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REVIEW: PART OF A SPECIAL ISSUE ON PLANT CELL WALLS

Back to the future with the AGP–Ca^{2+} flux capacitor

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• **Background** Arabinogalactan proteins (AGPs) are ubiquitous in green plants. AGPs comprise a widely varied group of hydroxyproline (Hyp)-rich cell surface glycoproteins (HRGPs). However, the more narrowly defined classical AGPs massively predominate and cover the plasma membrane. Extensive glycosylation by pendant polysaccharides O-linked to numerous Hyp residues like beads of a necklace creates a unique ionic compartment essential to a wide range of physiological processes including germination, cell extension and fertilization. The vital clue to a precise molecular function remained elusive until the recent isolation of small Hyp–arabinogalactan polysaccharide subunits; their structural elucidation by nuclear magentic resonance imaging, molecular simulations and direct experiment identified a 15-residue consensus subunit as a β-1,3-linked galactose trisaccharide with two short branched sidechains each with a single glucuronic acid residue that binds Ca^{2+} when paired with its adjacent sidechain.
• **Scope** AGPs bind Ca^{2+} ($K_d \sim 6$ μM) at the plasma membrane (PM) at pH ~5·5 but release it when auxin-dependent PM H^+-ATPase generates a low periplasmic pH that dissociates AGP–Ca^{2+} carboxylates ($pk_a \sim 3$); the consequential large increase in free Ca^{2+} drives entry into the cytosol via Ca^{2+} channels that may be voltage gated. AGPs are thus arguably the primary source of cytosolic oscillatory Ca^{2+} waves. This differs markedly from animals, in which cytosolic Ca^{2+} originates mostly from internal stores such as the sarcoplasmic reticulum. In contrast, we propose that external dynamic Ca^{2+} storage by a periplasmic AGP capacitor co-ordinates plant growth, typically involving exocytosis of AGPs and recycled Ca^{2+}, hence an AGP–Ca^{2+} oscillator.
• **Conclusions** The novel concept of dynamic Ca^{2+} recycling by an AGP–Ca^{2+} oscillator solves the long-standing problem of a molecular-level function for classical AGPs and thus integrates three fields: AGPs, Ca^{2+} signalling and auxin. This accounts for the involvement of AGPs in plant morphogenesis, including tropic and nastic movements.

Key words: Arabinogalactan proteins, plant cell wall protein, calcium signalling, hydroxyproline-rich glycoproteins, ion currents, AGP–Ca^{2+} flux capacitor.

INTRODUCTION

Arabinogalactan proteins (AGPs) have been subjected to intensive study ever since their discovery more than 45 years ago as arabinogalactan (AG) 'polysaccharides' in the growth medium of cell cultures (Aspinall *et al*., 1969). It soon became clear that these classical AGPs (Table 1), initially defined by their composition, contained a small amount (5–10 %) of a hydroxyproline (Hyp)-rich protein component (Lamport, 1970) and 90–95 % AG. Since then their biological role has remained elusive and variously described as enigmatic and mysterious (Albersheim *et al*., 2011; Pickard, 2013). Many papers have suggested a general signalling function for AGPs while extensive reviews have provided much interesting background information (Fincher *et al*., 1983; Bacic *et al*., 1996; Du *et al*., 1996*a*; Kreuger and van Holst, 1996; Nothnagel, 1997; Serpe and Nothnagel, 1999; Stone and Valenta, 1999; Clarke *et al*., 2000; Jose-Estanyol and Puigdomenech, 2000; Majewska-Sawka and Nothnagel, 2000; Schultz *et al*., 2000, 2002; Gaspar *et al*., 2001; Showalter, 2001; Qin and Zhao, 2004; Knox, 2006; Pal and Das, 2006; Seifert and Roberts, 2007; Driouich and Baskin, 2008; Ellis *et al*., 2010; Nguema-Ona *et al*., 2012, 2013). While most reviews ascribe a signalling role to AGPs, here we present both direct and indirect evidence for a specific role of AGPs in Ca^{2+} signalling, a novel aspect not previously considered.

The quest for AGP structure and function began with an approach based on 1,3,5-tris(4-β-D-glycopyranosyloxyphenylazo)-2,4,6-trihydroxybenzene as a specific precipitant of AGPs (Yariv *et al*., 1962); now known as the Yariv reagent, it has proved its versatility. Michael Jermyn exploited it beautifully to show that AGPs are ubiquitous in the plant kingdom (Jermyn and Yeow, 1975) and much subsequent progress has used the Yariv reagent to extend that pioneering work.

Yariv-agglutinated protoplasts isolated from various species were readily agglutinated by Yariv (Larkin, 1978); this gave the earliest indication of AGPs (then identified as β-lectins) primarily at the cell surface and was subsequently confirmed by their biochemical isolation from membrane preparations (Norman *et al*., 1990; Serpe and Nothnagel, 1996). Further use of Yariv as a histochemical reagent visualized AGPs at the cell surface of metabolically active tissues: styles (Gane *et al*., 1994), root cap and embryogenic cells (Samaj *et al*., 1999*a*; Thompson and Knox, 1998; Chapman *et al*., 2000), coleoptile epidermis (Schopfer, 1990), seedling roots and root epidermis (Willats and Knox, 1996; Lu *et al*., 2001), embryo (Tang *et al*.,

TABLE 1. *Classical AGPs: molecular properties*

Distribution:	~80 % periplasmic, ~20 % cell wall; T = M + S + W
	T = total AGPs; M = membrane-bound; S = soluble after cell breakage; M + S = periplasmic W = wall-bound.
	Quantification: e.g. BY-2 cells T = 600 μg AGPs g f. wt (Lamport *et al*., 2006)
Molecular Size:	~120 kDa = ~3 × 60 nm (Zhao *et al*., 2002)
Genes:	~19 in Arabidopsis (Schultz *et al*., 2000)
	13 in rice (Yang *et al*., 2007; Ma and Zhao, 2010; Showalter *et al*., 2010)
	At 17,18 & 19 (null; Coimbra *et al*., 2009) have a Lys-rich subdomain (Yang *et al*., 2007)
	eb1 is deficient in Gal synthesis (UDPGlc epimerase; Seifert *et al*., 2002)
	AtAGP17 (*rat1*) decreases *Agrobacterium* transformation (Gaspar *et al*., 2004)
Polypeptide:	87–739 aa residues in extended conformation (Showalter *et al*., 2010)
	Hyp, Ala, Ser, dominate
	Lack Tyr, Phe, Trp and Cys
	Subdomain often a 12-r Lys-rich (Gao *et al*., 1999; Zhao *et al*., 2002; Yang *et al*., 2005, 2007)
	Glycosylation motifs: SP AP TP VP (Tan *et al*., 2003)
Polysaccharide:	Arabinogalactan 'beads' or Hyp–AG glycomodules
	Size: 15–150 sugar residues
	Backbone: β-1,3-linked galactose trisaccharides β-1,6-linked (Tan *et al*., 2004, 2010)
	Sidechains: bifurcated $(Ara)_3$-Gal-(Rha-GlcU; Tan *et al*., 2010)
	Yariv reactivity: contentious; see text
Post-translational modifications:	N-terminal signal peptide (Schultz *et al*., 2000)
	C-terminal GPI lipid anchor (Oxley and Bacic, 1999; Svetek *et al*., 1999; Borner *et al*., 2003)
	Hydroxylation of peptidyl Pro via direct O_2 fixation (Lamport, 1963*a*)
	O-Hyp glycosylation rules (no N-glycosylation)
	Non-contiguous Hyp–AG polysaccharides (Zhao *et al*., 2002)
	Contiguous Hyp short arabino-oligosaccharides
	12 to 24 acidic Hyp–AGs (15–150 residues; Zhao *et al*., 2002)
	An Hyp–AG has 1 to 15 AG subunits
	AG subunit is a repetitive glycomotif of ~15 sugar residues
	Glycomotif consensus: Ara_6 Gal_5 $GlcA_2$ Rha_2
	AG bifurcated sidechain: Rha, GlcA, Ara_3, Gal
	Lack fucose with exceptions (Wu *et al*., 2008, 2010)
	Glycomotif linkage analysis (mol %):
	Main chain: 3,6-Gal ×2 (13·3 %) 6-Gal ×1 (6·7 %)*
	Sidechain: 3,6-Gal ×2 4-GlcA ×2
	3,5-Ara ×2 *t*- Rha ×2
	3-Ara ×2 *t*- Ara ×2
Calcium binding:	GlcU/Ca^{2+} molar ratio 2:1
	~30 Ca^{2+}-binding subunits/120 kDa AGP†

* 6-linked Gal connects repetitive subunits (glycomotifs).
† AGPs approx. 120 kDa bind approx. 1 % Ca^{2+} w/w = 1·2 kDa Ca^{2+}. Thus, moles bound Ca^{2+} = 1·2 kDa/40 Da.

