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Planta (1990) 180:524–529

Planta

Expression of genes for cell-wall proteins in dividing and wounded tissues of *Zea mays* L.

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Abstract. Hydroxyproline-rich glycoproteins (HRGPs) from *Zea mays* have been immunolocalized in the cell wall of root tip cells using ultrathin sections and antibodies ellicited against the purified protein. The accumulation of mRNA corresponding to this protein was studied using the cDNA probe. Maximum accumulation of the mRNA was found in tissues with a high proportion of dividing cells such as those in the root tip of young maize seedlings and a close relationship with cellular division was also observed in in-vitro cultures. However, the level of the mRNA in elongating tissues was minimal, as shown by studies carried out on the elongation zones of root tips and coleoptiles. The mRNA was induced by stress conditions, particularly by wounding young leaves and coleoptiles. It is concluded that in maize this group of proline-rich cell-wall proteins accumulates during cell division and not during cell elongation or differentiation, and participates in the stress-response mechanisms of the plant.

Key words: Cell division – Cell wall proteins – Glycoprotein – Hydroxyproline-rich glycoprotein – Wounding – *Zea* (cell wall proteins)

Introduction

A number of genes for cell-wall proteins have been characterized from dicotyledoneous species. These include proline-rich proteins such as carrot extensin (Chen and Varner 1985), nodulin-75 (Franssen et al. 1987) and an auxin-induced gene (Hong et al. 1987) from soybean, and glycine-rich proteins from *Petunia* (Condit and Meagher 1986) and bean (Keller et al. 1988). These studies have resulted in the elucidation of complete gene sequences and the determination of their pattern of expression in the plant. The genes coding for extensin, the best studied of cell-wall proteins, are expressed in different tissues of tomato and tobacco (Memelink et al. 1987) and they are also induced by wounding (Showalter et al. 1985). The gene corresponding to a glycine-rich protein from bean appears to have a high level of expression in the vascular system of the plant (Keller et al. 1988). Therefore cell-wall-protein genes are useful markers for the study of gene expression related to plant cell development and to plant defense mechanisms.

Little is known of the corresponding genes for cell-wall proteins from monocotyledoneous plants. A cell-wall protein fraction has been purified from maize cell-suspension cultures (Kieliszewski and Lamport 1987) and pericarp (Hood et al. 1988), and a complementary DNA (cDNA) has been isolated from maize coleoptiles (Stiefel et al. 1988). The cDNA codes for a highly repetitive, proline-rich protein that has been detected in cell-wall extracts from young coleoptiles by using antibodies against a synthetic peptide designed from the protein sequence deduced from the cDNA. Here, we present the subcellular localization of this protein using antibodies against purified protein and protein A-gold labelling. Our results demonstrate that the maize hydroxyproline-rich glycoprotein (HRGP) is located in the wall of root-tip cells. It was of interest to investigate the possible use of these probes as markers of defined periods of formation of the cell-wall during maize development. To this end, we have studied the expression of genes coding for the proline-rich cell-wall protein from maize in specific parts of the plant rich in dividing tissues. It appears that the expression of this gene may be correlated with cell division and that it is also induced by wounding.

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Abbreviations: cDNA = complementary DNA; 2,4-D = 2,4-dichlorophenoxyacetic acid; ELISA = enzyme-linked immunosorbent assay; HRGP(s) = hydroxyproline-rich glycoprotein(s); kDa = kilodaltons; PAGE = polyacrylamide gel electrophoresis; SDS = sodium dodecyl sulfate

Material and methods

Plant material. Seeds of *Zea mays* L. cv. W64A pure inbred line grown in a greenhouse at Barcelona, Spain, were germinated by imbibition in water at 25° C in the dark. Segments (0.5 cm long) of 3-d-old coleoptiles were obtained (after removing 0.2 cm of the apex) by cutting with a razor blade. They were incubated in the dark in a medium containing 5 mM phoshate, (pH 6.0), 1% sucrose, and 0.01% tetracycline in the presence or absence of 10^{-6} M 2,4-dichlorophenoxyacetic acid (2,4-D). Embryogenic and meristematic calli were obtained from the maize inbred line W64A (opaque version). Meristematic calli from caulinar meristems were cultured in a modified Murashige-Skoog (MS) medium (Torné et al. 1984). Embryogenic calli obtained from the scutellum of young embryos were cultured in the basal medium N6 as described by Chu et al. (1975). The 2,4-D concentration was 2 $mg \cdot l^{-1}$ in both cases, and culture conditions were as described by Torné et al. (1984).

