Developmental Expression and Localization of Petunia Glycine-Rich Protein 1

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An anti-petunia glycine-rich protein 1 (ptGRP1) antibody was used for biotin-streptavidin-alkaline phosphatase localization of this protein. In petunia stem and leaves grown under different light conditions, these studies revealed a complex pattern of cell localization for this protein. Levels of ptGRP1 were shown to decrease with developmental age of the tissue, appearing to correlate directly with expansive growth and inversely with lignification. Significantly, plants grown under low light (~32 μmol m⁻² sec⁻¹ at noon) showed at least an eightfold increased level of ptGRP1 protein throughout ptGRP1's expression period when compared to plants grown under higher light (~80 μmol m⁻² sec⁻¹ at noon). Evidence also indicated that for one cell type in which ptGRP1 is localized, this protein is imported rather than synthesized. In addition, confocal microscopy studies suggested that ptGRP1 is deposited at the cell wall/membrane interface rather than within the cell wall.

INTRODUCTION

Higher plants, like their animal counterparts, follow a specific developmental program that leads from a single cell to a complex, functional multicellular organism composed of a myriad of cell types, tissues, and organs. Following the development of the plant embryo, the formation of new cells, tissues, and organs becomes restricted almost entirely to the meristem. It is from the undifferentiated apical meristematic cells, located at the tips of shoots and roots, that all primary tissues of the plant are derived, including the lateral meristem and primary meristem tissue. The process by which the apical meristem cells divide and either remain meristematic or begin the developmental process leading to partially differentiated cell types, such as lateral meristem, vascular or cork cambium, protoderm, procambium, or cork cambium, and the process by which such cells further differentiate into mature differentiated cells are only now being elucidated. The contribution that structural proteins, whether cytoplasmic, membranous, or in the cell wall, make to the above series of events has yet to be determined. However, as more is learned about these classes of proteins, particularly those of the cell wall, it is becoming clear that they must participate in at least some of the events of development and morphogenesis, exhibiting as they do cell-, tissue-, and development-specific expression (Cassab and Varner, 1987; Keller et al., 1989; Koltunow et al., 1990; Pennell and Roberts, 1990; Stiefel et al., 1990; Marcus et al., 1991; Sheng et al., 1991; Ye and Varner, 1991; Ye et al., 1991; Chen et al., 1992; Ertl et al., 1992; Goldman et al., 1992; José-Estanyol et al., 1992; Ryser and Keller, 1992; Wyatt et al., 1992).

The immunocytochemical localization of the petunia glycine-rich protein 1 (ptGRP1) was undertaken in the hope that such a study would provide additional information on the role of cell wall proteins during development and differentiation. ptGRP1 was the first non-extensin-like gene isolated that could potentially code for a cell wall protein (Condit and Meagher, 1986). The assignment of the protein product of this gene as a cell wall protein was made based on the following data: (1) the N-terminal 27 amino acids contain an optimal signal sequence for transport and subsequent processing, (2) glycine is predicted to comprise 70% of the amino acids of the main body of the protein, (3) the tertiary structure of 90% of this protein is predicted to form an eight-stranded antiparallel β-pleated sheet, and (4) Varner and Cassab's (1986) isolation of a glycine-rich cell wall protein from pumpkin seed coats with a density of 1.58 g/mL, the predicted density of ptGRP1. The work presented here suggests that the assignment of ptGRP1 as a cell wall protein may not be correct; the primary evidence against such a functional role for this protein is that confocal microscopy localizes this protein to the cell wall/membrane interface and not to the cell wall. In addition, confocal microscopy clearly shows that in areas where the membrane has been displaced from the cell wall and is present as membrane fragments, ptGRP1 is found associated with these membrane fragments, indicating that this protein is more firmly attached to a membrane component than to a cell wall component. The data presented strongly support a membrane-associated location for ptGRP1; however, a cell wall location cannot be completely excluded.
RESULTS

Protein Gel Blot Analysis

We had previously made and characterized an anti-ptGRP1 antibody (Condit et al., 1990). This antibody was raised against the N-terminal portion of the mature ptGRP1 protein and recognized a single peptide of 23 kD in protein gel blot analysis. This antibody was unsatisfactory when used in immunocytochemistry experiments, as outlined in Methods. When compared to preimmune controls, this antibody caused an unconvincing, extremely light staining of primary phloem cells (data not shown). Thus, to perform immunocytochemistry experiments, a second anti-ptGRP1 peptide antibody was made (see Methods). This second antibody was raised against a synthetic peptide of the sequence CGGGSGHGGFGAGGV (a gift of the Monsanto Company, St. Louis, MO). This peptide, with an N-terminal-linked cysteine, represents amino acids 157 through 172 of the mature ptGRP1 protein. This sequence is directly repeated at positions 199 through 214, 241 through 256, and 279 through 294 in ptGRP1. After affinity purification, this antibody, designated α-157-172, was used in both protein and immunocytochemical analysis of ptGRP1 expression. As can be seen in Figure 1, this antibody and the previously characterized anti-ptGRP1 antibody both recognize a single peptide of ~23 kD.

