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Developmental and Hormonal Regulation of Genes Coding for Proline-Rich Proteins in Female Inflorescences and Kernels of Maize[1]

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The pattern of expression of two genes coding for proteins rich in proline, *HyPRP* (hybrid proline-rich protein) and *HRGP* (hydroxyproline-rich glycoprotein), has been studied in maize (*Zea mays*) embryos by RNA analysis and in situ hybridization. mRNA accumulation is high during the first 20 d after pollination, and disappears in the maturation stages of embryogenesis. The two genes are also expressed during the development of the pistillate spikelet and during the first stages of embryo development in adjacent but different tissues. *HyPRP* mRNA accumulates mainly in the scutellum and *HRGP* mRNA mainly in the embryo axis and the suspensor. The two genes appear to be under the control of different regulatory pathways during embryogenesis. We show that *HyPRP* is repressed by abscisic acid and stress treatments, with the exception of cold treatment. In contrast, *HRGP* is affected positively by specific stress treatments.

The use of recombinant DNA techniques has led to the identification and sequence determination of a number of structural protein components of the plant cell wall (Cassab and Varner, 1988; Showalter, 1993). These include different types of HRGPs and Pro-rich proteins, Gly-rich proteins, arabinogalactan proteins, and lectins. In most of these the protein sequence is highly repetitive. A good example in maize (*Zea mays*) is HRGP, one of the main proteins extractable from the cell wall (Kieliszewski and Lamport, 1987; Stiefel et al., 1988). Its mRNA is abundant in dividing maize tissues and highly accumulated in provascular cells (Stiefel et al., 1990). The *HRGP* gene responds to mechanical stress and to ethylene treatment by increasing its mRNA levels (Tagu et al., 1992). The protein is rich in Pro, Lys, and Thr, and these amino acids form the repetitive motifs that constitute most of its primary structure, which is polymorphic between different maize varieties (Raz et el., 1991). Homologous proteins have been described in related species such as teosinte, sorghum, and rice (Raz et al., 1991, 1992; Caelles et al., 1992).

Recently, a number of proteins other than HRGPs that are also rich in Pro or Gly have been described in several plant species. These proteins have characteristic signal peptides (probably necessary for the translocation of the protein to the cell wall), repetitive Pro- or Gly-rich domains, and a Cys-rich C-terminal domain that sometimes shows similarity to proteins with a defensive or a storage function. Because of the presence of two domains with such distinct features, these proteins have been called hybrid Pro-rich proteins (Josè and Puigdomènech, 1993, 1994). In at least two cases (Cheung et al., 1993; Domingo et al., 1994) proteins belonging to this class have been located in the cell wall using immunologic techniques.

A gene encoding a protein with a Pro-rich domain and a hydrophobic, Cys-rich C-terminal domain has been described in maize and named HyPRP (*zmHyPRP*) (Josè-Estanyol et al., 1992). Partially homologous sequences have been described in other plant species (Salts et al., 1992). The mRNA corresponding to this gene in maize accumulates mainly during embryogenesis. A low level of mRNA accumulation was also found in the pistillate spikelet before anthesis (Josè-Estanyol et al., 1992). Maize embryogenesis follows a characteristic pattern of development (Abbé and Stein, 1954). After anthesis, a single-celled zygote develops by division into the proembryo, which is radially symmetric. At the transition stage asymmetry is introduced by the formation of an internal, wedge-shaped meristematic region in the upper part of the embryo. At the coleoptilar stage this region gives rise to the shoot apex, the surrounding coleoptilar ring, and the root apex. After leaf primordium differentiation, subsequent stages follow. After the embryogenic period, ABA is induced and embryo maturity (during stage 6) is finally attained (Quatrano, 1987; Sheridan and Clark, 1987).

In situ hybridization studies indicated that at stage 2 of maize embryogenesis, the *HyPRP* gene is expressed in parenchymal cells surrounding the developing vascular system in the embryo axis, as well as in subepidermal cells, showing a high level of mRNA accumulation in the scutellum (Josè-Estanyol et al., 1992). In contrast, the accumulation of *HRGP* mRNA shows a pattern complementary to that observed for *HyPRP* in the sense that in the immature

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Abbreviations: HRGP, hydroxyproline-rich glycoprotein; HyPRP, hybrid Pro-rich protein.

embryo one gene is expressed in tissues in which the other is not expressed. In particular, *HRGP* mRNA is found abundantly in axis provascular cells, where *HyPRP* is not found (Josè-Estanyol et al., 1992; Ruiz-Avila et al., 1992). The mRNA of *HyPRP* disappears when ABA accumulation begins in the developing embryo with the initiation of the maturation and dessication phases (Jones and Brenner, 1987). We have proposed (Josè-Estanyol et al., 1992) that ABA could repress *HyPRP* expression, as its mRNA level is maintained in ABA-deficient maize mutants (*vp2*). In this paper we describe the cell types in which *HyPRP* and *HRGP* are expressed in the pistillate spikelet at different stages of ear development and during the first stages of embryo development before leaf primordia appearance. The effects of ABA and different physiological situations have also been studied.

MATERIALS AND METHODS

Biological Materials

Unless otherwise stated, the plant material used was derived from seeds of a maize (*Zea mays* cv W64A) pure inbred line grown in a greenhouse in Barcelona, Spain. Spikelets, kernels, and excised treated and untreated immature embryos were collected at different developmental stages and stored at −80°C. For stress treatments immature embryos excised at stage 2 of development were treated with a hormone solution (10 μM ABA), as described by Vilardell et al. (1990), a high-osmoticum solution (0.25 M NaCl), or cold treatment (4°C) for different times. As a control, excised embryos were placed in a humid chamber. Heterozygous caryopses of the *viviparous-2* (*vp2*) mutant of maize were obtained from Dr. R.J. Lambert (Maize Genetic Stock Center, University of Illinois, Urbana). Homozygous *vp2* kernels at stage 2 were selected on the basis of white, colorless endosperm.

