

# Wall Ingrowths in Epidermal Transfer Cells of *Vicia faba* Cotyledons are Modified Primary Walls Marked by Localized Accumulations of Arabinogalactan Proteins

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Despite the importance of transfer cells in enhancing nutrient transport in plants, little is known about how deposition of the complex morphology of their wall ingrowths is regulated. We probed thin sections of mature cotyledon epidermal transfer cells of *Vicia faba* with affinity probes and antibodies specific to polysaccharides and glycoproteins, to determine the distribution of these components in their walls. Walls of these transfer cells consist of the pre-existing primary wall, a uniformly deposited wall layer and wall ingrowths which are comprised of two regions; an electron-opaque inner region and an electron-translucent outer region. The primary wall reacted strongly with antibodies against esterified pectin, xyloglucan, the side chains of rhamnogalacturonan-1 and a cellulase–gold affinity probe. The electron-opaque inner region of wall ingrowths displayed a similar labeling pattern to that of the primary wall, showing strong cross-reactivity with all antibodies tested, except those reacting against highly de-esterified pectins. The electron-opaque outer layer of developmentally more mature wall ingrowths reacted strongly with anti-callose monoclonal and polyclonal antibodies, but showed no reaction for pectin or xyloglucan antibodies or the cellulase–gold affinity probe. The plasma membrane–wall interface was labeled strongly with anti-arabinogalactan protein (AGP) antibodies, with some AGP-reactive antibodies also labeling the electron-translucent zone. Nascent wall ingrowths were labeled specifically with AGPs but not anti-callose. A reduction in wall ingrowth density was observed when developing transfer cells were exposed to  $\beta$ -D-glucosyl Yariv reagent compared with controls. Our results indicate that wall ingrowths of transfer cells are primary wall-like in composition and probably require AGPs for localized deposition.

**Keywords:** Arabinogalactan proteins — Cell wall — Immunocytochemistry — Transfer cells — *Vicia faba* — Wall ingrowths.

Abbreviations: AGP, arabinogalactan protein; BSA, bovine serum albumin;  $\alpha$ GalY,  $\alpha$ -D-galactosyl Yariv reagent;  $\beta$ GleY,  $\beta$ -D-glucosyl Yariv reagent; GPI, glycosylphosphatidylinositol;

PBS, phosphate-buffered saline; RG, rhamnogalacturonan; SEM, scanning electron microscopy.

## Introduction

Transfer cells are frequently located at sites of intensive membrane transport of nutrients (Gunning and Pate 1969, Offler et al. 2003). The unique feature of these anatomically specialized cells is the presence of intricately invaginated wall ingrowths, the contours of which are followed by plasma membrane enriched in transporter proteins (Harrington et al. 1997). The increased surface area of plasma membrane, and concentration of transporter proteins, facilitates elevated rates of nutrient transport across the symplasmic–apoplasmic interface (Patrick and Offler 1995, Patrick 1997). The physiological relevance of such enhanced nutrient transport has been demonstrated in various seed models where an extensive body of structural (Offler and Patrick 1993), physiological (McDonald et al. 1996, Harrington et al. 1997) and molecular evidence (Tegeeder et al. 1999, Tegeeder et al. 2000) indicates that the development of transfer cells plays a pivotal regulatory role in maintaining seed growth rates during their development.

In developing seeds of *Vicia faba*, the abaxial epidermal cells of cotyledons trans-differentiate to form transfer cells. This trans-differentiation first involves deposition of a thin, uniform layer of wall material across the outer periclinal wall of the epidermal cells (Offler et al. 1997, Farley et al. 2000). Wall ingrowths emerge from this wall layer first as small papillate structures at discrete loci (Offler et al. 1997, Talbot et al. 2001). These papillate ingrowths then branch laterally and coalesce with neighboring ingrowths eventually to form a multilayered fenestrated network (Talbot et al. 2001). This so-called ‘reticulate’ (Talbot et al. 2001) pattern of wall ingrowth formation occurs in different transfer cell types, such as transfer cells in leaf minor veins (e.g. Haritatos et al. 2000) and nucellar projection transfer

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cells in wheat (Wang et al. 1994), and occurs in both monocotyledon and dicotyledon species (Talbot et al. 2002).

Whereas the physiological significance of transfer cells in plant growth and development is now widely realized (Patrick 1997), relatively little is known about the composition and ontogeny of wall ingrowths. Gunning and Pate (1969) speculated that wall ingrowths are highly pectinaeous and that this feature might impact on the transport properties of the ingrowths. DeWitt et al. (1999), however, concluded on the basis of comparative compositional analysis of maize endosperm cell lines that there were no major differences in the cellulose-, hemicellulose- or pectin-enriched fractions of transfer cell wall ingrowths vs. archetypal cell walls. Based on this analysis, DeWitt et al. (1999) suggested that major changes in wall morphology are possible in the absence of substantial differences in wall composition. These authors speculated that subtle changes in the organization of minor wall components may be sufficient to generate major differences in wall morphology, via the 'self-assembly' of wall polymers (DeWitt et al. 1999). Some support for this possibility is provided by observations of callose-rich domains at the base of wall ingrowths in young, but not mature, transfer cells of pea nodules (Dahiya and Brewin 2000).

Self-assembly, or interactions between wall polymers that influence wall morphology (Jarvis 1992), is suspected to play a role in regulating localized wall deposition in other cell types, such as xylem elements. In xylem, secondary wall deposition is partly regulated by a 'self-perpetuating cascade', in which localized cellulose deposition provides a framework for the localization of other cell wall components (Taylor et al. 1992, Taylor and Haigler 1993). Arabinogalactan proteins (AGPs), proteoglycans found in both the plasma membrane and cell wall (Nothnagel 1997), have also been implicated in coordinating cell wall deposition (Roy et al. 1998). Recently, immunolabeling of an AGP (PtaAGP6) was found to be associated with secondary wall deposition in xylem elements of *Arabidopsis thaliana* (Zhang et al. 2003).

As part of an ongoing study designed to understand regulatory processes involved in transfer cell wall ingrowth deposition, we have investigated the spatial and temporal organization of wall components in epidermal transfer cells of *V. faba* cotyledons using immunogold electron microscopy and a suite of antibodies and probes. We confirmed that the inner wall ingrowth region is similar in composition to the underlying primary wall, while the outer region is unique in composition. AGPs were predominantly localized at the interface between the plasma membrane and outer region, while callose was found within this region. Emerging wall ingrowths in younger transfer cells were distinguished by accumulation of AGPs at the tip of the papillate ingrowth, but little or no deposition of callose.

These findings, along with localization of cellulose, hemicellulose and pectic components, and results using an inhibitor of AGP function, are discussed in terms of possible mechanisms both for initiating sites of wall ingrowth emergence and for establishing wall ingrowth morphology.

