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A Proline-, Threonine-, and Glycine-Rich Protein Down-Regulated by Drought Is Localized in the Cell Wall of Xylem Elements[1]

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A cDNA clone encoding a proline-, threonine-, and glycine-rich protein (PTGRP) was isolated from a wild tomato species (*Lycopersicon chilense*) (L.X. Yu, H. Chamberland, J.G. Lafontain, Z. Tabaeizadeh [1996] Genome 39: 1185–1193). Northern-blot analysis and in situ hybridization studies revealed that PTGRP is down-regulated by drought stress. The level of the mRNA in leaves and stems of 8-d drought-stressed plants decreased 5- to 10-fold compared with that in regularly watered plants. The mRNA re-accumulated when drought-stressed plants were rewatered. Antibodies raised against a glutathione *S*-transferase/PTGRP fusion protein were used to elucidate the subcellular localization of the protein by immunogold labeling. In regularly watered *L. chilense* plants, PTGRP protein was found to be localized in xylem pit membranes and disintegrated primary walls. Examination of sections from drought-stressed plants revealed a significant decrease in the levels of labeling. In these samples, only a few scattered gold particles were detected in the same areas. In the leaf tissues of plants that had been rewatered for 3 d following an 8-d drought stress, the labeling pattern was similar to that of the regularly watered plants. To our knowledge, PTGRP is the first drought-regulated protein that has been precisely localized in the cell wall.

Plant response to drought stress is manifested by various changes in physiological and metabolical processes. It is well established that these changes are at the level of gene expression. During the past decade, several genes that are regulated by water stress and ABA have been identified (for review, see Tabaeizadeh, 1998). We were interested in understanding the molecular mechanism of drought tolerance in higher plants, so we focused our study on *Lycopersicon chilense.* This wild tomato species is a natural inhabitant of the desert areas of South America. During the course of our studies, three genes that are up-regulated by water stress were isolated from this species (Chen and Tabaeizadeh, 1992; Chen et al., 1993, 1994). Moreover, we identified a gene encoding a Pro-, Thr-, and Gly-rich protein (PTGRP) that is negatively regulated by drought (Yu et al., 1996).

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PTGRP has a high content of Pro (26%), Thr (19%), and Gly (18%). Furthermore, two large repeat motifs of Phe-Pro-Met-Pro-Thr-Thr-Pro-Ser-Thr-Gly-Gly-Gly-Phe-Pro-Ser and five $(Gly\text{-}X)_n$ repetitive units are distributed throughout the mature protein. PTGRP mRNA is significantly reduced when plants are subjected to water stress. Down-regulation of the PTGRP gene was also observed in desiccated cell suspensions of *L. chilense* and in those treated with NaCl and mannitol. Considering the common features of Pro-rich proteins (PRPs) (high Pro content, repeated motifs, and a putative signal peptide) and their association with the cell wall, we predicted that the PTGRP protein is targeted to the cell wall (Yu et al., 1996). Elucidation of the biological function of proteins encoded by drought-regulated genes is a challenging problem. Indeed, among the genes isolated so far, only a few encode proteins with known functions (for review, see Ingram and Bartels, 1996). To formulate a working hypothesis concerning the role of PTGRP, it was necessary to determine its precise location at the subcellular level. The results of the present investigation revealed that the PTGRP protein is indeed associated with the cell wall of xylem elements.

MATERIALS AND METHODS

Fusion Protein Construct, Expression, and Purification

The deduced PTGRP protein contains 105 amino acids (Yu et al., 1996). A nucleotide sequence from PTGRP cDNA encoding for amino acids 5 to 105 was produced by 12 cycles of PCR. The fragment was digested with *Eco*RI and *Xho*I and ligated into the corresponding sites of pGEX4T-1 vector (Pharmacia, Montreal) containing glutathione *S*-transferase (GST) to yield the pGEX-PTGRP plasmid. The GST/PTGRP junction was sequenced to ensure that this fragment was in the correct reading frame. The pGEX-PTGRP plasmid was used to produce the GST/PTGRP fusion protein according to the method of Domingo et al. (1994). *Escherichia coli* strain DH5 (BRL, Gaithersburg, MD) were transformed with pGEX/PTGRP and grown at 37°C until the A_{600} reached 0.7. They were then induced with 0.4 mM isopropyl-β-D-thiogalactoside for 4 h at 37°C. After the induction, cells were pelleted by centrifugation at 7,000*g* for 10 min, and then resuspended in 5 mL of ice-cold STE

