Salt stress upregulates periplasmic arabinogalactan proteins: using salt stress to analyse AGP function*

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Summary

• Arabinogalactan proteins (AGPs) are implicated in cell expansion by unknown mechanisms, thus AGP content and cell-expansion rate might be correlated.
• We used Yariv reagent to quantify release rates and distribution of AGP at the cell surface of tobacco BY-2 cells: plasma membrane (*M*); soluble periplasmic AGPs released by cell rupture (*S*); cell wall (*W*); and growth medium (G_{sink}).
• In contrast to earlier reports, we observed massive upregulation of AGPs in salt-stressed cells, and hence the absence of a simple, direct cause-and-effect relationship between growth rate and AGP release. There was a more subtle connection. A dynamic flux model, $M \rightarrow S \rightarrow W \rightarrow G_{sink}$, indicated that turnover was nondegradative, with little free diffusion of AGPs trapped in the pectic matrix of nonadapted cells where transmural migration of high molecular-weight AGPs occurred mainly by plug flow (apposition and extrusion). In contrast, however, an up to sixfold increased AGP release rate in the slower-growing salt-adapted cells indicated a greatly increased rate of AGP diffusion through a much more highly porous pectic network.
• We hypothesize that classical AGPs act as pectin plasticizers. This explains how β-D-glycosyl Yariv reagents might inhibit expansion growth by crosslinking monomeric AGPs, and thus mimic an AGP loss-of-function mutation.

Key words: arabinogalactan proteins (AGPs), cell-wall expansion, periplasm, salt-stress, Yariv reagent.

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Introduction

Arabinogalactan proteins (AGPs) are a subset of the hydroxyproline (Hyp)-rich glycoproteins (Lamport & Northcote, 1960) of the plant cell surface (Larkin, 1978), where they play vital but elusive roles in plant growth and development (Gaspar *et al.*, 2001). Hence AGPs are of current interest, but a particular challenge as there are no animal homologs to aid in their functional analysis.

In the broad sense, AGPs comprise numerous cell-surface glycoproteins that contain arabinogalactan polysaccharides. However, the physical bulk of AGPs consists of a well defined group of 'classical' AGPs (Mau *et al.*, 1995; Du *et al.*, 1996), represented by a relatively small multigene family (Schultz *et al.*, 2002) encoding extended polypeptides with numerous *O*-Hyp-arabinogalactan polysaccharide substituents that form a hyperglycosylated AGP domain (Zhao *et al.*, 2002) sandwiched between a secretory signal sequence at the N-terminus, and a C-terminal hydrophobic sequence that directs the addition of a glycosylphosphatidylinositol (GPI) lipid anchor (Schultz *et al.*, 2000). Unlike extensins, classical AGPs do not form covalent networks, but are initially tethered to the plasma membrane by the lipid anchor (Oxley & Bacic, 1999; Sherrier *et al.*, 1999; Svetek *et al.*, 1999). Thus, in rapidly growing cells, cleavage of the anchor continuously releases soluble AGP monomers that then migrate from the plasma membrane, through the cell wall, and into the growth medium that surrounds cultured cells, or into the middle lamella and intercellular space of cells *in planta*.

*A preliminary account of this work was presented at the Xth International Cell Wall Meeting.

A possible role of classical AGPs in cell expansion (Willats & Knox, 1996), for example as an 'intramural lubricant' (Schopfer, 1990; Schindler *et al.*, 1995; Van Hengel & Roberts, 2002), can in principle be tested by quantitative comparison of AGP-release rates in fast- and slow-growing cells. If growth rates and AGP production are coupled in a functional way, one might expect AGPs to be released more rapidly from fast-growing than from slowly growing cells. Therefore we quantified the release rates of AGPs from rapidly growing tobacco BY-2 cells for comparison with the slower-growing BY-2 cells when adapted to salt. These salt-stressed cells, reportedly with lower AGP levels of plasma membrane-bound AGPs (Zhu *et al.*, 1993a), seemed an attractive system for defining the precise quantitative distribution of AGPs and their role at the cell surface. As previous results of pulse-chase labelling to quantify AGPs are fraught with problems of interpretation (Takeuchi & Komamine, 1980; Gibeaut & Carpita, 1991; Darjania *et al.*, 2000), we chose to exploit the sensitivity, and versatility of the β-D-glycosyl Yariv reagent (Yariv *et al.*, 1962) which crosslinks 1,3-β-linked arabinogalactan proteins with a high specificity (Serpe & Nothnagel, 1995) and allows both sensitive colorimetric assay and gravimetric assay of total AGPs.

Here we report that Yariv reactivity enabled us to determine the quantitative distribution, release rate and turnover of AGPs in rapidly growing tobacco BY-2 cells for comparison with slower-growing salt-stressed cells. We also compared salt-adapted and nonadapted cells of *Acacia*, *Arabidopsis* and tomato (*Solanum lycopersicum*). Paradoxically, salt stress dramatically upregulated total AGPs four- to sixfold in the slower-growing, salt-adapted BY-2 cells which, however, contained much lower levels of plasma membrane-bound AGPs than nonadapted cells, apparently supporting a correlation between membrane-bound AGPs and growth rate (Zhu *et al.*, 1993a). However, this correlation did not hold for soluble AGPs. Indeed, we observed that sonic disruption released most of the cellular AGP from intact cells, but significantly not from protoplasts. Sonically solubilized AGPs were therefore major soluble components of the plasma membrane–wall interface, identified here as soluble periplasmic. Thus we propose a dynamic flux model for classical AGPs, based on their successive transfer from a GPI-anchored, membrane-bound pool (M) to the soluble periplasmic pool (S), that then enters a pool trapped in the expanding wall (W) until their release into the growth-medium sink: $M \rightarrow S \rightarrow W \rightarrow G_{sink}$.

Based on this model, we reconsider two general roles for classical AGPs. Firstly, as a periplasmic cushion they may stabilize plasma membranes subjected to high internal hydrostatic pressures (Serpe & Nothnagel, 1999). Secondly, we postulate a role for AGPs *in muro* as pectic plasticizers that loosen the pectic network, although insufficiently for rapid cell expansion of slow-growing salt-adapted cells, despite a turgor pressure severalfold higher than nonadapted cells (Iraki *et al.*, 1989b; Longstreth *et al.*, 2004). Presumably, crosslinked cellulosic and extensin networks are the major restraints to expansion growth of salt-adapted cells.

Mateials and Methods

Culture growth and adaptation to salt

We grew tobacco BY-2 cells in 25-ml aliquots of Murashige and Skoog (MS) medium containing 0.2 ppm 2,4-dichlorophenoxyacetic acid (Murashige & Skoog, 1962) on a rotary shaker at 120 rpm at 25°C under laboratory lighting, and adapted to 2% salt by initial transfer to media containing 1% NaCl for several weeks and then to 2% NaCl-yielding adapted lines cultured for at least 6 months. *Arabidopsis* cultures were transferred directly to MS medium containing 0.5% NaCl, but could not be adapted to 1% NaCl. Other species were transferred directly to MS medium containing 1% NaCl, and adapted well. Other cultures used were tomato (Bonnie Best), *Arabidopsis thaliania* (Columbia), and *Acacia senegal* from Professor J.-P. Joseleau (Centre de Recherches sur les Macromolecules vegetales, CNRS, Grenoble, France). All experiments with salt-adapted cells were performed in growth medium containing the appropriate NaCl content.

