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The differences in cell wall composition in leaves and regenerating protoplasts of *Beta vulgaris* and *Nicotiana tabacum*

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Abstract

Cell wall composition in leaves and regenerating leaf-derived protoplasts was studied in *Beta vulgaris* L. and *Nicotiana tabacum* L. Several oligosaccharides that characterize arabinogalactan proteins (AGPs) and pectins were visualized *in situ* by a series of immunocytochemical reactions. The most conspicuous difference between the two species involved the expression of AGP epitopes that bind LM2 and MAC207 in only sugar beet cells; both epitopes being characterized by the presence of glucuronic acid (GlcA). Moreover, *B. vulgaris* leaves and protoplast-derived cells showed lower content of pectin side-chains bearing (1→4)-β-D-galactose residues as compared to *N. tabacum*.

Additional key words: arabinogalactan proteins, pectins, sugar beet, tobacco.

Introduction

The successful application of protoplasts in plant improvement programs requires an efficient and reproducible method of protoplast-to-plant regeneration. Numerous studies are being conducted targeting identification of genotype-related factors responsible for the highly pronounced capacity for plant regeneration from protoplasts or single cells (Kwon *et al*. 2005). Among the factors thought to be involved in this process, the cell wall polysaccharides and their molecular organization are of a special focus (Majewska-Sawka and Münster 2003, Malinowski and Filipecki 2002).

We have compared cell wall components of two plants, which display very different responses to *in vitro* conditions. Sugar beet (*Beta vulgaris* L.) is considered a recalcitrant species in terms of new plant regeneration from mesophyll protoplasts and cells (Bhat *et al*. 1986, Jażdżewska *et al*. 2000, Krens *et al*. 1990, Lenzner *et al*. 1995); however, it shows an improved ability to form new plants when guard-cell-derived protoplasts are used (Hall *et al*. 1995, Wiśniewska and Majewska-Sawka 2007). The plating efficiency of sugar beet mesophyll protoplasts was proved to range between 0.005 % and 1.12 % depending on the genotype and the culture conditions used, whereas shoot organogenesis was observed for 1 - 20 % of calli (Jażdżewska *et al*. 2000, Krens *et al*. 1990, Lenzner *et al*. 1995). The plating efficiency of guard cell protoplasts (GCPs) has been estimated to be as high as 50 - 60 %, whereas regeneration frequency ranged between 1 - 10 %, depending on the genotype (Hall *et al*. 1997, Krens *et al*. 1998, Wiśniewska and Majewska-Sawka 2007). In contrast to sugar beet, the protoplasts of tobacco (*Nicotiana tabacum* L.) develop new shoots very easily. The process occurs with almost 100 % efficiency, regardless of the protoplast type (Wiśniewska 2006).

To characterize similarities and differences between the two species and between mesophyll and guard cells, we studied the composition and structural organization of their cell walls by immunocytochemical detection of specific oligosaccharidic epitopes. The presence and distribution of pectin and arbinogalactan protein (AGP) structural motives were examined within donor leaf tissues and in protoplasts that were isolated from mesophyll and guard cells. These studies provide insight related to basic compositional differences in the cell walls of *B. vulgaris* and *N. tabacum.*

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Abbreviations: AGP - arabinogalactan proteins; Gal - galactose; GC - guard cells; GCP - guard cell protoplasts; GlcA - glucuronic acid; MC - mesophyll cells; MCP - mesophyll cell protoplasts; MES - 2-(N-morpholino)ethanesulfonic acid.

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Materials and methods

Aseptic shoot cultures of sugar beet (*Beta vulgaris* L. line 0170) and tobacco (*Nicotiana tabacum* L. cv. Bursan), were used as donor material for protoplast isolation. Leaves were excised two weeks after shoot subculture and then subjected to preplasmolysis in salt solution (Frearson *et al*. 1973) with 0.45 M sorbitol to enable mechanical separation of the epidermis from the abaxial side.