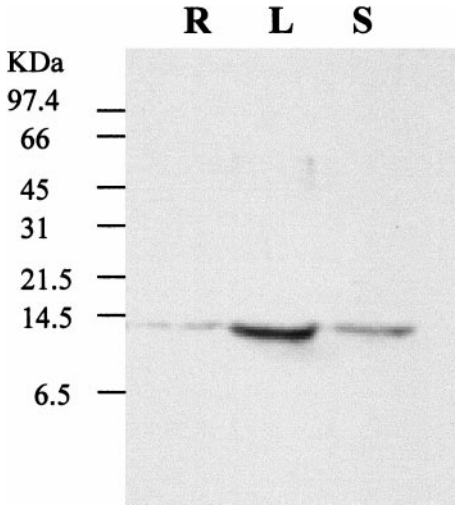

Figure 1. Western-blot analysis of anti-PTGRP antibody with protein isolated from root (R), leaf (L), and stem (S) of regularly watered *L. chilense* plants. Each lane contains 10 μg of protein. The size of the polypeptide is approximately 12.6 kD.

buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1 mM EDTA) containing 100 μg/mL lysozyme and incubated on ice. After 15 min, *N*-laurylsarcosine was added at the final concentration of 1.5% (w/v). After sonication and centrifugation at 10,000*g* (5 min, 4°C), the soluble fraction was boiled for 5 min, loaded on SDS-polyacrylamide gel, and subjected to electrophoresis at 150 V. To visualize the fusion protein band, the gel was stained with Coomassie Blue and rinsed several times with water. The band was recovered and resuspended in 300 μL of water through sonication. The purified protein was 12 kD larger than the 26-kD GST protein. This difference corresponds to the predicted molecular mass of amino acids 5 to 105 of PTGRP.

Production and Purification of the Anti-PTGRP Antibody

Purified protein (150 μg) was emulsified with 1 volume of Freund's complete adjuvant. The emulsion was injected subcutaneously into two New Zealand White rabbits. The second injection (150 μg) was performed 4 weeks after the primary injection. One week later, the blood was collected and centrifuged at 10,000*g* at 4°C to recover the serum.

Prior to antigen injection, a blood sample was taken to be used as preimmune serum. Anti-PTGRP antibodies and preimmune sera were purified by affinity chromatography using a gel column (Affi-Gel Blue, Bio-Rad, Hercules, CA) according to the manufacterer's instructions.

Protein Extraction, Gel Electrophoresis, and Immunoblotting

Two grams of leaves were ground to fine powder in liquid nitrogen. The powder was homogenized in 4 mL of 0.1 M Tris-HCl, pH 9.5, and 1 mM of PMSF, and incubated on ice for 30 min with occasional vortexing. The soluble fraction was recovered by centrifugation at 12,000*g* for 5 min, and the concentration of proteins was determined according to the method of Bradford (1976).

SDS-PAGE was performed as described by Laemmli (1970). Protein (10 μg) was loaded in each well. The transfer of proteins from SDS-PAGE gels to membrane (Immobilon N-C, Millipore, Bedford, MA) was carried out according to the method of Sambrook et al. (1989) using 25 mM Tris, 192 mM Gly, and 20% (v/v) methanol at 150 mA for 2 h. The membrane was incubated for 60 min in blocking solution containing PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.3) and 0.1% (v/v) Tween 20 (PBS/T) supplemented with 5% (w/v) nonfat dry milk. After rinsing briefly in PBS/T, the filter was incubated for 2 h in PBS/T containing anti-PTGRP antibody (1:3,000 dilution). After washing four times with PBS/T, peroxidase-labeled anti-rabbit antibody (Amersham, Montreal) diluted in PBS/T (1:15,000) was added and incubated for 60 min. The filter was then washed with PBS/T and the immunoreactive protein visualized by chemiluminescence (ECL, Amersham). All incubation and washing steps were performed at room temperature.

Treatment of Plants

Lycopersicon chilense plants (Chen et al., 1994) were drought stressed for 4 to 8 d. For rehydration treatment, 8-d drought-stressed plants were watered for 3 d. The leaf water potential was monitored during the treatment period.