RNA Preparation and Gel-Blot Analysis

RNA was extracted by the guanidinium-HCl procedure (Logemann et al., 1987). All RNAs were checked for RNA quality by nondenaturing electrophoresis on 1.5% agarose gels and by EtBr staining, and the concentrations were adjusted by reading the A_{260}. Total RNAs were separated on gels containing 1.5% agarose-formaldehyde (Lehrach et al., 1977). The gels were blotted onto nylon membranes (Hybond-N, Amersham), treated as described by the manufacturer, and hybridized at 65°C for at least 12 h in a phosphate solution (Church and Gilbert, 1984) with specific probes. Fragments from the *HyPRP* (Josè-Estanyol et al., 1992) and *HRGP* (Raz et al., 1992) coding regions and from the *RAB28* (Pla et al., 1991) and *H4* (Philipps et al., 1986) coding and 3′-nontranslated regions were labeled by random priming to a specific activity of 10^8 cpm/μg. After hybridization, filters were washed to 20 mM phosphate stringency at 65°C and autoradiographed. Experiments were repeated at least three times.

In Situ Hybridization

Ears or their excised spikelets, kernels, and treated and untreated immature embryos were collected at different developmental stages and submerged in 84:11:5 ethanol: formaldehyde:glacial acetic acid fixing solution. The whole procedure of in situ hybridization, including paraffin inclusion and tissue dissection in a microtome (Reichert, Germany), was performed essentially according to the method of Langdale et al. (1988). A 670-bp *Dde*I and a 660-bp FockI-SnabI fragment of the 5′-coding region of *HyPRP* and *HRGP* genes, respectively, were cloned in a pBluescript SK+ vector (Stratagene) and used as a template for the synthesis of sense and antisense riboprobes. Transcripts from T3 and T7 promoters were produced following the instructions of the manufacturers, using ^{35}S-CTP (37 TBq mmol $^{-1}$; Amersham). The final concentration of the probes was 0.1 μg/mL, and each slide was hybridized with 0.5 kBq of labeled probe. Hybridization was then performed as described previously (Langdale et al., 1988). The slides were exposed using Kodak NTB-2 emulsion, and stained after developing with 0.5% (w/v) fast green in 95% ethanol or 1% toluidine blue in 1% sodium tetraborate. The photographs were taken using an automated camera on a light microscope (Axiophot, Zeiss).

RESULTS

Developmental Expression of *HyPRP* and *HRGP* Genes in Maize Ears

The gene coding for the maize HyPRP is mainly expressed in young embryos and has a low level of expression in developing female inflorescences (Josè-Estanyol et al., 1992). *HRGP* mRNA accumulates throughout embryo development, mainly where the level of mRNA corresponding to genes expressed in proliferating tissues, such as histone genes, is very high (Ruiz-Avila et al., 1992). In this study the accumulation of *HyPRP* and *HRGP* mRNA was measured using RNA analysis and in situ hybridization techniques during the development of the pistillate spikelet, the immature female flower (Kiesselbach, 1980; Schmidt et al., 1993). Several stages of development were studied, from undifferentiated ear spikelet primordia to just before anthesis. At this stage differentiation is completed and silks are just longer than the ear (2.5-mm pistillate spikelet diameter). Nonpollinated pistillate spikelets up to 4.0 mm in size were also studied.

HyPRP mRNA accumulated in developing pistillate spikelets until anthesis (Fig. 1A). At this point, *HyPRP* expression decreased and disappeared when pollination did not occur (Fig. 1A). Following anthesis a low level of mRNA was observed in young kernels, which reached a maximum around 6 to 10 d after pollination, when the maize embryo begins to develop (Josè-Estanyol et al., 1992). *HyPRP* mRNA was not observed in the silk (Fig. 1A). Conversely, when the *HRGP* probe was used, a high level of mRNA accumulation was observed in the silk and, to a lesser extent, in the spikelet, where expression increased when anthesis did not occur (Fig. 1A). After pollination

