Instituto Juan March de Estudios e Investigaciones

101 CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Dynamics of the Plant Extracellular Matrix

Organized by

K. Roberts and P. Vera

A. Bacic
A-M. Boudet
N. C. Carpita
D. Cosgrove
S. R. Cutler
G. De Lorenzo
D. P. Delmer
C. Domingo
H. Höfte
B. Keller
B. D. Kohorn
C. Lamb
M. C. McCann
J. B. Nasrallah
P. Puigdomènech
R. S. Quatrano
J. Ralph
K. Roberts
L. G. Smith
P. Vera
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Instituto Juan March (Madrid)

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Introduction

P. Vera and K. Roberts

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Plant cells have evolved and retained at every stage of the cell cycle a complex extracellular matrix (ECM). The acquisition of this distinct ECM compartment is central to an explanation of the differences between plant and animal cell development. Because of its presence, plants have evolved a distinctly different mode of multicellular construction characterized by incomplete cell cleavage, continuous cytoplasmic connections, and absence of cell motility during morphogenesis. Therefore, the ECM of a differentiating plant cell is specifically adapted to the particular function of that cell type. Moreover, developmental events and exposure to any of a number of abiotic and biotic stresses further increase the compositional and structural variation of the ECM.

The plant ECM also plays important roles during pathogenesis because it is the place where the first host-pathogen interaction and recognition events take place, and where rapid cellular responses are executed in the form of local modification and signal transmission that set in motion subsequent transcriptional activation of defense-related genes.

The cell wall, by far the major component of the plant ECM, is also very dynamic and helps confer unique and distinctive features to plant cells. The construction and architecture of the cell wall varies in an orderly manner in accordance with the developmental stages of cells. This pliable character of cell walls is a direct consequence of changes in the proportion and degree of assembly of various structural polysaccharides, including cellulose microfibrils, pectin, and hemicellulose polymers. Also, differences between cell walls of defined cell types originate as a consequence of deposition of more specific polymers with defined functions (e.g., lignin, which serves to harden and stiffen the walls of tracheary elements).

Plant cell walls also contain structural proteins that participate in the flexible integration of environmental responses within plastic developmental programs. The genes encoding these structural proteins are tightly regulated, and their expression shows cell type specificity and developmental regulation. Collectively, it seems that plants have evolved regulatory mechanisms allowing genes encoding structural cell wall proteins to be expressed specifically and in accord with the different functions exerted by the different tissues. The expression of genes encoding some of these cell wall proteins can be altered by different cues (e.g., wounding, elicitor treatment, or pathogen attack), and the deposition of the encoded proteins in the cell wall results in alterations of the functional properties of this compartment.

Since it is increasingly apparent that cell-cell and cell-matrix interactions exert important influences upon gene expression and the development of cellular phenotypes in plants, the characterization of new molecules and signalling events in the plant ECM that could eventually participate in certain critical features of the plant lifestyle, either under normal or pathogenesis-related situations, are relevant to gain understanding of the importance of this extracellular cell compartment in plants.

This workshop brought together scientists actively engaged in the characterization and understanding of the plant ECM using different systems and with physiological, biochemical, molecular and cellular biology as well as genetic approaches. The workshop provided a unique opportunity for the participants to exchange and share information, to review and discuss new approaches, and to propose directions for future research.

Pablo Vera and Keith Roberts

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Session 1: Early signalling events

Chair: Keith Roberts

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Signaling at the pollen-stigma interface

June B. Nasrallah, Ram Dixit, Christel R. Schopfer, and Mikhail E. Nasrallah

Members of the crucifer family (the Brassicaceae) have evolved an elaborate genetic self-incompatibility system that controls mating in natural populations of nearly half the species belonging to this family. This system acts within minutes after the initial contact between pollen and stigma epidermal cell and inhibits hydration and germination of genetically related pollen. Specificity in this self-incompatibility is governed by a molecularly complex Mendelian locus, the *S* locus. The highly specific nature of pollen-pistil interactions underlying the self-incompatibility (SI) response and the importance of genetic self-incompatibility systems to flowering plant evolution have made SI systems attractive models for the study of intercellular recognition in plants. More specifically, the SI system of crucifers, and that of Brassica in particular, which is now known to be based on the activity of an *S* locus encoded transmembrane receptor protein kinase, has emerged as an excellent model system for investigating transmembrane receptor-mediated cell-to-cell signaling in plants. Indeed, the fact that molecular investigations into the basis of SI in Brassica are strongly rooted in the well-characterized genetic basis of SI have made the *S* locus receptor kinase (SRK) one of only a few plant receptor kinases with a known biological function.

Molecular studies of the Brassica SI system carried out in the early 1980's identified a highly polymorphic *S* locus gene, the S-locus gycoprotein (SLG) gene (Nasrallah et al., 1985; 1987) which encodes a secreted glycoprotein that accumulates specifically in the wall of cells of the stigma epidermis. This *SLG* gene provided a molecular marker for the Brassica *S* locus and a starting point for the detailed structural and functional characterization of the locus. Furthermore, the *SLG* gene, with its 10-12 cysteine residues arrayed in a precise order, has become the canonical member of a plant superfamily of genes, known as the *S* gene family, all members of which contain an *SLG*-like or *S* domain. This gene family includes another *S* locus-encoded and highly polymorphic gene, *SRK* (Stein et al., 1991), as well as a number of transmembrane protein kinases [such as *ZmpkI* which is expressed in maize roots (Walker and Zhang, 1990), the Arabidopsis *ARK* genes which are expressed in a variety of plant tissues (Tobias et al., 1992; Dwyer et al., 1994) and *RLK* genes (Walker, 1993)] and secreted glycoproteins [such as the Brassica *SLR1* gene (Lalonde et al., 1989), the Arabidopsis *AtS1* gene (Dwyer et al., 1992; 1994), the carrot *EP1* gene (van Engelen et al., 1993)]. Except for *SRK*, the biological functions of these *S* family genes are not known. However, because of their *SLG*-like domains, they are assumed to function in intercellular communication in different phases of plant development.

Based on molecular and genetic studies, the Brassica SI response is now known to require the activity of molecules encoded by the *S* locus and embedded in the extracellular matrices of the stigma epidermis and pollen grain. Accumulating evidence from genetic and transgenic studies implicates the highly polymorphic *SLG/SRK* gene pair as the stigmatic determinants of specificity in the SI response. Whereas the transmembrane SRK receptor kinase is thought to be the primary stigmatic determinant of specificity in SI, SLG appears to function, at least in part, by stabilizing SRK and allowing it to accumulate to wild type levels in the stigma, possibly by facilitating its proper folding (R. Dixit, M.E. Nasrallah, and J.B. Nasrallah, submitted for publication). Our working model of SI is that the SRK protein kinase is activated by contact between a stigma epidermal cell and self pollen (Nasrallah et al., 1994).

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By phosphorylating intracellular substrates, the SRK protein would couple the initial molecular recognition events at the papillar cell-pollen interface to the signal transduction chain that leads ultimately to pollen rejection. This model postulates the existence of a pollen-borne ligand for SRK, which would be highly polymorphic and also encoded within the *S* locus complex. This extracellular ligand would activate the receptor in an *S* haplotype-specific manner, thus providing the specificity inherent in "self" recognition.

Until recently, this postulated pollen determinant of SI has remained elusive. However, we have now identified a highly polymorphic, anther-expressed, *S*-locus linked gene designated *SCR* (*S* locus cysteine-rich protein gene), which fulfills the requirements for the pollen determinant (Schopfer et al., 1999). Loss-of-function and gain-of-function studies will be described which demonstrate that the SCR gene product is necessary and sufficient for determining pollen self-incompatibility specificity, possibly by acting as a ligand for the stigmatic receptor.

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Remodeling the extracellular matrix during plant development: the role of receptor protein kinases in floral abscission

John C. Walker, Tsung-Luo Jinn, Julie M. Stone*

Division of Biological Sciences, University of Missouri, Columbia, MO 65211, *Department of Molecular Biology, Massachusetts General Hospital and Department of Genetics, Harvard Medical School, Boston, MA 02114

All organisms must respond to intrinsic developmental as well as externally generated cues to persist. One predominant mechanism of signal transduction involves reversible protein phosphorylation to accomplish both innate and adaptive responses to these signals. In animals, receptor protein kinases (RPKs), characterized by large extracellular domains that function in ligand recognition, a single membrane-spanning segment, and a cytoplasmic protein kinase catalytic domain, are known to play diverse roles in development and stress responses (van der Geer et al., 1994). While less is known about these types of molecules in plants, plant receptor-like kinases (RLKs), so denoted because no ligands have been definitively identified yet, constitute a large percentage of the Arabidopsis genome, with current estimates of 1 to 2 % of genes encoding RLKs (JCW, unpublished data). In contrast to the majority of animal RPKs, which are tyrosine-specific protein kinases (with the notable exception of the TGFβ family), the plant RLK genes appear to encode serine/threonine-specific kinases. Despite the prevalence of RLKs in plants, the functions of only a few have been clearly established (reviewed in (Becraft, 1998; Lease et al., 1998). RLKs have been implicated in prevention of self-pollination, pathogen response, hormone perception and signaling, and plant development. The first RLK identified in plants, maize *ZmPK1*, displays homology in its putative ligand-binding extracellular domains to the S-locus glycoproteins (SLGs) required for sporophytic self-incompatibility in Brassica species (Walker and Zhang, 1990). A few of these so-called S-domain RLKs (SRKs) from Brassica were subsequently demonstrated to be required, in conjunction with their cognate SLGs, to prevent self-pollination (Nasrallah et al., 1994). The expression patterns of other S-domain RLKs, however, suggest that these proteins are involved in other processes as well (Tobias et al., 1992). The majority of RLKs with established functions revealed genetically by mutant phenotypes are involved in plant developmental processes. In maize, mutations in an RLK that exhibits some homology to tumor necrosis factor in its extracellular domain, *CRINKLY4*, causes a dramatic phenotype in which epidermal cells fail to differentiate properly (Becraft et al., 1996). Another class of RLKs designated by the presence of tandemly repeated leucine rich domains in their extracellular domains, the leucine rich repeat (LRR) RLKs, are also important in plant development. *CLAVATA1*, has been shown to control maintenance of the balance between cell proliferation and differentiation at the shoot apical meristem (Clark et al., 1997), whereas *ERECTA* controls organ shape (Torii et al., 1996). Perception and signal transduction of newly discovered plant hormones, brassinosteroids, is dependent on yet another LRR-RLK, Bri1 (Altmann, 1998; Li and Chory, 1997). LRR-RLKs are also important in other pathways unrelated to development. For example, the *Xa21* gene from rice functions as a plant disease resistance gene (Song et al., 1995). WAK1, a cell wall-associated receptor kinase with epidermal growth factor (EGF) repeats in its extracellular domain may mediate cell wall to cytoplasm signaling, is transcriptionally induced by pathogen infection (thereby designated a pathogenesis-related (PR) gene), and may be required for plant survival during the pathogen response (He et al., 1998). This diversity in function of plant RLKs is likely to extend to the myriad of RLKs whose function remain elusive.

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An integral element of plant development is the abscission of organ systems to accomplish shedding of senescing tissues, damaged organs and release of ripened fruits. Abscission zones are characterized by discrete bands of cytoplasmically dense cells, which undergo cell enlargement, disintegration of the middle lamella between cells, and subsequent differentiation into scar tissue. The abscission process is accompanied by the induction of genes encoding cell wall and pectin degrading enzymes (Bleecker and Patterson, 1997; Gonzalez-Carranza et al., 1998)

Our current studies have provided evidence that the Arabidopsis leucine rich repeat receptor kinase *Haesa* (formerly named RLK5) controls floral organ abscission. Native Haesa protein was detected it in the plasma membrane-enriched fraction and shown to possess serine/threonine protein kinase activity. *Haesa* is expressed at the base of the petioles and pedicels as well as in abscission zones of the floral organs, as assessed by both a *Haesa* promoter::β-glucuronidase reporter gene in transgenic plants and by in situ RNA hybridization. To assign a function for *Haesa* in abscission zones, transgenic plants expressing a constitutive antisense Haesa construct were generated, and abscission of floral organs were scored. Antisense-suppressed lines showed varying levels of suppression of the Haesa. The level of Haesa protein is inversely correlated with the degree of delay of floral organ abscission. This phenotype is due to the presence of the transgene, as individuals from segregating populations which had lost the transgene exhibit normal floral organ abscission. These results clearly demonstrate a role for Haesa receptor kinase in floral organ abscission, and afford new avenues for further analysis of the abscission process in plants.

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CELL WALL ASSOCIATED, RECEPTOR PROTEIN KINASES

Tanya Wagner, Cathy Anderson, and Bruce D. Kohorn.

DCMB/Botany, Duke University, Durham, NC USA 27708. kohorn@duke.edu

The plant cell wall is a dynamic structure that provides strength at the same time as flexibility to allow cell growth and response to external and internal stimuli. Physical connections between higher plant cell walls and the plasma membrane have been identified visually, but the molecules involved in the contact and signaling have only recently been suggested. We describe a family of 5 *Arabidopsis thaliana* protein kinases, designated WAKs for Wall Associated Kinases, that possibly provide a physical and a signaling continuum between the cell wall and the cytoplasm. All five WAKs contain a cytoplasmic protein kinase domain and span the plasma membrane to extend into the cell wall a domain that contains cysteine rich repeats. WAKs are tightly associated with the cell wall as they fractionate with insoluble material when plant tissue is ground in a variety of buffers and detergents, although WAKs can be released from the cell wall into a microsomal fraction by digestion of cells with pectinase. Immunocytochemistry confirms that WAKs are associated with both the cell wall and plasma membrane.

The five WAKs diverge significantly in the sequences of the extracellular domain, and thus may be functionally distinct. We wished to determine if Waks are expressed in the same or different cell type to predict if WAKs could possibly associate with each other. Based on Wak promoter – GUS fusions expressed in Arabidopsis, on in situ hybridization to sections of Arabidopsis and on Northern blots, we determined that each Wak gene is expressed in distinct but sometimes similar organs. Wak1 and Wak 2 are transcribed in a distinctive developmentaly regulated pattern at junctions of organs, the root apical meristem, and the cotyledon and leaf margins, somewhat reminiscent of ABA regulated genes. Wak1 and 2 expression are also induced by wounding and INA treatment, and this is consistent with our previously published work showing a requirement for WAK expression during the pathogen response. Thus WAK1 and WAK2 function may be associated with tissues or cells undergoing compression or expansion. Wak 3 expression is different in that it is restricted to apparently randomly spaced small clumps of cells in leaves and petioles and this expression is enhanced by INA. Wak4 appears to be restricted to very early seedlings and siliques. Wak5 expression is minimal relative to the other Waks and is barely detectable in leaves. Thus we conclude that there is overlapping expression and the Wak isoforms could bind to each other within one cell. Their expression patterns are suggestive of events related to cell expansion, and in addition with a role in the cellular response to pathogens.

We have generated a number of Arabidopsis lines that express antisense Wak alleles, and ones that separately have T-DNA insertions into the locus on chromosome 1 that contains the tandemly repeated Wak genes. These experiments provide results that are also consistent with a role of the WAKs in both cell expansion and separately pathogenesis.

While WAKs are tightly bound to the cell wall and can be released by pectinase treatment, it is possible that the extracytoplasmic domains bind protein ligands. Two hybrid analysis in yeast has identified a number of candidates for these ligands, and preliminary evidence suggests that each WAK may have interactions with different proteins in the cell wall, perhaps consistent with their sometimes cell type specific expression. The WAK1 cell wall domain appears to bind one class of Glycine Rich Protein (GRP) long thought to be a structural

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component of cell walls. Evidence suggests that this binding is activating. WAK2, on the other hand, has one extracellular domain that can bind a protein involved in carbohydrate synthesis, and another domain that binds an enzyme associated with the lignin biosynthesis pathway. We are in the process of testing these interactions genetically and biochemically.

Thus we find that WAK expression is distributed throughout the plant but each family member may have a distinct function. Genetic analysis implicates WAKs in some role during cell expansion, and in the maintenance of cell viability during a response to pathogens. How these two roles are related remains to be determined, and how the potential ligands for the various WAKs mediate these events is an obvious future direction.

WAKs continue to serve as candidates for a physical continuum between the cell wall and the cytoplasm and do indeed play a vital role in plant growth and development.

