

SHORT COMMUNICATION

## A role for arabinogalactan-proteins in plant cell expansion: evidence from studies on the interaction of $\beta$ -glucosyl Yariv reagent with seedlings of *Arabidopsis thaliana*

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### Summary

Seedlings of *Arabidopsis thaliana* were germinated and grown in medium containing  $\beta$ -glucosyl Yariv reagent ( $\beta$ GlcY), a synthetic phenyl glycoside that interacts specifically with arabinogalactan-proteins (AGPs), a class of plant cell surface proteoglycans. The effect of  $\beta$ GlcY on the seedlings was to reduce the overall growth of both the root and the shoot.  $\beta$ GlcY only accumulated in the root tissues and the reduced growth of the shoot appeared to be an indirect effect of impaired root growth. Reduced root growth was a consequence of a reduction in cell elongation during the postproliferation phase of elongation at the root apex and this was associated with extensive radial expansion of root epidermal cells.  $\beta$ GlcY penetrated roots as far as the endodermis and it is suggested that the interaction of  $\beta$ GlcY with AGPs in the load-bearing cell layers inhibited root elongation. When  $\beta$ GlcY was added to carrot suspension-cultured cells that had been induced to elongate rather than proliferate, cell elongation was inhibited. The AGP-unreactive  $\alpha$ -galactosyl Yariv reagent ( $\alpha$ GalY) had no biological activity in either of these systems.

### Introduction

Arabinogalactan-proteins (AGPs) are a heterogeneous class of proteoglycans found in higher and lower plants and many of their secretions (Fincher *et al.*, 1983). AGPs occur in leaves, stems, roots, floral organs and seeds, and are associated with the plasma membrane and the cell wall (Fincher *et al.*, 1983; Knox *et al.*, 1991; Schopfer, 1990). In addition, cultured plant cells secrete AGPs in to their medium, often in copious amounts (Knox *et al.*, 1991; Komalavilas *et al.*, 1991; Zhu *et al.*, 1993).

The carbohydrate moiety of AGPs contributes up to 98% of their mass and is characterized by a  $\beta(1\rightarrow3,1\rightarrow6)$ -

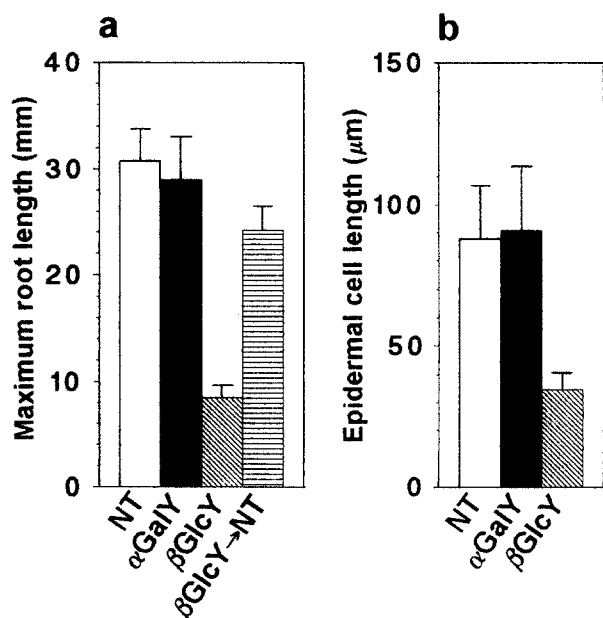
galactan framework substituted with a variety of other residues, principally arabinose and uronic acids, but composition can vary considerably between AGPs (Fincher *et al.*, 1983). The protein component comprises between 2 and 10% of the mass of AGPs and with the recent characterization of amino acid sequences it is now becoming apparent that it, like the carbohydrate moiety, is highly variable (Chen *et al.*, 1994; Du *et al.*, 1994; Mau *et al.*, 1995).

The function of AGPs is uncertain, and may be diverse (Chasan, 1994). There is evidence for their involvement in the regulation of somatic embryo development (Egertsdotter and von Arnold, 1995; Kreuger and Van Holst, 1993, 1995; Kreuger *et al.*, 1995), cell proliferation (Basile and Basile, 1993; Serpe and Nothagel *et al.*, 1994) and cell expansion (Schopfer, 1990; Zhu *et al.*, 1993). Evidence from immunolocalization studies has indicated extensive developmental regulation of AGP carbohydrate epitopes at the plasma membrane in relation to cell and tissue differentiation in roots (Knox *et al.*, 1991), flowers (Pennell and Roberts, 1990; Pennell *et al.*, 1991) and embryos (Stacey *et al.*, 1990).