Immunocytochemical Localization of ptGRP1

Immunocytochemical localization was first performed on petunia stems from greenhouse-grown plants under a light intensity of ~80 μmol m⁻² sec⁻¹ (measured at noon and defined for purposes of this paper as moderate light growth conditions). Young petunia stems and leaves grown under these light conditions exhibit maximum extension. Plants grown under higher light have shorter internodes and smaller leaf area, whereas plants grown under lower light, although retaining maximal internode distance, achieve a less radial expansion of their stems and exhibit a smaller leaf area, and the leaf itself is thinner. Figure 2 shows the expression of the ptGRP1 gene in petunia stem at various stages of development when grown under moderate light. As can be seen, ptGRP1 is highly expressed in the primary phloem, pith parenchyma, and cortex collenchyma and appears to localize to the cell wall but not to cell corners in young stem sections taken at 0.3 to 0.4 cm below the apical meristem. (The third internode of this plant was located at 1 cm below the apical meristem.) Close examination of the immunocytochemical staining pattern of ptGRP1 in these sections of petunia stem shows that the levels of this protein in the primary phloem, pith parenchyma, and cortex collenchyma change with development. In all sections, staining of the primary phloem cells is highest, whereas in the youngest stem sections (Figures 2B and 2C), the antigen appears to be deposited in the pith parenchyma cells in greater amounts than in the cortex collenchyma. Sections taken 1 mm below this level at 0.3 to 0.4 cm below the apical bud (Figures 2E and 2F) show an approximately equal staining intensity for this antigen in these two cell types, whereas sections taken 0.8 to 0.9 cm below the apical bud show higher deposition of the ptGRP1 protein in cortex collenchyma than pith parenchyma cells.

In sections of older stem tissue taken 2.0 cm (Figures 2K and 2L) and 5.0 cm (Figures 2N and 2O) below the apical bud, ptGRP1 also appears to localize to the cell walls of primary phloem, collenchyma, and pith parenchyma cells. However, there is a complete loss of ptGRP1 protein first from pith parenchyma cells and then from collenchyma cells of the cortex, whereas the presence of this protein in cells of the primary phloem remains high but does decrease in older cells (compare Figures 2B and 2C with Figures 2N and 2O). In sections ~10 cm below the apical bud, cells of the primary phloem barely stain for ptGRP1 (data not shown). In all sections, no staining is observed when preimmune IgG obtained from the same rabbit is used as primary antibody (Figures 2A, 2D, 2G, 2I, and 2M).

Although our ptGRP1 antibody was affinity purified and no staining was observed with preimmune IgG, the immune IgG fraction still contained antibodies specific for the carrier protein keyhole limpet hemocyanin (KLH) at a titer equal to that present against the ptGRP1 peptide in ELISA assays. Therefore, an antibody raised against a peptide of a putative capsid protein of human cytomegalovirus conjugated to KLH (Lahijani et al., 1991; and a kind gift of Dr. Steven St. Jeor, University of Nevada, Reno) was tested immunocytochemically against all petunia tissue sections used for immunocytochemical localization presented in this paper. Figure 3E shows a section of stem tissue with which the anti-KLH antibodies reacted particularly strongly; in this section, the anti-KLH antibodies have

Figure 1. Gel Blot Analysis of Total Protein Extracted from Cultivar Mitchell Petunia Leaves.

Lane 1 contains preimmune α-157-172 serum; lane 2, affinity-purified α-157-172 serum; lane 3, α-22-36 serum. Numbers at left indicate size standards in kilodaltons.
stained only the fragmented cytoplasmic remains of some protoxylem cells. In other sections tested, this antibody either stained some protoxylem cells, as shown in Figure 3E, or showed no reaction at all (data not shown).

In the stem, ptGRP1 thus appears to be primarily expressed in cells that are not lignified (the primary phloem and cortex collenchyma cells) or those with little lignification (the pith parenchyma) (Essau, 1965). This pattern of ptGRP1 expression is quite different from that seen for proteins homologous to the cell wall 33- and 28-kD proline-rich proteins in light-grown tomato, petunia, potato, tobacco, and soybean stems or for proteins homologous to the French bean GRP 1.8 or soybean seed coat hydroxyproline-rich glycoproteins in tomato, petunia, soybean, and tobacco stems (Keller et al., 1989; Ye et al., 1991). Homologs of these cell wall proteins have been shown to be primarily expressed within various specific cell types of the vascular tissue in a developmentally regulated manner and may be associated with cells that will become extensively lignified.

Relative Cell-Specific Levels of ptGRP1

Localization of ptGRP1 in these studies was via alkaline phosphatase using the substrates 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium. Unfortunately, the chromophoric products of this reaction inhibit the enzyme. Thus, cells staining equally for ptGRP1 might have very different levels of ptGRP1. Therefore, to determine the relative levels of ptGRP1 in the cells of petunia stem, all sections shown in Figure 2 were incubated with twofold decreasing levels of anti-ptGRP1 antibody. An example that typifies the results of this experiment is shown in Figures 3A through 3D. Stem sections taken 3 to 4 mm beneath the bud were incubated with primary antibody concentrations of 8 μg/ml (Figure 3A), 4 μg/ml (Figure 3B), 2 μg/ml (Figure 3C), and 1 μg/ml (Figure 3D). At the highest primary antibody concentration of 8 μg/ml, IgG, cells of the primary phloem, collenchyma, and pith parenchyma all appear to be equally stained (Figures 3A and 2E). Lowering the primary antibody concentration to 4 μg/ml decreases the intensity of staining in all cell types but to a much lower level in the pith parenchyma relative to the primary phloem and collenchyma cells (Figure 3B). At 2 μg/ml, only phloem cells remain stained (Figure 3C), whereas at 1 μg/ml no cells remain stained (Figure 3D). These results indicate that at this point in development the highest concentration of ptGRP1 is in the phloem, followed by collenchyma cells and then the pith parenchyma.

