

## Immunochemical Probes for Microscopy Study of the Plant Cell Walls

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**Abstract.** In the recent years immunochemical probes with specificity for a large number of the polymers that constitute plant cell walls have been developed. First, polyclonal and monoclonal antibodies with recognition specificity for various pectic and hemicellulosic polysaccharides have been prepared. The high specificity of primary antibodies and the advance in secondary markers and colloidal gold allowed identification and localization of cell wall polysaccharides in growing and differentiating cells. In conjunction with electron microscopy the method constitutes the most powerful tool for ultrastructural investigation of cell wall supramolecular organization. The immunological approach has been successfully extended to elucidate lignin and phenolic acids topochemical distribution in normal and transgenic plants. More recently, the novel technology of recombinant antibody phage display has emerged as an alternative to hybridoma technology for monoclonal antibodies. This shows a large potential for immunological probe production. All these techniques allied to the progress in microscopy instrumentation constitute an important step for a better understanding of the molecular architecture of the plant cell walls.

### Introduction

Describing plant cell wall structure is describing the structure of a supramolecular composite, i.e. the way its component macromolecules are organized and the way they interact. The fine description of plant cell walls must therefore ally the identification of the different molecular constituents with the observation of their respective distribution *in planta* at the highest scale of resolution. It has become compulsory, for a better understanding of the relations between composition and organisation of the walls to reach a scale of observation corresponding to the nanoscale organization, i.e. to obtain data concerning ordering of molecular chains within the cell wall [1]. Recent technological advances have made microscopy techniques highly powerful tools for visualization, qualitative identification and, in some cases, quantitative estimation of cell wall constituents. Microscope technology has seen improvement in resolution and sensitivity [2] and also has seen the emergence of novel microscopy techniques [3]. In combination to these technical progresses, new immunological probes with high specificity for many of the cell wall constituents have been developed that allow accurate and detailed topochemical investigation of plant cell walls. The continued advance in the available immunological probes and secondary marker systems to be used in fluorescent and electron microscopy has led to progress in the representation of the fine architecture of the plant cell walls. The purpose of the present review is to give an overview of the immunochemical probes that have been developed for *in situ* localization and identification of plant cell wall polymers.

### IMMUNOLABELING OF POLYSACCHARIDES.

The technique of immunocytochemistry was introduced by Coons et al. (1941) [4] who demonstrated that an antibody conjugated to a fluorochrome retained its ability to recognize its antigen [5].

**Primary wall.** Early use of antibodies for plant cell wall polysaccharides localization was reported by Vreeland (1970) [6] who was able to identify alginic acid and sulphated fucan in brown algae. The method involved fluorescent FITC-antibodies [6, 7, 8]. The immunochemical approach for the precise identification and localization of polysaccharides of land plants followed only later with the preparation, using hybridoma technology, of monoclonal antibodies binding to arabinogalactan-proteins (AGPs) with preference for  $\alpha$ -L-arabinofuranose and  $\beta$ -D-galactopyranose [9]. A limitation to the use of these monoclonal antibodies was that they would detect  $\alpha$ -L-arabinose and  $\beta$ -D-galactose whether they occurred in the targeted AGP or in any other polysaccharide. This was also the case for an anti- $\alpha$ -L-arabinofuranose antibody [10] used in the study of the cotyledon tissues of soy bean, rice endosperm and *Phaseolus vulgaris* cotyledon tissues [11]. Raising polyclonal antibodies from mono- and oligosaccharide-BSA conjugates, Northcote & al. [12] obtained several antisera that were used for immunolocalization by electron microscopy of the corresponding polymers in differentiating cells of *Phaseolus vulgaris* roots and cultured cells of *Zinnia elegans*.

An important aspect of the specificity of immunological characterization was given by the labeling provided by the antiserum against L-arabinofuranoside compared to the absence of labeling with the antiserum against L-arabinopyranoside [12]. *This shows that sugar ring conformation can be distinguished by immuno-electron microscopy. Taken together these early results acquired with polyclonal antisera demonstrate that immunocytochemical technique has the potentiality of identifying the main characteristic of cell wall polysaccharides including the type of sugar constituent, the mode of linkage (position and configuration) and the sugar ring conformation.*