A diagnostic feature of AGPs is their specific interaction with certain synthetic phenyl glycosides, known as Yariv reagents (Yariv *et al.*, 1962, 1967). These are coloured, multivalent compounds; the most commonly used being  $\beta$ -glucosyl Yariv reagent ( $\beta$ GlcY). The nature of this interaction is not fully understood but Yariv reagents have been used to purify AGPs as AGP- $\beta$ GlcY complexes (Jermyn and Yeow, 1975; Komalavilas *et al.*, 1991), as specific histochemical probes to localize the subcellular distribution of AGPs (Clarke *et al.*, 1978; Schopfer, 1990) and as stains to visualize AGPs following electrophoresis (Du *et al.*, 1994; Knox *et al.*, 1991). Moreover, it has recently become apparent that the addition of  $\beta$ GlcY to living systems may disrupt AGP function and provide an insight into the role(s) of AGPs *in planta*. Using this approach Serpe and Nothagel (1994) demonstrated that  $\beta$ GlcY reversibly inhibited the proliferation of suspension-cultured rose cells, and suggested that AGPs may be involved directly or indirectly in cell division. The non-AGP reactive  $\alpha$ -galactosyl Yariv reagent ( $\alpha$ GalY) had no activity in this system (Serpe and Nothagel, 1994).

We have studied the effects of  $\beta$ GlcY on whole plants and we report here the growth of seedlings of *Arabidopsis*

*thaliana* in medium containing both AGP-reactive and AGP-unreactive Yariv reagents. In this system,  $\beta$ GlcY caused a disruption of cell elongation in the root. We have also focused on the possible role of AGPs in cell expansion by determining the effects of Yariv reagents on a line of suspension-cultured carrot cells that can be induced to elongate, rather than proliferate, by manipulation of the culture conditions.



**Figure 1.** Bar graph showing the effects of  $\beta$ GlcY on root length (a) and root epidermal cell length (b) of 17-day old *Arabidopsis* seedlings.

Root growth and the elongation of root epidermal cells was reduced in seedlings grown in medium containing 30  $\mu$ M  $\beta$ GlcY compared with seedlings grown in 30  $\mu$ M  $\alpha$ GalY or seedlings that were not treated (NT). Some seedlings were grown with  $\beta$ GlcY for 12 days, then transferred to  $\beta$ GlcY-free medium for 5 days ( $\beta$ GlcY  $\rightarrow$  NT). All epidermal cell length measurements were made in a region of the root between 2.5 and 5 mm from the root tip.  $N > 20$  for all treatments. Error bars indicate the standard errors of the means.

## Results

### *The effects of $\beta$ GlcY on the growth of Arabidopsis thaliana seedlings*

The germination of *Arabidopsis* was not affected by sowing on medium containing 30  $\mu$ M  $\beta$ GlcY, with the radicles emerging at the same time as in the controls. However, during subsequent growth the  $\beta$ GlcY was taken up by the roots, colouring them red, and root growth was reduced greatly as shown in Figures 1(a) and 2(a). The roots of seedlings grown for 17 days in 30  $\mu$ M  $\beta$ GlcY were less than a third of the length of those of untreated seedlings, while the growth of roots of seedlings grown in the same concentration of  $\alpha$ GalY, a Yariv reagent that does not bind to AGPs, showed no significant difference compared with untreated seedlings (Figures 1a and 2a). The uptake and binding of  $\beta$ GlcY (as assessed by red colouration) was confined to the roots of seedlings, as indicated in Figure 2(b), even when shoots were deliberately exposed to  $\beta$ GlcY for several days.

Analysis of *Arabidopsis* root homogenates by separation by SDS-PAGE followed by probing with  $\beta$ GlcY revealed its reactivity with a smear of material of apparent molecular weight of 100–200 kDa, a characteristic of an AGP (Figure 3). A band of similar size and characteristics was present in homogenates prepared from shoots (Figure 3). Root preparations were also probed with the anti-AGP monoclonal antibodies LM2 (Smallwood *et al.*, 1996) and JIM16 (Knox *et al.*, 1991). LM2 bound to material at a similar position to  $\beta$ GlcY, whereas JIM16 bound to a smear of apparent molecular weight 80–130 kDa (Figure 3) suggesting that  $\beta$ GlcY-reactive AGPs are a subset of a larger AGP population.  $\alpha$ GalY did not bind to the root or shoot homogenates under the same conditions (data not shown).