2006) and cotyledons (Pal and Das, 2006). Yariv rapidly inhibited pollen tube growth (Roy *et al*., 1998); this suggested a direct involvement of AGPs in cell extension consistent with AGPs localized at the growing tips of pollen (Jauh and Lord, 1996; Coimbra *et al*., 2004; Castro *et al*., 2013). Most recently, Yariv assay adapted for whole cells (Lamport *et al*., 2006) enabled AGP distribution (Fig. 1) to be quantified in cell surface compartments: anchored to the plasma membrane; free in the periplasm; trapped in the cell wall matrix; and extruded into the growth medium where they provide a convenient source of mixed AGPs readily isolated by Yariv precipitation (Lamport, 2013*a*, *b*).

These approaches implicated AGPs in a huge range of processes. A consensus emerged that AGPs were signalling molecules, an idea consistent with the assumed great heterogeneity of their polysaccharide substituents. Both the potential signalling role and possible polysaccharide heterogeneity are discussed here.

First, a signalling role, i.e. as signalling molecules *per se*, lacks direct evidence. The single possible exception of 'xylogen' (Motose *et al*., 2004) remains to be corroborated, nor is it a classical AGP (Table 1) defined here as an Hyp-rich polypeptide backbone with a:

(1) N-terminal signal sequence for secretion;
(2) C-terminal sequence for glycosylphosphatidylinositol (GPI) addition;
(3) a classical AGP may contain as many as 24 O-Hyp-linked AG polysaccharides based on LeAGP-1 (Zhao *et al*., 2002) but the diversity of classical AGPs is well documented (Showalter *et al*., 2010);
(4) stoichiometric Ca^{2+} binding by Hyp AGs: GlcA:Ca^{2+} 2:1.

Classical AGPs have been variously modelled as a Wattle blossom, twisted hairy rope and most recently as a necklace (Fig. 2).

Secondly, AGP polysaccharide heterogeneity seems overemphasized; it does not include a wide variety of different sugars or glycosidic linkages (e.g. as in pectic RG-II) and is more accurately described as polydispersity due to the variable number of repetitive AG subunits (1–15). The AG consensus structure has the theoretical molar ratio: Gal_5 Ara_6 $GlcA_2$ Rha_2 (~2246 Da;

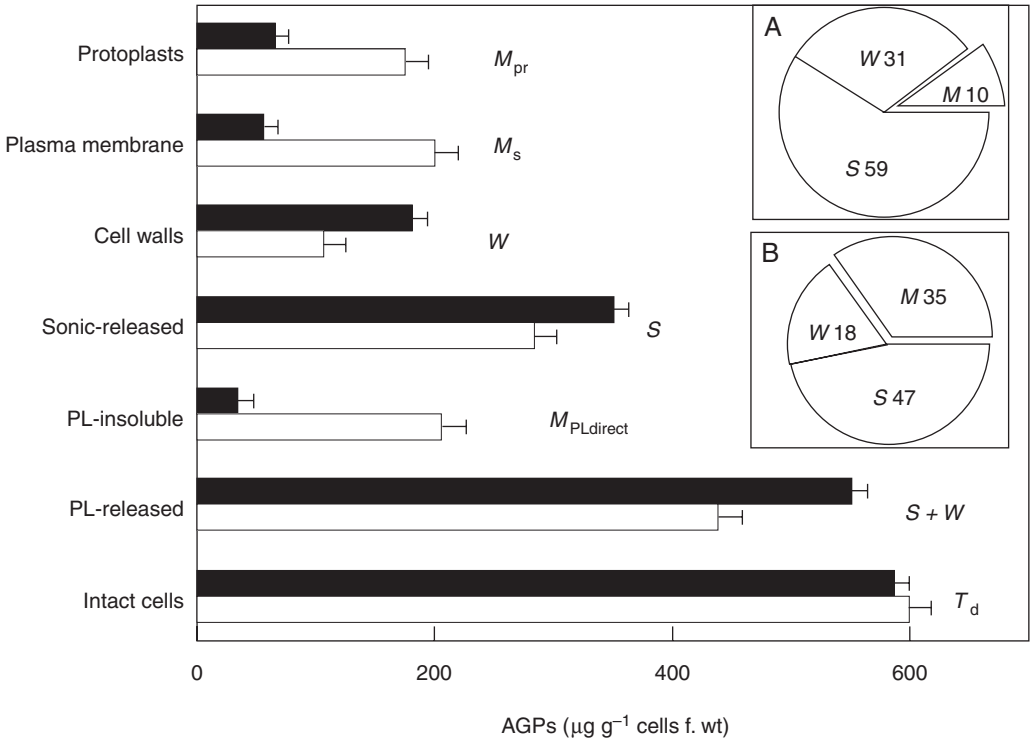

FIG. 1. Yariv reagent assay of AGP distribution in tobacco BY-2 cells adapted to growth in 2 % NaCl versus non-adapted controls. Solid columns, salt-adapted; empty columns, non-adapted controls. Protoplasts (M_{pr}), AGPs remaining bound after approx. 2 h treatment with cellulase/pectolyase. Plasma membrane (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 s.e. 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 non-adapted 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. [Reprinted from Lamport *et al.* (2006).]

Tan *et al.*, 2010), in agreement with an earlier conclusion of AG regularity (Churms *et al.*, 1983; Gane *et al.*, 1995b) although a somewhat larger subunit of ~8 kDa.

A relative few classical AGPs comprise the bulk of cell surface AGPs based on amino acid analyses of HF-deglycosylated AGP polypeptides separated by reversed-phase liquid chromatography (Gao *et al.*, 1999) and the narrow size distribution of AGPs separated by Superose-6 gel filtration (Lamport *et al.*, 2006). Many other AGP-like molecules exist (Borner *et al.*, 2002, 2003) but these make only a minor contribution to the total mass of AGPs; this includes the recently described classical AGP 'APAP1', At-AGP57C (a minor secreted component that crosslinks pectic RG-I *in muro*; Tan *et al.*, 2013) and the non-classical AGP31 (Liu and Mehdy, 2007).

Structural elucidation exemplifies a biochemical approach. For AGPs this involves in particular the C-terminal GPI that anchors AGPs to the outer leaflet of the plasma membrane (Youl *et al.*, 1998; Svetek *et al.*, 1999; Borner *et al.*, 2002) and the N-terminal signal sequence for secretion. However, the 90–95 % AG polysaccharide generally remained incompletely characterized due to its perceived overwhelming complexity. However, unlike most proteins polysaccharides derive their complexity from relatively simple repetitive subunits (Rees, 1977). Indeed, numerous earlier carbohydrate analyses clearly pointed to such an AG ground plan with small blocks of a β-1,3-linked galactan backbone separated by periodate-sensitive residues (Fincher *et al.*, 1983). Size heterogeneity of Hyp-polysaccharides released by alkaline hydrolysis (Pope, 1977) initially deterred further analysis. However, designing (Hyp)-rich cell surface glycoproteins (HRGPs) as green fluorescent protein (GFP) fusion proteins (Shpak *et al.*, 1999) solved the problem of purifying individual AGPs and novel AGP-like constructs (Xu *et al.*, 2007). Thus, the tenacity of Li Tan with the combined forces of genetic engineering and state-of-the-art nuclear magnteic resonance imaging (NMR) characterized a range of *small* Hyp–AG polysaccharides that yielded evidence of a consensus 15-residue *repetitive AG subunit* (Fig. 3; Tan *et al.*, 2004, 2010). Thus, variation in the number of repetitive AG subunits and minor variation in sugar composition may simply reflect AG polydispersity rather than true compositional AG heterogeneity.

Hyp–AG subunits (Fig. 3) have a relatively simple structure – a repetitive β-1,3-linked galactosyl trisaccharide backbone linked β-1,6 to successive galactosyl trisaccharides. Each repetitive galactosyl trisaccharide has two bifurcated sidechains: one branch an arabinofuranosyl trisaccharide, the other a rhamnosyl-glucuronic acid disaccharide. The five-residue sidechain structure is evidently widespread, first elucidated in gum arabic of *Acacia senegal* (Defaye and Wong, 1986). Such Hyp–AG conservation implies an essential role for AGP glucuronic acid residues, previously overlooked despite the 'known' approx. 1 % Ca^{2+} content of

