Université de Montréal

Le rôle des constituants pariétaux dans les propriétés biomécaniques du tube pollinique

Par

Élodie Parre

Département de Sciences Biologiques

Faculté des Arts et des Sciences

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Le rôle des constituants pariétaux dans les propriétés biomécaniques du tube pollinique

Présenté par :

Élodie Parre

a été évalué par un jury composé des personnes suivantes :

Président rapporteur : David Morse, PhD

Directeur de recherche : Anja Geitmann, PhD

Membre du jury : Denis Barabé, MSc

Résumé

Les tubes polliniques tout comme les hyphes fongiques ou encore les poils racinaires sont des cellules à croissance apicale. Cette désignation caractérise les cellules dont la croissance se limite à l'extrémité apicale avec une expansion unidirectionnelle. Le tube pollinique représente un modèle pour l'étude des propriétés mécaniques puisqu'elle est capable d'exercer des forces lors de son développement. La synthèse et l'insertion des précurseurs de la paroi est un événement essentiel pour la croissance du tube pollinique, elle doit être à fois suffisamment résistante pour retenir la pression interne mais également assez flexible pour permettre l'élongation.

Le but de mon projet de Maîtrise est de caractériser le rôle des principaux constituants pariétaux dans la biomécanique du tube pollinique, c'est-à-dire dans la capacité de cette cellule à résister, d'une part, au stress de tension exercé par la pression de turgescence interne et par d'autres forces de propulsion, et, d'autre part, aux forces de compression exercées par l'environnement de croissance. En utilisant une approche biomécanique nous avons démontré le rôle direct des pectines et de la callose dans la résistance au stress de tension impliqué dans l'initiation du tube pollinique lors de la germination, mais également le rôle essentiel des pectines lors de l'élongation apicale de la cellule. L'étude de la résistance aux forces de compression par un système unique de micro-indentation a révélé l'existence d'un gradient des propriétés physiques correspondant à un gradient de composition pariétale le long du tube pollinique; gradient que nous avons caractérisé par des marquages spécifiques, en utilisant différentes techniques de microscopie à fluorescence et immunomarquage.

Mots clés :

Callose – Croissance apicale – Cytomécanique – Forces de compression – Forces de tension – Grain de pollen — Paroi cellulaire – Pectine - Tube pollinique

Abstract

Pollen tubes are tip growing cells, similar to fungal hyphae and root hairs. The defining feature of tip growth is that cell expansion is unidirectional, restricted to the cell tip and results in a cylindrical cell shape. The pollen tube represents an excellent model system to investigate cellular biomechanical properties, since it has the capacity to exert forces and is exposed to deformation stress during its growth. Furthermore, these cells are encased in a cell wall thus representing typical living plant cells. Their rapid growth requires the continuous synthesis and deposition of an extracellular layer that is rigid enough to withstand internal turgor pressure and other putative growth driving forces, yet flexible enough to permit the cell to grow.

My Master's project investigates the physical properties of the pollen tube cell wall. I studied the role of its main components in producing the resistance to tension stress created by internal turgor pressure, and compression stress exerted by the surrounding tissue. Using a biomechanical approach, I found that pectin and callose are directly involved in resistance against tension stress during germination, whereas only the former plays a role in the subsequent elongation process. Using micro-indentation, a novel approach to caracterize resistance to compression forces on cellular level, I provide evidence that the pollen tube cell wall exhibits a gradient along the longitudinal axis regarding the mechanical properties. This corresponds nicely to the gradient in molecular composition evidenced with fluorescence microscopy.

Keywords :

Apical growth – Callose – Cell wall – Compression forces – Cytomechaniscs – Pectin – Pollen grain – Pollen tube – Tension forces

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The germination rate was assessed after 2h in liquid medium (♦), medium solidified with 20 $mg.mL^{-1}$ (○) and 40 $mg.mL^{-1}$ (▲) agarose. It was reduced in stiffer media, but moderate amounts of lyticase nevertheless had a significant stimulating effect. However, optimal enzyme concentrations and relative stimulation in stiff medium had a tendency to differ from those observed in liquid medium as summarized in Table V. **87**

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(g-j) *Solanum* pollen tubes labelled for methylesterified pectins with monoclonal antibody Jim 7 and corresponding DIC images. Pollen tubes grown in the presence of 1 $mg.mL^{-1}$ lyticase **(i,j)** showed significantly weaker label than the control cells **(g,h)**. The distribution pattern remained the same, however, with a higher concentration of methylesterified pectin at the pollen tube apex.
Bar = 10 µm. **89**

Figure 40: Force deformation graphs for micro-indentation experiments.

Local deformations were performed at the apex and at a distal location 30µm behind the tip of growing *Solanum* pollen tubes. The upper curve represents the deforming and the lower curve the retracting movement of the stylus. The slope of the linear part of the deforming curve indicates the stiffness whereas the surface area between the two curves expresses the delay upon retraction of the deforming stylus (hysteresis), which is an indication for the dissipated energy and thus the visco-elasticity of the deformed object. The deformation profiles at the distal pollen tube location show that the presence of 1 $mg.mL^{-1}$ lyticase caused a dramatic decrease in stiffness and an increase in visco-elasticity (**a**) compared to the control (**b**). At the apex, on the other hand, neither of these parameters was significantly affected by lyticase (**c**) if compared to the control situation (**d**). **93**

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Liste des sigles et des abréviations

$CaCl_2$:	Chlorure de calcium
$Ca(NO_3)_2$:	Nitrate de calcium
CCD:	*Charge-coupled device*
cf :	Conférer
cm:	Centimètre
cm/h :	Centimètre par heure
D :	Distance en micromètre
Da :	Déplacement de l'aiguille
ddH_2O :	Eau double distillée
DIC :	*Differencial interferencial contrast*
Dm :	Déplacement du moteur
Dr. :	Docteur
et al :	*et alia*
EGTA :	Acide éthylène Glycol-bis (ß-aminoethylether)-N,N,N',N'-tétraacétique
F :	Force en Newton
Fig :	Figure
FQNRT :	Fonds Québécois de la Recherche sur la Nature et les Technologies
GM :	*Growth Medium*
h :	Heures
H_2O :	Eau
H_3BO_3 :	Acide borique
IgG:	Immunoglobuline G
IRBV :	Institut de recherche en biologie végétale
K_2HPO_4 :	Phosphate de potassium
KNO_3 :	Nitrate de potassium
M :	Molaire
mdynes/µm :	Millidynes par micromètre
MES:	Acide 2-(4-Morpholino)-Éthane sulfonique

MET :	Microscopie électronique à transmission
$MgCl_2$:	Chlorure de magnésium
$mg.mL^{-1}$:	Milligramme par millilitre
$MgSO_4$:	Sulfate de magnésium
min :	Minutes
mM :	Millimolaire
n =:	Nombre d'expériences réalisées
N :	Newton
NSERC:	Natural Sciences and Enginnering Research Council of Canada
PBS :	*Phosphate buffered saline*
Pipes :	*Piperazine-N,N'-bis[2-ethanesulfonic acid]*
PME :	Pectine méthyle estérase
UK :	United Kingdom
US :	*United States*
UV :	Ultraviolet
°C :	Degré Celsius
% :	Pourcentage
$\mu g.mL^{-1}$:	Micro gramme par millilitre
µm :	Micromètre
$\mu m.s^{-1}$:	Micromètre par seconde

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Chapitre 1 :

Introduction générale

1.1 La reproduction des plantes à fleurs

La reproduction des plantes représente un champ de recherche incroyablement vaste ayant donné lieu à un très grand nombre d'études sur autant de différents aspects que les mécanismes d'auto incompatibilité (Franklin-Tong and Franklin 2003 ; Hiscock and McInnis 2003; Watanabe et al. 2003), le guidage du tube pollinique (Ra, et al. 1997 ; Malhò 1998), les facteurs biotiques ou abiotiques impliqués dans le transport des grains de pollen, ou encore la transgénèse par transformation du pollen (Vanderleedeplegt et al. 1995 ; Ramaiah and Skinner 1997).

Bien que les végétaux n'aient pas la même capacité que les animaux pour se mouvoir, le règne végétal n'est pas complètement dépourvu de mobilité, en effet, la reproduction des plantes à fleurs tout comme celle de la plupart des animaux nécessite une rencontre entre les deux partenaires sexuels c'est-à-dire, dans le cas des végétaux, entre le grain de pollen et la fleur réceptive ou plus précisément entre les cellules spermatiques du tube pollinique et les cellules du sac embryonnaire dans les ovules du pistil.

De manière schématique, nous pouvons résumer les différentes étapes aboutissant à la formation de la graine, étape ultime de la fécondation végétale, de la manière suivante:

1- L'androcée qui représente l'ensemble des pièces fertiles mâles, se compose des anthères et du filet (Fig. 1A). Les anthères renferment les grains de pollen immatures et les libèrent lors de la déhiscence.

2- Les grains de pollen matures sont transportés aléatoirement par différents vecteurs biotiques (insectes, etc.) ou abiotiques (vent, eau, etc.). Seul un très faible pourcentage des grains de pollen atteindra alors le stigmate d'une fleur réceptive.

3- L'adhésion entre le grain de pollen et le stigmate de la fleur est facilitée par la présence sur l'un et l'autre de molécules adhérentes. Au niveau du stigmate, des cellules papillaires produisent un exsudat qui va réhydrater le grain de pollen et lui fournir tous les nutriments nécessaires à sa germination.

4- La germination du grain de pollen en tube pollinique va lui permettre de parcourir de très longues distances à travers le style de la fleur dans le but d'atteindre l'ovaire et donc les ovules (Fig. 1A). L'anatomie stylaire varie suivant les espèces, et deux types structurels peuvent ainsi être dégagés : les styles solides, comme chez le *Solanum chacoense,* où le tissu de transmission représente un ensemble cellulaire relativement dense à travers duquel les tubes polliniques devront pénétrer (Lush et al. 2000); les styles creux où le tissu de transmission forme un canal, tel que décrit chez le *Lilium longiflorum* (Lord 2001 ; Park and Lord 2002).

5- Suivant la distance à parcourir, le tube pollinique atteindra l'ovule en un temps variable, ainsi la vitesse d'élongation de ces cellules peut atteindre 1cm/h. La livraison des cellules spermatiques contenues dans le tube pollinique représente l'étape ultime de la double fécondation des végétaux aboutissant, d'une part, à la production du zygote (2n) par fusion d'un noyau spermatique avec l'oosphère, et de l'albumen (3n) d'autre part par fusion avec le noyau polaire (Fig. 1B).

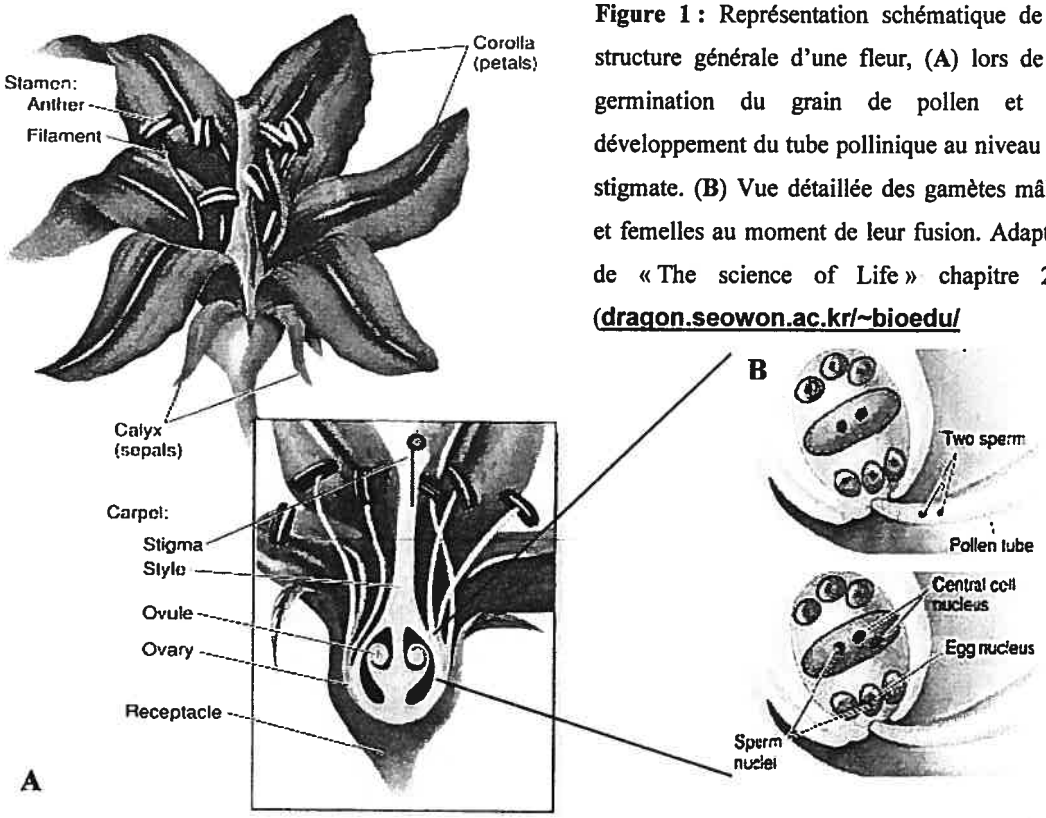

Figure 1 : Représentation schématique de la structure générale d'une fleur, (**A**) lors de la germination du grain de pollen et du développement du tube pollinique au niveau du stigmate. (**B**) Vue détaillée des gamètes mâles et femelles au moment de leur fusion. Adaptée de « The science of Life » chapitre 26. (dragon.seowon.ac.kr/~bioedu/

1.2 Le tube pollinique comme système d'étude modèle

Le tube pollinique, en tant qu'acteur de la fécondation végétale, est l'objet d'un grand nombre de recherches, aussi bien au niveau biochimique, physiologique, que microscopique. Certaines particularités de cette cellule en font un matériel tout à fait adapté pour l'étude des processus de croissance cellulaire, de flux ionique, de transport intracellulaire ou encore d'études cytomécaniques.

Citons-ici quelques caractéristiques qui font du grain de pollen et du tube pollinique un système d'étude particulièrement pertinent :

- Récolte abondante du matériel frais à partir des fleurs.

- Stockage simple et de longue durée : après déshydratation, entreposage à -20°C pendant plusieurs mois sans perte du pouvoir germinatif.

- Culture *in vitro* aisée et rapide dans un milieu de germination de composition très simple.

- Cellule isolée, n'appartenant pas à un tissu cellulaire d'où une élimination des risques de détérioration dus aux protocoles d'isolation.

- Accessibilité spatiale et temporelle de la cellule aux traitements physiques et chimiques (pas de délai de diffusion).

- Cellule avec croissance rapide et apicale: localisation du processus d'expansion cellulaire dans une zone bien précise, située à l'apex de la cellule, rendant ainsi les effets des différents traitements sont visibles immédiatement (arrêt de croissance/changement dans la forme de la cellule).

- Cellule haploïde: les mutations récessives sont observables.

Il existe néanmoins quelques inconvénients dans l'utilisation du pollen comme système d'études comme le montrent les points suivants :

- Au niveau d'une même plante ou encore d'une même fleur, les grains de pollen présentent des capacités de germination et de croissance différentes à cause de la ségrégation des allèles lors de la méiose.

- Certains facteurs externes difficilement contrôlables tels que la lumière, la température, l'humidité ou encore l'espace entre les grains de pollen, semblent avoir une influence significative sur les capacités de germination et d'élongation.

- En fonction des conditions *in vivo* ou *in vitro*, le pourcentage de germination ou encore la vitesse d'élongation varient sensiblement.

- Les grains de pollen de la plante modèle *Arabidopsis thaliana*, germent difficilement *in vitro* ce qui limite l'utilité de ce système modèle pour les différentes études portant sur le tube pollinique *in vitro*.

- Concernant l'aspect biomécanique, la forme cylindrique du tube pollinique en fait une cellule difficile à déformer de manière latérale (cf technique de micro-indentation sous chapitre 1.6.1.2.1).

1.3 La croissance apicale

Les tubes polliniques montrent des similarités morphologiques surprenantes avec les hyphes fongiques ou encore les poils racinaires (Fig. 2) et sont définis comme étant des cellules à croissance apicale (Hepler et al. 2001). Cette appellation sous-entend la capacité de ces cellules à croître dans une direction unique de part leur polarité structurelle (Rosen et al. 1964; Derksen 1996; Hepler et al. 2001). Ce type particulier de cellule est également caractérisé par une zone de croissance se limitant à l'apex de la cellule, suggérant l'existence de gradients morphologiques et physiologiques (Derksen 1996 ; Hepler et al. 2001). Plusieurs éléments cellulaires semblent impliqués dans cette organisation anisotropique : les vésicules de sécrétion sont transportées de leur site de synthèse (l'appareil de Golgi) jusqu'à l'apex de la cellule où a lieu l'exocytose, tandis que les plus gros organelles ne franchissent pas la zone sub-apicale. Le cytosquelette possède une organisation structurelle polaire particulière pour ce qui a trait aux filaments d'actine organisés en un réseau extrêmement dense dans la zone sub-apicale, tandis que de longs filaments parallèles à l'axe de croissance se trouvent dans la partie distale du tube (Steer and Steer 1989 ; Raudaskoski et al. 2001).

Enfin la paroi elle même montre des gradients structurels importants avec une zone apicale principalement constituée de pectines alors que la région distale est riche en callose (comme développé au sous chapitre 1.4). Ce qui nous amène à l'existence de gradients ioniques intracellulaires, dont le calcium représente l'exemple le mieux connu à ce jour (Feijo et al. 1995; Holdaway-Clarke et al. 1999; Holdaway-Clarke 2003; Holdaway-Clarke and Hepler 2003), particulièrement dans les phénomènes de croissance oscillatoire (Pierson, *et al.* 1996) mais également dans la formation de gel pectate rigide dans la zone distale du tube pollinique.

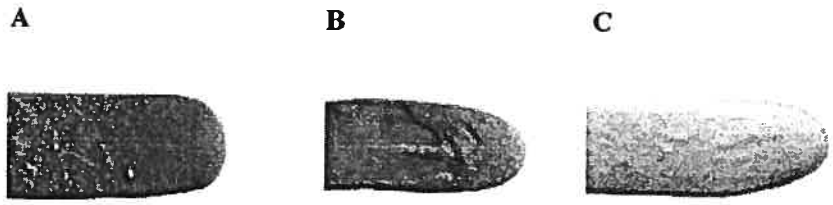

Figure 2 : Image DIC de la région apicale d'un tube pollinique de *Lilium longiflorum* (**A**), d'un poil racinaire de *Nicotiana tabacum* (**B**) et d'un hyphe fongique de *Rhizoctonia solani* en croissance (**C**) (Bartnicki-Garcia et al. 2000; Hepler et al. 2001).

Au niveau du tube pollinique, on peut distinguer quatre zones dont l'organisation structurelle diffère (Fig. 3) (Taylor 1997; Geitmann and Emons 2000; Lennon and Lord 2000) :

- zone claire riche en vésicules sécrétrices
- zone des organelles (Appareil de Golgi, mitochondries, réticulum endoplasmique)
- zones des noyaux
- zone vacuolaire

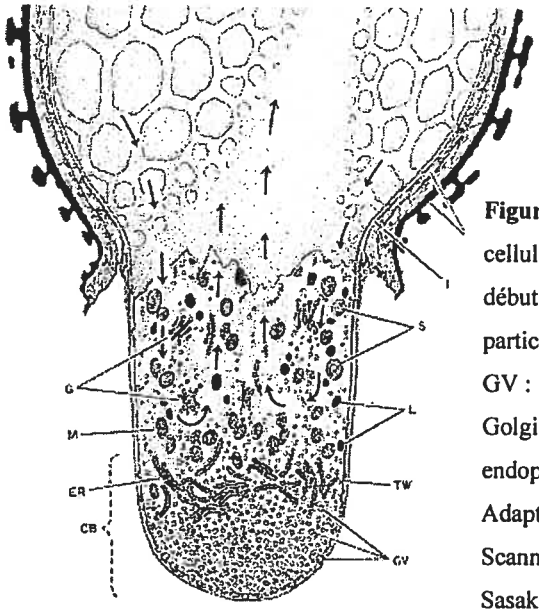

Figure 3 : Schéma représentant les éléments cellulaires d'un grain de pollen de lys en début de germination. E : exine. I : intine. L : particules lipidiques. TW : paroi du tube. GV : vésicules du Golgi. G : appareil de Golgi . M : mitochondries. ER : réticulum endoplasmique. CB : apex
Adaptée de « Pollen : Illustrations and Scanning Electromicrographs » Iwanami Y, Sasakuma T, Yamada Y. Copublished by KODANSHA LTD, and Springer Verlag.

L'expansion cellulaire est un processus complexe qui semble être contrôlé par deux facteurs mécaniques majeurs : la pression de turgescence interne d'une part, et d'autre part la synthèse et l'incorporation du matériel pariétal (Setterfield and Bayley 1961; Wilson 1964; Cleland 1971; Ray et al. 1972; Burström 1979; Taiz 1984; Masuda 1990; Boyer 1992; Cosgrove 1993a; Cosgrove 1993b).

Parce que la paroi entoure le protoplaste, celle ci ne peut pas s'étendre sans que le protoplaste augmente de volume, mais d'un autre coté, le protoplaste lui-même ne peut augmenter son volume sans une expansion de la paroi. Ainsi, la présence simultanée de ces deux facteurs semble être un pré-requis pour la croissance cellulaire bien que certains auteurs aient observé la croissance d'hyphes fongiques malgré une turgescence interne proche de zéro (Harold et al. 1996). La capacité d'une cellule à croître dépend ainsi, non seulement de la pression de turgescence interne mais également de la composition chimique des polymères pariétaux ainsi que de leurs interconnections. La croissance apicale nécessite donc l'apport continue de nouveau matériel pariétal par un système sécréteur actif ainsi que son incorporation.

Étant donné que la pression de turgescence interne s'exerce de manière égale en tous points de la surface cellulaire, il doit exister un gradient de composition pariétale et donc d'élasticité entre l'apex en croissance et le reste de la cellule qui doit être plus stable (Derksen 1996; Taylor 1997; Cosgrove 1999).

De plus, même en considérant la paroi comme identique tout le long du tube pollinique à la fois pour sa composition chimique et son épaisseur on aurait néanmoins une répartition des contraintes internes différente à l'apex et dans la région distale de telle manière que la tension circonférencielle équivaut à deux fois la tension longitudinale (Green 1962) (Fig. 4 et annexe 1). Ce qui souligne l'existence d'un gradient physique permettant d'un coté à la zone apicale de croître et de l'autre à la région distale de garder un diamètre uniforme.

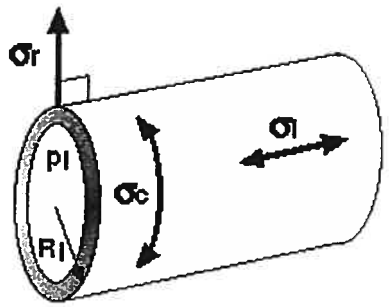

Figure 4 : Représentation des 3 contraintes pariétales vasculaires dépendant de la pression intraluminale, du rayon intraluminal, et de l'épaisseur pariétale. σ_r est la tension radiale, σ_c est la tension circonférencielle, σ_l est la tension longitidinale. Tirée de Biomécanique Coeur-Vaisseaux Travail du Service d'Hémodynamique Diagnostique et Thérapeutique Hôpital Cardiovasculaire et Pneumologique - Lyon
http://imagerie-cv.univ-lyon1.fr/WEB_CARDIO/documents/Documents_references/poly-biomeca/biomec_t.htm.

La paroi synthétisée doit être suffisamment résistante pour empêcher la cellule d'éclater sous la pression interne de turgescence, mais également assez flexible pour permettre à la cellule de croître (Steer and Steer 1989; Carpita and Gibeaut 1993; Darley et al. 2001; Hepler et al. 2001; Holdaway-Clarke 2003; Holdaway-Clarke and Hepler 2003).

De nombreux modèles ont été proposés pour illustrer le processus de croissance apicale, néanmoins on peut résumer ainsi celui partagé par le plus grand nombre d'auteurs (Burström 1979). La relaxation pariétale représente le premier événement, celle-ci entraîne alors une diminution du potentiel hydrique cellulaire accompagné d'une entrée d'eau.

En conséquence, le volume cellulaire augmente, simultanément avec la synthèse et l'incorporation de matériel pariétal, permettant à la cellule de croître (Fig. 5)

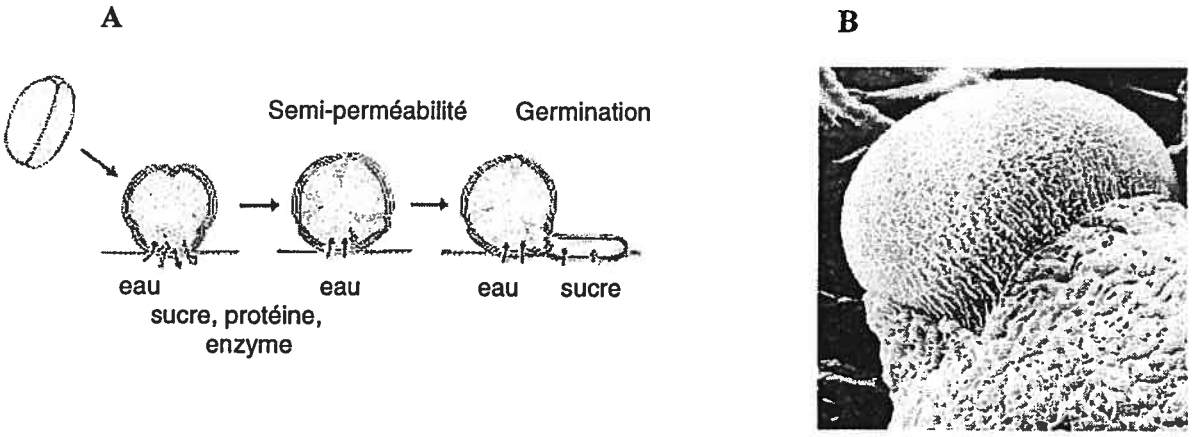

Figure 5 : Les premières étapes de la germination du grain de pollen. Hydratation et transfert de molécules (**A**), pour aboutir à la germination du tube pollinique (observée au microscope électronique à balayage chez *Pétunia*) (**B**). Adaptée de Pollen : Illustrations and Scanning Electromicrographs Iwanami Y, Sasakuma T, Yamada Y. Copublished by KODANSHA LTD, and Springer Verlag.

1.4 La paroi du tube pollinique

La paroi du tube pollinique peut être comparée à celle d'autres cellules végétales pour ce qui se limite au fait qu'elle consiste en plusieurs couches qui correspondent aux parois primaires et secondaires des autres cellules végétales. Néanmoins, le tube pollinique présente une composition pariétale très différentes des autres cellules végétales à la fois au niveau qualitatif mais aussi quantitatif (Cosgrove 1997), comme le démontre les pourcentages suivants obtenus pour différentes espèces (Tableau I) (Li et al. 1999; Raudaskoski et al. 2001).

	Cellules végétales (%)	Tube pollinique (%)
Cellulose	30	5-10
Hémicellulose	30	nd
Pectine	35	10
Callose	nd	80-85
Protéines structurelles	1-5	Nd

Tableau I : Pourcentages moyens des différents polymères pariétaux dans une cellule végétale et dans le tube pollinique.

De nombreuses études ont permis de localiser précisément les différents constituants de la paroi du tube pollinique, bien que, suivant l'espèce et la méthode utilisée, différentes organisations ait été mises en évidence (Heslop-Harrison 1987; Steer and Steer 1989).

Ainsi, la couche fibrillaire externe présente tout autour du tube pollinique est composée principalement de pectines (Heslop-Harrison 1987). Plus à l'intérieur, la couche intermédiaire, généralement peu distinguable de la couche externe, est majoritairement constituée de cellulose, bien que ce constituant reste l'un des moins bien caractérisé au niveau du tube pollinique (Steer and Steer 1989; Anderson 2002).

Enfin, la couche interne constituée de callose est absente de la partie apicale et devient de plus en plus épaisse dans la zone distale à partir d'environ 10 à 30 µm de l'apex (Meikle et al. 1991; Geitmann 1995; Ferguson et al. 1998). Cette répartition polaire caractéristique des tubes polliniques met en évidence l'existence d'un gradient physique déterminant pour la croissance apicale (Derksen 1996; Geitmann and Cresti 1998).