Another generation of antibodies directed against cell wall polymers or cell wall epitopes present in cell walls were prepared by the hybridomas technique against plant extracts (see Table 1). The principle guiding this approach is that antibodies to specific epitopes of complex macromolecules can be isolated [13]. This can even be done from ill-defined antigens such as crude extracts or whole cells. The time-consuming and difficult task following isolation of monoclonal antibodies is the characterization of their specificity. Although the precise epitope recognized in pectin molecules by the monoclonal antibodies JIM<sub>5</sub> and JIM<sub>7</sub> isolated by Knox and his collaborators [13] was unknown, they were largely applied to various plant materials in a number of laboratories to probe the low methyl-esterified *versus* the high methyl-esterified pectins, respectively [14,15], and to get indications about their regulation during growth and development [16]. A recent example of the use of a combination of six monoclonal antibodies to map the distribution of pectic epitopes in the cell walls of potato (*Solanum tuberosum*) tuber tissue, in light and electron microscopy, clearly demonstrated that the pectic composition of the walls of potato tubers is not homogeneous and that it is spatially regulated [17,18]. The monoclonal antibody 2F4 generated against a homogalacturonan conjugated to BSA recognized a homogalacturonan sequence with a minimum of nine contiguous galacturonic acid residues [19,20]. A typical feature of the recognized structure was its particular conformation maintained in cooperative association by calcium ions [21]. This is an important trait of this monoclonal antibody to show selectivity for galacturonan sequence in a precise conformation. To our knowledge, the only other immuno-microscopy probes sensitive to conformational parameters are the antibodies that recognize native linear and bulky lignin polymers [22] (*vide infra*). Visualization of the native conformation of polymers *in planta* is of interest for our understanding of cell wall ultrastructural organization and of the mode of association of polymers.

Table 1: Principal antibodies available for plant cell wall polysaccharides

Target component	Antigen	Mono-clonal	Poly-clonal	Reference
<b>PECTINS</b>				
<b>Homogalacturonans</b>				
	low ester pectins	JIM5	-	13,25,73
		PAM1	-	53,55
	high ester pectins	JIM7	-	25
		LM7	-	54
	Calcium complex	2F4	-	19,21
<b>Rhamnogalacturonans (RG I)</b>				
	RG I	-	yes	32
		CCRC M5	-	23
		CCRC M2	-	23
		CCRC M7	-	27
	Modified "hairy region"	1G 11	-	18
		6 B1	-	18
<b>Rhamnogalacturonan II (RG II)</b>				
	Borate dimer	-	yes	74,75
<b>Arabino-Galactan</b>				
	$\beta(1\rightarrow4)$ -galactan	LM5	-	76
	$\alpha(1\rightarrow5)$ -(Araf) <sub>7</sub>	LM6	-	77
<b>HEMICELLULOSES</b>				
<b>Xyloglucans</b>				
	XG-BSA conjugate	-	yes	32
	Purified XG	-	yes	33
	Heptasaccharide	-	yes	35
	Terminal $\alpha(1\rightarrow2)$ -linked Fuc	CCRC-M1	-	23
<b>Glucans</b>				
	$\beta(1\rightarrow3)$ -linked	LAMP	-	48
		2H1-2H7	-	49
<b>Xylans</b>				
	Wood xylans	-	yes	43-46
	Xylopentaose-BSA	-	yes	78
	MeGlcA-Xyl <sub>2-3</sub>	-	yes	78
<b>Glucomannans GM)</b>				
	$\beta(1\rightarrow4)$ -(Man) <sub>4</sub>	-	yes	42
	<i>Pinus</i> GM	-	yes	79
	Galactomannan	BGM C6	-	41
<b>CELL WALL GLYCOPROTEINS</b>				
<b>Hydroxyproline -rich HRGPs</b>				
	HR-Extensin	-	yes	80
	Purified extensin	11D2	-	81
		MC-1	-	82
	HR-glycoprotein	JIM-11	-	26
		JIM-12	-	26
		JIM-20	-	28
		JIM-19	-	13
		LM1	-	28

**Glycine-rich proteins**

Bean extract	-	yes	83
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**Undefined Cell Wall Proteins**

Oat root cap	40.1C2.8	-	84
Tyr- and Lys-rich	-	yes	85

**Arabinogalactan-Proteins (AGPs)**

Tobacco style AGP	PCBC3	-	9
Plasma membrane AGP	yes	-	13,27,86
Carrot AGP	ZUM-15;-18	-	87

Several monoclonal antibodies were generated from a rhamnogalacturonan I (RG1) purified from suspension-cultured *Acer pseudoplatanus* cells [23], each with a distinct pattern of recognition for cell wall polysaccharides. CCRC-M<sub>1</sub> was characterized to bind specifically terminal fucosyl residues in RG1 and wall xyloglucans. The amount of fucosylated xyloglucan in the cell walls of *Zinnia elegans* was probed with CCRC-M<sub>1</sub> and shown to vary during the transition from primary to secondary wall formation [24].