Tissue Processing

Leaf sections from regularly watered, 8-d drought-stressed and rewatered plants were diced in a fixative

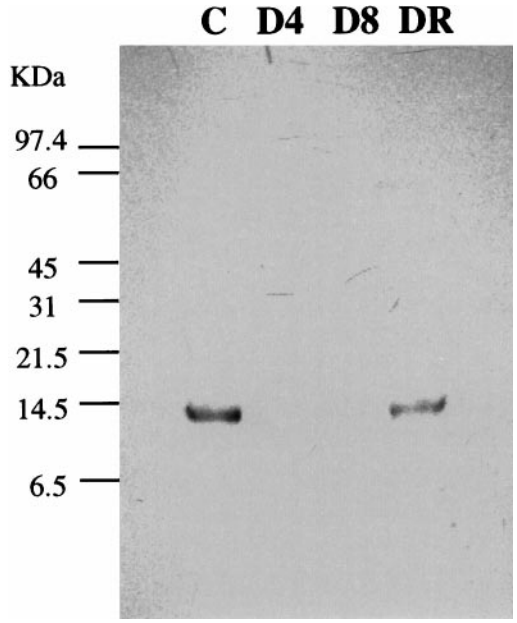

Figure 2. Western-blot analysis of anti-PTGRP antibody with leaf protein (10 μg) of regularly watered plants (C), 4-d drought-stressed (D4), 8-d drought-stressed (D8) plants, and plants rewatered for 3 d following 8 d of drought stress (DR). The size of the polypeptide is approximately 12.6 kD.