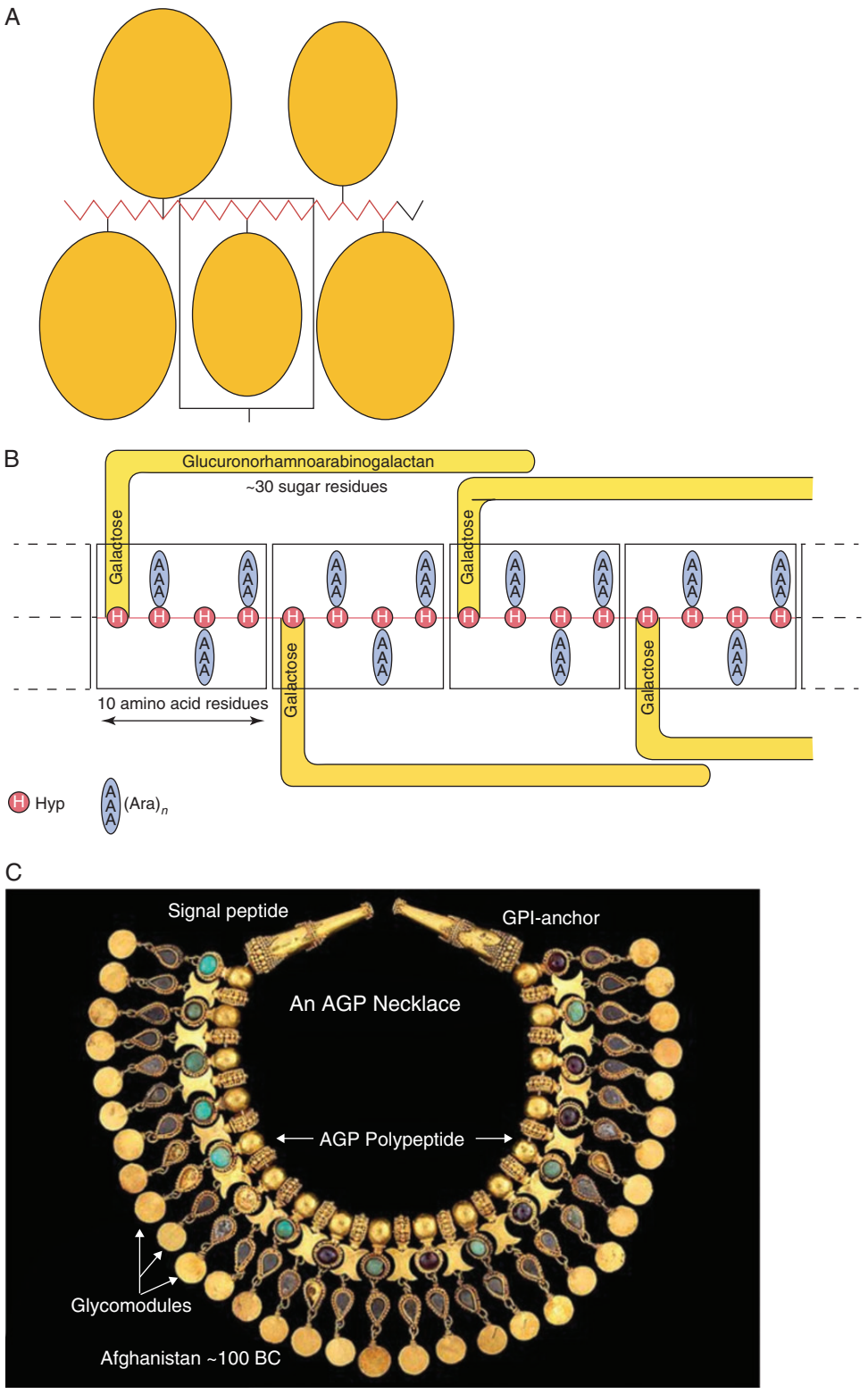

FIG. 2. AGP models: wattle blossom, hairy rope, necklace. (A) The 'wattle-blossom' model. Each Hyp residue of about 24 is attached to an arabinogalactan chain that contains from one to 15 repeats of a β-(l-3)-linked galactose (Gal) oligosaccharide with a degree of polymerization (d.p.) of 12. The 'wattle-blossom' model depicts 'the AGP as a whole is spheroidal': GlcA, glucuronic acid; Rha, rhamnose; Gal, galactose; Ara, arabinose; *p*, pyranose; *f*, furanose. [Reprinted from Du *et al.* (1996*a*).] (B) The 'twisted hairy rope' model of an AGP. Hypothetical block size of 7 kDa contains 10 amino acid residues (1 kDa), 30 sugar residues (4·4 kDa) and 3 hydroxyproline (Hyp)-triarabinosides (1·32 kDa). The glucuronorhamnoarabinogalactan probably has a galactan backbone with glucuronic acid (GlcA), rhamnose (Rha) and arabinose (Ara) side chains. [Reprinted from Qi *et al.* (1991) as adapted by Du *et al.* (1996*a*).] (C) AGP modelled as a necklace. An elaborate robe decoration in the form of a necklace, (Tillya Tepe), 100 BC – 100 AD; National Museum of Afghanistan; Photograph Thierry Ollivier, Musée Guimet (reproduced with permission).

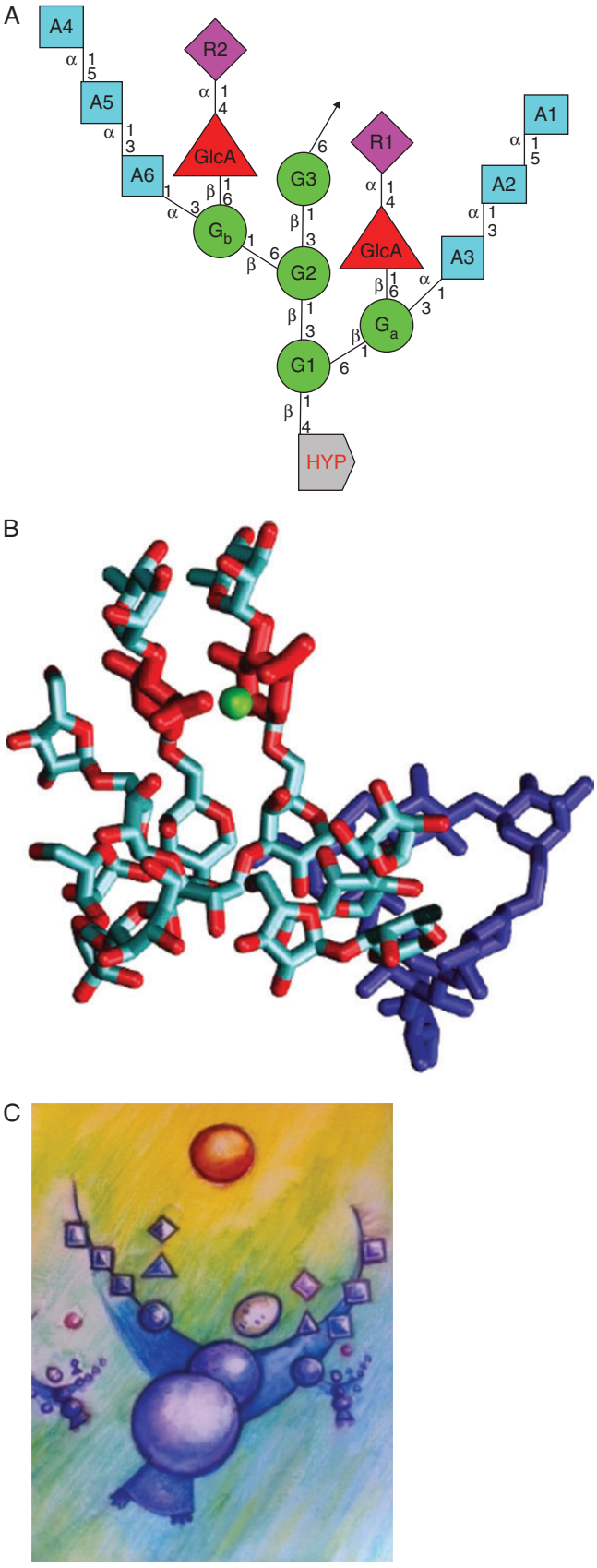

FIG. 3. Hyp-arabinogalactan (Hyp–AG) subunits. (A) The linkage connectivity of sugars involved in the repetitive 15-residue consensus Hyp–AG, a conserved structure that accounts for Ca^{2+} binding by classical AGPs (Lamport and Varnai, 2013). A_{1-6} arabinose residues; G_{1-3} galactose mainchain residues; G_a and G_b, galactose sidedechain residues; R_1 and R_2, rhamnose sidechain residues; GlcA, glucuronic acid sidechain residues; Hyp, hydroxyproline. (B) Three-dimensional molecular model simulating an Hyp–AG with bound Ca^{2+}. Hyp–AG interferon Hyp-polysaccharide-1 (IFNHP1) with Ca^{2+} ions (green) bound by two glucuronic acid (GlcA) sidechains (red); the galactan backbone is in dark blue and sidechains in light blue. [Reprinted from Lamport and Varnai (2013).] (C) 'Molly Cool' cartoon of an arabinogalactan subunit. Two hands are needed to catch a divalent Ca^{2+} ion. Credits: Amanda Dean and Freyja Dean.

gum arabic (Anderson and Brown Douglas, 1988; Lamport and Varnai, 2013), and its cytochemical location as membrane-bound Ca^{2+} (Slocum and Roux, 1982). Significantly, gum arabic does not form a stable complex with Mg^{2+} (Kunkel *et al.*, 1997).

3D computer models of the Hyp–AG subunit (Fig. 3b) showed a dramatically close approach of glucuronate carboxyls – a eureka moment that pointed to a specific biochemical role for the repeating Hyp–AG subunit in binding Ca^{2+}. Subsequent experiments confirmed the tight binding constant ($K_d \sim$ 6·5 μM) together with the 2:1 GlcA:Ca^{2+} binding stoichiometry at pH 5 (Lamport and Varnai, 2013) and pH-dependent dissociation (Fig. 4). These data also corroborate the repetitive subunit structure of the Hyp–AG deduced from NMR experiments (Tan *et al.*, 2010). Numerous methylation analyses of AGPs (Table 2) show approx. 7 % 1,6-linked Gal (i.e. one in every 15 residues of the consensus sequence) and thus further verify the consensus structure.