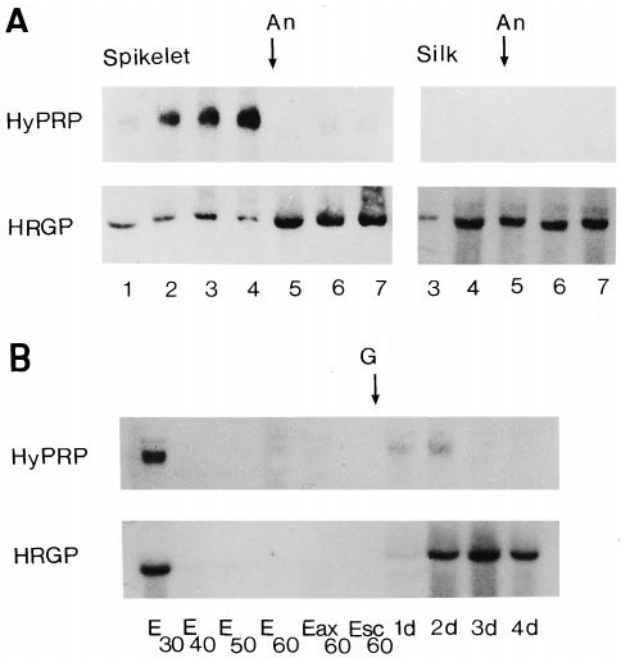

Figure 1. Accumulation of *HyPRP* and *HRGP* mRNA in different tissues. A, Accumulation of *HyPRP* and *HRGP* mRNA in maize female inflorescences. Total mRNA (30 μg per lane) from maize ears, from their isolated pistillated spikelets, or from their respective silks at different stages of ear development were subjected to electrophoresis on agarose-formaldehyde gels, transferred to nylon membranes, and hybridized with *HyPRP* and *HRGP* probes. Exposure time was 10 d for *HyPRP* and 1 d for the *HRGP* probe, with the exception of silk samples, which were exposed for only 2 h. 1, Ear length of 0.6 cm; 2, excised ear spikelets sharing 0.5-cm silk length; 3 through 5, excised ear spikelets without silks (at left) or their corresponding silks (at right). The stage of development is indicated by the length of the respective silks: 3, 4.5-cm silk length; 4, 10.5-cm silk length; 5, silks just extending above the ear; 6 and 7, excised ear spikelets without silks (at left) or their corresponding silks (at right), excised from an unpollinated ear after anthesis time. The stage of development is indicated by the number of days after anthesis time: 6, 5 d; 7, 12 d. The arrow indicates anthesis (An) time in ear development. B, RNA gel blot of mRNA extracted from excised mature, dehydrated, and germinated maize embryos. E_{30}, E_{40}, E_{50}, and E_{60}, immature embryos 30, 40, 50, and 60 d after pollination, respectively; Eax_{60} and Esc_{60}, embryo axis and scutellum, respectively, from dry kernels 60 d after pollination; 1d to 4d, excised coleoptile-nodular region from embryos 1 to 4 d after germination. Exposure time was 10 d for the *HyPRP* probe and 1 d for *HRGP*.

HRGP expression is maintained in the kernel (Ruiz-Avila et al., 1991).

Accumulation of *HyPRP* mRNA in maize embryo begins to decrease progressively after 18 d of anthesis (Josè-Estanyol et al., 1992), and accumulation of *HRGP* begins to decrease after 30 d (Ruiz-Avila et al., 1991). Neither *HyPRP* nor *HRGP* mRNA was detectable during maturation of the embryo (between 40 and 60 d after pollination) or in the dry embryo (Fig. 1B). Both genes began to be re-expressed in the embryo shortly after germination, when the growth of the plant began. *HyPRP* mRNA was transiently observed at a low level, but was no longer detectable during vegetative growth, whereas *HRGP* mRNA accumulated at high levels in the plantlet (Fig. 1B).

In situ hybridization was carried out to define the pistillate spikelet cell types responsible for *HyPRP* and *HRGP* mRNA accumulation. Figure 2 shows the results of such a study on a 2-mm longitudinal section placed in the middle of a 6-mm ear. In this zone it is possible to observe immature female flowers at different stages of development (Kiesselbach, 1980; Schmidt et al., 1993). *HyPRP* mRNA was accumulated at a very low level in parenchymal cells present at the border of each differentiating spikelet below the differentiating floral primordia (Fig. 2a). This is the region of the ear where *HRGP* expression is localized in the floret primordia below the epidermis and between growing spikelets (Fig. 2b).

Figure 2, c through g, shows the analysis of two different longitudinal sections of a young maize pistillate spikelet just before anthesis, when the different cells of the vascular tissue, placento-chalazal region, ovary, and embryo sac are well differentiated and the silks nearly extend above the ear (10.5 cm). In an internal longitudinal section containing the embryo sac, *HyPRP* mRNA (Fig. 2d) appeared to be nearly absent from the flower, where only a low level of expression remains around the placento-chalazal region, but not around the differentiated vascular tissue. In the accompanying abortive flower, which is in an earlier developmental stage similar to the one observed for 0.5-cm, silk-length spikelets (not shown), *HyPRP* mRNA was abundant in the cells surrounding the provascular emerging cells. When adjacent sections were hybridized with the *HRGP* probe (Fig. 2f), mRNA was observed around the embryo sac in the nucellus, in the ovary wall epidermis, and in the stylar canal. In the differentiated vascular tissue, expression was localized in cells accompanying fibers and xylem-differentiated structures.

When an external longitudinal section from the same stage of development was studied, *HyPRP* mRNA was only accumulated in the area surrounding the placento-chalazal region (Fig. 2e). In a similar section, *HRGP* mRNA (Fig. 2g) was accumulated in cells placed at the internal border of the placental side of the placento-chalazal region, as well as in the nucellus, in the epidermis of ovary wall and in the silk. The same results were observed when similar longitudinal sections of spikelets in an intermediate stage of development (silk lengths of 4.5 cm) were studied. The only difference was that at this stage starch grains in the cortex were present (data not shown).

mRNA Accumulation of *HyPRP* and *HRGP* in Maize Kernels

In situ hybridization (Fig. 3) of kernels between anthesis and 9 d after pollination showed that at the proembryo and transition phase of embryo development (Fig. 3, a and d), *HyPRP* mRNA was faintly detected in the embryo, whereas *HRGP* mRNA was already detectable in a polar manner in cells in which the suspensor was beginning to be developed (Fig. 3, b and e). *HRGP* mRNA continued to be highly