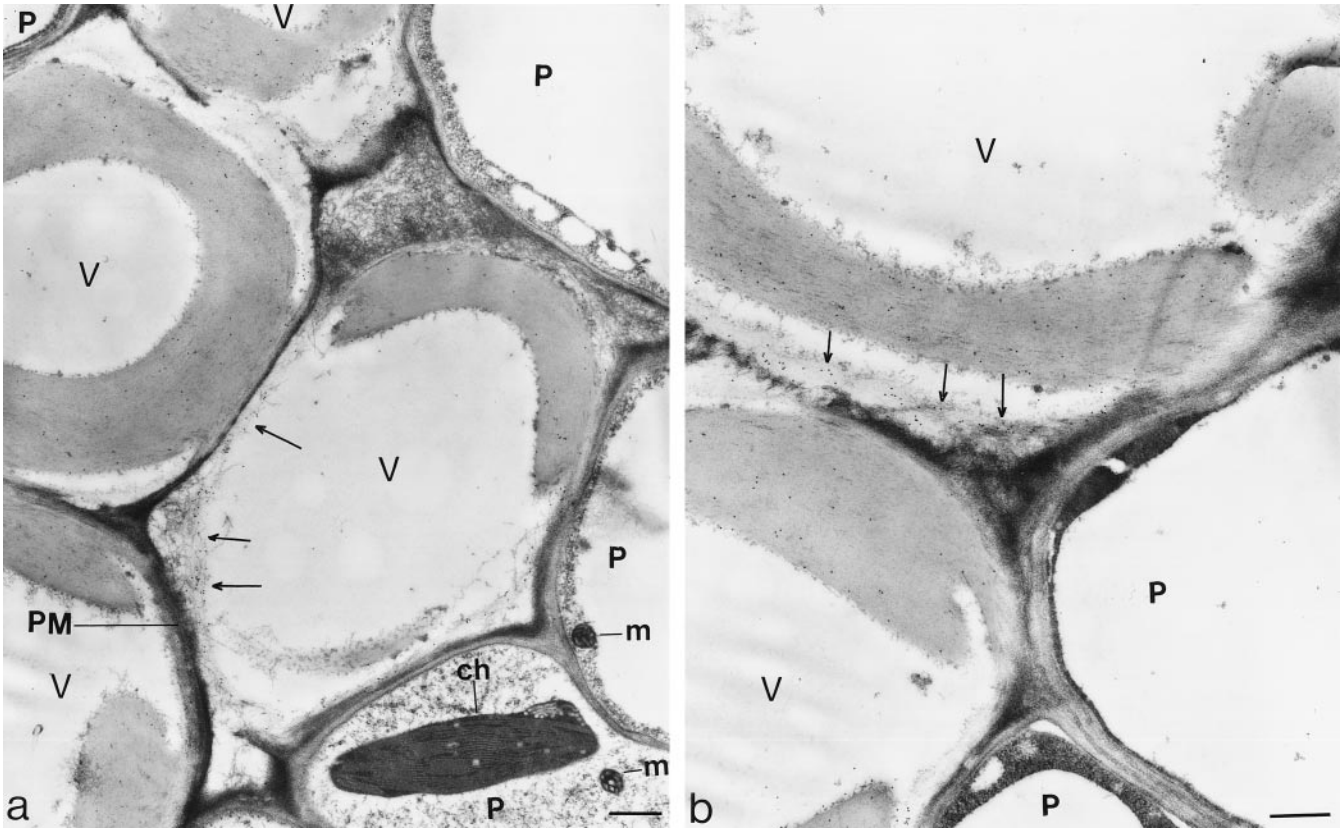

Figure 3. Electron micrographs of leaf sections from regularly watered plants demonstrating immunolocalization with anti-PTGRP antibody. Gold particles are more abundant in pit membranes (PM) and their disintegrated primary walls (arrows). A few gold particles are also present in the vessel (V) secondary walls. Parenchyma cell cytoplasm (P), chloroplast (ch), and mitochondria (m) are devoid of labeling. Scale bars = 500 nm.

solution consisting of 4% (w/v) formaldehyde and 0.5% (v/v) glutaraldehyde in 100 mM cacodylate buffer, pH 7.2. Specimens were vacuum infiltrated for 2 h at room temperature, and fixation was then extended overnight at 4°C. After fixation, the samples were washed for 1 h with the buffer (3 × 20 min), dehydrated in a graded series of ethanol, and embedded in LR White resin (Marivac, Halifax, Nova Scotia, Canada). Polymerization was carried out at 50°C. Thin sections were mounted on Formvar-coated nickel grids.

Immunogold Labeling

For immunolocalization, sections from regularly watered, drought-stressed and rewatered plants were simultaneously floated on drops of blotto blocking solution consisting of 5% (v/v) nonfat dry milk in PBS (Johnson et al., 1984) for 15 min. They were then transferred to drops of purified anti-PTGRP diluted 1:10 in blotto, and incubation was carried out overnight at 4°C. After washing with PBS, grids were placed on drops of goat anti-rabbit IgG coupled to 15-nm gold particles, which was diluted according to the manufacturer's recommendation (BioCell, Cardiff, UK). Incubation was carried out for 1 h at 37°C; grids were then rinsed with PBS and distilled water, stained with uranyl acetate and lead citrate, and examined under a JEOL (Tokyo) ×1,200 electron microscope. Control experiments consisted of replacing the anti-PTGRP with preimmune serum or PBS supplemented with 1% (w/v) BSA.

Quantitative Evaluation of Labeling

To evaluate labeling intensity, at least 10 micrographs of each sample (tissues from drought-stressed and control plants labeled with the PTGRP antibody or the preimmune serum) were scanned using Image 1.41 software (kindly provided by Dr. Wayne Rasband, National Institutes of Health, Bethesda, MD). After manually counting the gold particles, the density of labeling was calculated by dividing the number of gold particles by the surface areas. Statistical evaluations were carried out with a *t* test.

RESULTS

Immunoblot Analysis

In our previous study (Yu et al., 1996), PTGRP mRNA accumulation could be mainly observed in leaf tissues of regularly watered plants. The concentration in the stem was much lower than in the leaf and was barely detectable in the roots. Western-blot analysis using antibodies raised against PTGRP revealed the same trend for protein (Fig. 1).

