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Accumulation of cell wall hydroxyproline-rich glycoprotein mRNA is an early event in maize embryo cell differentiation

(cell division/histone H4/*in situ* hybridization)

LUIS RUIZ-AVILA, SHIRLEY R. BURGESS*, VIRGINIA STIEFEL†, MARIA DOLORS LUDEVID, AND PERE PUIGDOMÈNECH‡

Departament de Genètica Molecular, Centro de Investigacion y Desarrollo Consejo Superior de Investigaciones Cientificas, Jordi Girona 18, 08034 Barcelona, Spain

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ABSTRACT **The accumulation of the mRNA coding for a hydroxyproline-rich glycoprotein (HRGP), an abundant component of the wall from the cells of vegetative tissues, has been observed in maize embryo by *in situ* hybridization. The HRGP mRNA accumulates in the embryo axis and not in the scutellum and preferentially in dividing and provascular cells. The histone H4 mRNA is distributed in similar tissues but is restricted to defined groups of cells, indicating that these two gene products have a different steady-state level of accumulation during the cell cycle. The HRGP mRNA appears to be a useful marker for early formation of the vascular systems. The mRNA accumulation correlates in space and time with cells having a low content of cellulose in their walls, suggesting that the mRNA is produced in the early stages of cell wall formation before complete deposition of cellulose.**

Formation of the cell wall is an essential morphogenic event in plant cells. Different polysaccharide and protein components have been identified in the cell wall but the timing of deposition and assembly of these components is not known (1). The cell wall plays an important role in plant cell differentiation: some of its components, such as oligosaccharides, may induce specific developmental stages in tobacco thin layers (2) and a group of glycoproteins has been shown to complement mutations affecting defined steps during carrot somatic embryogenesis (3). Among the cell wall proteins, hydroxyproline-rich glycoproteins (HRGPs) are probably the best characterized. The extensins, HRGP components from dicotyledonous systems (4), have been characterized at the protein, cDNA, and genomic levels. In monocotyledonous species an analogous protein rich in threonine has been identified at the protein (5–7), cDNA (8), and genomic (9) levels in maize. Maize HRGP is a highly repetitive proline- and threonine-rich protein. Antibodies raised against the purified protein react with defined polypeptides in cell wall extracts, which have been localized in the cell wall by immunocytochemistry and immunoelectron microscopy (10, 11). Previous results indicate that the *Hrgp* gene is active in response to different situations during cell life. The expression of the *Hrgp* gene parallels histone mRNA accumulation in tissues rich in dividing cells. HRGP mRNA is also found in wounded young organs (10). The mRNA coding for the maize HRGP is transiently accumulated at new vascular sites in germinating embryos, leaves, and roots (9).

Tissue specificity has also been observed by immunological and Northern blot analyses in the maize embryo (11). In particular, the absence of both HRGP mRNA and protein in the scutellum has been observed. In accordance with these data, we have used a fragment of the coding region of the HRGP genomic clone as a probe to study the steps leading to formation of the cell wall in specific cell types during embryo development. We have analyzed the *in situ* hybridization patterns of HRGP mRNA accumulation in different stages of maize embryogenesis. The results we obtained allow us to conclude that the accumulation of HRGP mRNA occurs early in cell differentiation before acquisition of the final cell wall structure.

MATERIAL AND METHODS

Plant Material. Fresh embryos of a W64A+/+ pure inbred line originally obtained from the Department of Agronomy, University of Wisconsin, and grown in a greenhouse in Barcelona, were collected from hand-dissected kernels 13, 15, 17, and 20 days after pollination (DAP); they were immediately submerged in fixing solution.

Tissue Preparation. The embryos were fixed in a solution containing 80% ethanol, 3.5% formaldehyde, and 5% acetic acid for 1 h at room temperature, and then for a week at 4°C, with one change of fixative. The samples were then washed twice and stored at 4°C in 70% ethanol. Embedding of the embryos was carried out by Paraplast-plus (Monoject Scientific, Athy, Ireland) substitution. The embryos were dehydrated in an ethanol series at room temperature, plus further dehydration with increasing concentrations of *tert*-butanol (Merck). The last change of 100% *tert*-butanol was diluted 1:1 with molten paraplast and left overnight at 60°C. Two more changes of 12 h each were made with molten paraplast, and the blocks were made after a final change of 2 h in fresh paraplast at 60°C. Longitudinal and transverse sections (7 μm thick) of the embryos were sliced with disposable blades (Reichert) in a Reichert-Jung 2050 microtome. The sections were aligned on slides treated with poly(D-lysine) and allowed to air dry on a platform at 42°C. RNA and tissue conservation was confirmed by acridine orange staining.

