal antibodies have been generated subsequent to immunisation of rats with distinct isolates of RGs and XGs. We will present current knowledge of these new monoclonal antibodies in terms of epitope characterization and their recognition of plant materials using immunocytochemical methods.

Yves Verhertbruggen
Susan E. Marcus
Ash Haeger
J. Paul Knox

Verhertbruggen Yves
Centre for Plant Sciences
University of Leeds
Leeds LS2 9JT
UK
y.verhertbruggen05@leeds.ac.uk

Evolution of cell wall in grape skins during ripening.

P 41

Given that most of the aromatic and phenolic compounds are in grape skins, the quality of wine is directly correlated with the optimal maturity of that outlying tissue.

Even though fruit ripening is frequently accompanied by changes in cell wall material, studies performed on mesocarp (pulp) suggested there was no dramatic change in polysaccharide composition, but rather a sum of moderate modifications of specific components. Investigating the evolution and the extractability of polysaccharides from exocarp (skin) may be important for the determination of the optimal grape maturity.

Skin cell wall material was isolated from Shiraz grape berries from one to ten weeks after "*véraison*". Total amounts in cell wall polysaccharides were constant during ripening, about 4 mg/berry. Extractions by a HEPES/phenol buffered method enables the isolation of a soluble and an insoluble water fractions. During ripening, we could note a slight decrease in galactose content and also a significant de-esterification of methoxylated uronic acids. Concerning the soluble fraction, even though it represented a small fraction of the whole polysaccharides, its amounts increased more than two fold between the first and the last sample. Isolated walls were also analysed for their composition in amino acid.

The different modifications observed during ripening will be presented.

**Vicens Anysia
Sidhoum Louise
Williams Pascale
Fournand David
Doco Thierry**

Vicens Anysia
INRA-AGRO Montpellier
UMR Science Pour l'Oenologie,
bât 28
2, place Viala
34060 Montpellier Cedex 1,
France
vicens@ensam.inra.fr

Production, purification and characterization of 10 *Arabidopsis* xyloglucan endotransglycosylases/hydrolases (XTHs).

P 42

Xyloglucan endotransglucosylase/hydrolases (XTHs) are a class of enzymes that have the ability to cleave and rejoin xyloglucan chains and are considered to play a key role in both the construction and disassembly of the cell wall architecture.

The *Arabidopsis* genome encodes an XTH-family of 33 members that are highly similar. They share a conserved motif that is predicted to be necessary for their activity, as well as N-linked glycosylation sites and a putative signal sequence for translocation to the cell wall. The high degree of sequence similarity between these 33 XTH genes indicates that their proteins are likely to have analogous biochemical properties. However, the evolution of a large collection of related enzymes suggests the potential for diverse physiological functions.

Analysis has revealed that many members of the XTH gene family exhibit distinct organ- or tissue-specific expression profiles, but their exact functions remain largely unclear. To gain insight into their functional diversity we started the heterologous production in the yeast *Pichia pastoris* of 10 isozymes that are known to be predominantly expressed in roots.

The pPICZ α -expression vectors are constructed in a way so that the yeast cells secrete the XTH proteins into the medium. Media are analyzed by means of SDS-PAGE, western blotting and a specific fluorescent xyloglucan endotransglycosylase (XET) activity assay. When the protein mix contains the active heterologous XTH protein, the sample is subjected to ion exchange chromatography, followed by a size exclusion column for final purification. Once a proper purification level is reached, the proteins are tested on their enzymatic characteristics.

An Maris
Jean-Pierre Verbelen
Kris Vissenberg

Vissenberg Kris
University of Antwerp, Biology
Department, Groenenborgerlaan
171, 2020 Antwerp, BELGIUM
kris.vissenberg@ua.ac.be

Functional characterization of a recombinant *Selaginella* xyloglucan endotransglucosylase/hydrolase and its effect on cell wall mechanics

P 43

The plant cell wall is a solid, yet dynamic structure with a fibrous skeleton composed of cellulose microfibrils that are coated and tethered by xyloglucans. During cell elongation the cooperation of a set of enzyme families enables the wall to grow without losing its strength. Xyloglucan endotransglucosylase/hydrolases (XTHs) are believed to be involved in this 'wall loosening'. They break and rejoin xyloglucan chains, allowing the cellulose microfibrils to move apart driven by protoplasmic pressure.

Using a fluorescent *in vivo* assay we have located endotransglucosylase (XET)-action in the elongation zone of different vascular plants, down to the primitive spore plant *Selaginella*. With diverse PCR techniques based on conserved domains known in higher plant XTH-genes, a *Selaginella*-specific XTH-sequence has been isolated. *In silico* analysis revealed several features that are characteristic for XTH proteins, identifying the sequence as *SkXTH1*. The protein was heterologously expressed in *Pichia pastoris* to allow its functional analysis. SkXTH1 was found to be an XTH displaying XET activity over a broad pH and temperature range. XEH activity was not detected. The N-linked glycosylation and the presence of disulfide bridges seemed necessary for its 3-dimensional structure as interference resulted in the loss or strong reduction of XET activity. From results obtained by isoelectric focusing of *Selaginella* protein extracts combined with the fluorescent XET assay, SkXTH1 seems to be a member of a protein family in *Selaginella* of at least 4 XTH proteins displaying the endotransglucosylase (XET) activity.