Both types of tissues were digested in enzyme solutions for 8 - 12 h, with gentle shaking on a rotary shaker (20 rpm) at 26 °C in darkness. Guard cell protoplasts of sugar beet were obtained by digestion in solution composed of 1.9 % cellulase RS, 0.1 % macerozyme R-10 (both from *Yakult Honsha Co*., Tokyo, Japan) and 0.003 % pectolyase Y-23 (*Seishin Pharmceutical Co.,* Tokyo, Japan) dissolved in 0.45 M sorbitol solution supplemented with 20 mM 2-(N-morpho-lino)ethane-sulfonic acid (MES), and macro- and micro-elements according to Frearson *et al*. (1973). In case of tobacco guard cells the enzyme contained 2.0 % cellulase RS, 0.07 % macerozyme R-10, 0.003 % pectolyase Y-23 and 30 mM MES.

For mesophyll protoplasts isolation the solution contained 0.5 % cellulase R-10 and 0.4 % macerozyme R-10 (sugar beet), and 1.0 % cellulase R-10 and 1.5 % macerozyme (tobacco) dissolved in 0.45 M sorbitol solution containing 30 mM MES and salts of Frearson *et al*. (1973).

The guard cell-derived and mesophyll-derived protoplasts were purified by filtration through a nylon mesh (20 μm and 60 μm, respectively) followed by three washings in salt solution (Frearson *et al*. 1973) with repeated centrifugations. The protoplast pellets were resuspended in salt solution and mixed with 2.4 % calcium alginate (1:1), both containing 0.45 M sorbitol. After the polymerization of alginate beads, they were transferred to liquid Murashige and Skoog (1962; MS) medium supplemented with 2 μM BAP, 5 μM NAA, 100 μM n-propyl gallate, and sucrose (0.35 M for sugar beet guard cell protoplasts and 0.45 M for tobacco guard cell protoplasts and for mesophyll protoplasts of both species). The cultures were kept on the rotary shaker (20 rpm) at 26 °C in darkness for three-four weeks.

Leaves from donor shoot cultures were cut into fragments of *ca*. 0.25 cm^2 and fixed in a mixture of 4 % paraformaldehyde and 0.25 % glutaraldehyde in 0.05 M Pipes buffer, pH 7.2, for 24 h. Protoplast-derived microcalli were fixed in a solution of 4 % paraform-aldehyde, 1.5 - 2.0 % glutaraldehyde, and 0.15 M sodium citrate in 0.01 M phosphate buffered saline (PBS), pH 7.2, for 2 - 3 h. Then, all materials were dehydrated in ethanol series and embedded in *LR Gold* resin in gelatin capsules. After the resin had polymerized, the tissues were cut with an *RM 2155* microtome (*Leica Microsystems*, Nussloch, Germany) into 0.5-μm sections, which were collected on poly-L-lysine-coated glass slides and dried on the worm plate.

The tissues were used for *in situ* detection of selected oligosaccharidic antigens within newly formed walls of calli derived from guard cell or mesophyll protoplasts of both species. LM2, MAC207, JIM4, JIM8, JIM13 and JIM15 antibodies were used to detect AGPs epitopes, and JIM5, JIM7, LM5, LM6 and CCRC-M2 antibodies to detect pectin epitopes. The antibodies were characterized previously (Yates *et al*. 1996, Majewska-Sawka *et al*. 2004) and the immunocytochemical reactions were performed as described by Majewska-Sawka *et al*. (2004).

Results

The presence, spatial distribution, and relative abundance of several sugar epitopes characteristic for AGPs and pectins have been determined for donor leaves, as well as for regenerating guard cell- and mesophyll-derived protoplasts of *B. vulgaris* and *N. tabacum* (Fig. 1*A-F*, Table 1). Plant species studied display extremely different capacities for regenerating new plants in the course of *in vitro* culture.