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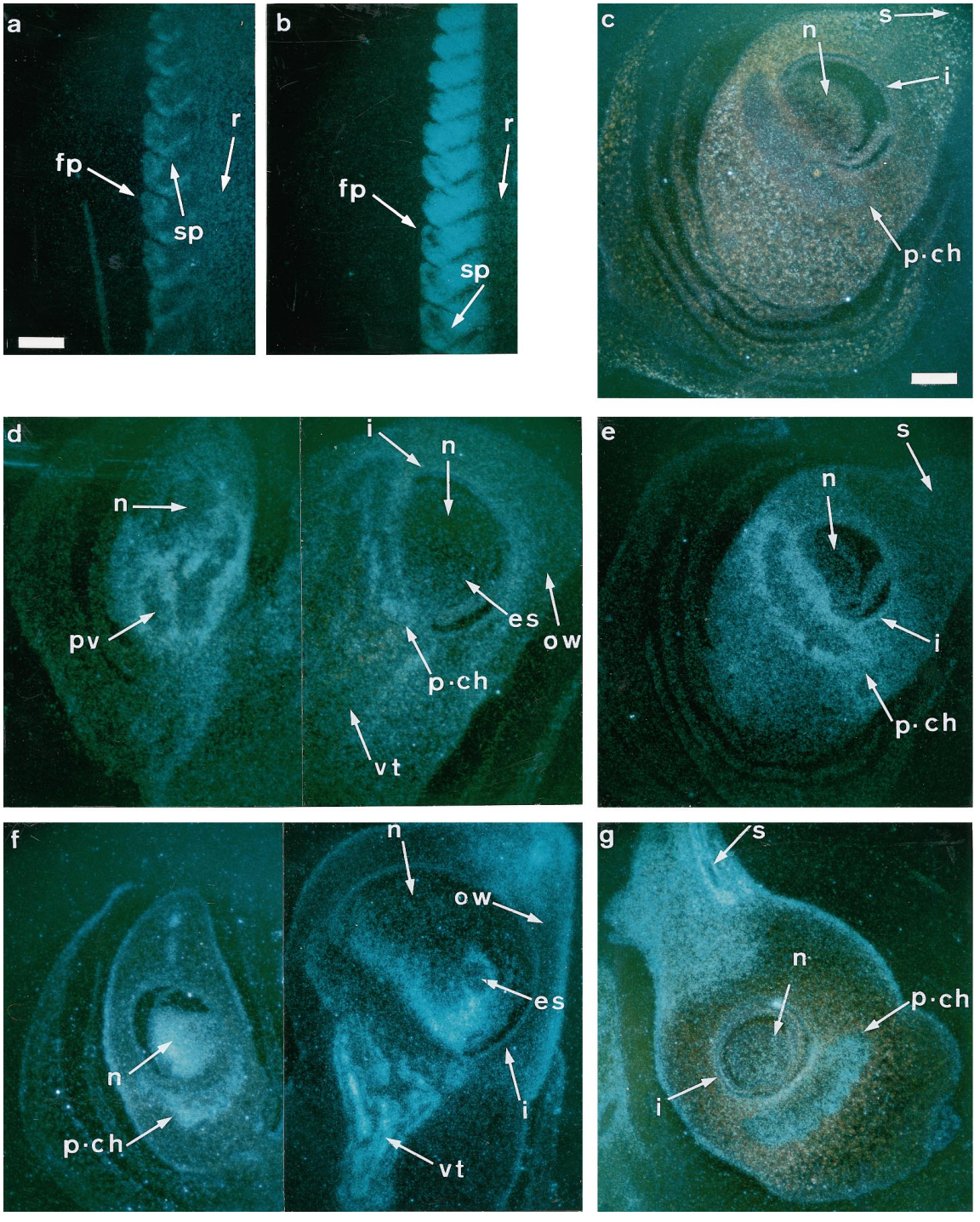

Figure 2. Cellular localization of *HyPRP* and *HRGP* mRNA in longitudinal sections from maize female inflorescences. a and b, Longitudinal section from maize ear at a very early stage of spikelet organ differentiation. In situ hybridization of an *HyPRP* (a) and an *HRGP* (b) antisense RNA probe with a longitudinal section from an ear 0.6 cm in length. *HyPRP* slides were exposed for 22 d, whereas *HRGP* was exposed for 7 d. c through g, Longitudinal sections from mature pistillate spikelets of maize just before anthesis time. c, In situ hybridization of an *HyPRP* sense RNA probe with a mature pistillate spikelet external longitudinal section. d through e, In situ hybridization of an *HyPRP* antisense RNA probe with a mature pistillate spikelet central and external longitudinal section, respectively. f through g, In situ hybridization of an *HRGP* antisense RNA probe with a mature pistillate spikelet and central and external longitudinal sections, respectively. Bars = 250 μm (a and b) and 200 μm (c–g). *HyPRP* slides were exposed for 18 d, whereas *HRGP* was exposed for 7 d. es, Embryo sac; fp, floral primordia; i, integuments; n, nucellus; ow, ovary wall; p.ch, placento-chalazal region; pv, provascular tissues; r, rachis; s, silk; sp, spikelet primordia; and vt, vascular tissue. All of the sections were examined under dark-field microscopy. Hybridization is visible as blue areas.