Similar analysis of all stem sections shown in Figure 2 revealed that the highest antigen concentration was always in the primary phloem (data not shown). In sections taken less than 1 cm beneath the apical bud, ptGRP1 could be detected in primary phloem down to a primary antibody concentration of 2 μg/ml, whereas in sections taken more than 1 cm beneath the apical bud, staining of these cells could only be seen at a primary antibody concentration of 4 and 8 μg/ml (data not shown). This latter result substantiates the conclusion made in the above section that the concentration of ptGRP1 in primary phloem cells decreases as these cells mature (Figures 2B and 2N). Staining of collenchyma and pith parenchyma cells in this analysis mirrored their relative concentrations as seen in Figure 2.

Low Light Growth Increases ptGRP1 Concentrations

Previously published work on the characterization of ptGRP1 transcripts and protein product was done on plant material grown under low light conditions rather than the moderate light growth conditions of the above work. To fully correlate the present immunocytochemistry studies with previous work, immunocytochemistry experiments identical to those illustrated in Figure 2 and Figures 3A through 3D were performed on tissue grown in low light (~32 μmol m⁻² sec⁻¹ at noon). Relative to their counterparts grown in moderate light, these low light–grown plants retain maximal internode distance, achieve less radial expansion of their stems, and exhibit a smaller leaf area; the leaf itself is also thinner.

Because the developmental cell-specific staining of low light–grown tissue was very similar to that of moderate light–grown tissue, only the results with one stem section taken 4 mm below the bud are shown (Figures 3F through 3H). As can be seen by comparing Figures 2E, 2F, and 3A with Figures 3F through 3H, plants grown under both light conditions express ptGRP1 in the primary phloem, collenchyma, and pith parenchyma cells. However, in stem sections grown under low light, all cells of the developing vascular system (with the exception of the protoxylem) also appeared to contain significant levels of ptGRP1 (Figures 3F to 3H). Furthermore, in low light–grown plants, there is at least an eightfold greater deposition of ptGRP1 in all cell types in which ptGRP1 is present than in plants grown under higher light. This is shown by the intensity of ptGRP1 staining being the same whether primary antibody was used at a concentration of 1 μg/ml (Figure 3H) or at 8 μg/ml (Figure 3G). As shown above (Figure 3D), the presence of ptGRP1 was undetectable in higher light–grown plants at the primary antibody concentration of 1 μg/ml. (There may be a slight diminution of staining in cells other than the primary phloem within the vascular system of low light–grown plants at the primary antibody concentration of 1 μg/ml.)

In more mature stem sections of low light–grown plants taken 10 cm beneath the apical bud, ptGRP1 remains present only in primary phloem cells (Figures 4A and 4B) in a manner similar to that of higher light–grown plants.

Presence of ptGRP1 in Leaves

Young immature low light–grown leaves of petunia were used to study the deposition of ptGRP1 in this organ. As shown in Figure 3I, the cell specificity of ptGRP1 within a vein is similar to that of the stem. High concentrations of the protein are seen in the primary phloem and outer collenchyma cells, whereas the parenchyma cells surrounding the vascular tissue again
Figure 2. Developmental Expression of ptGRP1 in Plants Grown under Moderate Light Conditions.
show no evidence of the presence of ptGRP1. As in light-grown stems, all cells of the vascular tissue with the exception of the protoxylem exhibit a slight staining (Figure 3I). The absence of ptGRP1 in leaf protoxylem can clearly be seen in Figure 3J, wherein the helical cells of the protoxylem are clearly visible within an area that does not stain for the antigen.

Figures 3I and 3J also show that ptGRP1 appears to be present at a higher concentration in leaf palisade cells than in the spongy parenchyma. In addition, Figure 3I shows one guard cell of a pair intensely stained for ptGRP1 (shown at a higher magnification in Figure 3K). In other leaf sections taken from different leaves or plants, both guard cells of a pair are often observed to stain for ptGRP1 (data not shown). However, in no sections are a majority of guard cells stained, and sometimes no staining of any guard cells is observed. In a similar manner, in stem sections a minority of lenticels stain for the presence of ptGRP1. The pattern of staining in these two last cell types may indicate an environmental induction of this protein.

ptGRP1 Is Transported into Mature Protoxylem Cells

In early development of petunia stems and leaves, there is no evidence for the deposition of ptGRP1 in protoxylem cells (Figures 1B, 1C, 1E, 1F, 1H, and 1I and Figures 2A through 2D, 2F, and 2H through 2J). In fact, in low light-grown petunia stems and leaves, the protoxylem elements appear to be the only cell type within the vascular system in which ptGRP1 is not deposited. However, late in development, when these cells begin to be crushed, deposition of ptGRP1 occurs within some but not all of these cells. Interestingly, deposition of ptGRP1 occurs exclusively at the interface between adjacent protoxylem elements and is not present at interfaces between protoxylem and other nonconducting cell types (Figures 2K, 2L, 2N, and 2O). In addition, only fully mature protoxylem cells stain for the presence of ptGRP1. Because mature protoxylem cells do not contain either cytoplasm or a nucleus, these results indicate that ptGRP1 must be imported into this cell type.