Although its binding was restricted to the roots, the presence of  $\beta$ GlcY reduced the overall growth of the seedlings with the relative lengths of the roots and the shoots equivalent to the untreated controls as shown in

**Figure 2.** The effects of  $\beta$ GlcY and  $\alpha$ GalY on the growth of *Arabidopsis* seedlings.

(a) Effects of Yariv reagents on the growth of seedlings. Treatments and labelling are as described for Figure 1. Scale bar = 1 cm.

(b) When *Arabidopsis* seedlings were grown in  $\beta$ GlcY the binding of  $\beta$ GlcY was restricted to the roots of the seedlings. Scale bar = 1 mm.

(c and d) Compared with untreated *Arabidopsis* seedlings (c) the epidermal cells [e] of the roots of seedlings grown in  $\beta$ GlcY (d) were shorter and had undergone extensive radial expansion resulting in dramatically bulged cells. Scale bars = 100  $\mu$ m.

(e) When *Arabidopsis* seedlings were transferred from untreated medium (NT) on to medium containing  $\beta$ GlcY, only the portion of the root grown post-transfer showed the morphological effects associated with  $\beta$ GlcY treatment. The point of transition from untreated to  $\beta$ GlcY-medium is indicated by the arrowhead. Scale bar = 100  $\mu$ m.

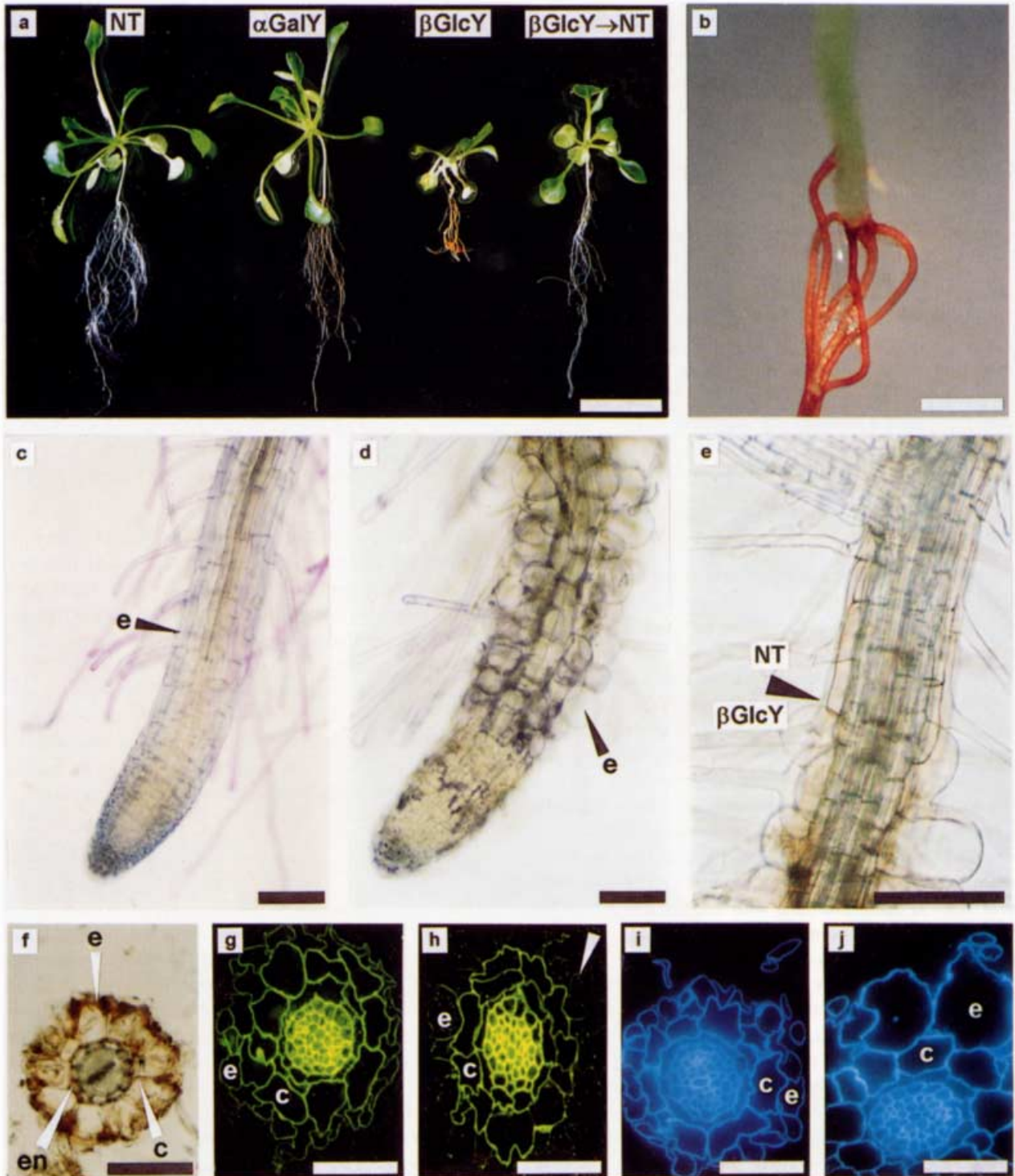
(f) Transverse cryosection of a root of an *Arabidopsis* seedling grown in the presence of  $\beta$ GlcY. The red coloured  $\beta$ GlcY penetrated roots and bound in the cell walls of epidermal [e], cortical [c] and endodermal [en] cells, but was excluded from the stele. The outer epidermal cell walls collapsed during cryosectioning. Scale bar = 50  $\mu$ m.