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Cell surface events in disease resistance signalling

Chris Lamb

John Innes Centre, Norwich NR4 7UH, UK

The extracellular matrix is the location for a complex array of signalling events associated with the perception of pathogen attack and activation of disease resistance mechanisms. The products of a number of disease resistance genes, e.g. Cf9 and Xa21, are located at the plasma membrane with putative ligand binding domains involved in perception of pathogen avirulence signals projecting into the extracellular matrix. In addition, emerging evidence indicates that the extracellular matrix is the site of several key events in downstream signalling pathways as well as the site of action of many effector defence mechanisms such as those involving some PR proteins, deposition and cross-linking of cell wall structural proteins and elaboration of lignin and lignin-like polymers. Novel signalling events include:

- Generation of reactive oxygen intermediates at the cell surface, mediated at least in part by a plasma membrane NADPH oxidase. These reactive oxygen intermediates drive wall cross-linking and signal gene activation, the hypersensitive response and induction of systemic immunity.
- By screening for a mutant that retains the ability to mount a local hypersensitive resistance response but is defective in the induction of systemic induced immunity we have cloned *DIR1* encoding a small cysteine-rich apoplastic lipid transfer protein. The DIR1 lipid transfer protein appears to function in the perception or transduction of the mobile signal for systemic acquired resistance.
- Using an activation tagging approach we have identified two related genes, *CDR1* and *CDS1* which when overexpressed lead respectively to constitutive disease resistance to normally virulent bacterial pathogens, and constitutive disease susceptibility reflected in a loss of gene-for-gene mediated resistance to normally avirulent pathogens as well as enhanced susceptibility to virulent pathogens. *CDR1* and *CDS1* encode related apoplastic aspartate proteinases and induced expression of *CDR1* apparently leads to the generation of a small, mobile signal peptide for systemic induction of PR protein genes and systemic acquired resistance.

These new data indicate that the extracellular matrix plays a key role in the signal networks involved in transducing pathogen perception into the concerted activation of disease resistance mechanisms for local defence against the invading pathogen and systemic induction of immunity to subsequent attack.

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Session 2: ECM remodelling and signalling

Chair: Pere Puigdomènech

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PGIPs: LRR cell wall proteins involved in defense and development

G. De Lorenzo, F. Cervone, D. Bellincampi, C. Capodicasa, C. Caprari, S. Ferrari, P. Giuli, M. Laurenzi , B. Mattei, A. Raiola, G. Salvi, F. Sicilia, D. Vairo, O. Zabotina

Dipartimento di Biologia Vegetale, Università di Roma "La Sapienza", Rome, Italy

Leucine-rich repeat (LRR) proteins represent a large family of molecules with different functions and cellular locations present in bacteria, fungi, animals and plants (Kobe, 1996). Polygalacturonase-inhibiting proteins (PGIPs), present in the cell wall of many plants, belong to the LRR protein family and show a close structural relationship to several resistance (*R*) gene products (Hammond-Kosack and Jones, 1997). PGIPs interact with endopolygalacturonases from phytopathogenic fungi with subsequent formation of elicitor-active oligogalacturonides, and play a role in defense (De Lorenzo et al. 1997; Leckie et al. 1999). To learn more about the function of PGIP, we have obtained tomato plants expressing a PGIP-1 from *Phaseolus vulgaris*. The transgenic plants exhibit interesting features in terms of morphological and physiological characteristics. Composition analysis of the cell walls of the transgenic plants shows that they contain a pectin fraction with a methylation degree much higher than the corresponding fractions isolated from the untransformed plants. This finding is consistent with results showing that PGIP *in vitro* interacts with methylated pectins better than with non methylated homogalacturonans. *In vivo*, PGIP binding to methylated pectins is likely to protect from demethylation, thus affecting the cell wall extensibility and ultimately the growth of the plant. Our results show that in plants, like in animals (Lemaitre et al. 1996), a single LRR protein plays a role in both defense and development, and support the notion that the dinamics of the extracellular matrix strongly influence the growth and development of plants.

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Extracellular Plant Subtilisin-like Proteases

Pablo Vera, Lucía Jordá

IBMCP. Universidad Politécnica-CSIC. Camino de Vera s/n.
46022-Valencia. Spain. vera@ibmcp.upv.es

Serine proteinases are of extremely widespread occurrence in living organisms. One of the largest families of this type of enzymes is that represented by the subtilisin-like (subtilase) family (EC 3.4.21.14). This clan represent an ancient family of proteins with homologues in such diverse organisms as Archae, bacteria, fungi, yeast, and higher eukaryotes and are classified into five families: subtilisin, thermitase, kexin, pyrolysin, proteinase K and lantibiotic peptidases (Siezen and Leunissen, 1997). Although more than 200 subtilisin-like enzymes are presently known, our information on the existence and role of this type of protease in plants is still scant.

We will report here the structure and genomic organization of six transcriptionally active genes encoding extracellular subtilisin-like proteases from tomato plants. The six members, named as P69A to P69F, are organized in clusters and are arranged in a tandem form. mRNA expression analysis as well as studies of transgenic plants transformed with promoter-reporter gene fusions for each of these genes revealed that they are tightly regulated by developmental and pathogenic signals, and in a tissue specific manner. From a comparison of all the P69 amino acid sequences, gene structure, expression profiles, and clustered organization a working model for P69 gene family evolution will be discussed.

Furthermore, since the P69-like enzymes are actively secreted outside the cell where they accumulate, we suggest that this type of proteinases may play critical roles in the remodeling or reprogramming of the extracellular matrix (ECM) during normal cell growth or during processes characteristic of pathogen-afflicted plants, and we will present approaches for the identification of function and isolation of proteolytic substrates.

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A NEW FAMILY OF *ARABIDOPSIS* PLASMA MEMBRANE RECEPTORS

Valerie Laval, Matthieu Chabannes, Marguerite Carriere, Jean-Philippe Galaud, and Rafaël Pont-Lezica

Wall to membrane attachments have been observed in plant cells and these cells adhesions may play a role in perception of mechanical signals. The involvement of extracellular matrix-plasmalemma-cytoskeleton (ECM-PM-CTK) continuum as mechanochemical transducer in animals is now well established (Eldelman et al 1993). Recent evidences suggest that plant cells may also have an ECM-PM-CTK continuum that functionally parallels that of animal system (Wyatt and Carpita, 1993). In *Arabidopsis thaliana*, wall to membrane attachments are arg-gly-asp (RGD) peptide sequence dependent. Several experiments clearly show that RGD sequence play a role in gravity sensing (Wayne et al, 1992) and cell multiplication (Schindler et al, 1989). In our laboratory, purified plasma membrane from Arabidopsis cells exhibits specific binding sites for RGD peptide. In addition, the binding was strongly inhibited by trypsin treatment, suggesting the protein nature of the RGD receptor. In animal system, the best characterized membrane receptors that recognize the RGD motif are integrins.

In order to clone an *Arabidopsis* cDNA, encoding an integrin like protein, an oligoscreening was performed. The oligonucleotide used was defined from a well conserved cytoplasmic domain of integrin beta subnit. One clone (AtELP-1) was isolated. It encodes a protein of 70 KDa presenting a single transmembrane domain, a short Cter region, a long Nter region and several structural analogies with integrin beta subnit.

Southern blot experiments and databank searches were performed and showed that AtELP-1 is a member of a multigenic family containing at least seven members (AtELP-1, 2a, 2b, 3, 4,5 and 6) located on chromosomes I, II and IV.

Northern blot analysis were performed on severals organs and after differents biotic and abiotic stresses with specific probes corresponding to AtELP-1, 2 and 3. The three mRNAs are accumulated in roots, fruits, stem and leaves and genes are over-expressed in response to water and osmotic stresses.

The localization of the AtELP-1 protein was carried-out. Two immunogenes regions were defined on AtELP-1 in the long N-ter region. The corresponding peptides were synthetized and used to rabbits immunization. Antibodies were tested on purified membrane fractions (plasmalemma, reticulum and tonoplast). AtELP-1 protein was mainly found in plasmalemma fraction. In vivo localization was also performed on sunflower protoplast and the immunoflorescent labelling was observed on plasmalemma indicating that the protein is a type I integral plasmamembrane protein.

Conclusions : **A new family of membrane protein receptors localized on plasmalemma and showing structural analogies with animal integrin was found in** ***Arabidopsis*****.** For the moment, neither a biological nor a physiological function was clearly established for the protein. In this way, sense and antisense transgenic plants were obtained and the search for ligands is actually in course.

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Arabinogalactan-proteins : key signalling molecule at the cell surface?

Bacic A[1,2], Oxley D[1], Gilson P[1], Qu, Y[1], Gaspar Y[1], Schultz C[2]

1. Plant Cell Biology Research Centre, School of Botany, University of Melbourne, Parkville 3052 Australia
2. CRC for Bioproducts, School of Botany, University of Melbourne, Parkville 3052 Australia

Arabinogalactan-proteins (AGPs), a class of hydroxyproline-rich proteoglycans, have been implicated in a number of key processes critical to plant growth and development including markers of cell fate, induction of somatic embryogenesis and cell proliferation/expansion. The dilemma is to explain how this structurally complex family of molecules can regulate such a diversity of cellular processes. The cloning of the genes encoding AGP protein backbones (for reviews see Nothnagel, 1997. Int. Rev. Cytol. **174**:195; Schultz *et al*, 1998, Trends in Plant Sci. **3**:426) and the discovery of GPI anchors on "classical" AGPs (Youl *et al*, 1998. P.N.A.S., USA. **95**:7921; Svetek *et al*, 1999. J. Biol. Chem. **274**:14724; Sherrier *et al*, 1999. Electrophoresis **20**:2027; Oxley and Bacic, 1999. PNAS [submitted]) have provided avenues to examine their mode of action at the molecular level. The functions of glycosylphosphatidylinositol (GPI)-anchored molecules and of cell surface proteoglycans are well documented in other eukaryotic systems and offer a framework to begin to design experimental approaches to establish the mode of action of AGPs. Three groups of genes involved in AGP synthesis/processing offer potential targets to disrupt AGP function, namely genes encoding (1) protein backbones ("classical"/"non-classical"), (2) GPI-anchor biosynthetic/processing enzymes and (3) glycosyltransferases responsible for elaborating the AG structures. Furthermore, identification of molecules interacting with AGPs at the cell surface will also contribute to our understanding of their mode of action as potential signalling molecules. We will discuss recent progress in each of these areas.

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AGPs as signalling molecules in plant embryogenesis

Arjon J. van Hengel*, Zewdie Tadesse, Ab van Kammen and Sacco C. de Vries

Laboratory of Molecular Biology, Agricultural University Wageningen, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

* Present address: Department of Cell Biology, John Innes Centre, Colney Lane Norwich NR4 7UH, UK

Zygotic plant embryos pass through characteristic globular, heart and torpedo shaped stages before being desiccated in the mature seed. Somatic embryos pass through the same stages, but lack a desiccation phase and develop directly into plantlets. The carrot cell line *ts11* is a temperature sensitive variant in which somatic embryogenesis is arrested in the globular stage at non-permissive temperatures (Lo Schiavo et al., 1990). The phenotype of *ts11* is pleiotropic and may result from a secretory defect (Baldan et al., 1997). Remarkably, addition of a carrot class IV endochitinase (designated EP3) allows further development of the embryos into plantlets (De Jong et al., 1992; Kragh et al. 1996). Endochitinases hydrolyse ß (1-4) linkages between at least three adjacent N-acetyl-D-glucosaminyl (GlcNAc) residues in chitin polymers (Molano et al., 1979; Usui et al., 1990). Plant cell walls are devoid of chitin polymers, suggesting that plants contain other, uncharacterised targets for chitinase activity.
AGPs are proteoglycans that can occur attached to membranes or in cell walls or are secreted into the culture medium, from which they can be selectively precipitated with 1,3,5-tri-(*p*-glycosyloxyphenylazo)-2,4,6-trihydroxybenzenes or Yariv reagent (Kreuger and Van Holst, 1993). One approach used to study the role of AGPs is the direct addition of these molecules to suspension cultures. The addition of mature carrot seed AGPs to a weakly embryogenic cell line has been shown to cause reinitiation of embryogenic cell formation (Kreuger and Van Holst, 1993) . This resulted in the presence of clusters of small cytoplasmic rapidly dividing cells, in line with the opposite effects reported for the addition of Yariv reagent to rose cells (Serpe and Nothnagel, 1994) . More recently, compounds were isolated from carrot cell conditioned medium, based on the ability to bind to JIM8, a monoclonal antibody that recognizes AGP epitopes. Addition of these JIM8 epitope containing compounds, to a JIM8(-) cell population, that was found to be unable to form somatic embryos, allowed the formation of somatic embryos (McCabe et al., 1997) . Therefore, a role for the JIM8 epitope containing AGPs in cell-cell communication during somatic embryogenesis was proposed.
AGPs consist of more than 90% carbohydrate with arabinosyl and galactosyl residues as the major sugar constituents (Nothnagel, 1997). In AGPs the polysaccharides that are attached to the protein core have many highly complex side chains with different terminal residues, while the monosaccharides in AGPs can be linked in different fashions (Mollard and Joseleau, 1994). The occurrence of oligomers of GlcNAc has not been reported (Baldwin et al., 1993; Komalavilas et al., 1991; Mollard and Joseleau, 1994; Serpe and Nothnagel, 1994; Serpe and Nothnagel, 1996; Smallwood et al., 1996; Van Holst et al., 1981), implying that it is neither a common nor an abundant constituent of AGPs. Because endochitinases (Van Hengel et al., 1998) and AGPs are found in immature seeds as well as in the culture medium, and both are active in embryogenesis, we investigated whether AGPs contain cleavage sites for endochitinases. We developed a new bioassay for demonstrating the effect of EP3

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endochitinases on wild type carrot cells, phenocopying the endochitinase-mediated increase in embryogenic cell clusters as observed in the *ts11* variant (De Jong et al., 1993). This bioassay was subsequently used to identifiy GlcNac-containing immature carrot seed AGPs as potent inducers of somatic embryogenesis (van Hengel et al., manuscript submitted)

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Session 3: The interface for cell determination and cellular interactions

Chair: Chris Lamb

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The Cell Wall and Cell-cell interactions

Professor Keith Roberts

John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, UK
(email: keith.roberts@bbsrc.ac.uk)

Individual plant cells are firmly cemented to their neighbours on all sides, connected in most cases only by plasmodesmata that come and go. Somehow, each cell knows who its neighbours are and can use this positional information to make key decisions about its future fate. Downstream, continuing cell-cell interactions help direct features of cell differentiation including cell shaping, polarity and local wall deposition. Later still, similar signalling at a local level will influence cell death. In plants, sadly, we know relatively little about the nature of the cell signalling events which underlie these issues of positional information, cell fate determination and size determination. I want to use this talk to explore some of the problems that the plant extracellular matrix creates for cell-cell communication, and to set this against emerging paradigms in the animal field. I will illustrate some of the issues with examples from my lab as well as others. These examples will include thoughts on the dynamic nature of plasmodesmata and their role in local signalling. I shall talk about the range of cell surface molecules that we know about, that could be involved in signalling, and the ways in which the activity inside one cell can be mirrored in its neighbour, for example through cytoskeletal activity. Cell death, either as a random natural event or artificially induced by laser ablation, has dramatic consequences on the subsequent behaviour of neighbouring cells. The activity of cells to grow into, and occupy, the spaces left by corpses is an intriguing insight into cell-cell signalling, and some thoughts on the role of the cell wall in these events will be presented. An important feature of cellular interactions is the degree to which they selectively let go and create intercellular spaces. This will be discussed in the context of local controls on cell growth.

Lastly I shall consider the possible role of arabinogalactan-proteins in local cell-cell interactions, and will describe some of our recent work to understand the molecular nature of the complex glycoproteins.

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Epidermal Cell Morphogenesis in the Maize Leaf: the Roles of *Brick* Genes

Laurie G. Smith, Mary Frank and Challe Woosley

Plant cells acquire a wide variety of shapes during development that are tailored to their specialized functions. In both monocots and dicots, leaf epidermal cells become lobed as they expand to create an interlocking network that forms the interface between shoot organs and the surrounding environment. We have identified 3 "*Brick*" genes in maize that are required for epidermal cell lobing without being required for cell expansion per se. We are studying how the cytoskeleton and cell wall contribute to the lobing of epidermal cells, and what features of this process are affected by *brk* mutations. As previously described for other species (1), we have found that banding of cortical microtubules is associated with the initiation of cell lobing, suggesting that non-uniform deposition of cellulose microfibrils contributes to lobe formation, but this feature of cell lobing is not affected in *brk* mutants. However, deposition patterns of certain cell wall components are altered in *brk* mutants, suggesting important roles for these components in the formation of specialized cell shapes. Although mutations in all three *Brk* genes produce essentially the same phenotypes, analysis of genetic mosaics demonstrates that these genes do not have equivalent functions: *Brk2* acts cell autonomously, while *Brk1* acts non cell-autonomously. These results suggest a role for intercellular signalling in the control of cell wall assembly as it relates to cell morphogenesis.