Significantly, AGPs bind Ca^{2+} more strongly than does pectin (Lamport and Varnai, 2013). Thus, the lower pK_a and non-methyl esterification of glucuronic acid rationalizes Nature's choice of glucuronic acid for AGPs rather than the methyl esterified galacturonic acid that typifies pectin. Furthermore, these biochemical data imply a biological role for tightly bound AGP–Ca^{2+} (pH 5) by creating a periplasmic reservoir of Ca^{2+} that can be dissociated by activated H^+-ATPase of the plasma membrane, thus feeding Ca^+ channels (Wheeler and Brownlee, 2008; Verret *et al.*, 2010) that supply cytosolic Ca^{2+} (Felle, 1988; Gehring *et al.*, 1990*a*; Shishova and Lindberg, 2004). Hence the suggestion that AGP–Ca^{2+} at the periplasmic interface is the major source of cytosolic Ca^{2+} (Lamport and Varnai, 2013). In one fell swoop this scenario connects AGPs with Ca^{2+} signalling – a unifying hypothesis that differs from previous models (Trewavas, 2000; Dodd *et al.*, 2010) but with considerable ramifications.

'The subtlety of Nature far surpasseth the subtlety of Man's understanding' Francis Bacon 1561–1621). Indeed, cell signalling molecules with their myriad interactions and interdependencies involving cross-talk, feedback, feed-forward and so on are of daunting complexity. A simplifying principle assumes that signalling networks do not operate independently but are integrated. Precisely how AGPs fit into this scheme we discuss below.

Ca^{2+} behaves as a universal signalling currency and acts as a 'second messenger' in plants (Hepler, 2005; Vanneste and Friml, 2013) and also in animals (Berridge, 1997) where it involves a huge range of processes most evident in muscle contraction (Ebashi and Endo, 1968) but also including cell migration (Tsai *et al.*, 2014) and skin homeostasis (Vandenberghe *et al.*, 2014). In plants, Ca^{2+} signalling involves an equally wide range of complex processes consistent with an earlier percipient comment that 'Perhaps in the phloem there is an electrical control of Ca^{2+} flux reminiscent of the well-known control by the sarco-

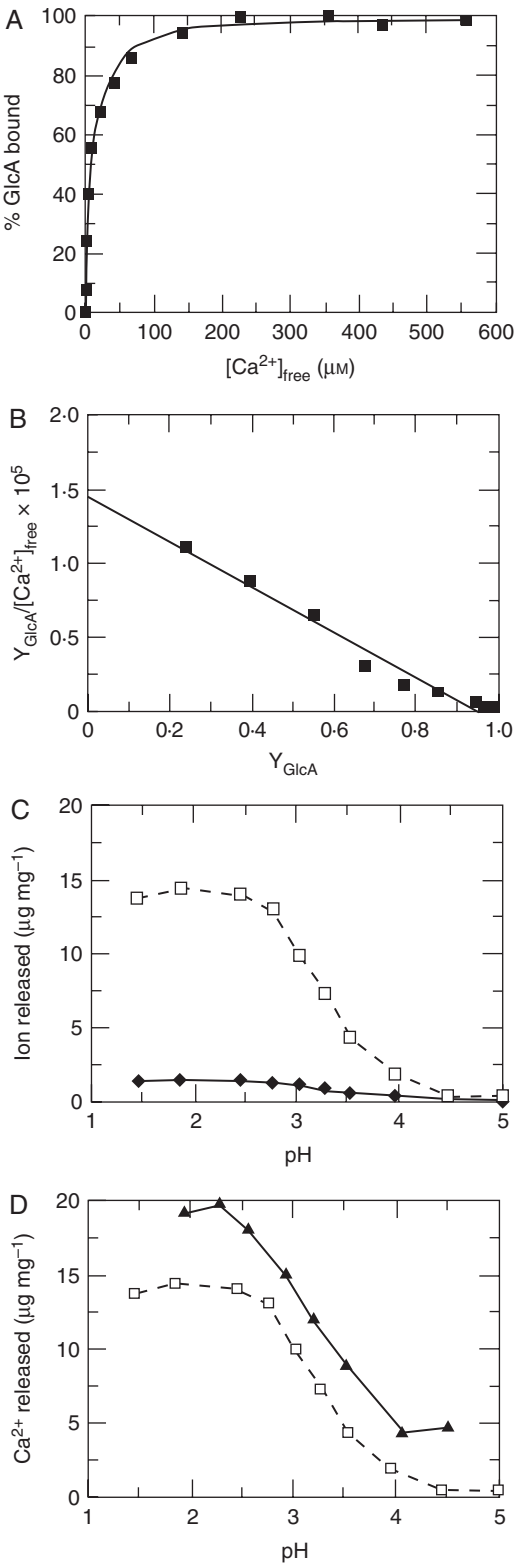

FIG. 4. AGP Ca^{2+} binding: titration of Ca^{2+}-depleted gum arabic with Ca^{2+} at pH 5. (A) Saturation binding data (black squares) with theoretical curve (line). (B) Scatchard analysis of binding data with best-fit line giving a K_d of 6·5 μM. Y_{GlcA} is the fraction of glucuronic acid bound. (C) pH-dependent cation release from Ca^{2+}-saturated gum arabic: Ca^{2+} (dashed line) and Mg^{2+} (solid line). (D) Release of Ca^{2+} from gum arabic (dashed line) compared with pectin (solid line). [Reprinted from Lamport and Varnai (2013).]

TABLE 2. *Content (mol %) of 6-linked galactose in AGPs*

Species	Source	6-Gal content (mol %)*	Reference(s)
Acer pseudoplatanus	Cultures	n.d.	Aspinall *et al.* (1969)
Acacia senegal	Gum exudate	1·2	Akiyama and Kato (1981)
Physcomitrella patens	Cultures	2	Lee *et al.* (2005)
Phleum pratense	Cultures	5	Sims *et al.* (2000)
Vitis vinifera	Grape juice	6	Saulnier *et al.* (1992)
Plantago major	Leaves	7	Samuelsen *et al.* (1998)
Rosa sp.	Cultures	7	Serpe and Nothnagel (1996)
Lolium multiflorum	Cultures	8	Bacic *et al.* (1987)
Nicotiana alata	Pistil (AGPNa3)	8	Du *et al.* (1996*b*)
Acacia erioloba	Gum exudate	12	Churms *et al.* (1986)
Raphanus sativus	Storage root	14	Kitazawa *et al.* (2013), Tsumuraya *et al.* (1988)
Nicotiana alata	Styles (GaRSGP)	14	Sommer-Knudsen *et al.* (1996)
N. alata	Stigma	21	Bacic *et al.* (1988)

* Identified as 2,3,4-trimethyl galactose.

plasmic reticulum in striated muscle!' (Pickard, 1973). However, the source of the Ca^{2+} signal involves dynamic Ca^{2+} storage by AGPs of the cell surface (Lamport *et al.*, 2006; Lamport and Varnai, 2013) and thus differs radically from the classical internal endoplasmic reticulum storage of animals.

AGPs strongly associated with so many plant processes (Table 3) led to the idea of AGPs as signalling molecules *per se*. However, specific AGP receptors remain elusive most likely because they are non-existent. As an alternative we propose that the AGP–Ca^{2+} oscillator integrates most signalling pathways that are downstream from the early Ca^{2+} signal (Ma *et al.*, 2013). This accounts for the ubiquity of AGPs where a trinity of primary messenger (e.g. auxin), secondary messenger (Ca^{2+}) and AGPs comprise a global signalling paradigm, with the evidence summarized in the following six sections.

THE AGP–Ca^{2+} OSCILLATOR: AUXIN AND EXTENSION GROWTH

AGPs and auxin are involved in most aspects of plant development; it is increasingly evident that *auxin generates Ca^{2+} signals* evidenced by increased cytosolic Ca^{2+} (Pickard, 1984; Gehring *et al.*, 1990*b*; Tretyn *et al.*, 1991; Irving *et al.*, 1992; Ayling *et al.*, 1994; Plieth and Trewavas, 2002; Shishova and Lindberg, 2010; Monshausen *et al.*, 2011), including a particularly insightful recent review (Vanneste and Friml, 2013). We propose that the AGP–Ca^{2+} oscillator generates those signals and is thus an integral component of the following AGP–Ca^{2+}–auxin signalling cascade (Fig. 5):

(1) auxin-activated plasma membrane H^+-ATPase releases protons at the surface;

TABLE 3. *Processes that involve auxin, Ca^{2+} and AGPs*

		Primary messenger auxin	Secondary messenger Ca^{2+}	AGP involvement
I. Auxin and extension growth		+++	+++	+++
II. Tropisms and mechanotransduction	Gravitropism	+++	+++	+
	Thigmotropism	–	+ +	+
	Pollen tube growth	–	+++	+++
	Stomatal movements	+	+++	+++
	Phototropism	+++	+++	?
III. Intracellular dynamics		+++	+++	+
IV. Morphogenesis	Seeds	?	?	+++
	Germination	+++	+++	+++
	Roots and lateral roots	+++	+	+++
	Shoots and branching	+++	+ +	+++
	Leaves	+++	?	?
	Flowering, fertilization and early embryogenesis	+++	?	+++
V. Stress, pathogenesis and symbiosis	Abiotic stress	?	+++	?
	Wound response	?	+	+++
	Salt stress	?	+++	+++
	Pathogenesis and symbiosis	+	+ +	+++

+, ++, and +++ indicate increasing evidence for involvement in a given process; see text for references.