Protein extraction and Western blots. Tissue samples from 6-d-old plantlets were obtained by sectioning with a razor blade, rapidly frozen in liquid nitrogen and homogenized in a cold mortar. The proteins were extracted from the resulting powder as described by Stiefel et al. (1988) for acid extraction. The acetone precipitates of the ethanolic acid extraction were resuspended in electrophoresis sample buffer. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was carried out as described by Laemmli (1970), the final acrylamide/bis-acrylamide ratio being 15/0.4. The gel was transferred onto nitrocellulose filters using a semi-dry blotter (Kyhse-Andersen 1984) and a replica stained with silver nitrate (Morrisey 1981). Filters were incubated with antisera at a 1:300 dilution. Goat anti-rabbit immunoglobulin G (IgG)-peroxidase conjugate (Dakopatts, Glostrup, Denmark) was used for antibody detection.

Antibodies against purified maize HRGP were obtained by injecting New Zealand rabbits essentially as described by Stiefel et al. (1988) except that 10 µg of protein were used in the first and second boosters and 100 and 1000 µg in the third and fourth booster, respectively. The protein was purified by ionic-exchange chromatography (Kiesliszewski and Lamport 1987). Enzyme-linked immunosorbent assay (ELISA) was performed as described by Craig et al. (1980). Purified protein (1 µg per well; saturating concentration for the two antisera) was used as immobilized antigen.

Immunocytochemistry. Root tips of 6-d-old maize plantlets were fixed for 1 h at room temperature in 4% (w/v) paraformaldehyde in 0.01 M phosphate, 0.15 M NaCl (pH 7.4) buffer (PBS), washed in the same buffer and dehydrated in a graded ethanol series. The samples were infiltrated with Lowicryl K4M (Agar Aids, Stansted, UK) at −35° C for 1 d and subsequently polymerized by ultraviolet irradiation at −35° C for 2 d and at room temperature for 1 d (Roth et al. 1981).

Ultrathin sections were obtained with an Ultracut (Reichert, Vienna, Austria) microtome and picked up on nickel grids. The grids were saturated with PBS containing 0.5% egg albumin (Sigma, München, FRG), 0.1% Tween-20 (Sigma) (buffer A) for 1 h at room temperature and then incubated overnight at 4° C with a 1/100 dilution of either pre-immune or immune anti-HRGP antiserum in buffer A. After washing with buffer A in the absence of antiserum and egg albumin, 20-nm Protein A-gold complexes (Sigma) diluted 1/20 in buffer A was used for antibody detection. The samples were treated with uranyl acetate and lead citrate for examination by electron microscopy. Observation was carried out in a Phillips (Eindhoven, The Netherlands) EM 301 electron microscope (Torrent et al. 1989).

Extraction of RNA and blot hybridization. Total RNA was isolated from calli and wounded young leaves (Dean et al. 1985) and from root and coleoptile sections (Logemann et al. 1987). Total RNAs were separated in 1.5% agarose-formaldehyde gels (Lehrach et al. 1977), and blotted onto nylon (Hybond-N; Amersham, Amersham, UK) or nitrocellulose (BA85; Schleicher and Schüell, Dassel, FRG) membranes (Alwine and Stark 1977). ^{32}P-labelled cDNA probes were prepared by random primer labelling to a specific activity of 10^8 $cpm \cdot \mu g^{-1}$. Prior to blotting and hybridization, the gels were stained with ethidium bromide to ensure that RNA samples contained approximately equal amounts of RNA.