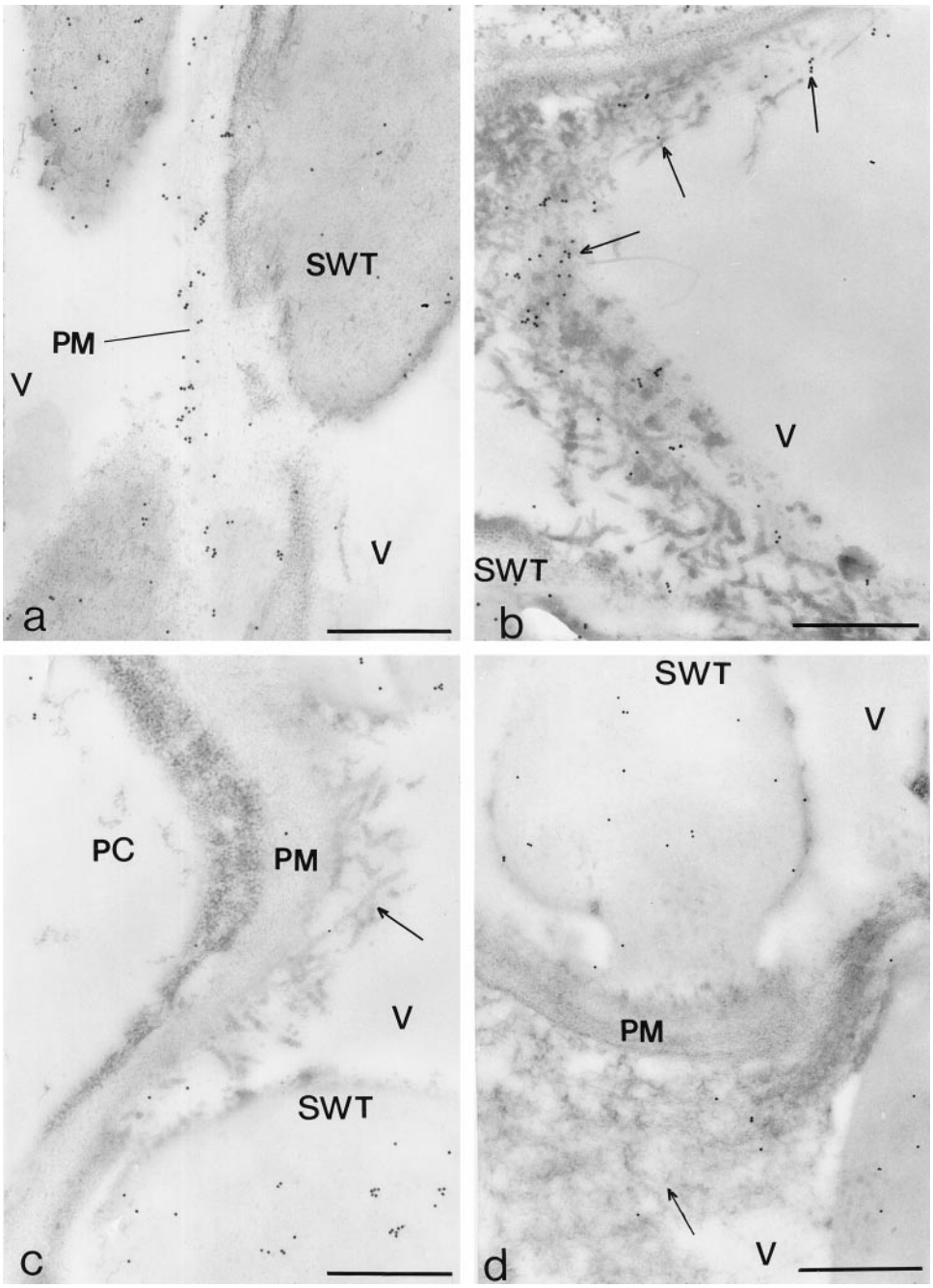

Figure 4. Electron micrographs of leaf sections from regularly watered (a–c) and 8-d drought-stressed (d) plants. a, b, and d, Immunolocalization with anti-PTGRP antibody. c, Control experiment with preimmune serum. a, Numerous gold particles are present in the pit membrane (PM) of a vessel element (V). A few particles are dispersed over the secondary wall thickenings (SWT). b, Gold particles are mainly associated with the lumen adjacent portions (arrows) of disintegrated primary walls of a vessel element. A few gold particles are present in the secondary wall thickenings. c, In this control experiment only few particles are seen in the pit membrane, including its disintegrated portion (arrow). A secondary wall thickening is covered by numerous gold particles. PC, Parenchyma cell. d, In this sample from an 8-d stressed plant, the pit membrane and its disintegrated portion (arrow) are only faintly labeled. A few gold particles are present in the secondary wall thickenings. Scale bars = 500 nm.