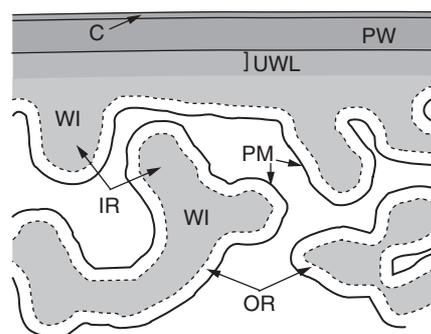
## Results

### *General structure of wall ingrowths*

Visualization of wall ingrowths by transmission electron microscopy (TEM) showed that they are comprised of two structurally distinct regions: an outer, more electron-translucent region, and an inner, more electron-opaque region resembling the density of the primary wall. A schematic diagram of a developing wall ingrowth labyrinth illustrating these features is shown in Fig. 1.

### *Immunocytochemical characterization of mature wall ingrowth labyrinths in abaxial epidermal cells*

Both primary walls and wall ingrowths reacted with each of the polyclonal and monoclonal antibodies used, although the structures labeled and the density of labeling varied between antibodies/probes (Table 1). Labeling density data (Table 1) indicate specific differences in composition between regions of wall ingrowths and the primary wall. Two basic labeling patterns were observed: antibodies labeling the primary wall and the electron-opaque interior region of wall ingrowths (Fig. 2), and those exclusively recognizing the plasma membrane interface and



**Fig. 1** Diagram illustrating wall layers and regions of wall ingrowths in abaxial epidermal transfer cells of *V. faba* cotyledons. The outer periclinal primary wall (PW) is thin and uniform and is bordered on the outer surface by a thin cuticle (C). Upon induction of transfer cell differentiation, a thin, uniform wall layer (UWL) is deposited evenly across the surface of the outer periclinal wall. Wall ingrowths (WI) are first deposited as single, papillate projections arising from the surface of the UWL. These ingrowths branch and fuse to form a reticulate labyrinth. Wall ingrowths themselves are comprised of an electron-opaque inner region (IR) and an electron-translucent outer region (OR). The border between these regions is indicated by a dashed line. The plasma membrane (PM) is also shown.

**Table 1** Densities of immunogold labeling in wall layers and wall ingrowths of abaxial epidermal transfer cells of *V. faba* cotyledons and adaxial epidermal cells of cotyledons cultured for 24 h

Antibody/probe	Abaxial epidermal transfer cells			24 h-cultured adaxial epidermal cells	
	Primary wall	Wall ingrowth inner region	Wall ingrowth outer region	Primary wall	Wall ingrowth
<b>Pectins</b>					
JIM5	14 ± 3	2 ± 2	0	14 ± 3	0
JIM7	116 ± 7	111 ± 14	2 ± 2	52 ± 1	47 ± 3
PGA polyclonal	8 ± 2	1 ± 1	0	8 ± 2	0
CCRC-M2	47 ± 6	35 ± 6	0	39 ± 3	31 ± 3
LM5	39 ± 4	27 ± 6	0	33 ± 6	18 ± 3
LM6	42 ± 2	38 ± 6	0	41 ± 5	22 ± 4
<b>Xyloglucans and cellulose</b>					
CCRC-M1	37 ± 3	32 ± 6	0	38 ± 3	36 ± 4
Xyloglucan polyclonal	87 ± 7	79 ± 6	1 ± 1	47 ± 3	40 ± 6
Cellulase gold	69 ± 6	60 ± 8	0	47 ± 7	40 ± 8
<b>Callose</b>					
Monoclonal	3 ± 1	14 ± 8	49 ± 3	0.07 ± 0	0.03 ± 0.02
Polyclonal	0	2 ± 1	52 ± 4	0.11 ± 0.01	0.08 ± 0.02
<b>AGPs</b>					
PCBC3	6 ± 4	17 ± 6	83 ± 7	0.06 ± 0.03	14 ± 4
JIM8	0	0	21 ± 3	0.03 ± 0.01	14 ± 3
CCRC-M7	0	0	47 ± 6	0.11 ± 0.06	17 ± 2

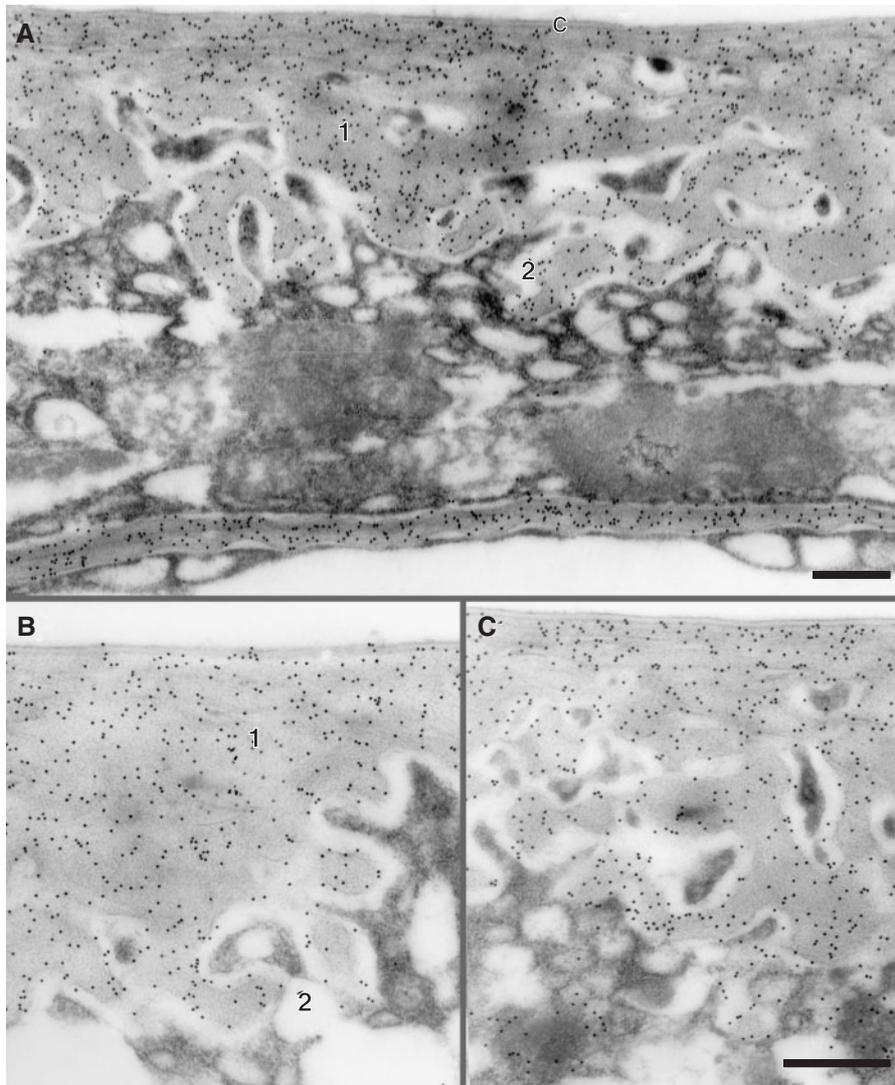
Data are densities of gold particles  $\mu\text{m}^{-2}$  ( $\pm\text{SE}$ ,  $n=3$ ) of the particular wall layer or region, calculated as described in Materials and Methods. Labeling of AGPs in abaxial epidermal cells is expressed as gold particles  $\mu\text{m}^{-1}$  ( $\pm\text{SE}$ ,  $n=3$ ), since labeling was restricted to the extreme periphery of the inner region of the wall ingrowth and also at the plasma membrane-wall ingrowth interface. In adaxial epidermal cells, AGP labeling is expressed as gold particles  $\mu\text{m}^{-1}$  ( $\pm\text{SE}$ ,  $n=3$ ) of plasma membrane covering either the periclinal wall or wall ingrowths protruding from the periclinal wall.

