Planta (2005) 221: 255–264
DOI 10.1007/s00425-004-1432-1

ORIGINAL ARTICLE

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A conserved functional role of pectic polymers in stomatal guard cells from a range of plant species

Received: 6 June 2004 / Accepted: 12 October 2004 / Published online: 2 December 2004

Abstract Guard cell walls combine exceptional strength and flexibility in order to accommodate the turgor pressure-driven changes in size and shape that underlie the opening and closing of stomatal pores. To investigate the molecular basis of these exceptional qualities, we have used a combination of compositional and functional analyses in three different plant species. We show that comparisons of FTIR spectra from stomatal guard cells and those of other epidermal cells indicate a number of clear differences in cell-wall composition. The most obvious characteristics are that stomatal guard cells are enriched in phenolic esters of pectins. This enrichment is apparent in guard cells from *Vicia faba* (possessing a type I cell wall) and *Commelina communis* and *Zea mays* (having a type II wall). We further show that these common defining elements of guard cell walls have conserved functional roles. As previously reported in *C. communis*, we show that enzymatic modification of the pectin network in guard cell walls in both *V. faba* and *Z. mays* has profound effects on stomatal function. In all three species, incubation of epidermal strips with a combination of pectin methyl esterase and endopolygalacturonase (EPG) caused an increase in stomatal aperture on opening. This effect was not seen when strips were incubated with EPG alone indicating that the methyl-esterified fraction of homogalacturonan is key to this effect. In contrast, arabinanase treatment, and incubation with feruloyl esterase both impeded stomatal opening. It therefore appears that pectins and phenolic esters have a conserved functional role in guard cell walls even in grass species with type II walls, which characteristically are composed of low levels of pectins.

Keywords Arabinans · Cell wall · Feruloyl esters · Pectins · Stomatal guard cells

Abbreviations FTIR: Fourier-transform infra-red

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Introduction

Whilst united by a common general architectural principle, the primary cell walls of higher plants have been classified as two distinct types based on wall composition (Carpita and Gibeaut 1993; Carpita and McCann 2000). Both cell-wall types are dominated structurally by cellulose microfibrils that form the framework for a composite material containing other non-fibrillar (matrix) polysaccharides along with a range of structural and enzymatic proteins. Type I and type II cell walls, as they are referred to, are distinguished from one another largely on the basis of their hemicellulose (or cross-linking glycans) and pectin content. Xyloglucans make up the predominant hemicellulose in type I walls, which are characteristic of most dicot and non-graminaceous monocot species, and these walls are also relatively rich in pectins, which make up 20–30% of wall dry mass. The major hemicelluloses in type II walls, characteristic of commelinoid plants, are glucuronoarabinoxylans and mixed-linkage β-glucans (found mostly in growing walls), and these walls typically contain significantly less pectin than type I walls.

Whilst such generalities regarding wall composition hold true, it is also clear that there is considerable variation in wall composition between species that possess either of these generalised wall types (Harris et al. 1997). In addition, wall composition also varies between different cell types within an individual plant reflecting either the developmental state, or specialised

nature of those cells. Such variations in wall composition have become particularly evident from immunolocalisation studies with antibodies that recognise specific wall epitopes. For example, a number of studies with LM5 and LM6, monoclonal antibodies recognising (1,4)-β-D-galactans and (1,5)-β-L-arabinans respectively, have shown very specific labelling of cells at different stages of development suggesting that wall composition undergoes dynamic changes related to the developmental state of the cells (Orfila et al. 2001; Willats et al. 2001; McCartney and Knox 2002).

Differences in wall composition also accompany and reflect the specialised functional roles of various cell types. Stomatal guard cells form, and regulate, the aperture of pores in the epidermis of most land plants. Guard cells are distinct from most other plant cells in that they undergo substantial reversible changes in shape and size in order to regulate pore aperture. The form and function of stomata and guard cells is generally conserved amongst the vascular plants. Within angiosperms there are essentially two distinct forms of guard cells, which are either kidney shaped, typified by most dicots and non-graminaceous monocots, or dumbbell-shaped, characteristic of the grasses (see Weyers and Meidner 1990). The functioning of these two morphologically distinct varieties of stomata is, however, based on the same principle. During opening, the turgor pressure in a guard cell can rise to levels of around 50 atm (Franks et al. 2001) causing the guard cell to increase its volume by around 70%. It is generally believed that differential thickening of the guard cell walls, and arrangements of microfibrils within the wall, determine the orientation of the deformation of guard cells causing them to bow apart, thus opening the stomatal pore. During stomatal closing, turgor decreases and the cells shrink to their resting size. To cope with the high internal pressure and reversible movements that guard cells undergo, their cell walls need to be both strong and elastic. We recently showed that pectins appear to play a key role in the flexibility of guard cell walls in *Commelina communis* (Jones et al. 2003). These results were perhaps unexpected given that *C. communis* is a commelinoid plant and thus thought to be characterised by a type II cell wall containing relatively low amounts of pectin. However, compositional analysis and immunolocalisation studies indicated that the cell walls from epidermal strips of *C. communis* are indeed relatively rich in pectin suggesting that the specialised nature of these cells might be reflected in their unexpected wall composition. In order to extend these observations, we have characterised and compared the composition of guard cell walls from a model dicot possessing a type I cell wall, *Vicia faba*, and a grass species having a type II wall, *Zea mays* with those of *C. communis*. We further investigate the effect of cell-wall modifying enzymes on stomatal function in the morphologically distinct guard cells of *V. faba* and *Z. mays* and compare results with those previously obtained with *C. communis*.