Cell fractionation and isolation of periplasmic AGPs

Cells were filtered on a sintered funnel and washed rapidly with 1% NaCl to remove loosely bound macromolecules. Weighed cell aliquots were transferred to 2-ml microtubes and broken by sonication in 1 ml 1% NaCl (ice-cold) at low power using a microprobe for 60 s. Walls were pelleted by low-speed centrifugation and washed exhaustively with 2% NaCl and distilled water to yield wall preparations (fraction I) essentially free from starch grains, organelles and other similarly sized particles, judging by optical microscopy; the salt washes contained no AGPs and were discarded. The initial low-speed supernatant was ultracentrifuged for 30 min at 150 000 ***g*** to yield a supernatant (fraction II) containing soluble periplasmic AGPs, which were then assayed colorimetrically by precipitation with the Yariv reagent as described below.

Weight recovery of classical AGPs

Classical AGPs for gel-filtration assays and for use as mixed AGP standards were recovered after clarification of tobacco BY-2 growth medium by high-speed centrifugation, precipitation with excess Yariv reagent in 2% v/v NaCl, and dissociation of the Yariv–AGP complex via reductive cleavage of the diazo linkage with sodium dithionite, followed by dialysis and freeze-drying as described previously (Gao *et al.*, 1999).

Gel filtration of AGPs via Superose-6

Recovered AGPs originating either from the periplasm or the growth medium were separated via analytical Superose-6 gel

filtration on a column (Pharmacia 30 × 1 cm) eluted with 0.2 M pH 7 phosphate buffer (0.05% sodium azide) at 500 µl min^{-1}, and monitored at 220 nm as described earlier (Everdeen *et al.*, 1988).

Colorimetric assay of AGPs with Yariv reagent

We used the readily available gum arabic as a standard AGP, and obtained a correction factor by comparing its average color yield of AGPs isolated from the growth medium of tobacco BY-2 cells.

We assayed the total cell surface AGP directly (designated T_d) by direct reaction of 30–100 (±0.1) mg f. wt intact washed cells incubated with 200 µg β-D-galactosyl Yariv diazo dye for 1 h in 1% NaCl, or with the α-D-galactosyl reagent as a control. Isolated cell walls, soluble fractions, pectolyase-treated cells, and protoplasts from similar weights of cells were similarly incubated. Yariv reagent does not permeate the intact plasma membrane, but associates specifically with the arabinogalactan side chains of cell-surface AGPs (Shpak *et al.*, 1999), forming a salt-insoluble complex that dissociates rapidly in 20 mM aqueous NaOH (Jermyn & Yeow, 1975). Thus, after adding 1 ml 20 mM NaOH to salt-washed AGP–Yariv complexes, the absorbancy of the soluble dye at 457 nm (chosen to avoid phenolic interference at shorter wavelengths) quantified the total cell-surface AGP content (T_d) of intact cells.

The relation $T = M + S + W$ defines the relative amounts of 'bound' cell-surface AGPs and tests the overall validity of the assay: T defines the total cell surface AGPs either measured directly by assay of intact cells (T_d) or indirectly (T_i) by separate assays of $M + S + W$; where M represents AGPs firmly bound to the plasma membrane; S represents soluble AGPs released by sonic disruption of intact cells; and W represents AGPs retained by an exhaustively washed cell-wall fraction. The assay sensitivity was approx. 25 ng.

Quantifying cell-surface AGP fractions

We weighed cell aliquots (30–100 ± 0.1 mg f. wt) in microtubes either for immediate assay (by reaction of the intact cells with 200 µg Yariv reagent for 1 h in 750 µl 1% NaCl at room temperature), or subsequent assay of the two major fractions, I and II, obtained after 60 s sonic disruption. Fraction I: cell walls (recovered quantitatively by low-speed centrifugation and washed by 10–12 centrifugations and resuspension in 1% NaCl). Fraction II: soluble AGPs (that remained in the sonicate supernatant after ultracentrifugation for 30 min at 150 000***g***).

We quantified soluble periplasmic (S) and membrane-bound (M) periplasmic AGPs (µg AGP g^{-1} cells, f. wt) by three methods.

Method 1: via pectolyase (M_{PL}) This involved the use of 100 µg ml^{-1} pectolyase (Seishin Co., Tokyo) to release both wall-bound (W) and soluble periplasmic (S) AGPs from intact cells within 60 min. Thus after centrifugation the cell-bound AGPs that remained insoluble were quantified with the β-D-galactosyl Yariv reagent to yield a direct estimate of plasma membrane-bound AGPs ($M_{PLdirect}$). An indirect estimate of M based on pectolyase-solubilized AGPs ($M_{PLindirect}$), could also be estimated from the relation: $M = T_d$ − (AGPs released by pectolyase: $S + W$).

Method 2: via protoplasting (M_{pr}) This method estimated M by direct assay of protoplasts with the Yariv reagent.

Method 3: via sonication (M_s) This involved the use of sonic disruption to release soluble AGPs (S) within 60 s; it also allowed assay of AGPs tenaciously bound to the wall fraction isolated after exhaustive (10–12) salt washes (W). Thus sonication further tested the relation: $M = T_d - (S + W)$, including the possibility that pectolyase might create an artifact by releasing GPI-anchored AGPs. However, assay of T_d followed by sonic disruption and separate assays of both S and W yields M_s, probably the most reliable estimate of M.

Protoplast preparation

We used a method optimized for BY-2 cells (Horemans *et al.*, 1998) to prepare protoplasts from nonadapted cells, and adapted BY-2 cells by incubation with 1% cellulase R-10 (Yakult Honsha Co., Tokyo) and 0.1% pectolyase Y-23 from *Aspergillus japonicus* (Seishin Co., Tokyo) in 0.4 M mannitol containing MS salts.

Yariv reagents

These were synthesized by the diazotization of phloroglucinol with the α- and β-D-galactosides, respectively, of *p*-aminophenol (Yariv *et al.*, 1962).

Osmotic shock

BY-2 cells were collected and rapidly washed by filtration and then resuspended in distilled water for 1 h before assay of AGPs released.

Cell-wall water content

Walls centrifuged at approx. 5000***g*** on preweighed microporous filters gave the hydrated weight. They were then freeze-dried to obtain the dry weight.

Hydroxyproline assay

This involved acid hydrolysis followed by oxidation with hypobromite and reaction with acidic Ehrlich's reagent, as described previously (Lamport & Miller, 1971).

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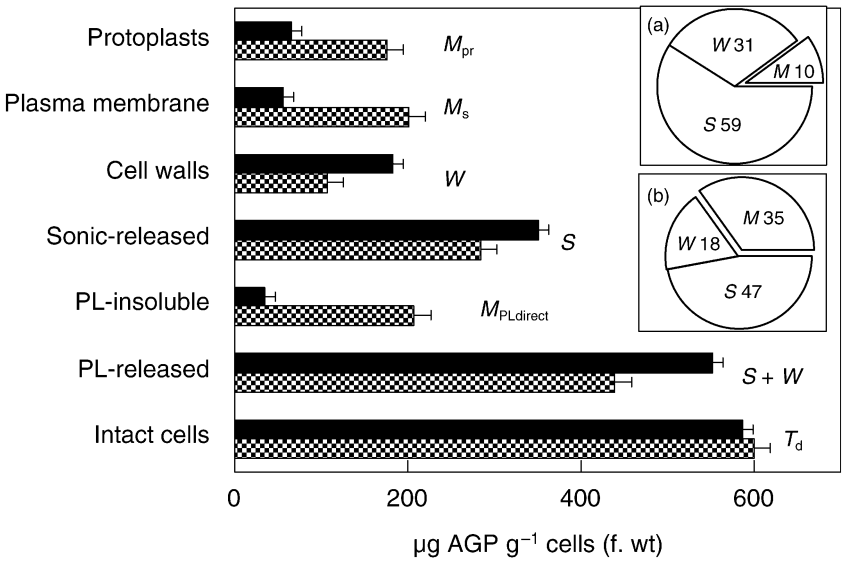