***In Situ* Hybridization.** Hybridization was performed as described (12). The slides were exposed for 10 days using Kodak NTB-2 emulsion and then stained with fast green 0.5% in 95% ethanol. Some were stained with hematoxylin in order to check the morphology of the sections.

Probes. The HRGP probe used is a 512-base-pair *Sna*BI fragment of the 3′ transcribed and translated region of the genomic clone of maize HRGP (9). The maize H4 histone probe (provided by Claude Gigot, IBMP, Strasbourg) is a 328-base-pair insert of H4 C14 clone (13), which covers the

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Abbreviations: DAP, days after pollination; HRGP, hydroxyproline-rich glycoprotein.

*Permanent address: Department of Agricultural Sciences, University of Bristol, Long Ashton Research Station, Long Ashton, Bristol, United Kingdom.
†Present address: Department of Biology, Osborn Memorial Laboratories, Yale University, New Haven, CT 06511-7444.
‡To whom reprint requests should be addressed.

whole coding sequence of the protein. Both were cloned in pBluescript SK+ (Stratagene) and used as templates for synthesis of sense and antisense RNA probes. Transcripts from T3 and T7 promoters were produced following the instructions of the manufacturers, using cytidine 5′-[α-[^{35}S]thio]triphosphate (1000 Ci·mmol^{-1}; 1 Ci = 37 GBq; Amersham) diluted 1:5 with nonlabeled rCTP as the radioactive precursor. Final concentration of probes was ≈0.25 μg·ml^{-1} and each slide was hybridized with ≈10^6 cpm of label.

Photographs were taken with a Zeiss automated camera on a Zeiss microscope under dark-field or bright-field illumination. Polarized light images were obtained in a Wild (Heerbrugg, Switzerland) microscope and camera.

RESULTS

HRGP mRNA Accumulation in Dividing and Provascular Cells from Maize Embryo. It has previously been reported that both Northern and Western blot analyses indicate that neither HRGP protein nor its mRNA is present in maize scutellum (11). However, mRNA levels comparable to those of vegetative tissues were observed in the embryo axis and the protein was detected by immunocytochemical analysis only in the embryo axis and not in the scutellum (11). *In situ* hybridization in the early leaves, coleoptile, and young roots showed an increased accumulation of the HRGP mRNA in cells associated with the vascular system and in sclerified cells. The correlation of divisional activity and HRGP mRNA accumulation in specific tissues has been observed in RNA gel blots hybridized with histone H4 probe, as a marker for cell division, and HRGP probe (10). From these data, it appears that maize HRGP mRNA accumulation is under the control of a number of factors, including divisional activity, cell type, and tissue specificity. Maize embryo offers the possibility to study by *in situ* hybridization the accumulation of HRGP mRNA in cells that are at different stages of differentiation and that belong to distinct cell types. In particular, it allows the study of HRGP mRNA in relation to formation of the elements that constitute the cell wall.

Transverse and longitudinal sections of the embryo were hybridized with the antisense strand of the HRGP probe. The results with 17 DAP embryos are shown in Fig. 1 and they agree with previous findings (10, 11). In the scutellum, there is some birefringence due to starch grains in dark field (see below), but only low levels of hybridization are detected in the procambium (better seen in the longitudinal section; marked P in Fig. 1) and in the zones near the coleorhiza. In the axis, the signal is mostly observed in the regions where a higher proportion of dividing cells are found, such as in the top of the coleoptile (Fig. 1*A*), and also where provascular cells are abundant. In particular, there is strong hybridization to the two provascular bundles in the coleoptile (Fig. 1*B*) and to the six main premetaxylem cells in the coleorhiza (Fig. 1*D*). The mRNA also accumulates in procambial cells of the vascular cylinder and in the pericycle, which marks the external border of hybridization with the HRGP probe. The pattern obtained with the transverse sections of the embryo is confirmed with the longitudinal sections where the provascular and actively dividing zones in the axis are clearly observed (Fig. 1*F*).