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A role for expansins in leaf abscission

Simon McQueen-Mason

Dept. of Biology. The Plant Laboratory, The University of York,
P.O. Box No. 373, York YO10 5YW (U.K.).

Expansins were first characterised by their ability to induce cell wall extension *in-vitro*, and appear to play a key role in the growth of plant cell walls (1, 2). The means by which expansins induce extension appears to involve the disruption of hydrogen bonds between cellulose microfibrils and the hemicelluloses that bind them to one another in the walls. Expansins have more recently been shown to be expressed in large quantities during fruit softening a process that does not involve wall elongation (3). During softening walls are being disassembled, and it is thought that expansins may play a role in this process by making wall polymers more accessible to hydrolytic enzymes. In this presentation, we show that expansin activity is induced during ethylene-induced leaf abscission. Several expansins have been cloned from leaf abscission zones and we will present data on their expression during this process. The role of expansins in leaf abscission will be discussed.

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Cytoskeletal and Cell Wall Interactions During the Establishment of Cell Polarity in the Fucus Zygote

Ralph S. Quatrano

Dept. Biology, Washington University, St. Louis, MO 63130-4899, USA

Zygotes of the brown alga Fucus provide a model system to study the basic mechanisms involved in the generation of cellular asymmetry and morphogenesis. A localized cortical domain, composed of F-actin, ion channels and free Ca, is the first asymmetry established in the zygote.

Stabilization of this polarity requires an actin-dependent polar secretion of Golgi vesicles into the cell wall at this site. Microfilaments also appear to provide "tracks" for vesicle targeting to this site of polar growth and are required for the polar redistribution of mRNA at the time of polar axis stabilization. Recent evidence indicates that the asymmetry in the cell wall at this cortical site provides signals to the underlying cytoplasm that orient the first asymmetric division plane perpendicular to the polar growth axis. Treatment with brefeldin A reversibly and selectively blocks polar secretion and results in the mis-orientation of the first division plane with respect to the light gradient. These results suggest that localized cell wall components may provide positional information for orienting the plane of cell division. Localized cortical complexes in the zygote, composed of actin and cell wall components, play key roles in the generation and stabilization of cellular asymmetries and in providing signals for proper orientation of the first cell division.

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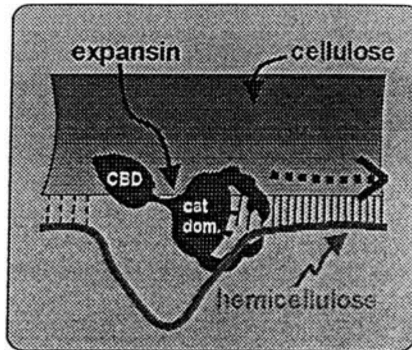
New Genes, New Roles for Expansins

Daniel Cosgrove

Department of Biology, Penn State University, University Park, PA 16802



Expansins were first identified as mediators of the "acid growth" response. These proteins bind to cell walls and catalyze wall extension in a pH-dependent manner. They do not exhibit hydrolytic activity against cell wall components, and indeed we have not been able to detect significant enzymatic activity in expansin preparations. Nevertheless, they act catalytically, not stoichiometrically, to induce wall expansion in vitro. They also have been shown to be capable of stimulating cell expansion in living cells when applied exogenously to *Arabidopsis thaliana* hypocotyls, cucumber root hairs, tomato meristems, and tobacco cell cultures.

We now recognize two families of expansins, called α-expansins and β-expansins. α-Expansins comprise the first-discovered family of expansins and have been studied in greatest detail. β-Expansins were more recently recognized when it was found that Zea m1, a member of a group of grass pollen allergens, has significant expansin activity. These grass pollen expansins probably function in loosening the cell walls of the stigma to aid pollen tube penetration. A number of related sequences that are present in GenBank have now been classified as β–expansins on the basis of sequence homology to Zea m1 and are presumed to have related wall loosening functions.

Between the α– and β– families there is relatively little sequence conservation, consisting most notably of a series of conserved cysteines in the N-terminal half of the protein, an HFD motif and an FRRV motif in the middle of the protein, and a series of tryptophans (W) near the carboxy-terminus. Despite the low sequence identity, the two groups of proteins have similar rheological effects on cell walls, namely induction of cell wall extension and stress relaxation). There are three likely domains in the expansin protein. The first ~22 amino acids encodes a classical signal peptide that directs the protein into the secretory pathway and is cleaved off to form the mature

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protein. A putative binding domain (~10 kDa) at the carboxy-terminus of the protein contains a series of conserved tryptophans (W) with spacing that resembles that of cellulose-binding domains of cellulases. The 15-kDa piece of the polypeptide between the signal peptide and the putative binding domain has distant, but significant, sequence similarity to the catalytic domain of family-45 glycosyl hydrolases [19]. The "HFD" motif and a second conserved aspartate (D) residue near the carboxy terminus make up the key residues for the catalytic site of the family-45 hydrolase, and these residues can be found in corresponding places in both α– and β–expansins.

α-Expansins make up a large multigene family, with at least 22 members in *A. thaliana* [see the expansin web site (http://www.bio.psu.edu/expansins/) for up-to-date listings of known expansin genes in *A. thaliana*, rice, tomato and other species]. Expression analysis indicates that expansin genes are selectively expressed in specific cell types and at specific developmental stages. I will present our latest results on expression analysis and the likely roles of specific members of this gene family.

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Session 4: Structural components of the cell wall

Chair: Sacco C. de Vries

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Regulation of genes coding for proline-rich proteins in maize.

Pere Puigdomènech.

Institut de Biologia Molecular de Barcelona. CSIC. Jordi Girona, 18. 08034 Barcelona. Spain

The genes or cDNAs coding for three different proline-rich proteins have been cloned from maize in our laboratory. They correspond to HRGP, a protein that appears to be one of the main components of the maize primary cell wall, HyPRP an embryo-specific protein that has a domain partially rich in proline and ZmPRP, a protein whose mRNA is accumulating in tissues where secondary cell wall is formed. Other genes related to maize cell wall have been cloned in our group, including genes coding for enzymes of the lignin biosynthesis pathway and other wall-related proteins such as expansins or XET.
The proteins encoded by the three genes have distinct features. They have in all cases signal peptides and the proline-rich domains are highly repetitive, although HyPRP has also a hydrophobic domain in its C-terminal half. The HRGP and HyPRP genes have a complementary pattern of expression in the maize embryo, the HRGP mRNA being accumulated in meristematic tissues and HyPRP mRNA in cells differentiating to parenchyma cells. The promoters of the two genes are active in transient expression assays by microbomabrding. The mRNA of the ZmPRP gene has a pattern of distribution similar to the one observed in genes coding for enzymes of the lignin biosynthesis pathway. The genes are controlled during development but also by hormone action. HRGP is induced by wounding, ethylene and fungal elicitors, while HyPRP is repressed by the action of ABA and ZmPRP is also induced by wounding as is the case of othe lignin-related genes.

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SECONDARY CELL WALL CROSSLINKED PROTEINS

Concha Domingo and Pablo Vera

IBMCP. Universidad Politécnica-CSIC. Camino de Vera s/n. 46022-Valencia. Spain.
cdomingo@ibmcp.upv.es

The wall is an important component of the plant cell with a central role in plant biology. It provides strength and shape to the cell and rigidity to the whole plant, controls the growth of the cell, participates in cell-cell communication and also participates in the protection from attack by pathogens and other environmental stresses. This great variety of roles suggests that the cell wall architecture must be tightly controlled.

Cell wall composition varies according to the developmental stage and the specialisation of each cell type. Proteins are part of the components of the plant cell wall and different classes of structural proteins have been characterised, some of them are the hydroxyproline-rich glycoproteins (HRGPs), the proline-rich proteins (PRPs), and the glycine-rich proteins (GRPs). The genes encoding these structural proteins are tightly regulated, and their expression patterns show cell type specificity and developmental regulation (Cassab, 1998). Also, the expression of genes encoding some of these cell wall proteins can be altered by different cues (e.g. wounding or pathogen attack) with the deposition of the encoded proteins in the cell wall resulting in alterations of the functional properties of this compartment (Showalter, 1993; Cassab, 1998). For a long time they have been considered as simple structural molecules, but now there are emerging data confirming that their role is more dynamic being involved in a variety of processes such as remodelling, stiffening and repair of cell walls.

A common characteristic of plant cell wall structural proteins is their ability to remain insolubilized in the cell walls in a durable fashion. For example, the extensins are basic proteins with ability to form an insoluble network that remains ionically bond to the cell wall pectins (Qi et al., 1995). The GRPs form a further group of wall-associated proteins associated with walls at the onset of lignification and they have been proposed to participate as nucleation sites for lignin formation as well as in wall repair processes (McDougall et al., 1996; Ryser et al., 1997). It has been reported a very rapid oxidative cross-linking mechanism for insolubilization of pre-existing cell wall structural proteins that seems to be under developmental control and mediated by the action of extracellular peroxidases (Bradley et al., 1992). However, the mechanism mediating the crosslinking of cell wall proteins as well as the structural requirements involved in this process remain poorly understood.

NtTLRP is a recently described tobacco cell wall protein that represents a member for a novel class of cell wall proteins associated with secondary cell walls. These proteins have in common the presence of a peptide motif rich in cystein and tyrosine amino acid residues. The NtTLRP gene is expressed at all stages of plant development and the amount of the corresponding mRNA is particularly high in developing seedlings. By using an specific antibody, it has been shown that NtTLRP is a cross-linked protein that localises in the developing protoxylem cells, following the pattern of lignin deposition in these cells, thus favouring the interpretation that this protein, as seems to be the case for the tomato TLRP (Domingo et al., 1994), is intimately and/or co-ordinately expressed during the process of lignification characteristic of tracheary element differentiation.

NtTLRP and its homologes share striking similarities at both the N-terminal signal peptide sequence and the C-terminal region. In the C-terminal region a highly conserved structural domain rich in cystein amino acid residues, and named cystein-domain (CD), can be identified. The CD domain appears to be a common signature in different cell wall structural proteins and consist of an arrangement of cysteine residues between which tyrosine and positively charged amino acid residues are interdigitated following the consensus sequence CXYXCC$(X)_6$C$(X)_{2-3}$CCSY. The conserved arrangement of cystein residues within the CD suggests that these residues (through the formation of intermolecular cystine bridges) may stabilise an appropriate conformation in the domain so that the side chains of the

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interdigitated residues (including tyrosine) will be exposed to facilitate interactions with other components of the cell wall.
By using a functional test in transgenic plants expressing a secreted and soluble protein (e.g., PR1 protein) to which the CD domain was translationally fused to the C-terminus (PR1-CD protein) we have demonstrated that the presence of the CD domain is *per se* sufficient to anchor this previous soluble protein into the cell walls. Although we still do not know what amino acids in the CD domain are responsible for the observed insolubilization, both cystein and tyrosine are likely candidates implicated in the crosslinking process.

High cysteine content is common to many cell wall proteins, and this amino acid residue has been proposed to be implicated in the insolubilization of different cell wall proteins due to their capability to form disulphide bonds under the proper redox conditions of the plant extracellular matrix, and thus may participate in major intermolecular interactions with other components of the cell wall. Clusters of cystein residues seem to be responsible also for the interaction of proteins with cell wall polymers as proposed for the chitin binding motifs present in potato lectins (Kieliszewski et al. 1994). Tyrosine is another amino acid residue proposed to participate in the cross-linking of proteins to cell walls through oxidative coupling of tyrosine residues mediated by a peroxidase/H_2O_2-catalized reaction. Such tyrosine-derived products has been reported to exist in plant cell walls in the form of isodityrosine (a dimmer of tyrosine which may form both intra-polypeptide loops and inter-polypeptide linkages), di-isodityrosine (a tetramer of tyrosine), and pulcherosine (a trimmer of tyrosine which could act as the natural intermediary between isodityrosine and di-isodityrosine) (Epstein and Lamport, 1984; Fry, 1986; Brady et al, 1996, 1998). In addition, tyrosine residues can also be linked to the aromatic side chains of lignin and thus facilitate the interaction of proteins with other components of the cell wall.

Both structural proteins and lignin are essential components for the differentiation and function of xylem vessels and their synthesis and deposition must be tightly co-ordinated in order to reinforce secondary cell walls to withstand the negative pressures that are generated in the xylem during the course of water transport. In this regard, the xylem-associated NtTLRP protein as a new component of xylem cells and the identification of a minimal structural domain in the protein that mediates the crosslinking to cell walls, will help increase our understanding of the molecular components and mechanisms mediating in the paradigmatic process of xylem differentiation. Furthermore, the CD motif can now be entertained as a minimal structural domain to approach biochemically and genetically the isolubilization process of cell wall proteins which is intimately associated to the differentiation of xylem.

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Interactions of structural proteins in the plant cell wall

Beat Keller, Nicolas Baumberger and Christoph Ringli.

Institute of Plant Biology, University of Zürich, Zollikerstrasse 107,
8008 Zürich. Email: bkeller@botinst.unizh.ch

GRP1.8 of bean (*Phaseolus vulgaris*) is a member of the class of glycine-rich structural cell wall proteins and is deposited in large amounts into the modified primary cell wall of protoxylem elements (Keller et al., 1988; Ryser and Keller, 1992). Modification of the primary cell wall of protoxylem elements includes the hydrolysis of the tension-bearing hemicelluloses that interconnect the cellulose microfibrills. This hydrolysis allows the passive stretching of the cell wall and thus enables the mature protoxylem to remain functional despite continuous growth of the plant. The deposition of GRP1.8 might be part of a repair mechanism leading to a modified cell wall with particular mechanical and chemical properties (Ryser et al., 1997). To get a better view on possible functions of GRP1.8, we have studied the biochemical properties of GRP1.8, in particular the chemical nature of non-covalent interactions established between GRP1.8 and components of the extracellular matrix. A reporter protein was expressed in transgenic tobacco either as wild-type protein or as a fusion protein with different domains of GRP1.8 and the solubility of these proteins in the extracellular matrix was studied. A chitinase originally isolated from the extracellular fluid of cucumber was used as the reporter protein (Boller and Métraux, 1988). A clear change in solubility was observed between the chitinase and the chitinase/GRP1.8 fusion proteins. While a low salt buffer was sufficient to extract the chitinase from tobacco cell wall preparations, an extraction buffer with a hydrophobic component such as SDS or Triton X-100 was required for the complete extraction of soluble chitinase/GRP1.8 fusion proteins. Thus, the GRP1.8 domains in the fusion proteins are involved in a hydrophobic interaction within the extracellular matrix. The same experiments with purified cell wall fractions of bean hypocotyl confirmed the observed hydrophobic property of GRP1.8. From these experiments, a model can be suggested in which, through self aggregation, GRP1.8 forms a hydrophobic layer in the modified primary cell wall to stabilize the protoxylem and to prevent water loss through diffusion.

We are studying a second class of structural proteins which is likely to have different interactions within the extracellular compartment. The studied proteins are chimeric extensins containing LRR (leucine-rich repeat) domains. Extensins form a specific class of cell wall hydroxyproline-rich glycoproteins in plants which is characterized by the presence of numerous $Ser(Pro)_4$ repeats. They

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are considered to be structural proteins because of their repetitive amino acid sequence and the lack of enzymatic activity. It has been postulated that their main function could be to fix the definitive shape of the cell at the cessation of growth and to increase resistance to tensile forces. This is consistent with the accumulation of certain extensins in vascular tissue when cell elongation stops. Furthermore, a strong binding to the extracellular matrix is clearly demonstrated since they are frequently recalcitrant to extraction, although the nature of this linkage is still a matter of considerable debate. An increasing number of proteins containing extensin motifs associated with various unrelated domains have been described recently. Their specific expression pattern, the identity of their additional domains (AGP-like, lectin-like, LRRs), and the resemblance of these chimeric extensins with algal cell adhesion molecules (algal- CAM) suggest that these proteins might play an important role in cell-cell recognition and adhesion during plant development.