Materials and methods

Plant material

Epidermal strips were peeled from the abaxial surface of mature leaves of 6-week-old plants of *V. faba* var. Aquadulce Claudia (Kings Seeds, Kelvedon, UK), *Z. Mays* var. Schumi (SOC, France) and *C. communis* that had been grown in a greenhouse at 25°C with supplementary lighting. Strips were placed in 10 mM KCl, 0.1 mM $CaCl_2$ and then trimmed and cut to size, approximately 5 mm^2.

FTIR micro-spectroscopy

Freshly peeled epidermal strips (~5 mm by 8 mm) of 2-week-old *V. faba*, *C. communis*, and *Z. mays*, were mounted on barium fluoride windows and left to dry at 4°C until use (about 2 h).

The barium fluoride disc with sample was mounted on the stage of an infra-red microscope (Spectra-Tech, IR-Plan), connected to a Bio-Rad FTS60 Fourier transform infra-red (FTIR) spectrometer. In all experiments, a background single-beam spectrum was collected of a clean, sample free area of the barium fluoride disc just before a spectrum of the sample was obtained. The same spectral acquisition parameters and aperture were used for both the sample spectrum and background spectrum. The incident light was arranged to be either perpendicular or parallel to a fixed axis of the sample plane.

Spectra were recorded with 64 interferograms being co-added before Fourier transformation to improve the signal to noise ratio. For epidermal and guard cells of *V. faba*, and *C. communis*, the aperture was set to 60×60 μm^2 due to signal to noise ratio. For *C. communis*, this included the inner lateral subsidiary cell pair. For *Z. mays*, the aperture was set to ~50 μm by 15 μm. Files of the recorded spectra were collected and imported into WINDISCRIM and principal components analysis (PCA) was carried out on the data files. Assignments of the main bands (cm^{-1}) in FTIR spectra were taken from a number of sources (Marchessault and Sundararajan 1983; Parker 1983; Colthup et al. 1990; McCann et al. 1994; Wellner et al. 1998; Wilson et al. 2000).

Cell-wall monosaccharide analysis

Epidermal strips were taken from the abaxial (lower) and adaxial (upper) side of leaves of 6-week-old *C. communis* plants and stirred slowly in liquid phenol (P-4682, Sigma) for 20 min and then overnight in DMSO to remove starch, before being rinsed four times in 95% ethanol and left to dry. For *V. faba* and

Z. mays, it proved extremely difficult to obtain epidermal strips from the adaxial surface, and instead, the leaf tissues remaining after removal of the lower epidermis were used. Duplicate 1-mg samples were hydrolysed with 2 M trifluoracetic acid (TFA) for 4 h at 100°C and separated by high performance anion exchange chromatography on a Dionex Carbopac PA-10 column with integrated amperometry detection. The separated monosaccharides were quantified using external calibration with an equimolar mixture of nine monosaccharide standards (arabinose, fucose, galactose, galacturonic acid, glucose, glucuronic acid, mannose, rhamnose and xylose) that were subjected to acid hydrolysis in parallel with the samples.

Stomatal opening experiments

For stomatal opening experiments, strips were incubated at room temperature in the dark for 1 h in 1 ml of 10 mM KCl, 0.1 mM $CaCl_2$ containing enzymes. Strips were then placed in 75 mM KCl, 1 μM fusicoccin and left to open for 2 h.

Enzyme treatments

Highly purified enzymes with well-defined properties were purchased from Megazyme Int., Ireland Ltd. These included: endo-1, 4-β-galactanase, endo-arabinanase, endo-polygalacturonanase, all reported to be purified to single bands as seen by SDS gel electrophoresis (see manufacturer's data sheets for details regarding purity, http://www.megazyme.com). Pectinesterase P5400 (Sigma-Aldrich), although this is not a purified enzyme, we found that it had no detectable effects on either stomatal aperture or in releasing reducing sugars from epidermal strips when used alone suggesting there was no significant contamination with polysaccharide hydrolases (Jones et al. 2003). Purified recombinant feruloyl esterase FaeA (Ralet et al. 1994) was a kind gift from Dr. Craig Faulds (IFR, Norwich, UK). All enzymes were used at 10 units per ml, except for feruloyl esterase, which was used at 1 unit per ml.

Image capture and analysis

Epidermal strips were examined by bright field microscopy with an Optiphot light microscope (Nikon 104). Stomatal apertures were measured using Lucia G version 3.52a software (Laboratory Imaging). Fluorescent images (excitation filter 365/10 nm) were captured using a CoolSnap digital camera (RS Photometrics) attached to a Microphot FXA Nikon microscope and analysed using CoolSnap 1.2 software (Roper Scientific).

Results

Guard cells are characteristically enriched in phenolic esters of pectin

Analysis of cell-wall composition was carried out in three distinct plant species in order to investigate whether the highly specialised role of stomatal guard cell walls is reflected by specialised wall structure and composition. The wall composition of guard cells is difficult to approach using conventional analytical chemistry methods, as it is not currently feasible to obtain purified guard cell-wall material in sufficient quantity. As an alternative, we decided to make use of the combined analytical power and spatial resolution of FTIR microspectroscopic studies. We used FTIR micro-spectroscopy to gather and compare spectroscopic data from guard cell pairs and from non-stomatal epidermal cells of *C. communis*, *Z. mays*, and *V. faba*. FTIR spectroscopy has the advantage that it can generate data related to the presence and interactions of wall polymers in situ from very small areas of plant tissue (McCann et al. 1992; Wells et al. 1994). Furthermore, FTIR has been used to detect alterations in composition and structure in the walls of mutant plants (Chen et al. 1998; Mouille et al. 2003). FTIR absorbance spectra from epidermal cells (solid lines) and guard cells (broken lines) of each species are presented in Fig. 1. There are clear differences in the spectra for each of the three species, particularly in regions corresponding to cellulosic (1,156–1,035 cm^{-1}) and pectic (approximately 1,738, 1,420, 1,371 cm^{-1}) polysaccharides. Peaks around 1,738 cm^{-1} are due to the ester groups of pectin and are present in all three species. Additional pectin peaks representing unesterified pectin (~1,420 cm^{-1}) are also evident in all three species whilst the peak at ~1,371 cm^{-1}, indicative

