ADVANCES IN PECTIN AND PECTINASE RESEARCH

Fons Voragen, Henk Schols and Richard Visser (Eds.)

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Advances in Pectin and Pectinase Research

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Preface

The second international symposium on Pectins and Pectinases was organized by Wageningen University and Research Centre and held in Rotterdam, May 6-10, 2001. This successful meeting was attended by around 130 participants from more than 20 countries representing almost all of the groups and industries working worldwide on pectins and pectinases. Following the first meeting on this subject held in December 1995, the symposium definitely forms a platform for researchers and industries working in the field, all within their own discipline and expertise. The symposium demanded a written account and this book is the result of that. It contains all keynote lectures and other oral presentations and provides an update about the current research. Significant progress has been made in the last 5 years.

This book provides an up-to-date insight into the research on pectin and pectic enzymes involved in their biosynthesis, degradation, modification or utilization. The progress in the elucidation of the chemical structure of pectin and mode of action and 3-D structure of the pectin degrading enzymes allows us to identify and influence the functionality of pectins and pectic enzymes, both *in vitro* after isolation as well in the plants themselves (*in planta*). Other contributions deal with new applications of both pectin and pectin-degrading enzymes, while more and more attention is paid to health and nutritional aspects of pectins.

The book provides a 'state of the art' account for both beginners and experienced researchers of almost all disciplines of pectin research.

We hope that it will satisfy your interests in this important and fast developing research field.

Fons Voragen, Henk Schols & Richard Visser, editors

Contents

INTERACTION OF A STYLAR PECTIC POLYSACCHARIDE AND A BASIC PROTEIN (SCA) MEDIATES LILY POLLEN TUBE ADHESION

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Abstract

Though pectins are implicated in cell adhesion in plants, this has never been tested directly. We developed an in vitro assay to study pollen tube adhesion to the stylar extracellular matrix (ECM) in lily. The adhesion of pollen tubes to the ECM of the stylar transmitting tract epidermis *in vivo* is proposed to be essential for a proper delivery of the sperm cells to the ovary. Using the assay, we identified two stylar molecules responsible for adhesion, a small protein and a pectic polysaccharide. The combination of at least these two molecules is required for this adhesion event. The 9-kD protein is cysteine-rich with some sequence similarity to lipid transfer protein. We named it stigma/style cysteine-rich adhesin (SCA). The second molecule has been isolated from the style using an imidazole extraction method and is mostly composed of galacturonic acid (70-75 mole%) with arabinosyl, galactosyl, rhamnosyl and glucuronosyl residues. This fraction reacts strongly with JIM5 (monoclonal antibody [MAb] to low esterifed homogalacturonans) and has some reaction with JIM7, LM5 and PAM1 (MAbs to esterified homogalacturonans, β-[1-4]-D-galactans, and blocks of 30 GalA repeat units). Pollen tube adhesion can be significantly reduced with a pre-treatment of this pectic fraction with endopolygalacturonase. All these data implicate a stylar pectic polysaccharide in lily pollen tube adhesion. *In vivo*, immuno-localization data show that SCA and low esterified homogalacturonan are co-localized at the transmitting tract epidermal surface where the pollen tubes adhere. Binding assays reveal that pectin and SCA bind each other in a pH dependent manner and that binding is necessary to produce pollen tube adhesion in the assay. Involvement of pectic polysaccharide and proteins in cell adhesion will be discussed.

1. Introduction

The new view of the plant cell wall as a cellular compartment rather than a rigid and inert network involved in protection and structural support is more and more accepted. This makes the plant cell wall, to some extent, comparable to the extracellular matrix (ECM) of animal cells in spite of their tremendous differences in structure and composition. Throughout plant development, the cell wall is subjected to many chemical and physical changes such as loosening during cell expansion and enzymatic degradation during fruit ripening. These defined wall modifications are spatially and temporally regulated [1], probably by their own cells and/or their neighboring cells.