We are currently investigating a new family of 8 *Arabidopsis* genes encoding extensin-like proteins which contain a leucine-rich-repeat (LRR) domain. As LRRs are known to mediate protein-protein interactions in eucaryotic cells, these genes could play a role in extracellular signalling or in the control of the activity of cell wall enzymes. These chimeric LRR-extensin genes (*Lrx*) seem to be widespread in the plant kingdom since other members are present in a *Solanaceous* species (*L. esculentum*) and in a monocot (*Zea mays*, Pex1 and Pex2, Rubinstein et al. 1995). The expression patterns of the 8 Arabidopsis genes were studied and very different patterns were found. Two genes (Lrx5 and 6) were ubiquitously expressed while two others were exclusively present in roots (Lrx1 and 2). The latter have been chosen for further investigation. Transgenic plants with promoter- *uidA* gene fusions show that Lrx1 and Lrx2 have different, although overlapping, expression patterns. The most striking feature is a stronger expression of Lrx1 in rhizodermal cells forming root hairs and a high expression of Lrx2 in meristematic cells. In order to address the question of the function of these genes, several mutants in these genes were isolated by transposon tagging. In addition, sense and antisense expression of the genes in transgenic plants is studied. Analysis of these mutants and transgenic plants is currently in progress.

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STRUCTURE AND DEVELOPMENT OF INFECTION THREADS

N.J. Brewin, E.A. Rathbun and J-P. Wisniewski

(*John Innes Centre, Norwich, NR4 7UH*)

In pea and other temperate legumes, *Rhizobium* gains entry to host root tissues through tip-growing infection threads. These are tubular ingrowths of the plant cell wall which originate from curled root hair cells. Having traversed the epidermis, the bacteria are once again released into the extracellular matrix. Infection is propagated because transcellular threads are reinitiated in root cortical cells and subsequently in the invasion zone of the developing nodule.

Bacteria within infection threads are normally surrounded by an extracellular matrix, apparently equivalent in composition to the intercellular matrix. A 110kDa plant matrix glycoprotein (MGP) was localised to the infection thread lumen by monoclonal antibody MAC265, which recognises a carbohydrate epitope on MGP. The glycoprotein was extracted from pea root exudate and concentrated by immunoaffinity chromatography using protein G sepharose coupled to MAC265. Immunopurified glycoprotein had a blocked N-terminus and was resistant to cleavage by many proteases. Fourier-transform infra-red spectroscopy indicated a high carbohydrate content (>60%). Following treatment with chymotrypsin, a 40 kDa polypeptide was isolated by gel electrophoresis. This fragment, which retained reactivity with MAC265, yielded 29 amino-acids by N-terminal sequencing. A 650 base-pair cDNA clone corresponding to MGP was obtained by 3'RACE PCR using mRNA isolated from *Rhizobium*-inoculated pea roots. The (incomplete) DNA sequence indicates that MGP-ER2 encodes an extensin-like glycoprotein with repeating (hydroxy)proline-rich domains. Interestingly, the sequence included four repeats of the motif SP_5.

....
TPHKKPYKYPSPPPPP
VHTYPHPHPVYHSPPPP
HKKPYKYSSPPPPP
VHTYPHPHPVYHSPPPPP
TPHKKPYKYPSPPPPP
AHTYPPHVPTPVYHSPPPP
VYSPPPP
AYYYKSPPPP
YHH*

There is very high homology between the MGP sequence from pea and the deduced amino acid sequence for VfNDS-E, a nodule-enhanced extensin from *Vicia faba*, previously isolated as cDNA in a subtractive hybridisation study (Perlick & Puhler 1993). Similarly, MtN12 from *Medicago truncatula* was obtained from a nodule-enriched cDNA library (Gamas et al., 1996). The repeated motif "VHTYPHPHPVYHSPPPP" is very strongly conserved. Of particular interest is that the 3'untranslated regions of the pea and *Medicago* sequences show even stronger homology than is seen in the coding sequence.

We have found that MGP can easily be insolubilised in a time-dependent manner by a process that is dependent on H_2O_2. We are investigating possible sources of peroxide in the extracellular matrix . These could include diamine oxidase, oxalate oxidase and NAPH oxidase. Hence, the regulation of these activities could be very important in symbiosis.

Our working model suggests that peroxide-induced hardening of MGP could influence both the initiation and the propagation of the infection thread. (1) In the curled root hair, solidification of the gum surrounding attached rhizobial cells could create a plug which would allow growing and dividing bacteria trapped on the inside to generate an inward thrust which could initiate infection thread development from the kink in the curled root hair. (2) In the lumen of the infection thread, hardening of the matrix material could explain why bacterial growth is only observed at the tip (Gage et al. 1998) where, presumably, the matrix is still fluid. (3) Finally, it is easy to imagine how the abortion of infection threads might be due to premature hardening of the luminal matrix in the region of the growing tip.

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Biochemical flexibility of the phenolic components of the cell wall

Alain-M. Boudet and Matthieu Chabannes

UMR CNRS/UPS 5546, Pôle de Biotechnologie végétale, BP 17 Auzeville, F-31326 Castanet Tolosan cedex, France, T. 33 5 62 19 35 21, F. 33 5 62 19 35 02, e-mail : amboudet@cict.fr

Lignin genetic engineering is a very active area of research which has been stimulated within recent years by the characterization of important genes controlling lignification. A significant number of transformed plants exhibiting qualitative and quantitative changes in their lignins has already been obtained (1, 2, 3) and, in a limited number of cases, preliminary data have demonstrated the industrial usefulness of the resulting products.

The modifications of lignin profiles which have been envisaged until now have dealt either with reduction in lignin content or changes in the lignin monomeric composition since this parameter is important for lignin extractability. However, it soon appeared that experiments aiming to decrease the lignin content indirectly altered the chemical composition of transgenic lignins (or of cell wall components).

As an example in severely CCR down-regulated tobacco plants an increase of cell wall linked phenolics released by mild alkaline hydrolysis occurred, the main enrichment concerning ferulic and sinapic acids and acetosyringone. It was suggested that the incorporation of ferulic acid into the cell wall was responsible for the brown orange coloration observed as a consequence of CCR silencing (4). Additional studies using 13C NMR revealed the presence of ferulate tyramine in cell walls of the CCR antisense line (5).

By crossing homozygous tobacco lines down-regulated for CCR and CAD, we have obtained hybrid lines down-regulated for both enzymes. The lignin content of the hybrid is surprisingly lower than for the CCR down-regulated homozygous line. However, in contrast to this parent line its size and morphology are not affected. In addition, a significant increase in cellulose and hemicelluloses was observed in the double transformants. This hybrid line with only 35% of residual lignin was processed in simulated kaft pulping experiments by CTP (Petit-Conil, personal communication) and exhibited very promising pulping chracteristics. These interesting results which have been confirmed several times on tobacco plants obtained in culture room conditions should be now extended to other species grown in natural conditions. They suggest that the CCR and CAD transgenes work in synergy in a still undefined way to give rise to new lignin profiles without altering plant morphology.

These results are comparable to those obtained by Chiang's group on transgenic aspens down-regulated for 4-coumarate CoA ligase which exhibited a substantial reduction in lignin quantity and, more surprisingly, a 15% increase in cellulose content (6).

Even if the rationale of the compensation mechanisms allowing plants with a low lignin content to have an apparent normal development is not understood, these studies demonstrate the high biochemical flexibility of the cell wall. In addition, the increase in polysaccharides observed in these transgenic lines is extremely interesting for future applications.

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Cell-Wall Development and Dynamics in Maize and Other Poales

Claudia E. Vergara[1], Marcos S. Buckeridge[2], Kim Findlay[3], Gareth Catchpole[3], Anna T. Olek[1], Maureen C. McCann[3], and Nicholas C. Carpita[1]

[1]Dept. of Botany & Plant Pathology, Purdue University, West Lafayette, IN, 47907 U. S. A.
[2]Inst. de Botânica, Secão de Fisiologia e Bioquímica Plantas, São Paulo, Brasil
[3]Dept. of Cell Biology, John Innes Centre, Norwich NR4 7UH, U. K.

The cell walls of grass species and commelinoid monocots are structurally and architecturally distinct from those of all other angiosperms. The major cellulose-tethering glycans are glucuronoarabinoxylans (GAXs) in place of xyloglucans, they are notably low in pectin, and aromatic substances replace structural proteins as the cross-linking agents in the primary wall (Carpita 1996). Also, Poales are further distinguished from the commelinoids by the ability to make a unique mixed-linkage (1→3),(1→4)-β-D-glucan (β-glucan). Unlike most angiosperms, the cell walls of grasses change markedly during cell division and elongation. Absent from meristematic cells, the β-glucans accumulate in the wall during cell elongation and turn over when elongation is complete. The highly substituted GAXs made during early stages of elongation lose a substantial portion of the arabinosyl side-groups as growth progresses. Novel, non-methyl esters of polygalacturonans also accumulate during the fastest rates of growth and decrease when growth wanes. We developed an efficient method to preserve the synthesis *in vitro* of β-glucan in isolated Golgi apparatus of maize seedlings (Gibeaut and Carpita 1993; Buckeridge *et al.* 1999). The mechanism of glucosyl transfer has several common features with cellulose synthesis, we proposed that the -glucan synthase genes are derived from ancestral cellulose synthase genes (Carpita and Vergara 1998). A search of cellulose synthase genes (*Ces*As) in rice identified several genomic *Ces*A and *Ces*A-like clones from rice. Sequencing of the EST clones revealed at three separate classes of *Ces*A genes. Experiments are underway to differentiate between cellulose synthase and β-glucan synthase by differential expression in seedling and endosperm and by immunolocalization of unique peptides of the β-glucan and cellulose synthase at the Golgi or plasma membrane, respectively.

As part of our studies to examine the turnover of β-glucans during growth, we purified a -glucan exohydrolase (ExGase) from the cell walls of developing maize shoots. Polyclonal antisera raised against the ExGase were used to select partial-length cDNA clones, and the complete sequence of 622 amino acid residues was deduced from a full length genomic clone. The seedling ExGase gene is expressed and the enzyme is enriched in the walls during all stages of maize coleoptile elongation. An antigenically related polypeptide is associated with plasma membrane. Unlike the wall enzyme, the membrane-associated ExGase is unable to cleave (1→4)-β-glucosyl linkages. We propose that the novel isoform of the ExGase has two potential functions at the membrane-wall interface other than β-glucan digestion.

We have used a multidisciplinary approach to determine how these polymers are assembled into architectural form in the primary walls of elongating maize coleoptiles. Isolated coleoptile walls and epidermal peels were examined by field-emission-scanning electron microscopy (FEG-SEM) and Fourier transform infrared (FTIR) microspectroscopy after sequential extractions of defined polysaccharides and aromatic substances. Microfibril impressions are observed through a dense blanket of material in unextracted isolated walls. Removal of the chelator-soluble pectins does not alter this structure substantially, but removal of the highly substituted GAXs increases the apparent pore size and permits deeper views into the wall. Oxidation of the phenolic network permitted removal of a substantial portion of the GAXs by dilute alkali. Such a treatment widened the pores further and permitted observation up

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to eight distinct microfibrils deep into the wall. β-Glucans still remaining in the wall after this treatment were removed subsequently with a specific endo-glucanase but the pore size was not increased further. Fourier transform infrared microspectroscopy was used to define the spectral changes associated with wall structure upon successive removal of the matrix components. By subtractive analyses of FTIR spectra catalog differences in the spectral behavior of isolated polymers such as GAXs and β-glucans and their impact on the FTIR absorbance when organized in an intact wall matrix.

We have developed discriminant analysis of Fourier transform infrared spectra as a high through-put and specific method to identify mutations in plant cell wall polysaccharide components and wall architecture (Chen *et al.* 1998). In collaboration with scientists from both public and private domains, we propose to employ this method to identify in mutagenized populations of maize and arabidopsis a broad range of mutants involved in the biogenesis and dynamic alteration plant cell wall architecture during growth and development. We have devised a systematic protocol that will employ biochemical, spectroscopic, and imaging methods to categorize defects in wall structure and architecture into one of six stages of wall biogenesis or disassembly. Disrupted genes of maize mutants identified based on the position of the insertional element in transposon-tagged maize populations. A major practical goal is to generate plants with genetically defined variation in composition and architecture to permit assessment of how specific modifications of the cell wall impacts cellular function, plant development, and biotic and abiotic stress interactions.

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Session 5: Dynamics of the primary and secondary cell walls

Chair: Daniel Cosgrove

Instituto Juan March (Madrid)

The Mechanism and Regulation of Cellulose Synthesis in Plants.

Deborah Delmer, Y. Kawagoe, D. Holland, N. Holland, L-C Peng.
Section of Plant Biology, University of California Davis, Davis CA 95616

The *CesA* genes of plants are believed to encode the catalytic subunit of the cellulose synthase. Evidence supporting this notion comes from their homology with the bacterial synthases, from ability to bind the predicted substrate, from expression patterns for at least some of the genes, and from genetic studies indicating that these genes are critical for the process (1,2,3). However, no one has demonstrated glycosyltransferase activity using recombinant protein. We have expressed an epitope-tagged version of the cotton *GhCesA-1* gene in the heterologous systems of yeast and green monkey kidney (COS) cells. In both cases, the proteins are expressed and inserted into membranes, but no glycosyltransferase activity can be detected. These systems are, however, useful for topological studies, results of which will be presented in this lecture. Expression of the *GhCesA-1* gene in either wild-type or mutated form in Arabidopsis or tobacco BY-2 cells leads to abnormal phenotypes that are presently being characterized.

Studies with the Novartis herbicide CGA 325'615 are shedding further light on the process of cellulose synthesis. In cotton fibers, this compound, at nanomolar concentrations, specifically inhibits the synthesis of crystalline cellulose. Concomitant with this, we see accumulation of some callose and an ammonium-oxalate soluble β-1,4-glucan that appears to be linked to protein; the nature of this protein and speculation on its role in cellulose synthesis will also be discussed.

The CesA genes comprise a relatively large gene family with at least 10 members in Arabidopsis and maize (4,5). In collaboration with Tim Helentjaris and K. Dhugga from Pioneer HiBred, 8 distinct full-length or near full-length *CesA* cDNA's from maize (*ZmCesA1-8*) have been identified and sequenced. The chromosomal locations of the relevant genes have been determined and expression patterns for the genes analyzed. A promoter-β-glucuronidase (GUS) fusion of the Arabidopsis *AtCesA-4* gene was also generated to add further information on the expression pattern for another member of this gene family in this plant. All of these genes are expressed in a variety of organs, although some show unique patterns of cell-type specific expression within those organs. Multiple sequence alignments of the deduced proteins were used to generate an unrooted cladogram in which the CesA proteins cluster in distinct groups. Most of these groups are not comprised of unique paralog from a single plant species, but rather cluster as orthologs; chromosomal location is also not correlated with the groupings. In at least some cases, the groups correlate with unique patterns of cell-specific expression as determined by a variety of methods. One group has members involved in primary wall synthesis (AtCesA-1; ZmCesA-1), another contains members that are expressed in tissues undergoing secondary wall synthesis (GhCesA's; PtCesA's, AtCesA-4). Mu-insertion mutants in *ZmCesA-8* have also been identified that show only a very mildly altered phenotype—a finding that might be explained by our observation that two other genes, *ZmCesA-6* and *ZmCesA-7*, may provide functional redundancy since they possess deduced amino acid sequences and patterns of tissue-specific expression that are very similar to those observed for *ZmCesA-8*.

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Identification of a rapid subcellular response to wounding by imaging plants expressing random GFP::cDNA fusions.

Sean R. Cutler[1,2], David W. Ehrhardt[2], Joel S. Griffitts, Chris R. Somerville[1,2]

[1]Department of Biological Sciences. Stanford University. Stanford CA 94305

[2]Carnegie Institution of Washington. Department of Plant Biology. 260 Panama Street, Stanford CA 94305

Many problems in plant cell biology can be informed by the direct observation of subcellular structures and their associated dynamics in living cells. To facilitate live-cell investigations, we have explored a high-throughput approach to examining subcellular organization by producing and imaging large numbers of plants that express random GFP::cDNA fusions. Fluorescence imaging of 5700 transgenic plants indicated that approximately 2% of lines expressed a fusion protein with a different subcellular distribution than that of soluble GFP. Our results suggest that a wide variety of subcellular structures and dynamic processes can be marked efficiently using random GFP::cDNA libraries.

The potential of this approach for identifying unique dynamic processes in living cells is demonstrated by the identification of a GFP fusion protein that displays a rapid subcellular redistribution after wounding. A fusion between GFP and the enzyme Nitrilase 1 was found to redistribute from the cytoplasm into granular structures minutes after delivery of a wound stimulus. Cells at a distance from the primary wound site display GFP::Nit1 granulation, suggesting that the fusion protein is responsive to a motile signal produced rapidly after mechanical damage to *Arabidopsis* cells.

The function Nitrilase performs in wound responses is being addressed using biochemical, genetic and cell biological approaches. To help elucidate the composition and function of GFP::Nitrilase granules, we have developed a granule purification protocol. Collectively, our observations suggest that the rapid formation of granules reveals a novel wound-induced signal and response pathway. The ability to visualize this process *in vivo* using GFP::Nitrilase will facilitate our analysis of this pathway.