The importation of ptGRP1 into protoxylem cells raises the question of whether (by analogy) ptGRP1 is made in all cell types of the primary phloem, or whether it is made only in certain cell types and imported into others. Figures 4A and 4B show the staining of the primary phloem in stem sections of low light-grown plants at a point 10 cm below the bud. As can be clearly seen, many companion cells stain intensely for the presence of ptGRP1. These cells stain not only at the cell wall but also within the cytoplasm. In addition, it can be argued that the intensity of staining of a primary phloem cell group is roughly proportional to the intensity and number of companion cells staining for ptGRP1 (Figures 4A and 4B), suggesting that ptGRP1 might be imported from the companion cells into other cell types of primary phloem.

ptGRP1 Localizes to the Cell Wall/Membrane Interface

The assignment of the functional role of the protein product of the ptGRP1 gene as a structural cell wall protein was made based on the following data: (1) the N-terminal 27 amino acids contain an optimal signal sequence for transport and subsequent processing, (2) glycine is predicted to comprise 70% of the amino acids of the main body of the protein, (3) the tertiary structure of 90% of this protein is predicted to form an eight-stranded antiparallel β-pleated sheet, and (4) Varner and Cassab's (1986) isolation of a glycine-rich cell wall protein from pumpkin seed coats with a density of 1.58 g/mL, the predicted density of ptGRP1. Studies of the steady state pattern of ptGRP1 RNA and soluble protein were consistent with a role for this protein as a cell wall structural constituent (Condit and Meagher, 1987; Condit et al., 1990). During this immunocytochemical analysis, however, it is observed that many cells (other than companion cells) appeared to be lightly stained in cytoplasmic regions. These areas of cytoplasmic staining appear to resemble torn or crumpled membranes (see in particular Figure 3G). This observation suggested the possibility that the ptGRP1 gene product might actually be located at the cell wall/membrane interface rather than in the cell wall itself. Therefore, to determine the exact location of ptGRP1 deposition, confocal microscopy was performed. In these experiments, reflectance optics were used to determine the location of the alkaline phosphatase reaction product, and autofluorescence

Figure 2. (continued).

(A) to (C) Immunocytochemical localization of ptGRP1 in stem sections 0.2 to 0.3 mm below the apical bud.
(D) to (F) Immunocytochemical localization of ptGRP1 in stem sections 0.3 to 0.4 mm below the apical bud.
(G) to (I) Immunocytochemical localization of ptGRP1 in stem sections 0.8 to 0.9 mm below the apical bud.
(J) to (L) Immunocytochemical localization of ptGRP1 in stem sections 2.0 cm below the apical bud.
(M) to (O) Immunocytochemical localization of ptGRP1 in stem sections 5 cm below the apical bud.
(A), (D), (G), and (M) Primary antibody is preimmune α-157-172 IgG.
(B), (C), (E), (F), (H), (I), (K), (L), (N), and (O) Primary antibody is affinity-purified α-157-172 IgG.
c, cortex collenchyma; cp, cortex parenchyma; ip, inner primary phloem; op, outer primary phloem; p, pith parenchyma; x, protoxylem. Arrows in (K), (L), (N), and (O) indicate deposition of ptGRP1 in mature protoxylem cells. All photographs in this figure were printed at f-stop 16 for 2.9 sec with Kodak 31Y, 24M filters.
Bar = 125 μm for (A), (B), (D), (E), (G), (H), (J), (K), (M), and (N); 50 μm for (C), (F), (I), (L), and (O).
Figure 3. Quantitative Analysis of ptGRPI in Stems of Plants Grown under Moderate and Low Light, and Immunolocalization of ptGRPI in Leaves of Low Light-Grown Plants.

(A) to (D) Stem sections taken 3 to 4 mm below the apical bud of moderate light-grown plants.
(E) Stem section 5 cm below the bud of plants grown in moderate light. Primary antibody, anti-KLH-cytomegalovirus serum.
(F) to (H) Stem sections taken 3 to 4 mm below the apical bud of low light-grown plants.
(I) to (K) Leaf sections from the first leaf of a plant grown in low light.