Fig. 1 Arabinogalactan protein (AGP) distribution in tobacco BY-2 cells adapted to growth in 2% NaCl and nonadapted controls. Solid columns, salt-adapted; hatched columns, nonadapted controls. Protoplasts (M_{pr}), AGPs remaining bound after approx. 2 h treatment with cellulase/pectolyase. Plasma membrane (M_s), PM-bound AGPs calculated from the relation $M = T_d - (S + W)$. Cell walls (W), AGPs assayed in the isolated wall fraction. Sonic-released (S), soluble AGPs released by ultrasonic cell disruption. PL-insoluble, AGPs remaining bound to cells after treatment with pectolyase also reflect PM-bound AGPs, hence $M_{PLdirect}$. PL-released, soluble AGPs released by pectolyase treatment of intact cells reflect soluble periplasmic AGPs plus AGPs *in muro* ($S + W$). Intact cells (T_d), AGPs that remain bound to washed cells. Error bars, 1 SE. Each data point represents a minimum of five separate experiments using 7-d cultures of salt-adapted cells and 7-d cultures for controls. Note similar values for AGPs in protoplasts, plasma membrane and the pectolyase-insoluble residue of nonadapted control cells, but significantly lower values for plasma membrane-associated AGPs in 2% salt-adapted cells (340 mM NaCl). Insets: M_s, S and W as a percentage of T_d: (a) salt-adapted; (b) control cells.

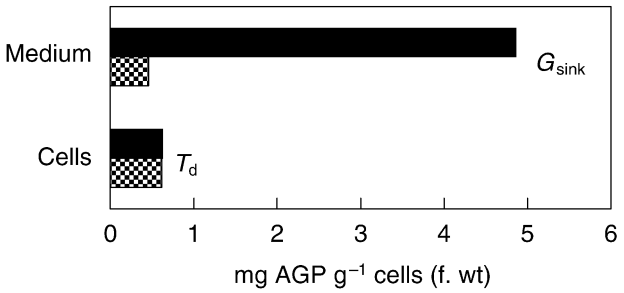

Fig. 2 Colorimetric assay of arabinogalactan proteins (AGPs) in tobacco BY-2 cells and growth medium. For comparison with the AGP content of intact cells (T_d), we expressed the AGP content of the growth medium (G). Note the dramatically increased biosynthesis and release of AGPs by 2% salt-stressed cells compared with nonadapted cells. Solid columns, salt-adapted cells; hatched columns, nonadapted controls. Data from single representative experiments.

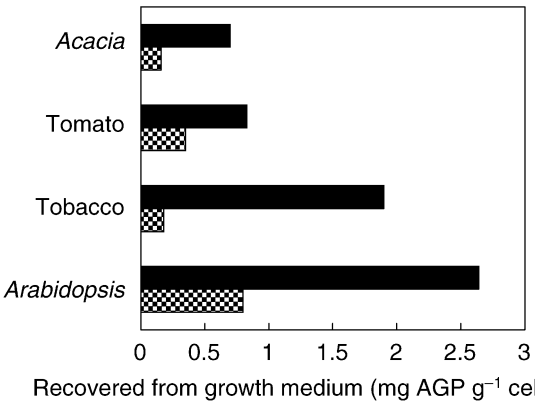

Fig. 3 Gravimetric assay of arabinogalactan proteins (AGPs) isolated from growth medium of salt-adapted compared with nonadapted controls. Weights of AGPs recovered from the growth medium of nonadapted and salt-adapted *Acacia* (1% NaCl), tomato (1% NaCl), tobacco (2% NaCl) and *Arabidopsis* (0.5% NaCl) cells. AGP yield expressed as mg AGP g^{-1} cells (f. wt) of cultures harvested after approx. 14 d. This enables a comparison of AGPs released by nonadapted and salt-adapted cells. Data from single representative experiments. Solid columns, salt-adapted; hatched columns, nonadapted controls.

Results

AGPs assayed colorimetrically and by weight recovery

Based on the known ability of the β-D-galactosyl Yariv reagent to bind both aqueous (Jermyn, 1978) and cell-bound AGPs (Majewska-Sawka & Nothnagel, 2000) (see Materials and Methods), we quantified freely soluble and 'bound' AGPs (Figs 1–3) of intact cells grown in suspension culture. Typically 20 µg tobacco AGPs and gum arabic gave color yields of approx. 530 and 800 milli-absorbance units (mAUs), respectively, at 457 nm, yielding a correction factor of 1.52 for conversion of absorption units to µg tobacco AGPs. Color

yields of AGPs in 5% trichloroacetic acid (TCA) were 5–10% lower.

Larch arabinogalactan (LAG) did not coprecipitate with the Yariv reagent; indeed, LAG actually decreased the color yield by 26% when added in 25-fold excess (500 μg) to 20 μg gum arabic.

For accurate total AGP weight recovery from the growth medium of BY-2 cells (Fig. 2) compared with other species (Fig. 3), we used one or more 25-ml cultures to yield 3–10 mg AGPs that were essentially free of other protein and polysaccharide, judging from Superose-6 gel filtration monitored continuously via diode array spectroscopy for peptide-bond absorbancy at 220 nm and scanned over a 190–500 nm range (data not shown). Hydroxyproline contents averaged 6.8 μg Hyp mg^{-1} AGP for *Acacia*, 6.9 μg for tomato, and 6.0 μg for tobacco AGPs.

Adaptation of cell suspensions to growth in salt

Judging from continued growth and enhanced AGP release rates, nonadapted tobacco BY-2 cells adapted to growth in MS media containing 1% NaCl in one passage (approx. 10 d) and adapted to growth in MS media containing 2% NaCl within another passage (data not shown). However, adapted cells were significantly smaller in diameter and grew more slowly, consistent with previous reports (Iraki *et al.*, 1989b). Most experiments involved BY-2 cells adapted to 1 and 2% NaCl for at least 6 months.

AGP distribution in cell-surface compartments: *M*, *S*, *W* and *T*

Figure 1 shows that salt-adapted and nonadapted cells had similar total cell-surface AGP content, but with significant differences in the percentage distribution of *M*, *S* and *W*.

AGPs of the cell surface appeared as both soluble and insoluble forms after cell disruption. Soluble AGPs were in the supernatant fraction (*S*). Insoluble AGPs were firmly attached to the cell wall (*W*) and membrane fractions (*M*). The average AGP content of the isolated cell-wall and the soluble cell fraction (150 000***g*** supernatant) was thus readily assayed on a μg g^{-1} cell fresh weight basis.

The soluble AGPs released by sonication accounted for 47 and 59% of total cell surface AGPs (T_d) in nonadapted and 2% salt-adapted cells, respectively (Fig. 1a,b insets). These soluble AGPs (*S*) were defined operationally as soluble periplasmic because sonically disrupted protoplasts did not release soluble AGPs, although the intact protoplasts retained their plasma membrane-bound AGPs. Furthermore, intact cells exposed to osmotic shock by transfer to distilled water, or gentle suspension in 5% TCA, also failed to release these soluble AGPs. However, control experiments involving disruption of frozen cells by grinding in a pestle and mortar (arguably less vigorous than sonication) also released soluble AGPs (data not shown).