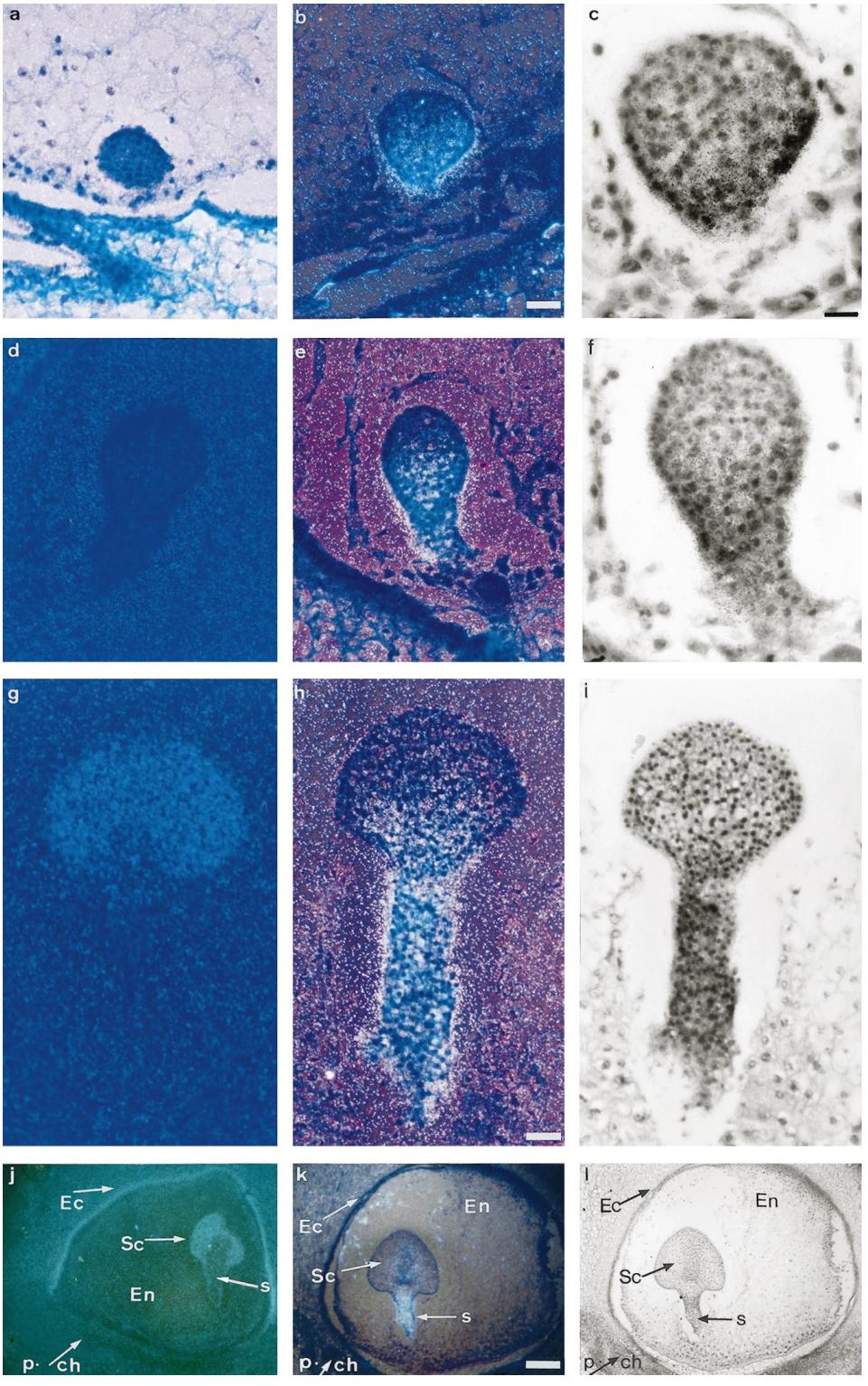

Figure 3. Cellular localization of *HyPRP* and *HRGP* mRNA in longitudinal sections from maize kernel. a through c, In situ hybridization with *HyPRP* (a) and *HRGP* (b–c) antisense probes with longitudinal sections of maize kernels at the proembryo stage of development. d through f, In situ hybridization with *HyPRP* (d) and *HRGP* (e–f) antisense probes with longitudinal sections of maize kernels at embryo transition phase of development. g through i, In situ hybridization with *HyPRP* (g) and *HRGP* (h–i) antisense probes with longitudinal sections of maize kernel at embryo initial coleoptilar stage of development. j through l, In situ hybridization with *HyPRP* (j) and *HRGP* (k and l) antisense probes with maize kernels at embryo coleoptilar stage of development. Bars = 50 μm (a, b, d, and e); 25 μm (c and f); 100 μm (g–i); and 250 μm (j–l). Sections were examined under light- or dark-field microscopy. Hybridization is visible as blue areas in a, b, d, e, g, h, and k, and as green areas in j.

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abundant in the suspensor until the coleoptilar stage was reached (Fig. 3, h and k). Dark (Fig. 3, b, e, h, and k)- and bright (Fig. 3, c, f, i, and l)-field exposures corresponding to the same sections for *HRGP* are presented for a better comparison of the anatomy of the region where the gene is expressed. In contrast, *HyPRP* mRNA was not clearly observed until the embryo reached the coleoptilar stage (Fig. 3, g and j). At this time *HyPRP* was accumulated in the regions differentiating to scutellum. *HyPRP* mRNA was also observed at this stage in the first layer of endocarp cells of developing pericarp (Fig. 3j). At this time the *HRGP* mRNA was accumulated in the suspensor and in cells surrounding the emerging shoot meristem (Fig. 3k), as well as in the developing exocarp (not shown).

Accumulation of *HyPRP* and *HRGP* mRNA in Maize Embryos under Different Physiological Conditions: Effect of ABA

HyPRP is expressed preferentially in immature embryos during morphogenesis, and its mRNA level disappears when the maturation stage starts after ABA begins to accumulate in the embryo (Quatrano, 1987; Josè-Estanyol et al., 1992). It has been proposed that ABA could repress *HyPRP* expression, as its mRNA levels are maintained until the onset of germination in maize *vp2* mutant plants that are deficient in ABA (Josè-Estanyol et al., 1992). An ABA-responsive element (Guiltinan et al., 1990) is present in its promoter (Josè-Estanyol et al., 1992). RNA blots were used to analyze the effect of exogenous ABA on *HyPRP* expression in freshly excised, immature maize embryos at stage 2 of development from wild type (Fig. 4) and in *vp2* mutant maize plants (Robertson, 1955) (Fig. 5).

HyPRP mRNA decreased dramatically in excised, immature wild-type embryos after 6 h of incubation with exogenous ABA; after 24 h it was undetectable (Fig. 4). In contrast, the mRNA levels of *RAB28*, induced by ABA in late embryogenesis (Pla et al., 1991), increased. When the effect of exogenous ABA on embryos from *vp2* mutant maize plants was studied a similar effect was found (Fig. 5). This indicates that the applied hormone simultaneously represses *HyPRP* and activates *RAB28*. A control experiment using the same conditions but in the absence of ABA is not possible because after 24 h in buffer conditions, the embryos germinate and the expression of *HyPRP* stops, as occurs during seed germination (see Fig. 1B). For this reason, we used embryos excised from the ear and maintained in a humid chamber without being subjected to any stress or damage as controls (Fig. 4). Under these conditions expression of *HyPRP* and *HRGP* was maintained and expression of *RAB 28* was increased. *HyPRP* mRNA accumulation at first decreased but in the absence of ABA the gene was not repressed (Fig. 4).