Une description plus détaillée de la nature et du mode de synthèse des différents composés pariétaux permet de mieux comprendre la zonation fonctionnelle existante entre l'apex en croissance, et la région distale plus stable.

Au niveau du grain de pollen, la structure pariétale généralement trouvée permet de distinguer deux couches : la couche interne pecto-cellulosique appelée intine, et la couche externe, nommée exine, composée principalement de sporopollenine (Tanaka et al. 1989; Van Aelst and Van Went 1992; Taylor 1997; El-Ghazaly 1999). Ainsi on trouve généralement des pectines acides dans l'intine proximale alors que la forme méthyle estérifiée est observée dans l'intine distale chez *Lilium longiflorum* (Aouali et al. 2001). Cependant la zone d'émergence du tube pollinique présente des caractéristiques particulières puisqu'elle montre une absence totale ou partielle de la couche la plus externe (exine) (Heslop-Harrison 1987; Taylor 1997; Ressayre et al. 1998; El-Ghazaly 1999). D'après une récente étude sur la plante modèle *Arabidopsis thaliana* (Johnson and McCormick 2001), une accumulation de callose s'effectuerait après l'hydratation des grains de pollen au niveau de la zone d'émergence du tube.

1.4.1 La cellulose

La molécule de cellulose est un polymère monotone uniquement constitué de cellobiose (glucoses liés en β 1-4) (Fig. 6).

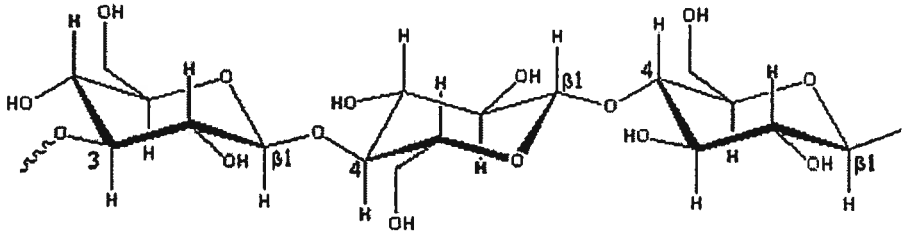

Figure 6 : Représentation chaise de la molécule de cellulose, les monomères sont liés entre eux par des liaisons β 1-4. http://www.lsbu.ac.uk/water/hycel.html.

Cette monotonie lui permet la répétition de liaisons faibles hydrogènes inter et intra caténaires.

Ainsi l'association de nombreuses molécules de cellulose permet la formation d'une microfibrille cellulosique aux propriétés de résistance remarquable (Albersheim et al. 1997; Carpita and Vergara 1998). La synthèse de la cellulose se fait à partir de structures nommées rosettes ou encore complexes terminaux, situés au niveau de la membrane plasmique et composés de plusieurs sous unités (Delmer and Amor 1995). Au niveau de la paroi du tube pollinique, la cellulose demeure assez mal caractérisée bien que Sassen dès 1964 l'ait étudié chez le pétunia (Sassen 1964). L'utilisation d'anticorps ou de fluorochromes spécifiques de la cellulose (calcofluor) ont permis de préciser la localisation de ce polymère pariétal généralement absent de l'apex pollinique et visible environ 5-15 µm derrière la zone apicale (Steer and Steer 1989; Ferguson et al. 1998). Même si la présence de cellulose dans la paroi du tube pollinique semble être confirmée par un certain nombre d'études basées sur différentes méthodes, il semble que ce polymère représente un constituant minoritaire dont le pourcentage varie de 2 à 10% dans le tube pollinique comparé à environ 20 à 30 % retrouvé dans les autres tissus végétaux (Steer and Steer 1989; Schlupmann et al. 1994).

1.4.2 La callose

La molécule de callose est un polymère de D-Glucose, tout comme la cellulose, à la différence que les monomères sont reliés entre eux par des liaisons β 1-3 (Fig. 7).

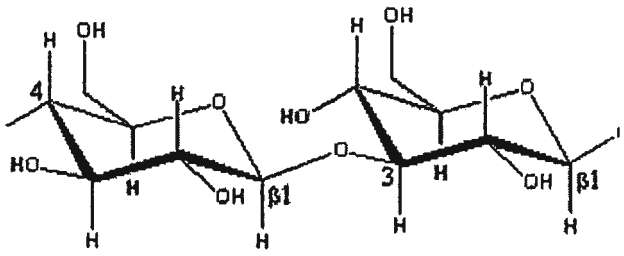

Figure 7 : Représentation chaise de la molécule de callose constituée de monomères de D-Glucose reliés entre eux par des liaisons β 1-3. http://www.lsbu.ac.uk/water/hycel.html.

La synthèse et le dépôt de ce polymère au niveau de la surface de la membrane plasmique par le biais de callose synthase semble remise en cause par de récentes études suggérant un rôle des rosettes de cellulose dans la synthèse callosique (Van Amstel 1994). Ainsi ces complexes terminaux seraient capables de synthétiser à la fois de la cellulose et de la callose en alternance. Dans la plupart des tissus végétaux, la synthèse de callose est le résultat d'une blessure mécanique ou d'un stress, contrairement au cas du tube pollinique qui présente une quantité inhabituelle de ce polymère en condition normale. En effet, environ 80 à 85% des polysaccharides pariétaux totaux sont de nature callosique (Rae et al. 1985). Ainsi le tube pollinique représente une cellule idéale pour l'étude de ce polymère.

Comme nous l'avons brièvement décrit précédemment, la callose est le constituant majeur de la paroi interne du tube. Des études de microscopie électronique à transmission (MET) ont permis de distinguer clairement cette couche callosique qui apparaît de manière translucide et homogène à environ 10 à 30 µm de l'apex, avec des dépôts de plus en plus épais vers les régions distales du tube (Knox 1984; Heslop-Harrison 1987; Mascarenhas 1993). L'utilisation en microscopie photonique d'un fluorochrome spécifique, l'aniline bleue décolorée, a permis de mettre en évidence la callose dans le tube pollinique chez de nombreuses espèces (Van Amstel 1994).

L'apex des tubes polliniques en croissance est normalement dépourvu de callose mais une accumulation visible est observable lorsque le tube cesse de croître ou lors d'une réaction d'auto incompatibilité (Rae et al. 1985; Van Amstel 1994; Geitmann 1995). Des dépôts significatifs de callose ont également été observés à intervalles réguliers le long du tube pollinique sous forme de bouchons transversaux (Hasegawa et al. 1998; Raudaskoski et al. 2001).

1.4.3 Les pectines

Dans la paroi des tissus végétaux, les pectines représentent le polymère le plus abondant et semblent impliquées dans diverses fonctions cellulaires comme par exemple l'adhésion entre les cellules ou encore la croissance plastique (Jarvis 1984; Levy and Staehelin 1992; Carpita and Gibeaut 1993).

Ce sont des polymères d'acides galacturoniques (Fig. 8) que l'on trouve sous deux formes distinctes : les pectines méthyle-estérifiées et les pectines acides. Ces macro molécules comportent à la fois des régions lisses (sans substitution) et d'autres hérissées. Elles sont souvent chargées, ce qui permet des interactions ioniques essentielles au sein de la paroi.

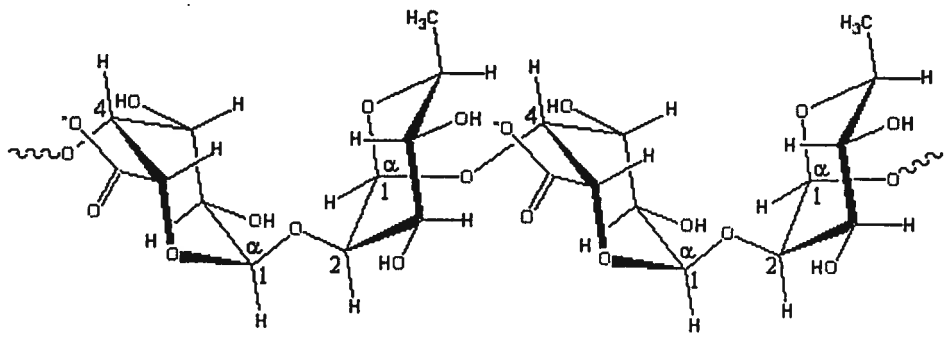

Figure 8 : Représentation chaise d'une molécule de pectine **http://www.lsbu.ac.uk/water/hycel.html**.

Comme nous l'avons mentionné précédemment, les pectines sont présentes tout le long du tube pollinique dans la couche externe de la paroi, et on peut clairement distinguer les deux formes méthyle-estérifiées et acides.

La synthèse des pectines a lieu au niveau de l'appareil de Golgi sous la forme méthyle-estérifiées (Levy and Staehelin 1992; Hasegawa et al. 1998). Ce n'est qu'après le transport (grâce au cytosquelette) et l'exocytose des vésicules de sécrétion à l'apex qu'une dé-estérification se produit, transformant les pectines estérifiées en pectines acides (Geitmann 1995).

Cette déméthylation est catalysée par l'enzyme pectine méthyle estérase (PME) (Kauss and Hassid 1967; Moore and Staehelin 1988; Zhang and Staehelin 1992; Carpita and Gibeaut 1993; Li 1997; Geitmann 1998).

Ainsi un gradient de méthylation existe au niveau du tube pollinique avec l'apex croissant riche en pectines méthyle-estérifiées et la région distale composée majoritairement de la forme acide (Li et al. 1994; Li et al. 1995; Jauh and Lord 1996; Li 1997; Stepka et al. 2000). Cette répartition caractéristique a été observée chez de nombreuses espèces par le biais d'anticorps monoclonaux Jim 5 et Jim 7, respectivement spécifiques de la forme acide et estérifiée (VandenBosch 1989; Knox 1990).

La présence d'ions Ca^{2+} entraîne une dimérisation des pectines acides qui forment alors une structure potentiellement plus rigide appelée gel pectate alors que les pectines méthyle-estérifiées demeurent solubles dans l'eau (Morris et al. 1982; Jarvis 1984; Carpita and Gibeaut 1993).

1.5 La biomécanique des cellules

La biomécanique est une science relativement récente ayant pour but l'étude des forces et des mouvements des objets biologiques. Depuis une dizaine d'années environ, cette approche suscite un intérêt particulier à la fois chez les animaux et les végétaux, bien que la plupart des études se soient jusqu'à présent concentrées sur les propriétés physiques au niveau des tissus et des organes. Ainsi une littérature abondante traitant de la biomécanique du bois est disponible pour ce qui a trait au règne végétal (Thibaut et al. 2001; Clair et al. 2003).

Au niveau cellulaire des études ont permis de caractériser la cytomécanique des érythrocytes chez les mammifères (Discher et al. 1988; Nash and Gratzer 1993; Knowles et al. 1994; Yuan et al. 1995). Néanmoins, à notre connaissance, aucune étude de ce type n'a encore abouti pour les cellules végétale. Ainsi notre projet de

recherche représente une approche totalement nouvelle pour la cytomécanique de la cellule végétale.

La stabilité mécanique des cellules dépend de plusieurs facteurs tels que les éléments du cytosquelette (microfilaments d'actine, microtubules) mais aussi de la membrane plasmique. Enfin, chez les végétaux, deux paramètres supplémentaires sont à considérer : la pression interne de turgescence et la paroi cellulaire, qui rendent ces études d'autant plus complexes.

Bien que nous nous intéressions en particulier au rôle de la paroi dans le processus de croissance cellulaire, celle-ci intervient aussi dans d'autres phénomènes cellulaires cruciaux comme par exemple les mécanismes de défense, les processus de différenciation ou encore la signalisation entre cellules.

Les propriétés mécaniques de la paroi végétale jouent un rôle essentiel pour la croissance cellulaire puisque l'expansion cellulaire implique une « relaxation » de la paroi pré-existante ainsi que la synthèse et l'incorporation du matériel pariétal néo-formé. Ainsi, du point de vue mécanique la croissance cellulaire est contrôlée par la capacité de la cellule à se déformer de manière élastique et plastique, ce qui, en fait, revient à dire que la capacité de croissance de la cellule dépend de la nature chimique des polymères pariétaux, de la présence d'ions, ou encore de leurs localisations et de leurs interconnections.

1.6 Objectifs

Le choix du tube pollinique comme cellule modèle nous permet d'envisager l'étude des propriétés biomécaniques d'un point de vue tout à fait novateur, étant donné que ce système consiste en une cellule unique isolée à protoplaste vivant.

In planta, le tube pollinique doit exercer d'une part des forces de pénétration et d'autre part des forces de résistance latérale pour permettre le passage des gamètes et le maintien de leur intégrité lors de la croissance à travers le style (Fig. 9).

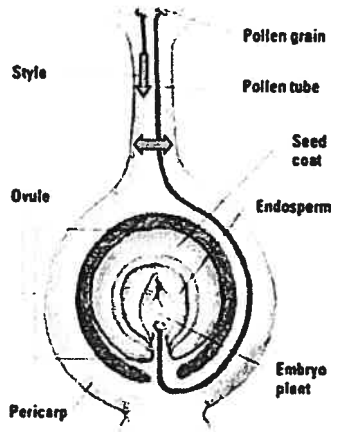

Figure 9 : Représentation schématique de la croissance du tube pollinique à travers le style d'une fleur réceptive. Le style est tapissé d'un tissu de transmission qui nécessite l'exercice par le tube pollinique de force de pénétration et de résistance latérale **www.homestore.com/.../Plants/ SNST_Fruits.asp.**

Le laboratoire de biologie cellulaire et cytoarchitecture végétale de Dr. Geitmann a pour objectif de caractériser de manière individuelle les différents paramètres qui contrôlent la stabilité mécanique des cellules végétales vivantes. Pour cela nous utilisons une cellule modèle : le tube pollinique de différente espèces végétales (*Papaver*, *Solanum*, *Lilium* ...). Évidemment il est absolument essentiel de garder en mémoire les relations existantes entre les différents acteurs de la stabilité mécanique cellulaire: paroi cellulaire, turgescence et cytosquelette. Ainsi mon projet de maîtrise s'inscrit dans cet objectif général en tentant de caractériser le rôle des différents polymères pariétaux du tube pollinique dans ses propriétés biomécaniques. Dans ce but j'ai concentré mes efforts sur les forces que le tube pollinique doit exercer lors de sa croissance *in vivo,* forces absolument indispensables pour aboutir à la livraison des cellules spermatiques c'est à dire à la fécondation.

L'accessibilité du tube pollinique cultivé *in vitro* m'a permis de modifier la structure pariétale de cette cellule de manière spécifique par ajout d'enzymes digestives avec le but de comprendre le rôle de chaque composé pariétal dans l'exercice des forces de pénétration et de résistance latérale que le tube pollinique est capable d'exercer.

1.6.1 Deux forces à caractériser, deux approches expérimentales

1.6.1.1 Force de pénétration

Dès l'arrivée du grain de pollen sur le stigmate d'une fleur réceptive, et jusqu'aux ovules, c'est à dire depuis les premières étapes de la germination jusqu'à celle de livraison des cellules spermatiques, le tube pollinique doit exercer des forces pour croître à l'intérieur du style. Comme nous l'avons dit précédemment (sous-chapitre 1.1), l'organisation du tissu de transmission qui tapisse le style peut être de deux types, solide ou ouvert, ce qui nous amène à envisager des forces de pénétration différentes suivant la structure du tissu à pénétrer, c'est à dire suivant l'espèce.
Dans le but d'étudier la capacité des tubes polliniques à exercer des forces de pénétration *in vitro*, j'ai utilisé des milieux de germination semi-solides, complémentés en agarose par comparaison avec un milieu liquide ne nécessitant pas l'exercice de forces.

1.6.1.2 Force de résistance latérale

L'exercice de force de résistance latérale est essentiel pour préserver l'intégrité des cellules à croissance apicale. Ceci est particulièrement important pour le tube

pollinique car il doit permettre le passage des cellules spermatiques lors de sa croissance à travers le style *in vivo*.

In vitro ces forces peuvent être caractérisées à l'aide de techniques de micro-indentation dont le fonctionnement est le suivant : de manière générale le principe est d'exercer des déformations mécaniques locales et de mesurer les forces exercées par le tube pollinique pour résister à ces déformations.

1.6.1.2.1 Le Micro-indenteur

Une aiguille de verre (c) dont le diamètre est de quelques microns est attachée à une fibre optique de verre (b), elle-même reliée à un moteur dont le mouvement vertical est contrôlable via une plate forme informatique (a). Les tubes polliniques en croissance sont contenus dans une chambre expérimentale (d) (Fig. 10).

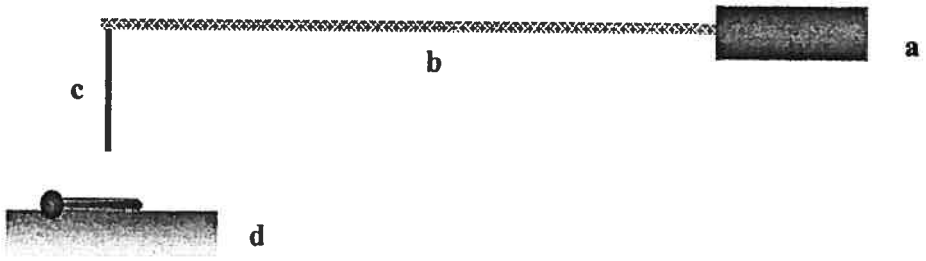

Figure 10 : Schéma du micro-indenteur, constitué du moteur se déplaçant verticalement (**a**), de la fibre optique flexible (**b**), de l'aiguille (**c**) et des tubes polliniques en croissance dans la chambre expérimentale (**d**).

L'aiguille est obtenue en digérant l'extrémité d'une fibre de verre dans de l'acide hydrofluorique, jusqu'à obtention du diamètre et de la forme souhaitée. Pour notre étude, nous nous sommes intéressés d'une part à la zone apicale et d'autre part à la région distale à environ 20-30 µm de l'apex, c'est pourquoi nous avons construit et utilisé une aiguille de diamètre égal à celui du tube pollinique. Il est essentiel que l'extrémité de l'aiguille soit le plus droite possible pour éviter les risques de transpercement du tube pollinique.

La fibre optique possède une constante de flexibilité connue par un calibrage précédent. Un logiciel permet de contrôler les mouvements du moteur, ainsi on fixe le déplacement vertical du moteur ainsi que la force qu'il exerce.

Un système de chambre expérimentale compartimentée permet de placer dans deux compartiments séparés, le moteur d'une part et une lamelle de verre recouverte de tubes polliniques en croissance d'autre part. Le système entier se place sur la plate-forme du microscope photonique inversé, permettant ainsi un placement exact de l'aiguille par rapport au tube pollinique.

La figure 11 permet de mieux visualiser la déformation mécanique. Lors d'une expérience de micro indentation, le moteur effectue un déplacement vertical de distance donnée (Dm). Si aucun objet n'entre en contact avec l'aiguille alors les déplacements du moteur et de l'aiguille seront identiques : Dm = Da. Par contre dans le cas où l'aiguille entre en contact avec un objet, son déplacement sera d'autant plus petit que l'objet exerce une résistance à la déformation d'où Dm > Da en fonction de la constante de flexibilité de la fibre optique connue. Ainsi la différence existant entre les distances de déplacement du moteur et celle de l'aiguille nous donne une information sur la rigidité locale de notre spécimen.

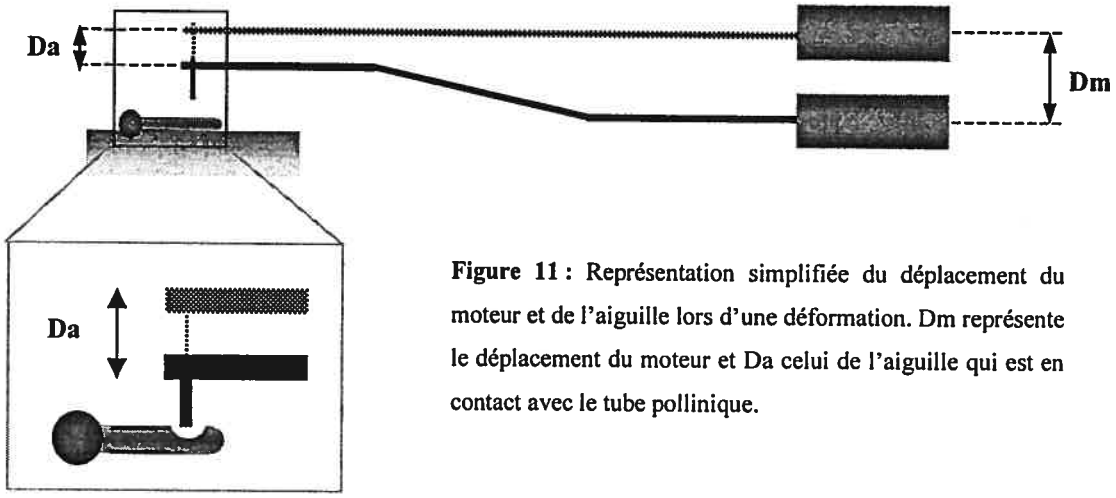

Figure 11 : Représentation simplifiée du déplacement du moteur et de l'aiguille lors d'une déformation. Dm représente le déplacement du moteur et Da celui de l'aiguille qui est en contact avec le tube pollinique.

On obtient ainsi, le graphique de la force exercée par l'aiguille en fonction de sa profondeur d'indentation pour déformer le tube pollinique ainsi que la force nécessaire pour que le spécimen se reforme après la déformation, comme illustré ci-dessous (Fig. 12)

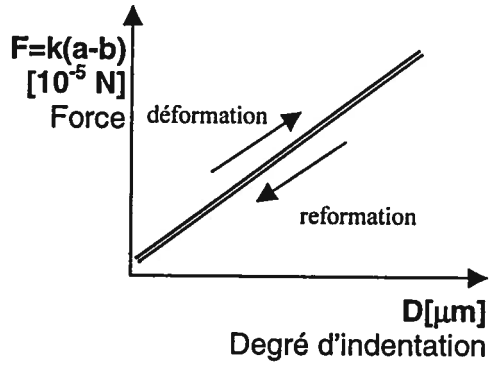

Figure 12 : Graphique de la force (en Newtons) en fonction du degré d'indentation (en micromètres) dans le cas d'un objet parfaitement élastique.

On peut alors en déduire deux paramètres comme le montrent les courbes typiques illustrées ci-dessous :

- la rigidité locale du spécimen

- la viscoélasticité locale

1.6.1.2.2 La rigidité locale du tube pollinique

La force nécessaire pour déformer le spécimen est représentée par une droite dont la pente représente la rigidité locale (Fig. 13). En effet, plus l'objet sera résistant et moins grande sera la profondeur d'indentation pour une force donnée F_0. Ainsi la pente de la droite 1, est plus élevée que celle de la droite 2, on en conclut que l'objet déformé en 1 est plus résistant que 2.

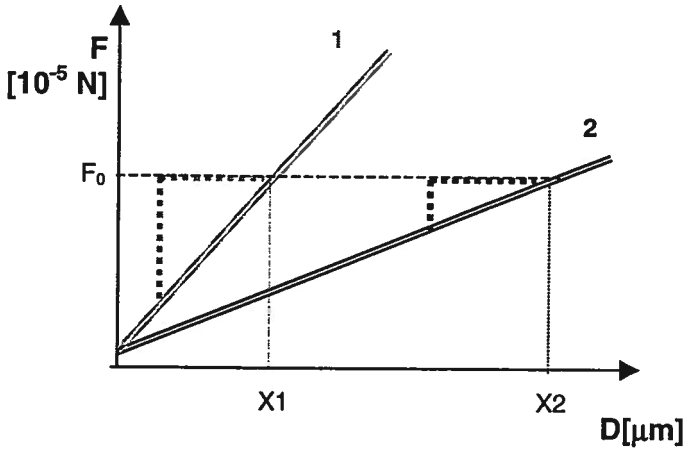

Figure 13 : Graphique de la force (en Newtons) en fonction de la profondeur d'indentation (en micromètres) pour deux objets élastiques de résistance différente. Comme la pente obtenue après déformation de l'objet 1 est plus forte que pour l'objet 2, ce dernier est moins résistant.

1.6.2.2.3 La viscoélasticité locale du tube pollinique

Littéralement la viscoélasticité est définie comme un « retard de l'effet sur la cause dans le comportement des corps soumis à une action physique » (définition du Petit Robert). Ainsi, le graphique de la force en fonction du degré d'indentation se compose de deux parties : la force nécessaire pour déformer l'objet et celle exercée pour que l'objet se reforme. La capacité d'un objet à reprendre sa forme initiale après une déformation mécanique dépend de ses propriétés physiques. Un objet sera défini comme élastique lorsqu'il possédera la capacité de se reformer immédiatement après la déformation, par contre un objet qui ne se reforme pas ou après un certain délai sera qualifié respectivement de plastique ou viscoélastique. Ainsi selon qu'un objet tend vers un modèle plutôt élastique, viscoélastique ou plastique, l'allure du graphique de la force en fonction de la profondeur d'indentation différera. L'aire géométrique comprise entre les deux courbes est une mesure de l'énergie qui est perdue (dissipée) par le système (le tube pollinique) pendant le processus d'indentation.

La figure 14 est une représentation typique d'un cas où l'objet déformé tend vers un modèle élastique. Dans un cas tel que celui ci, il revient à dire que l'objet se reforme immédiatement après l'indentation, les forces de déformation et de reformation sont égales et par conséquent le graphique représente deux droites confondues.

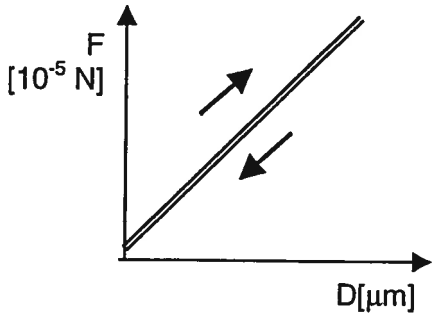

Figure 14 : Graphique de la force (en Newtons) en fonction de la profondeur d'indentation (en micromètres) pour un objet élastique. Les deux droites sont confondues puisque les forces pour déformer et reformer le tube sont identiques.

Dans le cas d'un objet viscoélastique (Fig. 15), la différence des deux parties du graphique forme une aire qui est d'autant plus grande que le temps de reformation de l'objet est grand.

L'aiguille remonte moins vite car la force qu'exerce sur elle le tube pollinique est moins grande que celle exercée par l'aiguille pour le déformer. Cette force est moins grande car il y a eu dissipation (perte) d'énergie.

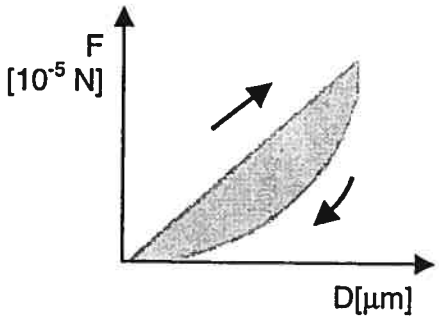

Figure 15 : Graphique de la force (en Newtons) en fonction du degré d'indentation (en micromètres) ; l'aire géométrique comprise entre les deux courbes représente la viscoélasticité locale du tube pollinique.

1.7 Résumés des résultats obtenus

La synthèse de mes travaux de recherche sera présentée sous forme d'articles scientifiques. Le chapitre 2 correspond à un manuscrit publié dans la revue *Sexual Plant Reproduction*, le deuxième chapitre a été soumis et est en révision pour la revue *Planta*, et enfin l'avant dernier chapitre (chapitre 4) vient d'être soumis à la revue *Plant Journal*.

Les principaux résultats obtenus sont présentés ci-dessous :

- Un gradient des propriétés physiques existe le long du tube pollinique de *Papaver rhoeas*, au niveau de la rigidité et de la visco-élasticité (Chapitre 2).