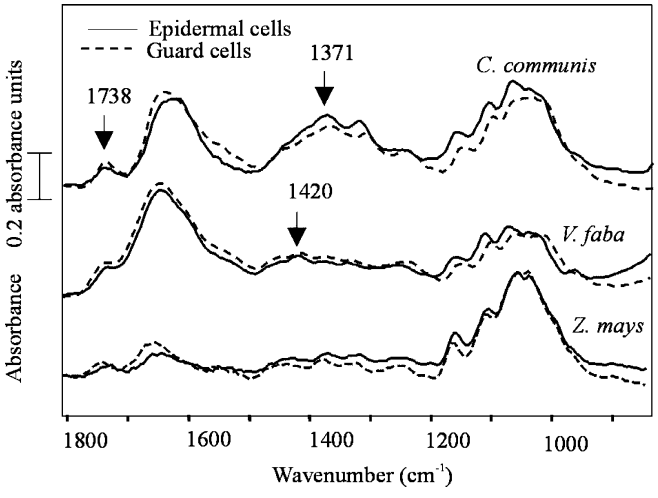

Fig. 1 Fourier-transform infra-red absorbance spectra for epidermal and guard cells of *Commelina communis*, *Vicia faba*, and *Zea mays*. Spectra are the average of ten epidermal cells, and ten guard cell pairs, for each species as indicated

of unesterified carboxyl groups of pectin is present in *C. communis* and *Z. mays* only.

Each species can be distinguished from the other on the basis of the relative peak heights of the cellulosic and pectic regions. For the monocots, *C. communis* and *Z. mays*, the proportion of pectin to cellulose is less than that for the dicot, *V. faba*. The exact wavenumber of absorbance for the pectin peaks is also different between species indicating different types of esterification, such as alkyl or aryl.

Despite the clear species-specific differences in these spectra, the distinctions between epidermal and guard cell spectra within each species appear subtle, consisting of small differences in the relative heights and positions of peaks throughout the spectra. Such subtle differences in spectra become more obvious when the differences are tested statistically through the use of PCA. PCA allows the data set to be reduced from several hundred data points in the original spectra to a fewer number of dimensions (Kemsley et al. 1994; Briandet et al. 1996). The variability in each individual spectrum relative to the mean for that population is then represented as a smaller set of values (axes) termed principal components (PCs). A plot of these PCs against one another can reveal clustering in the data set. It is possible to mathematically derive a spectrum, related to the PC score that represents an independent source of spectral variability in the data set. As a result the molecular basis for separation/clustering of groups of samples on the plot can sometimes be determined.

Figure 2a shows a PC plot for guard and epidermal cells of *C. communis*. Separation of the two cell types occurs on the basis of PCs 2 and 3. Mathematically derived plots of PCs 2 and 3 are shown in the lower panels of Fig. 2a. Each variate essentially corresponds to a wavenumber (*x*-axis) and the values of the loading vector or variate are plotted along the *y*-axis. Epidermal cells show a positive correlation with peaks that correspond to cellulosic polymers (1,034, 1,065, 1,099, and 1,161 cm^{-1}) and pectin (829 cm^{-1}) whereas guard cells of the same species show a negative correlation with these peaks, according to PC2 (Fig. 2a). The PC plot also shows that guard cells are positively correlated with peaks that represent protein (1,555, 1,655 cm^{-1} on PC2) and phenolic esters of pectin (1,717 cm^{-1} on PC2). In addition, epidermal cells are positively correlated with peaks that correspond to the carboxylic acid groups of unesterified pectin (949, 1,408, and 1,609 of PC2 and the peak at 1,613 on PC3, Fig. 2a). These data demonstrate that in the spectra for epidermal cells of *C. communis* the absorbance intensity of cellulosic polymers and unesterified pectins is greater, whilst guard cells appear more enriched in phenolic esters.

Figure 2b shows separation of epidermal cells and guard cells of *V. faba* when PC1 is plotted against PC2. Plots of these two PCs are shown in the lower panels and indicate that epidermal cells show a positive correlation with cellulose (according to peaks at, 1,011, 1,034, 1,065, 1,103, 1,157 cm^{-1} on PC2) and unesterified pectin