Our results indicate that screening GFP::cDNA libraries is a useful approach for identifying and visualizing components of subcellular structures in higher plant cells, and that it is capable of revealing novel insights into subcellular dynamics. Modifications of the approach to allow the identification of amino-terminal targeting information are being pursued to enable identification of secreted proteins or proteins that require free amino termini for proper targeting.

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Cellulose mutants in *Arabidopsis*

H. Höfte, M. Fagard, A. v. Tuinen, F. Goubet, A. Bichet, S. Pagant,
M. McCann and S. Vernhettes,

Laboratoire de Biologie Cellulaire, INRA, Route de St Cyr, 78026 Versailles cedex.

To identify genes involved in controlling directional cell expansion we have carried out a screen for mutants with reduced cell elongation and increased radial expansion in the hypocotyl. FTIR microspectroscopy in collaboration with Maureen McCann and coll. allowed 2 classes of mutants to be distinguished. Mutants with detectable defects in cell wall composition and those without. The latter class identified by 8 alleles of *botero*, shows defects in the organisation of the cortical microtubules. Cellulose defects were observed in alleles of *rsw1* (Arioli et al., 1998), *kor* (Nicol et al., 1998) and *prc*. Interestingly, in *prc* hypocotyls, the growth and cellulose defect is conditional: activation of phytochrome entirely reverts the phenotype. PRC maps to the bottom of chromosome V and was isolated through map-based cloning. The identity of the gene product will be presented. We are currently investigating the molecular basis of the conditionality of the phenotype. To our knowledge this is the first example of a molecular link between phytochrome action and cell wall synthesis in expanding cells.

Refs: Arioli, et al. (1998) **Science,** 279, 717-720 ; Nicol, et al (1998) **EMBO J.,** 17, 5563-5576.

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Characterisation of a novel secondary cell wall mutant in Arabidopsis.

Louise Jones and Simon Turner
University of Manchester

Although the components that comprise plant secondary cell walls, namely cellulose, xylan and lignin, are relatively well understood, little is known concerning the architecture of these cell walls and how the individual components contribute to the physical properties and functions of the cell walls.

Irx4 is a novel Arabidopsis mutant identified by its collapsed xylem phenotype (Turner and Somerville, 1997, Plant Cell, **9**, 689-701). Analysis of total cell wall phenolics, thioglycolic acid assays and solid-state NMR have demonstrated that the cell walls from *irx4* plant stems possess approximately 50% of the total lignin content compared to wild-type plants. Reduced lignin content has also been observed by histochemical studies using reagents considered as diagnostic stains for lignin, such as phloroglucinol and Maüles reagent. In contrast, analysis of hemicellulose levels indicated significantly increased proportions of xylan in *irx4* cell walls compared to the WT controls. Whether this reflects an actual increase in the total xylan content of *irx4* cell walls or an increase in their extractability remains to be determined. No significant differences have been observed in the cellulose content of stems from wild-type and *irx4* plants.

These alterations in the composition of *irx4* secondary cell walls are correlated with severe effects on the physical properties of *irx4* stems, as determined by three-point bending tests. In addition, examination of the ultrastructure of the cell walls of *irx4* xylem and interfascicular cells revealed hugely expanded and diffuse secondary cell walls with altered levels of staining.

Attempts to determine the nature of the mutation affecting lignin biosynthesis have indicated there is no disruption in the general phenylpropanoid pathway. However, there does appear to be an up-regulation in enzyme activity and transcript levels of several genes involved in the lignin biosynthetic pathway. Work is now underway to identify the gene responsible for the *irx4* mutation.

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Tracheary element formation: building up to a dead end

Maureen C. McCann, Dimitra Milioni, Nicola J. Stacey, Pierre Sado,
Maria Mourelatou, Concha Domingo and Keith Roberts

Mesophyll cells from the leaves of Zinnia elegans cv. Envy trans--differentiate into tracheary elements (TEs) when cultured in a medium containing a 1:1 ratio of auxin to cytokinin (Fukuda and Komamine 1980, Fukuda 1992). The differentiation process is irreversible and is characterised by a series of cytological changes. First, microtubules rearrange in a cortical banding pattern that reflects the position of future secondary cell-wall thickenings, then cellulose and other wall polymers are deposited in localized thickenings, then lignification occurs, and finally cell autolysis and cell death. This model system is unique among in vitro systems because of the readily inducible synchronous trans-differentiation of up to 80% of the cell population, making it ideal to study the processes involved in secondary wall formation.

In addition to the initial wound stimulus, exogenous auxin and cytokinin are absolutely required to induce TE formation in the Zinnia cell system. In the original system (Fukuda and Komamine 1980), these plant growth regulators are added at the start of the culture and TEs are induced by 96 h. However, we have discovered that these inductive signals can be present in the culture medium for as little as 10 min if added after 48 h of culture in a maintenance medium. By delaying the time at which the inducing hormones are added to the culture until 48 h, we have significantly improved the synchrony of the culture such that 80% of the cells trans-differentiate to TEs in 48 h after hormone addition. This feature makes it possible to dissect the chronology of molecular events even to minutes. We proposed that the first 48 h of culture represents a time in which the cells, freshly isolated from leaves, adapt to liquid culture and acquire the competence to respond to the hormones (McCann 1997). At 48 h, the auxin and cytokinin act as a switch to turn on the developmental pathway of TE formation. Other signals are then required to progress though the subsequent stages of terminal differentiation.

Other labs and ours have characterized a handful of genes involved in different stages of the developmental pathway to TE fate using the Zinnia system (Fukuda 1996, McCann 1997). In order to get a broader picture of the range of genes involved in this complex process, we require sequence information for comparison with known genes in databases and must identify both high- and low-abundance transcripts. One way to do this is to establish an EST database, such as the poplar and loblolly pine databases (Sterky et al. 1998, Lafayette et al. 1998). However, the EST databases are from many vascular cell types and asynchronously differentiating cells and, therefore, the order of differentiation-related events is difficult to establish. We have recently applied a novel method of RNA fingerprinting to allow the detection of DNA fragments derived from RNA using cDNA synthesis and subsequent PCR-amplified fragment length polymorphisms (cDNA-AFLP) (Vos et al. 1995, Bachem et al. 1996).

We have used cDNA-AFLP to screen the patterns of expression of about 7000 genes at time-points of 0 h, 30 min, 4 h, 24 h and 48h after hormone addition into the culture. We selected over 100 fragments whose transcription showed overt changes in abundance over time and obtained partial sequences of these. Database searches allow us to ascribe a putative identity to about 55% of the predicted gene products, although some are to genes of unknown function. About 10% of the partial sequences show no identity to any sequence in the databases. The remaining sequences show only some similarity to existing sequences. For those genes to which we can assign an identity,

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several are already known to be involved in processes related to TE formation, and specifically to secondary wall formation.

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PLANT RESPONSES TO DOWN- AND UP-REGULATION OF LIGNIN-BIOSYNTHETIC-PATHWAY ENZYMES

John Ralph

US Dairy Forage Research Center, USDA Agricultural Research Service, Madison WI 53706-1108, USA, and Department of Forestry, University of Wisconsin, Madison, WI 53706-1598, USA.

Introduction

Biogenetic manipulation of lignin content and/or structure in plants is seen as a route to improving the utilization of plant components, primarily the polysaccharides, in various natural and industrial processes. The aims range from enhancing cell wall digestibility in ruminants to reducing the energy demand and negative environmental impacts of chemical pulping and bleaching (papermaking). The major approach along these lines is to target enzymes of the monolignol biosynthetic pathway, as recently reviewed.[1-5] Various plants with deficiencies in lignin-biosynthetic-pathway genes (and consequent enzymes for monolignol synthesis) provide insights into the flexibility and dynamics of the lignification process and aid in our understanding of "normal" lignification.

The changes observed so far in a variety of mutants and transgenics from various groups have ranged from a simple downregulation of lignification to essentially normal lignification levels but with massive compositional shifts. It is anticipated that the flexibility of the lignification process can be exploited to produce plants with lignins that allow more extensive utilization of plant fiber.

Aspen Pt-4CL Transgenics

Chiang's group have generated transgenic aspen with suppressed expression of 4-coumaric acid:coenzyme-A ligase (Pt4CL1).[6] The enzyme functions early in the monolignol pathway and the researchers reasoned that this was a good target for effecting general lignin down-regulation. In fact, the downregulation of lignin was not only striking (up to a 45% reduction), but was also accompanied by a 15% increase in cellulose — the cellulose:lignin ratio nearly doubled![7] The benefits of vigorous plants with these properties for pulp and paper production are obvious.

Milled wood lignins from a control (42% of the total Klason lignin) and the most heavily down-regulated transgenic (28% of the total original Klason lignin) were examined by NMR.[7] What was initially disappointing was that the lignin in the transgenic was not significantly different from that in the control. The amounts of *p*-hydroxybenzoates were higher, but the lignin was otherwise quite normal. In fact this observation is significant in that it reinforces the choice of this strategy for effecting a general reduction in the amount of lignins in plants (without massively affecting the composition). The plant appears to be supplanting its reduced lignin with additional cellulose. If the required defense, water transport, and other properties survive, as they appear to in these particularly vigorous plants, the potential for improving plant utilization is enormous.

Arabidopsis F5H-Mutants and Transgenics

Chapple's group have produced an Arabidopsis mutant deficient in ferulate 5-hydroxylase (enzyme F5H, gene *f5h*), the first enzyme that controls the production of sinapyl alcohol and therefore syringyl units.[8-10] {Recent studies have shown that this enzyme may be inappropriately named — coniferaldehyde is the preferred substrate}.[11,12] The "fah1-2" mutant, derived from chemical mutagenesis, has virtually no syringyl component in its lignin, leaving essentially only guaiacyl (G) and perhaps *p*-hydroxyphenyl (H) units — a so-called G- or H/G-lignin. Such a lignin is typical of softwoods, but dicot (including hardwood) and monocot lignins all normally have a substantial syringyl component and are termed G/S-lignins; Arabidopsis is a dicot. More exciting still is the ability to up-

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regulate sinapyl alcohol production in the *fah1-2* mutant. When a suitably promoted *f5h* gene was introduced into the normally F5H-deficient mutant, up-regulation of sinapyl alcohol production was at such a high level that the lignin became more syringyl-rich that in any plant reported to date. This example is one in which massive compositional shifts have been effected, but in the traditional lignin components. As will be seen, compositional shifts can range considerably wider.

Pine CAD-deficient Mutant

Preliminary observations[13] on the lignins from a CAD-deficient pine mutant identified in Sederoff's group[14,15] appear to be being strengthened as more diverse evidence accumulates.[16-20] The plant, despite a reduction in CAD levels to less than 1% of normal levels, appeared to be producing normal levels of lignin (Klason lignin values were almost identical). The lignin in the CAD-deficient pine mutant however was highly colored and stained strongly with phloroglucinol, suggesting a higher aldehyde component. From NMR studies on isolated lignins, it was clear that aldehydes and dihydroconiferyl alcohol were substantial components of that lignin.[13] Others have claimed that the "lignin" was merely a poly-lignan artifact, but its lack of any discernable optical activity, its high molecular weight (~14 kDA) and its obvious formation incorporating DHCA monomer suggest that it should be regarded as lignin, although histochemical studies are still required.

Tobacco CAD- and CCR-Down-regulated Transgenics

Tobacco transgenics produced in Alain Boudet's group.[21,22] As with the pine, the lignin levels in a CAD-downregulated transgenic were similar to the control, and fibers had the brown coloration. And again, the CAD-down-regulated transgenics showed a significant buildup of aldehyde components.[23] No DHCA units were found, but there may be no pathway for their up-regulation — DHCA is seen less commonly in NMR spectra of lignins of plant materials other than softwoods, and is not at detectable levels in any of the tobacco isolated lignins.

Transgenics with CCR (cinnamoyl-CoA reductase) downregulated[22] by antisense methods had significantly lower (~50%) lignin contents in plants that were clearly less vigorous. The isolated "lignin" showed a striking increase in tyramine ferulate, which was shown to be incorporated in as an integral component of the polymer.[23] Tyramine ferulate is a logical sink-product for feruloyl-CoA when CCR is down-regulated in tobacco. However, it is a normal wounding response product and there remain issues of whether this component is truly incorporated into structural lignin, into suberized components,[24] or simply represents polymerized secondary metabolites similar to those observed in a wounding response.

OMT-Deficient Plants

Naturally occurring mutants (*e.g.*, the brown-midrib (bm3) mutants of maize and sorghum) and transgenic plants deficient in *O*-methyl transferase (OMT) contain significant amounts of units derived from 5-hydroxyconiferyl alcohol, as revealed in beautiful thioacidolysis studies.[25–28] We are just beginning NMR examination of such plant lignins but it seems logical that the unmethylated precursors to sinapaldehyde could be incorporated into lignins if they were to build up in the system due to reduced OMT activity. Is it also reasonable that 3,4-dihydroxycinnamyl alcohol could similarly incorporate if the earlier OMT were to be downregulated.

Normal Plants with Non-monolignol Lignin Precursors

There are many known lignins that are derived from precursors, or derivatives of the three monolignols, i.e. from components other than the monolignols themselves. The idea that other components are incorporated into lignins is nothing new. If they are truly foreign components from other

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pathways that are not laid down during lignification, then they should be distinguished But where the polymer so obviously contains intimately incorporated components, that can only arise from radical coupling reactions, particularly involving monomers, then nature may be defining them as lignins. Many lignins, for example, are biosynthesized by incorporating acylated monolignols into the lignification scheme. Thus, grasses utilize *p*-coumarates,[29-33] hardwoods and some dicots such as kenaf utilize acetates,[34,35] and some plants, notably bamboo, aspen and willow, use *p*-hydroxybenzoates as 'monomers' for lignification.[30,31,36] Ferulates and diferulates are found intimately incorporated into all grass and some dicot lignins, where they are equal partners in the free-radical polymerization process and may even be nucleation sites for lignification.[37,38] If one adds the aldehydes, tyramine hydroxycinnamates, dihydro-monolignols, 5-hydroxyconiferyl alcohol, and other precursors from the studies on mutants and transgenics, it becomes clear that a general definition of lignin must include more than the traditional three hydroxycinnamyl alcohols. If not, the phenolic polymers serving the structure and function of lignin in many plants, *e.g.*, in grasses, might not be considered lignin.

Conclusions

Recent studies have shown that manipulating specific lignin-biosynthetic-pathway genes produces profound alterations in the phenolic components of plants. Although the 'lignins' in mutant and transgenic plants may appear to be strikingly different from 'normal lignins,' findings indicate that they represent merely broad compositional shifts. All of the novel units that have been found to date appear to be minor units in lignins from 'normal' plants. The recognition that such minor units can incorporate into lignin provides significantly expanded opportunities for engineering the composition and consequent properties of lignin.

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POSTERS

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Bean callus cultures accommodated to grow on the herbicide isoxaben: modification of the cell wall and its relation to the mechanism of tolerance.

Díaz-Cacho, P., Encina, A., Moral, R., Alvarez, J., Acebes, J.L.
Departamento de Biología Vegetal, Laboratorio de Fisiología Vegetal, Universidad de León, E-24071 León, Spain. Corresponding author, e-mail: dbvjaa@unileon.es.

Isoxaben is a selective herbicide used for control of broad-leaf weeds in cereal crops. It inhibits the incorporation of glucose into cellulose[1]. This herbicide is extremely efficient at inhibiting the growth of different dicots, including seedlings and callus cultures of *Arabidopsis thaliana*[1,2], and soybean cell suspensions[3].

We have obtained bean callus cultures capable of growth in 12 μM isoxaben, a highly toxic concentration, 1200-fold greater than the I_{50} calculated for non-tolerant calli. Accommodated calluses grew more slowly than non-accommodated counterparts, formed large globular corpuscles on their surface, and were harder and browner. The altered pattern of growth of tolerant calluses seems to be related to modifications in the structure of the cell walls. A preliminary study of their cell walls confirmed this idea, since accommodated calluses showed a greater amount of dry cell walls isolated per gram of callus, and the volume of their cell walls in aqueous solution was also greater. FTIR spectra of cell walls from accommodated calluses showed an enrichment in pectins, and that a important proportion of these are unesterified. Furthermore, these cell walls had enhanced levels of uronic acids and lower contents of both cellulosic and non cellulosic neutral sugars.