Des expériences de micro-indentation le long du tube pollinique du Pavot ont permis de mettre en évidence l'existence d'un gradient des propriétés physiques à la fois au niveau de la rigidité et de la viscoélasticité en relation avec la répartition des éléments cellulaires.
La rigidité de l'apex du tube pollinique de pavot augmente fortement durant les 20 premiers micromètres, puis atteint un plateau. Ainsi l'apex du tube est moins rigide et plus visco-élastique que la région distale qui tend vers un modèle élastique.
Un marquage spécifique des constituants cellulaires (actine par immunofluorescence, cellulose par le fluorochrome spécifique Calcofluor White, callose par l'aniline bleue décolorée et pectines méthyle-estérifiées et acides par immunofluorescence) suggère un rôle clé des pectines dans l'augmentation de la rigidité des 20 premier micromètres de l'apex et plus particulièrement de leur degré d'estérification. Dans la partie distale du tube, la présence de callose et de pectines acides pourrait être responsable d'une plus grande rigidité qui permet une meilleure stabilité de cette zone du tube.

- La germination et la croissance apicale du tube pollinique de *Solanum chacoense* et *Lilium longiflorum* sont contrôlées par un équilibre entre les forces de pression de turgescence interne et celle de résistance de la paroi pectinique (Chapitre 3).

La digestion partielle des pectines acides et estérifiées par ajout de pectinase en concentration modérée entraîne une stimulation de la germination et de l'élongation chez les deux espèces testées, bien que des différences significatives soient mises en évidence. De plus, des concentrations élevées d'enzymes digérant les pectines entraînent un éclatement des grains de pollen suggérant un déséquilibre fatal entre les forces de pression interne et celle de résistance de la paroi.

- La quantité de pectines le long du tube pollinique de *Solanum chacoense* dépend de l'environnement dans lequel le tube croît (Chapitre 3).

Les effets de la pectinase sont différents d'une espèce à l'autre, suggérant un arrangement de ce polymère pariétal variable suivant la nature du tissu constituant le style de la fleur. L'utilisation de milieux semi-solides (complémentés en agarose, ou en Gel-Gro), en comparaison avec un milieu liquide en présence de pectinase, a mis en évidence une adaptation de la quantité de pectines en fonction de la rigidité du milieu de croissance.

- Le gradient de rigidité et de viscoélasticité existant le long du tube pollinique du *Solanum chacoense* correspond à un gradient du degré d'estérification des pectines (Chapitre 3).

Des déformations mécaniques au niveau de l'apex et de la zone distale (30 µm de l'apex) du tube pollinique de *Solanum chacoense,* après traitement avec l'enzyme pectinase et pectine méthyle estérase, ont révélé une corrélation entre le gradient d'estérification et la quantité des pectines le long du tube pollinique et ses propriétés mécaniques. Les tubes témoins sont caractérisés par un apex moins rigide et plus viscoélastique comparé à la zone distale.

La digestion des pectines entraîne une diminution de la rigidité à l'apex et en position distale, alors que la transformation des pectines méthyle estérifiées en pectines acides par la PME cause une augmentation de la rigidité de l'apex.

- La callose est un facteur déterminant de l'équilibre résistance de la paroi/pression interne de turgescence impliqué dans la germination (Chapitre 4).

L'utilisation d'une enzyme spécifique de la callose (la lyticase) a mis en évidence des résultats très différents chez le Lys et le *Solanum*. En concentrations élevées , cette enzyme inhibe la germination des deux espèces en causant l'éclatement des grains de pollen de *Solanum* uniquement. D'autre part, des concentrations plus faibles de lyticase stimulent la germination du *Solanum* contrairement au Lys.

- La constitution pariétale du grain de pollen diffère suivant l'espèce (Chapitre 4).

Un marquage spécifique de la callose, de la cellulose ou encore des pectines acides et estérifiées a mis en évidence une accumulation significative de callose et de pectines acides au niveau du site de germination chez *Solanum* alors que le Lys montre une accumulation de cellulose et de pectines acides.

- L'épaisseur et la structure de la paroi des grains de pollen varient suivant l'espèce (Chapitre 4).

L'observation de coupes ultra fines au microscope électronique à transmission a permis de mettre en évidence une paroi plus épaisse (3.6 µm) chez le Lys comparée au *Solanum* (0.5 µm). De plus, l'observation de la surface externe des grains de pollen au microscope électronique à balayage révèle une ornementation de type réticulée chez le Lys et scabrate chez le *Solanum*.

- La quantité de callose dans le tube pollinique de *Solanum* dépend de la rigidité du milieu de croissance (Chapitre 4).

La présence de lyticase en concentration faibles entraîne une stimulation de la germination des grains de pollen d'autant plus élevée que le milieu est dense. Un marquage spécifique de la callose par l'aniline bleue décolorée confirme que les grains et les tubes cultivés en milieu plus rigide possèdent moins de callose comparé au témoin liquide.

- La callose est impliquée dans les propriétés physiques de la région distale du tube pollinique de *Solanum* (Chapitre 4).

L'ajout de lyticase en quantité modérée entraîne une augmentation du diamètre des tubes polliniques traités. Des expériences de micro-indentation confirment que la rigidité et l'élasticité de la zone distale du tube pollinique est en partie due à la présence de callose, puisque les tubes traités avec de la lyticase présentent une rigidité distale diminuée de moitié.

Chapitre 2 :

The local cytomechanical properties of growing pollen tubes correspond to the axial distribution of structural cellular elements

Anja Geitmann and Elodie Parre

Cet article est le résultat combiné de mon travail et de résultats antérieurs de Anja Geitmann. La partie localisation des éléments structurels de la paroi cellulaire correspond à mon apport personnel. Il a été publié dans la revue Sexual Plant Reproduction.
Volume 17, pages 9 – 17 (2004)

Abstract

Morphological studies of pollen tubes have shown that the configuration of structural cellular elements differs between the growing apex and the distal part of the cell. This polarized cellular organization reflects the highly anisotropic growth behavior of this tip growing cell. Accordingly, it has frequently been postulated that physical properties of pollen tubes such as cell wall plasticity should show anisotropic distribution, but no experimental evidence for this has been published hitherto. Using micro-indentation techniques, we quantify pollen tube resistance to lateral deformation forces and analyze its visco-elasticity as a function of distance from the growing apex. Our studies reveal that cellular stiffness is significantly higher at the distal portion of the cell. This part of the cell is also completely elastic, whereas the apex shows a visco-elastic component upon deformation. To relate these data to the architecture of the particular pollen tube investigated in this study, *Papaver rhoeas*, we analyzed the distribution of cell wall components such as pectin, callose, and cellulose as well as the actin cytoskeleton in this cell using fluorescence label. Our data revealed that, in particular, the degree of pectin methyl esterification and the configuration of the actin cytoskeleton correlate well with the distribution of the physical properties on the longitudinal axis of the cell. This suggests a role for these cellular components in the determination of the cytomechanics of pollen tubes.

Keywords

Cell wall · Cytomechanics · Cytoskeleton · Pollen tube · Tip growth

2.1 Introduction

Pollen tubes are long tubular cells that are formed by pollen grains triggered to germinate *in vivo* (on a receptive stigma) or *in vitro* (in a liquid or solidified aqueous medium). Pollen tubes, similar to root hairs and fungal hyphae, are tip growing cells. This cell type is characterized by its highly anisotropic shape and behavior, since the cellular growth process occurs in one direction only and is confined to the dome-shaped apex of the cylindrical cell. Numerous cellular processes, such as ion fluxes and exocytosis, are known to be essential for this process (Hepler et al. 2001; Holdaway-Clarke and Hepler 2003), but the physical constraints that control this particular cellular morphogenesis are still largely unknown. Hypotheses range from models that explain confined apical expansion by local difference in cell wall plasticity (Dumais et al. MS submitted) to those based on a cytoskeleton-controlled direction of secretory activity (Ketelaar et al. 2003). Local liberation of secretory vesicles is doubtless one of the key elements, since continuous formation of new cell wall and plasma membrane is the main activity in the growing tip. In plant cells, intracellular transport of secretory vesicles is purported to be mediated by the actin cytoskeleton, the presence of which is a condition *sine qua non* for tip growth (Geitmann and Emons 2000; Raudaskoski et al. 2001). Next to vesicle transport, the rod-shaped, highly dynamic, actin filaments have also been postulated to play a physical role in the tip growth mechanism (Gibbon et al. 1999; Fu et al. 2001; Vidali et al. 2001; Gu et al. 2003); however, its precise structural function in this process is still elusive. It has been proposed that actin contributes to the propulsion of the tip (Steer and Steer 1989; Steer 1990; Baluska et al. 2000). Whereas in fungal hyphae there seems to be a high concentration of filamentous actin in the growing tip, thus providing support for this hypothesis, the very apex in growing pollen tubes and root hairs seems to contain fewer or only very fine and/or highly dynamic actin bundles (De Ruijter et al. 1999; Geitmann and Emons 2000). It is questionable whether these fine bundles would have the stability to provide a direct propelling force similar to pseudopod formation in animal cells (Holdaway-Clarke and Hepler 2003).

On the other hand, in both pollen tubes and root hairs the actin configuration is very dense in the subapical region—the most apical part of the cylinder-shaped portion of the cell. Control of cellular diameter is likely to be located in this region. In pollen tubes the diameter is an important cellular parameter, since the male gametes need to fit through the cylindrical tube while using it as a passageway on their path to the ovary. The abundance of actin in the structurally critical subapex indicates therefore that it might play a role in the control of tube diameter. We show that comparison of the cytomechanical properties of growing pollen tubes with the precise configuration of actin filament bundles in these critical apical and subapical regions provides a first indication that this might indeed be the case.

Next to the cytoskeleton, plant cell architecture is also determined by the cell wall and the turgor. The latter can be presumed to be identical in all parts of the living cytoplasm of the pollen tube, since the cell is a single compartment, though no accurate measurement of turgor in different pollen tube regions has been performed. The cell wall of pollen tubes on the other hand shows a structural gradient along the longitudinal axis (Li et al. 1994; Derksen et al. 1995; Taylor and Hepler 1997). Depending on the species, two or three layers can be distinguished. The inner callosic layer is mostly present in distal regions and absent at the growing tip (Meikle et al. 1991; Geitmann et al. 1995). Visible deposits of callose start around 30 μm from the apex and become thicker towards the distal part in *Nicotiana tabacum* (Ferguson et al. 1998); pollen tubes of other plant species are less precisely characterized. Similarly, the exact location of cellulose in the pollen tube cell wall is known for few species only (Steer and Steer 1989; Anderson et al. 2002). There is evidence that cellulose microfibrils co-localize with the inner callosic lining of *N. tabacum* pollen tubes, where they were detected approximately 5–15 μm behind the tip (Ferguson et al. 1998). The outer fibrillar layer of the pollen tube cell wall is present in distal and apical regions and contains mostly pectins (Heslop-Harrison 1987). In the apical region, pectins are known to be highly methyl-esterified, but their degree of esterification decreases towards the distal regions (Li et al. 1994, 1995, 1997; Jauh and Lord 1996; Stepka et al. 2000), thus making these polymers subject to gelation by calcium ions (Carpita and Gibeaut 1993).

This ion-dependent polysaccharide crosslinking presumably causes stiffening of the pectin layer in the distal regions (Morris et al. 1982). Together with the distribution of callose, this anisotropic cell wall composition has been hypothesized to result in a gradient in the physical properties of the cell (Derksen 1996; Geitmann 1998). The key aspect of this gradient is presumed to be increased plasticity at the apex that permits growth at this location (Roggen and Stanley 1969; Darley et al. 2001; Holdaway-Clarke et al. 2003). However, we lack experimental evidence for differences in stiffness, elasticity or plasticity of the cell wall along the longitudinal axis of the growing pollen tube.

We used local deformation experiments to study whether or not differences in cell wall and cytoskeletal configurations along the longitudinal axis of the pollen tube are reflected in its physical properties. Using a micro-indentation device we measured the local stiffness of the cell as a function of the distance from the growing apex. In addition, we determined local visco-elastic behavior by assessing the degree of delay during relaxation after mechanical deformation (hysteresis). We related these mechanical data to the cellular architecture and distribution of structural polymers and found significant correlation.

2.2 Materials and methods

2.2.1 *In vitro* growth of pollen tubes

Papaver rhoeas and *Petunia hybrida* plants were grown in the greenhouses of the Montreal Botanical Garden. Pollen was taken either fresh from dehisced anthers or stored dehydrated at -20°C. It was hydrated in a humid chamber at room temperature for at least 30 min before use. Hydrated pollen was grown on cover slips coated with poly-L-lysine (*Papaver rhoeas*) or stigmatic exudate (*Petunia hybrida*) and covered with liquid growth medium (GM), comprising: 100 μg ml^{-1} H_3BO_3, 300 μg ml^{-1} $Ca(NO_3)_2 \cdot H_2O$, 100 μg ml^{-1} KNO_3, 200 μg ml^{-1} $MgSO_4 \cdot 7H_2O$, 50 μg ml^{-1} sucrose after Brewbaker and Kwack (1963).

2.2.2 Aniline blue staining of callose

Living or fixed (Geitmann et al. 2000) pollen tubes were incubated with decolorized aniline blue solution (1 $mg.mL^{-1}$ aniline blue in 0.15 M K_2HPO_4) and immediately observed at UV light excitation.

2.2.3 Immunofluorescent labeling of pectins

Pollen tubes were fixed in 30 $mg.mL^{-1}$ formaldehyde in Pipes-buffer (1 mM EGTA, 0.5 mM $MgCl_2$, 50 mM Pipes) and subsequently treated with monoclonal antibodies Jim 5 or Jim 7 for 2 h. After washes in buffer, the secondary antibody, goat-anti-rat conjugated with alexa fluor 594, was applied overnight at 4°C in the dark. After several washes, pollen tubes were mounted and observed in a fluorescence microscope using a Texas red filter set. Controls were performed by omitting incubation with the primary or the secondary antibody.

2.2.4 Calcofluor white staining of cellulose

Fixed pollen tubes (see above) were incubated with 1 $mg.mL^{-1}$ Calcofluor White for 15 min at room temperature, then rinsed with ddH_2O and immediately covered and observed using UV light excitation.

2.2.5 Fluorescence microscopy

Specimens labeled for cell wall components were observed in a fluorescence microscope (Nikon TE2000) equipped with a Roper fx cooled CCD camera. Plots of maximum fluorescence intensity along the longitudinal axis of the pollen tube were obtained by applying the surface plot function of ImagePro (Media Cybernetics, Carlsbad, Calif.).

2.2.6 Alexa 488 phalloidin label of actin filaments

Pollen tubes were fixed and stained as described in Geitmann et al. (2000). Observations were performed with a Leica TCS confocal laser scanning microscope. Serial optical sections were taken at 0.2 μm intervals and a maximum projection of the z-stack was created. Reconstructions of xz cross-sections were obtained with the help of the Leica TCS software.

2.2.7 Micro-indentation

Pollen was incubated as described and, after germination had occurred, coverslips were submerged in the GM-containing experimental chamber of the micro-indenter. The design, and principles of operation, of the micro-indenter have been described previously (Petersen et al. 1982; Elson et al. 1983). The micro-indentation assemblies used here were mounted on either a Zeiss IM 35 inverted light microscope or a Nikon TE2000 inverted microscope. Briefly, the bending of a horizontal glass beam gauges the resistance to cellular deformation. A vertical glass stylus (tip diameter 3 or 4 μm) is mounted at the end of a 3-cm long horizontal vycor glass beam. The other end of the beam is mounted on a linear piezoelectric motor that moves vertically according to a programmed waveform. Optical sensors monitor the vertical positions of the stylus and the motor. The extent to which the beam is bent is proportional to the force exerted on the tip by the cell and is determined by comparing tip displacements in the presence and absence of cell contact. The force exerted by the cell on the stylus is determined with the help of the force constant of the beam, which is obtained by prior calibration. In the experiments reported in this paper, the motor was programmed to execute a single triangular waveform with a velocity of 4 μm s^{-1} and a total amplitude of 10 μm.

2.3 Results

2.3.1 Stiffness changes along the longitudinal axis of the pollen tube

To quantify the local stiffness of a growing pollen tube along its longitudinal axis, we performed mechanical deformations using a micro-indenter. Individual deformations were performed repeatedly on various positions of the tube; the depth of deformation was generally around 2 μm. From force-distance graphs of individual deformation experiments we calculated the stiffness and plotted it against the distance from the apex. Absolute values varied between experiments and batches of pollen, but the shape of the stiffness plot for individual tubes was consistent. Figure 16 is a typical example for measurements taken from one individual pollen tube. It illustrates that, starting from the apex, stiffness increased considerably in the first 20 μm of the tube, whereas it did not vary significantly at locations further distal except for regions containing large vacuoles, the male germ unit or callosic plugs (not shown). For poppy, the apex of an individual tube had a stiffness of 74.4±25.6% of the value measured for the distal region from the apex, which for poppy was 1,306.±678 dynes/cm. Other species showed a similar pattern (A. Geitmann, unpublished data for *Camellia* and *Arabidopsis* pollen). *Petunia* pollen tubes exhibited a distal stiffness of 655.8±237.0 dynes/cm, whereas stiffness at the apex of an individual tube was 85.0±15.0% of its respective distal value.

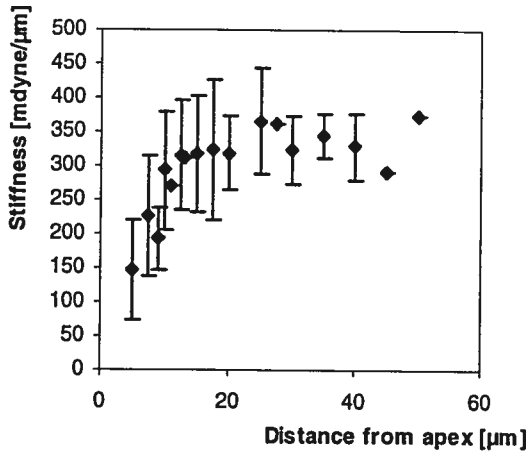

Figure 16 :Graph plotting stiffness versus distance from the apex of a typical growing *Papaver rhoeas* pollen tube. In the apical 20 µm a steep increase of stiffness can be noted whereas no significant variations occur in the distal area.

2.3.2 The growing pollen tube apex shows visco-elastic behavior

The force-distance graphs obtained for stiffness measurements revealed that distal regions of pollen tubes reacted almost perfectly elastically to mechanical deformation (Fig. 17B). In contrast, positions close to the apex typically showed a considerable delay in elastic recovery (hysteresis) upon retraction of the stylus, thus indicating the presence of a viscous component of cellular behavior at this location (a typical example is shown in Fig. 17A).

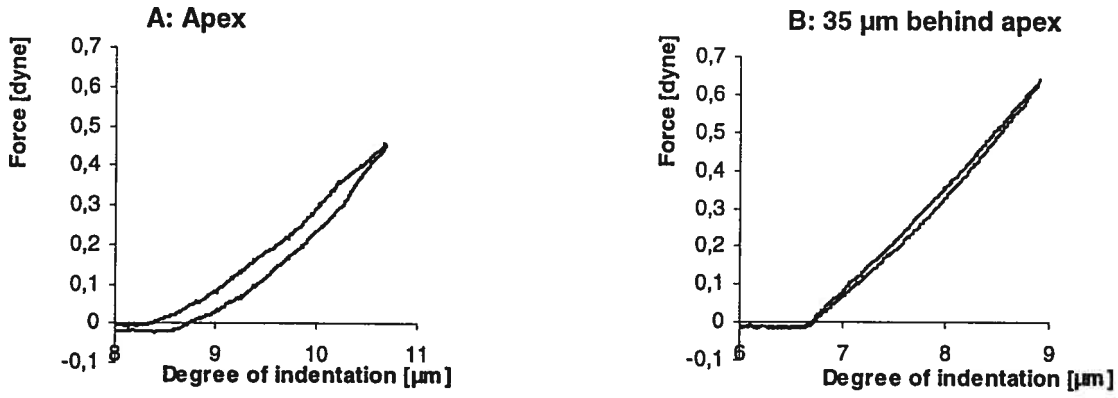

Figure 17 :Force-distance graphs of the apex (**A**) and the distal region 35 µm from the apex (**B**) of a growing *Papaver rhoeas* pollen tube. Whereas the distal region reacts almost perfectly elastically to deformation, the apex displays hysteresis upon retraction of the stylus, expressed by the area between the down- and upward path of the indenter.

2.3.3 The distribution of cell wall polymers in growing poppy pollen tubes

The mechanical deformation experiments showed that both stiffness and viscosity differ in the apical and distal parts of growing pollen tubes. We wanted to analyze whether we could identify the individual cellular components that might be responsible for the dramatic increase in cellular stiffness within the apical 20 µm of growing poppy pollen tubes. The main structural elements that contribute to pollen tube architecture and that are likely to show configurational differences along the longitudinal pollen tube axis are the cytoskeleton and the cell wall. It is known that several cell wall polymers show gradients in their distribution and chemical configuration along the longitudinal axis of pollen tubes. To relate the mechanical data with the exact distribution of these polymers in poppy pollen tubes we performed fluorescence labeling of individual cell wall components.

The cell wall polymer callose has been shown to be present immediately back from the tip, and then predominantly in the distal part of the pollen tube in many species. Fluorescence label with decolorized aniline blue showed clearly that no callose was present in the apex of growing poppy pollen tubes, as has been shown in many species. A plot of fluorescence intensity along the longitudinal axis revealed that in *Papaver rhoeas* a thin layer of callose started to be formed at 10 μm from the tip. The thickness of the layer increased gradually up to 30 μm, and with a smaller slope between 30 and 120 μm behind the apex. In more distal areas the thickness of the callose layer did not seem to change any further (Fig. 18).

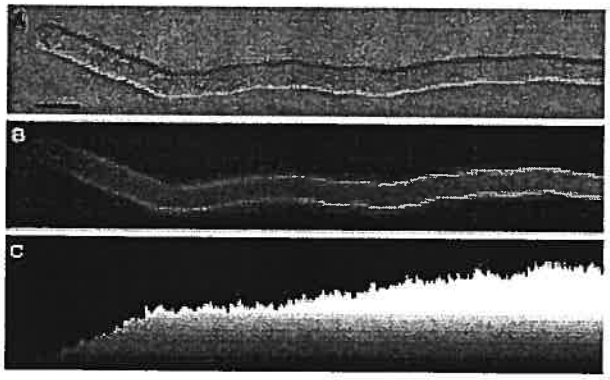

Figure 18 : Pollen tube of *Papaver rhoeas*. **A** Differential interference contrast microscopy (DIC) image. **B** Aniline blue staining for callose. **C** Plot of fluorescence intensity along the longitudinal axis of the pollen tube shown in **B**. Stain intensity increases gradually between the tip and 30 μm behind the tip. More distal areas show a slight increase in intensity until approximately 120 μm from the tip. *Bar* 10 μm.

The distribution of cellulose in the cell wall of growing pollen tubes varies between species and it is even absent in some. Fluorescence label using Calcofluor White revealed that in growing poppy pollen tubes cellulose seems to be present at low concentrations in the very apex, and a gradual increase of concentration takes place over the apical 50 μm or an even longer stretch of tube (Fig. 19).

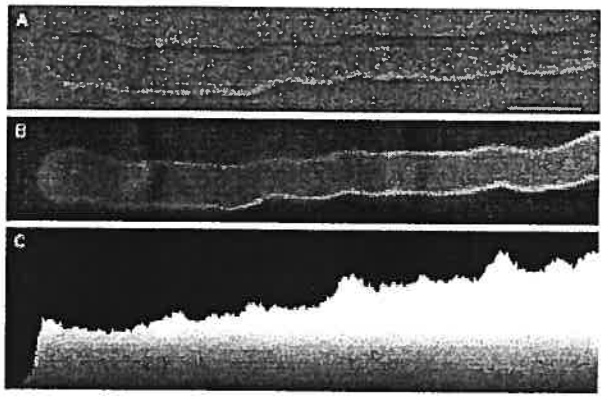

Figure 19 : Pollen tube of *Papaver rhoeas*. **A** DIC image. **B** Calcofluor white staining for cellulose. **C** Plot of fluorescence intensity along the longitudinal axis of the pollen tube shown in **B**. Stain intensity is very low at the apex and increases gradually towards the region 50–70μm behind the tip. *Bar* 10 μm.

Pectin is present in the pollen tube cell wall in varying degrees of methyl-esterification. We detected the presence of pectins with low and high degrees of methyl-esterification using monoclonal antibodies Jim 5 and Jim 7, respectively. As expected, Jim 7 label was intense at the growing apex and declined considerably in the region between 5 and 15 μm from the apex. Pectins with alow degree of methyl-esterification (as evidenced with Jim 5 label) were present starting 5 μm from the apex, and did not show a significant increase over the length of the tube (Fig. 20).

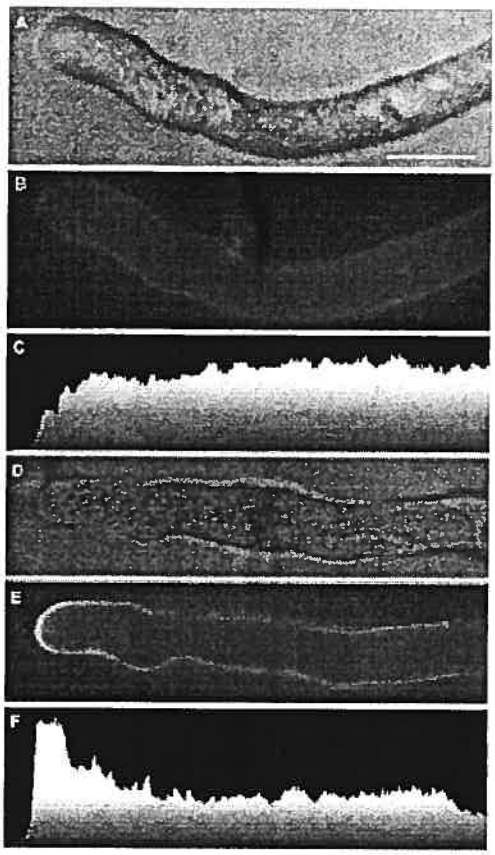

Figure 20A–F : Pollen tube of *Papaver rhoeas*. **A, D** DIC images. **B, E** Fluorescent label with monoclonal antibodies Jim 5 **(B)** and Jim 7 **(E)** for pectins with low and high degree of methyl-esterification, respectively. **C, F** Plot of fluorescence intensity along the longitudinal axis of the pollen tube shown in **B** and **E**. Stain intensity indicates that pectins are present in the apical and distal parts of pollen tubes. The degree of methyl-esterification at the apex is rather high and decreases rapidly in the region 5–15 µm from the apex *Bar* 10 µm.

2.3.4 The distribution of actin filaments in growing poppy pollen tubes

As expected, the very apex of growing poppy pollen tubes did not show strong phalloidin label (Fig. 21). This region, which coincides with the vesicle-filled "clear zone", was characterized by the absence of thick actin bundles.

In the region between 5 µm and 15 µm from the apex fluorescent label for actin was very dense, whereas further distal mainly axially oriented thick actin cables were present as published earlier for this species (Geitmann et al. 2000). The reconstruction of yz-cross sections from the z-stack of xy-sections obtained in the confocal laser scanning microscope allowed us to identify the density distribution of labeled actin on pollen tube cross sections (Fig. 22). This demonstrated clearly that the subapical dense mesh of actin filaments, the fine bundles of which were oriented anywhere from parallel to almost perpendicular to the longitudinal axis (Fig. 21), fills both the cortical and central parts of the lumen of the pollen tube in this region. On the other hand, the entire distal part of the cell contained mainly axially oriented thick actin cables that were located away from the plasma membrane, in a more central area of the tube, which reflects the reverse-fountain streaming pattern.

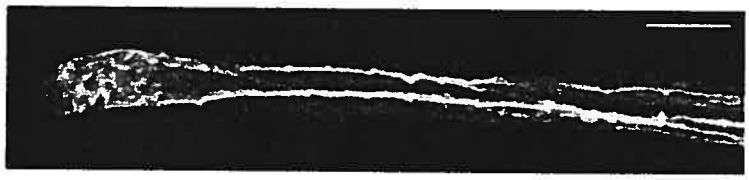

Figure 21 : Alexa 488-phalloidin label of actin in a *Papaver rhoeas* pollen tube observed by confocal laser scanning microscopy. Maximum projection of z-stack. Three zones can be distinguished: *a* The apex contains no thick actin cables but probably thin and/or dynamic actin bundles, *b* The subapex reaching from 5 to 15 µm behind the tip contains a dense mesh of fine actin bundles oriented in almost all directions from parallel to perpendicular to the longitudinal axis, *c* The distal region contains mainly axially oriented thick actin cables in the central area of the cytoplasm *Bar* 10 µm.