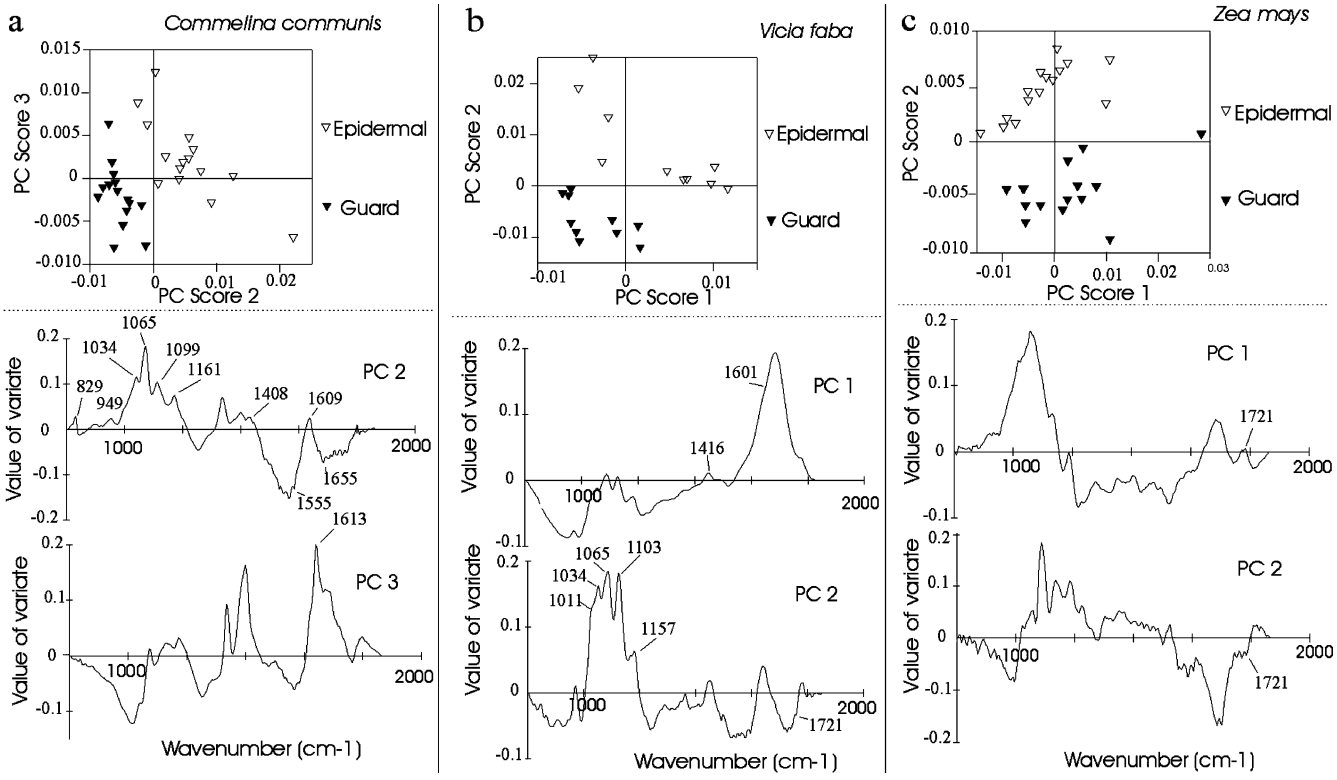

Fig. 2 a–c Exploratory principle components analysis (covariance matrix) separates guard cells from other epidermal cells in epidermal strips from three different plant species, *C. communis* (**a**), *V. faba* (**b**), and *Z. mays* (**c**)

(1,416, 1,601 cm^{-1} on PC1). Conversely, guard cells are negatively correlated with these peaks but positively correlated with peaks that represent phenolic esters of pectin (1,721 cm^{-1} on PC2). Figure 2c shows that, according to PC2, *Z. mays* epidermal cells are positively correlated with the carbohydrate region (1,000–1,200 cm^{-1}), which is mainly cellulosic, whilst guard cells are again positively correlated with phenolic esters of pectin (1,721 cm^{-1}).

There appear to be common characteristics that transcend species boundaries, by which guard cells can be distinguished from epidermal cells. These differences become even more evident when spectral data from epidermal and guard cells of two species are analysed together. For example, Fig. 3 shows a plot of epidermal and guard cells of *C. communis* and *V. faba* and reveals that cells from the two species are separated by PC1.

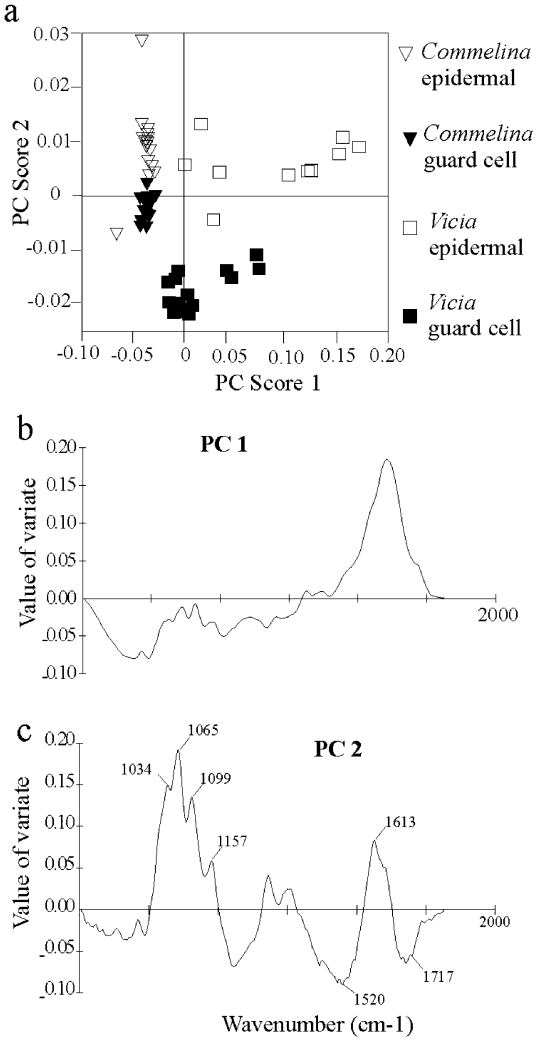

Fig. 3 a–c Exploratory principle components analysis (covariance matrix) separates guard cells and other epidermal cells from two different plant species; data from *C. communis* and *V. faba* are shown here. Note that guard cells from both species are clearly separated from non-guard cells by PC 1 and 2 (**b**, **c**)

More strikingly epidermal cells of both species are separated from guard cells of both species on the basis of PC2. An inspection of a plot of PC2 loadings (lower panel of Fig. 3) reveals that epidermal cells are positively correlated with cellulose (1,034, 1,065, 1,099, and 1,157 cm^{-1}), and unesterified pectin (1,613 cm^{-1}), whereas guard cells of both species are positively correlated with phenolic esters of pectin (1,717 cm^{-1}) and other phenolics (1,520 cm^{-1}).