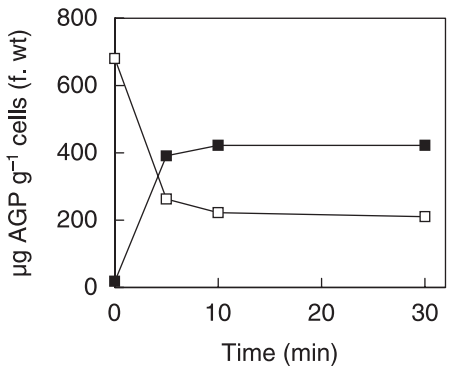

Fig. 4 Arabinogalactan proteins (AGPs) released by pectolyase but retained by protoplasts. Nonadapted tobacco BY-2 cells incubated with pectolyase (■) rapidly released soluble periplasmic and wall-bound AGPs. Cells incubated with cellulase/pectolyase (□) generated protoplasts that retained a bound AGP fraction; presumably these residual AGPs represent glycosylphosphatidylinositol (GPI)-anchored AGPs of the plasma membrane.

AGPs bound to the plasma membrane were estimated by three methods.

(i) M_{PL}: incubation of intact cells with pectolyase (Fig. 4) rapidly released soluble AGPs, presumably corresponding to $(S + W)$. The bound AGPs assayed in the pectolyase-treated cells were considered as plasma membrane-bound ($M_{PLdirect}$ in Fig. 1).

(ii) M_{pr}: AGPs that remained bound to the surface of protoplasts (Fig. 1).

(iii) M_s: membrane-bound AGPs calculated from the relation $M_s = T_d - (S + W)$ based on direct assay of T_d, *S* and *W*.

The three methods gave a range of values for *M* that varied within each cell line by ±15 μg AGP g^{-1} f. wt. However, the difference between nonadapted (M_s, 201 μg) and salt-adapted cell lines (M_s, 56 μg) was approx. 145 μg: the plasma membranes of nonadapted cells contained 3.5-fold more bound AGPs than the salt-adapted cells (Fig. 1), confirming the earlier reported loss of (bound) AGPs from the plasma membrane (Zhu *et al.*, 1993a). Determination of plasma membrane-bound AGPs as M_s, based on its indirect assay as $T_d - (S + W)$, is probably the most accurate as this method is based on the close agreement between T_d and T_i, when the latter is assayed as $(M_{PLdirect} + S + W)$.

Cytosolic AGPs

Intact cells pretreated for 60 min with the Yariv reagent (thus immobilizing all cell-surface AGPs), then washed with 1% NaCl to remove excess reagent, failed to release soluble (cytosolic) AGPs after sonic disruption of the pretreated cells.

AGP concentration in the periplasm of nonadapted cells

We estimated the aqueous concentration (w/v) of periplasmic AGPs from the cell-surface assays (Fig. 1) and the relative

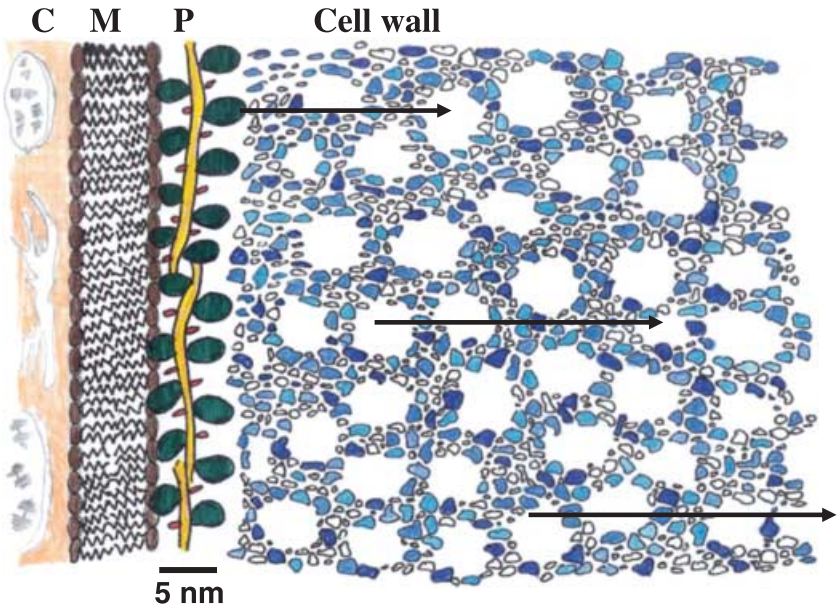

Fig. 5 Relative cross-sectional widths of primary cell wall, periplasmic arabinogalactan proteins (AGP) interface and plasma membrane. P, periplasm, containing arabinogalactan proteins with Hyp-arabinogalactan glycomodules shown as large side chains and Hyp-arabinosides as small side chains of the polypeptide backbone; M, plasma membrane; C, cytoplasm. Bar, 5 nm. Three considerations favor a periclinal orientation of periplasmic AGPs: (1) quantitative recovery of soluble AGPs after cell disruption; (2) the molecular dimensions of AGPs; (3) cell-wall porosity. Arabinogalactan polysaccharide glycomodules (Tan *et al*., 2004) approximate the limiting wall porosity and thus prevent the immediate entry of AGPs freshly cleaved from their glycosylphosphatidylinositol (GPI) anchor. This initially restricts soluble AGPs to the periplasmic space with their long axis parallel to the plasma membrane, ensuring maximal surface coverage. Presumably AGPs enter newly formed wall layers by apposition along with the pectic wall polymers, which leads to tenacious 'binding' of AGPs *in muro* resulting from the arabinogalactan glycomodule anchors trapped in the pectic network, and then move through the expanding wall by plug flow, indicated by arrows.

volumes of periplasm and wall, based on their relative widths (Fig. 5): an approx. 5-nm periplasmic space inferred from the approx. 5 nm width of a typical tobacco AGP (Tan *et al*., 2004) and approx. 100 nm wall (Iraki *et al*., 1989b). Hence, for equal masses of AGP in periplasm and wall, the periplasmic concentration would be 100/5, or 20-fold greater than the wall. But the total periplasmic AGP content of 485 μg g^{-1} f. wt in nonadapted cells (M_s, 201 μg + S, 284 μg) (Fig. 1) was approx. 4.6 times greater than the wall content (106 μg wall AGP g^{-1} cells, f. wt). Therefore the final periplasmic concentration was 20×4.6 or approx. 90-fold greater. The average AGP concentration of 0.2% w/v in hydrated walls was determined from quantitative recovery of walls (11% of cell d. wt); the AGP content of freeze-dried walls approx. 2% of wall d. wt consistent with earlier reports (Serpe & Nothnagel, 1995; Girault *et al*., 2000); and approx. 90% water content of the wall (data not shown). Hence a 0.2% AGP concentration in hydrated walls (×90) yields a final periplasmic concentration of approx. 18% w/v AGP, which is far greater than one can deduce from previous estimates of the AGP content in isolated plasma membranes (Komalavilas *et al*., 1991; Zhu *et al*., 1993a). This is a conservative figure as the centrifugal method is likely to overestimate the cell-wall water content, for example, walls containing 80% water would increase the estimate of periplasmic AGPs to 36% w/v.

In salt-adapted cells, although soluble periplasmic AGPs were significantly elevated (S = 350 μg), membrane-bound AGPs were much lower (M_s = 56 μg), hence the total periplasmic AGP concentration of 406 μg AGP g^{-1} cells (f. wt) (350 + 56 μg) was only slightly lower than the corresponding value of 485 μg AGP g^{-1} f. wt (284 + 201 μg) for nonadapted cells (Fig. 1). Thus salt-adapted cells had a somewhat lower overall periplasmic AGP concentration of approx. 15–30% w/v.