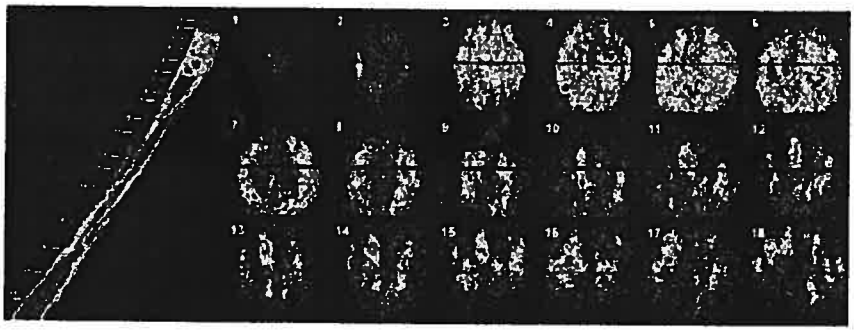

Figure 22 : Series of xz cross-sections reconstructed from the z-stack of images used to compose the xy image in Fig. 6. Fluorescence intensity is given in false colors—white and red represent the highest intensities. The positions of the cross sections are indicated on the figure showing the original z-projection in false colors. Cross-sections taken from the subapical region (5–15 μm from the apex) clearly show that a fine mesh of actin filaments is present across the entire diameter of the pollen tube. More distal regions show a few thick actin filament cables located in the more central areas of the tube.

2.4 Discussion

The configuration of structural features of pollen tubes as well as local confinement of the growth process to the apical dome suggest an anisotropic distribution of physical properties in this tip growing cell. For the first time, we have used a mechanical approach to show that this is indeed the case. Our experiments using micro-indentation revealed that local cellular stiffness is considerably reduced close to the growing apex compared to the distal part of the tube. At the same time, the cell showed visco-elastic behavior in the apical part of the tube, whereas distal regions behaved almost like perfect elastic bodies. This physical behavior corresponds well to the function of the different cellular regions. The distal part has to resist lateral deformation forces; maintenance of cellular diameter is pivotal for the biological function of pollen tubes—the passage of the male germ unit.

It is unlikely that the cytoskeleton contributes to the elastic deformability or the relatively high stiffness in this part of the cell, since actin filaments are oriented parallel to the longitudinal axis and are located mostly in the central area of the cell. They are therefore unlikely to be able to exert any supporting function in the direction perpendicular to the axis. Similarly, microtubules are oriented mostly parallel to the pollen tube longitudinal axis (Heslop-Harrison et al. 1988; Cai et al. 1997). Resistance to lateral deformation in the distal part of the cell is therefore likely to be provided by the equilibrium between cellular turgor and cell wall. The presence of callose in the distal region and/or the low degree of pectin methyl-esterification, and thus its putative gelation and resulting stiffening, are likely to be responsible for the elastic behavior of the cell. Further studies are underway to confirm the role of individual cell wall components. The apical and subapical portions of the pollen tube represent the most critical regions from the growth mechanical point of view. On the one hand, the tube has to be able to expand in the direction of the longitudinal axis, thus requiring a certain plasticity of the cell wall in the apical dome. On the other hand, the diameter of the cylindrical part of the cell has to be controlled and tightly maintained, a function that is likely to be localized in the subapical region adjacent to the apical dome. Our data show that stiffness of the cell in the apical region is reduced, and that the cell shows a visco-elastic component upon retraction of the stylus. It has been postulated that the cell wall at the apex shows plastic behavior as opposed to its elastic behavior in distal regions. We speculate that the observed viscosity component in the elastic behavior of the region near the tip is a strong indicator for this change of physical behavior towards the very tip. The subapical region also shows a steep increase in stiffness, which reaches a plateau at 20 μm from the apex. This steep gradient in stiffness corresponds to the hypothesized diameter-controlling function of this region. The question is, however, which cellular structure is responsible for this dramatic increase in stiffness and also the observed reduction in the viscous component of the behavior? Of course, we are aware of the fact that the cellular geometry might contribute to differences in measured stiffness, since the distal part of the cell is a cylinder, whereas the very apex consists of a half-sphere attached to this cylinder.

We are, however, confident that geometry does not affect our experimental data to a significant degree, since the average deformation applied by the micro-indenter was 2 μm and we started indenting only 5 μm from the apex. As Fig. 23 shows, even the most apical indentation should not be influenced to any great degree by the proximity of the half-sphere.

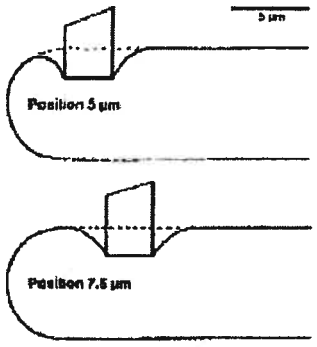

Figure 23 : Schematic illustration of the deformation on pollen tubes applied by the micro-indentation device. If there is any influence of geometry at all, only the indentation at 5 μm from the apex might be slightly affected by the proximity of the apical hemisphere. Data obtained from locations distal of this point are unlikely to be influenced by geometry.

The localization of polymers in the cell wall of growing poppy pollen tubes revealed that the thickness of the callosic cell wall layer increases only gradually in the subapical region, reaching its final thickness not before 120 μm from the apex. Similarly, the distribution of cellulose is extremely gradual in the subapical region. Whereas it is reasonable to propose that callose and cellulose contribute to the physical changes on the longitudinal axis, their very gradual increase in the subapical area indicates that they are unlikely to be the key factors responsible for the dramatic increase in stiffness in this region. On the other hand, the considerable decrease in the degree of pectin methyl-esterification observed coincides very well with the subapical increase in stiffness and decrease in viscosity in the subapical 5–20 μm of the pollen tube.

Experiments including the manipulation of the degree of pectin methyl esterification are therefore underway to further elucidate the role of this cell wall component in pollen tube cytomechanics. A remarkable correlation also exists between physical property gradients and the actin configuration in the subapex. The absence of prominent actin label in the very apex, and the dense actin mesh present in the subapical region, suggest that the cytoskeleton might be at least partly responsible for the steep increase in stiffness in the apical 20 μm. This could be the direct effect of a cytoskeletal scaffold formed by the dense actin mesh. However, another model that deserves consideration involves actin gel/sol transformations (Oster and Perelson 1994). By causing the formation of local gel/sol transitions in the cytoplasm, the subapical dense concentration of fine actin bundles might be responsible for the differences in the cytomechanical properties along the longitudinal axis. Studies using inhibitors of cytoskeletal functioning and polymerization are underway to further investigate this point. They prove to be anything but trivial, however, since elimination of the actin cytoskeleton leads to an arrest of pollen tube growth. This in turn is expected to rapidly affect the structural gradients present in the cell wall composition, since these are maintained only upon continuous insertion of new cell wall material (Li et al. 1997, 2002; Lennon and Lord 2000). The application of inhibitors of cytoskeletal functioning thus has undesired side effects that pose a conceptual problem for the isolation of the individual roles of the cytoskeleton and of the cell wall.

In summary, we confirm for the first time that the mechanical properties of growing pollen tubes exhibit a strong gradient along the longitudinal axis, both regarding stiffness and visco-elasticity. These differences are likely to be pivotal for the anisotropic growth behavior and biological functioning of these tip growing cells. As expected, the physical properties of the cell wall seem to be a determining factor in the cellular architecture, with pectins playing a key role. Interestingly, the configuration of the actin cytoskeleton suggests that next to its function in intracellular transport it might also have a structural role that could be significant for pollen tube growth.

2.5 Acknowledgements

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Chapitre 3 :

Pectin and the role of the physical properties of the cell wall in pollen tube growth

Elodie Parre, Anja Geitmann

Ce chapitre est actuellement en révision pour le journal Planta.

Abstract

The cell wall is one of the structural key players regulating pollen tube growth, since plant cell expansion depends on an interplay between intracellular driving forces and the cell wall. Pectin is the main cell wall component at the growing pollen tube apex. We therefore assessed its role for pollen tube growth and cytomechanics using the enzymes pectinase and pectin methyl esterase (PME). Pectinase activity was able to stimulate pollen germination and tube growth at moderate concentrations whereas higher concentrations caused apical swelling or bursting in both *Solanum chacoense* and *Lilium longiflorum* pollen tubes. This is consistent with a modification of the physical properties of the cell wall affecting its extensibility and thus the growth rate as well as its capacity to withstand turgor. To prove that the enzyme induced effects were due to the altered cell wall mechanics, we subjected pollen tubes to micro-indentation experiments. We observed that cellular stiffness was reduced and visco-elasticity increased in the presence of pectinase. These are the first mechanical data that confirm the influence of the amount of pectins in the pollen tube cell wall on the physical parameters characterizing overall cellular architecture. Cytomechanical data were also obtained to analyze the role of the degree of pectin methyl-esterification, which is known to exhibit a gradient along the pollen tube axis. This feature has frequently been suggested to result in a gradient of the physical properties characterizing the cell wall and our data provide for the first time mechanical support for this concept. The gradient in cell wall composition from apical esterified to distal de-esterified pectins seems to be correlated with an increase in the degree of cell wall rigidity and a decrease of visco-elasticity. Our mechanical approach provides new insights concerning the mechanics of pollen tube growth and the architecture of living plant cells.

Key Words

Cell expansion - Cell wall - Cytomechanics - Pectin - Pollen tube - Tip growth

Abbreviations

GM growth medium - PME pectin methyl esterase

3.1 Introduction

Pollen tubes are fast growing cells exhibiting tip growth. In flowering plants, pollen grains germinate on a receptive stigma and send pollen tubes down the style toward the ovary. This allows the delivery of the sperm cells to the ovules in order to perform fertilization. The growth process in pollen tubes is confined to the dome-shaped apex and occurs in one direction only (Derksen 1996; Hepler et al. 2001). Since the pollen tube grows rapidly and over long distances, the production of the cell wall, its composition and the configuration of its components are important features which regulate the physiology of pollen tube growth, and thus the fertilization process (Taylor 1997).

In general, plant cell growth is driven by internal turgor pressure and restricted by the ability of the cell wall to extend under this pressure. To which extent this equilibrium between turgor and cell wall determines the rate of tip growth in cells exhibiting polar elongation such as pollen tubes, root hairs or fungal hyphae, has been subject of vivid discussion (Money and Hill, 1997, and references therein). The importance of additional factors such as the putative propulsion force of the cytoskeletal elements and the regulation of insertion of new cell wall material is still largely unknown for tip growing cells in general and for pollen tubes in particular. There seems to be no doubt, however, that the force counteracting the internal driving force is provided by the cell wall. Even though the structure of the cell wall of pollen tubes has been described in detail, only few approaches have been made to assess its mechanics in a quantitative manner.

In the pollen tube cell wall, two or three layers can be distinguished depending on the plant species and the method of investigation (Heslop-Harrison 1987; Steer and Steer 1989). It consists of a primary pecto-cellulosic layer and a secondary callosic wall.

In the tip of the pollen tube, the cell wall lacks the callosic inner lining (Heslop-Harrison 1987), and thus pectins appear to be major component of the cell wall in this region crucial for cell expansion (Roggen and Stanley 1969; Li et al. 1994; Derksen et al. 1995a; Li et al. 1997; Li et al. 2002; Holdaway-Clarke and Hepler 2003).

Immunocytochemical studies have shown that pectins located at the tip of the growing pollen tube are highly esterified, while they are de-esterified in areas away from the tip (Li et al. 1994; Li et al. 1995; Jauh and Lord 1996; Li et al. 1997; Lennon and Lord 2000; Li et al. 2002). This gradient in the degree of esterification is the result of the tip-focused growth process. The wall components are secreted at the extreme apex as esterified pectin residues produced by the Golgi apparatus (Levy and Staehelin 1992; Hasegawa et al. 1998). Upon arrival in the cell wall, they become increasingly de-esterified during maturation through the activity of the enzyme pectin methyl esterase (PME) (Kauss and Hassid 1967; Li et al. 1997; Geitmann 1998). De-esterification permits Ca^{2+} ions to cross-link the acidic pectins to form a semi rigid pectate gel (Jarvis 1984; Carpita and Gibeaut 1993), that is presumed to provide mechanical support for the distal areas of the elongating tube by altering the physical properties of the cell wall. Esterified and thus non-gelated pectins, which are present predominantly at the apex, are presumed to allow turgor driven expansion of the tube (Morris et al. 1982; McNeil et al. 1984; Li et al. 1994; Geitmann et al. 1995; Derksen 1996; Jauh and Lord 1996; Li et al. 1997; Geitmann and Cresti 1998; Franklin-Tong 1999; Geitmann 1999). However, no experimental evidence has been brought forward to show that the physical properties of the cell wall are indeed different between the apex and the distal part of a growing pollen tube. A gradient of the cytomechanical properties along the longitudinal pollen tube axis measured by micro-indentation of *Papaver* pollen tubes is consistent with this concept (Geitmann and Parre 2004), but whether or not it is due to the physical properties of the cell wall or in particular to those of the pectin component remains to be shown.

Here we provide mechanical data that support two longstanding hypotheses that hitherto were largely based on structural observations: i) We show that pollen tube growth and germination are governed by physical properties of the apical cell wall thus supporting the importance of a equilibrium between inner driving and outer restricting forces in this cellular system. ii) Our data revealed that both the amount and the configuration of pectins are key factors determining the physical properties of the apical cell wall thus influencing pollen tube growth.

3.2 Materials and methods

3.2.1 Pollen tube growth

Solanum chacoense plants were grown in the Montreal Botanical Garden greenhouses and *Lilium longiflorum* flowers were obtained from a local flower shop. Pollen was collected after dehiscence, dehydrated and stored at –20°C. On the day of use pollen was rehydrated and cultivated in drops of liquid or solidified medium. The latter was obtained by addition of low melting agarose (Sigma, St. Louis, US) or Gel gro (Gellan gum; gel strength twice as high as that of agarose; ICN Biomedical Inc., Ohio, US). The growth medium (GM) contained 100 $\mu g\ mL^{-1}$ H_3BO_3, 300 $\mu g\ mL^{-1}$ $Ca(NO_3)_2 \cdot H_2O$, 100 $\mu g\ mL^{-1}$ KNO_3, 200 $\mu g\ mL^{-1}$ $MgSO_4 \cdot 7H_2O$, 50 $\mu g\ mL^{-1}$ sucrose (Brewbaker and Kwack 1963) for *Solanum* pollen and 0.29 $\mu g\ mL^{-1}$ MES, 0.01 $\mu g\ mL^{-1}$ H_3BO_3, 0.0147 $\mu g\ mL^{-1}$ $CaCl_2$ for lily pollen grains. Various concentrations of pectinase or pectin methyl esterase were added at the start of germination (to assess the effect on germination) or after 30 min of germination (to assess the effect on elongation). Controls were performed by adding enzyme that had been denatured by boiling for 10 min. Germination rate and pollen tube length were assessed in the light microscope after two hours of incubation on microscope slides.

3.2.2 Fluorescence label

For immuno-fluorescence label, pollen tubes were fixed after 2 hours of germination in 30 $mg.mL^{-1}$ freshly prepared formaldehyde in Pipes-Buffer (1 mM EGTA, 0.5 mM $MgCl_2$, 50 mM Pipes) for 30 min. To allow for quantitative comparison of fluorescence intensity, pollen tubes grown in solidified medium (Gel gro) were resuspended by adding 0.1M citrate buffer (55 mM citric acid, 125 mM sodium citrate, pH 6) at 30°C, to assure that the gel did not limit the access of antibodies to the cells (liquid medium controls were treated similarly). Subsequently, cells were incubated with monoclonal antibodies Jim 5 and Jim 7 (generously provided by Dr Paul Knox) diluted 1:50 in PBS buffer followed by an incubation with goat anti-rat IgG - alexa fluor 594 (diluted 1:100 in buffer) over night at 4°C. Jim 5 and Jim 7 recognize homogalacturonans with low (VandenBosch et al. 1989) and high degree of esterification, respectively (Knox et al. 1990). Pollen tubes were mounted and observed in the fluorescence microscope using a Texas red filter set. Controls were performed by omitting incubation with the primary or the secondary antibody.

Decolorized aniline blue and calcofluor staining (for callose and cellulose, respectively) were carried out on fixed pollen tubes (see above). After two washes, cells were incubated for 15 minutes with the staining agent (1 $mg.mL^{-1}$ aniline blue in 0.15M K_2HPO_4; 1 $mg.mL^{-1}$ calcofluor in ddH_2O), mounted immediately and observed at UV light excitation.

3.2.3 Brightfield and fluorescence microscopy

Specimens labeled for cell wall components were observed in the fluorescence microscope (Nikon TE2000) equipped with a Roper fx cooled CCD camera. Exposure times of images that had to be compared for fluorescence intensity were identical. These pictures were not corrected for brighness or contrast. Plots of the maximal fluorescence intensity along the longitudinal axis of the pollen tube were obtained by applying the Surface Plot function of ImagePro (Media Cybernetics).

3.2.4 Micro-indentation

Hydrated pollen was grown on cover slips coated with stigmatic exudate (to assure pollen adherence) and covered with liquid GM. After germination had occurred, cover slips were submerged in the GM containing experimental chamber of the micro-indenter. The design and principles of operation of the micro-indenter have been described previously (Petersen et al. 1982; Elson et al. 1983). The micro-indentation assemblies used here were mounted on a Nikon TE2000 inverted microscope. Briefly, the bending of a horizontal glass beam gauged the resistance to cellular deformation. A vertical glass stylus (tip diameter 10 μm) was mounted at the end of a 23mm long horizontal vycor glass beam. The other end of the beam was mounted on a linear piezoelectric motor, which moves vertically according to a programmed waveform. Optical sensors monitored the vertical positions of the stylus and the motor. The extent to which the beam was bent was proportional to the force exerted on the tip by the cell and was determined by comparing tip displacements in the presence and absence of cell contact. The force exerted by the cell on the stylus was determined with the help of the force constant of the beam, which had been obtained by prior calibration. In the experiments reported in this paper, the motor was programmed to execute a single triangular waveform with a velocity of 10 $\mu m.s^{-1}$ and a total amplitude of 5 μm.

3.3 Results

3.3.1 Pectinase affects pollen tube growth and germination rates

The cell wall composition of the pollen tube apex suggests that pectin is the main candidate for controlling the cell wall portion of the force equilibrium governing pollen tube growth. If we were able to affect the growth rate by reducing the amount of pectins at the pollen tube apex, we would confirm two hypotheses:

i) cell wall extensibility controls pollen tube growth and ii) the abundance of pectins determines cell wall extensibility. We therefore applied varying concentrations of pectinase thus partly degenerating the pectin component of the pollen tube cell wall. The distribution of other cell wall components such as cellulose and callose was not affected by this treatment as revealed by fluorescence label (Parre and Geitmann, unpublished data). Fig. 24 shows that pollen of both *Solanum chacoense* and *Lilium longiflorum* reacted in similar patterns to increasing pectinase concentrations, but that there existed considerable quantitative differences between the two species. In both species high pectinase concentrations inhibited pollen germination (Fig. 24A) and tube growth (Fig. 24b) by causing bursting, whereas certain optimal concentrations significantly increased both germination and elongation rate.

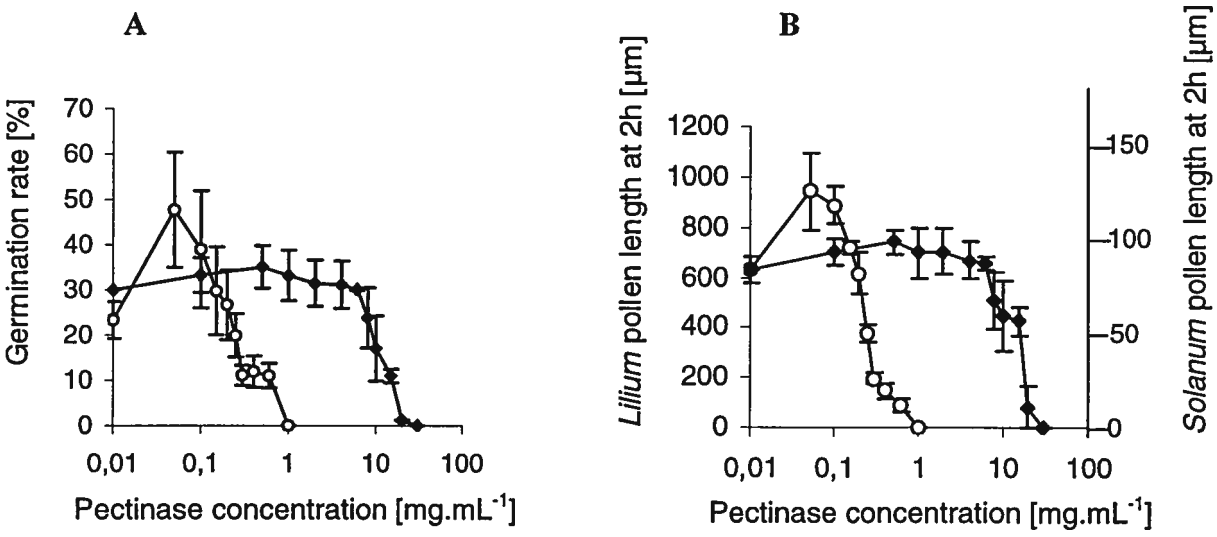

Figure 24: Effect of the presence of pectinase on germination rate (**A**) and pollen tube length at 2h (**B**) in *Solanum chacoense* (♦) and *Lilium longiflorum* (○) pollen grown in liquid medium. Both species show significant stimulation of germination rate and pollen tube length, but at different pectinase concentrations. High enzyme concentrations are inhibitory since they induce bursting of pollen grains.

The highest increase (17%) in pollen tube length measured after 2h for *Solanum* was accomplished by addition of pectinase at 0.5 $mg.mL^{-1}$ whereas an enzyme concentration of 0.05 $mg.mL^{-1}$ resulted in a maximal increase of pollen tube length by 50% in *Lilium*. Remarkably, the optimal concentrations for stimulation differed by a factor 10 and those for inhibition by a factor 30 between the two species with *Lilium* pollen tubes being much more sensitive to the enzyme (Table II).

	Solanum	***Lilium***
Pectinase concentration resulting in optimal stimulation of pollen germination and tube elongation	0.5 $mg.mL^{-1}$	0.05 $mg.mL^{-1}$
Pectinase concentrations resulting in bursting of 95% of pollen grains	30 $mg.mL^{-1}$	1 $mg.mL^{-1}$

Table II: Pectinase affects pollen tube growth in *Solanum* and *Lilium*.

Denaturing the enzyme by boiling for 10 min eliminated both the stimulating and inhibiting effects.

Since the enzyme was added from the beginning of germination, an increase in pollen tube length could be due to accelerated germination and/or an increase in pollen tube growth rate. In order to distinguish between these two variables, we applied growth enhancing concentrations of the enzyme on *Solanum* tubes 30 min after they had germinated under control conditions. After additional 90 min incubation in the presence of 0.5 $mg.mL^{-1}$ pectinase, pollen tubes treated with active enzyme were significantly longer (120±3 μm) than those treated with denatured enzyme (100±4 μm) (n=10). This indicates that the stimulating effect on pollen tube length was to a significant degree, albeit perhaps not exclusively, due to an effect on the pollen tube growth rate.

3.3.2 Sensitivity towards pectinase differs between species

The observed differences between *Lilium* and *Solanum* pollen tube reactions toward pectinase led us to believe that the amount of pectin in the pollen tube cell wall might vary significantly between species. To confirm this, we applied identical pectinase concentrations to growing *Lilium* and *Solanum* pollen tubes. We chose 1 $mg.mL^{-1}$ pectinase, since this concentration neither increased nor inhibited pollen tube growth in *Solanum*. Addition of 1 $mg.mL^{-1}$ pectinase caused 95% of the *Lilium* pollen tubes to burst at the apex within 10 sec whereas *Solanum* pollen tubes showed unaltered morphology at the same point of time (Fig. 25).

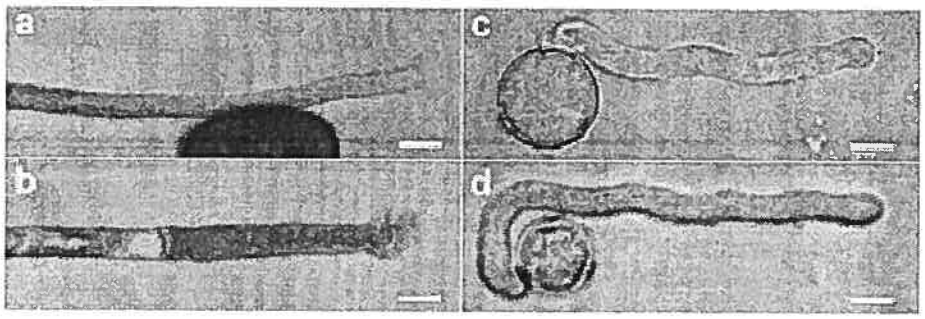

Figure 25: Effect of the addition of 1 $mg.mL^{-1}$ pectinase on pollen tubes grown under control conditions. Brightfield images of pollen tubes of *Lilium* **(a,b)** and *Solanum* **(c,d)** taken 10 sec after addition of enzyme deactivated by boiling **(a,c)** and of active enzyme **(b,d)**. *Lilium* pollen tubes have burst, whereas *Solanum* pollen tubes remained intact. Bars = 30µm (a,b), 10µm (c,d).

The pectinase concentration that was necessary to cause bursting in 95 % of *Solanum* tubes was 30 fold higher than the one effective in *Lilium* (Table II). This confirms the significant difference in sensitivity towards the enzyme between the two species.

3.3.3 Pollen tube apical swelling

We had observed that pectinase induced bursting occurred exclusively at the pollen tube apex. To identify the length of the region that seemed to be particularly sensitive to pectinase treatment, we assessed the effect of a moderately inhibitory concentration (8 $mg.mL^{-1}$) of pectinase on pollen tube morphology. *Solanum* pollen tubes grown in the presence of this enzyme concentration showed swelling of the apical region starting 30 µm from the apex. The diameter of the distal part of the tube was unchanged (Fig. 26).

Figure 26: Effect of the presence of 8 $mg.mL^{-1}$ pectinase in the germination medium on *Solanum* pollen tubes. The tube apex is swollen at the apical 30 µm. Bar = 10 µm.

3.3.4 Distribution of cell wall components

The morphology of the pectinase induced apical swelling could be explained with a local cell expansion driven by internal forces. The confinement of the phenomenon to the apex might be due to either a lower amount of pectins in this area, or to the presence of other cell wall polymers in the distal parts that keep resisting the internal forces once pectin is degenerated. To distinguish between these two alternatives we identified the distribution of two putative load-bearing cell wall components in the pollen tube cell wall: cellulose and callose. Figs. 27a-c illustrate that in *Solanum* pollen tubes callose was completely absent from the tip. Similarly, cellulose concentration was significantly lower at the tip compared to the distal part of the tube (Figs. 27d-f). Both polymers increased very gradually towards the distal parts of the tube. This is consistent with findings in other species.

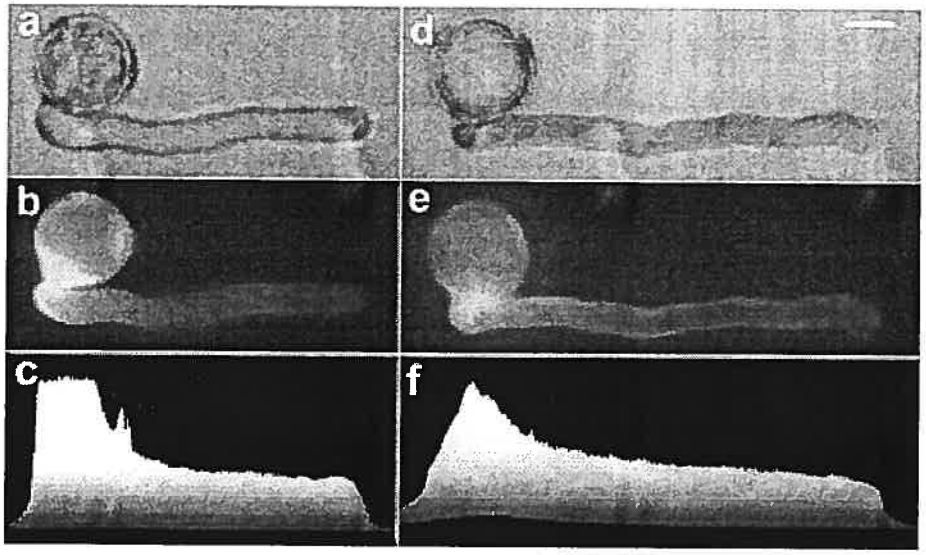

Figure 27: Fluorescent label for cell wall components in *Solanum* pollen tubes and pollen grains. Pollen tubes were fixed and subsequently stained for callose with decolorized aniline blue (**a-c**) or for cellulose with calcofluor white (**d-f**). Brightfield images are provided for reference (**a,d**). Relative fluorescence intensity along the longitudinal axis was plotted with the Surface Plot function of ImagePro (**c,f**). Label of both callose and cellulose was absent at the pollen tube apex. The intensity increased very gradually towards the distal portion of the tube. Bar = 10 μm.