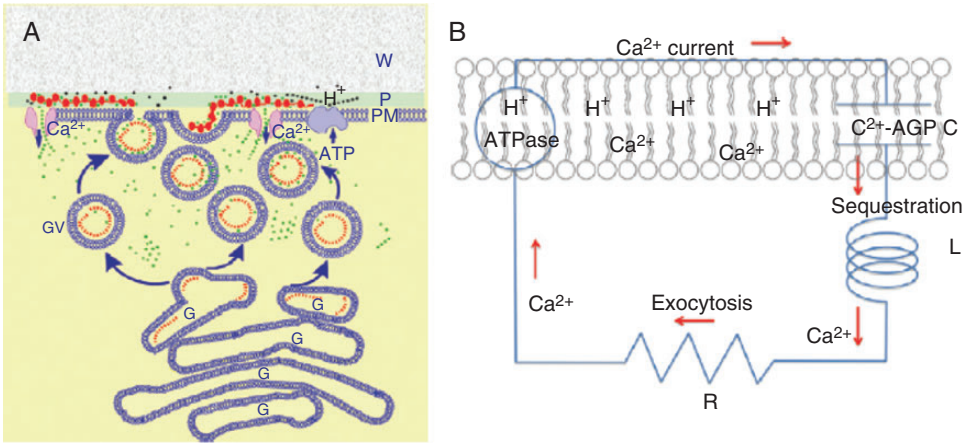

FIG. 5. The AGP–calcium oscillator. (A) Ca^{2+} release from periplasmic AGPs. The oscillator generates pulses of Ca^{2+} (green dots) whose influx coordinates exocytosis and rapid tip growth. This involves a pulse of H^+ (black dots) releasing Ca^{2+} from periplasmic AGPs (red beads) via stretch-activated Ca^{2+} channels into the cytosol, and then sequestration by exocytotic Golgi vesicles containing AGPs (red dots). Diffusion of the initial H^+ pulse to the wall domain restores the periplasmic pH. Ca^{2+} is recycled by fusion of vesicles with the plasma membrane. Ca^{2+} bound to periplasmic AGPs is now ready for the next oscillation. W, wall; P, periplasm; PM, plasma membrane; G, Golgi; GV, Golgi exocytotic vesicles. (B) Ca^{2+} current as a molecular clock: analogous to an electronic series RLC circuit where R is resistance, L is inductance and C is capacitance, with membrane-bound AGPs as the capacitor, C; Ca^{2+} sequestration as the inductance, L; vesicle exocytosis as the resistor, R, which limits the recycling rate. Hypothetically, C and L largely determine the oscillator frequency and amplitude of the Ca^{2+} current, I_{Ca}. Note the reported high efflux of Cl^- as a counterion may maintain electrical neutrality (Cosgrove and Hedrich, 1991; Zonia *et al*., 2002). [Reprinted from Lamport and Varnai (2013).]

(2) decreases pH of AGPs strategically located at the plasma membrane;
(3) low pH discharges AGP–Ca^{2+} (Fig. 5);
(4) Ca^{2+} enters the cytosol via plasma membrane Ca^{2+} channels (Wheeler and Brownlee, 2008; Verret *et al*., 2010);
(5) cytosolic Ca^{2+} increases;
(6) activates Golgi vesicle exocytosis.

While the above implies a role for AGPs in cell extension, there is also strong circumstantial evidence of AGP involvement in hypocotyl cell extension where a gibberellin-responsive gene (*CsAGP1*) encodes a classical AGP (Park *et al*., 2003).

The AGP–Ca^{2+} capacitor offers a new perspective on the source of cytosolic Ca^{2+} and its regulation

First, recycling Ca^{2+} via exocytosis of Golgi vesicles (Battey *et al*., 1999; Roy *et al*., 1999) recharges the AGP capacitor, and traps and conserves Ca^{2+}, thus avoiding the uncertainties of an external apoplastic supply of free Ca^{2+}.

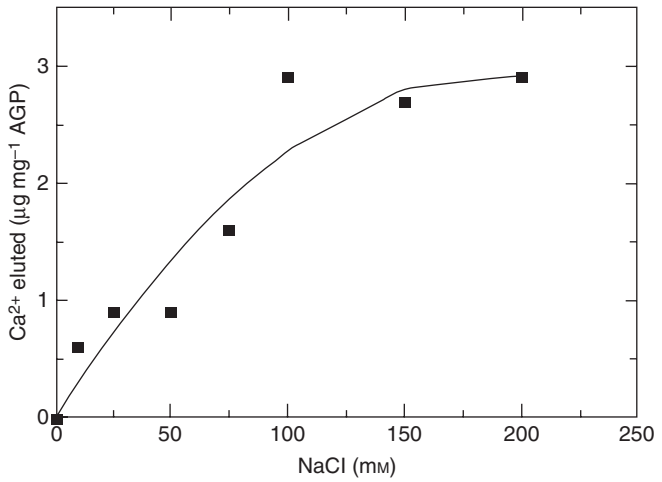

FIG. 6. High levels of Na^+ compete with AGP-bound Ca^{2+}. An experiment illustrating that competition by 75 mM Na^+ removed approx. 50 % of the Ca^{2+} from a carrot AGP–Ca^{2+} isolated from a carrot suspension culture. DTAL, unpublished data.

Secondly, classical AGPs at the plasma membrane are by definition close to Ca^{2+} channels. This confers a huge kinetic advantage to Ca^{2+} ions for entry into the cytosol when low pH dissociates AGP–Ca^{2+}.

Thirdly, plasma membrane-bound AGPs bind Ca^{2+}. This drastically decreases the Ca^{2+} electrochemical gradient until low pH dissociates the AGP–Ca^{2+}. [Note that pectic carboxyls will also bind free Ca^{2+}.]

Fourthly, cells can adjust the precise size of the capacitor by controlling surface AGP levels (section IV) but also by the size of O-Hyp-linked polysaccharides – these can vary by a factor of ten or more (Lamport, 1977; Pope, 1977; Xu *et al*., 2008).

Fifthly, the Hyp–AG Ca^{2+}-binding subunit is a *consensus* motif whose subtle variation presumably alters the K_d for Ca^{2+} and also the ability to discriminate against other divalent ions and monovalent ions, particularly Na^+, that compete at high levels (Fig. 6). Replacing glucuronic with 4-*O*-methylglucuronic acid is probably the most frequent variation. Minor sugars such as 3-*O*-methylgalactose are present in lower plants (Popper *et al*., 2001) and include 3-*O*-methylrhamnose (acofriose) in AGPs isolated from the moss *Physcomitrella* (Popper *et al*., 2004; Fu *et al*., 2007). However, fucose (6-deoxy-L-galactose), frequently reported as a minor component of AGPs (Tryfona *et al*., 2012) and a likely conservative replacement for rhamnose (6-deoxy-L-mannose), has not been detected in any Hyp–AG, but we have yet to explore this planet's vast AGP resources!

This review cannot do justice to all the ramifications of the AGP–Ca^{2+} capacitor. Here we discuss the correlation between AGPs, Ca^{2+} signalling and the primary messenger auxin with its classical effect on wall plasticity (Heyn, 1940). Increased wall plasticity or wall loosening, is pH-dependent ('proton excretion'; Rayle and Cleland, 1980), but a biochemical basis for the firmly entrenched 'acid growth' hypothesis (Rayle and Cleland, 1992) remains recalcitrant; its major postulate that low pH *in muro* activates wall loosening enzymes per se lacks convincing evidence (Schopfer, 1993) despite the clear involvement of plasma membrane H^+-ATPase in cell extension (Hager, 2003). However, the AGP–Ca^{2+} capacitor (Fig. 5) may resolve this problem as it identifies the source of cytosolic Ca^{2+} oscillations and shows how they are consistent '*with the general concept of calcium acting as a second messenger in hormone action in plants*' (Felle, 1988; Hepler, 2005): low pH dissociates carboxylate-bound Ca^{2+} of AGPs at the plasma membrane (Fig. 5). Remarkably, however, AGPs may perform a dual function by acting as a pectic plasticizer after their release from the plasma membrane (Lamport, 2001).

A dual role of classical AGPs in cell extension is consistent with the following observations:

(1) AGPs are strongly associated with rapid growth of cell suspension cultures and rapid cell extension during tip growth of moss protonema (Lee *et al*., 2005), pollen tubes (Jauh and Lord, 1996) root hairs (Samaj *et al*., 1999b) and coleoptile epidermal walls with AGPs suggested as 'an epidermal wall-loosening factor in auxin mediated coleoptile growth' (Schopfer, 1990).
(2) Growth retardation in double null AGP mutant pollen tubes (Costa *et al*., 2013*a*), and in the protonema of *Physcomitrella* AGP1 knockouts (Lee *et al*., 2005) and in root hairs of the *reb1* arabidopsis mutant (Ding and Zhu, 1997).
(3) Tropisms and mechanosensory mechanisms (Toyota and Gilroy, 2013).
(4) Auxin and Ca^{2+} signalling are connected (Vanneste and Friml, 2013).
(5) AGP cell wall plasticizers may contribute to the resistance of resurrection plants to desiccation (Moore *et al*., 2006, 2013).