An extensive investigation of the roles and structural variations of arabinogalactan-proteins was undertaken with the use of monoclonal antibodies [9,13]. Most of these antibodies had capacities to bind epitopes present in plasma membrane and cell surface molecules ([14, 24, 26 ]. Series of JIM antibodies with recognition for different epitopes of AGPs were generated by Knox and his collaborators (reviewed in [13]. Other monoclonal antibodies were isolated from plant extracts with specificities determined by ligand competition assays such as CCR-M7 which recognizes arabinosylated (1 → 6)-galactan [27] or LM<sub>2</sub> [28] which has recognition for β-D-glucuronosyl residues. These antibodies were used [11, 27] to localize some of these epitopes in the cell wall [29] and to show that AGPs were not simply present as soluble molecules in the cell wall space [30, 31].

Both monoclonal and polyclonal antibody strategies have been applied for immunolabeling of xyloglucans. These typical hemicelluloses from the primary wall of dicotyledonous plants have a complex primary structure basically made of a β(1 → 4)-linked glucan main chain carrying (1→6)-linked side chains of single xylose units, galactosyl-xylosyl and fucosyl-galactosyl-xylosyl side chains. From the point of view of immunology, the presence of four different sugar residues and four different glycosidic bonds together with three different types of side chains and of monosaccharides in terminal position constitutes a wealth of potential structural epitopes. Another interesting feature of xyloglucans for raising antibodies in view of cell wall immunolabeling is that it has many characteristic structural linkages that are not found in other cell wall polysaccharides. Polyclonal antibodies were obtained from a purified xyloglucan coupled to ovalbumin [32], and by direct immunization of rabbits with the unconjugated polysaccharide [33, 34].

Other polyclonal antibodies were prepared against xyloglucans using an heptasaccharide (XG07) and a nonasaccharide (XG09) [35] from tamarind seed (*Tamarindus indica*). These oligomers contained only glucose and xylose (4:3) and glucose, galactose and xylose (4:2:3), respectively, and could therefore recognize the main structural features of cell wall xyloglucans [36, 37]. These antibodies were used for three-dimensional image localization of xyloglucans in the cell walls of tobacco (*Nicotiana tabacum* L.) suspension culture by coupling rapid-freezing-deep-etching (RFDE) with immunogold labeling in electron microscopy [38]. This technique shows an interesting potential for three-dimensional images of the respective localization of various cell wall polymers.

The monoclonal antibody strategy has also been implemented for xyloglucan study in the cell walls [39]. The monoclonal series raised against RG-I [23] generated one group (group III) that bound more strongly to xyloglucan with specificity for terminal α-(1 → 2)-linked fucosyl residue [40].

**Secondary wall.** Immuno-microscopy of secondary wall polysaccharides mainly concerns xylan

and glucomannans. Most of the antibodies are polyclonal, raised against polymers and oligomers, usually conjugated to a carrier protein. One monoclonal (BGMC6) was obtained from a galactomannan-BSA conjugate and was shown to recognize  $\beta$ -(1  $\rightarrow$  4)-linked mannopyranosyl residues [41]. The antibody which had affinity to glucomannan and galactomannan was used for localizing galactomannans in the endosperm walls of normal coconut (*Cocos nucifera* L.) and a mutant at different developmental stages. A similar specificity for manno-oligosaccharides was demonstrated by a polyclonal antibody [42] that served for immunoelectron microscopic analysis of glucomannans in rice seed cell walls. A polyclonal antibody against a commercial 4-O-methyl-glucuronoxylan from birch has been prepared by Barry et al. [43] and used for studying the xylans in normal maize and bm3 mutants. To study the respective distribution of the highly substituted glucuronoarabinoxylans and low-branched xylans (modeled with xylopentaose) in *Zea mays*, Suzuki et al. [44] raised polyclonal antibodies against the two types of xylans.

Awano et al. [45] prepared an antibody against the glucuronoxylan from Japanese beech (*Fagus Crenata*) that they used for immunogold-silver enhancement in light microscopy and immunogold labeling in TEM. Xylan deposition in the secondary wall of *Fagus crenata* was examined in immunoelectron microscopy together with field emission scanning electron microscopy (FESEM). It was thus suggested that lignin deposition occurred simultaneously with xylan penetration [46].