Comparison of the species revealed that both similarities and differences exist between them in terms of cell wall composition. The similarities involve the widespread occurrence of epitopes recognized by anti-AGP JIM13 antibody and two pectic motives recognized by JIM7 and LM6 in leaf tissues, as well as in regenerating protoplasts and cells of beet and tobacco; only mesophyll protoplasts of sugar beet showed relatively lower pectin content (Figs. 2*A-G*, Table 1). Antigens binding JIM5 are present only in trace amounts, and those reacting with JIM4, JIM15, and CCRC-M2 antibodies could not be localized either in the leaves or in the protoplasts of both species examined (Table 1).

Observed differences between the cell wall composition of sugar beet and tobacco could be classified into two groups: *1*) the first involves epitopes present in sugar beet cells and absent or scarcely represented in tobacco cells, and *2*) the second involves epitopes expressed in both species but not equally abundant.

The first group concerns AGP epitopes that react with MAC207 and LM2 antibodies, which could be detected in high levels in all leaf tissues, as well as in both protoplast and cell types of *Beta* (Fig. 3*A*,*C*,*E,* Table 1). Conversely, no MAC207 (Fig. 3*B*,*D*,*F*) and only trace amounts of LM2-responding antigens (Table 1) could be localized in tobacco cells. The expression of epitopes recognized by JIM8 could only be identified in guard cells within the beet leaf tissues and, in relatively small amounts, in guard- and mesophyll protoplast-derived cells. In contrast, no JIM8 epitope could be found in

N. tabacum (Table 1).

The second group is represented by epitopes characteristic for pectin side chains bearing (1→4)-β-D-galactose residues. They could be detected only in trace amounts in guard cells of beet leaf (Fig. 4*A*) and in protoplast-derived cells (Fig. 4*C*,*E*), whereas they were conspicuously more abundant in all tobacco donor cells and protoplasts-derived cells (Fig. 4*B*,*D*,*F*). Control reactions performed by omitting the primary antibodies resulted in a complete lack of fluorescent signal (Fig. 2*G*).

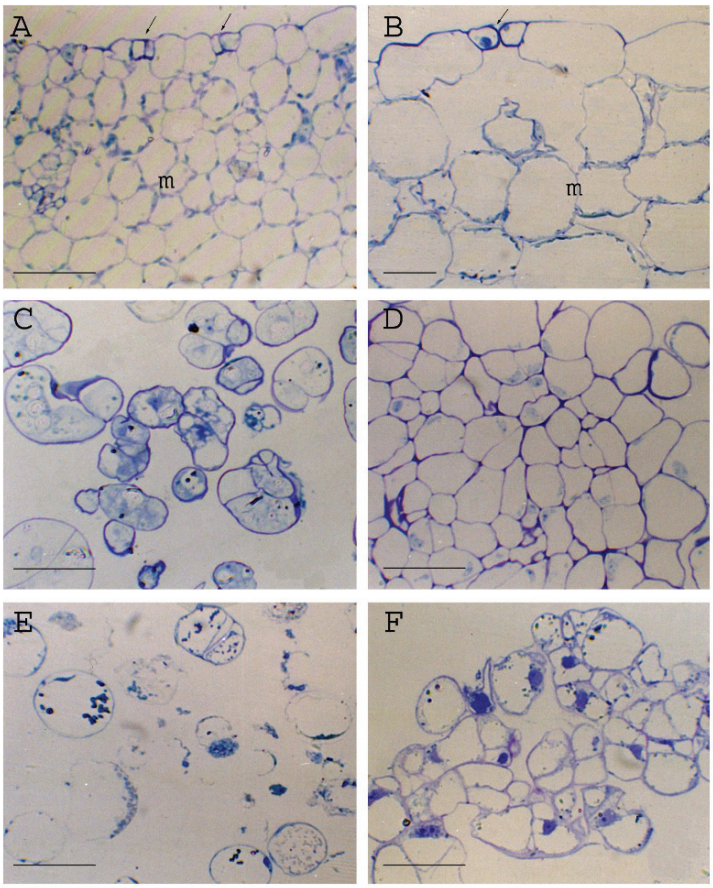

Fig. 1*A*-*F*. Morphology of sugar beet (*A*,*C*,*E*) and tobacco cells (*B*,*D*,*F*) stained with toluidine blue and Azur II. Leaf sections (*A*,*B*), guard protoplast-derived cells (*C*,*D*), and mesophyll protoplast-derived cells (*E*,*F*). Mesophyll cells (*m*), guard cells (→). *Bars* = 50 μm.