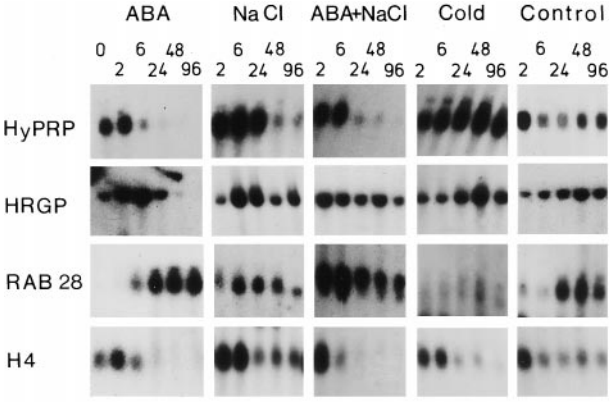

Figure 4. Accumulation of *HyPRP* and *HRGP* mRNA in young embryos after different stresses. Total RNAs (10 μg) from young, freshly excised embryos 16 d after pollination (at stage 2 of embryo development) subjected to the treatments indicated or not treated (control) were subjected to electrophoresis and hybridized with *HyPRP*, *HRGP*, *RAB28*, and *H4* probes. Hybridization and exposure times were similar for all blots. Numbers indicate hours of treatment before freezing for RNA extraction.

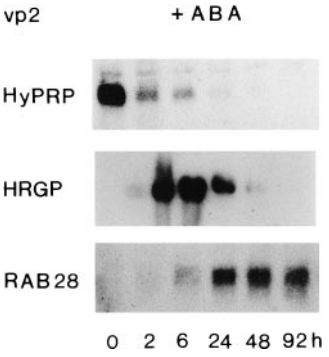

Figure 5. RNA gel-blot analysis of mRNA extracted from *vp2* embryos after ABA treatment. Total RNAs (10 μg) from *vp2* embryos at stage 2 of development treated for different times with ABA were subjected to electrophoresis and hybridized with *HyPRP*, *HRGP*, and *RAB28* probes. Numbers indicate hours of treatment.

Figure 4 also shows that *HRGP* expression can be arrested in excised, immature wild-type embryos after 24 h of exogenous ABA treatment, although expression increased from 2 to 6 h. Similar results were observed for *HRGP* when the same experiment was performed with immature *vp2* mutant embryos (Fig. 5). In both cases the mRNA levels increased slightly at the beginning of the ABA treatment and then disappeared. When H4, a marker of cell division (Philipps et al., 1986), was used cessation of H4 gene expression was observed (Fig. 4) as a result of the arrest of cell division in the induction of dormancy by ABA (Quatrano, 1987).

Aside from the regulation of *HyPRP* and *HRGP* mRNA levels related to embryo development, specific physiological conditions such as NaCl or cold treatment (4°C) may have an effect on mRNA accumulation. Results shown in Figure 4 indicate that NaCl treatment of excised embryos slightly increased *HyPRP* mRNA levels after 2 h of treatment, but that after 1 d these were reduced to less than zero and were also lower than the control. ABA plus NaCl treatments also increased *HyPRP* mRNA accumulation within 2 h of application, but after 6 h this was reduced to a low level, as was also observed with ABA treatment. Cold treatment gradually increased *HyPRP* mRNA (Fig. 4).