Pectins on the other hand were present in distal and apical regions. The antibodies Jim 5 (Figs. 28a-c) and Jim 7 (Figs. 28d-f) each label only subpopulations of pectin, but both labels taken together indicated that the amount of pectin was not reduced at the apex compared to the distal regions of *Solanum* pollen tubes.

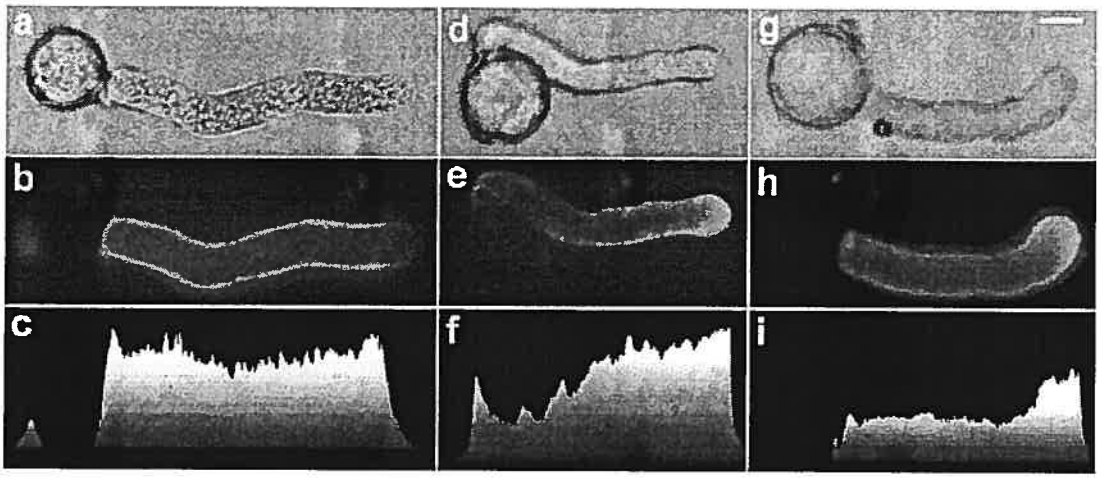

Figure 28: Immunofluorescent label for pectins in *Solanum* pollen tubes and pollen grains. Pollen tubes were fixed and subsequently labeled for acidic pectins with Jim 5 (**a-c, g-i**) or for methyl-esterified pectins with Jim 7 (**d-f**). Label for acidic pectins was absent from the pollen tube apex, the distribution in the distal regions was rather homogeneous. Label for methyl-esterified pectins was highest at the apex and decreased considerably towards distal regions. The pollen tube in Figs. g-i was grown in medium containing 0.5 $mg.mL^{-1}$ pectin methyl esterase to convert methyl-esterified pectins into the acidic variety thus presumably allowing Jim 5 to label the entire pectin population. Label intensity was highest at the apex thus indicating that the total amount of pectins is highest in this region. Bar = 10 µm.

This was confirmed by Jim 5 label of pollen tubes that had been treated with pectin methyl esterase. This enzyme treatment supposedly caused a transformation of methyl-esterified into acidic pectins and should thus make the entire pectin population recognizable for the Jim 5 antibody. Figs. 28g-i show that label was significantly stronger at the apex than at distal parts and thus indicates a high total pectin concentrations in this region. The labeling patterns for the three cell wall components pectin, cellulose and callose are consistent with the concept that pectin is the only cell wall component at the tip that resists the inner propulsion forces.

3.3.5 Pectinase affects pollen tube penetration growth

Our data suggest that pectins might play an important role in the equilibrium between pollen tube cell wall and the inner driving forces, which in turn is presumed to control the growth rate in liquid medium. Since *in vivo* pollen tube growth takes place within the transmitting tissue of the receptive flower, we wanted to investigate to which extent pollen tube growth through a solidified medium would be affected by pectinase. Solidification caused a reduction in germination and tube length at 2h compared to the liquid control. At low enzyme concentrations, pectinase stimulated germination and elongation of *Solanum* pollen tubes both in liquid and in solidified media (Fig. 29).

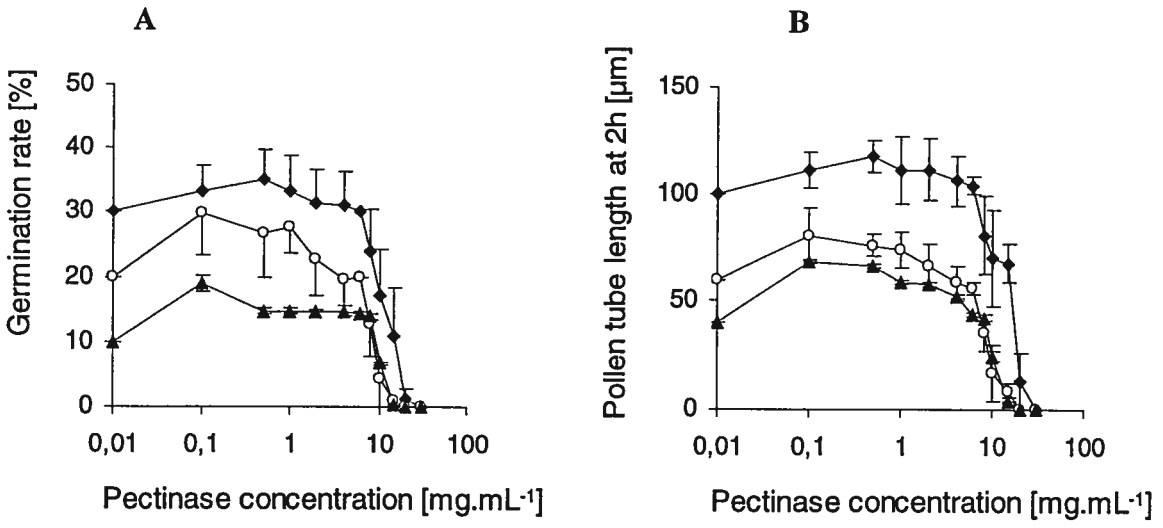

Figure 29: Effect of different pectinase concentrations on *Solanum* pollen germination (**A**) and tube length after 2h (**B**) of pollen grown in liquid medium (♦) or medium solidified with 20 mg.mL^{-1} (○) or 40 mg.mL^{-1} (▲) agarose. Both germination and pollen tube length were reduced in stiffer media. Pectinase nevertheless had a significant stimulating effect at moderate concentrations and an inhibiting effect at higher concentrations in stiff media. However, optimal enzyme concentrations and relative stimulation had a tendency to differ from those observed in liquid medium as summarized in Table III.

Surprisingly, the stimulating effect was relatively bigger in solidified than in liquid medium as summarized in Table III.

	Maximal germination rate		Maximal pollen tube length at 2h	
Medium stiffness	Relative increase compared to untreated control	Optimal pectinase concentration	Relative increase compared to untreated control	Optimal pectinase concentration
Liquid	17 %	0.5 $mg.mL^{-1}$	17 %	0.5 $mg.mL^{-1}$
20 $mg.mL^{-1}$ Agarose	50 %	0.1 $mg.mL^{-1}$	34 %	0.1 $mg.mL^{-1}$
40 $mg.mL^{-1}$ Agarose	90 %	0.1 $mg.mL^{-1}$	70 %	0.1 $mg.mL^{-1}$

Table III: Optimal pectinase concentrations that result in stimulation of *Solanum* pollen germination and tube elongation in liquid and solidified media.

The enzyme concentration necessary for optimal stimulation of both, germination and pollen tube elongation had a tendency to be lower in solidified media compared to liquid medium.

Interestingly, there was also a significant difference in the inhibitory pectinase concentration between the different media. Whereas 15 $mg.mL^{-1}$ pectinase almost completely inhibited pollen tube germination and growth in medium solidified with 20 or 40 $mg.mL^{-1}$ agarose, this concentration only had a partially inhibitory effect in liquid medium.

3.3.6 The abundance of cell wall pectin is affected by the stiffness of the growth medium

The shift in sensitivity upon pectinase treatment in pollen tubes grown in solidified media indicated that the stiffness of the surrounding medium might affect the equilibrium between inner and outer forces in the pollen tube. We hypothesized therefore, that lower growth stimulating and inhibitory pectinase concentrations might be the result of a reduced amount of pectins in the cell wall, thus enabling lower enzyme concentrations to be effective. To find out whether the pectin contents indeed differed between liquid and solidified media we compared pectin label of images taken at identical exposure times of pollen tubes grown in liquid medium and medium solidified by addition of 10 $mg.mL^{-1}$ Gel gro (corresponding to the gel strength of 20 $mg.mL^{-1}$ agarose). The use of Gel gro allowed us to resuspend the pollen after fixation, thus ensuring the antibody access to the cells was not limited by the stiffened medium - an essential prerequisite for comparison of fluorescence intensity.

Label with Jim 5 and Jim 7 revealed that the amount of both types of pectins decreased dramatically for pollen tubes grown in stiffer germination medium (Fig. 30). The distribution pattern between apex and distal parts for esterified and unesterified pectins remained approximately the same as that in cells grown in liquid medium.

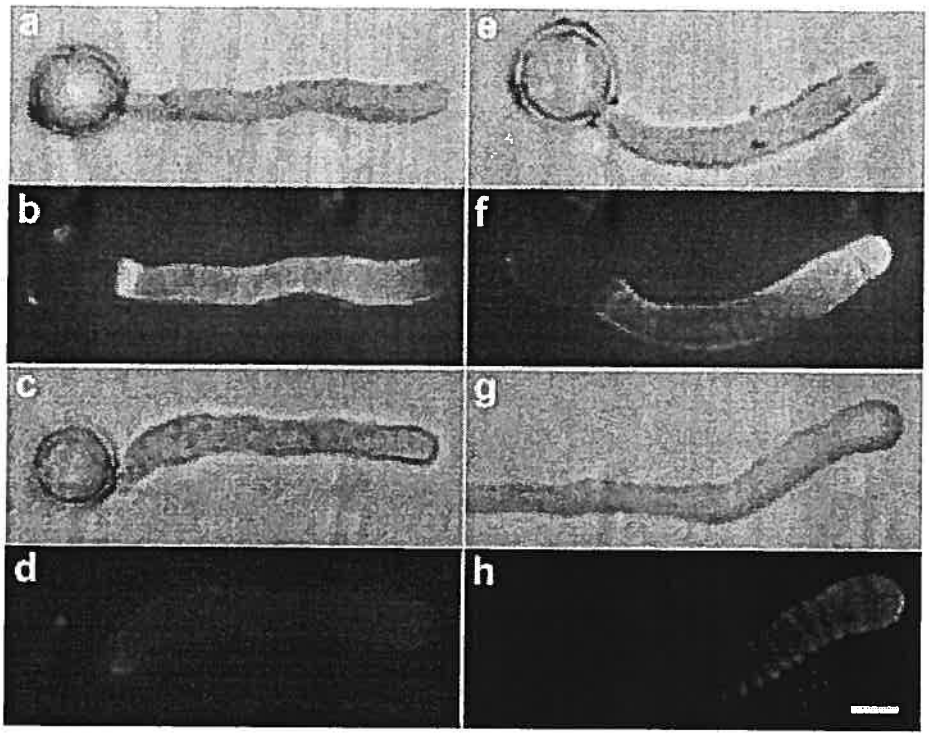

Figure 30: Comparison of immunofluorescence label intensity for pectins in pollen tubes grown in liquid and solidified medium. Within a single experiment exposure times were identical for all images. Both label with Jim 5 for acidic pectins (**a-d**) and Jim 7 for methyl-esterified pectins (**e-h**) evidenced that pollen tubes grown in liquid medium (**a,b,e,f**) were significantly stronger labeled than those grown in solidified medium (**c,d,g,h**). Bar = 10 µm.

3.3.7 Local stiffness and visco-elasticity of the pollen tube are affected by pectinase

Our observations revealed that depending on the enzyme concentration, pectinase treatment caused pollen tubes to swell or burst at the apex. Furthermore we showed that at the growing pollen tube apex, pectins seem to be the only cell wall polymer present in significant amounts to determine cellular behavior. Together, this indicates that pectins are a major factor in the force equilibrium that controls pollen tube growth.

We therefore attempted to assess the role of pectin for the mechanic properties of the cell wall in a quantitative manner.

Local cellular stiffness and visco-elasticity were measured using a micro-indentation device. Local deformations were performed at two positions of growing pollen tubes, at the apex and at a distal position between 20 and 30 µm from the apex. As shown earlier for other species (Geitmann and Parre 2004), normally growing *Solanum* pollen tubes were characterized by a difference in stiffness and degree of visco-elasticity between the growing apex and the distal region. Distal stiffness was $4760\pm417x10^{-5}$ N/cm whereas apical stiffness amounted to 85±5% of the value for the distal part of the same tube (n=7). The apex generally showed a bigger delay upon retraction of the deforming stylus (hysteresis) than the distal region thus indicating an increased visco-elastic behavior.

For pectinase treatment we used 6.4 $mg.mL^{-1}$, since this concentration neither stimulated nor inhibited pollen tube growth, but immunofluorescence label with Jim 5 and Jim 7 revealed that the amount of pectins was significantly reduced in the cell wall of pollen tubes grown in the presence of the enzyme. Images taken at the same exposure time as control tubes were completely black (not shown). Pollen tubes that were germinated in the presence of 6.4 $mg.mL^{-1}$ pectinase, showed a decrease in the stiffness for both the apex and the distal parts of the tube. The apical stiffness was reduced to $2438\pm151x10^{-5}$ N/cm whereas the ratio between apex and distal stiffness remained essentially the same with 82±5% (n=3). Both apical and distal regions showed an increase in the hysteresis upon retraction of the micro-indenter stylus thus indicating an increase in the degree of viscosity in the cellular structure. Figs. 31a,b illustrate the differences in the force-distance graphs concerning stiffness and hysteresis for the apex.

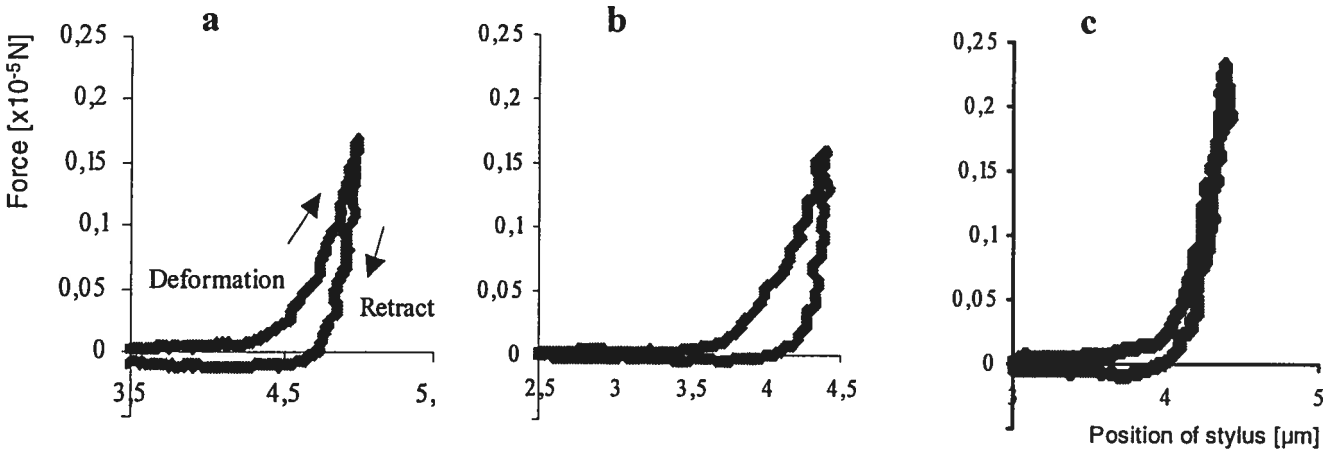

Figure 31: Force distance graphs for micro-indentation experiments performed at the apex of growing *Solanum* pollen tubes. The relative position of the stylus is given in positive values for downward movement. Zero position is approximately 1 µm above the pollen tube surface.

The upper curve represents the deforming- and the lower curve the retracting movement of the stylus. The slope of the curve indicates the stiffness whereas the surface area between the two curves expresses the delay upon retraction of the deforming stylus (hysteresis) which is an indication for the dissipated energy and thus the viscosity of the deformed object. The apex of pollen tubes grown in the presence of 6.4 $mg.mL^{-1}$ pectinase (**b**) showed an increase in hysteresis and a lower stiffness compared to that of the control pollen tubes (**a**). This indicates a higher viscosity component in the visco-elastic behavior of the enzyme treated cell. The apex of pollen tubes grown in the presence of 0.5 $mg.mL^{-1}$ PME (**c**) was stiffer than that of the control and reacted nearly completely elastic

3.3.8 Pectin methyl esterification influences the stiffness of the pollen tube cell wall

The degree of pectin esterification is known to influence the mechanical properties of the plant cell wall. To show that pectin configuration is indeed a critical factor for pollen tube growth we applied the enzyme pectin methyl esterase (PME) at varying concentrations.

This enzyme is supposed to transform methyl-esterified pectin into the acidic, gel-forming variety.

If a high degree of methyl-esterification is a prerequisite for cellular expansion at the pollen tube apex, PME treatment should be expected to decrease or inhibit pollen tube growth. Our results indeed confirmed a negative effect on germination and tube growth for concentrations of 0.1 mg.mL^{-1} and above (Fig. 32).

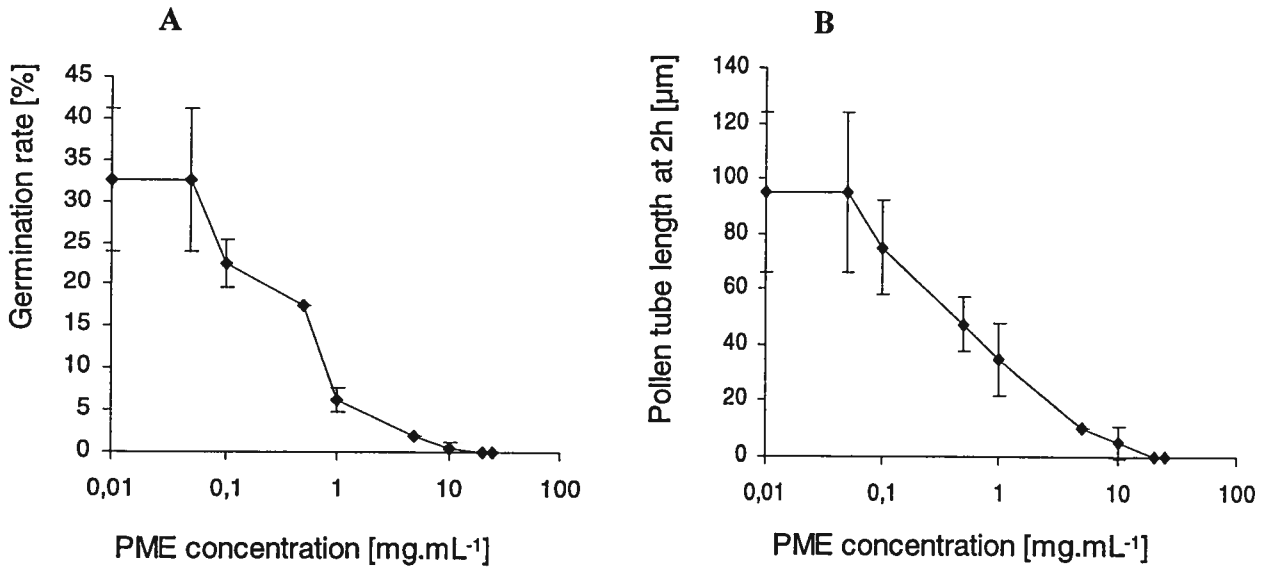

Figure 32: Effect of varying concentrations of pectin methyl esterase on *Solanum* pollen germination rate **(A)** and tube length at 2h **(B)**. Concentrations above 0.1 mg.mL^{-1} reduced both germination rate and tube length and complete inhibition was achieved at an enzyme concentration of 20 mg.mL^{-1}.

To demonstrate that de-esterification indeed rigidified the cell wall, we assessed pollen tube stiffness of PME treated pollen tubes with the micro-indenter. We chose a PME concentration that was not completely inhibitory, but that resulted in an increase of immunolabel for acidic pectins at the apex. PME at 0.5 mg.mL^{-1} was an appropriate concentration that fulfilled the requirement. Jim 5 label of pollen tubes grown in the presence of 0.5 mg.mL^{-1} PME revealed a strong label for acidic pectins at the apex (Figs. 28g-i), whereas Jim 7 label for methyl-esterified pectins was almost completely absent (the image is black at an exposure time identical to that of the control - data not shown).

We observed that the apex of pollen tubes treated with 0.5 $mg.mL^{-1}$ PME showed a dramatic increase in cellular stiffness reaching $6554\pm936x10^{-5}$ N/cm (n=6). This represents a 1.6-fold increase compared to the control samples.
Interestingly, the deformation at the apex of PME treated pollen tubes also generally revealed low hysteresis thus indicating a reduced viscous component in the visco-elastic behavior of the cell (Fig. 31c).

3.4 Discussion

3.4.1 Cell wall extensibility controls pollen tube growth

Tip growth is presumed to be regulated by an interplay between various forces that counteract each other. The force that drives cellular expansion is thought to be provided by one or several features on the inside of the cell: turgor, propulsive forces by the cytoskeletal elements and possibly other unidentified features. The relative contributions of the individual factors are still elusive and they are likely to differ between cell types and growth conditions (Money and Hill 1997). On the outside of the cell, the cell wall is the main structure that withstands the internal forces. Both plastic and elastic extension of the cell wall are purported to enter the equation determining plant cell expansion (Lockhart 1965; Ortega 1985). Studies on the mechanics of the cell wall on the single cell level are few however, and to our knowledge the role of the individual cell wall polymers has not been assessed quantitatively.
Early studies using pectinase have revealed an inhibitory effect of this enzyme on pollen tube growth at higher concentrations (Brewbaker and Kwack 1964) and on root hair elongation (Cormack 1956; Ekdahl 1957; Jackson 1959). Roggen and Stanley (Roggen and Stanley 1969) found, however, that *Pyrus communis* pollen tube growth could be stimulated by addition of 0.1 to 0.25 $mg.mL^{-1}$ pectinase. This observation encouraged us to look at the effect of pectinase in more detail and to use the enzyme to study the mechanical aspects of pectins in the cell wall of growing pollen tubes.

Our data confirmed that moderate concentrations of pectinase resulted in a stimulation of both pollen tube germination and growth in *Solanum* and *Lilium* pollen.

Micro-indentation of *Solanum* pollen tubes grown in the presence of the enzyme confirmed the softening effect on the entire pollen tube cell wall since cellular stiffness was reduced significantly in both the apical and distal regions. This is consistent with an increase in elastic deformability of the cell wall due to the enzyme activity. On the other hand, the increase in hysteresis upon retraction of the indenting stylus indicates that the viscosity component of the visco-elastic cell behavior has increased. The reaction is still elastic, since the cell returns to its original shape after deformation, but this is probably due to the presence of the internal turgor that presses the cytoplasm against the wall. The reduction in the speed of shape-recovery characterizing the visco-elastic response, however, could indicate an increase in plastic deformability of the cell wall and thus a higher plastic extensibility in the presence of pectinase. These data suggest for the first time based on the quantitative mechanical assessment of single cells that digesting the pectin component increases both the elastic and the plastic deformability of the cell wall. The observed increase in pollen tube growth rate indicated that this change in the cell wall properties lowered the cell wall's resistance, which in turn allowed the internal forces to push and expand the remaining cell wall more rapidly compared to the control conditions.

On the other hand, softening the cell wall too much resulted in bursting of pollen grains and tubes as the internal forces exceeded the cell wall resistance. Together these data corroborate the hypothesis that the physical properties of the cell wall influence the rate of the initial formation and of the continuous extension of the apically growing pollen tube. Albeit not being completely conclusive, this provides substantial support for the concept of a force equilibrium controlling tip growth in pollen tubes.

3.4.2 Pectin is a key component in the apical cell wall extensibility

We observed that addition of moderately inhibitory amounts of the digesting enzyme pectinase induced apical swelling whereas higher amounts resulted in the bursting of the pollen tubes at the apex causing a sudden release of the cytoplasm.

At other cellular locations on the pollen tube, swelling or bursting was never observed even though the pressure-induced circumferential tension stress in the cylindrical tube is twice as high as the longitudinal stress in the apex (Lockhart 1965). This indicated that only at the apex the digestion of pectins was sufficient to destabilize the cell wall in such a manner that the turgor was able to expand the wall or break it open completely. Apparently, other regions of the cell were protected from shape-changing or fatal pectinase effects. The protection could either be due to a massive amount of pectins or to the presence of other cell wall components that are responsible for retaining the internal forces upon digestion of the pectin component. To precisely relate the regions of swelling and those of unaltered morphology to the distribution of cell wall components in *Solanum*, we performed fluorescence label. As expected, pectin was the major component at the growing apex of the pollen tubes. Whereas the polymer was present in the distal regions as well, the amount was equal to or smaller than that surrounding the apex. Significant amounts of cellulose and callose, on the other hand, were present only in the distal parts of the cell that corresponded to the region that did not swell upon pectinase application. Cellulose and/or callose are therefore likely to be responsible for the increased stability of the distal parts. They presumably confer a mechanical stability against internal forces that might also serve to resist external deformation forces in the pollen tube shank, thus allowing the passage of the male gametes through the tube. The absence of these cell wall components at the tip on the other hand suggests that they play only a minor role in the control of the cell wall extensibility governing the growth process at the apex. This is consistent with data that address the role of these polymers in more detail (Parre, Geitmann submitted). Pectinase treatment at higher enzyme concentrations resulted also in an increase of the percentage of germinating pollen grains.

This finding suggests that similarly to the pollen tube apex, the *Solanum* pollen grain aperture must be rich in pectins. This has been observed for various species (Geitmann et al. 1995; Aouali et al. 2001; Suarez-Cervera et al. 2002) and our immunofluorescent label confirmed this to be the case for *Solanum* pollen as well (Parre, Geitmann, unpublished data).

Surprisingly, even though the *Solanum* pollen grain aperture also labels intensively for callose (Parre and Geitmann, in preparation), pectinase treatment at high enzyme concentrations was sufficient to induce bursting of pollen grains. Pectins do therefore seem to have a controlling function in retaining the turgor within the grain. This is consistent with the observation that the earliest visible cell wall alteration upon emergence of pollen tube is a loss of pectic material (Heslop-Harrison and Heslop-Harrison 1992; Taylor 1997).

3.4.3 Sensitivity towards pectinase differs between species

The comparison of the pectinase effect on two different species *Solanum* and *Lilium* revealed similarities since in both species pollen germination and pollen tube growth was stimulated at certain enzyme concentrations, whereas higher doses caused inhibition by inducing bursting. The remarkable difference between the two species lay in the sensitivity towards the enzyme. *Lilium* pollen was affected by concentrations that were one order of magnitude smaller than that for *Solanum* pollen which in turn were in the same range as those measured for *Pyrus communis* (Roggen and Stanley 1969).

Though we cannot exclude species-dependent differences in the accessibility of the enzyme to the polymers in the cell wall, different amounts of pectin per surface area are more likely to be the reason. It remains to be investigated whether the differences in pectin contents are in any way related to the physics of the *in vivo* growth environment of the cells: *Solanum chacoense* and *Pyrus communis* have a solid style whereas *Lilium longiflorum* possesses a hollow style. It has been purported previously that pectins might reinforce the pollen wall architecture in pollen tubes of solid styled species (Li et al. 1994; Li et al. 1995). As discussed further down, this is not consistent with our findings.