(A), (F), (G), (I), (J), and (K) Primary antibody is affinity-purified α-157-172 IgG used at a concentration of 8 μg/mL.
(B) Primary antibody is affinity-purified α-157-172 IgG used at a concentration of 4 μg/mL.
(C) Primary antibody is affinity-purified α-157-172 IgG used at a concentration of 2 μg/mL.
(D) and (H) Primary antibody is affinity-purified α-157-172 IgG used at a concentration of 1 μg/mL.

ab, abaxial phloem; ad, adaxial phloem; c, cortex collenchyma; cp, cortex parenchyma; g, guard cell; ip, inner primary phloem; l, leaf palisade cells; op, outer primary phloem; p, pith parenchyma; ph, phloem; s, leaf spongy parenchyma; v, vascular tissue; vb, vascular bundle; x, protoxylem. Arrows in (C) and (E) indicate the nonspecific staining of fragmented protoxylem cytoplasm by an anti-KLH cytomegalovirus antibody.

(A) to (D) were printed at f-stop 16 for 3.9 sec with Kodak 19Y and 12M filters. All others were printed as given in Figure 2.

Bar = 125 μm for (A) to (F) and (I); 50 μm for (G), (H), and (J); 10 μm for (K).
Localization of a Glycine-Rich Protein

B

Figure 4. Immunolocalization of ptGRP1 in Vascular Tissue 10 cm below the Apical Bud from Plants Grown under Low Light.

(A) and (B) Light microscopic image.

(C) Confocal microscopic image.

P, pith. Arrows in (A) and (B) indicate companion cells. Photographs in (A) and (B) were printed as described in Figure 2. Photograph in (C) was printed as described in Methods. Bar in (A), for (A) and (B) = 10 μm; bar in (C) = 1 μm.

was used to determine the location of the cell wall. Using a confocal microscope (MRC600; Bio-Rad), autofluorescence was set to give a green color, whereas reflectance, indicating the location of the ptGRP1 gene product, was set for a red-orange color. If the detected autofluorescence and reflectance overlapped, the system indicated this by giving that region a yellow color.

Figure 4C shows the result of one such experiment. The section of tissue in this figure is from the primary phloem. There is very little yellow color present, indicating that there is little overlap between the location of the ptGRP1 gene product and the cell wall and that the location of this protein (red color) appears to be internal to the cell wall (green color). Confocal microscopy of pith parenchyma and cortex collenchyma cells also shows little or no overlap of ptGRP1 with the cell wall, as shown in Figures 5A through 5F. In particular, it can be seen that areas within the cytoplasm of some cells (indicated by the white arrows in Figures 5A, 5C, 5D, and 5F) appear to contain high levels of ptGRP1 antigen. As visualized under light microscopy, these highly stained regions within the cell are membrane fragments. That one such region is indeed a membrane fragment can be seen in Figure 5C. Here (as indicated by the white arrow), high levels of ptGRP1 antigen (orange-red color) are shown to be contained within the edges of a membrane fragment (green color). (Visualization of the membrane fragment edges by fluorescent optics in this figure is most likely due to light scattering.) These confocal microscopy results indicate that in regions where the membrane has separated from the cell wall, the ptGRP1 antigen remains attached to the membrane rather than to the cell wall. In addition, these experiments show that ptGRP1 is not evenly distributed at this surface but accumulates at discrete points.

Stem sections reacted with our anti-KLH antibodies were also examined by confocal microscopy. One such stem section is shown in Figures 5G and 5H. In this section, the autofluorescence of the cell wall is clearly visible (Figure 5H), whereas only a small portion of some chloroplasts and nuclei show any reflectance (Figure 5G). In the combined reflectance and fluorescent image shown in Figure 5H, only the small reflecting areas of these chloroplasts and nuclei are colored yellow. These anti-KLH antibody experiments clearly show that the reflectance seen in Figures 5A, 5C, 5D, and 5F is due to the presence of the alkaline phosphatase reaction product rather than to nonspecific reflectance of cell structures and also give an indication of the discriminating power of this technique.

DISCUSSION

Tissue and Developmental Distribution of ptGRP1

The work presented in this paper indicates a complex pattern of ptGRP1 deposition in the vegetative plant body. In stems,
Figure 5. Confocal Microscopy Immunolocalization of ptGRP1 in Stem Tissue 0.6 cm below the Apical Bud.
deposition is highest in the primary phloem, followed by cortex collenchyma and pith parenchyma cells, the latter two cell types reaching their peak deposition of ptGRP1 at different times in the overall development program of the petunia stem. The pattern of expression of ptGRP1 in young stem is most similar but not identical to that of the glucuronidase A (gusA formerly uidA) gene under control of the French bean GRP 1.8 promoter containing a deletion that removes a negative regulatory element located between positions -205 and -186 of that gene (Keller and Baumgartner, 1991). This cis-acting element has been shown to limit gene expression in tobacco stems to vascular tissue (Keller and Baumgartner, 1991). Sequence comparison of the 5' flanking regions of the two genes shows that, although ptGRP1 contains sequence homology (properly spaced) to the stem-specific elements 1 and 2 of French bean GRP 1.8 found to be necessary for gene expression in that tissue (Keller and Baumgartner, 1991), no homology is present to the bean negative regulatory element.

In nonvascular tissue of leaves, ptGRP1 is found in highest concentration in palisade cells and then in the spongy parenchyma. In leaf vascular tissue, its deposition resembles that of stem tissue. All cell types in which ptGRP1 protein is present, in both leaves and stems, have in common the fact that they are either not lignified or not extensively lignified (Esau, 1965). This protein thus might play some role in determination of the extent of lignification of the cell wall, with its presence limiting lignification. It is noteworthy that this protein is not detectable in any developing or mature highly lignified cells other than some mature protoxylem cells wherein its pattern of distribution suggests that ptGRP1 is being transported into and through these cells (see below).