Cell wall fractionation of accommodated calli showed important differences in the CDTA and sn-CR fractions. Sugar analysis of these fractions revealed an increase in the amount of uronic acids and a lower molar percentage or Ara and Gal. By contrast, slightly decreased levels of total sugars were found in the TFA fraction, that was impoverished mainly in Ara, Gal and Glc, and in the KII fraction, that showed a decreased amount of Xyl and Glc. The xyloglucan content in KII fraction underwent a progressive reduction as the level of accommodation became higher.

The mechanism of tolerance to isoxaben in bean calluses is different to that described in accommodated soybean cell suspensions[3], and in three resistant mutants of *Arabidopsis thaliana*[2], in which the mechanism of tolerance was correlated with a reduced effect of the herbicide on cellulose synthesis. To our knowledge, this is the first time that tolerance to isoxaben has been linked to a modified cell wall. However, the composition of our accommodated cells resembles that obtained in tomato and tobacco cell suspensions tolerant to 2,6-dichlorobenzonitrile, another cellulose biosynthesis inhibitor[4]. Recently, we have obtained bean calluses accommodated to lethal concentrations of this herbicide and we have observed that tolerance also results from the ability to survive with a lower content of cellulose. However cell wall analysis of bean calluses showed that the mechanism of tolerance is not quite the same to both herbicides.

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THE USE OF RANDOM SEQUENCING IN THE ANALYSIS OF XYLEM CELL WALL FORMATION IN PINE

Isabel Allona

Secondary xylem formation is likely to involve some genes expressed rarely or not at all in herbaceous plants. To increase our understanding of xylem formation, and to provide material for comparative analysis of gymnosperms and angiosperm sequences, ESTs were obtained from immature xylem of loblolly pine. A total of 1097 single-pass sequences were obtained from cDNA libraries made from graviestimulated tissue from bent trees. About 10% of the recognised genes encode factors involved in cell wall formation. Sequences similar to cell wall proteins, most known lignin biosynthetic enzymes, and several enzymes of carbohydrate metabolism were found. A number of putative regulatory proteins also are represented. Two kind of studies have been performed. cDNAs coding for six novel cell wall associated proteins, as well as a homolog for a phytocyanin, have been identified and characterised from differentiating xylem of loblolly pine. Three cDNAs encode new putative loblolly pine arabinogalactan proteins based on their structural similarity to classical AGPs. One cDNA is related to proline-rich protein group and the other two to glycine-rich protein group and the mussel adhesive protein. Moreover, we have isolated and characterised, from pine xylem cDNA libraries, a set of clones representing a new subgroup of bHLH proteins. Basic helix-loop-helix (bHLH) proteins are an important class of regulatory proteins involved in control of cell fate in animals and yeast, and in the control of pigment synthesis in plants. Analysis of pine genomic DNA detects a single gene, suggesting that they are alternative splicing products derived from one gene.

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Extracellular proteins produced during pea (*Pisum sativum* L.) early fruit development induced by gibberellins.

Manuel Rodríguez-Concepción, Alicia Pérez-García and José Pío Beltrán

Instituto de Biología Molecular y Celular de Plantas (U.P.V.-C.S.I.C.). Camino Vera s/n, 46022 Valencia, Spain. E-mail: jbeltran@ibmcp.upv.es

The pea (*Pisum sativum* L.)is self-fertile and its flowers are usually self-pollinated. Pollination takes place one day before anthesis (day-1), and at anthesis (day o) fertilization of the ovules has ocurred. Induction of fruit set and development in pea is regulated by gibberellins (GAs) after fertilization of the ovules. Both, pollination and fertilization can be prevented by removing the anthers from the flower two days before anthesis (day-2). The resulting unpollinated carpels keep growing until day+2. Afterwards, both growth and assimilate import by the carpel cease and eventually carpels lose weight and degenerate. Before they enter senescence, unpollinated carpels can be stimulated to develop by exogenous application of gibberellic acid, giving rise to seedless parthenocarpic fruits with the same morphology as those arisen from fertilization. Treatment with GA3 transforms the carpel in a strong sink organ in which the activated metabolism supplies energy for cell division and expansion processes that result in fruit growth. The molecular mechanisms that control the transformation of carpels into fruit (fruit set) and the events that result in fruit growth and development are poorly understood despite these processes are relevant from the agricultural point of view.

The transition from the carpel of the flower to a developing fruit is not well characterized process despite its agricultural importance. We have identified three genes, *GIC17*, *GIC19* and *GIC4*, that are expressed after pea fruit set is induced by gibberellins. Northern analysis and *in situ* hybridization studies showed that *GIC17* and *GIC19* are predominantly expressed in growing carpels and young fruit, whereas *GIC4* transcripts are found in growing tissues throughout the plant. Analysis of their sequences and localization of fusion proteins with GFP indicate that GIC17, GIC19, and GIC4 are novel cell wall proteins. Like GIC17, GIC19 is a small proline-rich protein with no homology to other reported proteins. GIC4 belongs to a new family of proteins of unknown function. Our results support the current model of gibberellin mode of action during pea fruit set and development.

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ELI1, a gene required for correct secondary cell wall deposition and lignification in *Arabidopsis*

Ana I. Caño-Delgado and Mike W. Bevan

Xylem is the tissue transporting water and solutes in higher plants. Differentiation of xylem cells involves the formation of a secondary cell wall, which contains lignin. We are interested in understanding the molecular mechanism that leads to lignin deposition and regulates xylem formation in higher plants. The *Arabidopsis thaliana* primary root is an ideal model system to study xylem formation. The pattern for xylem development is established during embryogenesis and the number and organization of xylem elements is quite regular and conserved in the primary root.
Using a genetic approach, we screened for mutants with an altered pattern of lignification in the primary root of *Arabidopsis thaliana* seedlings. Different mutants have been isolated and complementation groups have been established. One of the mutants, *eli1* (ectopic lignification) has been extensively characterized. *Eli1* is a single locus recessive mutation, which has been mapped using SSLP microsatelites markers in chromosome five. When stained with phloroglucinol, *eli1*shows ectopic lignin in non-xylem cells. Transmission Electron Microscopy revealed that mosaic distribution of lignin in *eli1* occurs with an altered cell wall thickening. Confocal microscopy has shown that *ELI1* is required to maintain the continuity of the xylem strands in the primary root. Isolation of the *ELI1* gene will provide new insights in the molecular mechanism of cell differentiation during xylem development.

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Expansin controls the organ size and morphogenesis in Arabidopsis

Cho, Hyung-Taeg and Cosgrove, Daniel J.
Department of Biology, Pennsylvania State University, USA

Cell enlargement is one of the basic processes in plant growth and development. As expansins are likely to enlarge a cell by their wall-loosening ability, we have examined the role of expansins during development of *Arabidopsis thaliana* by using transgenic experiments. To avoid lethal or pleiotropic effect and specify the role of expansin on particular tissues or organs, *AtEXP10*, which is mainly expressed in the growing leaf, inflorescence stem, and silique, has been targeted. Various combinations of promoters and sense and antisense sequences from *AtEXP10* were tried to demonstrate gene-specific antisensing and over-expression effects on Arabidopsis development. Antisense lines showed small rosette size due to short leaf petiole and blade length and acquired curled or twisted leaf morphology. The *AtEXP10* promoter::*AtEXP10*-specific antisense construct induced a more severe morphological change in the leaf than the *AtEXP10* promoter::*AtEXP10*-coding region antisense construct. The small plant size and aberrant leaf morphology in *AtEXP10* promoter::antisense lines may result from the retardation of cell expansion in the growing leaf petiole and in the leaf veins where the expression pattern of *AtEXP10* was shown by *AtEXP10* 5'::GUS. On the other hand, sense plants have longer leaf petiole and bigger leaf blade. The *AtEXP10*-specific antisense construct driven by a strong CaMV 35S promoter resulted in even smaller rosette size and shorter silique than did other antisense constructs. Our results indicate that expansins are involved in determination of plant organ size and morphogenesis, most likely via their unique cell wall-loosening ability.

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Characterization of an extracellular protease preferentially expressed in young leaves

David Messdaghi, Dortje Golldack, Martina Holt, Karl-Josef Dietz

Lehrstuhl für Stoffwechselphysiologie und Biochemie der Pflanzen, Universität Bielefeld, Universitätsstraße 25, 33501 Bielefeld, Germany, e-mail: karl-josef.dietz@biologie.uni-bielefeld.de

Intercellular washing fluid (IWF) of leaves of all analyzed higher plants contained a protease activity cleaving the artificial substrate mca-Pro-Leu-Gly-Leu-Dnp-Ala-Arg (MCA substrate). However, the specific activity differed between the species. Inhibitor studies suggest that the protease belongs to the group of serine proteases. The most potent inhibitors were PMSF and chymostatin. Addition or chelation of Ca^{++} had no effect on protease activity. Extracellular compartmentation was established on the basis of a 26-fold increased specific activity of the MCA-dependent protease in IWF as compared to crude extracts. The studies then concentrated on tobacco and barley. Potease activity was maximal in young leaves and decreased with leaf age suggesting a preferential role in early leaf development. Chymostatin-sensitive MCA-cleaving activity was also detected in roots and shoots. Adverse growth conditions such as NaCl- and Cd-stress, high light or etiolation regimes slightly affected the protease activity. The molecular characterization was initiated. As determined by size exclusion chromatography the molecular mass of the protease(s) is 67 kDa. The protease protein(s) focuss(es) at a pH of 7.3. Presently, the purification of the tobacco enzyme is progressing in parallel with an analysis in respect to crossreactivity with anti P69 antibody raised against a pathogen induced serine protease in tomato (the antibody was kindly provided by P. Vera: Tornero P, Conejero V, Vera P J. Biol. Chem 272: 14412, 1997). These results in addition to first attempts to identify natural substrates will be presented.

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Functional cloning of protein substrates for p69A, a subtilisin-like protease from tomato plants.

Lucía Jordá, Mailin Sotolongo and Pablo Vera.
Instituto de Biología Molecular y Celular de Plantas (I.B.M.C.P). Universidad Politécnica de Valencia-C.S.I.C. 46022-Valencia, Spain.

Subtilisin-like proteases are an evolutionarily conserved type of enzimes. Recently, some subtilisin-like proteases have been described in plants but very little is known about their biological function.

In tomato, subtilisin-like proteases are represented by a multigene family called P69s. These P69-proteases are located in the plant extracellular matrix (ECM), where they presumably carry out its function. To understand P69 function in plants, the finding and characterization of protein substrates recognized and processed by this type of enzimes is mandatory.

Here we described a functional approach to directly isolate proteins recognized by P69 proteases. The method consist of the identification of individual cDNA clones that codes for a protein that after *in vitro* transcription and translation is specifically cleavaged by a member of the P69 clan of proteases.

We will present some preliminary characterization of a few of these protein substrates.

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Transient and stable specific expression of zmHyPRP promoter in maize and tobacco embryos respectively.

José-Estanyol, M., Puigdomènech, P.

Departament de Genètica Molecular. Centre d'Investigació i Desenvolupament. CSIC. c/. Jordi Girona Salgado, 18-26. 08036- Barcelona.

zmHyPRP is a proline-rich protein with a signal peptide to be exported to the cell wall, a central proline-rich domain and a C-terminal domain non repetitive, and with eight cysteines placed in a special pattern when compared with related proteins. *zmHyPRP* is expressed specifically in maize embryos until the maturation and desiccation stages are attained. The presence of an ABA responsive element in its promoter allows us to suggest that the gene is repressed by this hormone. Different experimental results appears to confirm this theory. In maize ABA deficient embryos as *vp2*, *zmHyPRP* is expressed until the germination process in the plant is initiated and when immature embryos are incubated with exogenous ABA, *zmHyPRP* expression is repressed. *zmHyPRP* by "*in situ*" hybridization shows to be expressed in the scutellum as well as in the axis cortex of maize embryo. We have studied the promoter elements able to allow specific expression of *zmHyPRP* by stable expression in maize and tobacco. In tobacco transformed plants GUS expression is initiated when cotyledon and cortex cells begin to be developed after heart stage differentiation. Transient expression studies by bombardment of maize embryos show that deletions of *zmHyPRP* promoter successisvely diminish the GUS expression. In maize embryos, at the beginning of the maturation stage, expression is clearly reduced three times with respect immature embryos. Similar results are obtained when immature embryos have been previously treated with exogenous ABA. Results indicate that 2Kb of *zmHyPRP* promoter are able to drive specific GUS expression in tobacco transgenic plants. Experiments in course will allow to better define the promoter specific regulatory elements and the immunolocalization of the protein.

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CELL WALL CHANGES ASSOCIATED WITH TRACHEID DIFERENTIATION

Milioni D., Stacey N.J., Sado P., Roberts K. and McCann M.C.
Cell Biology Department, John Innes Centre

Development of xylem involves several fundamental processes of plant growth and development, including cell division, cell expansion, formation of secondary cell walls (involving synthesis of cellulose, hemicellulose and lignin) and programmed cell death.

Our aim is the identification of differentially expressed genes during the period of cell fate determination and the systematic characterization of genes involved at early stages of xylem differentiation and wood formation. These genes would be of great importance for forestry, industry, paper and pulp products

Single mesophyll cells of Zinnia, in liquid culture, can transdifferentiate into dead lignified tracheary elements. Differentiation is induced by auxin and cytokinin, but nothing is known about the molecular mechanism involved. This system is unique in allowing access to a synchronized set of plant cells, that become determined and then differentiate with a high degree of reproducibility. This model system is extremely useful for studies of the precise sequence of events in tracheary element differentiation. An RNA fingerprinting method (cDNA-AFLP) is currently being used in order to obtain information about differential gene expression as a response to the growth factors immediately after the point of cell determination and at later time points when secondary walls are being deposited.

Preliminary results indicate that a large number of genes are either induced or suppressed during the period of cell fate determination and wood formation. Two hundred (200) different primer combinations have been tested (approximately 40% of total number of combinations) and; in order to choose genes for further analysis; 180 bands were isolated and sequenced. Almost 50% of the cDNA clones, analysed so far, can be assigned a cellular role on the basis of sequence similarity to proteins with known function using a BLASTX search.

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Two *Arabidopsis* Cinnamoyl CoA reductase genes are differentially regulated by abscisic acid and throughout development

Lars Østergaard, Henrik Næsted, Ole Mattsson, and John Mundy

Cinnamoyl CoA Reductase (CCR) is the first committed enzyme in lignin biosynthesis. Previous studies in *Eucalyptus* and tobacco have implicated a key role for this enzyme in regulating lignin production. Two tandemly repeated genes were identified on *Arabidopsis* chromosome II encoding CCRs (*Atccr1* and *Atccr2*) with 87% of amino acid identity. The promoter regions, however, are highly divergent indicating alternative regulation of the *ccr* genes. We initially analysed their expression by Northern blots and found strong *Atccr* induction by abscisic acid (ABA), drought, and heat shock, and a high expression level in seeds, thus resembling the expression pattern of late embryogenic abundant (*lea*), ABA-responsive genes. Differential regulation of the two CCR promoters was observed in transgenic plants expressing GUS behind the promoters and the regulation of both was found to be under tight developmental control: No activity of the AtCCR1 promoter was detected in lignified tissue of GUS-stained stem sections. However, the promoter was highly responsive to ABA and heat shock and showed a dramatically high activity in embryos of dormant seeds. *Atccr2* on the other hand did not respond to the above mentioned stimuli and was not expressed in seeds; however, GUS-staining of 10 days old plants and stem sections of four weeks old plants revealed constitutive expression in lignified tissue. We therefore propose that whereas AtCCR2 is involved in lignification of the vascular tissue in older tissue, AtCCR1 is involved in synthesis of the vascular tissue of germinating embryos.

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Characterisation of an *Arabidopsis thaliana* mutant affected in cell elongation.

Pagant, S. [1], Bichet A. [1], Fagard, M. [1], McCann M. [2], Desprez T. [1], Durand N. [1], Vernhettes S. [1], Höfte H. [1]
pagant@versailles.inra.fr

[1] Laboratoire de Biologie Cellulaire, INRA, Route de Saint-Cyr, 78026 Versailles cedex, France.
[2] Department of cell Biology, John Innes centre, Norwish Research park, Colney, Norwich, NR4 7UH, UK.