AGP release rates and upregulation in salt-adapted cells

AGPs were released into the growth medium by nonadapted cells and slower-growing salt-adapted cells at markedly different rates, expressed as μg AGP h^{-1} g^{-1} cells (f. wt). Thus typical short-term (approx. 3-h) release rates of approx. 18 μg AGP h^{-1} g^{-1} cells (f. wt) in rapidly growing (log-phase) nonadapted BY-2 cells contrasted with 74 and 114 μg AGP h^{-1} g^{-1} cells (f. wt) in 1 and 2% NaCl-adapted cells, respectively (Fig. 6), indicating a dramatic four- to sixfold increased rate of AGP biosynthesis in adapted cells. Significantly, nonadapted tobacco BY-2 cells transferred directly to growth medium containing 1% NaCl increased their AGP release rate within one passage of 7 d (data not shown). AGP yields from the growth medium, assayed gravimetrically by weight recovery of AGPs obtained by Yariv precipitation, dithionite

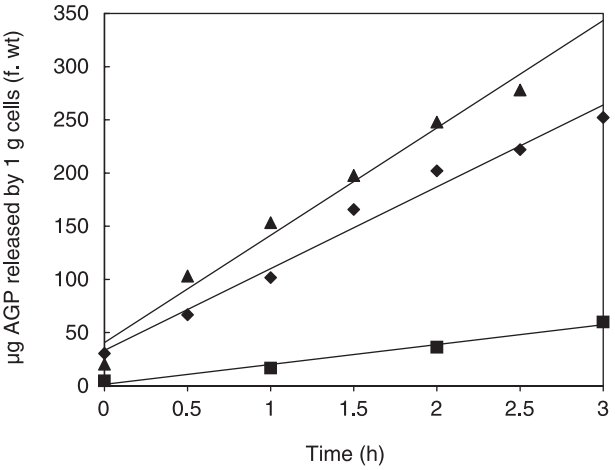

Fig. 6 Time course of arabinogalactan protein (AGP) release from tobacco BY-2 cells adapted to growth in ■, 0; ◆, 1; ▲, 2% NaCl. Results of three representative time-course experiments. Washed cells (100–500 mg aliquots) harvested from nonadapted and adapted cultures were incubated in growth medium salts with appropriate addition of 0, 1 or 2% NaCl. After appropriate times, cells were centrifuged and the supernatant assayed with Yariv reagent.

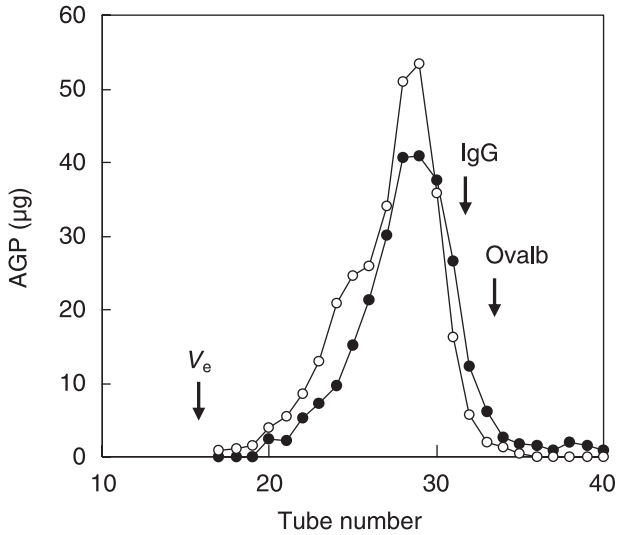

Fig. 7 Superose-6 gel-filtration profiles of arabinogalactan proteins (AGPs) from salt-adapted cells. AGPs isolated from the periplasm (○) and growth medium (●), respectively (see Materials and Methods), of 2% NaCl-adapted tobacco BY-2 cells, were injected on a Superose-6 column (also monitored at 220 nm) Fractions of 500 µl were collected and assayed by adding 100 µl β-D-galactosyl Yariv reagent to each fraction. The bulk of Yariv-precipitable AGPs peaked at a relative retention time of 2 relative to the void and with standards: IgG (167 kDa), 2.3; ovalbumin (45 kDa), 2.6; vitamin B12 (1 kDa), 3.3. Column load was approx. 300 µg AGP obtained from approx. 1 g cells (f. wt) and 2 ml growth medium, respectively. Calculated post-column recoveries of periplasmic AGP and growth medium AGPs were 91 and 101%, respectively. Similar size profiles (not shown) were obtained using nonadapted cells.

reduction, dialysis, freeze-drying and weighing, also corroborated the massive upregulation of AGPs in salt-adapted tobacco cells, showing a 10-fold increase (Fig. 3). These AGPs were further authenticated by Superose-6 fast protein liquid chromatography (FPLC) (Fig. 7).

AGP-turnover rates

Protein turnover usually implies complete degradation and recycling. However, our data argue strongly against degradative turnover of classical AGPs (see Discussion), but are consistent with nondegradative turnover involving AGP flux through successive pools $M \rightarrow S \rightarrow W \rightarrow G_{sink}$ as follows:

An observed release rate of 18 µg AGP h^{-1} g^{-1} f. wt (Fig. 6) corresponds to approx. 1.5% of the total AGPs g^{-1} cells (f. wt) (medium + cells ≈ 1200 µg g^{-1} f. wt, which includes bound and soluble AGPs in the growth medium). This value coincides with the overall biosynthetic rate of 1.5% cell mass increase per h in BY-2 cells, which corresponds to a mean generation (doubling) time of approx. 48 h. Thus nonadapted cells exhibited balanced growth: AGPs increased at about the same rate as total biomass. On the other hand, salt-adapted cells exhibited unbalanced growth, with lower growth rates but much higher short-term AGP release rates, typically 74 and 114 µg AGP h^{-1} g^{-1} cells (f. wt) for 1 and 2% NaCl-adapted cells, respectively (Fig. 6). Assays of total cell-surface AGPs and total weights of AGPs recovered from the growth medium (Fig. 3) corroborated these short-term release rates, confirming the AGP contribution to balanced growth of nonadapted cells. Thus, from the AGP release rates and pool sizes, we calculated approximate nondegradative turnover rates expressed as AGP residence times in the periplasm and wall, as follows:

Under balanced growth conditions, when $G_{sink}/T_d = 1$ the average residence time for AGPs at the cell surface roughly approximates the cell mean generation time (approx. 48 h) before release. In a steady-state system, a molecule remains in a pool for a time that is proportional to the pool size. Therefore the total residence time of a cell-surface AGP is the sum of the times in each cell-surface pool. Thus in nonadapted cells, where an AGP distribution of M = 33%, S = 47% and W = 18% (Fig. 1) corresponds to a total residence time of 48 h, the approximate residence times for each pool correspond to: 16 h for GPI-anchored AGPs (33% of 48 h); 23 h for soluble periplasmic AGPs; and 9 h for AGPs *in muro*.

On the other hand, slower-growing 2% NaCl-adapted cells showed an approx. sixfold increased AGP release rate in short-term experiments (Fig. 6). This much faster AGP turnover in salt-adapted cells is consistent with recovery experiments (Fig. 3) where AGP release by salt-adapted cells ($G_{sink}/T_d = 10$) was approx. 10-fold greater than that of nonadapted cells ($G_{sink}/T_d = 1$). Thus in salt-adapted cells, the total AGP residence time spent at the cell surface before its release into the medium is only 10% (15 h) of the mean generation time

(approx. 150 h). Therefore the AGP distribution in salt-adapted cells, where $M = 10\%$, $S = 60\%$ and $W = 30\%$ (Fig. 1), indicates approximate residence times of 10, 60 and 30% of the total 15-h residence time, equivalent to 1.5 h for GPI-anchored AGPs; 9 h for soluble periplasmic AGPs; and 4.5 h for AGPs *in muro*. These AGP residence times demonstrate much faster transit of AGPs through the walls of salt-adapted than nonadapted cells.