Results

Subcellular localization of maize HRGP. Two polypeptides of apparent molecular mass (M_r) in the range 50–70 kilodaltons (kDa) were previously identified as maize HRGP in total maize cell-wall extracts by using antisera against a synthetic peptide designed after the cDNA sequence of the HRGP (Stiefel et al. 1988). These antisera react more strongly with deglycosylated antigen than with purified antigen that is modified in the proline residues by hydroxyl groups and by glycosylation with arabinosyl residues (Kieliszewski and Lamport 1987). To try to locate these polypeptides in the cell-walls by immunochemical labelling, we have produced antibodies against the purified maize HRGP. The antigen was iso-

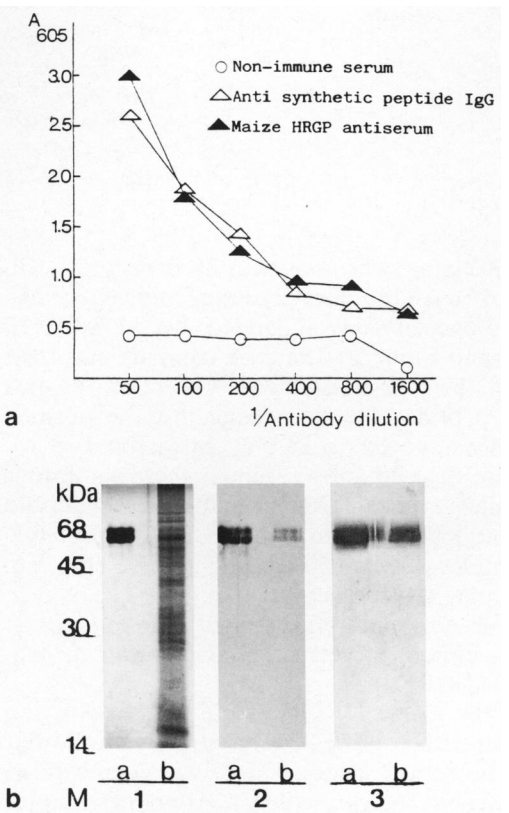

Fig. 1a, b. Specificity of antisera against synthetic peptide and against purified maize HRGP. **a** Antisera reaction at different dilutions against 1 µg of purified maize HRGP as measured by ELISA. **b** Analysis by SDS-PAGE (*1*) and immunoblotting (*2, 3*) of purified maize HRGP (*a*) and total maize cell-wall extracts (*b*). Nitrocellulose filters were incubated with serum against synthetic-peptide (*2*) or against purified maize HRGP (*3*) and developed with peroxidase conjugated to goat anti-rabbit IgG. *M* = molecular-weight markers