If ptGRP1 protein does play some role in the extent of lignification, one would expect that it might also play some role in expandability of the cell. At the very least, it would be expected that this protein would be present in higher amounts in young cells than in old and would not be present in fully expanded cells. This is the exact pattern of deposition that is seen for this protein. In all cell types in which this protein is expressed, it is only present transitorily and the amount of protein present decreases as the cell expands and ages. This is true whether the plant is grown in moderate or low light conditions. However, if the only role of this protein is to limit lignification and allow expansion, it is difficult then to correlate this role with the fact that low light–grown plants have at least an eightfold higher level of this protein than do their moderate light–grown counterparts, because, as can be seen by comparing Figure 3F with Figures 3A through 3D, individual cells in both types of stem seem to be equally well expanded at this point in development. One hypothesis to explain this discrepancy is that this protein might be necessary in higher amounts in low light–grown plants to allow them to retain the potential of expansion rather than be fixed in their partially expanded form.

In petunia development after the seedling emerges, small petunia plants phenotypically most resemble their older low light–grown counterparts during their first 4 to 6 weeks of growth (grown under a light intensity of ~200 μmol m⁻² sec⁻¹ at noon; C. Condit, unpublished data). Only after a certain period of growth do the leaves and stems of these small plants begin to thicken and resemble their older counterparts grown under high light. It is thus conceivable that the high levels of ptGRP1 seen in low light–grown plants might have an actual functional significance in the normal development of petunia.

ptGRP1 Is Transported

Evidence has been presented in this paper that strongly indicates that ptGRP1 is transported into protoxylem cells. As shown in Figures 2K, 2L, 2N, and 2O, ptGRP1 appears in protoxylem cells after they have undergone autolysis and do not contain either a nucleus or a cytoplasm. In addition, the deposition of ptGRP1 appears to be localized in the cell wall and occurs only between adjacent faces of protoxylem cells, rather than on all faces of these cells, a further indicator that ptGRP1 is being transported into and through these cells and occurs, perhaps coincidentally, at a point in development when other cells of the stem show a significant decrease in their levels of ptGRP1. Recently, evidence has been presented that it is highly likely that discrete proteins can be transported into highly

Figure 5. (continued).

(A) to (C) Confocal microscopy of one stem section.
(D) to (F) Confocal microscopy of a second stem section.
(G) and (H) Confocal microscopy of a third section.
(A), (D), and (G) Reflectance images.
(B) and (E) Autofluorescence images.
(C), (F), and (H) Reflectance and autofluorescence images combined.
(A) to (F) Primary antibody is affinity-purified α-157-172 IgG used at a concentration of 8 μg/mL.
(G) and (H) Primary antibody is anti-KLH-cytomegalovirus serum.
Abbreviations are as given in the legend to Figure 3. The yellow/green arrows in (A), (C), (D), (F), and (H) indicate an artifact of reflectance microscopy due to a reflection from the surface of the turret eyepiece that was picked up by the photomultiplier tube of the instrument. White arrows in (A), (C), (D), and (F) indicate cellular membrane fragments that show strong reflectance, indicating the presence of ptGRP1 within these subcellular structures. Bar in (C) = 20 μm for (A) to (C); bar in (F) = 20 μm for (D) to (F); bar in (H) = 20 μm for (G) and (H).
lignified cells. Ryser and Keller (1992) have shown that French bean cell wall protein GRP 1.8 is most probably synthesized in xylem parenchyma cells and deposited into all faces of the primary walls of protoxylem only after their death.

Currently, the possibility that ptGRP1 import into protoxylem cells of petunia has a functional significance for these cells cannot be excluded. It is possible, however, that ptGRP1 is instead being transported to another functional site within the plant via the protoxylem or it could be in the process of being exported from the plant.

**Subcellular Location of ptGRP1**

Previous assignment of the ptGRP1 protein as a structural component of the cell wall was based, as stated above, on protein structural considerations and the fact that Varner and Cassab (1986) had isolated from pumpkin seed coats a glycine-rich cell wall protein with a density of 1.58 g/mL, the predicted density of ptGRP1. Cell fractionation experiments showed that ptGRP1 could be solubilized by 0.4 M NaOH but not by 4% Nonidet P-40 (Condit et al., 1990), supporting the conclusion that ptGRP1 was unlikely to be either an integral or peripheral membrane protein but rather a cell wall protein. This cell fractionation work was performed using tissue that had been previously frozen, which, thus, could possibly have altered the localization of this protein. Also, extraction with Nonidet P-40 was performed at 0°C (necessitated by the instability of this protein). Extraction at 0°C using this level of Nonidet P-40 could have caused the coprecipitation of detergent and solubilized proteins. Thus, the cell fractionation work leaves open the possibility that ptGRP1 is associated with the cell membrane. However, a role of ptGRP1 as an integral membrane protein is not as likely as that of a peripheral membrane protein, because in tissue prints some percentage of ptGRP1 was extracted from fresh tissue using 0.4 M NaOH.