Discussion

Here we discuss the criteria for quantifying cell-surface pools, release rates of AGPs, and the average AGP flux through each pool. This flux is modelled by the logical precursor–product relationship $M \rightarrow S \rightarrow W \rightarrow G_{sink}$: Classical AGPs anchored to the plasma membrane (M) are continuously released by cleavage of their GPI anchor, initially as soluble AGPs (S) in the periplasmic region, before they enter and migrate through the wall (W) until their final release into the growth medium (G_{sink}). We consider how the kinetic data support nondegradative AGP turnover, but not the degradative turnover (recycling) of AGPs proposed previously (Gibeaut & Carpita, 1991). We then relate the distribution and flux of AGPs, in both nonadapted and salt-adapted cells, to their structural and functional analysis at the molecular level. This includes the likely orientation of AGPs at the outer surface of the plasma membrane, and the mechanism of AGP transmural migration. Finally, we discuss specific roles for the large pool of AGPs in the periplasmic region of the plasma membrane–wall interface, and the smaller pool *in muro*.

Yariv assay of free and 'bound' AGPs

Earlier, we applied the relatively simple growth energetics of microbial systems to cell-suspension cultures as a reductionist model of higher plant growth at a cellular level that is comparable with some green algae (Lamport, 1964). Here we extrapolate that approach by quantifying AGPs in three cell-surface compartments by using the expression $T = M + S + W$. Its accuracy depends on the following observations and inferences.

The β-D-galactosyl Yariv reagent is specific for templated AGP glycomodules Although the β-D-glucosyl and α-D-galactosyl Yariv reagents adsorb nonspecifically to cotton that contains secondary cell-wall cellulose (Triplett & Timpa, 1997), nonspecific staining of primary cell-wall cellulose by either the β-D-galactosyl or α-D-galactosyl Yariv reagent was not apparent under our reaction conditions. While the β-D-galactosyl reagent is a good precipitant of AGPs (Jermyn & Yeow, 1975), it did not precipitate the related β-1,3-linked larch arabinogalactan, contrary to an earlier report (Fincher *et al.*, 1983). Larch arabinogalactan (Chandrasekaran & Janaswamy, 2002) differs significantly from AGPs in two respects: firstly, it is not a glycoprotein; secondly its arabinogalactan backbone lacks the acidic side chains (Defaye & Wong, 1986) that typify classical arabinogalactans (Tan *et al.*, 2004). Nor does Yariv reagent precipitate small Hyp-arabinogalactan polysaccharides. Thus for effective crosslinkage by Yariv reagent, arabinogalactan polysaccharides must be templated by attachment to a polypeptide. Most recent work (J. Xu and co-workers, unpublished data) also suggests that rare AGPs with very small glycomodules lacking rhamnose may also be unreactive.

GPI-anchored AGPs are tightly bound to membranes Early addition of the GPI anchor in the endoplasmic reticulum (ER) (Barz & Walter, 1999) results in membrane-bound proteins that are not easily extracted (Grogan *et al.*, 2002) and thus account for the membrane-bound AGPs that resist release by high salt, sonication or hypotonic osmotic shock (Norman *et al.*, 1990). Most cell-surface AGPs are initially GPI-anchored (Eisenhaber *et al.*, 2003; Schultz *et al.*, 2004).

Soluble periplasmic AGPs comprise the bulk of cell surface AGPs The surprising observation that sonically disrupted protoplasts did not release soluble AGPs prompted us to reassess earlier conclusions (Lamport, 1970; Gibeaut & Carpita, 1991) that soluble AGPs released by breakage of intact cells represented a cytosolic or cytoplasmic fraction. As enzymic or mechanical rupture of the cell wall was essential for rapid release of these soluble AGPs, we designate this soluble pool as periplasmic. These AGPs are present at a high concentration and are clearly distinct from firmly bound AGPs of the plasma membrane and AGPs tenaciously retained by exhaustively salt-washed cell wall preparations (Fig. 1). The data indicated that soluble periplasmic AGPs were the largest cell-surface pool, thus providing a significant layer of AGPs at the membrane–wall interface. Soluble AGPs are 'periplasmic' by analogy with the biochemical definition (Mitchell, 1961) of the membrane–wall interface in bacteria (Singleton & Sainsbury, 1987), which is also usefully applied to metaphytes (Iraki *et al.*, 1989a; Herman & Lamb, 1992; Desveaux *et al.*, 1998; Ramassamy *et al.*, 1998; Roy *et al.*, 1998; Samajova *et al.*, 1998; Vincent, 1999; Funke & Edelmann, 2000; Lord *et al.*, 2000; Crews *et al.*, 2003; Schultz *et al.*, 2004).

Additional experiments tested the periplasmic hypothesis by pretreating intact cells with Yariv reagent. This dye rapidly forms an insoluble complex with AGPs at the cell surface, but does not permeate through the plasma membrane. Hence subsequent sonic rupture after washing cells to remove excess Yariv reagent should have released any *bona fide* cytosolic AGPs – but the release of Yariv-precipitable AGP was negligible. This rules out the possibility of cytosolic or vacuolar AGPs as the major source of soluble AGPs released from disrupted cells, and is consistent with tight binding of GPI-anchored proteins and the addition of GPI anchors at an early stage in the ER.

The large pool of soluble AGPs in plant cells therefore further identifies the periplasm as a dynamic cell compartment, study of which has been relatively neglected despite its crucial role in self-assembly of the wall (Vincent, 1999). Presumably other soluble periplasmic components are also amenable to biochemical analysis using the methods described here, particularly those that involve loosening of the pectic matrix.

Absence of ionically bound AGPs *in muro* We also considered the possibility that the soluble AGPs in cell sonicates were released from 'wall sites' (Darjania *et al.*, 2002), for example by ionic desorption. However, salt-washed intact cells suspended in 5% TCA, which should release ionically bound hydroxyproline-rich glycoproteins, did not release AGPs. Thus ionically bound AGPs do not contribute significantly to the soluble AGPs released by sonication; indeed, the greater level of soluble (periplasmic) AGPs in 370-mM salt-adapted cells compared with nonadapted cells (Fig. 1) is also inconsistent with ionic binding, as such binding should decrease with increasing ionic strength. On the other hand, cells treated with pectolyase, which increases pectic porosity, released soluble AGPs rapidly (Fig. 4) and quantitatively equal to $M + S$ (Fig. 1), that is, AGPs were released by increased cell-wall porosity, not by ionic desorption.

Pools with similar AGP profiles give a similar absorbancy with Yariv reagent The sizing data presented here (Fig. 7) and the earlier peptide profiles of AGPs isolated from cell suspension cultured cells (Gao *et al.*, 1999; Sun *et al.*, 2004) show similar profiles of AGPs isolated from the periplasm and growth medium, respectively, for both nonadapted and salt-adapted cells. Although the reaction stoichiometry of individual classical AGPs may vary, the reproducibility and internal consistency of the data suggest a similar overall average Yariv reactivity for the major AGP pools. For example, T_d assayed directly using intact cells, and T_i assayed indirectly as the sum of M, S and W, agreed. In other words, the stoichiometry of AGPs 'bound' at the cell surface and of free AGPs in aqueous solution is similar.