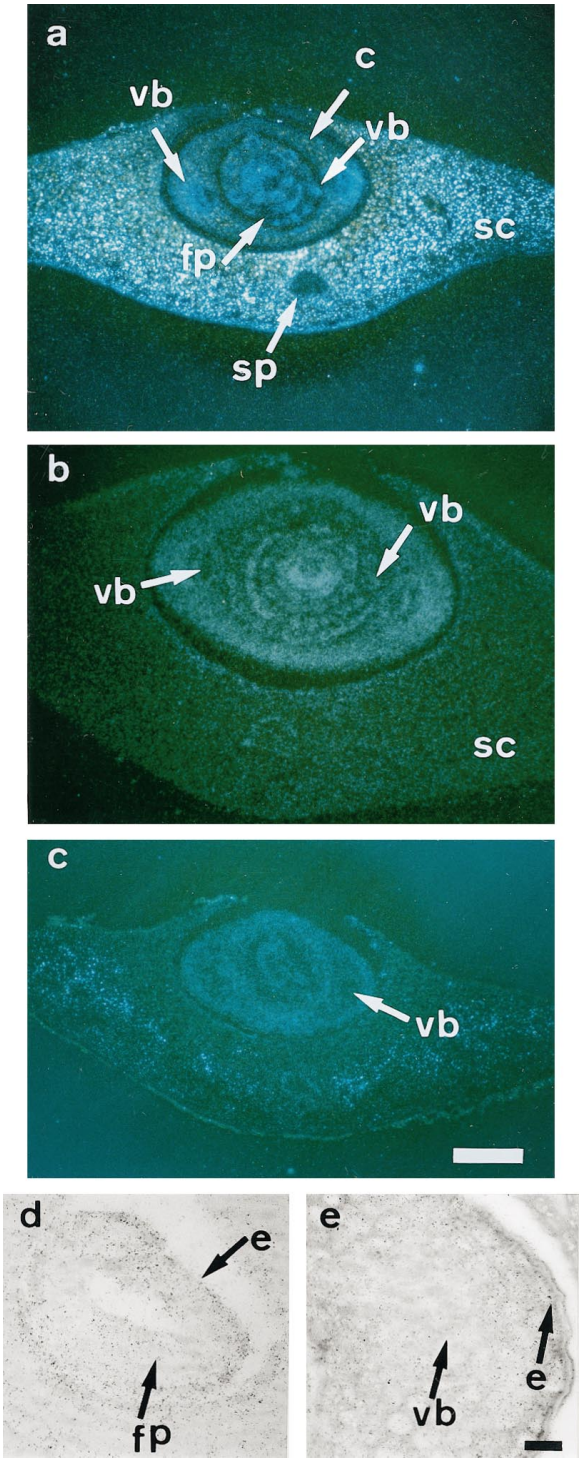

Figure 6. In situ hybridization of three transverse sections of maize embryo coleoptiles. a, Location of HRGP mRNA in maize embryos 16 d after pollination (at stage 2 of development) examined under dark-field microscopy. b, Location of *HRGP* mRNA in maize embryos 25 d after pollination (at stage 3 of embryo development) examined under dark-field microscopy. c, Location of *HRGP* mRNA in embryos 16 d after pollination (at stage 2 of development) after 24 h of cold treatment at 4°C examined under dark-field microscopy. d and e, Details of the same section shown in c are examined under bright-field microscopy. d, Foliar primordium detail. e, Vascular bundle detail of the coleoptile. c, Coleoptile; e, epidermis; fp, foliar primordium; sc, scutellum; sp, scutellar procambium; and vb, vascular bundle. Bar = 250 μm for a through c, and 25 μm for d and e. Hybridization is visible as blue-green areas in a through c, and as dots in d through e.

HyPRP was never induced by cold, wounding, or fungal attack in germinated or young plants (results not shown). Salinity and cold treatment induced an increased level of *HRGP* mRNA to a maximum around 6 h of NaCl and 48 h of cold treatment. A simultaneous treatment with ABA plus NaCl led to a maximum expression of *HRGP* after 2 h (Fig. 4).

The results described were correlated to those obtained from two other genes expressed in the axis and scutellum of immature maize embryos, *RAB28*, an ABA-responsive gene, and histone *H4*, a marker of cell division. *RAB28* gene expression was induced after NaCl treatment. ABA plus NaCl showed a cooperative effect, which reached a maximum only 2 h after treatment, but after cold treatment only low levels of mRNA accumulation were observed (Fig. 4). In contrast, *H4* expression was always arrested after cold or NaCl plus ABA treatment and markedly diminished after NaCl treatment, which confirmed that after stress treatment the embryo cell division program was arrested, as it was after ABA treatment (Fig. 4).

Previous in situ hybridization studies (Josè-Estanyol et al., 1992) indicated that *HyPRP* mRNA in immature embryos at stage 2 of development is observed mainly in parenchymal cells surrounding the developing vascular system in the axis and in cells with a high level of mRNA transcription in the scutellum. In contrast, the *HRGP* gene shows a pattern of expression mainly associated with tissues involved in vascular development on the axis of immature embryos but not in the scutellum. These two genes thus appeared to be expressed in adjacent tissues. We wondered whether the changes of expression that are shown by nothern analysis after stress treatment have an effect on the tissue distribution revealed using in situ hybridization. For cellular localization analysis, only results with the *HRGP* probe are presented, as *HyPRP* mRNA usually disappeared after stress treatment and no changes were observed after cold treatment (not shown).

In situ hybridization studies showed that when we compare the hybridization pattern of *HRGP* on sections of embryos at stage 2 of development without exogenous ABA treatment (Fig. 6a) with the one observed after 24 h of exogenous ABA treatment (not shown) or after the appearance of high ABA levels in the embryo (at stage 3) (Jones and Brenner, 1987), the *HRGP* mRNA accumulation disappeared from the developing vascular bundles of the coleoptile and foliar primordia (Fig. 6b), although it was still observed in the parenchymal cells surrounding them. No change was observed in a similar transverse section corresponding to the radicular region. After cold treatment of immature maize embryos at stage 2 of development, a modified pattern of *HRGP* expression was also observed in a transverse section at the coleoptilar level. *HRGP* mRNA levels increased in parenchymal cells surrounding the vas-

cular coleoptile bundle and below the foliar primordium epidermis (see Fig. 6c and bright-field microscope plates for a more detailed analysis).

DISCUSSION

HRGP and *HyPRP* belong to an expanding family of plant genes encoding proteins with a Pro-rich domain (Josè and Puigdomènech, 1993, 1994). In immature maize embryos, *HyPRP* mRNA has been shown by in situ hybrization to accumulate in cells having the features of parenchyma around the emerging provascular cells of the embryo axis. In this organ *HyPRP* mRNA is accumulated in tissues adjacent to but not coincident with tissues accumulating *HRGP* mRNA, which is expressed in axis provascular cells. In this organ, expression of these genes appears to be mutually exclusive. Although the immature embryo is the location of maximum levels of *HyPRP* mRNA, it has been shown that *HyPRP* also appears to be expressed at low levels in nonpollinated pistillate spikelets prior to anthesis as well as in kernels 6 to 10 d after pollination. Another feature of this gene is that its mRNA disappears (Josè-Estanyol et al., 1992) from the embryo when the maturation stage is attained and the ABA content increases (Jones and Brenner, 1987). The two main aims of the present report were: (a) to analyze the cell types accumulating *HyPRP* and *HRGP* mRNA in developing female inflorescences and kernels of maize at the first stages of development to understand how the expression of these genes is regulated as a function of the development of specific cell types; and (b) to analyze the response of these genes under hormonal and stress treatments in the embryo.