The hybridization pattern of HRGP observed in 17 DAP embryos is the same in younger embryos (Fig. 2). Fig. 2 *A* and *B* shows regions of the embryo similar to Fig. 1 *A* and *B*. A control section hybridized with a sense RNA probe (Fig. 2*C*) and a section at the level of the radicle stained with hematoxylin in order to observe the morphology in this zone (Fig. 2*D*) are shown. Early embryos appear to have a less-defined morphology than more mature embryos, but the pattern of

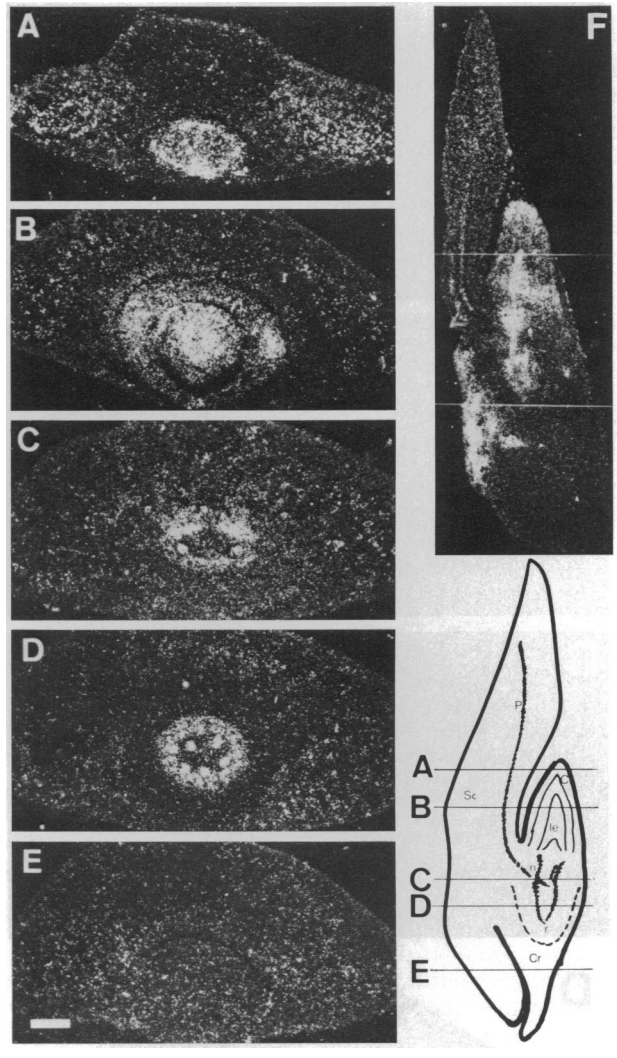

FIG. 1. Pattern of maize HRGP mRNA accumulation in immature embryos, 17 DAP under dark-field microscopy. Transverse and longitudinal sections of 17 DAP embryos were hybridized to a maize HRGP antisense RNA probe. (*A–E*) Transverse sections of the corresponding lines in the scheme on the right. (*F*) Longitudinal section. Controls with the sense probe gave no hybridization (data not shown). P, procambial strand; C, coleoptile; le, leaf primordia; n, scutellar node; Sc, scutellum; r, radicle; Cr, coleorhiza. (Bar = 200 μm.)

hybridization also corresponds to actively dividing zones where the vascular system is beginning to form.

HRGP and Histone H4 mRNA Accumulation in Maize Embryo. The correlation of HRGP mRNA accumulation with organs having a high rate of cells undergoing division has already been shown (7). In particular, correlation of the HRGP mRNA level in different organs with that of histone H4 mRNA has been described (10). To observe this correlation at the cellular level, *in situ* hybridization with a histone H4 probe was carried out on sections of maize embryo equivalent to those used for hybridization with the HRGP probe.