electron-translucent outer wall ingrowth region (Fig. 3). Antibodies to highly esterified pectins, xyloglucans, rhamnogalacturonan (RG)-1 side groups and the cellulase-gold affinity probe (Fig. 2 and Table 1) labeled the primary wall and electron-opaque interior region of wall ingrowths. Several monoclonals that recognize AGPs and antibodies that recognize callose labeled only the electron-translucent outer ingrowth region (Fig. 3 and Table 1). Each of these groups of wall components is discussed separately below.

**Pectins.** The JIM7 monoclonal antibody recognizing primarily esterified pectins strongly labeled both the primary wall and the electron-opaque inner wall ingrowth region (Fig. 2A). The density of labeling was similar between these two wall layers (Table 1). In contrast, the electron-translucent outer region of the ingrowth did not label either esterified (JIM7) or de-esterified (JIM5) pectins. Labeling patterns for esterified and primarily de-esterified pectin epitopes were mutually exclusive in the epidermal cell walls. Primarily de-esterified pectins recognized by JIM5 occurred only as a thin layer along the cuticle-wall interface (Fig. 3D). An identical pattern was obtained

(data not shown) using a polyclonal antiserum to PGA that recognizes highly de-esterified pectin epitopes (Moore et al. 1986). Both de-esterified pectin antibodies strongly labeled the middle lamellae in *V. faba* cotyledons (data not shown), as is typical of labeling with these antibodies in other species (Moore et al. 1986, Sabba et al. 1999).

The LM5 and LM6 antibodies recognize respectively the (1 → 4)- $\beta$ -galactan and (1 → 5)- $\alpha$ -arabinan residues of RG-I (Willats et al. 1999). Both antibodies (Fig. 3A for LM6, data not shown for LM5) labeled the primary wall and inner wall ingrowth regions, indicating that both RG-I modifications were present throughout the pectin fraction in these walls (Table 1). A slightly higher labeling density of the LM6 antibody compared with LM5 in the wall ingrowths relative to the primary wall may reflect the relative age of the ingrowths compared with the primary wall. Generally, LM6 labeling is associated with rapidly growing cell walls, whereas LM5 is associated with more mature tissues (e.g. Vaughn 2003). Neither of these antibodies labeled the middle lamellae (data not shown) or the outer region of the wall ingrowths (Table 1).

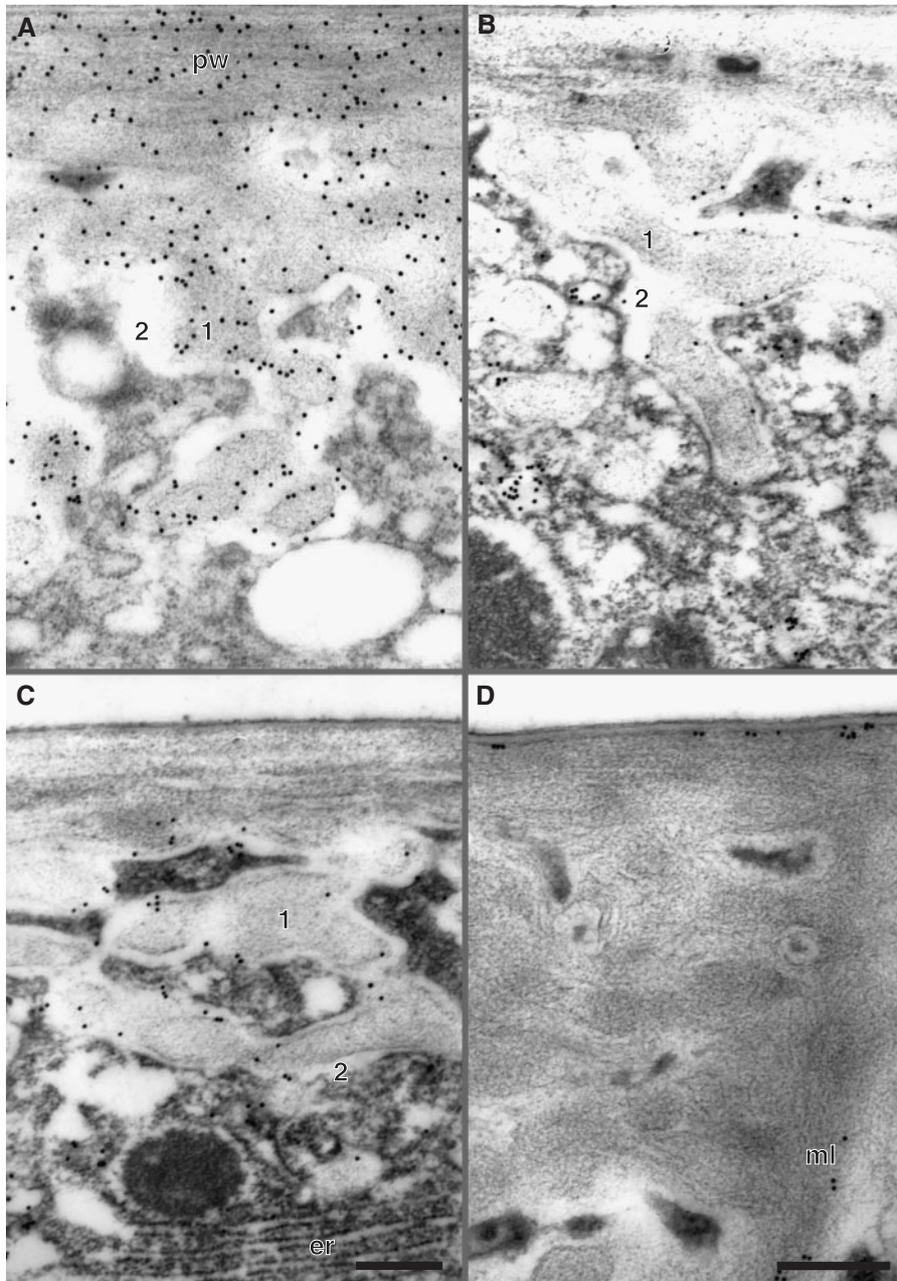


**Fig. 2** Immunogold and affinity gold labeling of mature wall ingrowths with (A) the primarily de-esterified homogalacturon antibody JIM7, (B) the cellulase-gold affinity probe and (C) the fucosylated xyloglucan monoclonal CCRCM-1. The electron-opaque inner region (1) and electron translucent outer region (2) of the wall ingrowths are indicated. Each of these antibodies/probes strongly labels the inner region of the wall ingrowths. c, cuticle. Bars = 500 nm (bar shown in C is for B and C).