3.4.4 The exertion of penetration forces requires a change in the equilibrium between inner driving forces and the cell wall

Pollen tube growth in solidified medium in the presence and absence of pectinase revealed two striking results: Firstly, the stimulation of germination and pollen tube growth was relatively bigger in solidified medium, and secondly, the optimal pectinase concentration for both, stimulation and inhibition differed between solidified and liquid media. Both effects could be explained if the solidification of the medium is taken into the equation formed by the equilibrium between inner propulsing forces and the cell wall. Without attempting to discuss the equations for plant cell growth established by Lockhart (1965) and modified later (as summarized by Money and Hill 1997), pollen tube expansion necessitates probably the following rather simplified condition:

Inner driving force > Cell wall stiffness

The difference between the two opposing forces is small, presumably, yet probably above a certain yield threshold (Taiz 1984; Cosgrove 1986) during normal growth, whereas if the value attains a certain fatal threshold, bursting occurs. Let us now assume that in a pollen tube that penetrates a solidified medium, the inner driving force also has to act against the stiffened medium. We therefore include it in the equation:

Inner driving force > Cell wall stiffness + Medium stiffness

Assuming that the inner driving force is unaffected by the substance responsible for the solidification of the medium, the cell wall stiffness could thus be lowered without leading to bursting upon replacement of the liquid by a stiff medium. Indeed, the cell wall stiffness might necessarily *have* to be lowered to allow the inner driving force to be higher than the sum of cell wall and medium stiffness in order to allow apical expansion. To accomplish the reduction in cell wall stiffness, one solution would be to reduce the amount of pectins.

As a consequence, lower amounts of pectinase would be sufficient to achieve either stimulation or inhibition since the available pectins would be digested more quickly. The observed shift in sensitivity is consistent with this hypothesis.

An alternative explanation for this sensitivity shift or the relatively higher stimulation in stiffened medium would be a reduced diffusion of pectinases secreted by the pollen tubes proper. Pollen tubes are known to produce pectin degrading enzymes (Dearnaley and Daggard 2001 and references therein), PME (Mu et al. 94 and references therein, Li 2002) and expansins (Darley et al. 2001) to be able to soften the transmitting tissue. While we think that the concentration of pollen produced pectinase is neglegible compared to the concentrations applied externally we can certainly not exclude that they play into the equation, especially if they accumulate due to reduced diffusion. The comparative analysis of the immunofluorescence label for pectins on pollen tubes grown in liquid and solidified medium is not conclusive in this case. It revealed that the amount of pectins was indeed dramatically lower in the tubes grown in stiff medium, but whether this was due to lowered production and/or secretion of cell wall components or a post-secretion degeneration by the pollen tube's own enzymes remains unclear. As a third hypothesis one might even postulate that production of cell wall degenerating enzymes is stimulated by stiffened medium. This topic most certainly warrants further research. Nonetheless, our data provide an indication for the mechanism that allows tip growing cells to penetrate a solid substrate: a tight control of the amount of driving force retaining cell wall polymers in the growth zone, localized either pre- or post-secretion. This necessity to be able to control the physical properties of the cell wall is not surprising since cell wall softening to allow tip growth upon lowered turgor pressure has been observed previously in fungal hyphae (Money and Harold 1992).

These results provide clear evidence that pectins have load-bearing capacities to withstand tension stress. Compression stress on the other hand, does not seem to be a function of the pectin component. Since the stiffened medium should be expected to exert lateral deformation forces on the pollen tubes, one would have expected that the amount of pectin increases in medium with higher stiffness, if pectins were used to resist compression stress. Since the contrary was the case, our findings are not consistent with a compression load-bearing function of pectin in pollen tubes.

3.4.5 The pectin configuration determines the mechanical properties of the pollen tube apex

The physical properties of the cell wall are not only controlled by the total amount of pectins, but also by their configuration. Since immunofluorescence localization has first revealed a high apical concentration of methyl-esterified pectin (Li et al. 1994) it has been suggested that this pectin configuration is responsible for a higher extensibility of the apical cell wall. This concept is based on the fact that in the esterified configuration the pectin polymers are not gelated by calcium ions (Jarvis 1984; Li et al. 1994).

Here we provide for the first time mechanical evidence on cellular level that shows that transformation of methyl-esterified pectins into the de-esterified variety caused the cell wall to stiffen and to lose the viscosity component in the visco-elastic behavior. In other words, both elastic and plastic deformability of the cell wall seem to be reduced by the de-esterification of the pectin polymers. The micro-indentation experiments therefore contributed important cytomechanical information to the cell biology of pollen tube growth.

Consequently, the inhibitory effect of pectin methyl esterase on pollen tube germination and pollen tube growth above 0.1 $mg.mL^{-1}$ can be explained with a hardening of the cell wall at the aperture and the pollen tube apex, respectively. This is consistent for observations made for other species (Geitmann 1997). Our data therefore provide strong mechanical evidence for the concept that a low degree of pectin esterification is essential for pollen tube expansion at the apex.

3.5 Conclusion

In summary, our data demonstrate that the cell wall pectin represents a crucial component for the mechanical properties of growing pollen tube. Our results provide evidence that pollen tube expansion is controlled by the extensibility of the apical cell

wall. We showed that while pectin is able to resist compression stress, this function might not play a role in pollen tubes. For the first time mechanical data corroborate the long-standing hypothesis that the amount of pectins and their degree of esterification are essential structural factors for pollen tube cytomechanics.

3.6 Acknowledgements

This research was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC), the Canadian Fondation for Innovation (CFI) and the Fonds Québecois de la Recherche sur la Nature et les Technologies (FQRNT) to A. Geitmann. The generous gift of monoclonal antibodies Jim 5 and Jim 7 from Keith Roberts, John Innes Centre, Norwich, UK, and from Paul Knox, Leeds University, UK, is gratefully acknowledged. We would like to thank William B. McConnaughey, Washington University, St. Louis, Missouri, for assistance with the micro-indentation setup.

Chapitre 4 :

More than a leak sealant:

The mechanical properties of callose in growing plant cells

Elodie Parre and Anja Geitmann

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Summary

While callose is a well known permeability barrier and leak sealant in plant cells, it is largely unknown whether this cell wall polymer can also serve as a load-bearing structure. Since callose occurs in exceptionally large amounts in pollen, we assessed its role for resisting tension and compression stress in this cell. The effect of callose digestion in *Solanum* and *Lilium* pollen grains demonstrated that, depending on the species, this cell wall polymer represents the only or a major stress bearing structure at the aperture area of germinating grains. In the pollen tube it is involved in the cell wall resistance to circumferential tension stress, and despite its absence at the growing apex, callose is indirectly involved in the establishment of tension stress resistance in this area. To investigate whether or not callose is able to provide mechanical resistance against compression stress, we subjected pollen tubes to local deformation by micro-indentation. The data revealed that lowering the amount of callose resulted in reduced cellular stiffness and increased visco-elasticity thus indicating clearly that callose is able to resist compression stress. Whether this function is relevant for pollen tube mechanics, however, is unclear as, unexpectedly, stiffened growth medium caused a decrease in callose deposition. Together our data provide clear evidence for the capacity of cell wall callose to resist tension and compression stress thus demonstrating that this amorphous cell wall substance can have a mechanical role in growing plant cells.

4.1 Introduction

β-1,3-Glucan (callose) is one of the most dynamic components of the plant cell wall. It is known to be synthesized and deposited at the surface of plasma membrane by callose synthases which are localized in the membrane (Carpita and Gibeaut 1993). The synthesis of this amorphous polymer is an important part of plant cell responses to pathogen attacks as well as physical and chemical stresses (Coffey 1976; Currier 1957; Delmer and Amor, 1995; Esau and Cronshaw, 1967). While injured plant cells use the polymer as a leak sealant, in certain plant cell types callose is produced during normal development (Currier 1957; Esau 1948; Heslop-Harrison 1964; Morrison and O'Brien 1976; Scott et al.. 1967; Stone and Clarke 1992; Waterkeyn 1981). From the location of these callose deposits it has been concluded that the polymer acts as permeability barrier, as in pollen mother cell walls (Heslop-Harrison 1964) and muskmelon endosperm envelopes (Yim and Bradford 1998); as a matrix for deposition of other cell wall materials, as in developing cell plates and sieve-plate pores; as a sealing or plugging material at the plasma membrane of pit fields, plasmodesmata, and sieve-plate pores (Eschrich 1975). Despite the widespread occurrence of callose, its functions other than a leak sealing cement or permeability barrier are not well understood (Stone and Clarke 1992). One of the few suggestions of callose playing a role in cell wall mechanics was made based on circumstantial evidence in cotton seed hairs (Maltby et al. 1979). The abundance of callose in pollen grains and pollen tubes and the interesting growth behaviour of these cells suggest that it might have a structural function in these cells (Geitmann and Cresti 1998). While indirect evidence has been described, the mechanical aspect of callose in pollen has not been assessed by any kind of quantitative approach hitherto.

In recent years it has been increasingly obvious that for the functional analysis of structural cell components the knowledge of their mechanical characteristics is pivotal. Both *in vitro* and *in vivo* approaches have been applied by physicists and biologists to investigate various structural molecules such as the cytoskeletal elements actin and microtubules (Dogterom and Yurke 1997; Ingber 2003). These studies used in particular mammalian cells such as fibroblasts and erythrocytes (Discher et al. 1988; Knowles et al. 1994; Nash and Gratzer 1993; Sokabe et al. 1991; Yuan et al. 1995). The physical properties of the plant cell wall components have been studied intensively, but mostly at tissue level (Buntemeyer et al. 1998; Edelmann 1995; Nolte and Schopfer 1997; Showalter 1993), or in dead cells (Wimmer et al. 1997). Callose is primarily functional in living plant cells and therefore, to assess its mechanical function, an *in vivo* system is essential. Here we investigated the role of callose for cell wall resistance to tension and compression stresses on cellular level.

Our model system is pollen tube formation and growth. In addition to producing abundant amounts of callose during normal cell development (Geitmann 1998), these cells grow individually and are thus readily manipulated for mechanical studies *in vitro*. In flowering plants, pollen grains germinate on a receptive stigma and send pollen tubes down the style towards the ovules with impressive growth rates, thus allowing the delivery of the sperm cells to perform fertilization. The pollen tube could therefore be compared to a tunnel transport system for the male gametes. The pollen tube cell wall is generally composed of two or three layers: an outer pecto-cellulosic sheath and an inner callosic lining (Heslop-Harrison 1987; Steer and Steer 1989). It has to resist both, tension stress and compression stress: Tension stress in the cell wall is created at the growing hemisphere-shaped apex of the tube and at the pollen grain aperture since cell growth is governed by a tightly controlled equilibrium between inner driving forces, the expansion of the existing cell wall, and the continuous insertion of new cell wall material (Taylor 1997). While there is an ongoing discussion about the nature of growth driving forces in apically growing cells (Money 1997; Money 2001), there seems to be no doubt, that the key factor counteracting these forces is the apical, expanding part of the cell wall.

It must be able to continuously yield to the growth propulsing forces, while at the same time it needs to provide enough resistance to prevent bursting. Tension forces are also established in the cylindrical parts of the tubular cell due to the action of the turgor pressure. The mechanics of thin wall pressure vessels dictates that the turgor induced circumferential stress in the cylindrical part is twice as high as the tension stress in the wall of the apical hemisphere (Green 1962; Lockhart 1965). Therefore, maintenance of the cylindrical shape is certain to require tension-withstanding properties in the distal cell wall. At the same time, in the *in planta* situation, compression stress is presumably exerted on the cylindrical cell by the surrounding tissue thus adding additional requirements to the structural characteristics of the cell.

To understand the mechanical properties of callose we analyzed which role it plays for the cell wall's capacity to resist the different types of stresses present in the growing pollen tube. To do so we related structure and function combining two approaches: 1. Localization of callose in pollen grains and tubes in two different species. 2. Analysis of the effect of enzymatic callose degeneration on the germination and growth behaviour (tension stress) and on the resistance to lateral deformation (compression stress).

4.2 Results

4.2.1 Callose digestion affects pollen germination

To establish whether callose is a structural factor in the resistance of the cell wall to the cytoplasmic germination driving forces we tested whether lowering its amount influenced germination behaviour and pollen tube growth rate in *Solanum chacoense* and *Lilium longiflorum*. To do so we used the enzyme lyticase, which specifically hydrolyses callose. Figure 33 reveals that high lyticase concentrations reduced the rate of pollen germination and also resulted in a shorter pollen tube length after 2h. The sensitivity to the enzyme differed between the two species, however.

The lyticase concentration that was necessary to completely inhibit germination of *Solanum* pollen grains was 10 fold higher than the one effective in *Lilium*. Surprisingly, only in *Solanum* pollen the inhibitory effect was attributable to visible bursting (95% of pollen grains burst upon addition of 20 $mg.mL^{-1}$ lyticase), whereas *Lilium* pollen grains seemed to have unaltered morphology at inhibitory enzyme concentrations.

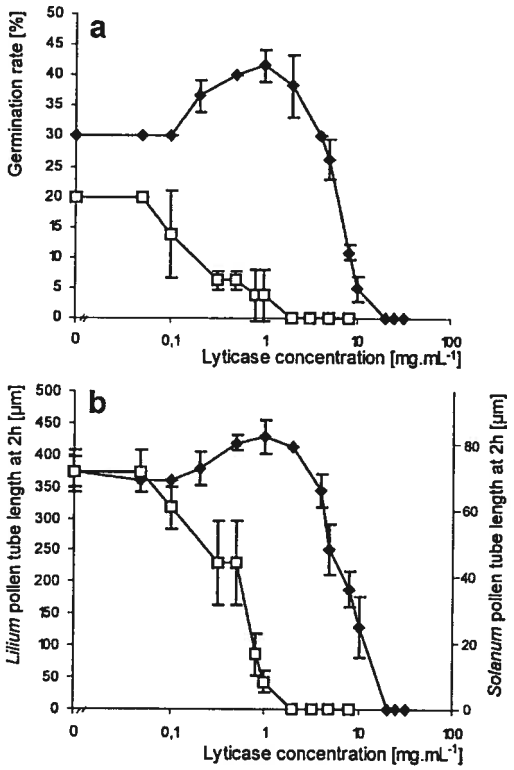

Figure 33: Effect of lyticase on germination rate and pollen tube length. Germination rate (**a**) and pollen tube length at 2h (**b**) in *Solanum* (♦) and *Lilium* (□) pollen grown in liquid medium are plotted against lyticase concentration. High enzyme concentrations were inhibitory for both species but *Lilium* had a tenfold higher sensitivity compared to *Solanum*. Only germination in *Solanum* was stimulated at moderate enzyme concentrations.

Moderate enzyme concentrations caused stimulation of both germination rate (Fig. 33a) and pollen tube length at 2h (Fig. 33b) in *Solanum* pollen. The highest increase in pollen germination (35%) and pollen tube length (15%) was accomplished by addition of lyticase at 1 $mg.mL^{-1}$. In *Lilium,* on the other hand, no stimulatory effect was observed at 2h for any of the enzyme concentrations tested. We hypothesized that the increase in tube length observed in *Solanum* pollen that had germinated in the presence of the enzyme could be due to either of two factors or a combination of both: an earlier onset of the germination process and/or an increase in pollen tube growth rate.

To distinguish between these two variables, we applied stimulating concentrations of the enzyme 30 min after *Solanum* pollen had germinated under control conditions. After additional 90 min incubation in the presence of 2 or 1 $mg.mL^{-1}$ lyticase, pollen tubes treated with the enzyme had a length of 159±3 µm, which was not significantly different from the control cells treated with denatured enzyme, which had a length of 160±3 µm. This indicates that the stimulating effect on pollen tube length was due to an acceleration of the onset of germination but not to a change in pollen tube growth rate.

4.2.2 *Solanum* and *Lilium* pollen grains show different patterns of callose distribution

Since the lyticase effect on germination differed between *Solanum* and *Lilium* pollen we investigated whether this was due to the distribution of the polymer in each of the species. As expected, fluorescent label with decolorized aniline blue revealed significant differences in the callose patterns of the two species. In ungerminated *Solanum* pollen grains (Figs. 34a,b) callose label was present uniformly in the cell wall. Shortly prior to the onset of germination one of the three apertures was strongly labelled for callose and later featured an accumulation at the base of the emerging pollen tube (Figs. 34c,d). *Lilium* pollen grains showed weak overall label and some callose accumulation at the entire colpus area. No significant accumulation was observed at the base of emerging tube, however (Figs. 34i,j). This might indicate that callose at the aperture plays a less important role in *Lilium* germination compared to *Solanum*.

To confirm the effect of lyticase on the amount of callose in *Solanum* and *Lilium* pollen grain cell wall we applied enzyme concentrations that reduced pollen germination to approximately a third (8 $mg.mL^{-1}$ for *Solanum* and 0.5 $mg.mL^{-1}$ for *Lilium*). This treatment resulted in a significant loss of callose label in both *Lilium* and *Solanum* pollen grains while causing bursting in *Solanum* grains only (Figs. 34g,h,k,l).

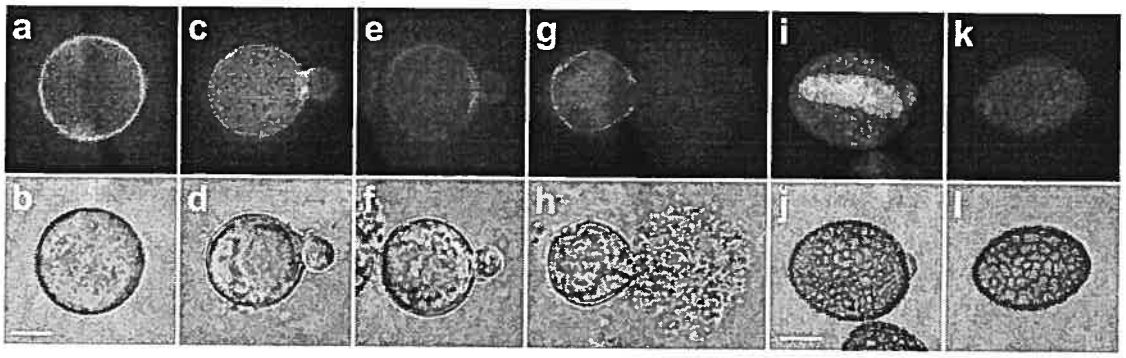

Figure 34: Effect of lyticase on the abundance of callose in *Solanum* and *Lilium* pollen grains observed after label with decolorized aniline blue and corresponding DIC images.

(a,b) *Solanum* pollen at the beginning of imbibation in liquid control medium. Callose is distributed evenly around the grain.

(c,d) *Solanum* pollen after 30 min of imbibation in liquid control medium. Callose accumulations are visible at the base of the emerging pollen tube.

(e,f) *Solanum* pollen grain germinating in solidified medium show significantly weaker callose label.

(g,h) *Solanum* pollen after 30 min of imbibation in medium containing 8 $mg.mL^{-1}$ lyticase. The pollen grain has burst and callose label at the functional aperture is weak.

(i,j) *Lilium* pollen after 45 min of imbibation in control medium. Callose label is present in the colpus, but no accumulation is visible at the base of the emerging pollen tube.

(k,l) *Lilium* pollen after 45 min of imbibation in 0.5 $mg.mL^{-1}$ lyticase. Callose label is very weak; the pollen grain remains intact. Bars = 10µm (a-h), 30 µm (i-l).

4.2.3 Cell wall thickness influences the stability of pollen grain architecture

Our data showed that exposure to lyticase at high concentrations inhibited germination in both *Solanum* and *Lilium* pollen, but only in the former bursting was induced. This raised the question whether pollen grain architecture or other cell wall components might withstand the turgor pressure in *Lilium* but not in *Solanum* once callose was digested. To analyze the effect of pollen grain architecture we compared the cell wall thickness and exine ornamentation in both species.

Transmission electron microscopy revealed that the cell wall of *Lilium* pollen grains was 3.6 µm thick, whereas that of *Solanum* pollen grains had an average thickness of 0.5 µm. In particular, the intine and the sexine where considerably thicker in *Lilium* pollen grains, whereas the nexine thickness was comparable between the two species (Figs. 35a,b). Furthermore, scanning electron microscopy showed that the *Lilium* sexine had a course reticulate structure, whereas the surface of *Solanum* pollen grains was characterized by fine scabrate surface ornamentation (Figs. 35c,d). These observations are consistent with but not conclusive for *Lilium* pollen grain wall having higher structural resistance against internal turgor forces thus providing a mechanical fortification against tension stress.

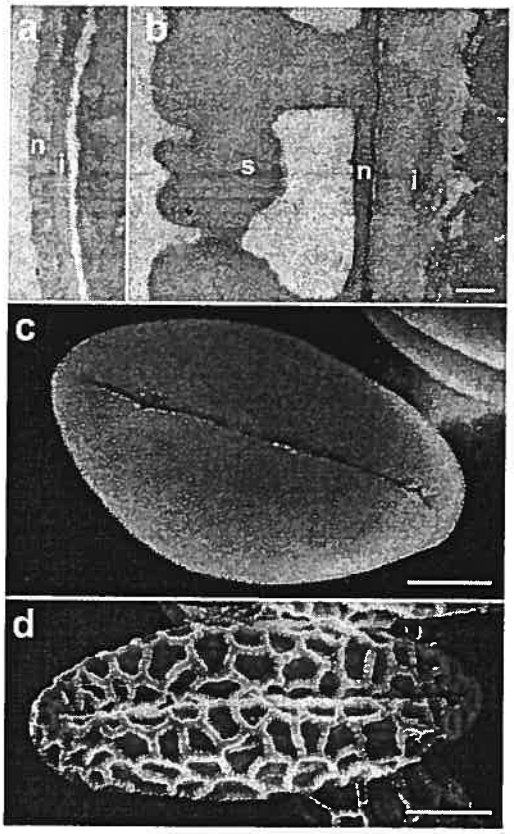

Figure 35: Electron micrographs of the pollen grain cell wall structure.
Transmission electron micrographs reveal that *Solanum* pollen grains (**a**) have a considerably thinner intine (i) than *Lilium* grains (**b**). While nexine (n) thickness is comparable, the sexine (s) of *Lilium* is extremely thick. Scanning electron micrographs show also that the *Lilium* sexine is structured in a course reticulate pattern (**d**), whereas *Solanum* is ornamented by very small scabrate structures (**c**).
Bars = 0.5µm (a,b), 5µm (c), 20µm (d).

4.2.4 *Solanum* and *Lilium* pollen grains show different patterns of cellulose and pectin distributions in the pollen grain aperture

To investigate whether other cell wall components were responsible for preventing *Lilium* but not *Solanum* pollen grains from bursting upon callose digestion, we evidenced the distribution of other major cell wall polysaccharides in both species. Immunolabel for pectins revealed that in *Solanum* pollen grains, acidic pectins (labelled with monoclonal antibody Jim 5) were present at the apertures (Figs. 36a,b), whereas methyl-esterified pectins (labelled with monoclonal antibody Jim 7) were absent from the pollen grain (data not shown, since no label visible; compare with Fig. 39g). Calcofluor label for cellulose was rather weak and evenly distributed in *Solanum* pollen grains (Figs. 36c,d). Callose and acidic pectins therefore seemed to be the main cell wall components at the *Solanum* pollen grain apertures. In *Lilium* pollen grains on the other hand, label for methylesterified was absent (not shown) and that for acid pectins was extremely weak in the entire grain (Figs. 36e,f). Calcofluor label revealed weak label all around the grain, but a considerable accumulation of cellulose at the base of the germinating tube (Figs. 36g,h).

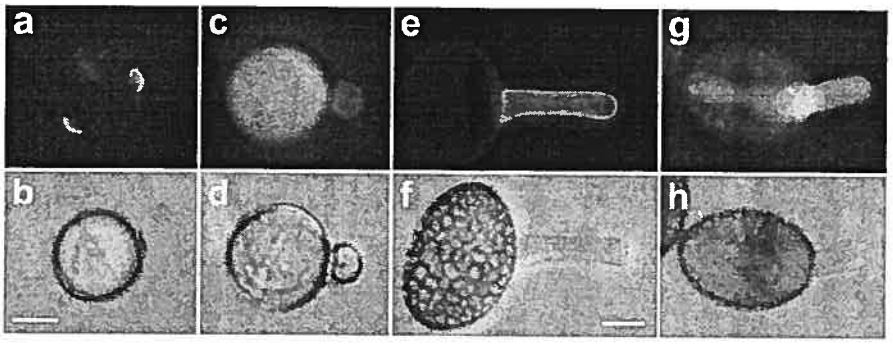

Figure 36: Fluorescent label for acidic pectins (monoclonal antibody Jim 5) and cellulose (calcofluor white) in germinating pollen grains and corresponding DIC images.

(a,b) *Solanum* pollen grain showing intensive label for acidic pectins at all three apertures.

(c,d) Germinating *Solanum* pollen grain showing weak and evenly distributed label for cellulose.

(e,f) *Lilium* pollen grain showing very weak label for acidic pectins. The prominent label in the pollen tube serves as a control for the success of the label technique.

(g,h) *Lilium* pollen grain showing weak cellulose label around the grain and a considerable accumulation at the base of the newly formed pollen tube.

Bars = 10μm (a-d), 30 μm (e-h).

The presence of cellulose in addition to callose at the aperture of *Lilium* pollen grains might therefore provide an explanation for the absence of bursting events upon callose digestion in these grains. To test this hypothesis we added β-glucanase, an enzyme that digests both callose and cellulose, as well as cellulase, an enzyme that is specific for cellulose, to *Lilium* pollen. We observed that at 3 $mg.mL^{-1}$ β-glucanase inhibited germination of *Lilium* by causing bursting of 95% pollen grains. Furthermore, moderate amounts of β-glucanase significantly stimulated germination rates in *Lilium* (Fig. 37), whereas this effect was never observed when cellulase and lyticase were added separately as summarized in Table IV. These results clearly show that either component, callose or cellulose, at the *Lilium* aperture is sufficient to prevent bursting of pollen grains and both need to be softened to stimulate germination.

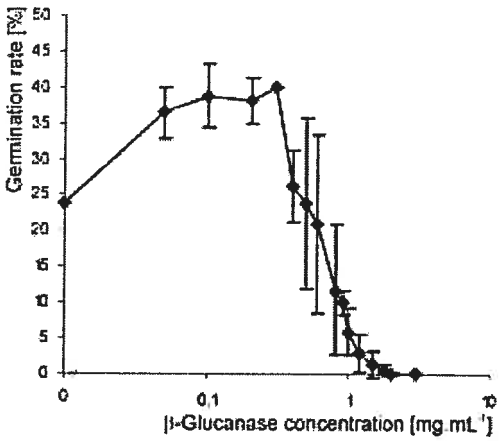

Figure 37: Effect of ß-glucanase on *Lilium* pollen germination.
Moderate concentrations of the enzyme are able to stimulate the germination rate.

	Stimulation of germination rate		**Inhibition of germination**	
Enzyme	Relative increase compared to untreated control	Optimal enzyme concentration	Enzyme concentration achieving complete inhibition	Pollen grain morphology
Lyticase	0 %	n/a	2 $mg.mL^{-1}$	Intact
Cellulase	0 %	n/a	50 $mg.mL^{-1}$	Intact
β-Glucanase	70 %	0.05 $mg.mL^{-1}$	2 $mg.mL^{-1}$	Burst

Table IV. Effect of cell wall digesting enzymes lyticase, cellulase and β-glucanase on germination rate and pollen grain morphology in *Lilium*.
Addition of moderate amounts of β-glucanase stimulated germination rate and higher concentrations caused bursting whereas neither of these effects was observed after addition of cellulase or lyticase.

4.2.5 The lyticase effect depends on the stiffness of the medium

Previous studies using pectinase have shown that the stiffness of the growth medium affects the abundance of pectin polymers in the cell wall of pollen grains and tubes (Parre and Geitmann, submitted). This finding has implications for the comparison between the *in vitro* and *in vivo* situations.

Since in *Solanum in vivo* pollen tube growth takes place within the solid style of the receptive flower, we wanted to investigate whether the stiffness of the medium influenced the pollen response to lyticase. As shown earlier (Parre and Geitmann, submitted) solidification of the medium reduced the germination rate in this species (Fig. 38).