AGP turnover involves transfer through successive pools: $M \rightarrow S \rightarrow W \rightarrow G_{sink}$ The bulk of AGPs accumulating in the growth medium originate from classical AGPs that are transiently attached to the plasma membrane (Gaspar *et al.*, 2001; Schultz *et al.*, 2004; Sun *et al.*, 2004) by a GPI anchor. Phospholipase cleaves the anchor, further evidenced by the detection of C-terminal ethanolamine in released AGPs (Darjania *et al.*, 2002; Sun *et al.*, 2004). Thus release of classical AGPs with approximate parity between AGPs in the growth medium and cell-surface AGPs ($G_{sink}/T = 1$) indicates an AGP flux through cell-surface pools in a logical precursor–product relationship: $M \rightarrow S \rightarrow W \rightarrow G_{sink}$, with measurable steady-state turnover kinetics. Comparison of residence times for plasma membrane-bound AGPs in nonadapted (16 h) and 2% salt-adapted cells (1.5 h) was particularly instructive, as it showed a 10-fold increase in the AGP turnover rate in salt-adapted cells that was reasonably close to the sixfold increase observed by direct assay of short-term release rates (Fig. 6). We note that a turnover rate of 6 h for plasma membrane-bound AGPs in cultured *Arabidopsis* cells was reported earlier (Darjania *et al.*, 2002).

AGP turnover is nondegradative For valid conclusions about AGP function, we need to make a clear distinction between degradative and nondegradative turnover. By definition, protein turnover involves complete degradation and metabolic recycling of the components. A frequently cited paper (Gibeaut & Carpita, 1991) proposed a novel degradative 'turnover cycle for AGPs' in cell cultures where 'a substantial portion must turn over at the cell wall with its sugars returned to the cytosol for synthesis of new polymers'. However, that work was based on cytoplasmic misidentification of the 'buffer-soluble' polysaccharide/AGP fraction released on cell rupture, rather than periplasmic, and was not strictly quantified, with a 3 h pulse-chase that was too brief to quantify AGPs released into the growth medium.

On the other hand, our flux calculations assume AGP stability evidenced by AGP release rates and yields; their large size; relatively narrow size range; and absence of cytosolic AGPs or their lower molecular-weight degradation products (Fig. 7). All these data are consistent with AGP migration through cell-surface pools and their quantitative accumulation in a sink without substantial degradative turnover.

Periclinal orientation of AGPs at the plasma membrane Crowded acidic polysaccharide side-chain substituents constrain polypeptides in an extended conformation (Gottschalk, 1960; Tan *et al.*, 2004). Thus AGPs are highly asymmetrical. For a typical classical AGP such as LeAGP-1, we calculate a length of approx. 60 nm compared with approx. 150 nm measured for the unusually large gum arabic glycoprotein (Qi *et al.*, 1991). Previously we (and many others) had assumed that AGPs diffuse by reptation (Qi *et al.*, 1991) through the wall to the exterior (Zhao *et al.*, 2002). However, the 5 nm pectic porosity approximates the size of small AGP Hyp-polysaccharide glycomodules (Tan *et al.*, 2004). Anticlinal (end-on) insertion would physically trap classical AGPs such as LeAGP-1. Thus pectic porosity excludes the large soluble periplasmic pool of AGPs because they are periclinally oriented (Fig. 5). However, after their addition to the wall by apposition they are quantitatively retained (Fig. 1).

Salt stress upregulates AGPs

Tobacco cells adapt rapidly to salt-induced osmotic stress by increasing their vacuolar salt concentration, ultimately yielding turgor pressures severalfold higher than nonadapted

cells (Iraki *et al.*, 1989b; Zhu *et al.*, 1993a). We assayed the AGP content of these stressed cells, initially prompted by the report that salt stress decreased the growth rate of tobacco BY-2 cells and downregulated AGPs (Zhu *et al.*, 1993a). Although others had reported that 'NaCl-adapted cells released about the same amount of total sugar, with almost equal proportions of AGP as the nonadapted cells' (Iraki *et al.*, 1989a), they did not make the most revealing comparison based on AGP yields per unit of biomass (μg AGPs g^{-1} cells, f. wt). Our data (Fig. 1) confirm the reported decrease in plasma membrane-bound AGPs (Zhu *et al.*, 1993a), but not the decreased AGP accumulation in the culture medium (Fig. 2). On the contrary, salt tress dramatically increased AGP release assayed either as short-term release rates (Fig. 6) or as actual weights recovered (Fig. 3); these data were also corroborated by the 10-fold difference in residence times of plasma membrane-bound AGPs: 16 h for nonadapted, but only 1.5 h for salt-adapted cells, for which much lower levels of membrane-bound AGPs (Fig. 1) are consistent with the short residence time (high AGP-turnover rate) at the membrane needed to sustain the massive increase of AGPs released into the growth medium. Judging from the four cultured species subjected to salt stress (Fig. 3), such upregulation of AGPs in response to hyperosmotic salt stress may be quite a common phenomenon in glycophytes, and is verified by unpublished microarray data for salt-stressed *Arabidopsis thaliana* (J. Kudla, https://www.genevestigator.ethz.ch). This raises the questions of why, and how.

Do periplasmic AGPs stabilize the plasma membrane?

Periclinally oriented AGPs would maximize the surface coverage consistent with the insightful suggestion that 'plasma membrane-AGPs may help to maintain the integrity of the plasma membrane when it is pressed against the wall by turgor pressure' (Serpe & Nothnagel, 1999). This raises the question of precisely how AGPs might protect the membrane.

The large soluble periplasmic pool identified here suggests larger amounts of AGPs associated with the plasma membrane than generally assumed, but is consistent with their cytochemical localization (Schopfer, 1990), including the description of membrane-bound AGPs as a 'plasmalemmal reticulum' (Gens *et al.*, 2000). An extraordinarily high content of very long-chain fatty acids, comprising 22% of total fatty acids (Matthes & Boger, 2002), particularly the C24 lignoceric acid component that characterizes GPI-anchors (Oxley & Bacic, 1999; Svetek *et al.*, 1999), agrees with a high level of plasma membrane loading by AGPs, although AGP levels actually decreased in salt-adapted cells with $M + S = 406$ μg compared with 485 μg in nonadapted cells (Fig. 1). This decrease is consistent with the concomitant increased membrane–wall adhesion (observed when salt-adapted cells are plasmolysed in hypertonic salt; Zhu *et al.*, 1993b) and supports the idea that periplasmic AGPs act as a buffer zone, stabilizing the membrane by electrostatic cushioning (Seitz *et al.*, 1999) that prevents direct interaction of the naked membrane with the wall matrix. Comparative biochemistry also supports a protective role, as animal cells are isotonic and lack AGPs or their homologs, while increased hydrostatic pressure upregulates analogous macromolecules such as aggrecan in animal chondrocytes (Toyoda *et al.*, 2003).

Finally, judging from their nondegradative migration through the wall to the exterior, classical AGPs do not function only at the membrane surface. This raises further questions, notably the mechanism of their upregulation and release from the plasma membrane; the mechanism of their migration; and the role of these migratory molecules *in muro*.

Components of the AGP upregulation cascade

The precise sequence of events leading to the upregulation of AGPs by NaCl is not clear. However, the much higher AGP release rate of salt-adapted cells suggests increased AGP diffusivity via increased pectic porosity that allows loss of soluble periplasmic AGPs to the growth medium. Homeostasis replenishes the depleted pool, probably via activation of cell-surface phospholipase C or D to cleave the GPI anchor, thus releasing AGPs to the exterior while the detached anchor acts as a signal to the interior. This scenario is consistent with rapid activation of phospholipase by hyperosmotic (Munnik *et al.*, 2000; Zhu, 2002) or cold stress (Ruelland *et al.*, 2002), while its inactivation inhibits root-hair growth and radicle emergence (Gardiner *et al.*, 2003). Thus phospholipase activity may regulate the AGP content of polymer blends that characterize different wall types. For example, tip-specific addition of AGPs may enable rapid tip growth of pollen tubes up to 1 cm h^{-1} (Taylor & Hepler, 1997; Roy *et al.*, 1998). Such growth is also correlated with release of an acid phosphatase (Ibrahim *et al.*, 2002), possibly corresponding to phospholipase-dependent release of AGPs from germinating maize pollen (K. Anderson and D.T.A.L., unpublished data). Phospholipase may therefore be a key enzymic component of the AGP upregulation cascade that controls not only AGP release under conditions of salt stress and wound stress (Qi *et al.*, 1991), but also super-fast tip growth.