Therefore, leaf tissues were chosen as the material in this study. In the immunoblot analysis (Fig. 2) the protein isolated from leaf tissues of regularly watered plants strongly cross-reacted with the antiserum, while no cross-reaction was observed with the protein isolated from 4-d and 8-d drought-stressed plants. The protein isolated from rewatered plants showed the same intensity of cross-reaction with the antibody as that from regularly watered

Table I. *Quantitative evaluation of the labeling over xylem pit membrane and disintegrated primary wall using anti-PTGRP antibody and preimmune serum*

The difference in labeling density between anti-PTGRP antibody and preimmune serum was significant for both control plants ($P < 0.001$) and drought-stressed plants ($P < 0.05$). The difference in labeling density with anti-PTGRP antibody between control plants and drought-stressed plants was also significant ($P < 0.01$).

Plants	Preimmune Serum	Anti-PTGRP Antibody
	gold particles/μm^2	
Control	3.18 ± 0.95	26.58 ± 5.34
Treated	3.02 ± 0.84	10.32 ± 3.8

plants (Fig. 2). As is clear in both Figures 1 and 2, only one band was detected in the immunoblot analysis. This polypeptide was approximately 12.6 kD, which corresponds to the size of predicted PTGRP protein.

None of the protein samples cross-reacted with the preimmune serum (data not shown).

Subcellular Localization of PTGRP

Examination of sections from regularly watered plants revealed an accumulation of numerous gold particles in the xylem elements, while almost no particles were seen in the vascular parenchyma cell cytoplasm (Fig. 3) or in the mesophyll. In the xylem, gold particles were mainly associated with the vessel pit membranes (Fig. 4a) and the more external portions of disintegrated primary walls of pit membranes (Fig. 4b, arrows). In control experiments in which the anti-PTGRP antibody was replaced with the preimmune serum, the labeling was always very low or absent in the disintegrated portions of the xylem pit membranes (Fig. 4c), suggesting that it was specific to these areas. This specificity was also confirmed by quantitative evaluation (Table I).

Gold particles were also present in the secondary wall thickenings; however, this labeling was found to be nonspecific, since observations of numerous wall thickenings in the control experiments (in which preimmune serum was used instead of anti-PTGRP antibody) revealed a similar labeling in the secondary walls (Fig. 4c). Further statistical analysis comparing the labeling observed in the secondary walls of vessels in which anti-PTGRP antibody was used with that of those with preimmune serum revealed that the difference was not significant (data not shown). Other control experiments in which the antibody was replaced with PBS supplemented with 1% (w/v) BSA resulted in the absence of labeling in all cell compartments (results not shown).

In leaf sections from 8-d drought-stressed plants, only a few gold particles were seen in the xylem pit membranes and other leaf tissues (Fig. 4d). Similar to the regularly watered plants, however, the secondary wall thickening was also labeled. As deduced from Figure 4, the distribution of the protein in the primary walls was higher in control plants (Fig. 4a) than in drought-stressed plants (Fig.