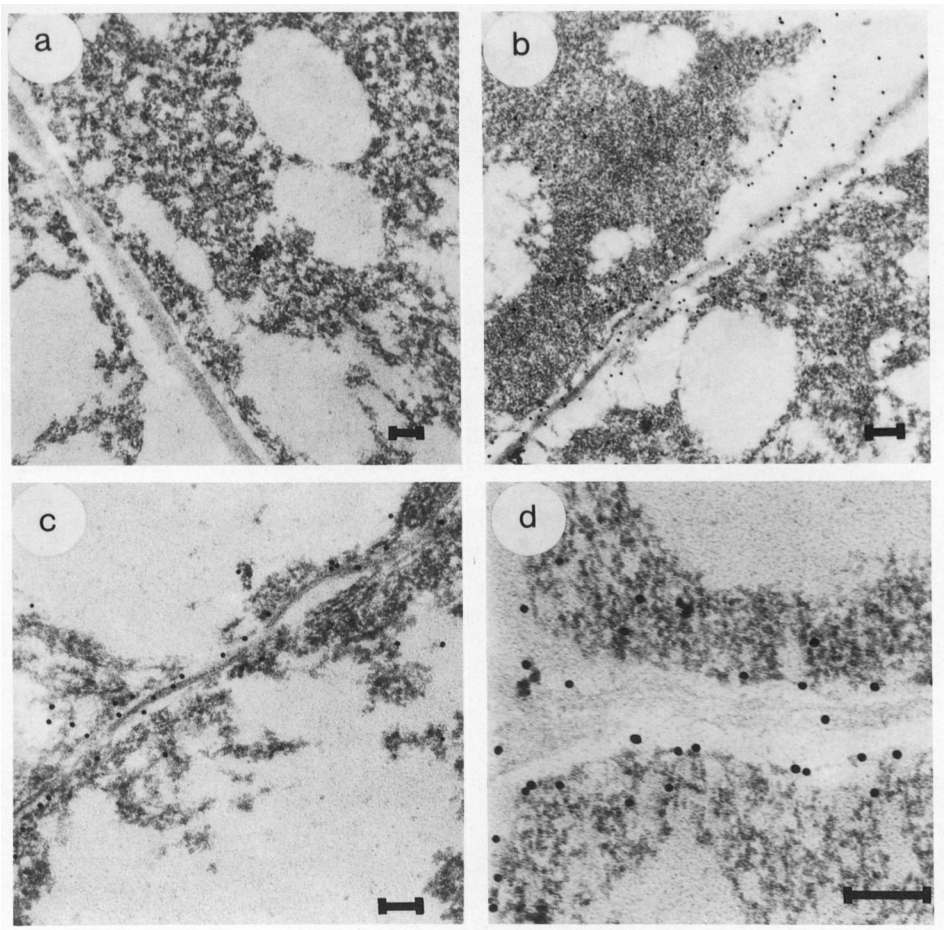

Fig. 2a–d. Immunocytochemical labelling of HRGP in maize root tips. Root-tip sections from 6-d-old maize seedlings were fixed in 4% paraformaldehyde, dehydrated in a graded ethanol series and embedded in Lowicryl K4M. Ultrathin sections were incubated with anti-maize HRGP (**b–d**) and with pre-immune serum (**a**) and developed with protein A-gold complexes. *Bars* = 0.1 μm

lated by ion-exchange chromatography from total cell-wall extracts of 6-d-old maize seedlings and purified to apparent homogeneity, as indicated by SDS-PAGE (Kieliszewski and Lamport 1987). A comparison of the specificities of the two antisera (the one raised against the synthetic peptide and the one against the purified protein) as measured by ELISA is shown on Fig. 1a. The reaction of the two antisera under these conditions was very similar. Maize HRGP purified from 6-d-old coleoptiles could be resolved by SDS-PAGE into two main polypeptides of M_rs 65 kDa and 70 kDa (Fig. 1b, gel 1). When analyzed by Western blot (Fig. 1b, blots 2, 3), the two antisera immunoreacted with the same polypeptides from either extracts of total cell-wall protein or from purified HRGPs.

To locate the maize HRGP within maize cells, the antiserum against the purified polypeptides was allowed to react with ultrathin sections of Lowicryl-embedded root tips of 6-d-old maize seedlings. After the samples had been processed with protein A-gold particles, the antigens were found mainly in the cell-wall (Fig. 2). Non-immune serum was used as a control to confirm the presumptive localization of maize HRGP polypeptides in cell-walls.

Accumulation of the maize cell-wall-protein mRNA in tissues rich in dividing cells. The mRNA hybridizing with the cDNA coding for the HRGP from maize has been measured by RNA blot. Only one band (shown in the following figures) of 1500 nucleotides hybridized with the HRGP probe. Previous results indicated that the regions showing the highest level of RNA hybridizing with this probe are the coleoptile (especially the coleoptile node) and the root tip (Stiefel et al. 1988). These are regions of the plant rich in meristematic tissues. A more detailed study was devised in order to measure the expression of the gene in defined zones of these regions.

The root tip has been widely used for studies of tissues that are in particular stages of differentiation. Three zones were cut from 6-d-old roots: zone 1, 2-mm-long segments from the tip; zone 2, 4-mm-long segments at 1 cm from the tip; and zone 3, 20-mm-long segments at 3 cm from the tip. From morphological studies, these zones appear to be rich in dividing, elongating and maturating cells, respectively. The results of the RNA blot analysis on RNA extracted from these three regions is shown in Fig. 3. To determine whether the steady-state level of HRGP mRNA may be correlated with cell division, histone DNA probes were used. In Fig. 3, the result of the RNA blot is shown using both the maize HRGP and histone H4 probes (a kind gift of Dr. Claude Gigot, Strasbourg, France; see Philipps et al. 1986). Figure 3 also shows a quantitation of the results for the two

probes using the expression in the root tip as a standard. It is clear that the maximum accumulation of mRNA is found in the root tip, the zone richer in dividing cells.