We have studied the effect of SkXTH1 on the mechanical properties of the epidermis of growing onion bulb scales in constant-load experiments. The epidermis of onion bulb scales is an adequate model to study anisotropic extension. Its thick outer periclinal wall defines the tissue's mechanical strength and has a clearly defined mean or net cellulose microfibril orientation. Addition of SkXTH1 to heat-inactivated onion epidermal cell walls restored on average more than 70% of the protein-dependent creep transverse to the cellulose microfibrils that was lost during boiling. Parallel to the net cellulose orientation, no changes in extensibility was measured. These results suggest that XTH can act as a cell wall-loosening enzyme.

Vicky Van Sandt
Dmitry Souslov
Jean-Pierre Verbelen
Kris Vissenberg

Vissenberg Kris
University of Antwerp, Biology
Department, Groenenborgerlaan
171, 2020 Antwerp, BELGIUM
kris.vissenberg@ua.ac.be

Primary plant cell walls are a complex structure composed of various polysaccharides, phenolics and structural proteins. They are built by two polysaccharide networks, cellulose microfibrils cross-linked by xyloglucan chains, and pectins, both networks contributing to their functional properties. The xyloglucan-cellulose complex has been considered as the load-bearing structure of the primary walls, and thus the responsible for controlling the extension capacity of primary cell walls. Xyloglucan binds with high specificity and affinity to cellulose microfibrils, thereby producing a cell shape-determining cellulose-xyloglucan network and being responsible for controlling the rate of cell expansion. It consists of a linear β -(1-4)-linked D-glucan backbone that carries α -D-xylosyl, β -D-galactosyl-(1-2)- α -D-xylosyl, and α -L-fucosyl-(1-2)- β -D-galactosyl-(1-2)- α -D-xylosyl side chains attached to the OH-6 of β -glucosyl residues. Molecular modelling of the binding of xyloglucan to the surface of cellulose microfibrils suggested that a binding site is initiated by the trisaccharide sidechain (i.e., fucosylated) that flattens out an adjacent region of the xyloglucan backbone. Upon contacting a cellulose microfibril this region spreads by step-wise flattening of successive segments of the backbone. Changes in the structure of the side-chains of xyloglucan may modulate its interaction with microfibrils and then its functionality in cell walls.

Several exo-glycosidases are able to modify xyloglucan and have been found in *Arabidopsis* plant cell walls. An α -fucosidase gene (*AtFXGI*), whose product can hydrolyse fucosylated xyloglucan, and an α -xylosidase (*AtXYLI*) that cleave *t*-xylosyl residues attached to the glucosyl residue farthest from the reducing end of xyloglucan oligosaccharides have already been cloned by our group. Although numerous β -D-galactosidases have been reported in plants, few have been shown to be active against xyloglucan and not any gene responsible for this activity has been cloned. The *Arabidopsis* genome contains 12 genes whose products are predicted to be family 35 β -galactosidases and to be secreted to the wall. Several of these proteins may be involved in xyloglucan degradation. Finally, we used a β -glucosidase from *Tropaeolum* able to remove unsubstituted β -glucosyl residues at the non-reducing end of xyloglucan as a reference to search for its *Arabidopsis* functional homologs. Four putative β -glucosidases from family 3 closest to the one from *Tropaeolum* were selected as the best candidates to act on xyloglucan or its oligosaccharides.

Since the cellulose-xyloglucan network is the key determinant of wall extensibility and at some extent of the wall digestibility, the role of the above mentioned glycosidases on the functional properties of the walls is currently under study in our lab. *Arabidopsis thaliana* was used as experimental material. A preliminary screening on the glycosidases putatively involved in the xyloglucan metabolism was carried out studying the phenotype as well as the xyloglucan structure from the knockout mutants of each gene of interest. Those mutants with interesting characteristics in relation with xyloglucan metabolism were further studied at the biochemical and molecular level.

The significance of the four glycosidases acting on the xyloglucan side chains and how all these activities they are coordinated as well as their effect on the functionality of plant cell walls will be presented.

Natalia Iglesias
Gloria Revilla
María Teresa Herrera
Ignacio Zarra

Zarra Ignacio
Depto. Fisiología Vegetal
Fac. Biología
Univ. Santiago de Compostela
15782 Santiago de Compostela
Spain
bvzarra@usc.es