Table 1. The presence and relative abundance of sugar epitopes in leaves and protoplast-derived cells of sugar beet and tobacco. GC - guard cells, MC - mesophyll cells, GCP - guard cell protoplasts, MCP - mesophyll cell protoplasts. (np - antigen not present, tr - antigen present in trace amounts, + - antigen present, ++ - antigen present in abundance)

Antibody	Dilution	Sugar beet				Tobacco			
		GC	MC	GCP	MCP	GC	MC	GCP	MCP
JIM7	1:20	++	++	++	+	++	++	++	++
JIM5	1:5	tr	np	tr	np	tr	np	tr	np
LM6	1:5	++	++	++	+	++	++	++	++
LM5	1:5	+	++	+	tr	++	++	++	++
CCRC-M2	1:5	np	np	np	np	np	np	np	np
LM2	1:5	++	++	+	+	tr	tr	tr	tr
MAC207	1:5	++	++	++	++	np	np	np	np
JIM4	1:5	np	np	np	np	np	np	np	np
JIM8	1:5	tr	np	tr	np	np	np	np	np
JIM13	1:5	++	++	++	++	++	++	++	++
JIM15	1:5	np	np	np	np	np	np	np	np

Discussion

The detailed examination of cell walls deposited by regenerating guard cell-derived and mesophyll-derived protoplasts of sugar beet and tobacco revealed several important features that may reflect species-specific differences. Two protoplast types have been involved in studies with the aim to establish: *1*) whether the same protoplast/cell types from two different species are characterized by the presence of the same cell wall structural domains, which would suggest its cell type-specific feature; or *2*) whether the same protoplast/cell types from two different species are characterized by the presence of different structural domains, which would indicate its species-specific feature, possibly related to *in vitro* response.