These results demonstrate that guard cells show compositional features that are significantly different from those of other epidermal cells and that these differences are conserved across plant species with significantly different overall wall compositions. Epidermal cells of all three species are positively correlated with cellulosic polymers whereas guard cells are positively correlated with protein and phenolic esters. In *V. faba* and *C. communis*, epidermal cells are also positively correlated with unesterified pectin. This conservation of specific wall compositional features may be related to the specialised function of guard cell walls.

Epidermal cell-wall composition indicates a typical type I wall in *V. faba* and type II wall in *Z. mays*, whilst *C. communis* composition combines features of both

In order to ascertain whether epidermal cell walls from each of the three species show compositions typical of type I or type II cell walls, we carried out an analysis of their TFA-soluble monosaccharide composition. Lower (abaxial) epidermal strips were hydrolysed in 2 M TFA at 100°C. Such mild acid hydrolysis will release monosaccharides from wall matrix polysaccharides but is not strong enough to hydrolyse crystalline polysaccharides such as cellulose (Fry 1988). Data from these experiments are shown in Fig. 4. Wall sugar composition in *V. faba* (Fig. 4a) appears quite representative of type I cell walls with galacturonic acid comprising the major monosaccharide in non-cellulosic polysaccharides, indicating pectin-rich walls. The monosaccharide profiles from TFA-digested walls of *Z. mays* (Fig. 4b) are dominated by xylose and have very low levels of galacturonic acid, as is typical of type II walls (Smith and Harris 1999). Although *C. communis* is a commelinoid plant and therefore expected to have a type II wall, the monosaccharide composition profile (shown in Fig. 4c), although dominated by xylose, also includes substantial quantities of galacturonic acid, indicating an abundance of pectin more in line with type I cell-wall composition. Arabinose makes a notable contribution to the monosaccharide profiles of all three species, accounting for between 13 and 22% of total sugars. Another notable difference is in the relative quantities of galactose that accounts for some 10% of wall sugars in *V. faba*, but less than 5% in the two commelinoid plants. In an attempt to identify any unique compositional characteristics of guard cell walls, analysis was also carried out on upper epidermal strips (*C. communis*) and

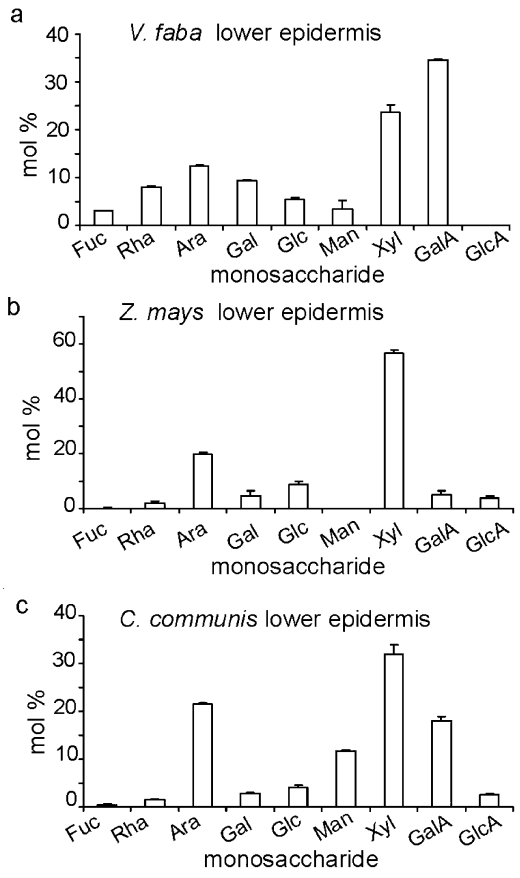

Fig. 4 a–c Monosaccharide composition of non-cellulosic polysaccharides from epidermal cell walls of *V. faba* (**a**), *Z. mays* (**b**) and *C. communis* (**c**). Cell walls from epidermal strips were hydrolysed in TFA and the monosaccharides released analysed by anion exchange chromatography. Results are averages ±SE from three samples. Overall experiments were repeated twice with similar results

leaf tissue minus the lower epidermis (*V. faba* and *Z. mays*). Since stomata are significantly more abundant in the lower epidermis (for *C. communis* we found on average 40.4 guard cell pairs per mm^2 on the lower epidermis compared to 7.6 per mm^2 for the upper epidermis), any major differences in monosaccharide composition between the tissue types might reflect the presence of these specialised cell types. However, no obvious differences in wall monosaccharide compositions between the two tissue types within each species could be detected (data not shown), indicating that any differences in composition between guard cell walls and those of other epidermal cells may be quite subtle.

Autofluorescent cell walls are characteristic of guard cells

The FTIR data described here demonstrated that one of the key features that distinguished guard cells from epidermal cells was the relative abundance of phenolic esters of pectins. Previous work with *C. communis* demonstrated the presence of bright blue autofluorescent material, which is likely to correspond to phenolic esters in guard cell walls. Such autofluorescence under UV light is a characteristic of guard cells in a number of species, as evident in Fig. 5, which shows epidermal strips from *C. communis*, *V. faba* and *Z. mays* viewed under UV light. Guard cell walls in all three species fluoresce; however, the pattern, colour and intensity of the autofluorescence vary between the species. Type II cell walls are typified by the presence of significant quantities of phenolic compounds, and indeed all epidermal cell walls are clearly autofluorescent in both of the two Commelinoid species (*C. communis* and *Z. mays*; Fig. 5a–d). However, in both species autofluorescence is clearly most intense in the guard cell walls particularly in the ventral walls adjacent to the stomatal pore in *C. communis*, and the dorsal walls of guard cells in *Z. mays*. In these two species, autofluorescent material is also evident at the opposing poles of the stomata where the