To detect hemicellulosic  $\beta$ -(1  $\rightarrow$  3), (1  $\rightarrow$  4)-glucans which are unique to grasses, polyclonal antibodies were used [47]. More specificity was found for the monoclonal antibody BG 1 that showed strict specificity for the alternate sequence of (1  $\rightarrow$  3) - and (1  $\rightarrow$  4) linkages typical to cereal glucans [48]. An anti-callose probe [49] was an essential tool for studying the deposition of  $\beta$ -(1  $\rightarrow$  3) glucan in *Nicotiana glauca*.

**Recombinant antibody phage display technology.** High resolution electron microscopy associated with immunolabeling has provided interesting information about the fine architecture of plant cell walls. However, as mentioned previously, monoclonal antibodies against plant cell wall polysaccharides have been obtained from purified polysaccharides or structural elements of a polysaccharide, and also in several instances from complex ill-defined cell wall extracts [13]. In most cases, the epitopes recognized are not known or not precisely defined. These monoclonal are produced using the hybridoma technique. Recently, the novel technology of recombinant antibody phage display has emerged as an alternative to hybridoma technology for plant cell wall investigation. Immunoglobulin variable (V) genes are amplified from hybridomas using polymerase chain reaction [50]. The Fab genes are expressed as fusion proteins with a phage coat protein, and the expressed antibody fragment is assembled on the surface of the phage into which the corresponding DNA is packaged. This leads to construction of large and highly diverse combinatorial repertoires of antibody fragments from which the antigen-specific antibodies can be selected by affinity techniques [51]. This approach was implemented by Williams and collaborators [52] for production of an antibody Fab (CCRC-R<sub>1</sub>) that had specific recognition for RG II in suspension-cultured sycamore cells. Other technologies for phage display may be used that by-passes the immunization procedure, and allow tailoring of the affinity and specificity of the antibodies. These "single-pot" phage display approaches lead to construction of the so-called *naive* V-gene libraries and synthetic V-gene libraries, respectively [51]. From a naive phage display library, Willats and collaborators [53] generated antibodies binding specifically to a series of oligogalacturonides, highly representative of polygalacturonic acid since they required 30 de-esterified galacturonic acid residues for optimal binding. With the recognition of such a large epitope, these antibodies (PAM<sub>1</sub> and PAM<sub>2</sub>) are remarkable tools for identifying and localizing blocks of polysaccharides in the architecture of the cell wall. *An important step in the selection of the antibodies is the characterization of their recognition pattern by immuno-dot blot analysis, a technique that requires the use of a large number of pure and well-characterized epitope molecules.* This analysis was recently developed for the selection of the new monoclonal antibody LM 7 [54] that allowed to

show that pectin methyl esterases generate blockwise and non-blockwise distribution of methylated pectin in cell wall micro-domains [55].

The progress in production of antibodies together with the improvement in the characterization of the recognized epitopes are important for the development of immuno-microscopy. *The formidable potentiality of antibody phage display associated to micro-array selection of specificity represents a powerful advance. Although the technique takes time and efforts to construct an antibody repertoire and to isolate antibodies with precise binding affinity, it appears a most promising tool for a greater understanding of the molecular architecture of plant cell walls.*

## IMMUNOLABELING OF LIGNINS AND LIGNIN-LIKE COMPOUNDS

The structural diversity of lignins being related to their monomeric composition together with the type of linkages between monomer units, it is of prime importance to have markers that could specifically distinguish monomers and linkage types. This is crucial in many instances where lignin deposition must be visualized in relation to biosynthesis conditions, or whenever an accurate mapping of lignin distribution is needed that can depict cell wall topochemistry in terms of lignin structural characteristics in immature as well as mature tissues.

The first attempt to obtain an antibody against lignin was from a chemically extracted lignin preparation [56], but was not used for immunocytochemistry. In 1997, Kim and Koh [65] produced a polyclonal antibody from milled wood lignin (MWL) preparation from spruce (*Picea abies*) and could infer that MWL originated from secondary wall, in agreement with the results obtained by the radio-tracer method [66] contrary to the preceding suggestion by Lee et al. [67]. The first antibodies raised against totally pure lignin polymers were raised by Ruel *et al.* in 1994 [57] against synthetic dehydrogenative polymers (DHPs). The choice of DHPs as antigens, rather than extracted lignins was because there are no report of isolation of lignins totally devoid of contaminating carbohydrates. Therefore, synthetic polymers guarantee the chemical purity of the antigen [57]. The choice of polyclonal antibodies was to have multifunctional probes with the potentiality to recognize several structural features of the corresponding DHP antigens, that are also present in the natural lignin polymers (protolignin) [58]. The first antibodies were directed against DHPs of guaiacyl, *p*-hydroxyphenyl propane, and a mixed guaiacyl-syringyl polymer, respectively. The specificity of the polyclonal antibodies was difficult to assay by the conventional immunological tests that require solubilization of the antigen because of the lack of solubility of DHPs in solvents compatible with ELISA tests and other classical immunological tests. To overcome this difficulty an affinity test was developed in TEM allowing to assay insoluble compounds [34, 22]. This test may be of general utilization for all polymeric antigens to be assayed.