In Fig. 3*A* hybridization with the histone H4 probe of a section of a 17 DAP embryo in the central region of the coleoptile is presented. The pattern of hybridization is similar to that observed using the HRGP probe (see Figs. 1*B* and 4*A*). In particular, there is hybridization in the primordial leaf and in the vascular bundles of the coleoptile. This result confirms that the *Hrgp* genes are expressed during cell division. However, there are two interesting differences when the

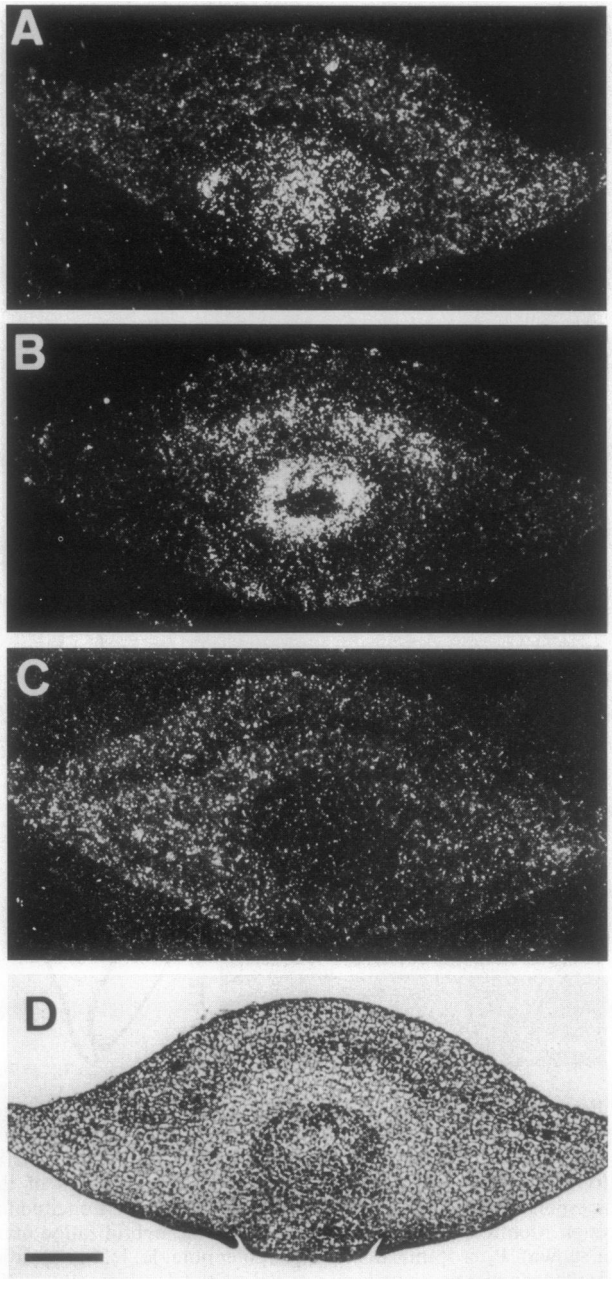

FIG. 2. Maize HRGP mRNA accumulation in 13 DAP embryo. (*A* and *B*) Transverse sections of 13 DAP embryo hybridized to a maize HRGP antisense RNA probe. These sections are equivalent to those of Fig. 1 *B* and *D*. (*C*) Same region as in *B* hybridized to the sense RNA probe as a control. (*D*) Section equivalent to *B* and *C* stained with hematoxylin for morphological examination. (*A–C*) Dark-field photographs. (Bar = 100 μm.)

results of the two probes are compared. First, hybridization of the HRGP probe appears homogeneously distributed, while the histone H4 probe seems to hybridize in patches. Using a higher magnification, it is possible to observe that the patches of histone H4 hybridization in the general view correspond to a small group of cells, between one and three. It seems that a subgroup of cells among those showing accumulation of the HRGP mRNA hybridizes more intensely to the histone H4 probe (Fig. 3*B*). This is in accordance with a very short half-life of H4 histone transcripts, restricted to the S phase of the cell cycle. Second, in the scutellar cells, there is hybridization with the H4 probe (Fig. 3*C*), but none is detected with the HRGP probe (see Figs. 1 and 2). Therefore, the absence of HRGP hybridization is due not to