THE AGP–Ca^{2+} OSCILLATOR: TROPISMS AND MECHANOTRANSDUCTION

Gravitropism

Calcium signalling underlies all tropisms (Toyota and Gilroy, 2013), so the question devolves into the mechanism that elevates cytosolic Ca^{2+} (Toyota *et al*., 2008). Frequently this involves mechanotransduction exemplified by gravitropism where the biochemical mechanism now seems to involve the redistribution of auxin transporter activity and hence auxin itself (Baster *et al*., 2013). Furthermore, Ca^{2+} is well represented by stretch-activated Ca^{2+} channels in plants and algae (Ding *et al*., 1993; Verret *et al*., 2010) suggesting that the ultimate gravity sensor is the membrane and its stretch-sensitive receptors; this would include the calcium mechanosensitive receptors described by Pickard (Ding and Pickard, 1993) rather than starch grain 'statoliths' (Caspar and Pickard, 1989). Thus, 'Although Ca^{2+} is usually discussed as a cytoplasmic regulator, apoplastic fluxes of this ion may also play a key role in gravitropism' via stretch-sensitive Ca^{2+} channels that regulate Ca^{2+} flux (Toyota and Gilroy, 2013). Hence the term 'flux capacitor' borrowed from 'Doc' Brown immortalized by Christopher Lloyd in the Sci-Fi movie *Back to the Future* (Zemeckis, 1985) where Doc's invention of the aptly named capacitor was an integral component of the time machine powered by the continuous flux of a capacitor in an oscillating circuit; the allusion to time travel is a reminder that plant evolution is a form of time travel that depends on the AGP–Ca^{2+} and other biochemical oscillators!

Thigmotropism

The AGP–Ca^{2+} oscillator does not exclude a role for AGPs in mechanotransduction based on adhesion of plasma membrane to the cell wall by a 'plasmalemmal reticulum' involving AGPs and wall-associated kinases (WAKs; Gens *et al*., 2000; Pickard, 2007). Although precise details of such 'tensegrity' are lacking, stretch receptors with associated kinases and AGPs (Telewski, 2006) and more specifically mechanosensitive (MS) Ca^{2+} channels (Nakagawa *et al*., 2007; Swarbreck *et al*., 2013) are clearly involved in thigmotropism, which includes a wide range of processes ranging from the rapid movements of insectivorous plants and the tendrils of climbing plants to root growth (Weerasinghe *et al*., 2009) particularly root tips (Pickard, 2007).

Pollen tube growth

Ca^{2+} is essential to pollen tube growth (Mascarenhas, 1993; Chen *et al*., 2008; Chebli and Geitmann, 2012) and pollen tube directionality (Franklin-Tong, 1999); not surprisingly, AGPs are implicated as chemotropic agents (Cheung *et al*., 1995; Wu *et al*., 2000) although not corroborated by others (Sommer-Knudsen *et al*., 1998). This discrepancy may be resolved by considering the *in vitro* growth of millet pollen tubes that are directed by a polygalacturonic acid-calcium gel which forms a Ca^{2+} gradient (Reger *et al*., 1992). Thus, the signal guiding a pollen tube may be the Ca^{2+} gradient generated by AGP–Ca^{2+} dissociation in the transmitting tissue of female but not in male flowers lacking AGPs (Coimbra and Duarte, 2003) rather than AGPs themselves. Indeed, the apparent increase in AGP glycosylation from stigma to ovule (Wu *et al*., 1995) supports this interpretation and strongly hints at the ways in which AGPs may be involved in tip growth dependent on the essential ions: Ca^{2+}, protons (H^+) and borate ($B(OH)^{4-}$; Holdaway-Clarke *et al*., 2003). One could, for example, view the tip as an exquisitely sensitive living Ca^{2+} electrode whose AGPs integrate local Ca^{2+} levels and thus enable directional growth by discriminating between small but highly localized changes in Ca^{2+}. This is consistent with the early observation of a specfic chemotropic response to Ca^{2+} by growing pollen tubes (Mascarenhas and Machlis, 1962).

Stomatal movements

Stomata also exemplify the AGP–Ca^{2+} oscillator hypothesis. Not only are they replete with oscillator components but changes in cytosolic pH and calcium of guard cells precede stomatal movements (Irving *et al*., 1992; Kim *et al*., 2010). This is consistent with stretch-activated Ca^{2+} channels (Cosgrove and Hedrich, 1991) and the marked abundance of guard cell AGP epitopes (Majewska-Sawka *et al*., 2002) also demonstrated by cytochemical location of the 'Lys-rich' classical AGP AtAGP18 expressed as a GUS construct (fig. 3j in Yang and Showalter, 2007).

Phototropism

Finally, phototropism involves blue light receptors that initiate lateral auxin fluxes (Gehring *et al*., 1990*b*; Friml *et al*., 2002; Christie *et al*., 2011; Ding *et al*., 2013) and lead to increased cytosolic Ca^{2+} (Folta *et al*., 2003), indicating yet another possible role for the AGP–Ca^{2+} oscillator.

THE AGP–Ca^{2+} OSCILLATOR: INTRACELLULAR DYNAMICS

Early work with gibberellin-induced secretion of α-amylase first identified a specific Ca^{2+}-dependent biochemical process (Chrispeels and Varner, 1967). Since then it has become clear that Ca^{2+} is directly involved in many processes: cell cycle regulation (Himanen *et al*., 2002; Vanneste *et al*., 2005), membrane trafficking including the transport of auxin efflux proteins (Baster *et al*., 2013), the balance between exo- and endocytosis (Paciorek *et al*., 2005; Robert *et al*., 2010; Vanneste and Friml, 2013), apoptosis (Levine *et al*., 1996) and programmed cell death (PCD; Jones, 2001; Chaves *et al*., 2002).

Most if not all of these processes involve the universal Ca^{2+} signal transducer calmodulin and calmodulin-like proteins (comprehensively reviewed by Bouche *et al*., 2005).

THE AGP–Ca^{2+} OSCILLATOR: MORPHOGENESIS

It is convenient to discuss morphogenesis beginning with the seed, as Jermyn first noted that AGPs were released from virtually all seeds by extraction of the seed meal with mild aqueous buffer (Jermyn and Yeow, 1975). This raises several questions: Where are these AGPs located in the seed? Are they identical to the classical AGPs of actively growing plant cells? Do they bind Ca^{2+}? What is their functional significance in metabolically inactive seed tissues? And what role do they play during seed maturation and germination?

Seeds

Yariv as a cytochemical reagent identifies the cytochemical location of AGPs in seeds such as coffee (Sutherland *et al*., 2004) specifically in the thickened cell walls of the coffee endosperm where they are notably concentrated at the interface between the wall and the plasma membrane (fig. 2 in Redgwell *et al*., 2002; Redgwell *et al*., 2006). However in seeds of *Jatropha curcas* AGPs are particularly evident in vessels of the cotyledon and in the procambium ring of the embryo (Fig. 7.) but 'no AGPs were detected in the endosperm' (Sehlbach *et al*., 2013).

Significant structural differences between the AGPs of seeds (Tryfona *et al*., 2010), storage roots (Tsumuraya *et al*., 1988; Table 2) and growing tissues (Tan *et al*., 2004, 2010) suggest different roles for AGPs in metabolically inactive versus metabolically active tissues. AGPs of resurrection plants reportedly contribute to the viability of their desiccated tissues (Moore *et al*., 2013); so by analogy, seed AGPs may also enhance the viability of dehydrated seed tissue, particularly as such AGPs (Jermyn and Yeow, 1975) may be surprisingly abundant and may include both classical AGPs and the much smaller AG peptides (Fincher *et al*., 1983; Fincher and Stone, 1974) that comprise, for example, >0·3 % d. wt of wheat flour (Loosveld *et al*., 1997; Tryfona *et al*., 2010) while classical AGPs account for approx. 0·8 % d. wt of tomato seeds (Lu *et al*., 2001) similar to BY-2 cells when adjusted to a fresh weight