The specificities of the antibodies directed against DHPs not only were related to the monomeric units, but were also related to the inter-unit linkages, i.e., whether these are *non-condensed* ( $\beta$ -O-4 aryl-alkyl linkages) or *condensed* (essentially C-C linkages). This distinction arose from the chemical structure of the synthetic DHPs in which the frequencies of the type of inter-unit linkage varies according to the mode of polymerization *in vitro* [58].

In a study correlating FTIR,  $^{13}\text{C}$ -NMR CP/MAS spectroscopy and immunocytochemical localization of H, G and GS epitopes along a growing maize internode [22] the immunological probes were shown to have the potentiality of distinguishing between inter-unit substructures in terms of condensed *versus* non-condensed linkages. Such a discriminative ability of antibodies raised against model DHPs was further demonstrated in the study of transgenic tobacco plants [59] modified in their monolignol biosynthesis pathway [60].

**Table 2. Antibodies to localize lignin and phenolic components**

Target component	Antigen	Epitope	Mono-clonal	Poly-clonal	Reference
<b>Lignin</b>	MWL	unknown	-	yes	56
	MWL	unknown	-	yes	65
	DHPs	H units	-	yes	57
	"	G units	-	yes	57
	"	GS units	-	yes	57
	"	S units	-	yes	Ruel (unpublished)
	"	Condensed/Non-Condensed subunits	-	yes	22
<b>Z and E-Coniferyl alcohol</b>	E-Coniferyl alcohol		-	yes	70
	Z-Coniferyl alcohol		-	yes	70
<b>p-coumaric acid</b>	p-coumaroyl-BSA "	p-coumaric acid and phenolic acids	-	yes	71
<b>Ferulic acid</b>	Feruloyl-arabinose	Ferulic acid, p-coumaril acid, L-Arabinose	-	yes	88
<b>Dibenzodioxocin</b>	BSA-conjugate	Dibenzodioxocin structure	-	yes	68
<b>5-5 Biphenyl</b>	BSA conjugate	Biphenyl, G-ring	-	yes	69
Agatharesinol (Lignan)	BSA-conjugate	Agatharesinol	-	yes	72

The antiserum against non-condensed GS subunits was determinant for demonstrating *in situ* the early formation of lignin-like compounds in primary walls of maize coleoptiles [61], and to distinguish between suberin and lignin at different stages of development of primary roots of maize [62]. Differential lignification in the walls of fibers and vessels was clearly illustrated in maize, wheat straw and the wood from poplar and eucalyptus trees [63].

Antibodies against monomeric or dimeric units of lignin were also obtained. Two different antibodies against BSA-p-coumaric acid conjugates, one coupled *via* the carboxy group, the other attached to the aromatic ring, were produced [71]. Using a BSA-conjugate of agatharesinol, a norlignan from *Cryptomeria japonica*, Nagasaki et al. [72] produced polyclonal antibodies that were used for immunolocalization of the lignan epitope in light microscopy, showing its cell wall localization. The 8-membered dibenzodioxocin ring structure could be conclusively identified and localized by TEM-immunogold labeling in the xylem of young norway spruce soon after the onset of lignification in the primary and secondary wall layers [68]. Another dimer, dehydrodivanillin was coupled to bovine serum albumin to obtain a polyclonal antibody [69].

Through the various antibodies developed for immunolocalization *in muro* of lignin and phenolic acids, it appears that several levels of specificities have been obtained. The specificity could be related

to the distinction between constitutive monomers [71, 57] or for the stereochemical configuration Z and E of coniferyl alcohol [70], to the inter-unit linkages [22], to the chemical functionality on the sequence, e.g. homoguaiacyl *versus* mixed guaiacyl-syringyl [22]. All these different aspects related to specific affinity with respect to many essential structural features of lignin macromolecules constitute targets that should be further developed to permit establishing more complete topochemical characteristics of lignins in cell walls.

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