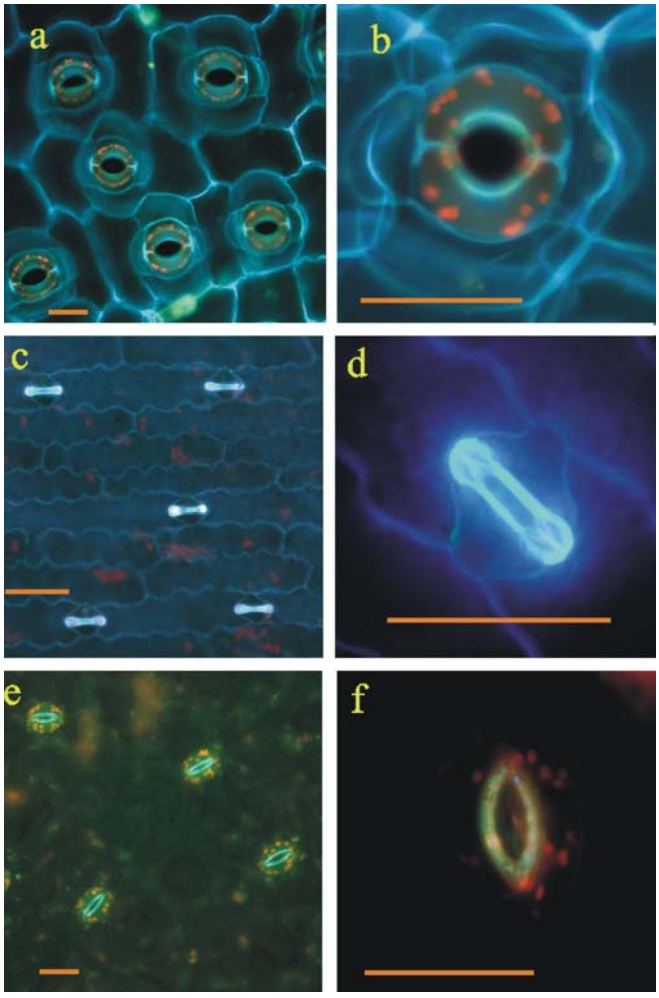

Fig. 5 a–f Distinctive UV autofluorescence associated with guard cells in three plant species, *C. communis* (**a, b**), *Z. mays* (**c, d**), and *V. faba* (**e, f**). Epidermal strips were visualised with a Microphot FXA microscope (Nikon) illuminated with a mercury lamp in the presence of a 365/10 nm excitation filter

two guard cells are fixed to one another. In contrast, in the dicot plant (*V. faba*) the only autofluorescence apparent throughout the epidermis is restricted to the guard cell walls, where it forms a bright ring in the walls bordering the stomatal pore (Fig. 5e, f).

Arabinanase and feruloyl esterase treatment impairs stomatal opening in *V. faba* and *Z. mays*

The observation that guard cell walls can be distinguished from other epidermal cell walls on the basis of pectic components using FTIR analysis may suggest that pectins have a conserved functional role in guard cells of all three species examined here. Previous work has shown that stomatal guard cell function in *C. communis* was dependent on the properties of pectins in the cell walls (Jones et al. 2003), and that treatment of epidermal strips with arabinanase or feruloyl esterase inhibited stomatal opening. In contrast, in strips treated with pectin methyl esterase (PME) and endopolygalacturonase (EPG), the stomata opened wider than those in untreated strips. To assess whether these treatments were similarly effective in *V. faba* and *Z. mays*, epidermal strips from both species were incubated with enzymes prior to being treated with fusicoccin to induce stomatal opening following experimental procedures described in Jones et al. (2003). Stomatal apertures were recorded after 2 h in the presence of fusicoccin. Treatment with either PME or EPG alone had little effect on the ability of stomata to undergo fusicoccin-induced opening in either *V. faba* (Fig. 6a) or *Z. mays* (Fig. 6b). In contrast, incubation with a combination of PME and EPG caused the stomatal pore to open wider when treated with fusicoccin than those of control strips. This effect was observed in both species but to a lesser extent in *Z. mays*. These data indicate that depolymerisation of methyl-esterified homogalacturonan is responsible for the observed increases in stomatal aperture seen in both species. In contrast to this observation, treatment of epidermal strips with (1-5)-α-L-arabinanase (arabinanase) severely impeded the ability of the stomata to open in response to fusicoccin in both species. Similarly, treatment with feruloyl esterase also hindered fusicoccin-induced opening but this effect was not as severe as that seen following arabinanase treatment. These results are very similar to those previously reported for experiments with epidermal strips of *C. communis* (Jones et al. 2003). However, whilst galactanase treatment in *C. communis* had no significant effect on stomatal function, this was not the case in *V. faba* and *Z. mays* (Fig. 6a). In both species, galactanase treatment consistently led to a very slight but significant reduction in stomatal aperture following fusicoccin-induced opening.

These results demonstrate a striking commonality in the effects of wall-modifying enzymes on stomatal functioning in all three species, suggesting conservation in the importance of specific wall polymers in guard cell function.