The data presented in this paper support a membrane-associated location of ptGRP1, because confocal microscopy (Figures 4C and 5A through 5F) localizes ptGRP1 to the cell wall/membrane interface at discrete points rather than to the cell wall itself. In addition, confocal microscopy clearly shows that in areas where the membrane has been displaced from the cell wall and is present as membrane fragments, ptGRP1 is found associated with these membrane fragments, indicating that this protein is firmly attached to some membrane component (Figures 5A through 5F). This latter finding strongly supports the conclusion that ptGRP1 is not a cell wall protein but is in some manner associated with the membrane. However, other interpretations of the data consistent with a cell wall location of ptGRP1 are possible.

It can be postulated that the ptGRP1 antigen associated with membrane fragments is actually present in vesicles at the membrane surface. This is possible, even though previous electron microscope work that localized two cell wall proteins, carrot extensin and French bean GRP 1.8, to that structure (Statstrøm and Staehelin, 1988; Ryser and Keller, 1992) did not show accumulation of either protein at the inner surface of the membrane in vesicles (but did find a portion of the French bean GRP 1.8 protein within the Golgi complex). Those experiments detected their respective antigens via secondary antibody conjugated to gold particles, whereas our experiments detected ptGRP1 using secondary antibodies conjugated to biotin and then reacted with streptavidin conjugated to alkaline phosphatase. Use of this latter detection system allows for a much greater amplification of signal than does the former system. Thus, it can be argued that use of the biotin-streptavidin-alkaline phosphatase system would allow for the detection of large amounts of ptGRP1 at the membrane surface in vesicles, whereas use of the former system would not.

The finding by confocal microscopy that the ptGRP1 antigen localized at the cell wall/membrane interface does not overlap with autofluorescence of the cell wall, inconsistent with a cell wall location of ptGRP1, could be interpreted to be due to the possibility that confocal microscopy may not be able to discriminate between deposition at the membrane and that at the newly deposited cell wall surface. In addition, since the anti-ptGRP1 antibody used in these experiments is an antibody to an epitope on the protein, it can be argued that the small number of epipetopes which this antibody recognizes could become masked within more developed regions of the cell wall. This latter consideration would seem to be obviated by the detection of the transport of ptGRP1 within and through the highly structured cell walls of mature protoxylem cells, indicating that these same epitopes should be detectable in other cell walls if present. However, it is now clear that cell walls of different cell types have different chemical compositions and structural components. Thus, although it is possible that ptGRP1 epitopes might be exposed in one type of cell wall, it can be argued that these same epitopes might not be exposed in other cell wall types.

From the above discussion, it is obvious that a conclusive determination of the site of deposition of the ptGRP1 protein cannot yet be made, although, on balance, a membrane-associated site appears more likely than a cell wall site. It would not be completely surprising if ptGRP1 is found to be a membrane-associated protein rather than a cell wall protein, because, unlike all other characterized cell wall proteins, ptGRP1 contains no tyrosine and thus cannot participate in the formation of isodityrosine linkages. These linkages are believed to be involved in determining the architectural structure of the cell wall. Additionally, ptGRP1, unlike all other characterized cell wall proteins, is extremely unstable after lysis of the cell (Condit et al., 1990). Furthermore, the protein structure of ptGRP1 is compatible with a function as a connector protein between the membrane and the cell wall. The first 42 N-terminal amino acids of the mature ptGRP1 protein are extremely hydrophilic, whereas the C-terminal 36 amino acids are composed of a 23–amino acid hydrophobic region followed by an 11–amino acid hydrophilic region (Condit and Meagher, 1988). Thus, the N- and C-terminal ends of ptGRP1 could comprise two separate protein interactive regions separated by a β-pleated sheet region, the whole protein spanning the periplasmic space between the cell membrane and wall. It is,
of course, possible that pGFR1 might perform some type of connector function within the cell wall.

A definitive determination of the actual functional site of pGFR1 must await electron microscopy localization. Use of a polyclonal antibody or several peptide antibodies made to show whether or not pGFR1 is a membrane-associated protein.

**METHODS**

**Preparation and Purification of Anti-pGFR1 IgG**

A synthetic peptide of the sequence CQGSGHGGFGAGGVS was a generous gift of the Monsanto Company. This peptide, with an N-terminal-linked cysteine, represents amino acids 157 through 172 of the mature pGFR1 protein. In addition, this sequence is repeated at positions 199 through 214, 241 through 256, and 279 through 294 in pGFR1. The peptide was linked to keyhole limpet hemocyanin (KLH) as previously described (Condit et al., 1990). Crude IgG of preimmune and immune sera was prepared by caprylic acid and ammonium sulfate precipitations (Harlow and Lane, 1988). Preimmune IgG was further purified by affinity chromatography, using a Bio-Rad Affi-Gel protein A agarose column according to the manufacturer's instructions. Anti-pGFR1 IgG was affinity purified using the peptide linked to Sepharose 4B via CNBr (Pharmacia LKB Biotechnology) according to the manufacturer's instructions. Anti-pGFR1 (α-157-172) antibody was eluted from this column using 100 mM glycine, pH 2.5.