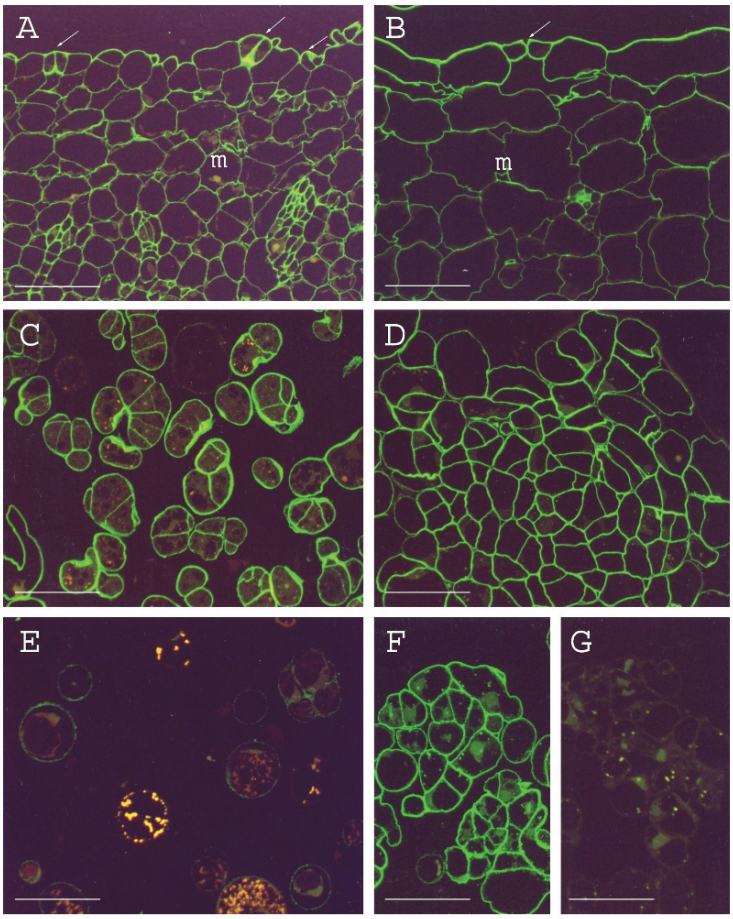

Fig. 2*A*-G. Immunolabeling of sugar beet (*A*,*C*,*E*) and tobacco cells (*B*,*D*,*F*,*G*) with JIM7 antibody resulted in strong signal in almost all cells examined; only mesophyll protoplasts of sugar beet showed trace content of pectins. Control reaction resulted in the lack of the signal (*G*). Leaf sections (*A*,*B*), guard protoplast-derived cells (*C*,*D*), and mesophyll protoplast-derived cells (*E*,*F*,*G*). Mesophyll cells (*m*), guard cells (→). *Bars* = 50 μm.

The most outstanding difference is the presence and widespread distribution of two AGP epitopes (MAC207 and LM2) in all sugar beet cell types examined and, in contrast, a complete absence of MAC207 and traces of LM2-binding epitopes in tobacco cells.

The noteworthy feature of both epitopes is the presence of *β*-D-Glc*p*A residues (Yates *et al*. 1996). Several authors have demonstrated the significance of uronic acids in plant morphogenesis. Mutant callus - *nolac-H18* of *Nicotiana plumbaginifolia* deficient in the glucoronosyltransferase gene (Iwai *et al*. 2002) exhibited reduced GlcA levels within the rhamnogalacturonan II polysaccharide, weakened intercellular attachments, and an inability to form shoots. Similarly, *Arabidopsis Fragile Fiber8* (*fra8*), which encodes a member of glycosyltransferase family 47, showed a defect in the addition of glucuronic acid residues into xylans, and consequently in biosynthesis of secondary cell wall (Zhong *et al*. 2005). Mutation of a family 8 glycosyl-transferase in the *Arabidopsis* genome also caused changes in cell wall sugar composition resulting in a dwarf phenotype (Lao *et al*. 2003).

Arabinogalactan proteins have been previously shown to play an important role in protoplast regeneration of both *B. vulgaris* and *N. tabacum*, as evidenced by very early synthesis and deposition of these compounds within newly formed walls (Butowt *et al*. 1999, Majewska-Sawka and Münster 2003); in addition, there is a cessation of growth in response to the addition of anti-AGP antibodies or Yariv reagent to the culture media (Butowt *et al*. 1999, Vissenberg *et al*. 2001). MAC207 and LM2-binding epitopes, both being differentially expressed in two species studied, are undoubtedly involved in plant morphogenesis, as evidenced previously for processes occurring both *in planta* and *in vitro* (Pennell and Roberts 1990, Showalter 2001). In some cases cell differentiation is accompanied by disappearance of definite epitope(s) (Pennell and Roberts 1990), whereas in other models described so far, cell specialization occurs concomitantly with the enhanced expression of the same AGP epitope(s). The presence of LM2-binding motives has been frequently correlated with the embryogenic potential of callus tissue and formation of embryos (Borderies *et al*. 2004, Chapman *et al*. 2002a,b, Saare-Surminski *et al*. 2000). It is noteworthy, however, that the same epitope could also be found in vegetative organs, such as roots (Showalter 2001). In *Beta vulgaris*, LM2 and MAC207 epitopes were abundantly present in all cell types studied, regardless of their morphogenetic properties. This observation provides an additional argument for the discussion on the mechanisms involved in AGPs biological function. It supports the previous results, according to which the presence of particular structural motif cannot be considered as the only factor responsible for cell differentiation, but some other criteria are also important, *e.g*. the conformational structure of polysaccharide molecules that contain definite epitope, as well as the presence and composition of AGPs protein core (Shawalter 2001).