Transmural migration of AGPs via plug flow and diffusion

Superose-6 FPLC confirms a large M_r that prohibits rapid diffusion of AGPs into the gel or wall matrix, thus accounting for the sizeable soluble periplasmic pool. AGP size profiles of both periplasm and growth medium are similar (Fig. 7), consistent with the similar AGP polypeptide profiles and compositional data reported earlier (Lamport, 1970; Gao *et al.*, 1999; Sun *et al.*, 2004).

Cultured cells present an apparent paradox of transmural migration by a tenaciously retained wall component (Fig. 1), even though it is generally considered to be ionically bound and to migrate by passive diffusion (Gaspar *et al.*, 2001). Nevertheless, the large size and shape of classical anionic AGPs (typified by the approx. 164-kDa glycoprotein LeAGP-1 with a 16.4-kDa polypeptide and approx. 90% carbohydrate) indicates that AGPs are physically trapped *in muro* by a pectic network (Titel *et al.*, 1997) whose limiting porosity approximates the 5-nm axial diameter of AGPs possessing small Hyp-arabinogalactans (Tan *et al.*, 2004). Pectolyase directly increases wall porosity (Baron-Epel *et al.*, 1988) and rapidly released the large periplasmic AGP pool (Figs 4 and 5), supporting the conclusion that pectin porosity *per se* prohibits rapid transmural diffusion of AGPs; similarly also for EDTA (data not shown) that disrupts Ca^{2+}-stabilized pectic networks (Fleischer *et al.*, 1999). How, then, are AGPs released into the growth medium during growth?

A small AGP pool *in muro* with an approximate residence time of 9 h (calculated from the pool size and mean generation time) suggests that anchored AGPs migrate from the innermost wall layer to the outer wall layers largely by an extrusion mechanism that involves 'plug flow' generated by turgor pressure and apposition; as cell expansion thins the wall, addition of new wall layers restores the original width. Thus molecules originally at the inner wall surface move passively through the stretched wall layers until reaching a sufficiently stretched and porous outer wall that finally allows rapid diffusion of AGPs and other soluble polymers into the growth medium.

On the other hand, salt-adapted cells with a much slower growth rate gave surprisingly high AGP release rates, indicating a much more loosely crosslinked pectic matrix, so that AGP transmural migration occurs here more by diffusion than plug flow. This conclusion is consistent with the increased amounts of EDTA-extractable pectin in salt-adapted BY-2 cells (Iraki *et al.*, 1989b, 1989c). We infer a functional role for these transmural migratory AGPs as plasticizers.

AGPs as pectate plasticizers *in muro*

Although consensus on AGP involvement in cell expansion is clear (Schopfer, 1990; Serpe & Nothnagel, 1994; Willats & Knox, 1996; Ding & Zhu, 1997; Park *et al.*, 2003), precisely how such small amounts of AGPs might affect *in muro* properties is unknown. However, the suggestion that binding of AGPs 'might reduce the formation of pectate gels and result in a more extensible wall' (Serpe & Nothnagel, 1999) raises the question of mechanism.

We estimate an AGP-to-pectin weight ratio of approx. 1 : 100 in control cells and approx. 1 : 50 in adapted cells. These figures are significant, however, because very small amounts of monomer plasticize synthetic polymer blends by disrupting the regularity of polymer alignment (Wypych, 2003), hence the suggestion that 'AGPs are normally involved in *the proper alignment of pectins in the wall…*' (Lord *et al.*, 2000) is pertinent. The primary cell wall is a polymer blend and is truly plastic, as it acquires considerable strain before cracking. Thus a previous suggestion of monomeric AGPs as candidates for a plasticizing role (Lamport, 2001) can now be rationalized as a more specific hypothesis: the porosity of the pectic network *in muro* increases when AGPs are upregulated because they decrease pectic alignment and crosslinking. Thus AGPs should enhance cell expansion.

Slow-growing, salt-adapted cells might seem to contradict that simple hypothesis. However, Yariv reagent causes prompt cessation of tip growth (Jauh & Lord, 1996) in rapidly growing pollen tubes (Holdaway-Clarke & Hepler, 2003). This seminal experiment dramatically confirms a role for AGPs in cell expansion. The simplest explanation suggests that Yariv reagent abolishes the plasticizing effect of AGP monomers by converting them into a multimeric AGP–Yariv network that also enhances the load-bearing properties of the cell wall. This mechanism would account for retention of turgor and resumption of pollen tube tip growth on removal of Yariv. It may also account for the deposition of AGPs particularly at the growing tips of moss protonema (Lee *et al.*, 2005), root hairs (Samaj *et al.*, 1999) and pollen tubes (Coimbra *et al.*, 2004), including the specific deposition of LeAGP-1 in tomato pollen tubes. Finally, it may account for defective pollen-tube extension in GPI mutants (Lalanne *et al.*, 2004).

We suggest that fast tip growth may help resolve the paradox of upregulated AGPs in slowly growing cells. Wall expansion is generally a complex function of three interpenetrating networks – cellulosic, pectic and protein – each of which must be loosened to allow extension or expansion growth. However, the hyper-extensible pollen tube tip is a largely pectic network enriched in AGPs. Control of the pectin sol–gel transition point by AGPs would provide a simple mechanism for regulating the rate of tip growth (Jauh & Lord, 1996), but inoperative in AGP-deficient mutants such as *Reb1* with inextensible root hairs (Ding & Zhu, 1997) (Andeme-Onzighi *et al.*, 2002). Thus Yariv effectively mimics an AGP loss-of-function mutant.

Role of AGPs *in planta*

A role for classical AGPs as plasticizers *in muro* seems plausible in cultured cells. However, AGPs are not restricted to the periplasm and primary cell walls of cell-suspension cultures, but are readily extractable from a wide range of plant tissues with both primary and secondary cell walls (Jermyn & Yeow, 1975), including the xylem of flax (Girault *et al.*, 2000), loblolly pine (Loopstra & Sederoff, 1995; Zhang *et al.*, 2003), and tomato (Gao *et al.*, 1999). These AGPs may also be viewed as plasticizers if they facilitate limited lateral movement of cellulose microfibrils, thus enabling woody tissues to flex; an AGP-defective mutant exhibits a brittle

phenotype (McCann *et al.*, 2003) reminiscent of polymers deficient in plasticizer.

There are further ramifications *in planta*, as upregulation of classical AGPs has additional physiological roles. Wound-inducible gum exudates create both interior (Crews *et al.*, 2003) and exterior (Qi *et al.*, 1991) defensive barriers to pathogens; for example, storage carbohydrates of *Taro* tubers are exceptionally rich in AGPs (Jiang & Ramsden, 1999). Furthermore, xylem sap contains AGPs (Lamport, 1977) that appear on the inner surface (G layer) of newly formed tension wood (Lafarguette *et al.*, 2004). These may correspond to the 'xylem conditioners' that influence xylem hydraulic conductance (Zwieniecki *et al.*, 2001); salt-induced AGP upregulation, in conjunction with pectin hypertrophy, may perhaps even contribute to desalination of xylem sap in mangroves (Zimmermann *et al.*, 2002) as well as the fleshy growth habit that typifies maritime plants (Paramonova *et al.*, 2003).

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