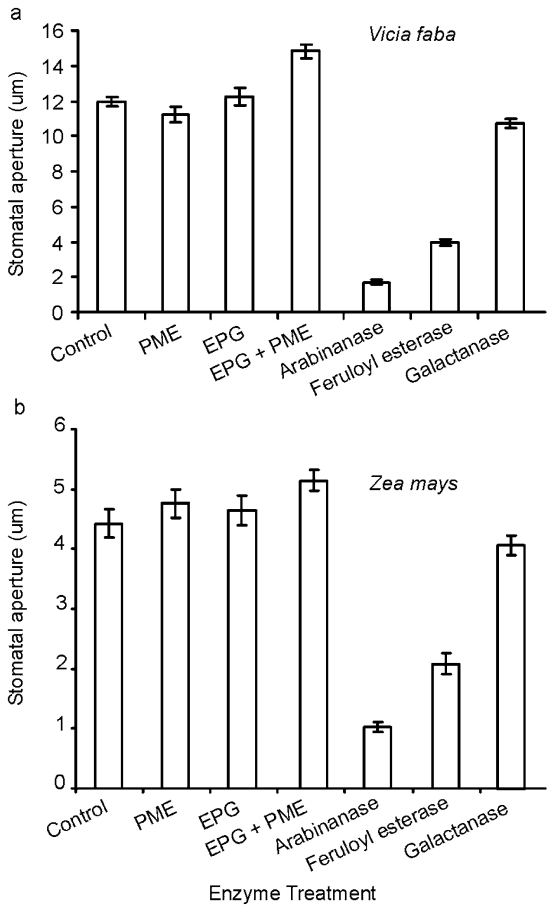

Fig. 6 a, b The effects of pectin-modifying enzymes on stomatal opening in epidermal strips from *V. faba* (**a**) and *Z. mays* (**b**). Epidermal strips were incubated in the dark with PME, EPG, arabinanase, feruloyl esterase, galactanase or buffer only (control) for 1 h before being treated with fusicoccin to induce stomatal opening. All incubations used 10 units per ml of enzyme, except for feruloyl esterase, which was used at 1 unit per ml. Values presented are for stomatal apertures following fusicoccin treatment; data are averages and standard errors from measurements of a total of 100 guard cell pairs (from three separate strips) for each. Overall experiments were repeated three times with similar results

Discussion

Comparisons of FTIR spectra revealed that in three divergent angiosperm species guard cell walls could be distinguished from the walls of other epidermal cell walls on the basis of being specifically enriched in phenolic esters. Previous work has shown that enzymatic disruption of arabinans, feruloyl esters and homogalacturonan in guard cell walls of *C. communis* has direct effects on the ability of stomata to open or close, indicating the importance of pectins and phenolics in these cell walls (Jones et al. 2003). Here, we show that very similar effects to those seen in *C. communis* are also seen in the morphologically distinct guard cells of a dicot (*V. faba*) and a monocot (*Z. mays*).

Given the clear effects of pectin-modifying enzymes on stomatal function in all species examined so far,

this may suggest that the specialised function of guard cells is intimately associated with a unique wall composition.

Fourier-transform infra-red micro spectroscopy allows the comparison between wall composition and structure at the level of single cells and is therefore ideal for pinpointing any clear differences between guard cell and non-guard cell walls. FTIR micro spectroscopic analysis showed that guard cell walls could be separated from other epidermal cells on the basis of similar spectral components in all three species indicating a common specialisation in wall structure in these cells. In all three species, phenolic esters appeared to be significantly more abundant in guard cell walls than in those of other epidermal cells.

Because of the relative abundance of stomata on the lower epidermis, the upper epidermis contains approximately 85% fewer stomatal pores in comparison to the lower epidermis; we compared the monosaccharide composition of non-cellulosic polysaccharides from cell walls of the upper and lower epidermis in *C. communis*. Any dramatic difference in wall composition between guard cell walls and non-guard cell epidermal cell walls should become apparent when directly comparing these two different types of tissue. This, however, was clearly not the case as no striking differences in overall wall composition were evident between upper and lower epidermal cell walls for this species. The general similarities between wall composition of guard cells and epidermal cells were also evident from the FTIR studies (see Fig. 1). What was apparent from these compositional analyses however, was that whilst the components of the *V. faba* walls were consistent with a type I cell wall and those of *Z. mays* with a type II wall, the cell walls of *C. communis*, appeared to share features characteristic of both types of wall.

An obvious characteristic common to guard cells in all three species is the presence of autofluorescent material in their cell walls. In the commelinoid species this is manifested as bright blue fluorescence under UV light characteristic of ferulic acid esters (Harris and Hartley 1976). Ferulic acid esters are common constituents in type II primary cell walls and both *C. communis* and *Z. mays* exhibit UV autofluorescence throughout their walls. This fluorescent material is, however, particularly prominent in the guard cell walls. Phenolic esters are more minor constituents of type I cell walls and it is notable that when epidermal strips from *V. faba* are viewed under UV light, the only obvious autofluorescence is emitted from stomatal guard cells and is present along the walls facing the pore, effectively forming a ring of blue/green fluorescence. Fry (1982, 1983) showed that pectins from spinach cells are decorated with phenolic esters on terminal arabinosyl and galactosyl residues of neutral side chains of RGI and suggested that these might play a role in crosslinking these polymers, and similar roles for feruloyl esters in the formation of crosslinks between wall polymers have also been proposed by Ishii (1997). Recently, Levigne et al. (2004) showed that arabinan from sugar beet pectins can contain both *O*-5 and *O*-2 linked feruloyl esters, and Jones et al. (2003) predicted that effects caused by arabinanase and feruloyl esterase treatment on *C. communis* guard cells indicated that arabinans in RGI may be linked through feruloyl diesters. The fact that feruloyl esterase treatment has a similar inhibitory effect on guard cell movements in all three species examined suggests a comparable functional role for phenolic groups in these walls.