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Thus, cell contact or cell adhesion sites might be involved in the transfer of information, communication and/or signaling between cells. The most studied case of signaling via cell contact is probably the recognition and rejection of self-incompatible pollen that involves interacting molecules from the male and female parts [2, 3]. To determine the importance of adhesion in cell development, we have developed an *in vitro* adhesion bioassay, using lily pollen and stylar extracts, that mimics the adhesion of pollen tubes observed within the style. Using this assay we have isolated the molecules required for this adhesion event. Based on the results obtained with our adhesion system, cell adhesion in general will be discussed with regard to the molecules involved.

2. Pollen tube adhesion in lily

During pollination in lily, pollen grains land on the surface of the stigma, hydrate and produce pollen tubes that travel along the hollow style to deliver the two sperm cells to the ovule (Figure 1). Pollen tubes grow by tip growth ie. secretion of wall material via vesicles targeted to the tip. After passing through the stigma, the growing pollen tubes come in contact with the transmitting tract epidermis (TTE) that lines a canal filled up with an exudate secreted by the TTE cells and composed of lipids, proteins and carbohydrates [4].

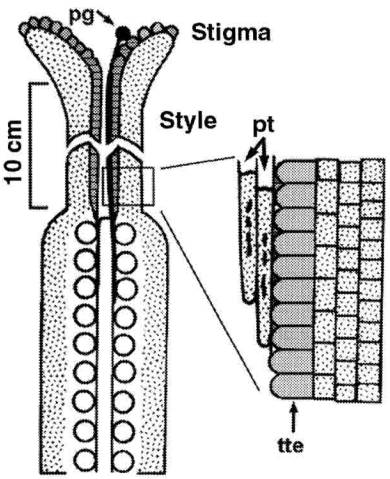

Figure 1. Pollination and adhesion of lily pollen tubes on the transmitting tract epidermis of the stigma and style. pg. pollen grain, pt. pollen tube, tte. transmitting tract epidermis. Reprinted with permission from Elsevier Science, *Trends in Plant Science* [5].

Adhesion between the pollen tube walls and the TTE surface can be observed, after cryo-preservation of pollinated styles preserving the extracellular matrix or ECM (Figure 2A-B). Adhesion occurs also between pollen tubes themselves (Figure 2B). This adhesion between the pollen tubes and the TTE cells has been proposed to be, at least in part, responsible for the fast growth rates of *in vivo* grown pollen tubes compared to the pollen tubes grown in an artificial liquid medium [6].