Author index

Acebes J. L. – León, Spain	P1, 71
Arroyo J. – Madrid, Spain	L8, 16
Azuma M. – Osaka, Japan	L24, 32
Bacic A. – Melbourne, Australia	L13, 21
Beauvais A. – Paris, France	P2, 72
Bermejo C. – Madrid, Spain	F1, 59
Berrin Jean-Guy – Marseille, France	P3,73
Boone C. – Toronto, Canada	L28, 37
Bottin A. – Castanet-Tolosan, France	P4, 74
Bulone V. – Stockholm, Sweden	L12, 20
Bussey H. – Montreal, Canada	L1, 9
Calderone R. – Washington DC, USA	L30, 39
Danchin E. – Marseille, France	F2,60
Douich A. – Mont Saint Aignan, France	P5, 75
Dupree P. – Cambridge, UK	L25, 34
Eijsink V. – As, Norway	L37, 46; P6, 76
Faber K. – Frederiksberg, Denmark	P7, 77
Farkas V. – Bratislava, Slovakia	P8, 78
Fasmer Hansen S. – Grenoble, France	P9, 79
Fincher G. – Adelaide, Australia	L26, 35
Firon A. – Paris, France	P10, 80
Fontaine T. – Paris, France	P11, 81
François J.M. – Toulouse, France	L29, 38
Fry S. – Edinburgh, UK	L22, 30
Fugelstad J. – Stockholm, Sweden	P12, 82
Fukuda H. – Tokyo, Japan	L33, 42
Gastebois A. – Paris, France	F3, 61
Gille S. – East Lansing, MI, USA	P13, 83
Gomez A. – Salamanca, Spain	F4, 62
Gonneau M. – Versailles, France	P14, 84
Gow N.A.R. – Aberdeen, UK	L20, 28
Guillon F. – Nantes, France	P15, 85
Hahn M. – Athens, USA	L4, 12
Helbert W. – Roscoff, France	L44, 53
Henrissat B. – Marseille, France	L11, 19
Höfte H. – Versailles, France	L31, 40
Horiuchi H. – Tokyo, Japan	L23, 31
Hurtado Guerrero R. – Dundee, Scotland, UK	F5, 63
Immerzeel P. – Golm Germany	P16, 87
Keegstra K. – East Lansing, USA	L2, 10
Klis F. – Amsterdam, The Netherlands	L5, 13
Knox J.P. – Leeds, UK	L6, 14

Kogan G. – Bratislava, Slovakia	P17, 87
Konomi M. – Tokyo, Japan	L9, 17
Kumar V. – Mysore, India	F6, 64
Lahaye M. – Nantes, France	P18, 88
Latgé J.-P. – Paris, France	L3, 11
Lerouge P. – Villenave d'Ornon, France	P19, 89
Lerouxel O. – Versailles, France	F7, 65
Linghuo J. – Beijing, China	P20, 90
López M. – Grenoble, France	P21, 91
Martinez-Ruboco F. – Frankfurt, Germany	P22, 92
Marty I. – Avignon, France	P23, 93; P24, 94
McQueen-Mason S. – York, UK	L42, 51
Merino S. – Copenhagen, Denmark	L43, 52
Merzendorfer H. – Osnabrück, Germany	L36, 45
Meulewaeter F. – Ghent, Belgium	L45, 54
Mohnen D. – Athens, GA, USA	L18, 26
Moïse A. – Avignon, France	P25, 95
Molina M. – Madrid, Spain	L34, 43
Morris V. – Norwich, UK	L10, 18
Mouyna I. – Paris, France	P26, 96
Negishi T. – Chiba, Japan	P27, 97
Nogami S. – Chiba, Japan	P28, 98
Øbro J. – København Denmark	P29, 99
Ohno N. – Tokyo, Japan	L28, 47; P30, 100
Ohya Y. – Tokyo, Japan	L27, 36
Oka T. – Ibaraki, Japan	P31, 101
Pauly M. – Golm, Germany	L15, 23
Perlin D. – Newark, USA	L41, 50
Persson S. – Stanford, USA	L17, 25
Poulain D. – Lille, France	L39, 48
Ragni E. – Milano, Italy	F8, 66
Ralet M.C. – Nantes, France	L7, 15
Ribas J.C. – Salamanca, Spain	L14, 22
Robic A. – Nantes, France	P32, 102
Romano J. – Tel-Aviv, Israel	F9, 67
Roncero C. – Salamanca, Spain	L21, 29
Sado P.-E. – Nantes, France	P33, 103
Schott A. – Heidelberg, Germany	P34, 104
Seidl V. – Edinburgh, Scotland, UK	F10, 68
Shimma Y. – Aist, Japan	P35, 105
Shimoi H. – Higashi-Hiroshima, Japan	L40, 49
Soulié M.-C. – Paris, France	P36, 106
Steinbach W. – Durham, NC, USA	L48, 57
Strahl S. – Heidelberg, Germany	L47, 56
Terrak M. – Liege, Belgium	L35, 44
Turner S. – Manchester, UK	L19, 27
Vaaje-Kolstad G. – Ås, Norway	P37, 107; P38, 108.
Van Aalten D. – Dundee, UK	L46, 55
Van Orden J. – Antwerp, Belgium	P39, 109
Vazquez de Aldana C.R. – Salamanca, Spain	L16, 24
Verherbruggen Y. – Leeds, UK	P40, 110
Vicens A. – Montpellier, France	P41, 111
Vissenberg K. – Antwerp, Belgium	P42, 112; P43, 113
Vos A. – Amsterdam, The Netherlands	F11, 69
Wasteneys B.G. – Vancouver, Canada	L32, 41
Zarra I. – Santiago de Compostela, Spain	P44, 114