*Cellulose and xyloglucans.* Cellulase-gold affinity probe labeled the primary wall, the uniform wall layer deposited prior to wall ingrowth deposition and the inner wall ingrowth region (Fig. 2B). A similar pattern was evident for fucosylated xyloglucan labeling with CCRC-M1 monoclonal (Fig. 2C) or anti-xyloglucan polyclonal serum (Table 1). All other walls in the epidermal cells, and walls of other cells of the cotyledon, were labeled with these antibodies, with the exception of plasmodesmata and middle lamellae areas (data not shown). This same labeling pattern has been observed in other species with these antibodies and probes (Freshour et al. 1996, Vaughn et al. 1996, Sabba et al. 1999, Vaughn 2003, Meloche et al. 2006). A similar pattern of cellulose labeling within the inner

region of the wall ingrowth was also observed (data not shown) using a cellulose-specific carbohydrate-binding module, CBM2a (McCartney et al. 2004).

*AGPs.* In contrast to the pattern of labeling described above, labeling with anti-AGP antibodies was associated exclusively with the electron-translucent outer region of wall ingrowths and plasma membrane. There were two distinctive labeling patterns for AGPs. For the group of monoclonal antibodies that recognize a variety of AGP epitopes (e.g. PCBC3 and CCRC-M7), labeling was present at the plasma membrane-wall interface and throughout the electron-translucent outer region of the ingrowth (e.g. Fig. 3B). The monoclonal JIM8 antibody recognizes more lipophilic AGPs (Gaspar et al. 2001), and in this case



**Fig. 3** Immunogold labeling of mature wall ingrowths with the (A) LM6, (B) CCRCM-7, (C) callose and (D) JIM5 monoclonal antibodies. (A) The LM6 antibody recognizes an arabinan side chain of RG-1 and strongly labels the pre-existing epidermal primary cell wall (pw) as well as the electron-opaque inner wall region (1) of the wall ingrowths. No label is present in the electron-translucent outer wall region (2). (B) Labeling of the outer wall region (2) of the wall ingrowths with the anti-AGP antibody CCRCM-7. No labeling is seen in the inner region (1) of the wall ingrowths. (C) Labeling of the outer region (2) of the wall ingrowths with antibodies to callose. No labeling of the inner region (1) is seen (er, endoplasmic reticulum). (D) JIM5 monoclonal antibody to highly de-esterified pectin labels only the area of the primary cell wall just beneath the cuticle and along the middle lamellae (ml). Bars = 500 nm (A–C are at the same magnification).

the label was more tightly associated with the plasma membrane (data not shown). Other wall surfaces in both epidermal and non-epidermal cells were either labeled sparsely or not at all with the AGP antibodies; labeling that was found was associated only with the plasma

membrane (data not shown). Although the CCRC-M7 antibody also recognizes the middle lamellae in some species (e.g. Freshour et al. 1996, but see Vaughn 2003, Ligrone et al. 2002), it did not label the middle lamellae in the *V. faba* epidermal transfer cells.

*Callose.* Both monoclonal antibodies (Meikle et al. 1991) and polyclonal serum (Northcote et al. 1989) have been used extensively to characterize the distribution of callose in plant cells (e.g. Northcote et al. 1989, Vaughn et al. 1996, Sabba et al. 1999). In our experiments, both antibodies strongly labeled the electron-translucent outer region of wall ingrowths, and labeling appeared to be distributed uniformly throughout this region (Fig. 3C). In addition to this labeling pattern, electron-translucent wall areas surrounding plasmodesmata were also strongly labeled with both antisera (data not shown).

#### *Analysis of developing wall ingrowths*

Functional transfer cells can be induced to form in adaxial epidermal cells of *V. faba* cotyledons cultured on agar plates containing various sugars (Offler et al. 1997, Farley et al. 2000). Since wall ingrowth morphology and ontogeny in these induced transfer cells are similar to those in abaxial epidermal cells (Talbot et al. 2001), induced transfer cells have been used to investigate the composition of wall ingrowths during early stages of their development. To compare wall composition in mature vs. developing wall ingrowths, adaxial epidermal cells of cotyledons cultured for 24 h were probed with the same suite of antibodies used for the abaxial epidermal transfer cells. Although the densities of immunogold labeling were reduced for some antibodies in nascent wall ingrowths compared with the more mature ingrowths in abaxial epidermal transfer cells (see Table 1), the pattern of esterified pectin (Fig. 4A), xyloglucan (Fig. 4B) and cellulase-gold (Fig. 4C) labeling in both the primary wall and inner region of nascent wall ingrowths was similar to that for mature wall ingrowths. A notable difference was reduced labeling of JIM7 in nascent wall ingrowths compared with mature wall ingrowths (Table 1). Labeling of highly de-esterified pectins was restricted to the cell wall-cuticle interface, as was observed in mature wall ingrowths (data not shown; see Table 1). The cellulose-specific CBM2a probe (McCartney et al. 2004) labeled the inner region of wall ingrowths in a similar manner to the cellulase-gold probe (data not shown).

Interestingly, analysis of the quantitative data presented in Table 1 shows that the relative composition of wall ingrowths changes across their development. Wall ingrowths induced in adaxial epidermal cells of 24 h cultured cotyledons displayed higher relative percentages (of total immunoreactive wall content excluding AGPs) of cellulose (~2%) and xyloglucans (~6%) than the underlying walls. Furthermore, relative to the underlying primary wall in each case, LM5- and LM6-reactive pectins were less abundant in developing wall ingrowths of adaxial epidermal cells compared with the inner region of the more mature wall ingrowths in abaxial epidermal

cells (Table 1). These data demonstrate that the relative levels of pectins increase as wall ingrowths mature.

#### *AGPs but not callose are associated with emerging wall ingrowths*

Antibodies recognizing AGPs, such as CCRC-M7 (Fig. 4D, E) and PCBC3 (Fig. 4F), strongly labeled the plasma membrane-wall interface of nascent wall ingrowths. The localization of AGPs to the plasma membrane and outer region associated with emerging wall ingrowths was specific, as indicated by the >150-fold increase in gold particles per  $\mu\text{m}$  of plasma membrane surrounding a wall ingrowth compared with adjacent regions of plasma membrane between ingrowths, scored for each anti-AGP antibody (Table 1). Despite similarities of labeling between nascent and mature wall ingrowths for most of the polysaccharide components investigated, no callose labeling was detected in the outer region of nascent wall ingrowths (Table 1). However, plasmodesmatal collars in these same sections were labeled at densities similar to those with mature wall ingrowths (data not shown).