How cell wall polysaccharides are synthetised and assembled in growing cells is poorly understood. We have conducted screens for EMS and T-DNA mutants with reduced hypocotyl elongation, which has yielded so far over 150 mutants. We are now focusing our analysis on mutants with reduced cell elongation and increased radial expansion and most of these mutants have a reduced cellulose content. In this poster we present the characterisation of T-DNA mutant called *cgu9* that shows a dwarf phenotype in light and dark conditions. Microscopic analysis of this mutant revealed an abnormal hypocotyl structure with thin cell walls and tissue disruptions.The *cgu9* mutant also presents particular and specific responses to phytohormones involved in cell elongation. The most striking phenotype is obtained in the presence of brassinosteroïds. This mutant which is not deficient in BR synthesis, shows an abnormal exaggerated lateral expansion at the basis of the hypocotyl leading to a loss of internal tissues.
In order to know if the cell wall defects were provoked by an abnormal organisation of the microtubules, immunolabelling studies have been realised and showed that microtubules orientation are not affected. Using micro-FTIR spectroscopy, preliminary results suggest that cgu9 is deficient in cellulose content. These data must be confirmed by more accurate FTIR and biochenical analysis of the cell wall.
Genetic analysis revealed that the mutation is recessive, monogenic and closely linked to the T-DNA insertion which is between the markers mi403 and Fad7 on the chromosome 3. A plamid-rescue strategy allowed us to obtain and sequence the genomic sequence flanking the insertion. Homology has been found in data sequences with a putative protein of Arabidopsis coming from the systematic genomic sequencing. To better understand the involvment of CGU9 in cell elongation, cgu9 expression and localisation will be investigated.

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Possible functions of *O*-acetyl substituents on homogalacturonan: Inhibition of degradation by *endo*-polygalacturonases and pectinmethylesterases

Markus Pauly[1], Carl Bergmann[2], Peter Albersheim[2], Alan Darvill[2], and Henrik Vibe Scheller[1]

[1]Plant Biochemistry Laboratory, The Royal Agricultural and Veterinary University, Thorvaldsensvej 40, 1871 Frederiksberg, Copenhagen, Denmark
[2]Complex Carbohydrate Research Center, University of Georgia, 220 Riverbend Rd., Athens, GA, 30602-4712, USA

The pectic polysaccharides in cell walls are often *O*-acetylated. *O*-acetyl residues have been found in some homogalacturonan preparations as well as on the backbone galacturonosyl-residues of rhamnogalacturonan I. Little is known about the function of the *O*-acetyl substituents *in vivo*. Research on *O*-acetylated rhamnogalacturonan preparations has shown that *O*-acetylation significantly inhibits the degradation of this polysaccharide by *endo*hydrolases and endolyases. Studies on chemically *O*-acetylated homogalacturonans demonstated that *O*-acetyl groups inhibit degradation by endohydrolases. However, the *O*-acetyl substituents on chemically *O*-acetylated pectins may be randomly distributed, in contrast to pectins in the walls, where a blockwise distribution is possible. Furthermore, chemically *O*-acetylated homogalacturonans usually have a much higher degree of *O*-acetylation than homogalacturonan found in cell walls.

In an attempt to shed further light on the function of *O*-acetyl substituents on native pectins, homogalacturonan, prepared from sugarbeet walls, was subjected to methyl- as well as acetylesterase treatment, removing the substituents and resulting in homogalacturonans with varying degrees of methylesterification and acetylation. Using these esterases it could be shown that *O*-acetylation affects the action of pectinmethylesterase and vice versa that methylesterification affects the action of acetylesterase. The homogalacturonan preparations with varying degrees of methylesterification and acetylation were then further digested with various *endo*polygalacturonases (EPGs) that exhibit *endo* as well as *endo*/*exo* attack cleavage modes. Analysis of the digestion products using HPAEC-PAD demonstrates that *O*-acetylation affects degradation of the native wall homogalacturonans. Possible biological interpretations of the obtained results are discussed.

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Xyloglucan Fucosyltransferase, a Model Plant Cell Wall Biosynthetic Enzyme

Perrin R.M.*, Sarria R.*, Faik A.*, Zeng W.*, Vanzin G.˙, Reiter W.-D.˙, Norambuena L.†, Orellana A.†, Raikhel N.V.*, Keegstra K*.

* Michigan State University-Department of Energy Plant Research Laboratory

† University of Chile, Santiago

˙ University of Connecticut

Arabidopsis thaliana fucosyltransferase 1, or *AtFT1*, is a plant cell wall biosynthetic gene which synthesizes the α-1,2 fucose:galactose linkage on xyloglucan, a major hemicellulose of dicot and non-graminaceous monocot cell walls. This gene was identified when pea fucosyltransferase was purified biochemically, allowing us to obtain partial amino acid sequence. The pea FTase peptides were not significantly homologous to enzymes of known function in the databases but did allow the identification of an *Arabidopsis* EST that contained four of the pea FTase peptides with 63%-83% identity. Demonstration of the functionality of AtFT1 as a xyloglucan fucosyltransferase was accomplished by 1) immunoprecipitation of FTase activity from solubilized *Arabidopsis* microsome proteins using antibodies directed against recombinant AtFT1, and 2) heterologous expression of *AtFT1*in Cos-7 mammalian cells, which resulted in XG FTase in these cells but not in controls lacking *AtFT1* expression. These data were recently published (Perrin *et al.*, 1999, Science 284:1976.) Studies to investigate the expression of *AtFT1* and the effects of altering its expression *in vivo* are underway. Several other *Arabidopsis* ESTs have been identified which are highly homologous to *AtFT1* and which may represent other fucosyltranferases. The expression patterns and functions of these homologs are under investigation. In addition, *mur2-1*, a cell wall mutant that has a 50% overall reduction in fucose levels in its cell wall, has been identified as having a point mutation in *AtFT1*. The effect of this point mutation is to cause a D to N change at the amino acid level, creating an N-glycosylation consensus sequence in the mutant where there is none in the wild type. The lack of fucose residues appears to be specific to xyloglucan (Vanzin and Reiter, unpublished results.) It is hoped that studies of this gene may ultimately allow the identification of other genes involved in plant cell wall biosynthesis.

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GRADIENTS OF TENSILE CELL WALL STRAIN ALONG GROWING ZONES IN HIGHER PLANTS

Winfried S Peters
AK Kinematische Zellforschung, Biozentrum der JW Goethe Universitaet
Marie-Curie-Str. 9, D-60439 Frankfurt, GERMANY
☎ +49 (0)69 79829482 Fax +49 (0)69 79829607 e-mail w.s.peters@zoology.uni-frankfurt.de

In plants growth usually is restricted to defined growing zones. It is here shown that the growing zones of maize seedlings (root tip, subnodular mesocotyl, whole coleoptile, and primary leaf base) correlate with zones, in which turgor-induced tensile strain peaks. Since available evidence suggests that turgor is practically constant along growing zones, it is concluded that high elastic cell wall extensibility correlates with high growth rates. The existence of gradients of tensile strain along growing zones is discussed in various contexts: **1)** Gradients of turgor-dependent strain might be responsible for artifacts in the determination of spatial growth patterns from histological data. The discrepancy between "flat-max" *versus* "single-peak" interpretation of grass leaf growing zones is probably caused by such an artifact. **2)** If we define growth as *irreversible* size increase, growth rates might be underestimated in growing zone portions, that coincide with decreasing strain gradients. Model calculations performed on data from root growing zones will be presented. **3)** The derivation of the famous "Lockhart-equation" rests on the assumption, that relative growth rates with respect to turgescent (i.e. strained) and plasmolized sizes are equal. This implies that no strain gradients exist along growing zones. It will be evaluated under which circumstances Lockhart's approach can still be applied to higher plant organs. **4)** The general significance of the existence of strain gradients for the cellular mechanism of plant organ growth will be briefly discussed.

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Modified Levels of Pectin Methyl Esterase in Transgenic Potato Plants Affect Stem Elongation and Ion Partitioning

Pilling, J., Gipmans, M., Willmitzer, J. and Fisahn, J.
Max-Planck-Institute of Molecular Plant Physiology, Karl-Liebknecht-Str.25, 14476 Golm, Germany

Pectin methyl esterases (EC 3.1.1.11) are enzymes which catalyse the de-esterification of pectin. They release methanol, increase the charge density of the cell walls, are thought to facilitate degradation of pectin and, thus, have been discussed to be of great importance for the physiology of plants. However, so far use of trangenic plants to elucidate the exact physiological role of PME *in vivo* was restricted to fruit ripening and root border cell separation.
This present study describes two complementary approaches which aimed at both the ubiquitous reduction and the ubiquitous elevation of PME activity in potato plants. Two cDNAs encoding potato PMEs were isolated and used to inhibit enzyme activity in transgenic plants. Selected lines displayed reduced stem elongation rates throughout their development, an increase in cell wall galacturonic acid as well as modifications in the ion binding capacity of their walls. Moreover, apoplastic and total ion concentrations differed between leaves of PME inhibited and wild-type plants. Interestingly, juvenile potato plants which constitutively overexpressed a *Petunia inflata* PME showed enhanced stem elongation rates. Cell walls of these plants also had modified ion binding capacities while no structural changes of their pectin was detected.

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The glycine-rich structural protein GRP1.8 of bean shows hydrophobic interactions in the cell wall

Christoph Ringli and Beat Keller
University of Zurich, Institute of Plant Biology, 8008 Zurich, Switzerland

Glycine-rich proteins (GRPs) represent a major class of plant structural proteins and are often localized in the cell wall matrix. GRP1.8, a glycine-rich protein of bean (*Phaseolus vulgaris*), has been shown to be expressed in the vascular tissue and is an abundant protein in the modified primary cell wall of protoxylem elements. In this study, we have investigated the interaction of GRP1.8 in the cell wall. Different domains of GRP1.8 were fused to a reporter protein and the fusion proteins were expressed in the vascular tissue of transformed tobacco. Through different extraction procedures we have investigated the interaction of the fusion proteins in the cell wall matrix compared to the reporter protein alone. Our results suggest a hydrophobic interaction of GRP1.8 in the cell wall. The different GRP1.8 domains conferred the same property to the respective fusion protein, reflecting the homogeneous, repetitive amino acid sequence of the GRP1.8 protein. The extraction of bean cell wall fractions and subsequent immunoblot analysis with anti-GRP1.8 antiserum suggests hydrophobic properties of the endogenous GRP1.8, confirming the conclusions made from the analysis of the fusion proteins in tobacco. These results reveal a so far unknown characteristic of GRP1.8 and are in agreement with the proposed role of this protein in a repair mechanism of protoxylem elements.

Instituto Juan March (Madrid)

Andrea Celia Porchia, Nicolai Obel, Markus Pauly, and Henrik Vibe Scheller

Characterization of xylan xylosyltransferase and arabinosyltransferase from wheat.

The biosynthesis of arabinoxylan was investigated in seedlings of wheat (*Triticum aestivum* L.). Initially it was determined that in very young seedlings (3 days old), the non-cellulosic wall polysaccharides are comprised principally of highly substituted arabinoxylan (Ara/Xyl ≈0.8). The arabinoxylan appeared to contain significant amounts of ester-linked hydroxycinnamates. Xylan 4-ß-xylosyltransferase activity involved in the formation of arabinoxylan was identified in microsome preparations from the wheat seedlings. Incubation of wheat membranes with UDP-[^{14}C]xylose resulted in an incorporation of [^{14}C]xylose into a product insoluble in 70% ethanol. Incorporation reached a maximum at day 3 during development of the seedlings. Optimal synthesis of product occurred at a pH of 6.7-7.5 and at 30 °C. The presence of 1 mM $MgCl_2$ or $MnCl_2$ yielded maximal stimulation of activity while no effect of $CaCl_2$ was detected. Different analytical techniques were used to show that polymeric xylan accounts for at least 70 % of the ^{14}C-labeled product formed. The product contained only negligible amounts of radiolabelled arabinose indicating that epimerization of UDP-xylose did not take place to any significant extent. The TLC migration patterns obtained for xylanase-treated product and xylanase plus arabinofuranosidase-treated product resulted in a migration of radioactivity to positions similar to xylobiose, xylotriose and xylotetraose. These results show that the radioactive xylose was incorporated into regions devoid of arabinosyl substituents. The radioactive product was also solubilized by ß-xylosidase but it was not digested with *endo*-polygalacturonase, *endo*-xyloglucanase or proteinase-K which can attack other xylose containing polymers. The solubilization by ß-xylosidase suggests that xylan chains grow at the non-reducing end. Radiolabelled UDP-L-arabinose was produced from UDP-D-xylose using an preparation of soluble UDP-xylose-4-epimerase from wheat germ. The UDP-arabinose was purified by a new chromatographic procedure and shown by NMR to be in the pyranose configuration. Preliminary investigations of xylan arabinosyltransferase will be presented.

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MOLECULAR ANALYSIS OF *AtCelA1*, A MEMBER OF THE CELLULOSE SYTHASE CATALYTIC SUBUNIT GENE FAMILY

Xuan Wang, Rudy Vanderhaeghen, Gerda Cnops, Sabine De Block, Dimitri Tack, Marc Van Montagu, and Mieke Van Lijsebettens. Department of Genetics, affiliated to the Flanders Interuniversity Institute for Biotechnology (VIB), University of Gent, K. Ledeganckstraat 35, 9000 Gent, Belgium. Email: milij@gengenp.rug.ac.be

Cellulose is the most abundant biopolymer being part of the primary and secondary cell wall of plant cells. Recently, experimental proof for the cloning of cellulose synthase catalytic subunit (*CelA*) genes has been obtained for two cotton fiber-specific cDNA's (Pear et. al., 1996) and for the *RSW1 Arabidopsis* genomic sequence of which T-DNA tagging resulted in radial swelling of cells. (Arioli et al., 1998). Data base analysis revealed multiple celA genes in the *Arabidopsis* genome represented by at least 15 different cDNA clones (EST's) closely related to the cotton *CelA* cDNA's (Cutler and Somerville, 1997).

We cloned a member of the *CelA* gene family by T-DNA tagging. The gene, *Arabidopsis thaliana CelA1* (*AtCelA1*), was mapped by using a set of 100 Recombinant Inbred Lines. The Southern analysis showed that one T-DNA copy is present in the genome. A specific mutant phenotype in the root epidermis has been identified in this line. A 12 kb genomic region corresponding to the *AtCelA1* gene was cloned and sequenced. The *AtCelA1* gene consists of four exons and three introns as shown by the gene prediction program GeneMark, the merged open reading frame has a high homology with *CelA* genes of *Arabidopsis*, cotton and poplar. The 12 kb genomic fragment as well as a 5.38 kb sub-fragment, which only contained the *AtCelA1* gene, were transformed into the mutant and restoration of the wild type phenotype was obtained, showing that the phenotype is due to T-DNA insertion into the *AtCel1* gene. A few mutants in *Arabidopsis* have been described with a cell-specific defect due to mutation in secondary cell wall cellulose synthesis, such as the *trichome birefringence* mutation (Potikha and Delmer, 1995) and the *irregular xylem3* mutant (Taylor et al., 1999). These mutants and ours indicate that *CelA* genes might be regulated at the transcriptional level. Expression studies are being done to study cell specificity of the *AtCelA1* gene activity.

With these studies we will make a significant molecular-genetic contribution to the postulation that the plant cell wall is very important in cell morphogenesis processes.

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Side chains of pectic polysaccharides are regulated in relation to cell proliferation and cell differentiation

William G. T. Willats, Clare G. Steele-King, Sue E. Marcus, J. Paul Knox

Centre for Plant Sciences, Leeds Institute for Plant Biotechnology and Agriculture, University of Leeds, Leeds, LS2 9JT, United Kingdom.

Pectic polysaccharides consist of a galacturonan-rich backbone to which side chains rich in neutral sugars are attached, principally at domains in which the backbone contains alternating residues of galacturonic acid and rhramnose (rhramnogalacturonan, RG I). Whilst the structure and biological roles of backbone domains of pectin have been extensively investigated, the roles of the side chains of pectic polysaccharides are poorly understood.

The occurrence and function of the side chains of pectic polysaccharides were investigated during carrot cell development using defined monoclonal antibodies to (1→4)-β-D-galactan and (1→5)-α-L-arabinan. Immunolocalization studies of carrot root apices indicated that cell walls in the central region of the meristem contained higher levels of (1→5)-α-arabinan than the cell walls of surrounding cells. In contrast, (1→4)-β-galactan was absent from the cell walls of meristematic cells but appeared abundantly at a certain point during root cap cell differentiation and also appeared in cell walls of differentiating stele and cortical cells. This developmental pattern of epitope occurrence was also reflected in a suspension-cultured carrot cell line that can be induced to switch from proliferation to elongation by altered culture conditions. (1→4)-β-galactan occurred at a low level in cell walls of proliferating cells but accumulated rapidly in cell walls following induction, before any visible cell elongation, while (1→5)-α-arabinan was present in cell walls of proliferating cells but was absent from cell walls of elongated cells. Immunochemical assays of the cultured cells confirmed the early appearance of (1→4)-β-galactan during the switch from cell proliferation to cell elongation. Anion-exchange chromatography confirmed that (1→4)-β-galactan was attached to acidic pectic domains and also indicated that it was separate from a distinct homogalacturonan-rich component. The observations reported here present a new perspective on the biological roles of pectic polysaccharides and demonstrate that heterogeneous populations of RG I are developmentally regulated and may have important roles in plant cell development.