In all three species treatment with a combination of pectin methyl esterase and endopolygalacturonanase caused stomata to open to a significantly greater extent following fusicoccin-induced opening in comparison to untreated cells. In contrast, arabinanase treatment severely impaired opening and feruloyl esterase similarly, but less severely, restricted opening. As has been previously proposed (Jones et al. 2003), these data highlight the importance of the pectic network in the functional properties of guard cell walls, indicating that homogalacturonan interactions serve to constrain wall extensibility and that arabinans maintain fluidity in the pectin network. It is interesting to note that Majewska-Sawka et al. (2002) observed, using immuno-localisation studies, that esterified homogalacturonan epitopes were abundant in sugar beet guard cell walls whilst no epitopes for blocks of de-esterified pectins were detected in these walls. In our studies, both pectin methylesterase and polygalacturonanase in combination were required in order to obtain the gaping effects observed following homogalacturonan degradation. This strongly suggests that the homogalacturonans underlying this effect exist in a methyl-esterified form in guard cell walls as polygalacturonanase can only efficiently hydrolyse de-esterified homogalacturonan (Chen and Mort 1996). Taken together these data indicate similar roles for homogalacturonan, arabinans and phenolic esters in guard cell function in all three species. The only exception to the observed similarities was that galactanase treatment also impaired opening of *V. faba* and *Z. mays* stomata although not as severely as arabinanase or feruloyl esterase treatment. It is generally held that both arabinans and galactans are commonly present as side chains of RGI (Ridley et al. 2001; Willats et al. 2001; Vincken et al. 2003) and such "hairy regions" in pectins might prevent the association of neighbouring regions of homogalacturonan. It seems feasible that galactans play a similar role to arabinans, although less influential, in guard cell walls of *V. faba* and *Z. mays*. In this context, it is interesting to note that Skjot et al. (2002) recently found that the expression of a fungal arabinanase gene in transgenic potato plants had a severe impact on growth and development, whereas the expression of a galactanase gene had no such detrimental effects, indicating, perhaps, a more significant role for arabinan degradation than of galactan degradation on the functional properties of the walls. The fact that galactanase had no detectable effects on stomatal function in *C. communis* may simply reflect the low abundance of

galactans in these tissues as suggested by the analysis of wall monosaccharides.

Previous studies have shown correlations between the presence of arabinan epitopes and specific cell types and stages of development. For example, Willats et al. (1999) showed that LM6 monoclonal antibodies recognising linear arabinans labelled proliferating cells in carrot roots but not those that had ceased elongation. Similar studies in tomato fruit showed that the LM6 epitope was greatly reduced in *cnr* mutant fruit as compared to wild type (Orfila et al. 2001). Fruit of *cnr* mutant plants exhibit greatly reduced cell to cell adhesion and it was suggested that arabinans might have a role in adhesion. A more recent study (Iwai et al. 2001) showed that pectic arabinan levels were much lower in the cell walls of loosely attached tobacco callus cells as compared to more tightly associated cells. Whilst such correlations may suggest a role for arabinans in cell-to-cell adhesion, this has yet to be directly demonstrated.

Feruloylated oligosaccharides have been shown to have inhibitory effects on growth and were proposed to act as signalling compounds in plants (reviewed in Ishii 1997). Similarly, it has also been shown that oligogalacturonides can cause reductions in stomatal apertures mediated through changes in calcium and H_2O_2 levels (Lee et al. 1999). Thus, it is possible that our observed effects of enzyme treatments on stomatal aperture may result from the signalling properties of oligosaccharides released during wall digestion. Two observations suggest it unlikely that the effects on stomatal aperture that we report are due to oligosaccharide-induced effects. Firstly, the effects reported for oligogalacturonides by Lee et al. (1999) involved reductions in stomatal aperture. In our experiments, such reductions in aperture from treatments likely to generate oligogalacturonides (polygalacturonase treatments) were not observed, rather our treatments led to increased aperture following an opening treatment. Secondly, experiments in *C. communis* showed that when epidermal strips were treated with arabinanase and then treated with osmotica, they failed to close, whereas pores in control strips closed readily (Jones et al. 2003). Osmotic treatments induce stomatal closure by reducing cell turgor by physical means independent of signal transduction and cell physiology implying that the effects observed result directly from modifications of the cell wall.

The data described here demonstrate that the effects of cell-wall modification on guard cell function previously reported in *C. communis* are not a peculiarity of this species, and more importantly shows that these effects are common to plants with either type I or type II cell walls. This is a significant observation since pectins form only minor components in type II cell walls and as such might not be considered to be as important from a functional point of view as they are in type I walls. Indeed, compositional analysis of wall sugars confirmed that galacturonic acid represents greater than 30% of non-cellulosic wall monosaccharides in the dicot *V. faba* with a typical type I wall. In contrast, the grass species *Z. mays* with a type II wall is composed of less than 5% galacturonic acid.

In conclusion, we have shown that there appears to be a conserved functional role for pectins in determining guard cell-wall properties throughout contrasting Angiosperm species and that this role is also evident in plants with type II cell walls, which characteristically contain only low levels of pectin. Whilst no obvious differences in overall sugar composition of wall polysaccharides were evident from tissues enriched with guard cells, FTIR analysis revealed clear enrichment of pectin phenolic esters in guard cells of all three species. Both arabinanase and feruloyl esterase treatment impaired stomatal opening in all three species, and phenolic groups are often associated with wall arabinosyl or galactosyl residues (Oosterveld et al. 2001). Our data suggest that the functional role of pectic arabinan and phenolic esters in guard cell walls is conserved in morphologically distinct stomata and in different angiosperm species with opposing cell-wall types. We suggest that both components are important in maintaining pectin fluidity in guard cell walls.

Acknowledgements Louise Jones and Jennifer L. Milne contributed equally to this research. This work was supported in part by funding to CNAP by The Garfield Weston Foundation and in part by a Ph.D. Studentship from the BBSRC. We thank Dr. Craig Faulds (Institute of Food Research, Norwich, UK) for kindly supplying us with recombinant feruloyl esterase.

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