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Involvement of a maize proline-rich protein in secondary cell wall formation as deduced from its specific mRNA localization

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Abstract

A clone encoding a proline-rich protein (*ZmPRP*) has been obtained from maize root by differential screening of a maturing elongation root cDNA library. The amino acid sequence deduced from the full-length cDNA contains a putative signal peptide and a highly repetitive sequence containing the PEPK motif, indicating that the *ZmPRP* mRNA may code for a cell wall protein. The PEPK repeat is also found in a previously reported wheat sequence but differs from the repeated sequences found in hydroxyproline-rich glycoproteins (HRGP) and in dicot proline-rich proteins (PRP). In the maize genome, the ZmPRP protein is encoded by a single gene that is expressed in maturing regions of the root, in the hypocotyl and in the pericarp. In these organs, the *ZmPRP* mRNA accumulates in the xylem and surrounding cells, and in the epidermis. No *ZmPRP* mRNA was found in the phloem. The pattern of mRNA accumulation is very similar to the one observed for genes coding for proteins involved in lignin biosynthesis and, like most cell wall proteins, *ZmPRP* synthesis is also induced by wounding. These data support the hypothesis that ZmPRP is a member of a new class of fibrous proteins involved in the secondary cell wall formation in monocot species.

Introduction

Proline-rich proteins are considered to be structural components of the cell wall. Several classes of these proteins have been described [17, 30]. The best known are HRGPs (hydroxyproline-rich glycoproteins), also called extensins in dicot species. However, other sequences containing proline-rich repetitive motifs have been described. Examples are PRPs (proline-rich proteins) [15] and HyPRPs (hybrid proline-rich proteins) [18]. PRPs have been mostly described in dicot species. In soybean, three genes have been observed that are expressed in different cell types during the development of the germinating plant [15]. These proteins have typical signal peptides but the repetitive unit differs from the one (SPPPP) found in HRGPs. In the case of the soybean PRP, the most frequent repetitive elements are PPVXK. Other proteins having repetitive sequences that are considered to be structural components of the cell wall are glycine-rich proteins (GRPs). They have been described in a number of different dicot species [33]. In some cases, these GRPs are thought to take part in the formation of the secondary cell wall [19].

The composition of the structural cell wall proteins in cereals has been studied in a few species (for review, see [17]), but sequence data on the proteins extractable from the cell wall are available mostly for maize. In this species, the main protein observed when analysing cell wall proteins is a proline/threonine-rich protein that has been considered as a member of the HRGP

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number Y17332.

family, as a consequence of its post-translational modifications [16, 20, 31]. Using full-length cDNA and genomic clones corresponding to this maize HRGP, the complete protein sequences in maize [26], sorghum [27] and rice [4] have been obtained. The maize HRGP protein has been shown to be present in the cells of most of the organs analysed but its mRNA accumulates mostly during cell division [28, 29] and in the pericarp [16]. It has therefore been considered as a structural component of the primary cell wall. The only PRP described in cereals until now is WPRP whose mRNA is mainly accumulates in the growing tissues of wheat [25].

In the course of screening for genes related to the secondary cell wall, we isolated a cDNA (*ZmPRP*) encoding a new proline-rich protein from the maturation region of maize roots. Its full-length sequence was cloned and the expression of the corresponding gene was analysed. Here we report the molecular characterization of the *ZmPRP* cDNA whose sequence indicates that the ZmPRP protein could be a member of the PRP family. In addition, evidence for the correlation of *ZmPRP* mRNA accumulation with cells undergoing formation of secondary cell wall is provided.

Materials and methods

Plant material

Dry seeds of *Zea mays* L. inbred line W64A were germinated in a growth chamber on wet Whatmann paper under 16 h light/8 h dark conditions at 25 °C. Plants used for northern blot studies were dissected at different growth time points and immediately put in liquid nitrogen. Coleoptiles of 3-day old seedlings used for the investigation of the wound response were subjected to small cuts with a razor blade and frozen in liquid N_2 at different times after wounding. For the construction of a tip-less root cDNA library, some 300 nine-day old maize seedlings were hand-dissected involving the removal of the cap and the apical meristematic tissue.

cDNA library construction

Total RNA was isolated from 9-day old tip-less roots as described previously [34] followed by gentle phenol-chloroform extraction. Poly(A)$^+$ RNA was isolated from total RNA using the PolyATrack kit (Promega). 5 μg of poly(A)$^+$ were used for the synthesis of double-strand cDNA using the λZAPII-cDNA synthesis kit (Stratagene). Ligation of cDNA and packaging (Gigapack, Stratagene) were performed according to the manufacturer's instructions.

Differential cDNA library screening and sequencing

The tip-less root cDNA library (300 000 clones) was plated on *Escherichia coli* MRF$^-$ XL1-blue at low density (5000 pfu/plate). The screening for genes related to the secondary cell wall formation was performed by hybridizing duplicate filters from the library with two different probes. The positive probe corresponded to cDNAs obtained after reverse transcription of 500 ng poly(A)$^+$ isolated from 9-day old tip-less roots, while the negative probe corresponded to cDNAs obtained by reverse transcription of the same quantity of poly(A)$^+$ isolated from the remaining root tips. Both positive and negative probes were labelled with ^{32}P by random priming as described previously [10] and used, at the same specific activity, for hybridization to a series of duplicate filters. The hybridization conditions were those previously described [7]. The plaques giving a signal only with the positive probe were subjected to further screening. The clones of interest were then excised *in vivo* to produce pBluescript phagemids containing the cDNA inserts. Phagemids were sequenced by the dideoxy chain termination using an ABI 377 automatic sequencer (Applied Biosystem) to limit the use of primers on the highly repetitive *ZmPRP* sequence.