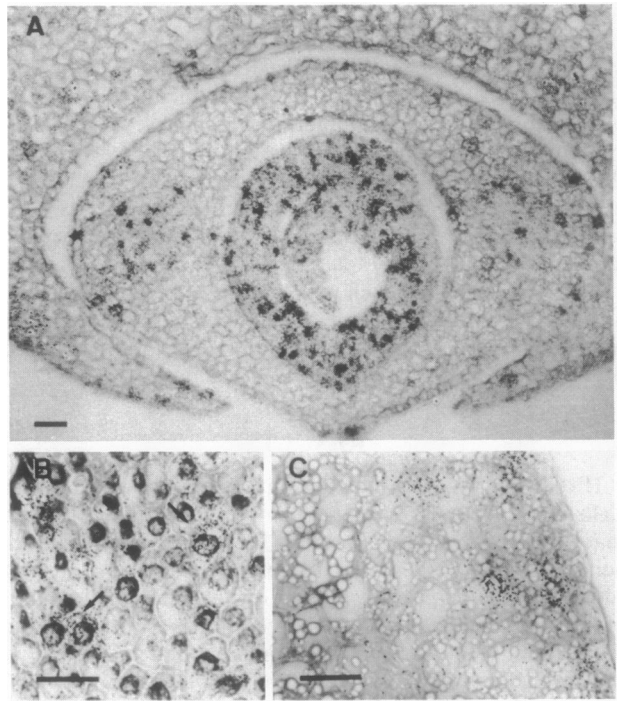

FIG. 3. Maize H4 histone pattern of mRNA accumulation. The 17 DAP embryo sections were hybridized to a maize H4 histone antisense RNA probe. (*A*) Transverse section of coleoptile (see Figs. 1*B* and 4*A*). (*B*) Detail of a test section stained with hematoxylin to examine the number of cells that hybridize to the H4 antisense RNA probe (arrows). (*C*) Transverse section of a 17 DAP embryo showing a detail of hybridization to scutellar cells. Bright-field photographs. (Bars = 50 μm.)

an experimental artefact but to the existence of a tissue-specific control of HRGP mRNA accumulation. Using the H4 probe, there are small groups of cells in the scutellum that give rise to a pattern of hybridization similar to that observed in the embryo axis.

Relation of HRGP mRNA and Cellulose Accumulation in Maize Embryos, as Observed Under Polarized Light Microscopy. Polarized light microscopy was used to examine two effects: the difference between hybridization and birefringence of starch granules in dark field and the relationship between the sites of HRGP mRNA accumulation and the thickness of the cell wall. In Fig. 4, the result of hybridization of transverse sections of a maize embryo 17 DAP at the level of the coleoptile and coleorhiza is shown by polarized light microscopy. In Fig. 4*C* it is possible to observe the different type of birefringence produced by starch granules in the preparation and silver grains from the photographic emulsion that gives a similar image under dark field in black and white prints.

In Fig. 4*A*, hybridization of a coleoptile section observed under polarized light shows the cellulose fibrils of the cell walls. The cells showing the strongest hybridization with the HRGP probe are small cells with thin walls. This is apparent in the leaf primordia, where an almost homogeneous pattern of hybridization occurs with a slight increase in cells located around the vascular bundles. This is also the case in the vascular bundles of the coleoptile. This morphology is demonstrated in a control, nonhybridized section (Fig. 4*B*) observed under polarized light. Cells having strong birefringence from cellulose show little or no hybridization with the possible exception of mature cells in the coleoptile that are probably the precursors of protective sclerified cells of the vascular bundles. In younger embryos, the correlation of hybridization with small and thin-walled cells also occurs, as