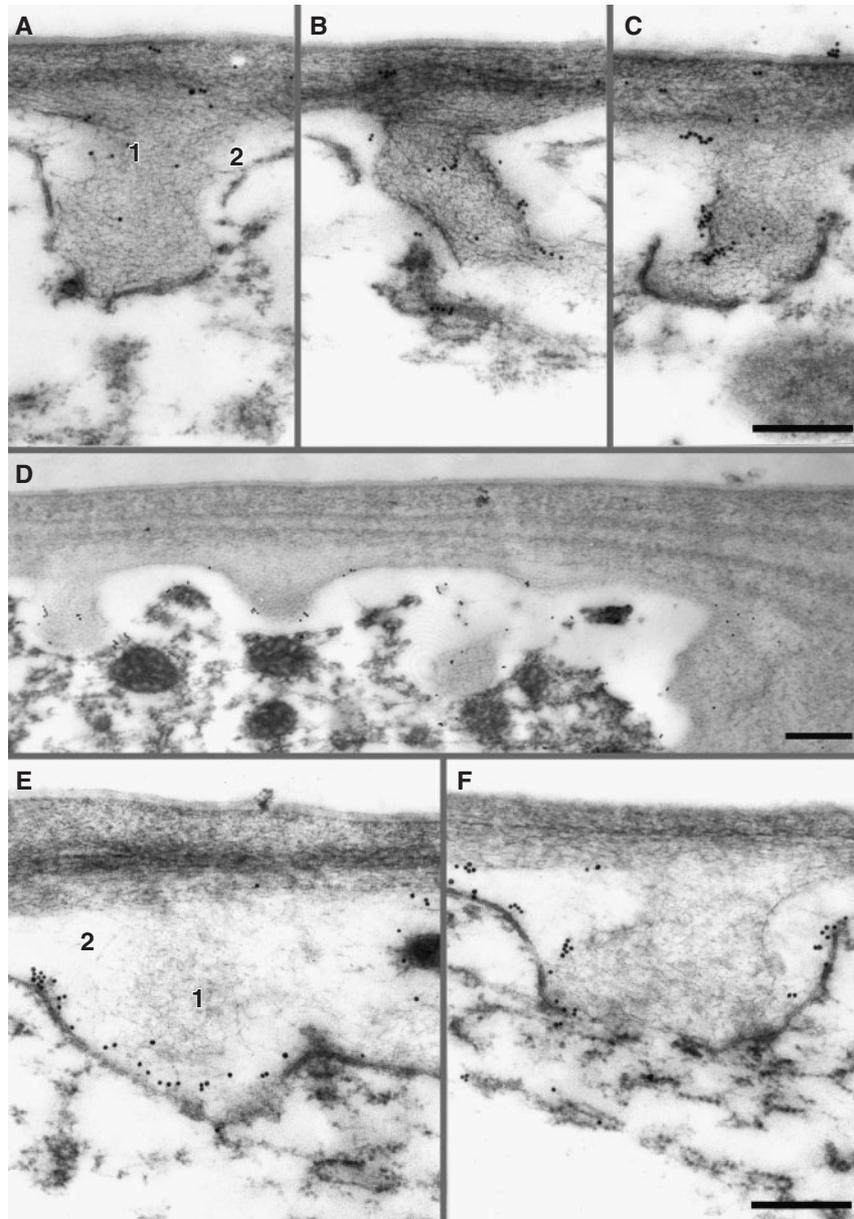
#### *AGPs are involved in wall ingrowth deposition*

The localization of AGPs associated with emerging wall ingrowths suggested a role for these proteoglycans in wall ingrowth deposition. We tested this possibility by culturing cotyledons for 24 h in the presence of either  $\beta$ -D-glucosyl Yariv reagent ( $\beta$ GlcY), a synthetic phenylglycoside that specifically binds to AGPs, or an inactive control,  $\alpha$ -D-galactosyl Yariv reagent ( $\alpha$ GalY) (Nothnagel 1997). Culturing cotyledons in the presence of 60  $\mu\text{M}$   $\beta$  GlcY over 24 h culture caused a 52% ( $\pm 5.6$ ;  $n = 4$ ) reduction in density of wall ingrowths in adaxial epidermal transfer cells, compared with ingrowth deposition occurring in the presence of  $\alpha$ GalY. The densities of wall ingrowth deposition in these experiments were determined from scanning electron microscopy (SEM) observations (see Materials and Methods; Talbot et al. 2001).

## Discussion

#### *Wall ingrowths are modified primary walls*

Transfer cell differentiation in *V. faba* epidermal cells first involves deposition of a thin, uniform wall layer over the pre-existing primary wall (Farley et al. 2000, Wardini et al. 2006; Fig. 1). A labyrinth of wall ingrowths is then progressively built from this layer, beginning with the emergence of papillate wall ingrowths (Offler et al. 2003). Wall ingrowths are composed of two distinct regions: an electron-opaque inner region and an electron-translucent outer region. Our study has revealed that the inner region of mature wall ingrowths is compositionally similar to the primary wall and the uniformly deposited wall layer,



**Fig. 4** Immunogold labeling of nascent wall ingrowths with (A) JIM7 monoclonal, (B) anti-xyloglucan antibody and (C) cellulase gold probe, reveals a similar pattern of labeling to that in more mature wall ingrowths (see Fig. 1). Label is restricted to the inner region (1) of the wall ingrowths, leaving the outer region (2) essentially unlabeled. Extensive labeling of the plasma membrane/outer region (2) is seen with the (D and E) CCRCM-7 (low magnification view shown in D, higher magnification view shown in E) and (F) PCBC3 anti-AGP monoclonals. No label is present in the inner wall ingrowth region (1). Bars = 200 nm (A–C, E and F are at the same magnification).

while the electron-translucent outer region of wall ingrowths is compositionally distinct (Table 1). The conclusion that the inner regions of wall ingrowths are primary wall like is supported by the observation that they do not stain with phloroglucinol-HCl (M.T., unpublished observation), a stain used to detect lignification of secondary walls (e.g. Gritsch and Murphy 2005).

Across their development, however, the relative percentage of components of the wall ingrowths changes, such

that the inner region of developing wall ingrowths contains less pectin, compared with the underlying primary wall, than that seen in more mature wall ingrowths. Therefore, we have adopted the term ‘modified primary wall’ to describe the chemical nature of the inner region of wall ingrowths. Overall, while this conclusion is broadly consistent with the findings of DeWitt et al. (1999) and Dahiya and Brewin (2000), the quantitative immunogold/affinity probe approach used in our study has clearly

established that wall ingrowths are compositionally and developmentally heterogeneous.