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Arabidopsis thaliana α-xylosidase. Gene sequence and developmental regulated expression.

J. Sampedro[1], C. Sieiro[2], G. Revilla[1], T. González-Villa[1] and I. Zarra[1].

[1]Universidad de Santiago de Compostela. [2]Universidad de Vigo. Spain.

Xyloglucan oligosaccharide α-xylosidase is an enzyme that specifically cleaves terminal α-xylosyl residues attached to the C-6 of glucose on the non-reducing end of the molecule. It is involved both in the mobilisation of seed xyloglucan and in the catabolism of the xyloglucan present in primary cell walls. In this later case it could also have a noteworthy role in the complex control mechanism of plant cell growth.

An α-xylosidase active against xyloglucan oligosaccharides was purified from cabbage leaves. Its Mr was 89.000. The N-terminus and an internal fragment of the amino acid sequence were determined. Two *Arabidopsis* ESTs (G10B11T7 and H8A7T7) were identified as fragments of a gene encoding *Arabidopsis* α-xylosidase. The entire sequence of the longer clone (H8A7) was obtained and submitted to GENBANK (accesion no. AF087483). Furthermore, an IGF-BAC clone (F22C21) that encompasses the entire α-xylosidase gene has been localised and mapped to chromosome I, between markers pCITf117 (100.44 cM) and mi462 (107.48 cM). We propose to call this gene XYL-1. A 4288 bp fragment comprising the coding region was sequenced and submitted to GENBANK (accesion no. AF144078). The product of XYL-1 has 915 amino acids, a MW of 102 kD, and a potential signal peptide. From the N-terminal sequence of cabbage α-xylosidase, it seems that after excision of the signal peptide the enzyme loses a 96 amino acids propeptide. It belongs to family 31 of glycosil hydrolases and it is closely related to plant acidic α-glucosidases.

We have found α-xylosidase to be an apoplastic enzyme, specially abundant in actively growing tissue. The gene promoter includes sequences with high similarity to light regulated elements. The first eight true leaves from 19 days old *Arabidopsis* plants were divided in four groups according to their appearance order. Xyl-1 expression, as measured by Northern blots, is higher in younger, fast-growing leaves, as would be expected for an enzyme involved in xyloglucan metabolism. Similar results were obtained for α-xylosidase specific activity.

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LIST OF INVITED SPEAKERS

Antony Bacic Plant Cell Biology Research Centre, School of Botany, University of Melbourne, Parkville 3052 (Australia). Tel.: 61 3 9344 5041. Fax: 61 3 9347 1071. E-mail: A.Bacic@botany.unimelb.edu.au

Alain-M. Boudet UMR CNRS/UPS 5546, Pôle de Biotechnologie végétale, BP 17 Auzeville, F-31326 Castanet Tolosan cedex, (France). Tel.: 33 5 62 19 35 21. Fax: 33 5 62 19 35 02. E-mail: amboudet@cict.fr

Nicholas C. Carpita Dept. of Botany & Plant Pathology, Purdue University, West Lafayette, IN. 47907 (USA). Tel.: 1 765 494 4653. Fax: 1 765 494 0363. E-mail: carpita@btny.purdue.edu

Daniel Cosgrove Department of Biology, Penn State University, 208 Mueller Lab., University Park, PA. 16802 (USA). Tel.: 1 814 863 3892. Fax: 1 814 865 9131. E-mail. dCosgrove@psu.edu

Sean R. Cutler Dept. of Biological Sciences, Stanford University, and Carnegie Institution of Washington, Dept. of Plant Biology, 260 Panama Street, Stanford, CA. 94305 (USA). Tel.: 1 650 325 1521. Fax: 1 650 325 6857. E-mail: cutlers@Andrew2.stanford.edu

Giulia De Lorenzo Dipartimento di Biologia Vegetale, Università di Roma "La Sapienza", Piazza A. Moro 5, 00185 Rome (Italy). Tel.: 39 06 499 12 454. Fax: 39 06 499 12 446. E-mail: delorenzo@axrma.uniroma1.it

Deborah P. Delmer Section of Plant Biology, University of California Davis, One Shields Avenue, Davis, CA. 95616 (USA). Tel.: 1 530 752 5561. Fax: 1 530 752 5410. E-mail: dpdelmer@ucdavis.edu

Concha Domingo IBMCP, Univ. Politécnica-CSIC, Camino de Vera s/n., 46022 Valencia (Spain). Fax: 34 96 387 78 59. E-mail: cdomingo@ibmcp.upv. es

Herman Höfte Laboratoire de Biologie Cellulaire, INRA, Route de St Cyr, 78026 Versailles cedex (France). Tel.: 33 1 308 33 390. Fax: 33 1 308 33 099. E-mail: hofte@versailles.inra.fr

Beat Keller Institute of Plant Biology, University of Zürich, Zollikerstrasse 107, 8008 Zürich (Switzerland). Tel.: 41 1 634 8230. Fax: 41 1 634 8204. E-mail: bkeller@botinst.unizh.ch

Bruce D. Kohorn DCMB/Botany, Duke University, Durham, NC. 27708 (USA). Tel.: 1 919 613 8182. Fax: 1 919 613 8177. E-mail: kohorn@duke.edu

Instituto Juan March (Madrid)

Chris Lamb	John Innes Centre, Norwich Research Park, Colney Norwich, NR4 7UH (U.K.). Tel.: 44 1603 45 25 71. Fax: 44 1603 45 68 44. E-mail: Chris.Lamb@bbsrc.ac.uk
Maureen C. McCann	Dept. of Cell Biology, John Innes Centre, Colney, Norwich NR4 7UH (U.K.). Tel.: 44 1603 45 2571. Fax: 44 1603 45 6844. E-mail: maureen. mccann@bbsrc.ac.uk
June Nasrallah	Dept. of Plant Biology, Cornell University, Ithaca, NY. 14853 (USA). Tel.: 1 607 255 81 89. Fax: 1 607 255 5407. E-mail: jbn2@cornell.edu
Pere Puigdomènech	Institut de Biologia Molecular de Barcelona, CSIC, Jordi Girona 18, 08034 Barcelona (Spain). Tel.: 34 93 204 06 00. Fax: 34 93 204 59 04. E-mail: pprgmp@cid.csic.es
Ralph S. Quatrano	Dept. of Biology, Campus Box #1137, Washington University, One Brookings Drive, St. Louis, MO. 63130-4899 (USA). Tel.: 1 314 935 6850. Fax: 1 314 935 8692. E-mail: rsq@wustl.edu
John Ralph	US Dairy Forage Research Center, USDA Agricultural Research Service, Madison WI. 53706-1108, and Dept. of Forestry, Univ. of Wisconsin, Madison, WI. 53706-1598 (USA). Tel.: 1 608 264 5407. Fax: 1 608 264 5147. E-mail: jralph@facstaff.wisc.edu
Keith Roberts	John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH (U.K.). Tel.: 44 1603 45 2571. Fax: 44 1603 45 6844. E-mail: keith.roberts@bbsrc.ac.uk
Laurie G. Smith	Biology Dept. 0116, U.C. San Diego, 9500 Gilman Drive, La Jolla, CA. 92093-0116 (USA). Tel.: 1 858 822 2558. Fax. 1 858 534 7108. E-mail: lsmith@biomail.ucsd.edu
Pablo Vera	IBMCP, Univ. Politécnica de Valencia, Camino de Vera s/n, 46022 Valencia (Spain). Tel.: 34 96 387 78 64. Fax: 34 96 387 78 59. E-mail: vera@ibmcp.upv. es
Sacco C. de Vries	Laboratory of Molecular Biology, Agricultural University Wageningen, Dreijenlaan 3, 6703 HA Wageningen (The Netherlands) Tel.: 31 317 48 4325. Fax: 31 317 48 3584. E-mail: Sacco.deVries@Mac. MB.WAU. NL
John C. Walker	Div. of Biological Sciences, University of Missouri, 308 Tucker Hall, Columbia, MO. 65211 (USA). Tel.: 1 573 882 3583. Fax: 1 573 882 0123. E-mail: WalkerJ@missouri.edu

Instituto Juan March (Madrid)

LIST OF PARTICIPANTS

José Luis Acebes Dpto. de Biología Vegetal, Lab. de Fisiología Vegetal, Universidad de León, E-24071 León (Spain). Tel.: 34 98 729 14 82. Fax: 34 98 729 14 79. E-mail: dbvjaa@unileon.es

Isabel Allona Dpto. de Biotecnología, E.T.S. I. De Montes, UPM, Ciudad Universitaria s/nº, 28040 Madrid (Spain). Tel.: 34 91 336 64 08. Fax: 34 91 543 95 57. E-mail: iallona@etsi.montes.upm.es

Daniela Bellincampi Dipt. Di Biologia Vegetale, Universitá di Roma "La Sapienza", P.le A. Moro 5, 00185 Roma (Italy). Tel. and Fax: 39 06 499 12 446. E-mail: Bellincampi@axrma.uniroma1.it

José Pío Beltrán Instituto de Biología Molecular y Celular de Plantas (U.P.V. - C.S.I.C.). Camino de Vera s/n. 46022 Valencia (Spain). Tel.: 34 96 387 78 50. Fax: 34 96 387 78 59. E-mail: jbeltran@ibmcp.upv.es

Nicholas J. Brewin Dept. of Genetics, John Innes Centre, Norwich NR4 7UH (U.K.). Tel.: 44 1603 452 571. Fax: 44 1603 456 844. E-mail: nick.brewin@bbsrc.ac.uk

Ana I. Caño-Delgado Molecular Genetics Dept., John Innes Centre, Norwich NR4 7UH (U.K.). Tel.: 44 1603 452 571. Fax: 44 1603 505 725. E-mail: ana.cano-delgado@bbsrc.ac.uk

Felice Cervone Dipt. Di Biologia Vegetale, Universitá di Roma "La Sapienza", Lab. di Fisiologia Vegetale, Piazza A. Moro 5, 00185 Roma (Italy). Tel. and Fax: 39 06 499 12 446. E-mail: Cervone@axrma.uniroma1.it

Hyung-Taeg Cho Dept. of Biology, Pennsylvania State University, 208 Mueller Lab., University Park, PA. 16802 (USA). Tel.: 1 814 865 3752. Fax: 1 814 865 9131. E-mail: hxc31@psu.edu

Karl-Josef Dietz Lehrstuhl für Stoffwechselphysiologie und Biochemie der Pflanzen, Universität Bielefeld, Universitätsstraße 25, 33501 Bielefeld (Germany). Tel.: 49 521 106 5589. Fax: 49 521 106 6039. E-mail: Karl-josef.dietz@biologie.uni-bielefeld.de

Louise Jones School of Biological Sciences, 3.614 Stopford Bldg., Oxford Road, University of Manchester, Manchester M13 9PT (U.K.). Tel.: 44 161 275 3857. Fax: 44 161 275 3938. E-mail: Ljones@man.ac.uk

Instituto Juan March (Madrid)

Lucía Jordá	IBMCP, Universidad Politécnica de Valencia- C.S.I.C., 46022 Valencia (Spain). Tel.: 34 96 387 77 30. Fax: 34 96 387 78 59. E-mail: ljorda@ibmcp. upv.es
Matilde Josè-Estanyol	Departament de Genètica Molecular, Centre d'Investigació i Desenvolupament, CSIC, c/ Jordi Girona Salgado 18-26, 08036 Barcelona (Spain). Tel.: 34 93 204 06 00. Fax: 34 93 204 59 04. E-mail: mjegmp@cid.csic.es
Mª Carmen Marqués	Instituto de Biología Molecular y Celular de Plantas, CSIC, Univ. Politécnica de Valencia, Camino de Vera s/nº, 46022 Valencia (Spain). Tel.: 34 96 387 78 64. Fax: 34 96 387 78 59. E-mail: mmarques@ ibmcp.upv.es
Simon McQueen-Mason	Dept. of Biology, The Plant Laboratory, The University of York, P.O. Box No. 373, York YO10 5YW (U.K.). Tel.: 44 1904 43 43 18. Fax: 44 1904 43 43 12. E-mail: sjmm1@york.ac.uk
Dimitra Milioni	Cell Biology Department, John Innes Centre, Norwich Research Park, Norwich NR4 7UH (U.K.). Tel.: 44 1603 45 25 71. Fax: 44 1603 50 17 71. E-mail: Dimitra.Milioni@bbsrc.ac.uk
Lars Østergaard	Dept. Plant Physiology, Inst. Molecular Biology, Univ. Copenhagen, Øster Farimagsgade 2A, DK-1353 Copenhagen K (Denmark). Tel.: 45 35 32 21 36. Fax: 45 35 32 21 28. E-mail: larsoe@biobase.dk
Silvère Pagant	Laboratoire de Biologie Cellulaire, INRA, Route de Saint-Cyr, 78026 Versailles cedex (France). Tel.: 33 1 30 83 30 21. Fax: 33 1 30 83 30 99. E-mail: pagant@versailles.inra.fr
Markus Pauly	Plant Biochemistry Laboratory, The Royal Agricultural and Veterinary University, Thorvaldsensvej 40, 1871 Frederiksberg, Copenhagen (Denmark). Tel.: 45 352 83 335. Fax: 45 352 83 333. E-mail: mp@ kvl.dk
Robyn M. Perrin	Michigan State University-Department of Energy Plant Research Laboratory, 110 Plant Biology Bldg., East Lansing, MI. 48824 (USA). Tel.: 1 517 353 7876. Fax: 1 517 353 9168. E-mail: perrinr1@pilot.msu. edu
Winfried S. Peters	AK Kinematische Zellforschung, Biozentrum der JW Goethe Universität, Marie-Curie-Str. 9, D-60439 Frankfurt (Germany). Tel.: 49 69 798 29 482. Fax.: 49 69 798 29 607. E-mail: w.s.peters@zoology.uni-frankfurt. de

Instituto Juan March (Madrid)

Jens Pilling	Max-Plant-Institute of Molecular Plant Physiology, Karl-Liebknecht-Str. 25, 14476 Golm (Germany). Tel.: 49 331 5678 8221. Fax: 49 331 5678 8408. E-mail: pilling@mpimp-golm.mpg.de
Rafael Pont-Lezica	UMR 5546 CNRS-UPS, 24 chemin de Borde-Rouge, BP 17 Auzeville, 31326 Castanet-Tolosan (France). Tel.: 33 5 62 19 35 16. Fax: 33 5 62 19 35 02. E-mail: lezica@cict.fr
Christoph Ringli	University of Zürich, Institute of Plant Biology, Zollikerstr. 107, 8008 Zürich (Switzerland). Tel.: 41 1 634 8233. Fax: 41 1 634 8204. E-mail: chringli@botinst.unizh.ch
Henrik V. Scheller	The Royal Veterinary and Agricultural University, Dept. of Plant Biology, 40 Thorvaldsensvej, DK-1871 Frederiksberg C, Copenhagen (Denmark). Tel.: 45 35 28 33 32. Fax: 45 35 28 33 10.
Mieke Van Lijsebettens	Department of Genetics, University of Gent, K. Ledeganckstraat 35, 9000 Gent (Belgium) Tel.: 32 9 264 51 84. Fax: 32 9 264 53 49. E-mail: milij @gengenp.rug.ac.be
Samantha Vernhettes	Laboratoire de Biologie Cellulaire, INRA, Route de Saint CYR, 78026 Versailles (France). Tel.: 33 1 30 83 30 41. Fax: 33 1 30 83 30 99. E-mail: vernhett@tournesol.versailles. inra.fr
William G. T. Willats	Centre for Plant Sciences, Leeds Institute for Plant Biotechnology and Agriculture, University of Leeds, Leeds LS2 9JT (U.K.). Tel.: 44 113 233 31 68. Fax: 44 113 233 31 44. E-mail: pab5wgtw@bmb.leeds.ac.uk
Ignacio Zarra	Universidad de Santiago de Compostela, Fac. de Biología, Lab. Fisiología Vegetal, 15706 Santiago de Compostela (Spain).Tel.: 34 981 56 31 00. Fax: 34 981 59 69 04. E-mail: bvzarra@usc.es

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Instituto Juan March de Estudios e Investigaciones
Castelló, 77 • 28006 Madrid (España)
Tel. 34 91 435 42 40 • Fax 34 91 576 34 20
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