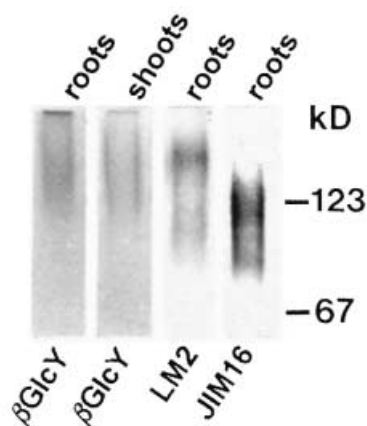
(g–j) Transverse sections of resin-embedded untreated roots (g) and (i) and roots grown in the presence of 30  $\mu$ M  $\beta$ GlcY (h) and (j). Sections shown in (g) and (h) were probed with the anti-pectin antibody JIM7 and sections (i) and (j) were probed with Calcufluor. Cortical cells [c] did not appear to be affected by  $\beta$ GlcY treatment while epidermal cells [e] had undergone extensive radial expansion and showed reduced reactivity with JIM7 — note the faint labelling arrowed in (h). Scale bars = 50  $\mu$ m.

Figure 2(a). Reduction in growth was proportional to the concentration of  $\beta$ GlcY supplied in the medium. The lowest concentration of  $\beta$ GlcY that resulted in reduced growth was 5  $\mu$ M. As the seedlings grew,  $\beta$ GlcY was progressively removed from the medium and incorporated into the roots. At low concentrations of  $\beta$ GlcY the

medium was eventually depleted of  $\beta$ GlcY, after which seedling growth proceeded as normal (not shown). It was with 30  $\mu$ M  $\beta$ GlcY that the reduction in seedling growth was most apparent and all subsequent experiments were performed using this concentration.

In order to test if the effects of  $\beta$ GlcY were reversible





**Figure 3.** SDS-PAGE analysis of material from shoots and roots of seedlings of *Arabidopsis* probed with  $\beta$ GlcY or the anti-AGP monoclonal antibodies LM2 and JIM16.

Twenty micrograms of protein were loaded per lane.

some seedlings were transferred from medium containing 30  $\mu$ M  $\beta$ GlcY on to untreated medium and vice versa. When seedlings were grown in 30  $\mu$ M  $\beta$ GlcY for 12 days, then transferred to  $\beta$ GlcY-free medium for 5 days, root growth rapidly increased (within 24 h) to that of untreated seedlings, with a concomitant increases in shoot growth (Figure 2a).