#### *The roles of AGPs and callose in transfer cell wall ontogeny*

The electron-translucent outer region of wall ingrowths is both compositionally and structurally unique compared with the primary walls and other regions of the wall ingrowths of epidermal transfer cells as well as compared with all other cell walls in the cotyledon. This region labels strongly for AGPs, which are present at either the plasma membrane–wall interface and throughout the electron-translucent outer region of wall ingrowths, or only at the plasma membrane–wall interface. This latter region also labels for callose in mature transfer cell wall ingrowth labyrinths. No other cells in the cotyledon possess a wall of such composition.

The abundance and localized distribution of AGPs in transfer cell walls of *V. faba* indicate a role for these molecules in wall ingrowth formation. Based on their relatively high abundance over the nascent wall ingrowth, AGPs are well positioned to participate in defining loci for deposition. Their localization at the plasma membrane might infer that the AGPs recognized by antibodies used in this study contain glycosylphosphatidylinositol (GPI) lipid anchors, implicating roles in signaling pathways (Schultz et al. 2000). The identification of an AGP with a predicted GPI anchor ('xylogen') that mediates inductive cell–cell signaling of tracheary element differentiation (Motosé et al. 2004) provides support for such a role.

AGPs have also been implicated in coordinating cell wall assembly (Dolan et al. 1995, Roy et al. 1998) and maintaining the solubility of wall polymers before addition into the wall (Carpita and Gibeau 1993). The AGPs found at sites of wall ingrowth formation might therefore ensure that xyloglucans and pectins remain soluble until their intercalation into the wall matrix.

Support for a role for AGPs in directing wall ingrowth deposition comes from the inhibitor experiments using  $\beta$ GlcY, a synthetic dye that specifically binds and precipitates AGPs (Nothnagel 1997, Serpe and Nothnagel 1999). Culturing cotyledons in the presence of  $\beta$ GlcY caused an approximately 50% reduction in the density of wall ingrowths in adaxial epidermal cells. While other studies using  $\beta$ GlcY have shown extensive disruption of wall formation, particularly in pollen tubes (Roy et al. 1998), the substantial reduction in the extent of wall ingrowth formation seen in the presence of this inhibitor suggests that AGPs have a role in this process. The observation that wall ingrowth formation is not completely disrupted in the presence of  $\beta$ GlcY might reflect the finding that not all AGPs are fully complexed by this inhibitor (Nothnagel 1997), and that Yariv reagents generally display low solubility and often poor intracellular uptake

into plant tissues (Willats and Knox 1996). Collectively, the data reported here strongly support current ideas that AGPs are critical components in denoting new sites of wall synthesis (Roy et al. 1998). Interestingly, in the accompanying manuscript, we show that normal wall ingrowth formation is essentially abolished when developing transfer cells are exposed to the cellulose synthesis inhibitors isoxaben and 2,6-dichlorobenzonitrile. Under these conditions, however, immunogold labeling established that AGPs were distributed evenly along the plasma membrane (K.V., unpublished observation).

Callose was not detected in the electron-translucent outer region of nascent wall ingrowths but was a prominent constituent of this region in mature wall ingrowths. Callose deposition can be induced by aldehyde fixation (Hughes and Gunning 1980); however, its absence in developing wall ingrowths indicated that the callose detected in this region of the mature wall ingrowths was not an artifact of fixation. Normally, the appearance of callose is associated with 'plugging' transport of molecules, as in phloem sieve plate pores and plasmodesmata (Stone and Clarke 1992). However, callose is also found as a thin layer just below the plasma membrane at very specific stages of development, such as during the formation of cell plates (Samuels et al. 1995) and throughout the differentiation of a number of cell types, including cotton fibers (Stone and Clarke 1992) and xylem elements (Gregory et al. 2002). In cell plates, it is thought that the callose provides a mechanical role, possibly a 'spreading' influence on the cell plate as it expands throughout the cytoplasm (Samuels et al. 1995, Vaughn et al. 1996). A similar situation could be envisaged for the outer region of wall ingrowths in transfer cells, where the callose might aid the progressive intrusion of wall ingrowths into the cytoplasm. Alternatively, or in addition, callose could provide a matrix through which assembly of wall ingrowth components occurs. It is thought that callose, which can form gel-like matrices, would provide a suitable environment into which other molecules of the wall, including polysaccharides and proteins, can be deposited (Stone and Clarke 1992).

In conclusion, we have shown using a suite of cell wall-specific antibodies and probes that wall ingrowths are compositionally similar to the primary wall in epidermal cells of *V. faba* cotyledons. The original prediction that wall ingrowths are pectin rich (Gunning and Pate 1969) is not supported in that xyloglucan antibodies and cellulase–gold affinity probes also clearly label the wall ingrowths. The electron-translucent outer region of wall ingrowths, however, is compositionally unique compared with other epidermal cell wall layers. This region is marked early in development with antibodies that recognize a diverse array of AGPs. The highly localized distribution of AGPs found over areas of wall ingrowth emergence,

and the reduced density of wall ingrowth formation on exposure to an AGP inhibitor, suggests that AGPs may function to mark sites for new wall ingrowth biogenesis. The developmentally regulated deposition of callose may account for the extended, labyrinthine morphology of maturing wall ingrowths.

## Materials and Methods

### *Plant material and conditions*

*Vicia faba* (Faba bean) plants were grown in environmentally controlled growth cabinets. Methods for inducing transfer cell differentiation in adaxial epidermal cells were as described by Offler et al. (1997). Briefly, cotyledons were surgically removed from the seed coat, separated, and transferred adaxial epidermal surface-down onto agar culture plates containing 50 mM glucose, 50 mM sucrose and 100 mM betaine. Plates were sealed with Parafilm and incubated in darkness at 25–27°C for 24 h.

### *Fixation and embedding*

Whole cotyledons were fixed for 4 h on ice in 2.5% (w/v) glutaraldehyde and 2.5% (w/v) paraformaldehyde in 10 mM sucrose buffered to pH 7.0 with 25 mM sodium cacodylate. Some tissue pieces were post-fixed overnight at 4°C in 1% (w/v) osmium tetroxide in 25 mM sodium cacodylate. Following three rinses in buffer and three rinses in double-distilled water, the tissue was dehydrated on ice through a 10% step graded ethanol series, changed at 45 min intervals. Infiltration was at room temperature through a 20% step graded series to 100% LR White resin (Polysciences, Inc., Queensland, Australia) over 9 h. After daily changes of 100% resin for 6 d, the tissue was embedded in gelatin capsules by polymerization at 60°C for 24 h. Specimens were trimmed and re-mounted to give cross-sections through the adaxial epidermis.