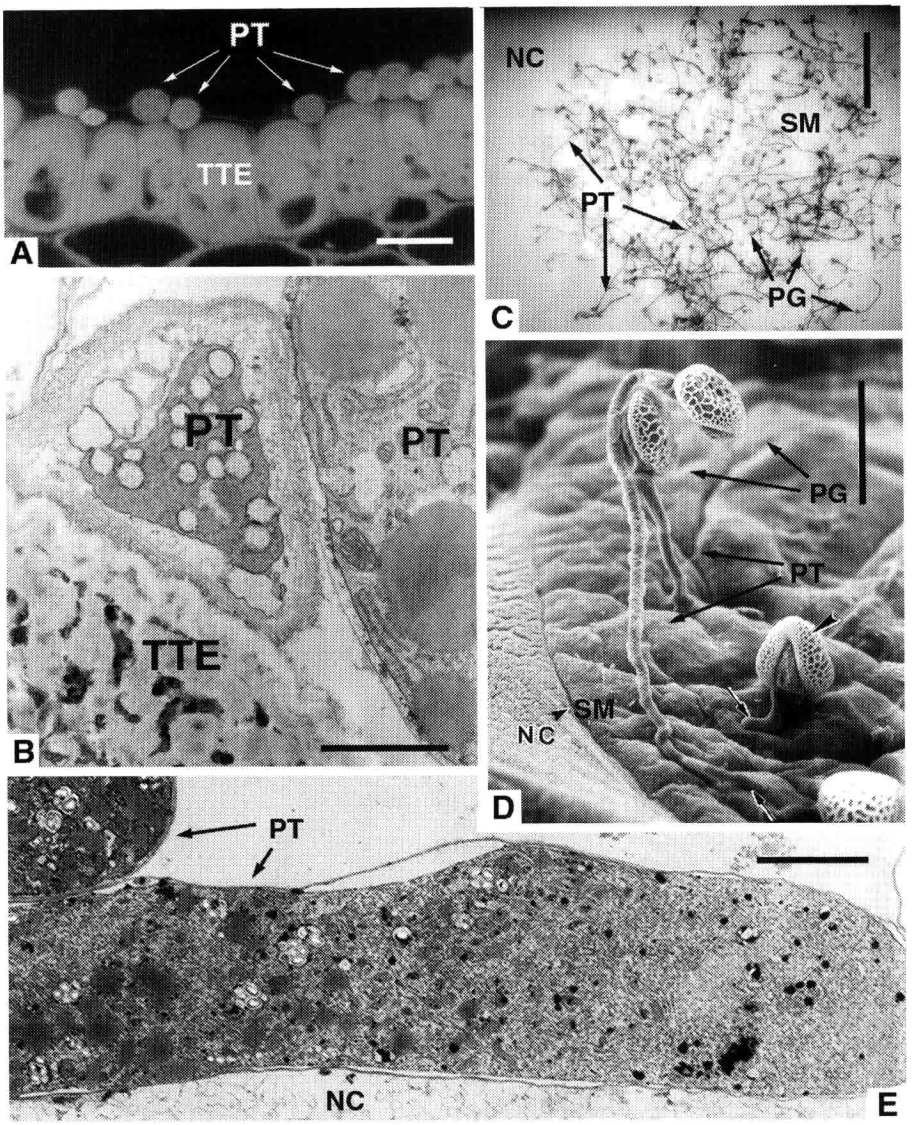

Figure 2. In vivo (A-B) and *in vitro* (C-E) adhesion of lily pollen tubes to the TTE and the *in vitro* artificial stylar matrix, respectively. A. Cross section of a lily pollinated style showing the pollen tubes adhering to the TTE surface. Scale bar = 25 μm. B. TEM view of pollen tubes in cross section adhering to the TTE surface and to each other. Scale bar = 5 μm. Reprinted with permission from Springer, *Sexual Plant Reproduction* [7]. C. Stereomicroscope view of the *in vitro* adhesion assay after staining with coomassie blue. Pollen tubes adhere to the stylar matrix but not to the surrounding nitrocellulose membrane. Scale bar = 1 mm. D. SEM view of pollen tubes adhering to the stylar matrix. Note the pollen tubes adhere at the tip. Scale bar = 50 μm. Reprinted with permission from Springer, *Sexual Plant Reproduction* [8]. E. TEM view of pollen tube tip adhering to the *in vitro* stylar matrix. Scale bar = 5 μm. Reprinted with permission from ASPB, *Plant Cell* [9]. NC. nitrocellulose membrane, PG. pollen grain, PT. pollen tube, SM. stylar matrix composed of SCA and stylar pectin, TTE. transmitting tract epidermis.

To determine the nature of the stylar molecules involved in this adhesion, an *in vitro* adhesion bioassay was developed [8]. Pre-germinated pollen tubes are incubated with nitrocellulose membranes impregnated with the molecules isolated from styles and/or stigmas as shown in Figure 3. If adhesion occurs, the number and length of pollen tubes can be measured under a stereomicroscope after staining with coomassie blue (Figure 2C). Typically, pollen tubes adhere via their tips to this artificial matrix within 2h of incubation, while the pollen grains and the pollen tubes back from the tips float in the liquid medium (Figure 2D). In this assay, pollen tubes adhering to the stylar matrix are also able to adhere to each other and grow (Figure 2E), as observed *in vivo*.