**Protein Gel Blot and Immunocytochemical Analysis**

Greenhouse-grown *Petunia hybrida* cv. Mitchell was used as a source of tissue for these studies. Protein gel blot analysis was performed using preimmune and affinity-purified α-157-172 and our previously characterized anti-pGFR1 antibody (Condit et al., 1990). This latter antibody was raised against amino acids 22 through 36 of the mature pGFR1 protein and was designated α-22-36. Protein extraction, gel electrophoresis and transfer, incubations, washes, and color development were as previously reported (Condit et al., 1990) with the following exception: primary preimmune and immune α-157-172 antibodies were used at a concentration of 3 μg/mL, whereas α-22-36 was used at a concentration of 10 μg/mL; secondary antibody was goat anti-rabbit IgG conjugated to alkaline phosphatase (Southern Biotechnology, Birmingham, AL) used at a dilution of 1:3000.

Plant tissue used for immunocytochemical analysis was from plants with stems ~20 cm in length. Tissue was cut into 1-mm slices while immersed in fixation solution (2% [w/v] glutaraldehyde in 50 mM Tris, pH 7.2, 150 mM NaCl; Keller et al., 1989) and fixed in this same solution for 2 hr at 4°C. Tissue was washed in 50 mM KPO4, pH 7.2, 150 mM NaCl at 4°C for 60 min with three changes of buffer. Tissue was then dehydrated in a series of ethanol (EtOH), xylene steps, each for 30 min at room temperature as follows: once in 10% EtOH, 20% EtOH, 30% EtOH, 50% EtOH, 70% EtOH, and 85% EtOH; twice in 95% EtOH, 100% EtOH, and xylene. Dehydrated tissue was then infused at 58°C for 30 min in a 1:1 mixture (v/v) of xylene/Paraplast (Fisher Scientific, Santa Clara, CA), followed by three 30-min vacuum infusions of 100% Paraplast at 58°C.

Tissue was embedded and cut in 10-μm sections and attached to slides using a 1:30 dilution of a 0.2% polyvinyl alcohol, 0.2% vinyltriethoxysilane solution (Fink, 1987). Paraffin was removed from tissue by incubating the slides at 60°C in xylene twice for 10 min each and then once in 100% EtOH for 10 min. Rehydration of the tissue was accomplished by 5-min incubations of the slides at room temperature as follows: once each in 100% EtOH, 95% EtOH, and 70% EtOH; three times in distilled H2O; once in PBS, 10 mM glycine; once in PBS, 10 mM glycine, 0.5 mg/mL sodium borohydride; once in PBS, 10 mM glycine; and once in TBS (50 mM Tris, pH 7.4, 200 mM NaCl).

Tissue attached to slides was blocked at room temperature in 100% normal goat serum (Sigma) for 1 hr. Normal goat serum was removed from the slides using Kimwipes, and primary antibody at a concentration of 8 μg/mL in a solution (unless otherwise specified in the Results section) of TBS, 0.2% Tween 20, 1.5% BSA, 2.5% normal goat serum was placed over the section. Tissue was covered with a coverslip, and the slides were incubated in a moist chamber overnight. After incubation, coverslips were removed by placing the slides briefly in a solution of TBS, 0.2% Tween 20. Slides were then washed six times in the same solution for 10 min each, with shaking at 50 rpm. After washing and removal of excess moisture, slides were incubated as above for 15 min in 100% normal goat serum, followed by a 45-min incubation with secondary antibody, goat anti-rabbit IgG conjugated with biotin (Southern Biotechnology, Birmingham, AL). Secondary antibody was diluted 1:1500 in TBS, 0.2% Tween 20, 3% BSA, 5% normal goat serum. Removal of coverslips and washing was performed as for the primary antibody. Slides were incubated with streptavidin conjugated to alkaline phosphatase (Southern Biotechnology), as was done for the secondary antibody. To remove excess streptavidin conjugate, slides were washed at room temperature in TBS, 0.2% Tween 20 three times for 10 min each, once in TBS, 0.2% Tween 20, once in TBS, and once in alkaline phosphatase reaction buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM MgCl2).

Color reaction was in alkaline phosphatase reaction buffer containing 0.66 mg/mL nitro blue tetrazolium and 0.12 mg/mL 5-bromo-4-chloro-3-indolyl phosphate. The color reaction was allowed to proceed for 5 min and was stopped by placing the slides in TBS for 5 min. Tissue was then dehydrated by washing for 5 min three times in distilled H2O, followed by 30-sec washes once in 70% EtOH, 95% EtOH two times, 100% EtOH two times, and xylene two times. Slides were air dried overnight and then mounted in Permount (Fisher Scientific). Sections were visualized using a microscope (Zeiss Standard, Oberkochen, Germany) under bright-field illumination and photographed using Kodak Inter-negative 6011 film.

The intracellular localization of pGFR1 was determined using a scanning laser confocal microscope (MRC600; Bio-Rad) using reflectance optics for detection of the alkaline phosphatase reaction product and fluorescence optics for detection of cell wall position using an excitation energy of 488 nm and an emission window of greater than 505 nm. Confocal microscopy pictures were photographed directly from the computer screen using Kodak Kodacolor Gold 100 ASA film.

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