### *Immunolabeling*

Thin sections were cut with a Delaware Diamond Knife on a Reichert Ultracut ultramicrotome at ~100 nm (pale gold reflectance) and mounted on uncoated 300 mesh gold grids. The grids were then floated, specimen side-down, on 4 µl drops of the following reagents: 1% bovine serum albumin fraction IV in phosphate-buffered saline (PBS-BSA), 30 min; primary antibody diluted 1:10 to 1:80 depending upon source, for 4 h; four drops of PBS-BSA, 10 min; 1:20 dilution of secondary antibody–gold or protein A–gold (EY Labs, San Mateo, CA, USA), 30 min; four drops of PBS, 10 min. The grids were then washed with double-distilled water and post-stained in 2% aqueous uranyl acetate for 2 min and Reynold's lead citrate for 30 s. Specimens were examined on a Zeiss EM 10CR electron microscope operating at 60 kV. All localization experiments were repeated a minimum of three times using different tissue samples.

### *Quantification of immunolabeling*

To ensure randomness, only sections nearest to the 500/500 centering point on the Zeiss EM 10CR were sampled and all epidermal tissue within that section was photographed for analysis. Three separate experiments were performed using different cotyledon tissue, and 18–20 negatives for each immunolocalization experiment were collected. Prints from negatives were enlarged to 37,500× magnification and the numbers of gold particles over a given section were counted manually. For determination of

gold particle density on a µm<sup>2</sup> basis, the area of wall containing the label was cut out from the print and the area determined gravimetrically compared with a piece of photographic paper of 1 µm<sup>2</sup>. Linear µm lengths of plasma membrane were calculated using a manual map reader.

### *Antibody specificity*

A majority of the antibodies and probes used in this study have been previously described in reports from the first author's laboratory (Vaughn et al. 1996, Sabba et al. 1999, Ligrone et al. 2002, Vaughn 2003). Many of these antibodies were generous gifts from Andrew Staehelin, Michael Hahn, Keith Roberts and Paul Knox, or were purchased from Biosupplies Australia (Melbourne, Victoria, Australia) or Plant Probes (Leeds, UK). A full set of control experiments for each of these antibodies and probes is described in Vaughn (2003) and are the same sets of controls used in this study. In none of the studies was background label over areas of plastic or vacuoles greater than 0.3 gold particles µm<sup>-2</sup>.

### *Yariv reagent treatment and image analysis*

α-Galactosyl and β-glucosyl Yariv reagents (Biosupplies Australia Pty, Ltd, Victoria, Australia) were prepared as 5 mM stock solutions in water and were added to agar culture medium to achieve the required final concentration (60 µM). Cotyledons were cultured (see above) in the presence of Yariv reagents for 24 h and then prepared for SEM (for details, see Talbot et al. 2001). To quantify wall ingrowth deposition, SEM images were inverted in Adobe Photoshop 7.0 to convert wall ingrowths to black, and the cell background to white. Cells were outlined with the pen tool, and any obstructing cell debris (including large pieces of remnant cytoplasm or starch grains) was removed using the eraser tool. The resultant image was then analyzed in ImageJ v1.31 using the 'analyze particles' command. Wall ingrowth density was calculated by dividing the number of particles (wall ingrowths) by the measured cell area (µm<sup>2</sup>). Fifty cells were scored for each of four cotyledon replicates, with densities compared within matched cotyledon pairs.

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## References

- Carpita, N.C. and Gibeaut, D.M. (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J.* 3: 1–30.
- Dahiya, P. and Brewin, N.J. (2000) Immunogold localization of callose and other cell wall components in pea nodule transfer cells. *Protoplasma* 214: 210–218.
- DeWitt, G., Richards, J., Mohnen, D. and Jones, A.M. (1999) Comparative compositional analysis of walls with two different morphologies: archetypical versus transfer-cell-like. *Protoplasma* 209: 238–245.
- Dolan, L., Linstead, P. and Roberts, K. (1995) An AGP epitope distinguishes a central metaxylem initial from other vascular initials in the Arabidopsis root. *Protoplasma* 189: 149–155.
- Farley, S.J., Patrick, J.W. and Offler, C.E. (2000) Functional transfer cells differentiate in cultured cotyledons of *Vicia faba* seeds. *Protoplasma* 214: 102–117.