#### *$\beta$ GlcY acts in the region of cell elongation and results in changes to epidermal cell morphology*

$\beta$ GlcY did not appear to bind to cells of the root cap or to the root meristem and the cells in this region did not differ morphologically from those in the root tips of untreated roots (Figure 2c and d). Swelling of the tip of root hairs was occasionally seen in  $\beta$ GlcY-treated plants although the extent of this was variable. The epidermal cells of the roots of  $\beta$ GlcY-treated roots were in the order of over 60% shorter than the equivalent cells in untreated roots (Figure 1b) and underwent a dramatic radial expansion which resulted in the extensive bulging of the outer epidermal cell walls (Figure 2d, e and j). In some cases the epidermal cells of  $\beta$ GlcY-treated roots were distended so that the radial diameter was up to sixfold that of untreated cells. Bulging was seen in epidermal cells with and without root hairs (Figure 2d and e). The morphology of the root epidermal cells was not altered by  $\alpha$ GalY.

When seedlings were grown in medium without Yariv reagents for 12 days, then transferred to medium containing 30  $\mu$ M  $\beta$ GlcY for several days, the portion of the root newly grown after transfer had all the morphological changes previously described for seedlings grown continuously in 30  $\mu$ M  $\beta$ GlcY, but  $\beta$ GlcY had no effect on the epidermal cells of roots already grown in untreated medium (Figure 2e). Therefore, the effects resulted from the activity of  $\beta$ GlcY during the period of cell elongation.

Analysis of transverse sections of  $\beta$ GlcY-treated and untreated roots indicated that only the epidermal cells underwent extensive radial expansion in response to  $\beta$ GlcY (Figure 2g–j). The walls of the bulged cells appeared to be thinner than the adjacent cortical cells. The anti-pectin monoclonal antibody, JIM7 (Knox *et al.*, 1990) is an effective probe for all cell walls at the root apex of *Arabidopsis* (Figure 2g) but it showed virtually no binding to the bulged epidermal cells of  $\beta$ GlcY-treated seedlings (Figure 2h). The cellulose-reactive fluorescent probe Calcufluor was an effective probe to visualize the cell walls of the bulged in resin-embedded sections of these roots as shown in Figure 2(j).

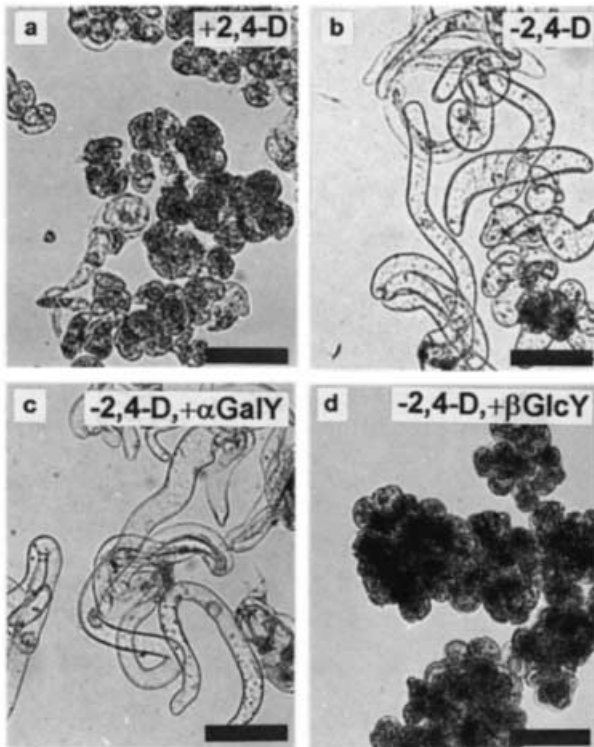
In order to understand the action of  $\beta$ GlcY in reducing root growth it is important to determine the extent of  $\beta$ GlcY penetration into the root. The red  $\beta$ GlcY could not be seen in resin-embedded sections of treated roots, but could be seen in cryosections cut to a thickness of 100  $\mu$ m (Figure 2f). The bulged epidermal cells collapsed during cryosectioning to leave a layer of intense staining surrounding the cortical cells.  $\beta$ GlcY was associated with the epidermal, cortical and endodermal cells but did not appear to enter the stele (Figure 2f).

#### *The effects of $\beta$ GlcY on the elongation of carrot suspension culture cells*

To further investigate the direct effect of  $\beta$ GlcY on expanding and elongating plant cells, it was added to elongating cultured carrot cells (McCann *et al.*, 1993). When carrot cells were diluted 100-fold into fresh medium without 2,4-D, cell proliferation was arrested and at least 70% of the cells elongated unidirectionally by as much as 20-fold of their original diameter (Figure 4b). This cell elongation was completely inhibited if 30  $\mu$ M  $\beta$ GlcY were added to the cells immediately after dilution and removal of 2,4-D (Figure 4d). Under the same conditions,  $\alpha$ GalY had no effect on cell elongation (Figure 4c).