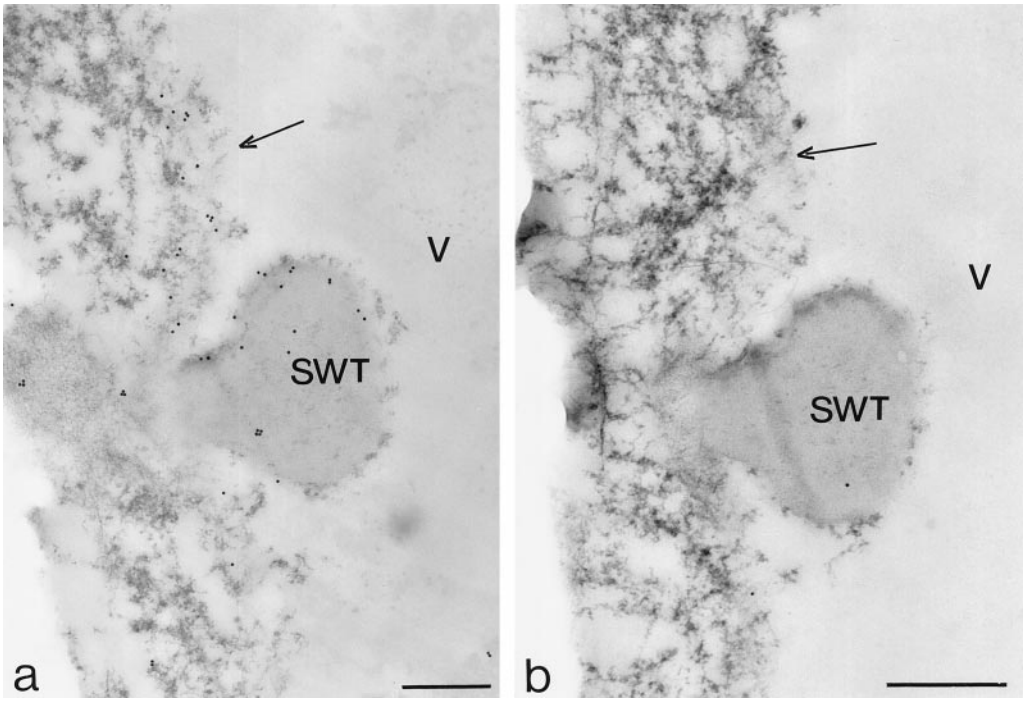

Figure 5. Electron micrographs showing adjacent leaf sections from a plant rewatered for 3 d following 8 d of drought stress. a, After incubation with the anti-PTGRP antibody, gold particles are associated with the disintegrated primary wall (arrow). A few particles are also present over the secondary wall thickening (SWT). V, Vessel. b, In a control experiment in which the section was incubated with the preimmune serum, almost no labeling is seen in the disintegrated wall or the secondary wall thickening. Scale bars = 500 nm.

4d). This difference was found to be statistically significant (Table I).

Observations of samples from plants that had been rewatered for 3 d following 8 d of drought stress revealed a labeling pattern similar to that observed in the regularly watered plants, with numerous gold particles in the pit membranes and disintegrated primary walls (Fig. 5a, arrow). This labeling was found to be specific, since control experiments in which the antibody was replaced with preimmune serum showed a near absence of gold particles in these areas (Fig. 5b, arrow). The labeling observed in the secondary wall thickenings was considered to be nonspecific, even though it was occasionally lower in control experiments (Fig. 5b), since most of these cell walls were covered by numerous gold particles.

DISCUSSION

This study was conducted to elucidate subcellular localization of the protein encoded by the PTGRP gene, a *L. chilense* gene that is down-regulated by drought. In our previous study (Yu et al., 1996), we demonstrated that the PTGRP gene belongs to a small family. It is not known if the PTGRP antibody produced in this study interacts with other members of the family; however, only one band was detected in western-blot analysis. The size of the band corresponded to the size of predicted PTGRP protein. Therefore, it is possible that all of the members code for the same protein or similar proteins of the same size.

The protein recognized by the PTGRP antibody was found to be localized in the walls of xylem elements in *L. chilense.* Several different proteins have been previously identified in the cell wall. These proteins can be classified in four groups: PRPs, Hyp-rich proteins (HRGPs), Gly-rich proteins (GRPs), and arabinogalactan proteins (AGPs). The first three groups are particularly well characterized. HRGPs are characterized by the repeated pentapeptide sequence Ser-(Hyp)4. These proteins are also rich in Lys, Tyr, Ser, and Val (Showalter and Varner, 1989; Keller, 1993; Showalter, 1993). The accumulation of HRGPs is developmentally regulated (Ye and Varner, 1991). PRPs are the second class of proteins identified in the cell wall. PRPs isolated from different plant species show significant heterogeneity in their respective amino acid sequences, but are all rich in Pro or Hyp. PRPs localized in the cell wall of xylem elements have been identified in soybean, tomato, petunia, and tobacco (Ye et al., 1991; Wyatt et al., 1992). GRPs are another class of well-characterized cell wall proteins, containing repetitive $(Gly\text{-}X)_n$ motifs in which the X is often Gly (Keller et al., 1988; Keller, 1993). Two GRP proteins, grp-1 (Condit et al., 1990) and GRP1.8 (Keller et al., 1988), have been clearly localized in the cell walls of vascular tissues of petunia and bean, respectively. Keller and Baumgartner (1991), while studying the regulation of GRP1.8, identified a negative regulatory element in a promoter region that controls vascular specific expression of the gene. The protein encoded by GRP1.8 was suggested to be produced by xylem parenchyma cells and then exported to the walls of protoxylem vessels (Ryser and Keller, 1992).