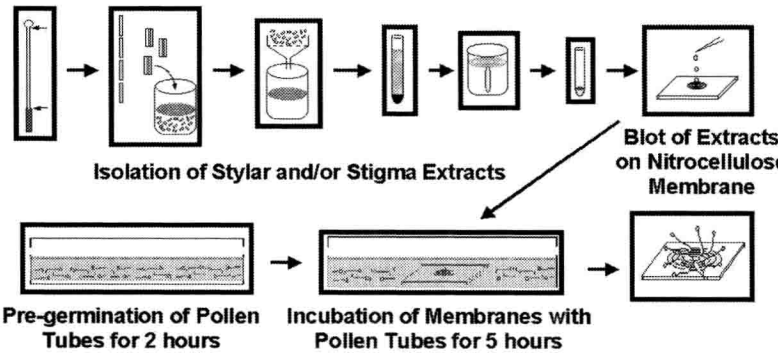

Figure 3. Diagram of the *in vitro* pollen tube adhesion assay method

3. Stylar molecules required for lily pollen tube adhesion

3.1. A 9-KD POLYPEPTIDE (SCA)

Preliminary data suggested that at least two molecules of different molecular weights were involved in pollen tube adhesion based on a size fractionation of a stylar extract. The low molecular weight molecule was purified from stigmas and styles following the method shown in Figure 4. It corresponds to a 9 kD, basic (pI 8.6), cysteine-rich protein with some sequence similarity with plant lipid transfer protein including eight conserved cysteine residues (LTP, Figure 5) [9]. We named this protein SCA for style/stigma cysteine-rich adhesin. *In vitro*, LTPs are able to transfer lipids between organellar membranes [10] but there is no evidence of this function *in vivo*. Since LTPs are secreted into the plant ECM [11], it is unlikely that they play a role in cytoplasmic lipid transfer [12]. *In vivo* and *in vitro*, LTPs have been shown to have anti-microbial activity [13]. We are currently testing if SCA has antimicrobial activity. SCA is not produced by the male gametophyte, ie pollen tube and pollen grain [14]. However, immunolocalization of SCA on pollinated styles reveals that SCA is detected on the wall of the TTE but also on the wall of the pollen tubes [9]. SCA is secreted by the TTE cells and probably binds to the pollen tube walls during their travel along the style. The exact role of SCA in adhesion is still unclear. SCA is not adhesive alone but needs another large molecule to allow pollen tube adhesion.

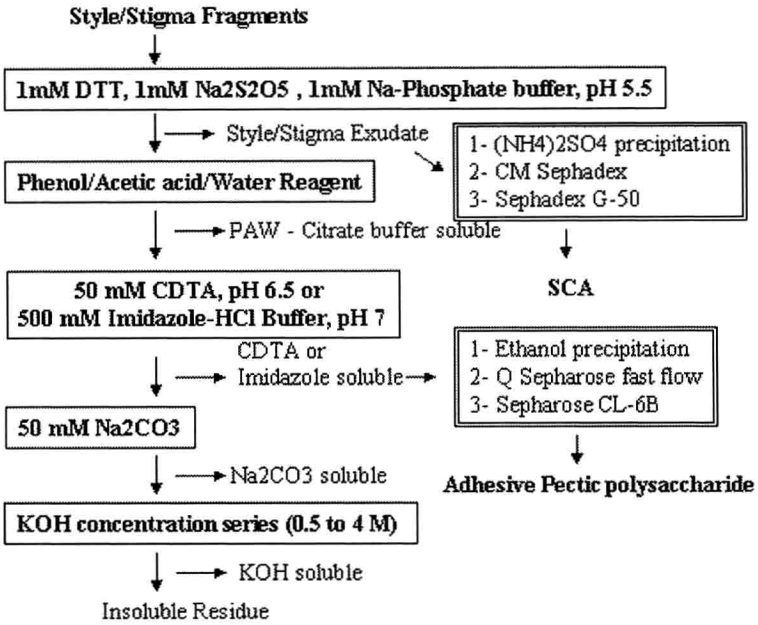

Figure 4. Flow chart of the method used for the fractionation and purification of SCA and pectin from lily styles and/or stigma

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Lily 9 kD                    MAR-SS-AVCF--LLLLAF-LIGIA-S-A
Rice                         :::AQLVL:AVVAA::::A--PH:A-V-:
Maize                        :::TQQL::VATAVV--:LV:LAA:T:E:
Arabidopsis                  ::GVMKLA-:L--::ACMIVA-:PIT:N:

                             ▼
Lily 9 kD                  -ITCGQVDSDLTSCLGYARKGGVIPP-GCCAGVRTLNNLAKTTPDRQ
Rice                       -::::::N:AVGP::T:::G:AGPSAA-::S:::S:KAA:SN:A::R
Maize                      A:S::::A:AIAP:IS:::GQ:AG:SA:::S:::S:::A:R::A::R
Arabidopsis                ALS::S:N:N:AA:I::VLQ::::::A-::S::KN::SI::::::::