Calli can also be used for developmental studies, and have a high proportion of dividing cells when grown in the usual culture media; however, in a medium deprived of auxin, tissue differentiation occurs. The accumulation of HRGP mRNA was measured in two types of calli, embryogenic and meristematic (both from a W64A genetic background), each having a different type of regeneration pattern. The mRNA levels of these two cultures at different times after depriving them of auxin are shown in Fig. 4. Both embryogenic and meristematic calli showed a decrease in the expression of the HRGP gene in the absence of auxin in a way parallel to that observed for the histone H4 mRNA, indicating a good correlation of the expression of this gene with cell division. The presence of auxin in the culture medium allows the calli to maintain a high proportion of cells in a continuously dividing state. Cell division stops when the calli are cultured in an auxin-depleted media for a long period of time. Under these conditions, the division rates become progressively lower and cells differentiate. The lowest level of mRNA corresponds to 16 d of culture without auxin. At this stage embryogenic calli were surrounded by embryoids and meristematic calli by young roots and leaves. The proportion of fully differentiated cells was becoming increasingly larger.

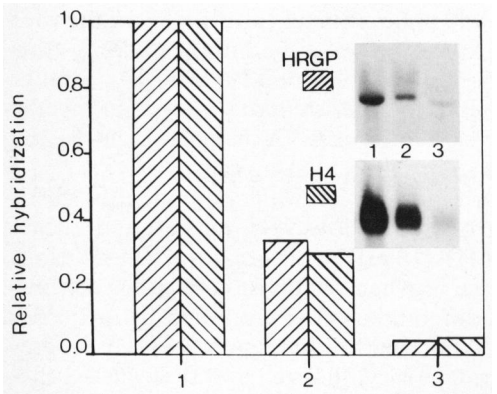

Fig. 3. Accumulation of maize HRGP mRNA in root segments with different cellular division rates. The RNAs were extracted from different zones of the roots of 6-d-old maize plantlets. Three zones from each root were assayed: *1*, a 2-mm segment at the root tip; *2*, a 5-mm segment at 1 cm from the tip; and *3*, a 20-mm segment at 3 cm from the root tip. The RNAs (50 µg per lane) were separated in formaldehyde-containing agarose gels, blotted onto nylon membranes and hybridized with maize HRGP and histone H4 cDNA probes. In the histogram, the densitometric analysis of the autoradiograms (*inset*) is plotted as the value obtained for HRGP and H4 probes relative to that of the distal root segment sample (zone *1*)

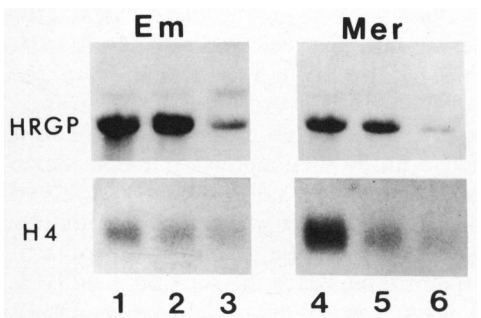

Fig. 4. Accumulation of HRGP mRNA in maize callus cultures. Total RNAs (15 µg per lane), purified from embryogenic (*Em*) and meristematic (*Mer*) calli cultured in the presence (*lanes 1, 4*) or in the absence of 2,4-D for 9 d (*lanes 2, 5*) or 16 d (*lanes 3, 6*), were subjected to electrophoresis on agarose-formaldehyde gels, transferred to nylon membrane and hybridized with cDNA probes corresponding to maize HRGP or histone H4

Expression of the HRGP gene in elongating coleoptiles. Induction by wounding. We have presented evidence that the accumulation of HRGP mRNA correlates with cell division. After division and during cell growth, cell-wall loosening occurs. An experiment was devised to determine whether maize HRGP gene expression is involved in the process of cell elongation. The mRNA analysis of elongating tissues was carried out using segments of maize coleoptile (3-d-old), a classic tissue for this kind of study (Zurfluh and Guilfoyle 1982; Carpita 1986).