#### **Discussion**

When seedlings of *Arabidopsis* were grown in the presence of  $\beta$ GlcY overall growth was reduced. This effect was not induced by the AGP-unreactive  $\alpha$ GalY indicating that the action of  $\beta$ GlcY was AGP-dependent. The reduction in root length correlated with a reduction in cell elongation in the postproliferation zone of elongation. The observation that  $\beta$ GlcY did not enter the shoot tissues suggests that its effect on shoot growth was an indirect effect, probably due to reduced uptake of water and nutrients by the aberrant roots. SDS-PAGE analyses indicated that the leaves contained a  $\beta$ GlcY-reactive AGP of similar abundance and size to that found in roots and it is possible that



**Figure 4.** The inhibition of elongation of suspension-cultured carrot cells by  $\beta$ GlcY.

- (a) Untreated cells.  
 (b) Cells 2 days after 1/100 dilution into 2,4-D-free media.  
 (c) Cells 2 days after 1/100 dilution into 2,4-D-free media containing 30  $\mu$ M  $\alpha$ GalY.  
 (d) cells 2 days after 1/100 dilution into 2,4-D-free media containing 30  $\mu$ M  $\beta$ GlcY.

Scale bar = 100  $\mu$ m.

the lack of binding was due to the physical exclusion of  $\beta$ GlcY from the leaves by the cuticle.

The driving force of plant cell expansion is turgor pressure, but the fine control is provided by the regulation of the loosening of cell walls of specific cell layers (Pritchard, 1994). There is compelling evidence that shoot extension is modulated by the epidermis, whereas in roots the load-bearing layer is located in the underlying deeper tissues and the epidermis plays no major role in controlling root growth (Burstrom, 1971; Pritchard, 1994). Although we did not measure epidermal cell volume, it was clear that the shorter, bulged epidermal cells of  $\beta$ GlcY-treated plants had in many cases, a greater volume than the epidermal cells of untreated plants. Therefore the elongation, but not the expansion, of these cells was reduced. Our observations indicate that  $\beta$ GlcY penetrated roots up to and including the endodermis. We suggest that  $\beta$ GlcY reduced the extension of internal load-bearing cell walls but not the extension of the outer epidermal cell wall. The load-bearing cell walls could be the inner cell wall of epidermal cells, the cell walls of the cortex or of the endodermis, or a combination of these. Whatever the site of action of  $\beta$ GlcY our observa-

tions also indicate that it disrupted the normal controls that ensure that expansion is radially synchronized across the root. In the case of the suspension-cultured carrot cells,  $\beta$ GlcY completely inhibited expansion in any direction. The carrot cell walls are load-bearing and these combined observations may indicate that  $\beta$ GlcY-reactive AGPs are involved in the regulation of cell wall extension in load-bearing cell walls.  $\beta$ GlcY-reactive AGPs may therefore be involved in expansion *per se* and/or in the directional control of expansion necessary for elongation.

Some evidence for the involvement of AGPs in cell expansion already exists. Zhu *et al.* (1993) demonstrated that the expansion of NaCl-adapted cultured tobacco cells was reduced compared with unadapted cells, and that this reduction was correlated to the amount of  $\beta$ GlcY-reactive AGP. The inhibition of the proliferation of rose cell suspension cultures by  $\beta$ GlcY (Serpe and Nothnagel, 1994) may be a direct effect implying a role for AGPs in signal transmission across the plasma membrane affecting cell cycle machinery. Clearly, cell division and cell expansion are linked processes and inhibition of the latter by  $\beta$ GlcY may indirectly prevent the former. In maize coleoptiles, a  $\beta$ GlcY-reactive AGP has been localized to the inner and outer faces of the growth-controlling outer epidermal cell wall (Schopfer, 1990) although there is a negative correlation between coleoptile growth and the amount of  $\beta$ GlcY-reactive AGP (Schindler *et al.*, 1995). There are indications in *Arabidopsis* that hypocotyl length is correlated with the amount of AGP present and a mutant *Arabidopsis* has been generated, known as *dim*, that is characterized by a severe reduction in cell length in the longitudinal axis in numerous tissues and also has reduced amounts of  $\beta$ GlcY-reactive AGP (Takahashi *et al.*, 1995). *Arabidopsis* mutants designated *reb* (root epidermal bulger) show similar root morphologies to that induced by  $\beta$ GlcY, with conspicuously bulging root epidermal cells (Baskin *et al.*, 1992). The AGPs of *reb* plants have not been characterized although analysis of this and other mutants that show aberrant cell expansion, such as *shr*, *cob*, *lit*, and *sab* (Benfey *et al.*, 1993) could be highly instructive.