The *HyPRP* mRNA during the development of the pistillate spikelet (Kiesselbach, 1980; Schmidt et al., 1993) is mainly associated with parenchymal cells surrounding initial floral meristems, initial developing vascular tissue, or the placento-chalazal region; when anthesis is achieved it disappears. The *HRGP* mRNA is very abundant in the floral meristem during spikelet differentiation and then decreases until pollination takes place. In the silk HRGP mRNA levels are always very high (Fig. 1). During development of the pistillate spikelet, *HRGP* mRNA is observed in the provascular cells, where it has been observed in other organs (Stiefel et al., 1990). After anthesis, *HRGP* mRNA is observed in the exocarp cell layer of kernel pericarp in differentiation, whereas in the embryo its mRNA is especially abundant in the suspensor. During the first stages of kernel development (Kiesselbach, 1980; Sheridan and Clark, 1987; van Lammeren, 1987), *HyPRP* mRNA is observed in the first cell layer of ectodermic tissue, which originates from maternal ovary wall that differentiates into the pericarp after anthesis and in the embryo proper after the first scutellar cells are differentiated. The study of *HyPRP* and *HRGP* cell expression during female inflorescence and kernel development indicates that these genes always show a different pattern of expression.

The mRNA levels corresponding to *HyPRP* and *HRGP* decrease after stages 3 and 4 of development, respectively, and no mRNA can be detected in the dry embryo. Both genes are activated during germination, but in the case of *HyPRP* this is a short, transient effect followed by a definitive ending of *HyPRP* gene expression, whereas *HRGP* is actively expressed during the growth of the seedling. This transient expression of *HyPRP* is consistent with the hypothesis that embryo maturation and dessication are in fact a temporal interruption of embryogenesis, which is restarted in early germination. Immediately after germination, normal vegetative growth appears and *HyPRP* is no longer detected in the adult plant.

The expression of *HyPRP* is interrupted when the concentration of ABA in the embryo increases at stage 3 of development, and expression of *HRGP* is interrupted at stage 4 (Jones and Brenner, 1987; Quatrano, 1987). Although both genes are repressed during embryo development, they are probably under the control of different regulatory signals. It is shown here that *HyPRP* is under the negative control of ABA, as observed after exogenous ABA treatment of excised, wild-type maize embryos. Moreover, in excised *vp2* maize embryos deficient in ABA and supplied with exogenous ABA, the *HyPRP* mRNA level decreased in parallel to the increase in a typical ABA-induced gene, *RAB28*. It may then be proposed that the same mechanisms or signals that act upon RAB28 to increase its mRNA level also act on *HyPRP*, but in the opposite direction. In addition, in the 5′-flanking region of the *HyPRP* gene a consensus ABA-responsive element is present, although it is not known whether it regulates this effect (Guiltinan et al., 1990). In contrast, the *HRGP* mRNA level in the wild type and in *vp2* mutants is first transiently increased and then repressed in response to ABA application. In maize *HRGP* arrest might be associated with the arrest of cell division in the induction of dormancy occurring as a result of ABA action (Quatrano, 1987). This behavior is specific for maize *HRGP*. In rice plantelets the homologous gene (Caelles et al., 1992) responds positively to ABA stress but not to wounding (Guo et al., 1994), indicating that maize and rice *HRGP* genes are differentially regulated and respond to different stresses. The rice gene promoter includes a sequence in agreement with the ABA-responsive element described by Guiltinian et al. (1990), whereas this is lacking in maize.

NaCl treatment reduces *HyPRP* mRNA accumulation to a low level following a pattern that is opposite to the induction of the *RAB28* gene. In this situation the *HRGP* mRNA level is increased, in contrast to what was observed after ABA treatment. This result may indicate that *HRGP* is sensitive to salt stress in an ABA-independent manner. The correlation of the expression of the *HRGP* and *H4* genes in the seedling indicated the need for *HRGP* in the new cell walls after division (Ludevid et al., 1990; Ruiz-Avila et al., 1991). *HRGP* expression after NaCl treatment is increased, although cell division has been arrested, as judged by the low histone *H4* mRNA level. We conclude that after NaCl treatment, as was also observed after ethylene treatment (Tagu et al., 1992), *HRGP* expression is not correlated with the accumulation of histone H4 mRNA, and therefore the response is independent of cell division.

Cold treatment increases *HyPRP* expression. Cold responses are mediated in some cases by ABA (Klee and Estelle, 1991), but this is apparently not the case for *HyPRP*

expression, in which the cold response is clearly ABA independent, as indicated by the lack of RAB28 induction. This finding is consistent with the hypothesis that after cold treatment the signal necessary for *RAB28* induction and *HyPRP* repression is absent, and for this reason, *HyPRP* expression is not reduced and RAB28 is not induced. Other genes responding to dehydration or cold treatment in an ABA-independent manner have been described (Guerrero et al., 1990; Yamaguchi-Shinozaki and Shinozaki, 1994). Different results are obtained with the *HRGP* gene. After cold treatment or during ABA induction or treatment, cells accumulating *HRGP* mRNA are different from those accumulating the same mRNA at normal temperatures, or in the absence of ABA, as seen by in situ hybridization (Fig. 6). *HRGP* behavior indicates that after the process of cold or initial ABA treatment the maize embryo is not maintained in the previous state but, rather, is now in a new state, which has an effect on either the transcriptional status of the *HRGP* gene or its mRNA stability.

In conclusion, the two genes coding for Pro-rich proteins in maize studied here allow the study of specific processes occurring during female inflorescence and at the first stages of maize kernel development. *HRGP* marks the regions where meristematic and cell division activities are high. Its mRNA accumulates particularly in the provascular cells and the silks in female inflorescences, and in suspensor cells of immature embryos. *HyPRP* follows the formation of parenchymal cells surrounding the provascular system of pistillate spikelets and scutellum formation in maize embryos. The expression of both genes is reduced by ABA treatment. However, there is fast repression by ABA in the case of *HyPRP*, whereas there may be an indirect effect for *HRGP* due to the cessation of meristematic activity, which appears first to be transiently induced by the hormone.

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