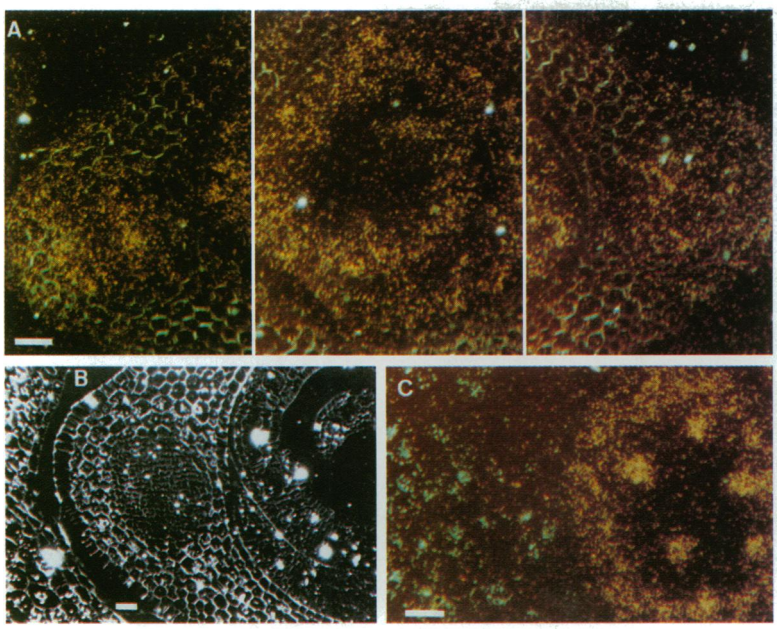

FIG. 4. Maize HRGP mRNA and cellulose accumulation under polarized light microscopy. mRNA hybridization (yellow dots), cellulose (white birefringence), and starch (white grains) may be distinguished. (*A*) Transverse section of coleoptile and first leaf primordia, 20 DAP embryo, hybridized to antisense HRGP RNA. (*B*) Same as in *A*, nonhybridized section. (*C*) Radicle section with strong hybridization to premetaxylem cells and procambium on the vascular cylinder. Note starch accumulation in the scutellum and the absence of detectable cellulose in the walls of the radicle cells. (Bars = 50 μm.)

birefringence in the wall is hardly detectable, whereas mRNA accumulation is high (result not shown).

DISCUSSION

Previous results, essentially from Northern blot analyses, indicate that accumulation of the mRNA coding for maize HRGP in young tissues of the plant correlates with the abundance of cells undergoing division. The parallel accumulation of histone H4 mRNA was also observed in different parts of the developing root and in maize calli (10). This correlation is lost when HRGP mRNA accumulates after wounding (10) and also in the scutellum, which has much lower levels of accumulation of HRGP mRNA. The results obtained by *in situ* hybridization confirm the previous conclusions and also give more information on the temporal and spatial expression of the *Hrgp* gene, demonstrating that HRGP mRNA accumulates in the early stages of cell differentiation.

The correlation of expression of the genes coding for HRGP and histone H4 already observed by Northern blot analysis is confirmed by *in situ* hybridization. Histone mRNA is synthesized periodically during the cell cycle of HeLa cells (14), accumulates mainly during the S phase, and displays a very short half-life. On the other hand, the need for a mRNA encoding a component of the cell wall during cell division seems obvious. However, there are two main differences between the pattern of mRNA accumulation of these two genes during maize embryogenesis. One is the tissue specificity of *Hrgp* gene expression in the axis, which is lacking in the histone gene. This result confirms that, in defined parts of the embryo, mainly in the scutellum, the cell wall does not contain HRGP and therefore it probably has a different structure. The composition and properties of this embryo-specific cell wall are not known but genes coding for other proline-rich proteins specific to the embryo could be assumed. The second difference is that, although the regions of the embryo axis showing hybridization with the HRGP and histone H4 probes are in general the same, the histone H4 probe hybridizes to small groups of cells. This result may be interpreted as reflecting the expression of the histone gene in defined stages of the cell cycle. According to this assumption the observed pattern could be the combination of an asynchronous cell cycle, with a faster turnover of H4 histone mRNA. Comparing the expression of the two genes, either the expression of the *Hrgp* gene is not restricted to a defined stage of the cell cycle or its mRNA has a higher stability than the histone H4 mRNA, or both.

The HRGP probe hybridizes to regions where cell division is occurring but with enhanced accumulation in the provascular tissues as has been proposed (9). In fact, both in the embryo and in the coleoptile a higher level of HRGP mRNA is transiently observed in differentiation of cells in which mechanical strength of the wall is a key property (9). This makes it an interesting marker for the formation of vascular tissues during maize embryogenesis.

Little is known of the function of HRGP in plant cell walls. The possibility that extensins form a protein network through ionic or covalent bonds has been proposed (15). In maize, the accumulation of HRGP mRNA is found in cells that are small and show a low birefringence in their walls. This result and the correlation of expression of the genes coding for HRGP and histones would indicate that the HRGP mRNA is needed in the early stages of cell wall formation. No data are available on the stability of HRGP mRNA in different cell types, on posttranslational modifications and intra- and extracellular transport, and on the polymerization and dynamics of these proteins in the wall. Nevertheless, the presence of the mRNA in the early stages of cell morphogenesis would agree with the participation of HRGP in the initial formation of the basic architecture of the cell wall.

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