Lily 9 kD                  TACNCLKSLVNP-SLGLNAAIVAGIPGKCGVNIPYPIRMQIDCNKVR
Rice                       :::::::NAARG-IK::::GNA:S::S::::SV::T:SASIH:SR:S
Maize                      A::::::NAAAG-VS::::GNA:S::S::::S:::T:STS:::SR:S
Arabidopsis                Q::::IQGAARALGS::::GRA::::KA:::::::K:STS:N:KT::
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Figure 5. Amino acid sequence alignment of SCA deduced from the cDNA compared to several plant LTPs. The arrow indicates the N terminus of the mature protein. Identical amino acids are indicated by colons and dashes represent spaces introduced to maximize alignment. Cysteine residues are in bold, lysines are double underlined and arginines are single underlined - Reprinted with permission from ASPB, *Plant Cell* and modified from [9].

3.2. A STYLAR PECTIN

The second molecule involved in adhesion has been chemically extracted from style fragments using the method described in Figure 4. None of the stylar extracts allow pollen tube adhesion on their own. In combination with SCA, the most adhesive stylar extract was detected in the imidazole-HCl fraction (Figure 6).

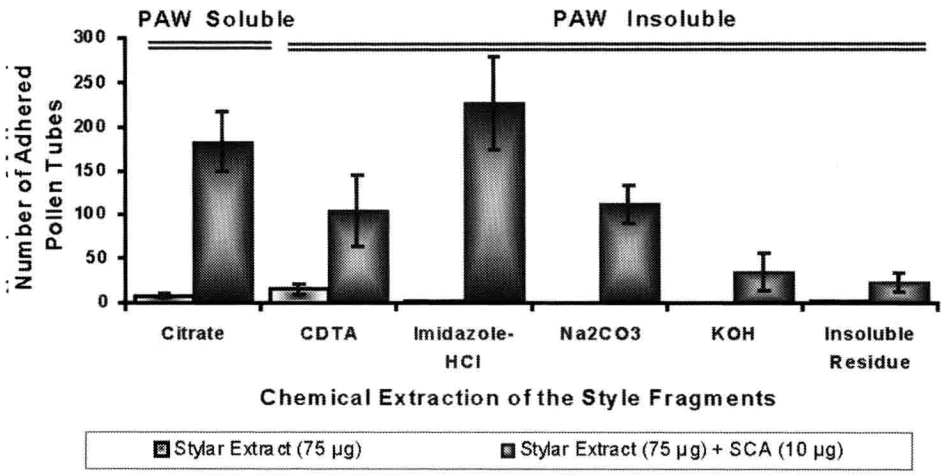

Figure 6. Fractionation of the stylar adhesive molecule using an in vitro adhesion assay. The different fractions were obtained by sequential chemical extraction as shown in Figure 4. Reprinted with permission from ASPB, *Plant Cell* and modified from [15].

The imidazole extract was further fractionated by ethanol precipitation, anion exchange (Q sepharose fast flow) and size exclusion (Sepharose CL-6B) chromatography using 100 mM imidazole-HCl, pH 7 as eluent. Adhesion assays, in combination with SCA, demonstrated that the most adhesive fractions were detected between 40-60% ethanol precipitation and eluted between 300-400 mM NaCl from the Q Sepharose [15]. After size exclusion chromatography, 50 µg of the most adhesive fraction in combination with 5 µg of SCA are sufficient to allow 401 ± 53 pollen tubes to adhere to the artificial matrix (Table 1). The estimated size of this fraction on a sepharose CL-6B was 1,500,000 MW compared to dextran standards. This fraction is a galacturonic acid enriched polysaccharide (Table 2) with a molar ratio of GalA to Rha of 11. It reacts strongly with JIM5 (MAb to low esterified homogalacturonan) and has some reaction with JIM7, LM5 and PAM1 (MAbs to esterified homogalacturonan, β-[1-4]-D-galactan and a stretch of 30 GalA units, respectively) (Figure 7) indicating the presence of homogalacturonan and rhamnogalacturonan 1 regions. The pectic fraction reacts also with JIM13 and JIM8 (Mabs to carbohydrate epitopes of arabinogalactan-proteins) but does not react with the Yariv phenylglycoside, a diagnostic tool to detect arabinogalactan-proteins. This implies that arabinogalactan side chains may also be part

of the RG1 region. Proteinase K pre-treatment on the pectic fraction does not affect the number of pollen tubes adhering to the matrix [15]. Endopolygalacturonase treatments on the pectic fraction [15] or on the pectic fraction combined with SCA result in a significant decrease in the number of pollen tubes adhering to the nitrocellulose membrane (Table 1). All these data suggest that the second molecule required for lily pollen tube adhesion is a pectic polysaccharide.