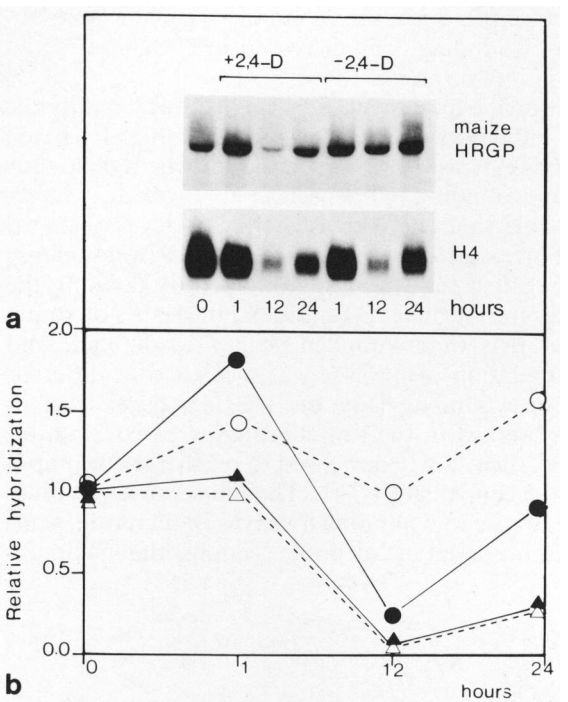

Fig. 5a, b. Accumulation of HRGP mRNA in elongating sections of maize coleoptiles. **a** Autoradiographs of total RNAs (50 µg per lane) of 3-d-old maize coleoptile segments, incubated for different times (1, 12 or 24 h) in the presence (+2,4-D) or in the absence (−2,4-D) of 2,4-dichlorophenoxyacetic acid, separated in agarose-formaldehyde gels, transferred to nylon membrane and hybridized with maize HRGP and histone-H4 cDNA probes. The data at zero time are for control coleoptiles, prior to sectioning and incubation. **b** Densitometric quantitation of autoradiographs of HRGP (●, ○) and H4 histone (▲, △) hybridizations, in the presence (*solid line*) or in the absence (*dotted line*) of 2,4-D. Relative hybridization was calculated with reference to the densitometric value of the control sample

The abundance of HRGP mRNA in tissues enriched in elongating cells was measured in coleoptile segments from which the apices had been removed. These sections were incubated for various times in the presence or the absence of the synthetic auxin, 2,4-D. After 24 h of incubation, coleoptile sections increased threefold in length (1.5 cm ± 2 mm final length). The HRGP-mRNA level was measured at different times and compared with the accumulation of histone H4 mRNA (Fig. 5). In Fig. 5, the RNA blot is shown for both probes at 1, 12 and 24 h of incubation in the presence and in the absence of 10 μM 2,4-D. The intensity of the signal for the HRGP probe relative to the control level is shown in the graph. It is clear that by 1 h of incubation the level of HRGP mRNA had increased relative to the level of histone H4. However, the level of expression of both genes sharply decreased at 12 h and then increased after 24 h of incubation. It is also clear that, while the accumulation of histone H4 mRNA did not seem to be affected by the presence of the auxin analogue, the accumulation of HRGP mRNA decreased with 2,4-D. It is interesting that the time of minimum gene expression corresponds with that of maximum elongation activity (Carpita 1986). During the first hour of incubation, with or without 2,4-D, HRGP mRNA increased compared with the control. Since the coleoptile segments were obtained by wounding, this increased mRNA level could be a wounding response.

Additional experiments were carried out to study the possible induction of the gene by wounding. It is well known that extensin genes are induced in dicotyledonous species by wounding (Chrispeels et al. 1974). It is therefore possible that the increased mRNA level observed at the shorter incubation times is caused by a defense reaction against the wounding inflicted by dividing the coleoptiles into segments. To this end the leaves of young maize plantlets were wounded using a razor blade and the accumulation of mRNA was measured at different times after wounding (Fig. 6). The low basal level of mRNA observed in the leaf sharply increased 2 h after wounding, then slowly decreased to reach a level comparable to the control after 24 h. The time course of induction in response to wounding in leaves is, therefore, similar to that observed in coleoptile sections, the maximum level of mRNA being attained within 2 h after wounding.