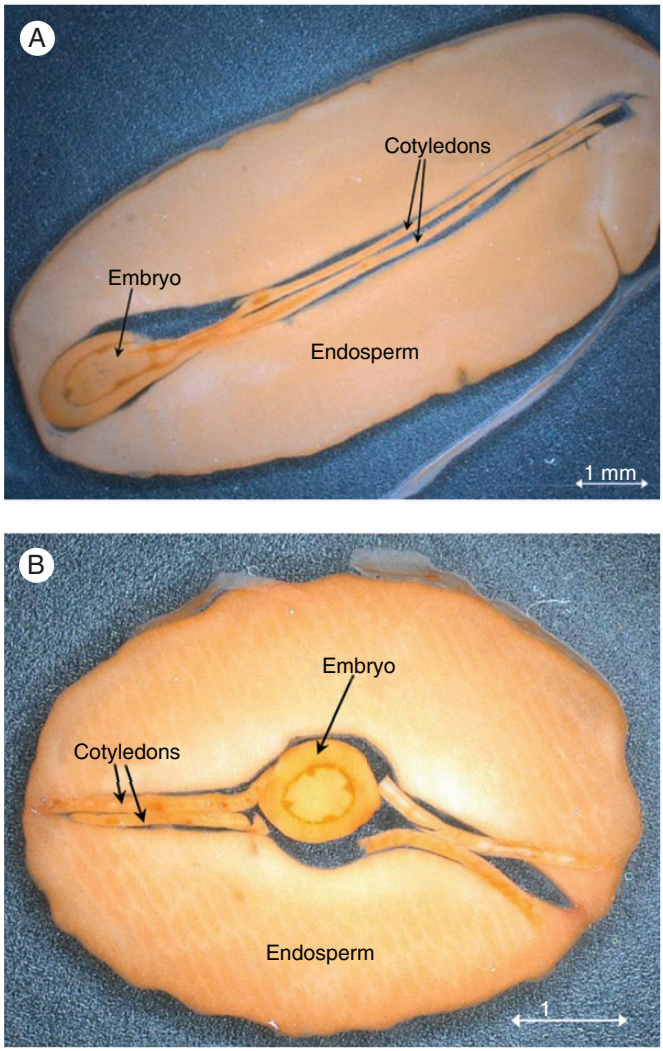

FIG. 7. *Jatropha curcas* staining with Yariv. *J. curcas seed* sections after staining with β-D-glucosyl Yariv: (A) sagittal and (B) transverse section. [Reprinted from Sehlbach *et al.* (2013).]

basis although there are clearly wide variations in different species (Boudjeko *et al.*, 2009). Despite possible structural differences between classical and seed AGPs some evidently bind Ca^{2+} in germinating barley seeds, as described below.

Germination

Gibberellin (GA) induces Ca^{2+}-dependent secretion of α-amylase by barley aleurone cells (Chrispeels and Varner, 1967) and also by their protoplasts (Suzuki *et al.*, 2002). As the β-D-glucosyl Yariv reagent inhibits Ca^{2+}-dependent amylase secretion by the protoplasts, a direct role for AGPs during germination is likely (Mashiguchi *et al.*, 2008). Curiously, however, the Yariv reagent does not inhibit amylase secretion by *intact* aleurone cells or the germination of tomato seeds (Lu *et al.*, 2001) possibly due to a permeability barrier as Yariv reagent markedly inhibits subsequent seedling growth (Lu *et al.*, 2001). Indeed, judging from the role of auxin in root tip cell differentiation (Ding and Friml, 2010), lateral root initiation (Himanen *et al.*, 2002; Lavenus *et al.*, 2013), root hair formation (Jones *et al.*, 2009; Ikeda *et al.*, 2009) and root gravitropism (Toyota and Gilroy, 2013), the AGP–Ca^{2+} oscillator operates consistently during seed germination and seedling growth.

Roots and lateral roots

AGP epitopes that appear very early, within one or two cells of the apical initials, are notably associated with the determination of cell fate during root development. Thus, the AGP monoclonal JIM4-labelled developing pericycle cells in carrot root (Knox *et al.*, 1989). A JIM13 AGP epitope confirmed differentiation at 'the earliest stage of development' (Dolan *et al.*, 1995) in species-specific patterns (Casero *et al.*, 1998) that were related to lateral root initiation (De Smet *et al.*, 2006). Inhibition of auxin transport also inhibits lateral root initiation (Casimiro *et al.*, 2001) and branching (Lavenus *et al.*, 2013); thus, by increasing the size of their AGP–Ca^{2+} capacitor, pericycle cells may predestine their response to auxin during development. Interestingly, the *reb1* mutant yields defective root epidermal cell walls with lower levels of AGPs (Ding and Zhu, 1997) although more recent work identified *reb1* as a UDP–D-Glc epimerase defective mutant, and hence a galactose deficiency with concomitant pleiotropic effects on xyloglucan, pectin and AGP synthesis according to Nguema-Ona *et al.* (2006). Nevertheless, the location of AGPs precisely where Ca^{2+} signals will be needed seems entirely consistent with the AGP–Ca^{2+} oscillator paradigm.

Shoots and branching

Morphogenesis of the shoot, including its vascular system (Fukuda, 2004), branching (Shinohara *et al.*, 2013) and leaf development (Scarpella *et al.*, 2006), is more complex than the root, so the search for principal determinants has great appeal (Smolarkiewicz and Dhonukshe, 2013). However, such determinants depend greatly on the level studied: morphological, cytological, genetic, physiological and molecular.

AGPs provide a new perspective with their novel role as regulators of Ca^{2+} signalling and metabolism. The Ca^{2+} oscillator rationalizes this by relating the size of the AGP–Ca^{2+} capacitor to the amplitude of the Ca^{2+} signal, and hence cellular response. Significantly the size of the AGP–Ca^{2+} capacitor depends not only on AGP concentration at the protoplast surface but also on variation in the number of Hyp–AGs and their size in any given AGP (Lamport, 1977; Pope, 1977; Qi *et al.*, 1991; Xu *et al.*, 2008). Presumably, cells with an AGP deficit will not react to signals that require release of Ca^{2+}. This may be relevant to the profound problem of branching where 'apical dominance' suppresses axillary buds but far less so in bushy growth. This involves complex interactions between auxin transport, and rapid redistribution of auxin efflux transporter PIN proteins by other growth substances, including cytokinins and the recently discovered branching repressors, strigolactones (Coombs, 2013; Jiang *et al.*, 2013; Shinohara *et al.*, 2013; Smith, 2013; Zhou *et al.*, 2013). Intriguingly, over-expression of a single AGP gene dramatically altered the phenotype of tomato plants from tall to bushy (Sun *et al.*, 2004). We tentatively suggest that overexpression of AGPs in axillary buds makes them more responsive to auxin signals by increasing the availability of Ca^{2+}. There is a parallel here with phenotypic variation in

Streptocarpus caused by the β-Yariv reagent and the conclusion that AGPs play a pivotal role in pattern formation during plant morphogenesis (Rauh and Basile, 2003). How far this simple scenario accounts for the activation of lateral buds after terminal shoot decapitation remains to be seen.

Leaves

Morphogenesis of the leaf is currently of great interest. In 1606 Adrian Spieghel wrote 'But what is the leaf' (Arber, 1950), answered by Nehemiah Grew (~1666) co-founder of plant anatomy: 'The skin of the leaf is only the amplification of that of a branch' (Arber, 1950). Not surprisingly leaves also involve similar PIN-directed auxin gradients that mediate morphogenesis (Benkova *et al*., 2003; Scarpella *et al*., 2006; Barkoulas *et al*., 2008) by the formation of auxin maxima at sites of tissue outgrowth (Di Giacomo *et al*., 2013; cf. Fig. 3B). Again this implies involvement of the AGP–Ca^{2+} capacitor.

Flowering, fertilization and early embryogenesis

The transition from leaf morphogenesis to flowering (Taoka *et al*., 2013) and reproductive growth is essentially a study in leaf modification. Again AGPs are involved at every developmental stage evidenced by histochemical detection (Yariv) of AGPs in ovaries (Gane *et al*., 1995*a*), immunodetection of a non-classical AGP in styles (Sommer-Knudsen *et al*., 1996), direct isolation of classical AGPs from styles and stigmas (Gane *et al*., 1995*b*) and indirect evidence from auxin-dependent patterning of the ovule (Pagnussat *et al*., 2009). Indeed, 'AGPs are essential for somatic embryogenesis' (Kreuger and van Holst, 1993) although that idea was based on the assumption that AGPs are freely diffusible between cells.

AGP appearance may be dynamic or static. For example, AGP epitopes localized in unfertilized tobacco egg cells disappear rapidly after fertilization (Qin and Zhao, 2006) and the stylar transmitting tissue accumulated AGPs in response to pollination (Qin *et al*., 2007) while differential expression of AGPs during early embryogenesis (Costa *et al*., 2013*b*) suggests that asymmetric delivery of AGPs determines the fate of the basal cell (Souter and Lindsey, 2000). Indeed, specific AGPs associated with directional pollen tube tip growth are abundant along the pistil transmitting tissue pathway based on its reactivity with monoclonals MAC207 and JIM13 but not JIM8 (Coimbra and Duarte, 2003). In contrast, cells of the micropyllar nucellus pathway were JIM8- and MAC207-reactive (Coimbra and Salema, 1997). Finally, an arabidopsis double null mutant of two pollen-specific AGPs (*agp6 agp11*) decreased pollen tube growth with concomitant altered expression levels of calcium- and signalling-related genes. The suggested AGP calcium interaction via calmodulin (Costa *et al*., 2013*a*) is consistent with our proposal in section II that during pollen tube growth AGPs and AGP–Ca^{2+} dissociation may be a crucial determinant of Ca^{2+} gradients and pollen tube guidance.