Another result of our studies provides evidence of the differences in relative content of (1→4)-β-D galactose residues in the sugar beet and tobacco cells, extremely abundant in the latter species. Several lines of evidence suggest that pectins are responsible for the strength of intercellular attachments, which in turn might be related

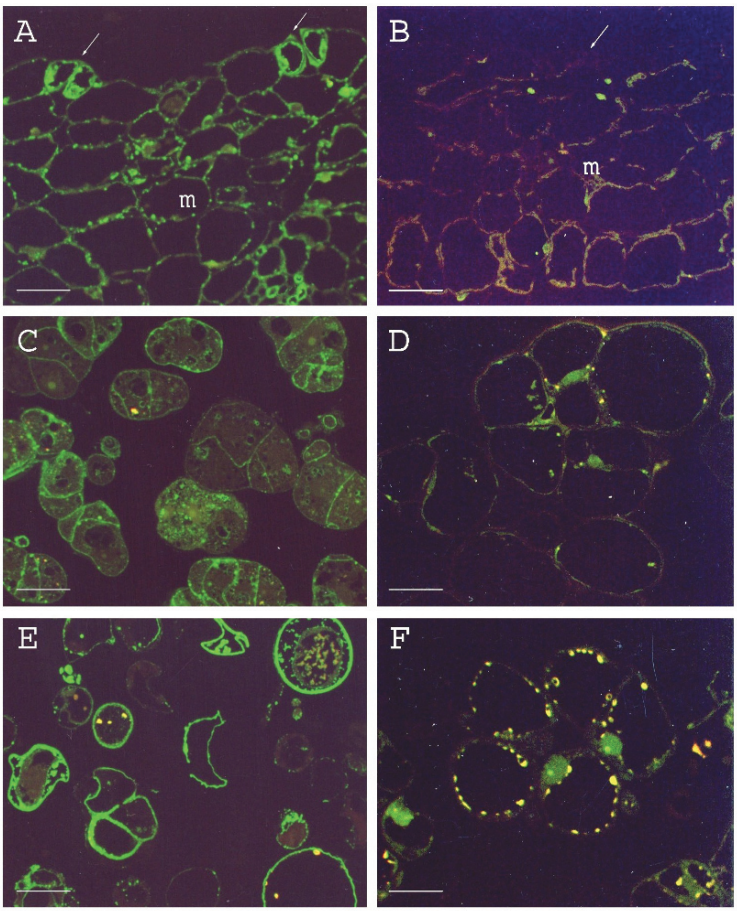

Fig. 3*A*-*F*. Immunolabeling of sugar beet (*A*,*C*,*E*) and tobacco cells (*B*,*D*,*F*) with MAC207 antibody resulted in strong labeling of all beet cells, whereas no signal could be detected in tobacco leaves and protoplast-derived cells. Leaf sections (*A*,*B*), guard protoplast-derived cells (*C*,*D*), and mesophyll protoplast-derived cells (*E*,*F*). Mesophyll cells (*m*), guard cells (→). *Bars* = 50 μm.