As for the function of these proteins, different hypotheses have been formulated based on experimental observations. It has been shown that PRPs are insolubilized in the cell wall during different stages of plant development (Datta et al., 1989; Keller, 1993). This insolubilization has been suggested to be mediated by Tyr residues through the formation of isodi-Tyr cross-links between PRP molecules or sometimes between PRPs and GRPs or HRGPs. The insolubility of PRPs has also been demonstrated to occur rapidly in plants after fungal infection and wounding. In soybean cell cultures treated with fungal elicitor and in soybean tissues subjected to wounding (Bradley et al., 1992), the insolubilization initiates 2 min after treatment and terminates within 10 min. These results have led the authors to speculate that, aside from their role in plant development, PRPs also have a role in strengthening the cell wall for plant defense. *PvPR1*, a bean gene coding for a PRP, was reported to be down-regulated by fungal elicitors (Sheng et al., 1991). The authors hypothesized that this likely cell wall protein is reduced because of its low Tyr content and therefore its low potential for wall strengthening during the defense response (Sheng et al., 1991). The decrease in the *PvPRP1* mRNA level in cells treated with elicitors was demonstrated to be due to destabilization, which is dependent on the synthesis of new RNA and protein (Zhang et al., 1993).

The association of PRPs with lignification has also been reported. For example, in soybean root, PRPs are detected in primary xylem cell walls that are lignified (Ye et al., 1991). In tomato, petunia, and potato, PRPs were also localized in lignified cell walls. More recently, Ryser et al. (1997) reported that the secretion of a soybean PRP protein correlates with lignification of the cell wall of xylem elements.

The high content of PTGRP and its presence in the wall of xylem vessels indicate its functional importance. Since it was localized in the primary xylem wall, which is lignified, its implication in lignification cannot be excluded. As clearly demonstrated by our results using western-blot analysis and immunolocalization studies, PTGRP was noticeably reduced during drought stress. It is well documented that water stress affects the mechanical properties of cell walls (Sakurai et al., 1987; Sakurai and Kuraishi, 1988). For example, in osmotically stressed wheat coleoptiles, cell wall stiffening is reduced significantly (Wakabayashi et al., 1997). Moreover, during the same period, the activity of Phe alanine ammonia-lyase, which is implicated in lignin biosynthesis, substantially decreases. Lignin can make cross-links with many cell wall components such as polysaccharides and proteins (Liyama et al., 1994), which reinforce mechanical support. It is likely that PTGRP is also one of the proteins that provide mechanical support under normal conditions in conjunction with lignin, and that during drought stress it is reduced as lignin is reduced. It has been suggested that remodeling of the cell wall as part of the plant defense response during pathogen invasion not only requires synthesis of some new proteins that can act as a barrier against attack, but also the reduction of some proteins that are more suitable for cell wall function during normal conditions (Sauer et al., 1990). It is therefore possible that the reduction of PTGRP is related to the remodel-

ing of vessel cell walls during drought conditions. The presence of drought-induced proteases can demonstrate the necessity of protein hydrolysis as an adaptive mechanism during water stress (Tabaeizadeh, 1998). Indeed, TDI65, a drought-induced protein from tomato (Tabaeizadeh et al., 1995), was recently identified to be a protease (H. Harrak and Z. Tabaeizadeh, unpublished data). The TDI65 antibody also cross-reacts with the protein isolated from drought-stressed *L. chilense* plants.

Different groups of cell wall proteins have been studied, mostly with regard to plant development (Ye et al., 1991). Only a few studies involving GRPs and PRPs have so far been carried out in relation to stress, mainly caused by pathogen attack and wounding (for review, see Showalter, 1993). There is also a report on MsPRP2, a salt-inducible-gene encoding a PRP isolated from alfalfa (Deutch and Winicov, 1995). Based on the homology of MsPRP2 with cell wall PRPs, the authors suggested that the protein is targeted to the cell wall. Two GRPs from maize (Gomez et al., 1988) and rice (Mundy and Chua, 1988) have been shown to be regulated by water stress. However, since none of these GRPs contains a signal peptide, their association with the cell wall is unlikely. Indeed, rice GRP was localized in the cytosol (Mundy and Chua, 1988) when the antibody raised against the protein was used in an immunoblot analysis with different cell fractions. Therefore, we believe that PTGRP is the first example of a drought-regulated protein that has been clearly localized in the cell wall.

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