THE AGP–Ca^{2+} OSCILLATOR: STRESS

The pervasive presence of Ca^{2+} oscillations and Ca^{2+} signalling in stress-related plant growth and development (Bose *et al*., 2011) is consistent with the major role of Ca^{2+} as a central node in the overall signalling web (Tuteja and Sopory, 2007) and, as inferred here, involvement of the AGP–Ca^{2+} oscillator as follows.

Abiotic stress and wound response

Abiotic stress such as drought, heat shock, cold shock, wound response and salinity increase cytosolic Ca^{2+} mostly due to influx from the apoplast (Knight *et al*., 1997; Neill *et al*., 2002; Lecourieux *et al*., 2006). Indirect evidence involves AGPs in these responses to stress: the Yariv reagent leads to abrupt cessation of pollen tube growth with massive accumulation of the Yariv–AGP complex at the tube tip (Roy *et al*., 1998). The Yariv reagent also triggers wound-like responses in cultured cells (Guan and Nothnagel, 2004) and PCD (Gao and Showalter, 1999; Chaves *et al*., 2002). PCD also involves Ca^{2+} influx (Groover and Jones, 1999). As the Yariv complex undoubtedly binds Ca^{2+}, accumulation of a periplasmic AGP–Yariv complex represents a potentially larger pool of available Ca^{2+}.

Acacia senegal exemplifies AGP upregulation in response to wounding even though the AGP (Qi *et al*., 1991; Goodrum *et al*., 2000) and AGP-like gum arabic polysaccharides (Siddig *et al*., 2005) function as a plastic wound sealant rather than an AGP–Ca^{2+} oscillator. Although known for many years, the significance of Ca^{2+} bound by the uronic acids of gum arabic only became clear when 3D molecular modelling of the Hyp–AG structure revealed the mechanism and rationale for specific Ca^{2+} binding by the Hyp–AG subunits of periplasmic AGPs (Lamport and Varnai, 2013).

Salt stress

Salt stress is of particular interest with huge economic and ecological significance. The salt overly-sensitive (SOS) signalling pathway (Zhu, 2001) enhances tolerance to saline conditions via SOS1 the Na^+/H^+ antiporter (Na^+ efflux) activated via the SOS2 kinase in conjunction with the SOS3 Ca^{2+} sensor that detects elevated cytosolic levels of Ca^{2+} (Ishitani *et al*., 2000). An extracellular source of Ca^{2+} is critical (Tuteja and Sopory, 2007). However, high levels of Na^+ compete with AGP–Ca^{2+} (Fig. 6) and may thus dissipate Ca^{2+} availability. Upregulation of AGP biosynthesis by salt-stressed tobacco cells (Lamport *et al*., 2006) may reflect a homeostatic adaptation for Ca^{2+} retention. Halophytes may have solved disruption to Ca^{2+} signalling by using AGPs that discriminate more effectively against Na^+ by subtle variation of the Ca^{2+}-binding subunit. This might account for the occurrence of 3-*O*-methylgalactose in desiccation-resistant lycophyte genera such as *Lycopodium* and *Selaginella* (Popper *et al*., 2001) and 3-*O*-methylrhamnose (acofriose) in AGPs isolated from the moss *Physcomitrella* (Popper *et al*., 2004; Fu *et al*., 2007) and Charophytes such as *Chara* and *Coleochaete*, but not observed (yet!?) in higher plants.

Pathogenesis and symbiosis

'Hold your friends close but your enemies closer' reflects the progress from parasite to symbiont. Both relationships involve calcium signalling (Lecourieux *et al*., 2006) with associated AGPs in root–microbe interactions comprehensively reviewed

(Nguema-Ona *et al*., 2013). Frequently these AGPs appear as markers or predictors of potential cell fate that is presumably finally triggered by an auxin signal (Grunewald *et al*., 2009) or its repression (Navarro *et al*., 2006).

ONLY CONNECT . . . STRUCTURE WITH FUNCTION . . . BACK TO THE FUTURE OF AGPs

Hydroxyproline was first identified in plants in the late 1940s as a minor (secondary) amino acid (Joslyn and Stepka, 1949; Maehly and Paleus, 1950; Hunt, 1951; Steward *et al*., 1951). Later its main location bound to the primary cell wall (Dougall and Shimbayashi, 1960; Lamport and Northcote, 1960; Lamport, 1963*b*) suggested a structural protein by analogy with collagen, the major structural protein of animals. Subsequent work recognized two major families of cell surface HRGPs: the extensins crosslinked (Held *et al*., 2004) to the wall itself and classical AGPs primarily located at the surface of the plasma membrane. Both families are characteristically extended polypeptides rich in glycosylated Hyp. Despite this similarity in molecular design, differences in their glycosylation underpin quite different roles – extensins, self-assembling rod-like amphiphiles stabilized by short arabinooligosaccharides, are scaffolding proteins that template new cross-wall deposition (Cannon *et al*., 2008). By contrast, the exquisitely designed AG polysaccharides of classical AGPs possess repetitive subunits whose paired glucuronic acid residues bind substantial amounts of Ca^{2+} at the plasma membrane: hence, a Ca^{2+} signalling role. Nevertheless, this work only scratches the surface regarding our understanding of AGPs and their possible multifunctional role. There is much to do at all levels of AGP function from molecular to environment:

The challenge to dissect the molecular role of each AGP subdomain includes both protein and glycosubstituents each with their own fascinating problems.

The N-terminal signal sequence and C-terminal GPI-addition signal are well known. However, the 12-residue basic subdomain of LeAGP1, the most abundant and best known AGP of BY-2 cells, remains a mystery (Pogson and Davies, 1995; Li and Showalter, 1996). Such lysine-rich subdomains in other AGPs (Yang *et al*., 2005, 2007) may enable binding to phospholipid headgroups or pectate carboxylates and would contribute to orientation and cell surface ordering of AGPs (Gens *et al*., 2000; Pickard, 2007).

Molecular dissection of AGP glycosylation presents intriguing questions: the size and precise composition and spacing of Hyp–AGs along the AGP polypeptide as well as the total number of Hyp–AGs in an AGP are most likely determined or encoded by the primary amino acid sequence, especially the AP and SP motifs, whose numbers and clustering vary widely between different classical AGPs. Are Hyp–AGs 'tuned' to discriminate between Ca^{2+} and other cations such as Al^{3+} and high levels of Na^{+} (Fig. 6)? Besides the Ca^{2+}-binding role of glucuronic acid residues (how 'essential' is the terminal rhamnose?) can we dissect the role of the β,1–3-linked AG backbone and its sidechain substituents? What does the triarabinosyl branch add? Some suggest that the arabinosyl sidechain is essential for binding of the Yariv reagent (Komalavilas *et al*., 1991; Serpe and Nothnagel, 1994; Classen *et al*., 2000).

This suggests a molecular role for the α-L-linked triarabinosyl sidechain *in muro*; based on the similar sterochemical configuration of α-L- and β-D-sugars, α-L-linked arabinosyl sidechains might dock with the terminal β-D-galacturonic acid of pectic RG-II sidechain-A; competitive disruption of the apiosyl borate crosslink (O'Neil *et al*., 2004) would thus plasticize the pectic network. However, others suggest that the Yariv reagent binds to the AG β-linked galactan backbone, a discrepancy that may reflect the different binding assays used (Kitazawa *et al*., 2013).

This raises further questions about Hyp–AG biosynthesis, which requires a minimum of eight or nine AGP glycosyltransferases to build a repetitive Hyp–AG; currently four have been identified: AtGALT2 (*At4g21060*), and Hyp-*O*-galactosyltransferase of the GT31 family (Basu *et al*., 2013); AtGALT31A (*At1 g32930*), a β-1,6-glactosyltransferase also in the GT31 family (Geshi *et al*., 2013); AtGlcAT14A (*At5g39990*), a β-glucuronosyltransferase of the GT14 family (Knoch *et al*., 2013); and AtFUT4 (*At2g15390*) and AtFUT6 (*At1g14080*), α-(1,2) fucosyltransferases of the GT37 family (Wu *et al*., 2010; Liang *et al*., 2013). The evolution of a functional Hyp–AG Ca^{2+}-binding glycomotif of such elegant complexity (or simplicity?) harks back to the past origin of glycosylated Hyp in photosynthetic protists (Gotelli and Cleland, 1968; Lamport and Miller, 1971; Miller *et al*., 1972; Bollig *et al*., 2007). Tantalisingly, however, protists lack classical AGPs critically identified by chemical characterization. Indeed, we have been unable to isolate classical AGPs from *Coleochaete* despite the presence of Yariv-reactive material (Buglass *et al*., 2007)! The appearance of classical AGPs in bryophytes (Lamport, 1970) reflects the sea change that enabled the transition to terra firma and a new challenging environment (Popper and Fry, 2003).

The journey from HRGP structure to function begun half a billion years ago, in human terms only half a century ago, has finally arrived at molecular-level roles for both extensins (Lamport *et al*., 2011) and AGPs with the paradoxical conclusion that although often perceived, both figuratively and literally, as peripheral glycoproteins, in fact AGPs and extensins play a central role in plant growth and development as originally surmised (Lamport, 1963*b*).

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