TABLE 1. Adhesion of lily pollen tubes on a matrix coated with stylar pectin and SCA after their treatments with endopolygalacturonase.

Fraction and Enzymatic Treatment	Number of Adhered Pollen Tubes	
Stylar Pectin [a] (50 µg) + SCA (5 µg)	401 ± 53	(n = 5)
PGase treatment on the stylar pectin [b]		
Stylar Pectin 50 µg (PGase treated) + SCA (5µg)	51 ± 16	(n = 3)
Stylar Pectin 50 µg (boiled PGase) + SCA (5 µg)	311 ± 43	(n = 2)
PGase treatment on the stylar pectin combined with SCA [c]		
[Stylar Pectin (50 µg) + SCA (5 µg)] PGase treated	18 ± 6	(n = 2)
[Stylar Pectin (50 µg) + SCA (5 µg)] boiled PGase	356 ± 62	(n = 2)

[a] Stylar pectin was obtained after ethanol precipitation, Q sepharose and Sepharose CL-6B chromatography of the imidazole extract.
[b] Stylar pectin was incubated with endopolygalacturonase 2 (PGase treated) or boiled endopolygalacturonase 2 (boiled PGase) and combined with SCA. Reprinted with permission from ASPB, *Plant Cell* and modified from [15].
[c] Stylar pectin and SCA were combined and incubated with endopolygalacturonase 2 (PGase treated) or boiled endopolygalacturonase 2 (boiled PGase).
(n) number of replicates.

TABLE 2. Chemical composition of the stylar pectic polysaccharide from the imidazole extract fractionated by ethanol precipitation, Q Sepharose fast flow and Sepharose CL-6B chromatography.

Carbohydrate	Protein	Glycosyl composition (mol%)								
% (w/w)	% (w/w)	Ara	Rha	Fuc	Xyl	Man	Gal	Glc	GlcA	GalA
98.6	0.5	4.6	6.6	0.8	1.5	0.8	4	0.8	7.6	73.3

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4. Pectin in adhesion

Pectins and especially low esterified homogalacturonan have been implicated in intercellular adhesion at the middle lamella (see review [24]) based mainly on immuno-localization data. An antibody specific for blocks of 30 galacturonic acids (PAM1) bound in the contact zone between two cells in *Arabidopsis* suspension cultures [17]. Mutants deficient in cuticle showing abnormal fusion of organs revealed low esterified pectic polysaccharide in the junction zone [25, 26], as well. However, methyl esterified homogalacturonan [27] and neutral side chains of RG1 [28] have also been implicated in cell attachment. Decrease in arabinose and galactose content has also been reported during the dis-adherence of walls during the fruit ripening [29]. All these genetic, immuno-localization and biochemical data demonstrate that pectins are good candidates as adhesive molecules but the region of the pectic polysaccharide involved is still a question of debate. In our assay system, the adhesive pectic fraction contains low esterified homogalacturonan and RG1 regions. The presence of RG2 and xylogalacturonan is not known yet. The determination of the fine structure of our adhesive pectic polysaccharide with specific enzymes will help to define the region that is clearly involved in cell adhesion.