- Freshour, G., Clay, R.P., Fuller, M.S., Albersheim, P., Darvill, A.G. and Hahn, M.G. (1996) Development and tissue-specific structural alterations of the cell wall polysaccharides of the *Arabidopsis thaliana* root. *Plant Physiol.* 110: 1413–1429.
- Gaspar, Y., Johnson, K.L., McKenna, J.A., Bacic, A. and Schultz, C.J. (2001) The complex structure of arabinogalactan proteins and the journey towards understanding their functions. *Plant Mol. Biol.* 47: 161–176.
- Gregory, A.C., Smith, C., Kerry, M.E., Wheatley, E.R. and Bolwell, G.P. (2002) Comparative subcellular immunolocalization of polypeptides associated with xylan and callose synthases in French bean (*Phaseolus vulgaris*) during secondary wall formation. *Phytochemistry* 59: 249–259.
- Gritsch, C.S. and Murphy, R.J. (2005) Ultrastructure of fibre and parenchyma cell walls during early stages of culm development in *Dendrocalamus asper*. *Ann. Bot.* 95: 619–629.
- Gunning, B.E.S. and Pate, J.S. (1969) 'Transfer cells': plant cells with wall ingrowths, specialized in relation to short distance transport of solutes: their occurrence, structure and development. *Protoplasma* 68: 107–133.
- Haritatos, E., Medville, R. and Turgeon, R. (2000) Minor vein structure and sugar transport in *Arabidopsis thaliana*. *Planta* 211: 105–111.
- Harrington, G.N., Franceschi, V.R., Offler, C.E., Patrick, J.W., Tegeder, M., Frommer, W.B., Harper, J.F. and Hitz, W.D. (1997) Spatial and temporal expression of sucrose transport-related genes in developing cotyledons of *Vicia faba* L. *Protoplasma* 197: 160–173.
- Hughes, J.E. and Gunning, B.E.S. (1980) Glutaraldehyde-induced deposition of callose. *Can. J. Bot.* 58: 250–258.
- Jarvis, M.C. (1992) Self-assembly of plant cell walls. *Plant Cell Environ.* 15: 1–5.
- Ligrone, R., Vaughn, K.C., Renzaglia, K.S., Knox, J.P. and Duckett, J.G. (2002) Diversity in the distribution of polysaccharides and glycoprotein epitopes in the cell walls of bryophytes: new evidence for the multiple evolution of water conducting cells. *New Phytol.* 156: 491–508.
- McCartney, L., Gilbert, H.J., Bolam, D.N., Borastan, A.B. and Knox, J.P. (2004) Glycoside carbohydrate-binding modules as molecular probes for the analysis of plant cell wall polymers. *Anal. Biochem.* 326: 49–54.
- McDonald, R., Fieuw, S. and Patrick, J.W. (1996) Sugar uptake by the dermal transfer cells of the developing cotyledons of *Vicia faba* L.: experimental systems and general transport properties. *Planta* 198: 54–63.
- Meikle, J., Boning, I., Hoogenraad, N., Clarke, A.E. and Stone, B.A. (1991) The location of (1→3)- $\beta$ -glucans in the walls of pollen tubes of *Nicotiana glauca* using a (1→3)- $\beta$ -glucan-specific monoclonal antibody. *Planta* 185: 1–8.
- Meloche, C.G., Knox, J.P. and Vaughn, K.C. (2006) A cortical band of gelatinous fibers causes the coiling of redvine tendrils: a model based upon cytochemical and immunocytochemical studies. *Planta* (in press).
- Moore, P.J., Darvill, A.G., Albersheim, P. and Staehelin, L.A. (1986) Immunogold localization of xyloglucan and rhamnogalacturonan I in the cell walls of suspension-cultured sycamore cells. *Plant Physiol.* 82: 787–794.
- Motose, H., Sugiyama, M. and Fukuda, H. (2004) A proteoglycan mediates inductive interaction during plant vascular development. *Nature* 429: 873–878.
- Northcote, D.H., Davey, R. and Lay, J. (1989) Use of antisera to localize callose, xylan and arabinogalactan in the cell plate, primary and secondary walls of plant cells. *Planta* 178: 353–366.
- Nothnagel, E.A. (1997) Proteoglycans and related components in plant cells. *Int. Rev. Cytol.* 174: 195–291.
- Offler, C.E., Liet, E. and Sutton, E.G. (1997) Transfer cell induction in cotyledons of *Vicia faba* L. *Protoplasma* 200: 51–64.
- Offler, C.E., McCurdy, D.W., Patrick, J.W. and Talbot, M.J. (2003) Transfer cells: cells specialized for a special purpose. *Annu. Rev. Plant Biol.* 54: 431–454.
- Offler, C.E. and Patrick, J.W. (1993) Pathway of photosynthate transfer in developing seed of *Vicia faba* L.: a structural assessment of the role of transfer cells in unloading from the seed coat. *J. Exp. Bot.* 44: 711–724.
- Patrick, J.P. (1997) Phloem unloading—sieve element unloading and post-sieve element transport. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48: 191–222.
- Patrick, J.P. and Offler, C.E. (1995) Post-sieve element transport of sucrose in developing seeds. *Aust. J. Plant Physiol.* 22: 681–702.
- Roy, S.J., Yuh Jauh, G., Hepler, P.K. and Lord, E.M. (1998) Effects of Yariv phenylglycoside on cell wall assembly in the lily pollen tube. *Planta* 204: 450–458.
- Sabba, R.P., Durso, N.A. and Vaughn, K.C. (1999) Structural and immunocytochemical characterization of the walls of dichlobenil-habituated BY-2 tobacco cells. *Int. J. Plant Sci.* 160: 275–290.
- Samuels, A., Giddings, T. and Staehelin, L.A. (1995) Cytokinesis in tobacco BY-2 and root tip cells: a new model for cell plate formation in higher plants. *J. Cell Biol.* 130: 1345–1357.
- Schultz, C.J., Johnson, K.L., Currie, G. and Bacic, A. (2000) The classical arabinogalactan protein gene family of *Arabidopsis*. *Plant Cell* 12: 1751–1767.
- Serpe, M.D. and Nothnagel, E.A. (1996) Heterogeneity of arabinogalactan-proteins on the plasma membrane of rose cells. *Plant Physiol.* 112: 1261–1271.
- Stone, B.A. and Clarke, A.E. (1992) Chemistry and physiology of higher plant (1–3)- $\beta$ -glucans (callose). In *Chemistry, and Biology of (1–3)- $\beta$ -Glucans*. Edited by Stone, B.A. and Clarke, A.E. pp. 365–429. La Trobe University Press, Melbourne.
- Talbot, M.J., Franceschi, V.R., McCurdy, D.W. and Offler, C.E. (2001) Wall ingrowth architecture in epidermal transfer cells of *Vicia faba* cotyledons. *Protoplasma* 215: 191–203.
- Talbot, M.J., Offler, C.E. and McCurdy, D.W. (2002) Transfer cell wall architecture: a contribution towards understanding localized wall deposition. *Protoplasma* 219: 197–209.
- Taylor, J.G. and Haigler, C.H. (1993) Patterned secondary cell-wall assembly occurs in a self-perpetuating cascade. *Acta Bot. Neerl.* 42: 153–163.
- Taylor, J.G., Owen, T.P.Jr, Koonce, L.T. and Haigler, C.H. (1992) Dispersed lignin in tracheary elements treated with cellulose synthesis inhibitors provides evidence that molecules of the secondary cell wall mediate wall patterning. *Plant J.* 2: 959–970.
- Tegeder, M., Thomas, M., Hetherington, L., Wang, X.-D., Offler, C.E. and Patrick, J.W. (2000) Genotypic differences in seed growth rates of *Phaseolus vulgaris* L. Factors contributing to cotyledon sink activity and sink size. *Aust. J. Plant Physiol.* 27: 119–128.
- Tegeder, M., Wang, X.-D., Frommer, W.B., Offler, C.E. and Patrick, J.W. (1999) Sucrose transport into developing seeds of *Pisum sativum* L. *Plant J.* 18: 151–161.
- Vaughn, K.C. (2003) Dodder hyphae invade the host: a structural and immunocytochemical characterization. *Protoplasma* 220: 189–200.
- Vaughn, K.C., Hoffmann, J.C., Hahn, M.G. and Staehelin, L.A. (1996) The herbicide dichlobenil disrupts cell plate formation: immunogold characterization. *Protoplasma* 194: 117–132.
- Wang, H.-L., Offler, C.E., Patrick, J.W. and Ugalde, T.D. (1994) The cellular pathway of photosynthate transfer in the developing wheat grain. I. Delineation of a potential transfer pathway using fluorescent dyes. *Plant Cell Environ.* 17: 257–266.
- Wardini, T., Wang, X.-D., Offler, C.E. and Patrick, J.W. (2006) Induction of wall ingrowths of transfer cells occurs rapidly and depends upon gene expression in cotyledons of developing *Vicia faba* seeds. *Protoplasma* in press.
- Willats, W.G., Steele-King, C.G., Marcus, S.E. and Knox, J.P. (1999) Side chains of pectic polysaccharides are regulated in relation to cell proliferation and cell differentiation. *Plant J.* 20: 619–628.
- Willats, W.G. and Knox, J.P. (1996) A role for arabinogalactan-proteins in plant cell expansion: evidence from studies on the interaction of beta-glucosyl Yariv reagent with seedlings of *Arabidopsis thaliana*. *Plant J.* 9: 919–925.
- Zhang, Y., Brown, G., Whetten, R., Loopstra, C.A., Neale, D., Kieliszewski, M.J. and Sederoff, N. (2003) An arabinogalactan protein associated with secondary wall formation in differentiating xylem of loblolly pine. *Plant Mol. Biol.* 52: 91–102.

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