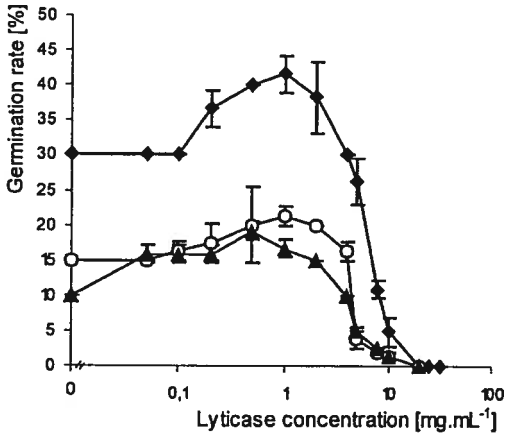

Figure 38: Effect of various lyticase concentrations and medium stiffness on *Solanum* pollen germination.

The germination rate was assessed after 2h in liquid medium (♦), medium solidified with 20 mg.mL^{-1} (○) and 40 mg.mL^{-1} (▲) agarose. It was reduced in stiffer media, but moderate amounts of lyticase nevertheless had a significant stimulating effect. However, optimal enzyme concentrations and relative stimulation in stiff medium had a tendency to differ from those observed in liquid medium as summarized in Table V.

Lyticase had a stimulatory effect in both, medium containing 20 and 40 mg.mL^{-1} agarose, but interestingly, the relative stimulation of the germination rate was bigger in solidified medium compared to the liquid control. Furthermore, the enzyme concentration necessary to accomplish this was 2 to 5 fold lower compared to liquid medium as summarized in Table V. Optimal concentrations for the stimulation of pollen tube length at 2h were also shifted with the highest increase of tube length reaching 100% in agarose complemented media compared to liquid medium in which a maximal increase of length of 14% was achieved. Similarly to germination, lyticase concentration necessary to increase pollen tube length at 2h were 2 to 5 fold lower in solidified than in liquid medium (Table V).

	Maximal germination rate		Maximal pollen tube length at 2h	
Medium stiffness	Relative increase compared to untreated control	Optimal lyticase concentration	Relative increase compared to untreated control	Optimal lyticase concentration
Liquid	36 %	1 $mg.mL^{-1}$	14 %	1 $mg.mL^{-1}$
20 $mg.mL^{-1}$ Agarose	41 %	1 $mg.mL^{-1}$	19 %	0.5-1 $mg.mL^{-1}$
40 $mg.mL^{-1}$ Agarose	91 %	0.5 $mg.mL^{-1}$	100 %	0.2-0.5 $mg.mL^{-1}$

TableV: Optimal lyticase concentrations that result in stimulation of *Solanum* pollen germination and tube length at 2h in liquid and solidified media.

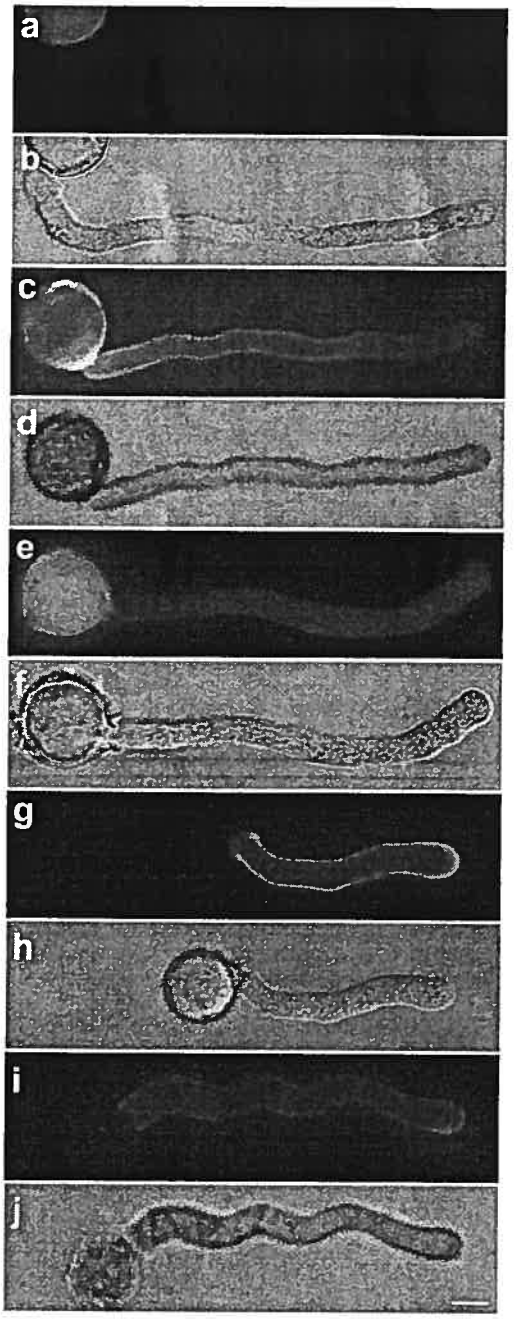

Figure 39: Callose and pectin content in pollen tubes.

(a-f) *Solanum* pollen tubes labelled for callose with decolorized aniline blue and corresponding DIC images. Tubes grown in solidified medium **(a,b)** showed reduced abundance of callose compared to the control tubes grown in liquid medium **(c,d)**. The presence of 1 $mg.mL^{-1}$ lyticase reduced the abundance of callose in the pollen tube **(e)** and caused an increase of the pollen tube diameter **(f)** compared to the control tubes **(c,d)**

(g-j) *Solanum* pollen tubes labelled for methylesterified pectins with monoclonal antibody Jim 7 and corresponding DIC images. Pollen tubes grown in the presence of 1 $mg.mL^{-1}$ lyticase **(i,j)** showed significantly weaker label than the control cells **(g,h)**. The distribution pattern remained the same, however, with a higher concentration of methylesterified pectin at the pollen tube apex

Bar = 10 µm.

4.2.6 The amount of callosic cell wall is affected by the stiffness of the growth medium

The effect of medium stiffness on pollen sensitivity toward lyticase treatment suggests that the physical properties of the environment in which the pollen germinates might affect the force equilibrium governing pollen germination and growth by altering cell wall thickness. To investigate whether the abundance of callose in the pollen grain indeed differed between liquid and solidified media, we compared the fluorescence intensity of callose label. Pollen grains were germinated either in liquid medium or in medium solidified by addition of Gel gro to allow for resuspension of pollen prior to label. Label with decolorized aniline blue revealed that the amount of callose was significantly reduced in pollen grains grown in solidified medium, although the distribution pattern remained the same, featuring an accumulation of callose at the functional aperture (Figs. 34e,f). Furthermore, we observed that in stiff medium pollen tubes as well showed a reduction of callose content (Figs. 39a,b) while the distribution remained the same, featuring the characteristic absence of callose at the tube apex as observed in control pollen tubes (Figs. 39c,d).

4.2.7 Lyticase affects the pollen tube diameter

It is well known, and our data confirm that callose is absent from the growing pollen tube apex, whereas significant amounts of the polymer are present in the distal part of the cell. We wanted to investigate whether in these distal parts callose plays a role in the resistance to circumferential tension stress in the cell wall created by the internal turgor pressure. To do so we assessed whether the pollen tube diameter changes in the presence of various concentrations of lyticase. At 0.1 $mg.mL^{-1}$ and above, lyticase caused an increase in pollen tube diameter both at the apex and the distal regions compared to control pollen tubes as summarized in Table VI. Decolorized aniline blue label for callose confirmed that after addition of 1 $mg.mL^{-1}$ lyticase the amount of cell wall callose in the pollen tube wall was reduced compared to the control sample (Figs. 39c-f).

Lyticase concentration	Distal diameter	Apical diameter
0 $mg.mL^{-1}$	6.96 ± 0.56 µm	6.08 ± 0.42 µm
0.1 $mg.mL^{-1}$	7.73 ± 0.50 µm	6.83 ± 0.85 µm
1 $mg.mL^{-1}$	8.19 ± 0.42 µm	7.28 ± 0.61 µm

Table VI: Effect of lyticase on pollen tube diameter at the apex and at distal locations of *Solanum* pollen tubes. The presence of 0.1 $mg.mL^{-1}$ lyticase and above, caused a significant increase in diameter at both locations.

4.2.8 Callose digestion has an indirect effect on pectin distribution

Since the pollen tube apex showed an increase in diameter in the presence of lyticase even though no visible amounts of callose are present in the pollen tube tip, we suspected that other cell wall components were affected by the continuous digestion of callose. To confirm this we labelled cellulose and pectins in pollen tubes treated with lyticase and compared fluorescence intensity and localization with that of control pollen tubes. Calcofluor white label for cellulose revealed that the amount and distribution of cellulose remained the same after callose digestion by lyticase at 1 $mg.mL^{-1}$ (not shown). Similarly, immunolabel with Jim 5 for acidic pectins was comparable between treated and untreated pollen tubes. Surprisingly, immunolabel with Jim 7 revealed that the amount of methyl-esterified pectins was significantly reduced in pollen tubes treated with 1 $mg.mL^{-1}$, although the distribution pattern remained the same with methylesterified pectin present mainly at the pollen tube apex (Figs. 39g-j). Since alteration of the pectin contents caused apical swelling in pollen tubes (Parre and Geitmann, submitted), the observed indirect effect of lyticase on the abundance of methyl-esterified pectins most likely provides the explanation for the increased apical diameter visible after application of 1 $mg.mL^{-1}$ lyticase.

4.2.9 Callose plays a role in the resistance to compression stress

To investigate whether callose provides compression stress resistance on the individual cell level, we assessed local cellular stiffness and visco-elasticity with micro-indentation, a technique that has proven useful for the assessment of pollen tube cytomechanics (Geitmann *et al.*, 2004; Geitmann and Parre, 2004). Local deformations were performed at two positions of the tube, at the growing apex and at a distal position around 30 μm from the apex, where visible amount of callose were observed. As shown earlier (Geitmann and Parre, 2004), normally growing *Solanum* pollen tubes were characterized by a difference in stiffness and in the degree of visco-elasticity between the growing apex and the distal region with the apex being more visco-elastic and having a stiffness of 85±5% of that of the distal region in an individual tube. For lyticase treatment we used 1 $mg.mL^{-1}$, since this concentration caused an increase in pollen tube diameter and a significant reduction in aniline blue fluorescence label intensity. We observed that pollen tubes that were germinated in the presence of 1 $mg.mL^{-1}$ lyticase showed a dramatic decrease in distal stiffness to $2329\pm250x10^{-5}$N/cm (Fig. 40a), compared to control pollen tube reaching $4970\pm170x10^{-5}$N/cm (Fig. 40b), whereas apical stiffness ($4021\pm385x10^{-5}$N/cm, Fig. 40c) remained virtually identical to that of untreated pollen tubes ($4061\pm197x10^{-5}$N/cm, Fig. 40d). As a consequence, the ratio between apex and distal local stiffness in individual tubes increased to 177±14%.

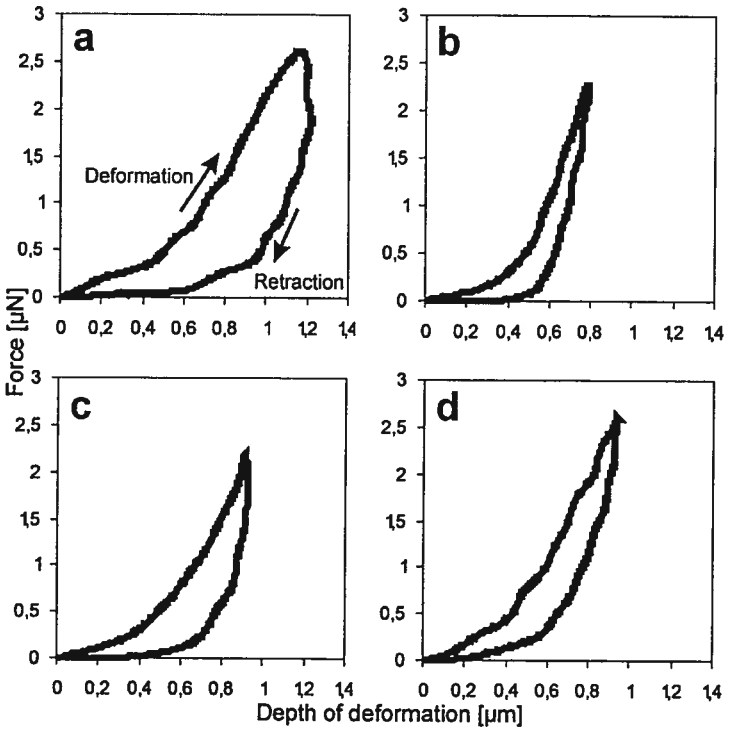

Figure 40: Force deformation graphs for micro-indentation experiments.

Local deformations were performed at the apex and at a distal location 30µm behind the tip of growing *Solanum* pollen tubes. The upper curve represents the deforming and the lower curve the retracting movement of the stylus. The slope of the linear part of the deforming curve indicates the stiffness whereas the surface area between the two curves expresses the delay upon retraction of the deforming stylus (hysteresis), which is an indication for the dissipated energy and thus the visco-elasticity of the deformed object. The deformation profiles at the distal pollen tube location show that the presence of 1 $mg.mL^{-1}$ lyticase caused a dramatic decrease in stiffness and an increase in visco-elasticity (**a**) compared to the control (**b**). At the apex, on the other hand, neither of these parameters was significantly affected by lyticase (**c**) if compared to the control situation (**d**).

Interestingly, contrary to the control samples, the deformation at the distal area of lyticase treated pollen tubes also generally revealed high hysteresis upon the retraction of the stylus thus indicating an increase in the overall cellular visco-elasticity. Figs. 40a-d illustrate the differences in the force distance graphs concerning stiffness and viscosity for both the apex and distal regions of the tube.

4.3 Discussion

4.3.1 Cell wall resistance to tension stress at the pollen grain aperture depends on callose

The pollen grain aperture has several interesting functions. It provides routes for transfer of water and other substances and allows for harmomegathy, the process by which pollen grains change in shape to accommodate variations in the volume of the cytoplasm caused by changing hydration. From a mechanical point of view the most interesting function is the emergence of the pollen tube. During this process the aperture covering cell wall has to yield to germination driving forces to allow pollen tube emergence. At the same time it has to withstand these same forces to avoid bursting of the cell at this weakened location in the otherwise rigid shell protecting the pollen grain protoplast (El-Ghazaly 1999; Johnson and McCormick 2001; Li et al. 1999; Ressayre et al. 2002; Roggen 1969). The pollen grain and in particular its aperture provide therefore an excellent experimental system to test whether or not callose is able to resist tension forces in living plant cells.

Previous studies using β-1,3-glucanase, an enzyme digesting callose, revealed a stimulatory effect on pollen germination in *Pyrus communis* at moderate concentrations (Roggen 1969). This was a first indication for callose playing a role in controlling the equilibrium between the germinating driving forces and the resisting cell wall. In turn, this would imply that callose has the capability of resisting tension stress in the cell wall thus attributing it a mechanical function that has not been described hitherto. Our data corroborate the findings by Roggen (1969) and provide more detailed information by relating the mechanical functioning of callose to its distinct distribution patterns in two species, *Solanum chacoense* and *Lilium longiflorum*. Germination of *Solanum* pollen grains was stimulated by lyticase, and higher enzyme concentrations caused bursting of the grains at the aperture. It seems therefore, that slight softening of the cell wall allowed the driving forces to act earlier and more effectively causing a higher germination rate and earlier onset of the process.

On the other hand, digesting the callosic cell wall too much destroyed the load bearing structure resulting in bursting, as the internal turgor pressure exceeded the cell wall resistance. Both findings are consistent with the fact that callose is abundant at the functional aperture in pollen grains of *Solanum*, whereas most other major cell wall components were present only in small amounts at this cellular location. Similar compositions of aperture cell walls have been described for other species (Aouali et al. 2001; Geitmann et al. 1995; Suarez-Cervera et al. 2002). The fact that lyticase treatment was sufficient to induce bursting in *Solanum* indicated that acidic pectins, the most abundant component next to callose, was not able to withstand the cellular turgor once callose was digested. Callose does therefore seem to be the main tension stress-bearing structure in the aperture cell wall of *Solanum* pollen grains. Further support for this concept was provided by the observation that *Solanum* pollen grains germinating in stiffened medium showed a shift in the sensitivity towards lyticase, which was caused by a reduced amount of callose in the functional aperture. This is consistent with the concept of the stiff medium counteracting the cellular turgor thus reducing the tension stress in the aperture cell wall which in turn needs less callose.

Pollen grains of *Lilium*, on the other hand, lacked prominent callose accumulations at the site of the emerging pollen tube. Consistent with this, germination in this species was not stimulated by any of the lyticase concentrations tested. Moreover, *Lilium* pollen grains remained morphologically intact when treated with high lyticase concentrations, even though germination was inhibited. We cannot exclude that the thicker, coarser overall cell wall architecture provided mechanical fortification against turgor forces in pollen grains of this species. However, the observed lower amount of callose at the site of pollen tube emergence is consistent with it playing a less important role in aperture cell wall mechanics thus making the grain less prone to bursting upon lyticase treatment. The abundance of cellulose in *Lilium* pollen grain apertures suggests that the task to resist turgor forces is shared by both callose and cellulose in this species. This is corroborated by the fact that only simultaneous digestion of both polymers by β-glucanase was able to stimulate germination, whereas neither lyticase nor cellulase alone could achieve this effect.

What remains puzzling is the fact that higher concentrations of lyticase were nevertheless able to inhibit germination in *Lilium*. Since the grains did not burst in presence of the enzyme, it is unclear which mechanism led to inhibition in this species. It might be that the development of a critical amount of callose deposit at the aperture is critical for pollen germination by controlling water influx and continuous digestion of the polysaccharide disturbs the regulation mechanism of the process.

4.3.2 Callose is indirectly involved in tension stress resistance at the growing pollen tube apex

As has been observed in numerous species (Derksen 1996; Ferguson et al. 1998; Hasegawa et al. 1998; Holdaway-Clarke and Hepler 2003; Li et al. 2002; Li et al. 1997; Li et al. 1994; Roggen 1969), in *Solanum* pollen tubes callose was detected only starting approximately 20 to 30 µm behind the growing apex. This suggests that callose is unlikely to be involved directly in the pollen tube elongation process (Roggen 1969). While moderate pectin digestion resulted in an increase in pollen tube growth rate (Parre and Geitmann, submitted), no growth stimulation occurred when 30 min old pollen tube were treated with lyticase. This confirms that the increased pollen tube length observed in samples with continuous presence of lyticase was primary the consequence of an accelerated germination resulting from the enzyme dissolving the callose inside the pore intine and initial pollen tube (Roggen 1969). Lyticase was not completely without effect on the pollen tube apex, however, since surprisingly, a significant increase in apical diameter was observed when pollen was germinated in presence of lyticase. Consistent with this, application of lyticase on older pollen tubes caused apical swelling (Parre, unpublished results). Unless callose was present in the pollen tube apex in invisible amounts, this finding suggests an indirect effect of callose digestion on the deposition and/or configuration of other cell wall polymers.

The labelling pattern for the two other main cell wall components cellulose and pectin showed clearly that cellulose distribution and abundance was not affected in the presence of lyticase. However, the amount of methylesterified pectin deposition was reduced after callose digestion.

Earlier studies have shown that a reduction in the amount of pectins in the cell wall caused apical swelling in pollen tubes (Parre and Geitmann, submitted) thus being consistent with the data presented here. This intriguing result suggests that, although absent from the growing apex, callose seems to play an indirect role in the turgor/cell wall equilibrium involved in apical expansion by interacting with the deposition of methylesterified pectin.

4.3.3 Callose is a crucial factor in the cell wall resistance to circumferential tension stress

For geometrical reasons, the circumferential tension stress in the cell wall of the cylindrical part of the tube is twice as high as that in the wall of the dome shaped apex (Green 1962; Lockhart 1965). Yet, in normally growing pollen tubes it is the apex that yields to allow expansion, whereas the cylinder remains stable (Derksen 1996; Geitmann and Cresti 1998; Hepler et al. 2001). The distal cell wall must therefore be strong enough to resist considerable tension forces created by the internal pressure. Our previous experiments have shown that application of pectinase caused the swelling of the apical part of the pollen tube, whereas the diameter of the distal tube is maintained, thus indicating that pectin is not a major structural factor in creating this resistance to tension stress in the cylindrical part (Parre and Geitmann, submitted).

The presence of callose in the distal regions of the pollen tube suggested that this polymer might possibly be a load-bearing component. The observed increase of cellular diameter in pollen tubes grown in the presence of lyticase confirms this hypothesis. The fact that bursting was never observed in the distal part of the tube indicates, however, that callose is not the only cell wall component resisting to the circumferential tension stress. Cellulose is likely to play an important role as well.

4.3.4 Callose is able to resist compression stress but does not necessarily have that function in pollen tubes

Micro-indentation studies have shown that normally growing pollen tubes of *Solanum chacoense* and *Papaver rhoeas* are characterized by differences in the mechanical parameters of the cell between the growing apex and the cylindrical distal part of the cell (Geitmann and Parre 2004; Parre and Geitmann, submitted). The apex is generally less stiff than the distal part of the same tube with the apical stiffness amounting to 85% of the value for the distal region in *Solanum* (Parre and Geitmann, submitted). Furthermore, the distal region reacts almost completely elastic to deformation whereas the apex shows visco-elastic behaviour.

The data presented here show clearly that digesting the callosic cell wall was sufficient to dramatically reduce the cellular stiffness and increase the cellular visco-elasticity in the distal part of *Solanum* pollen tubes. On the other hand, no significant change to cellular mechanical parameters was observed in the apex of lyticase treated pollen tubes thus confirming, that callose does not play an important role in the cell wall mechanics of this part of the cell.

These results provide clear evidence that callose has load-bearing capacities, and thus could theoretically have a stabilising function in the mature cylindrical part of the pollen tube. One would presume that *in planta* pollen tubes have to exert mechanical resistance forces to withstand the compression stress exerted by the surrounding transmitting tissue. Of all tip growing cell types this function would seem to be particularly important in the pollen tube, since it serves as a delivery tunnel for the male gametes on their way from the pollen grain to the ovule and, therefore, must not collapse before their passage has taken place. It was therefore puzzling to observe that pollen tubes grown in stiffened medium revealed an increase in sensitivity to lyticase due to a reduced amount of callose in their cell walls as confirmed by fluorescence label. Since the stiffened medium should be expected to exert lateral deformation forces on the pollen tubes, this finding is not consistent with a compression load-bearing function of callose in pollen tubes.

On the other hand, this corroborates earlier observations which revealed that cellular stiffness at pollen tube locations featuring callosic plugs was not higher than that at locations in adjacent turgescent parts of the cell (Geitmann, unpublished results). Only in distal parts of pollen tubes which had lost turgor pressure were callosic plugs stiffer than the adjacent parts of the cell. This indicated that the hydroskeleton established by the turgor pressure is an important if not the only factor in compression resistance.

In summary, the most important structural function of callose in pollen seems to be the control of the cell wall - turgor equilibrium at the functional pollen grain aperture and the resistance towards circumferential tension stress in the distal pollen tube cell wall. Our results therefore show conclusively that callose does not only have a function as a leak sealant or as a layer controlling water permeability. It is able to resist tension stress and it is used in that function, albeit perhaps not exclusively, in pollen grains and pollen tubes. We showed that callose is also able to resist compression stress, but this function does not seem to play a role in pollen tubes. Whether or not other cell wall or cytoplasmic components are involved in the resistance to lateral deformation stress in these cells, or whether the hydrostatic pressure established by the turgor is the only structural feature remains to be elucidated.

4.4 Experimental procedures

4.4.1 Pollen tube growth

Solanum chacoense plants were grown in the Montreal Botanical Garden greenhouses and *Lilium longiflorum* was obtained from a local flower shop. Pollen was collected after dehiscence, dehydrated and stored at –20°C. On the day of use pollen was rehydrated and cultivated in drops of liquid or solidified media. The latter was obtained by addition of low melting agarose (Agarose Type I-B Low EEO Sigma, St. Louis, US) or Gel gro (Gellan gum; gel strength twice as high as that of agarose; ICN Biomedical

Inc., Ohio, US). The growth medium (GM) contained 100 $\mu g.mL^{-1}$ H_3BO_3, 300 $\mu g.mL^{-1}$ $Ca(NO_3)_2 \cdot H_2O$, 100 $\mu g.mL^{-1}$ KNO_3, 200 $\mu g.mL^{-1}$ $MgSO_4 \cdot 7H_2O$, 50 $\mu g.mL^{-1}$ sucrose (Brewbaker and Kwack, 1963) for *Solanum* pollen and 0.29 $\mu g.mL^{-1}$ MES, 0.01 $\mu g.mL^{-1}$ H_3BO_3, 0.0147 $\mu g.mL^{-1}$ $CaCl_2$ for lily pollen grains. Various concentrations of lyticase (332 units/mg solid, Sigma, St. Louis, US) were added at the start of imbibation (to assess the effect on germination) or after 30 min of imbibation (to assess the effect on elongation). Cellulase (6.3 units/mg solid, Sigma, St. Louis, US) and β-glucanase (1 unit/mg solid, Sigma, St. Louis, US) were added at the beginning of imbibation. Controls were performed by adding enzyme that had been denatured by boiling for 10 min. Germination rate and pollen tube length were assessed in the light microscope after two hours of incubation on microscope slides. The results are mean values from eight repetitions of the experiment.

4.4.2 Fluorescence label

For fluorescence microscopy, pollen tubes were fixed after 2 hours of germination in 3% freshly prepared formaldehyde in Pipes-Buffer (1 mM EGTA, 0.5 mM $MgCl_2$, 50 mM Pipes) for 30 min. To allow for quantitative comparison of fluorescence intensity, pollen tubes grown in solidified medium (Gel gro) were resuspended by adding 0.1 M citrate buffer (55 mM citric acid, 125 mM sodium citrate, pH6) at 30°C, to assure that the gel did not limit the access of fluorochrome to the cells (liquid medium controls were treated similarly). Decolorized aniline blue and calcofluor staining (for callose and cellulose, respectively) were carried out on fixed pollen tubes. After two washes, cells were incubated for 15 minutes with the staining agent (0.1% aniline blue in 0.15M K_2HPO_4; 0.1% calcofluor in ddH_2O), mounted immediately and observed at UV light excitation.
For immuno fluorescence label fixed cells were incubated with monoclonal antibodies Jim 5 and Jim 7 (generously provided by Dr Paul Knox, Leeds, UK) diluted 1:50 in PBS buffer followed by an incubation with goat anti-rat IgG - alexa fluor 594 (diluted 1:100 in PBS buffer) over night at 4°C. JIM5 and JIM7 recognize homogalacturonans with low and high degree of esterification, respectively (Knox et al. 1990; VandenBosch et al.

1989). Tubes were mounted and observed in the fluorescence microscope using a Texas red filter set. Controls were performed by omitting incubation with the primary or the secondary antibody.

4.4.3 Brightfield and fluorescence microscopy

Specimens labelled for cell wall components were observed in the fluorescence microscope (Nikon TE2000) equipped with a Roper fx cooled CCD camera. Exposure times of images that had to be compared for fluorescence intensity were identical. These images were not manipulated for contrast or brightness before reproduction.

4.4.4 Transmission electron microscopy

Samples for transmission electron microscopy were fixed in 2% formaldehyde and 2,5% glutaraldehyde solution in 0,05 M phosphate buffer, pH 7.2 for 2 h at room temperature. They were washed in 0,05 M phosphate buffer, pH 7.2 and post-fixed with 1% osmium tetroxide in the same buffer for 2 h. This was followed by dehydration in acetone and embedding in Spurr's resin. Ultrathin sections were cut with a Reichert OM U2 ultramicrotome, collected on formvar coated copper grids, and stained with 2% uranyl acetate and lead citrate (Reynolds). Specimens were observed with a JEOL 100-S transmission electron microscope operated at 80 kV.

4.4.5 Scanning electron microscopy

Unfixed dehydrated *Solanum* and *Lilium* pollen grains were sputtered with gold using a Technics Hummer II sputter coater and observed with a JEOL JSM 35 scanning electron microscope operated at 15 kV.