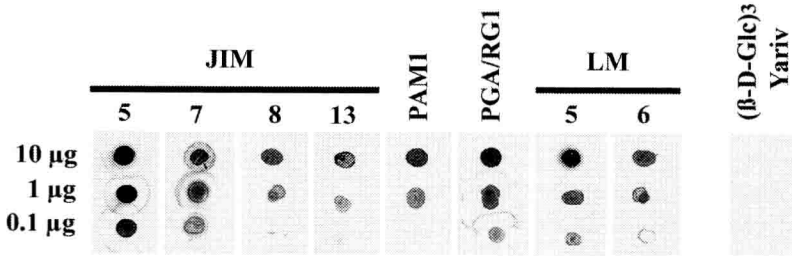

Figure 7. Dot-Blot immuno-assays of the stylar pectin fraction isolated from lily styles with the imidazole method after ethanol precipitation, Q sepharose fast flow and sepharose CL-6B. JIM5, MAb to low esterified homogalacturonan [16]; JIM7, MAb to high esterified homogalacturonan [16]; JIM8 and JIM13, MAbs to carbohydrate epitopes of arabinogalactan-proteins [17, 18]; PAM1, phage against blocks of 30 GalA [19]; PGA/RG1, polyclonal Ab to RG1 regions [20]; LM5, MAb to (1-4)-β-D-galactan [21]; LM6, MAb to (1-5)-α-L-arabinan [22] and $(ß\text{-D-Glc})_3$ Yariv phenylglycoside, a red synthetic probe that binds arabinogalactan-proteins [23].

The cohesion of homogalacturonan in cell corners is thought to be maintained by calcium bridges [30]. It appears that pectin behavior in solution with calcium may be more complex than the "egg box" model [31]. In our assay, the role of calcium is difficult to evaluate as it is a requirement in the germination medium to promote pollen tube growth, a prerequisite for adhesion. In addition, an important component in our adhesion assay is a small basic polypeptide (SCA).

5. Protein - pectin interaction

5.1 SITE OF PROTEIN - PECTIN BINDING

In addition to the structural polysaccharides, a wide range of proteins, enzymes and glycoproteins exist (see review [32]) that can be bound to the wall by covalent linkages or anionic interactions. For example, extensins can be cross-linked in the wall to pectins via rhamnogalacturonan [33]. In our system, a 9 kD basic protein and a pectic polysaccharide act together to promote lily pollen tube adhesion. SCA and the stylar pectin appear to bind to each other by ionic interaction rather than covalent cross-linking as the binding can be disrupted by a slight modification of pH over the isoelectric point of SCA [15]. Recently, two cationic amino acids (lysine and arginine) from two different wall enzymes (pectate lyase and peroxidase) have been shown to be responsible for the binding of these proteins to the homogalacturonan region of pectins [31, 34]. SCA possesses five lysines and five arginines (Figure 5) that may also be involved in the binding of SCA to the stylar pectin. In solution, basic proteins can also modify the physico-chemical properties of pectins as does calcium with homogalacturonan [35]. SCA may have a similar action on the adhesive stylar pectin but probably in a more specific manner, as substitution of SCA with other small basic peptides like cytochrome c or polylysine did not allow for pollen tube adhesion [15]. Other reports mention that cysteine-rich domains of proteins can mediate specific carbohydrate binding [36] as do lectins through "deep or shallow pockets" in their three dimensional structure [37]. We are using a variety of methods, ie synthesis of commercial peptides for competition studies, to determine the binding site of SCA and pectin.

5.2 SIGNALING BY PROTEIN- PECTIN INTERACTION

Other proteins or proteoglycans located at the cell wall/plasma membrane interface have also been reported to bind to pectins (arabinogalactan-proteins [AGPs] and wall associated kinases [WAKs]). These two molecules might be involved in signaling at the plasma membrane. AGPs have been reported to bind and/or to be co-purified with pectin [38] and they can be anchored to the plasma membrane through a glycosylphosphatidyl inositol anchor (GPI anchor) [39, 40] that may transfer signals to the cell [41]. WAKs have been also reported to be covalently linked to pectin [42]. These data suggest that pectins and other wall polysaccharides might be somehow linked together to form a network able to perceive or transmit signals.

6. Pollen tube wall and enzymes

The pollen tube cell is highly polarized. Even the wall at the tip, the site of secretion and growth, is different from the wall back from the tip (Figure 8). Based on immuno-localization using monoclonal antibodies, low and esterified pectins were found to be the main components at the tip in lily [43] and in other species [44]. In *Arabidopsis*, only esterified pectins were detected at the tip of *in vivo* grown pollen tubes [45]. In lily, arabinogalactan-proteins have been also detected in the tip region [43]. Back from