## Experimental procedures

### Materials

Yariv reagents,  $\beta$ GlcY and  $\alpha$ GalY, were obtained from Biosupplies Australia. In certain cases  $\beta$ GlcY was synthesized as previously described (Yariv *et al.*, 1962).

Seedlings of *Arabidopsis thaliana* (ecotype C24, Lehle) were grown for up to 30 days in tissue culture petri dishes (Falcon) on a solid medium consisting of 2.2 g l<sup>-1</sup> Murashige and Skoog basal medium (Sigma) supplemented with 10 g l<sup>-1</sup> sucrose and 2 g l<sup>-1</sup> phytigel (Sigma). They were grown with a 16 h photoperiod (50  $\mu$ mol m<sup>-2</sup> sec<sup>-1</sup>) at 22°C. To assess the effects of Yariv reagents on plant growth, either  $\beta$ GlcY or  $\alpha$ GalY (sterilized by autoclaving) were added to molten media to a final concentration of 30  $\mu$ M.

A carrot (*Daucus carota*) cell suspension culture derived from the line known as L2 or 'Oxford' (Knox *et al.*, 1991; McCann *et al.*, 1993) was cultured in 4.43 g l<sup>-1</sup> Murashige and Skoog basal medium supplemented with 25 g l<sup>-1</sup> sucrose and 0.5 mg l<sup>-1</sup> 2, 4-dichlorophenoxyacetic acid (2,4-D) and subcultured by 10-fold dilution every 7 days. To induce elongation in the cell cultures, cells were washed twice in fresh medium containing no 2,4-D, then diluted 100-fold into medium containing no 2,4-D. To assess the effects of Yariv reagents on elongation, either  $\beta$ GlcY or  $\alpha$ GalY (sterilized by autoclaving) were added to cultures immediately after washing and dilution at a final concentration of 30  $\mu$ M.

### Microscopy and histochemistry

Roots of *Arabidopsis* seedlings were prepared for microscopy by rinsing briefly in distilled water to remove excess phytigel prior to mounting on slides with cover slips and photography on an inverted microscope (Olympus CK-2). Photomicrographs of suspension-cultured carrot cells were taken with an Olympus BH-2 microscope. For sectioning and immunohistochemistry *Arabidopsis* roots were fixed in glutaraldehyde and embedded in LR White resin and sections were prepared as described previously (Smallwood *et al.*, 1995). Cellulose was localized in the cell walls of the sections by incubation with the fluorescent probe Calcufluor at 1 mg l<sup>-1</sup> in phosphate-buffered saline for 2 min and pectin with the anti-pectin monoclonal antibody, JIM7 (Knox *et al.*, 1990) as described previously (Smallwood *et al.*, 1995). In certain cases *Arabidopsis* roots from seedlings grown in the presence of  $\beta$ GlcY were fixed in glutaraldehyde and 100  $\mu$ m thick cryosections produced that were directly examined using bright field optics.

### Gel electrophoresis and electroblotting

The roots and shoots of *Arabidopsis* seedlings grown in untreated medium were homogenized in liquid nitrogen, immediately suspended in an equal volume of electrophoresis sample buffer and then heated at 100°C for 5 min. All samples were centrifuged at 10 000 g for 5 min before supernatants were loaded on to gels. SDS-PAGE on 8.5% polyacrylamide minigels (Bio-Rad) and electroblotting were performed as described previously (Smallwood *et al.*, 1996).  $\beta$ GlcY-reactive AGPs were visualized in gels by staining directly with 30  $\mu$ M  $\beta$ GlcY in 1% NaCl for 3 h at room temperature and de-staining (50% v/v methanol, 7.5% v/v acetic acid in distilled water) for 12 h. Blots were probed with the anti-AGP monoclonal antibodies LM2 and JIM16 as described previously (Knox *et al.*, 1991; Smallwood *et al.*, 1996).

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