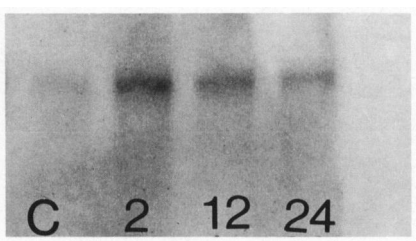

Fig. 6. Accumulation of HRGP mRNA in maize leaves in response to wounding as measured by RNA blot. Total RNAs (10 μg per lane) from leaves of 10-d-old maize plants were subjected to electrophoresis, blotted onto nitrocellulose filters and hybridized with maize HRGP cDNA probe. Wounds were made by incising the leaves with a razor blade. *C*, Control (non-wounded) leaves; *2, 12, 24*, leaves 2, 12 or 24 h after wounding

Discussion

For dicotyledonous species, data concerning the expression of genes for cell-wall proteins have been obtained using extensin probes. It has been shown that these genes are under developmental control and that they are induced in defense reactions (for a review, see Cassab and Varner 1988; Varner and Lin 1989). In the present study, data on the accumulation of mRNA coding for a cell-wall protein from maize indicate a correlation of the expression of the corresponding genes with tissues rich in dividing cells. Induction of these genes by wounding has also been found.

The highest levels of the mRNA corresponding to maize HRGP have been found in root tip (present results) and coleptile nodes (Stiefel et al. 1988). In the root tip, maximum expression was found in the dividing zone for both the HRGP and histone-H4 mRNA. Similar results were found for meristematic and embryogenic calli, higher levels of the mRNA being present in the dividing cells than in differentiating cells.

The need for a protein cell-wall component in dividing cells seems obvious. However, our results indicate that the level of HRGP mRNA decreases when cell elongation takes place or when cell division in callus cultures is arrested and differentiation is promoted by auxin depletion. It is possible that in dividing maize cells, a precursor of the protein is synthesized and that this synthesis is transcriptionally controlled. Indeed active synthesis of HRGP mRNA takes place in the coleoptile, an organ in which the corresponding gene is highly expressed, as has been shown by a run-on transcription experiment using nuclei from 7-d-old maize coleoptiles (data not shown). During the cell-elongation step the protein could either be secreted or polymerized using already-synthesized precursors. The cDNA used here (Stiefel et al. 1988) corresponds to a protein having the same amino-acid composition as a maize cell-wall protein isolated from maize cell-suspension cultures (Kieliszewski and Lamport 1987) and maize pericarp (Hood et al. 1988), indicating that it is one of the main extractable protein components of maize cell walls. Therefore, it is probable that once the protein is deposited in the cell wall it has a very low turnover.

The immunocytochemical studies on sections of root tip indicate that the antibodies against purified maize HRGP recognize the cross-linked proteins in the cell wall. The gold particles are mainly found in the matrix of the wall, but some label can be seen in the cytoplasm near the plasma membrane. Since the root tip is a dividing tissue, this situation may indicate that maize HRGP is still being secreted in the recently formed primary wall. It has been reported (Stafstrom and Staehelin 1988) that the incorporation of extensins is a late event (after cellulose has been laid down) in the development of carrot cell walls, indicating that a similar situation may happen in maize root.

The induction of extensin by wounding has often been reported in dicotyledonous species but, to our knowledge, no such effect has been reported in a monocotyledonous species. In the experiments on coleoptile segments, an increased expression of the HRGP gene was observed shortly after excision. This effect was probably the result of wounding, as is indicated by the similar results with wounded leaves. It is interesting that other stress situations such as desiccation or high temperature promote the accumulation of HRGP mRNA (data not shown). These results indicate that maize HRGP-gene expression could be dependent upon mediators of a more general stress response.

Gene-dosage (Stiefel et al. 1988) results indicate that a low number of genes (less than five) code for the maize HRGP. It is not known whether different genes are expressed coordinately or, alternatively, whether distinct genes are responsible for the various effects here described. In any case, they offer an interesting set of markers for specific steps of maize development and for defense reactions.

The authors are grateful to Drs. J.A. Martínez-Izquierdo and M. Pagès (Dep. Genética Molecular, CID-CSIC, Barcelona, Spain) for critical revision of the manuscript and Plan Nacional and the European Communities for continuous financial support. This work was partially supported by the Comissió Interdepartemental de Recerca i Innovació Tecnològica. L.R.-A. and M.P.V. are recipients of the Plan de Formación de Personal Investigador (Biotechnology) fellowships. Facilities used in the electron-microscopy study were provided by the Servei de Microscòpia Electrònica de la Universitat de Barcelona.

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Received 5 August; accepted 26 October 1989