4.4.6 Micro-indentation

Hydrated pollen was grown on cover slips coated with poly-L-lysine or stigmatic exudate and covered with liquid GM. After germination had occurred, cover slips were submerged in the GM containing experimental chamber of the micro-indenter which was mounted on a Nikon TE2000 inverted microscope. The design and principles of operation of the micro-indenter have been described previously (Elson et al. 1983; Petersen et al. 1982). Briefly, the bending of a horizontal glass beam gauges the resistance to cellular deformation. A vertical glass stylus (tip diameter 10 μm) is mounted at the end of a 23mm long horizontal vycor glass beam. The other end of the beam is mounted on a linear piezoelectric motor, which moves vertically according to a programmed waveform. In the experiments reported in this paper, the motor was programmed to execute a single triangular waveform with a velocity of 14 $\mu m.s^{-1}$ and a total amplitude of 7 μm. Optical sensors monitor the vertical positions of the stylus and the motor. The extent to which the beam is bent is proportional to the force exerted on the tip by the cell and is determined by comparing tip displacements in the presence and absence of cell contact. The force exerted by the cell on the stylus is determined with the help of the force constant of the beam, which is obtained by prior calibration. The stiffness is obtained by calculating the slope of the linear part of the graph obtained by plotting force against depth of deformation.

4.5 Acknowledgements

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Chapitre 5 :

Discussion générale

5.1 Les propriétés biomécaniques du tube pollinique de *Papaver rhoeas* en fonction de la répartition des éléments cellulaires.

La présence de zones fonctionnelles (l'apex en croissance et la région distale stable), mais aussi structurelles (par une répartition hétérogène des différents constituants pariétaux, des filaments d'actine, etc.) suggère l'existence d'une distribution anisotropique des propriétés physiques de long du tube pollinique.

L'utilisation pour la première fois de techniques de micro-indentation sur une cellule végétale unique, vivante et en croissance a permis de démontrer l'existence d'un gradient physique correspondant parfaitement aux fonctions des différentes parties de cette cellule.

En effet, le tube pollinique en tant que cellule à croissance apicale possède la caractéristique de croître dans une direction unique, avec une zone de croissance limitée à l'apex de la cellule.

La croissance apicale du tube pollinique nécessite l'apport continu de matériel pariétal à l'apex, par un système d'exocytose des vésicules de sécrétion contenant le matériel pariétal néo-synthétisé. Les vésicules de sécrétion sont transportées de l'appareil de Golgi à l'apex de la cellule où elles doivent être assimilées pour permettre l'expansion de la cellule. La relaxation pariétale, en simultanée avec une pression de turgescence significative, semble donc être l'une des premières étapes nécessaires à l'expansion cellulaire.

Ce pré-requis suggère donc la capacité de la zone apicale à se déformer de manière plastique pour permettre l'insertion de la paroi néo-synthétisée et donc la croissance. De plus, par l'existence même d'une zone de croissance limitée à l'extrémité apicale de la cellule il semble évident que cette zone devrait posséder une rigidité moindre que la zone distale qui ne croît pas.

In planta, le tube pollinique doit résister aux forces latérales exercées par le tissu de transmission à travers duquel il croît, afin d'assurer l'intégrité des cellules spermatiques lors de leur passage vers l'ovule. Cela suggère la nécessité d'une plus grande stabilité mécanique de la région distale de la cellule comparée à l'apex en croissance. De plus, sachant que la pression de turgescence interne s'exerce de manière identique tout au long du tube pollinique, la géométrie même de cette cellule nous permet d'affirmer que les forces de tension exercées au niveau de la partie cylindrique du tube pollinique sont deux fois plus élevées que celle exercées au niveau de l'apex sphérique (Green 1962). De cette manière il apparaît clairement que la région distale du tube pollinique doit être significativement plus stable que la zone apicale afin de résister aux forces de tension exercées par la pression de turgescence interne et de maintenir intacte la morphologie du tube.

Nos expériences de micro-indentation ont confirmé, pour la première fois, l'hypothèse répandue depuis de nombreuses années selon laquelle, l'apex en croissance serait moins rigide et moins élastique comparé aux régions distales de cette cellule. En plus de fournir une preuve quantitative des propriétés physiques du tube pollinique en croissance, nos résultats démontrent l'existence d'un gradient de rigidité et d'élasticité le long de la cellule avec une augmentation drastique de ces paramètres dans les 20 premiers micromètres apicaux, suivie d'une zone de plateau où la rigidité et l'élasticité maintiennent une valeur constante dans la partie distale.

Cette approche quantitative des propriétés physiques du tube pollinique représente la première véritable preuve du concept longtemps supposé d'un apex moins rigide capable de se déformer de manière plastique, comparé aux régions matures stables et plus résistantes de la cellule. Cependant, la constitution cellulaire anisotropique de cette cellule complexe permet d'établir une relation entre les propriétés mécaniques observées et la distribution des éléments cellulaires au sein du tube pollinique.

La stabilité mécanique d'une cellule dépend de plusieurs facteurs distincts comme la paroi ou encore le cytosquelette, et chacun de ces éléments est susceptible d'expliquer les propriétés physiques de cette cellule. L'utilisation de marqueurs spécifiques des différents constituants cellulaires du tube pollinique nous a permis de dégager le rôle clé de la paroi dans le gradient physique existant le long du tube pollinique.

Les fonctions physiologiques des filaments d'actine ont été largement étudiés et leur rôle dans le transport des vésicules de sécrétion prouvé. Ainsi au niveau du tube pollinique de *Papaver rhoeas* comme chez d'autres espèces (Hepler et al. 2001; Raudaskoski et al. 2001; Vidali and Hepler 2001; Vidali et al. 2001), les filaments d'actine sont orientés de manière longitudinale dans la zone subapicale du tube pollinique, avec un réseau particulièrement dense et dynamique à environ 5-15 µm de l'apex. Dans la zone subapicale, leur orientation à la fois périphérique et centrale suggère leur rôle potentiel dans le gradient de rigidité. Par contre, dans la région distale, les filaments sont répartis exclusivement dans le centre de la cellule, ce qui suggère qu'ils ne contribuent probablement pas à la stabilité de cette région. De façon similaire, les microtubules sont centrés dans la cellule suggérant alors un rôle primordial de la paroi dans les propriétés mécaniques du tube pollinique.
La balance existant entre l'élasticité et la plasticité le long de l'axe longitudinal du tube pollinique semble donc être contrôlée principalement par la paroi cellulaire ; autrement dit, par la synthèse des différents polymères pariétaux et leur insertion. Ainsi, la paroi néo-synthétisée devra être suffisamment rigide pour contenir les forces de pression exercées par la turgescence interne mais également assez flexible pour permettre à la cellule de croître (Steer and Steer 1989; Carpita and Gibeaut 1993; Darley et al. 2001; Hepler et al. 2001). Autrement dit, la paroi semble jouer un rôle clé dans le processus de croissance du tube pollinique sachant que l'équilibre entre la pression de turgescence et la résistance de la paroi détermine son extensibilité et donc sa capacité à croître (Cosgrove 1993) .

L'utilisation de marqueurs spécifiques de la callose et de la cellulose permet d'écarter ces deux polymères comme responsables de l'augmentation de la rigidité de l'apex.
En effet la cellulose semble pratiquement absente de l'apex du tube pollinique du pavot avec une augmentation significative des dépôts de ce polymère à partir

d'environ 50 à 70 µm de l'apex. De la même manière, la callose est totalement absente de l'extrémité apicale avec un épaississement définitif de cette couche interne à partir de 120 µm. D'un autre côté, les pectines représentent les polymères pariétaux les plus aptes à expliquer l'augmentation de rigidité des 20 premiers micromètres du tube.

L'utilisation d'anticorps monoclonaux spécifiques des deux formes de pectines méthyle-estérifiées et acides existant au sein de la paroi du tube pollinique a permis de confirmer que les pectines méthyle-estérifiées représentent le constituant majoritaire de l'apex croissant. Durant l'élongation du tube pollinique, les pectines sont déposées à l'apex de la cellule sous la forme estérifiées, et ce n'est qu'ensuite qu'une dé-estérification de ces pectines en pectines acides s'effectue grâce à l'action de la pectine méthyle estérase (PME), créant ainsi un gradient de dé-estérification le long du tube pollinique.

Ce gradient semble, d'après nos résultats de micro-indentation, jouer un rôle important dans les changements dynamiques de la viscoélasticité et de la rigidité de la paroi indispensable à la croissance. Ceci confirme les observations précédentes mettant en évidence la capacité des pectines acides à former une structure plus stable et plus rigide par l'intermédiaire de ponts calcium (Carpita and Gibeaut 1993).

Étant donné que les différents polymères constituant la paroi des tubes polliniques ne peuvent raisonnablement être considérés indépendamment les uns des autres, il semble que la rigidité des parties distales du tube pollinique de *Papaver rhoeas* puisse être le résultat de la présence simultanée de callose, cellulose et pectines alors que les propriétés mécaniques de la zone apicale semble être le résultat direct de la présence de pectines méthyle-estérifiées.

Pour résumer, la présence constitutive de longs filaments d'actine, de microtubules et d'une paroi cellulaire complexe semble confirmer la plus grande stabilité de la zone distale du tube pollinique comparée à l'apex en croissance, soulignant ainsi l'existence d'un gradient physique permettant d'un coté à la zone apicale de croître et, de l'autre, à la région distale de garder un diamètre uniforme.

5.2 Résistance aux forces de tension dans la zone apicale durant la germination et l'élongation : le rôle des pectines et de la callose.

Les cellules à croissance apicale sont caractérisées par une zone d'élongation limitée à l'apex de la cellule (Roggen and Stanley 1969; Derksen 1996; Hepler et al. 2001). Ainsi dans le cas des tubes polliniques, des hyphes fongiques ou encore des poils racinaires, le processus d'expansion cellulaire semble contrôlé par une force de propulsion se composant vraisemblablement de différentes forces exercées par la pression de turgescence interne, le cytosquelette ou encore l'insertion de composants pariétaux (Taiz 1984; Cosgrove 1986; Money 1997). Lors de sa germination et de son élongation, la paroi cellulaire du tube pollinique doit exercer des forces de résistance au stress de tension, provoquée par la force de propulsion, pour permettre sa croissance. Nos expériences de marquage ont révélé que le grain de pollen et, plus particulièrement, la zone d'émergence du tube du *Solanum* étaient majoritairement constitués de callose et de pectines acides. D'un autre côté, la zone de croissance des tubes polliniques est constituée majoritairement de pectines. Cette répartition anisotropique de la paroi suggère, d'une part, un rôle essentiel de la callose et des pectines acides dans le processus de germination, et d'autre part, un rôle prépondérant des pectines dans l'élongation de la cellule (Roggen and Stanley 1969; Li et al. 1994; Derksen 1996; Li et al. 1997; Li et al. 2002). L'apport du matériel pariétal vers l'apex de la cellule s'effectue par exocytose des vésicules de sécrétion, suivie de leur incorporation.

Nos résultats mettent en évidence l'existence d'un équilibre entre les forces de propulsion et celles de résistance de la paroi que l'on peut simplifier ainsi :

Forces de propulsion > Résistance de la paroi

Pour que les processus de germination et d'élongation puissent avoir lieu, on considère que les forces de propulsion doivent être légèrement supérieures à celles de résistance de la paroi.

En effet, une digestion poussée des pectines ou de la callose modifie l'équilibre de telle manière que la paroi n'est plus assez résistante pour retenir la tension causée par les forces de propulsion, aboutissant alors à un éclatement de la cellule (le grain ou le tube). L'équilibre peut être alors écrit ainsi :

Forces de propulsion >>>> Résistance de la paroi

D'autre part, l'ajout de concentrations faibles d'enzymes digestives spécifiques de la callose ou des pectines entraîne une digestion modérée de ces polymères augmentant ainsi la plasticité de la paroi ce qui pourrait favoriser l'insertion de matériel néosynthétisé tout en permettant l'expansion, ainsi l'équilibre se simplifie comme suit.

Forces de propulsion >> Résistance de la paroi

Cette équation représente l'équilibre optimal entre les forces internes et externes, permettant donc une stimulation de la germination et de l'élongation des tubes polliniques. Les pectines étant présentes à la fois au niveau du grain de pollen mais aussi de l'apex du tube pollinique, leur digestion entraîne une stimulation de la germination mais aussi de l'élongation. La digestion de la callose n'entraîne pas de stimulation de l'élongation comme suggérait son absence à l'apex, par contre on observe une augmentation du taux et une accélération de la germination. Bien que la callose soit absente de l'apex des tubes polliniques, nous avons observé un gonflement de l'apex après digestion modérée de la callose, ainsi qu'une diminution de la quantité de pectines méthyle-estérifiées à l'apex. Ces résultats suggèrent l'existence d'une interaction entre la callose et les pectines estérifiées et donc d'un rôle indirect de la callose dans la résistance aux forces de tension exercée au niveau de l'apex. Une autre explication de ce phénomène pourrait être la présence, d'après certains auteurs, de légers dépôts de callose sous forme peu polymérisées au niveau de l'apex des tubes pollinique (Hasegawa et al. 1996), ces molécules de callose pourraient interagir avec les pectines méthyle estérifiées et ainsi l'application de lyticase entraînerait un renflement de l'apex.

5.3 Résistance aux stress de circonférence dans la région cylindrique distale

La répartition des différents polymères pariétaux au sein du tube pollinique de différentes espèces révèle que la callose représente le constituant le plus abondant dans la paroi de cette cellule particulière (Nakumura et al. 1980, Rae et al. 1985). Alors que la callose est synthétisée et déposée généralement en réponse à un stress mécanique ou chimique dans la plupart des tissus (Currier 1957, Esau and Cronshaw 1967, Coffey 1976, Delmer and Amor 1995), elle est le résultat d'un développement normal dans le cas des tubes polliniques. Ainsi cette cellule représente un modèle idéal pour l'étude des propriétés cytomécaniques de la callose.

Il a été démontré que les contraintes physiques dues à la turgescence ne sont pas réparties uniformément le long du tube pollinique. Ainsi le stress de tension créé au niveau de la zone cylindrique distale du tube pollinique est deux fois plus élevé que celui appliqué au niveau de l'apex sphérique. Cette observation suggère donc une plus grande stabilité et résistance des zones distales du tube pollinique comparées à l'apex en croissance dans le but d'assurer le maintien d'un diamètre uniforme et le passage des cellules spermatiques.

Les premiers dépôts de callose étant visibles à partir d'environ 30 µm de l'extrémité apicale, ce polymère semble impliqué dans la stabilité des régions distales du tube pollinique.

Le stress de tension dans cette zone étant plus élevé que celui perçu à l'apex, la présence de pectines acides plus rigides et/ou de callose permettrait à cette zone une plus grande résistance aux forces de pression internes. Ainsi l'application de pectinase entraîne un gonflement de l'apex alors que le diamètre du tube demeure inchangé, prouvant que les pectines acides ne sont pas exclusivement responsables de la résistance aux stress de tension dans la partie cylindrique du tube pollinique. À l'inverse, la digestion de la callose entraîne une augmentation du diamètre du tube pollinique confirmant pour la première fois le rôle de ce polymère pariétal dans le maintien et la stabilité du diamètre pollinique.

5.4 Résistance au stress de compression latérale

Lors de la croissance *in planta*, le tube pollinique pénètre les cellules qui tapissent le style pour atteindre les ovules et libérer les cellules spermatiques. L'environnement dans lequel le tube croît exerce donc des forces sur la cellule, forces auxquelles le tube pollinique doit résister afin d'assurer le passage des gamètes mâles. Ces forces de compression exercées par le style i*n vivo* peuvent être étudiées en appliquant des déformations mécaniques locales à l'aide du micro-indenteur et en accédant aux mesures de rigidité et de viscoélasticité.

La pectinase agit en digérant à la fois les pectines estérifiées mais aussi les pectines acides, on peut modifier le gradient de dé-estérification existant le long du tube pollinique par traitement avec la pectine méthyle esterase (PME) qui transforme les pectines estérifiées, majoritairement présentes à l'apex, en pectines acides capables de former un gel pectate (Kauss and Hassid 1967; Li et al. 1997; Geitmann 1998). Nos expériences de micro-indentation ont démontré pour la première fois le rôle, non seulement de la quantité, mais aussi du degré d'estérification des pectines dans les propriétés biomécaniques de l'apex du tube. La digestion non-spécifique des deux types de pectines par traitement à la pectinase entraîne une diminution caractéristique de la rigidité apicale avec une augmentation du comportement viscoélastique.

Tout comme il a été observé chez de nombreuses espèces, une augmentation du degré d'estérification semble être corrélé avec la croissance cellulaire, alors que la dé-estérification entraîne un arrêt de la croissance (Kim and Carpita. 1992; McCann and Roberts 1994). Ainsi l'ajout de PME entraîne une augmentation de la rigidité de l'apex en transformant les pectines méthyle-estérifiées en pectines acides, confirmant ainsi la plus grande résistance des parties distales du tube pollinique par formation de gel pectate (Jarvis 1984; Carpita and Gibeaut 1993; Derksen 1996; Li et al. 1997; Li et al. 2002).

Des expériences de micro-indentation sur des tubes polliniques en croissance en présence de lyticase confirment le rôle de la callose comme agent stabilisateur, puisque la digestion spécifique de la callose entraîne une diminution importante de la rigidité de la région distale alors que les propriétés mécaniques de l'apex restent inchangées.

Ceci suggère que la callose pourrait avoir un rôle de résistance contre le stress de compression, ce qui semble contredit par un marquage spécifique de la callose dans des tubes germant en milieux semi-solides. Ces marquages ont démontré une diminution de la quantité de callose dans les tubes germant en milieu dense comparé au liquide, et des observations identiques ont été faites pour les pectines. Ainsi il semblerait que la callose et les pectines acides puissent être des facteurs de résistance aux forces de compression dans les régions distales bien que ce rôle précis ne semble pas être avisé dans le cas précis des tubes polliniques.

5.5 Conclusion générale

Par une approche novatrice de micro-indentation nous avons confirmé l'hypothèse longtemps proposée d'un gradient des propriétés physiques le long du tube pollinique qui correspondrait à un gradient structurel pariétal. Ainsi la présence de pectines sous formes méthyle estérifiées à l'apex de cette cellule particulière, semblerait déterminante dans l'équilibre entre la pression interne de turgescence et la résistance de la paroi qui contrôle à la fois le processus de germination et l'élongation. Enfin, l'abondance de callose dans la zone distale du tube pollinique de *Solanum chacoense* semble conférer une plus grande stabilité à cette région permettant ainsi le maintien du diamètre du tube.

5.6 Perspectives futures

Mon projet de maîtrise a permis de répondre à un certain nombres d'interrogations pour ce qui a trait du rôle de la paroi dans la biomécanique du tube pollinique en utilisant différentes approches dont la technique de micro-indentation qui représente une innovation remarquable.

Le micro-indenteur est un matériel unique au Canada ayant du faire l'objet d'une adaptation intensive à notre but de recherche. Ainsi l'amélioration des différentes fonctions possibles de cet appareil représente un défi majeur à relever pour le futur proche.

Le but de ce sous chapitre n'est pas de dresser une liste exhaustive de toutes les perspectives futures se rapportant à mon projet de recherche, mais de présenter de manière brève quelques axes pertinents.

5.6.1 Micro-indentation

- Mesures de la rigidité et de la viscoélasticité locales sur des tubes polliniques après leur germination, avant et après l'ajout d'enzymes digestives spécifiques de la paroi, dans le but de caractériser l'effet de l'hydrolyse des constituants pariétaux en fonction du temps.

- Mesures des paramètres physiques des tubes polliniques en fixant soit la position de l'aiguille, soit la force appliquée (une fonction particulière a été crée dans ce but) avec pour objectif de caractériser plus précisément le comportement cellulaire.

- Mesure de la rigidité du tissu de transmission tapissant le style, et du milieu semi-solide complémenté en agarose dans le but de les comparer.

5.6.2 Études structurelles

- Caractérisation et approfondissement du rôle des pectines sous formes d'anneaux (Fig. 41), après ajout de concentrations modérées de pectinase (cette observation a déjà été faite mais la correspondance avec une croissance pulsée ou un environnement de germination devrait être poursuivie).

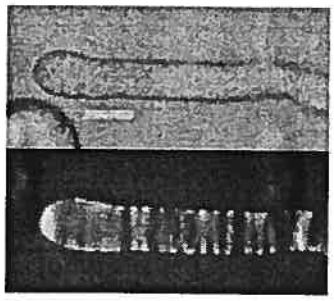


Figure 41 : Mise en évidence de la présence d'anneaux de pectines méthyle-estérifiées après ajout de pectinase. (A) photo DIC, (B) marquage par l'anticorps Jim 7.

- Approfondissement du rôle de la cellulose dans les propriétés mécaniques du tube pollinique (l'effet de la cellulase sur la germination et l'élongation des tubes polliniques à été caractérisée ainsi que l'aspect micro-indentation mais une étude approfondie serait nécessaire).

- Utilisation d'inhibiteurs de la synthèse de la paroi, comme par exemple l'isoxaben ou le 2,6-Dichlorenzonitrile pour la cellulose, dans le but de comparer leurs effets avec ceux des enzymes digestives.

- Étude de la répartition des différents polymères pariétaux en fonction de la densité du milieu de croissance en faisant germer un même tube pollinique dans deux milieux de rigidité différente.

- Caractérisation au niveau microscopie électronique à transmission des effets des enzymes digestive et des inhibiteurs de la synthèse pariétale (Fig. 42).

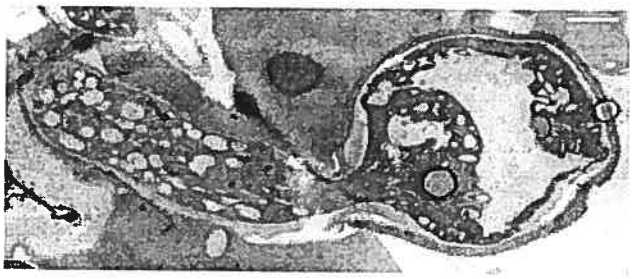


Figure 42 : Grain de *Solanum chacoense* en germination observé au microscope électronique à transmission.

- L'utilisation de mutants pariétaux de l'espèce modèle *Arabidopsis thaliana* représente une approche très prometteuse (voir annexe 2 : liste de mutants pariétaux connus à ce jour).

5.6.3 Modélisation par la méthode des éléments finis

Cette partie se fait actuellement en collaboration avec le département de Physique de l'Université de Montréal (Laurent Lewis, Jean-François Bolduc) et l'École Polytechnique (Geneviève Lebeau, Carl-Éric Aubin). Le but de cette approche est de mieux interpréter les résultats expérimentaux provenant du micro-indenteur. L'utilisation de modèle par éléments finis permet de comprendre le rôle d'un constituant cellulaire (i.e. la paroi) sur les propriétés de résistance à la déformation des tubes polliniques en faisant varier un seul paramètre à la fois (i.e. épaisseur de la paroi).

L'analyse par éléments finis est une technique de modélisation numérique utilisée pour calculer le comportement mécanique de structures. Le principe de la méthode est simple et consiste à subdiviser les structures complexes en un ensemble de structures simples dont le comportement peut être modélisé mathématiquement. Ces structures seront appelées les *éléments*. Ils sont liés les uns aux autres par les *nœuds*, points commun entre deux ou plusieurs structures simples. L'ensemble des éléments et des nœuds constitue le *maillage* (Fig. 43).

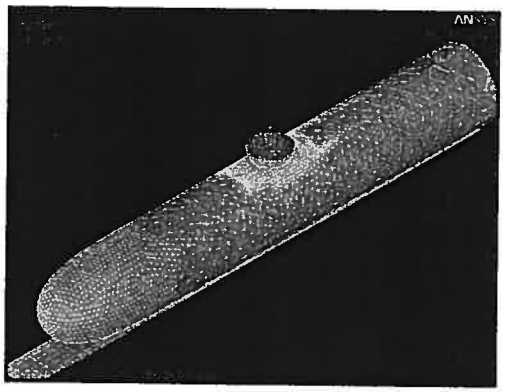


Figure 43 : Représentation par éléments finis d'un tube pollinique soumis à une micro-indentation. La finesse du maillage est différente suivant les régions de la cellule. Tirée des travaux de Jean-François Bolduc.

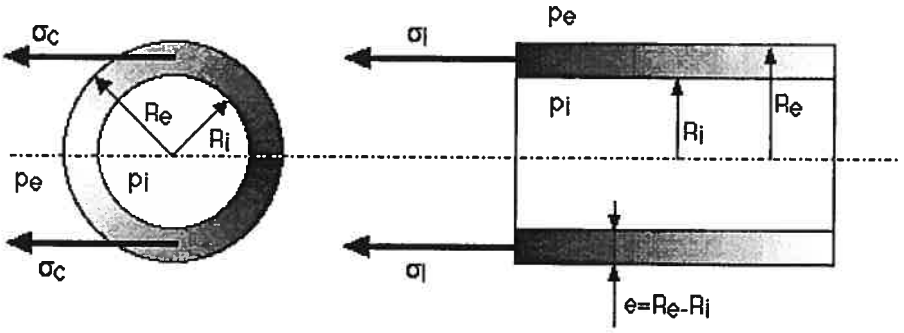
Dans notre cas précis, les modèles conçus comprennent trois composantes principales: la pointe de l'aiguille du micro-indenteur, le tube pollinique et le substrat rigide sur lequel repose le tube. Pour tous les modèles, le substrat rigide et la base du tube sont fixés, le second ayant pour but de simuler la présence du grain de pollen à partir duquel le tube croît

Le tube pollinique sera représenté par un modèle incluant plusieurs constituants cellulaires; il sera construit et adapté en fonction des questions auxquelles nous voudrons répondre.

La pointe du micro-indenteur est modélisée par une surface rigide dont le déplacement est contrôlé. Un déplacement est donc appliqué à cette surface qui entrera en contact avec le tube et s'enfoncera dans celui-ci jusqu'à une profondeur d'indentation imposée et déterminée à l'avance par l'utilisateur. La force de résistance exercée sur cette surface par la cellule est alors calculée.

Les premiers résultats de cette étude montrent que l'épaisseur de la paroi joue un rôle influant dans l'étude de la biomécanique du tube pollinique ce qui laisse entrevoir d'intéressantes avancées caractérisant le rôle clé de la paroi dans les propriétés physiques de cette cellule.

Annexe 1 : Le stress de tension est deux fois plus élevé dans le cylindre que dans l'apex sphérique



La **tension radiale** est égale à :

$$\sigma_r = p_i R_i$$

La **tension circonférencielle** est égale à :

$$\sigma_c = \frac{p_i R_i}{e}$$

La **tension longitudinale** est égale à :

$$\sigma_l = \frac{p_i R_i}{2e} = \frac{1}{2}\frac{p_i R_i}{e} = \frac{1}{2}\sigma_c$$

Annexe 2: Principaux mutants de la paroi chez *Arabodopsis thaliana*

Mutant	Modification of cell wall	Visible phenotype	Gene mutated
Mur5-6-7	Reduced arabinose	Not reported	?
Mur4	Reduced arabinose (50%)	Not reported	UDP-D-Xyl-4-epimerase
Mur8	Reduced rhamnose	Not reported	?
Mur 0	Reduced fucose and xylose	Slow growth, dark-green leaves, low seed-set	?
Mur11	Reduced Rhamnose, fucose and xylose	Not reported	?
Cnr	Reduced de-esterified homogalacturonan	Disturbed fruit ripening, reduced cell-to-cell adhesion and non-swollen cell walls in the pericarp	?
Emb30	Abnormal localization of the pectin	Seeds unable to pass through normal embryogenesis, abnormal plants	?
Irx1, 2, 3	Reduced cellulose	Collapsed xylem walls, adult plants slightly smaller than wild type	Cellulose synthase catalytic subunit
Keu	Incomplete cell walls in embryo	Seedling lethal, cytokinesis defective	?
Kn	Incomplete cell walls in embryo	Seedling lethal, cytokinesis defective	?
Kor (acw1)	Reduced cellulose in primary walls	Hypocotyl elongation defect;dwarfed	?
Mur1	Reduced fucose	Slightly dwarfed; brittleelongating flower stems	?
Mur9	Reduced rhamnose	Slow growth during rosette stage, normal inflorescence	?
Mur2-8	Reduced fucose or arabinose	Not observed	?
Rsw1	Reduced cellulose, increase pectin	Radial swelling	CesA1
Qrt1,2	Absence of pectin degradation in walls of pollen mother cells	Pollen produced in tetrad	?

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