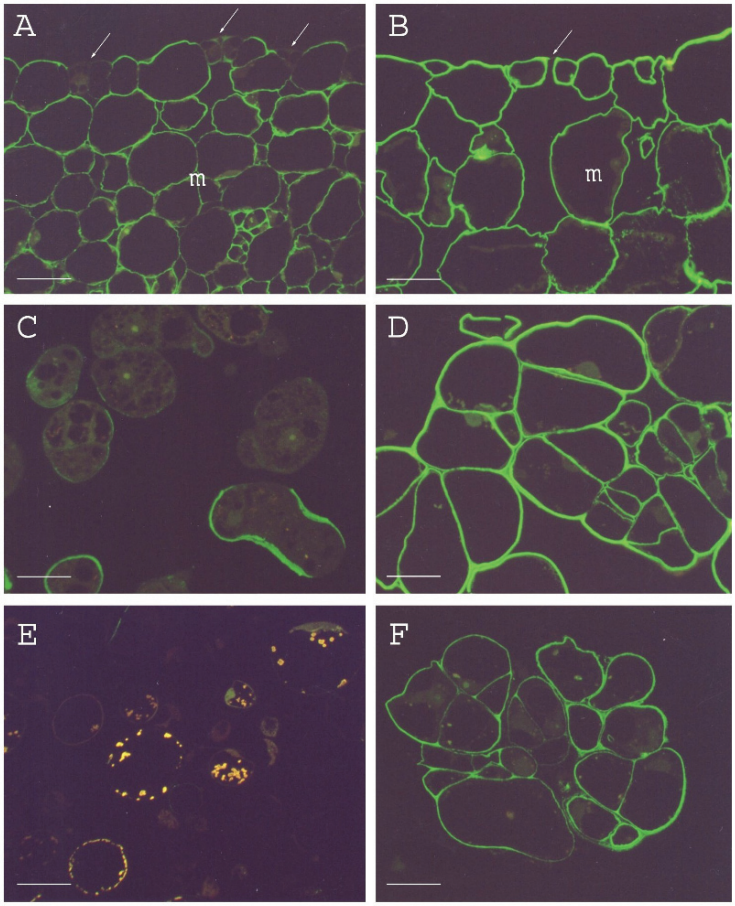

Fig. 4*A-F*. Immunolabeling of sugar beet (*A*,*C*,*E*) and tobacco cells (*B*,*D*,*F*) with LM5 antibody produced strong labeling of cells within the leaves of both species (except guard cells in beet, *arrows*), and also of guard protoplast- and mesophyll protoplast-derived cells of tobacco. Regenerating cells of beet show only weak signals. Leaf sections (*A*,*B*), guard protoplast-derived cells (*C*,*D*), and mesophyll protoplast-derived cells (*E*,*F*). Mesophyll cells (*m*), guard cells (→). *Bars* = 50 μm.

to tissue compactness and morphogenetic properties (Bouton *et al*. 2002, Brummell *et al*. 2004, Liners *et al*. 1994, Malinowski and Filipecki 2002, Saher *et al*. 2005, Sobry *et al*. 2005). Similarly, the structure of other cell wall oligosaccharides has been shown to determine their biological activity in terms of cells or organs growth (Kollárová *et al*. 2006).

The synthesis and deposition of galactose-rich pectin chains affect galactose:arabinose ratio in cell walls, and in consequence may influence greatly plant morphogenesis. This supposition seems to be supported by analyses of mutants or transgenic plants that display disturbances in pectin biosynthesis, secretion, degradation, or subcellular localization (Brummell *et al*. 2004, Oomen *et al*. 2002, Shevell *et al*. 2000, Vanzin *et al*. 2002).

Galactose-rich pectin side-chains have mainly be found in differentiating cells (McCartney *et al*. 2000, 2003, Willats *et al*. 1999). In *Arabidopsis* suspension cells, the change in cell development from the proliferation into the elongation phase is accompanied by degradation of (1→4)-β-D-galactan and a decrease in cell adhesion (Leboeuf *et al*. 2004). The results of our studies are consistent with the latter observation; we found that galactose-rich epitopes are poorly represented in sugar beet guard cells and in both types of protoplast-derived cells, but are extremely abundant in all tobacco cells that easily differentiate and form new organs when cultured *in vitro*.

Our results and those presented by other authors suggest that the amount of GlcA and Gal, along with the structural involvement of both residues in specific cell wall polysaccharidic domains, might be factors playing an important role in the developmental pathway of cells and their morphogenetic properties *in planta* and *in vitro*. Future verification of this hypothesis will involve studies of the activity of several enzymes of the glycosyltransferase family in sugar beet and tobacco tissues.

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