Genomic DNA gel blot analysis

Genomic DNA was isolated from W64A pure inbred maize seedlings as described [9], restricted with the appropriate endonucleases, subjected to electrophoresis in 0.7% agarose gel (10 μg per lane), and transferred onto Hybond-N membranes (Amersham). Hybridization was performed at 65 °C in a phosphate solution [7] using the full-length *ZmPRP* cDNA as a probe.

RNA gel blot analysis

RNA for northern blot analyses was extracted using guanidiniun-HCl as described [23]. RNA (10 μg) was electrophoresed through 1.5% formaldehyde-containing agarose gels and blotted onto Hybond-N membranes (Amersham) with 20× SSC, as described by the manufacturer. RNA deposition and transfer

were estimated by ethidium bromide staining. Hybridizations were performed at 65 °C in a phosphate buffer [7] using probes labelled by random priming (see above). Full-length *ZmPRP* and caffeic acid *OMT* (*O*-methyltransferase) [8] cDNAs, and a fragment of the *HRGP* (hydroxyproline-rich glycoprotein) coding region [26] were used as probes with the same 10^8 cpm/μg DNA specific activity. After hybridization, the filters were washed twice (15 min per wash) in 40 mM phosphate solution, and twice in a stringent 20 mM phosphate solution at 65 °C. All the RNA blot analyses were repeated three times.

In situ *hybridization*

Hypocotyls and maturing root (1 cm) sections, excised from 9-day old germinated maize plantlets, were fixed, embedded in paraffin and sectioned using a microtome [2]. A 230 bp *Msp*II fragment of the *ZmPRP* cDNA coding region was cloned in a pBluescript SK+ vector (Stratagene) and linearized for use as the DNA template for the synthesis of sense and antisense riboprobes. These probes were synthesized *in vitro* from pBluescript T3 and T7 promoters, by incorporation of digoxygenin (DIG)-UTP using the RNA labelling kit (Boehringer Mannheim) according to the manufacturer's instructions. Hybridizations were performed as previously described [22] using the RNA colour kit for non-radioactive *in situ* hybridization (Amersham). The photographs were taken using an automated camera on a light microscope (Axiophot, Zeiss).

Results

cDNA cloning and sequence analysis of the maize proline-rich protein ZmPRP

The mRNA accumulation corresponding to genes coding for proteins involved in the secondary cell wall formation has a characteristic pattern in the maize root [8]. In particular, these genes do not seem to be expressed in the root tip where the proportion of meristematic cells is high, while they are abundant in the more differentiated radicular zones.

In order to detect genes highly expressed in the maturing region of the maize root and which might be related to the biosynthesis of the secondary cell wall, a cDNA library from this zone was constructed in λZAPII and screened with single-stranded labelled cDNA from the same region. Ubiquitously expressed genes were eliminated by an additional screening using cDNAs corresponding to genes expressed in the root-tip as negative probe. Among the positive clones, one cDNA (*C105*, 1100 bp) contained a partial nucleotide sequence coding for a proline-rich protein. The *C105* partial cDNA was used for further screening of the library and a full-length cDNA (termed *ZmPRP*) was selected for analysis.

The *ZmPRP* cDNA is 1496 bp long. The first ATG codon of the deduced protein sequence occurs at position 88, with the longest open reading frame encoding 378 amino acids. The protein structure of the maize ZmPRP begins with a hydrophobic segment having the features expected for a signal peptide as shown in Figure 1A, followed by a small glycine-rich stretch. The remaining amino acid sequence is composed of a highly repetitive, proline-rich hydrophilic polypeptide. The most frequent segment is a PEPK sequence which is sometimes interrupted by glutamine residues forming a PX sequence that forms 88% of the polypeptide. This repetitive motif is different from the typical SPPPP element found in dicot HRGPs [6]. It also differs from the repetitive elements forming the threonine-rich proteins present in maize HRGPs [21, 31, 32] and from the PPVXK of soybean PRP [13, 14]. This PEPK block has been described in a wheat PRP [25] where the PEPK, PEPMK and PMPK motifs were also observed. This wheat PRP is the most similar protein sequence to ZmPRP found in databases although the length of the protein chain is different and contains other repetitive elements besides the common PEPK motif. The highly repetitive and hydrophilic nature of the protein is easily visualized in the hydropathy plot of ZmPRP as shown in Figure 1B. The long stretch of continuous alternating polar amino acid residues that gives a hydrophilic flat line in the profile is particularly interesting.

Gene copy number of ZmPRP

Southern blot analysis of maize genomic DNA digested with six different restriction endonucleases was carried out with the full-length *ZmPRP* cDNA as probe. The pattern obtained with all the enzymes indicates that *ZmPRP* is a single-copy gene (Figure 2). This result is consistent with the absence of similar sequences among the expressed sequence tags (ESTs) available up to now in the data banks.

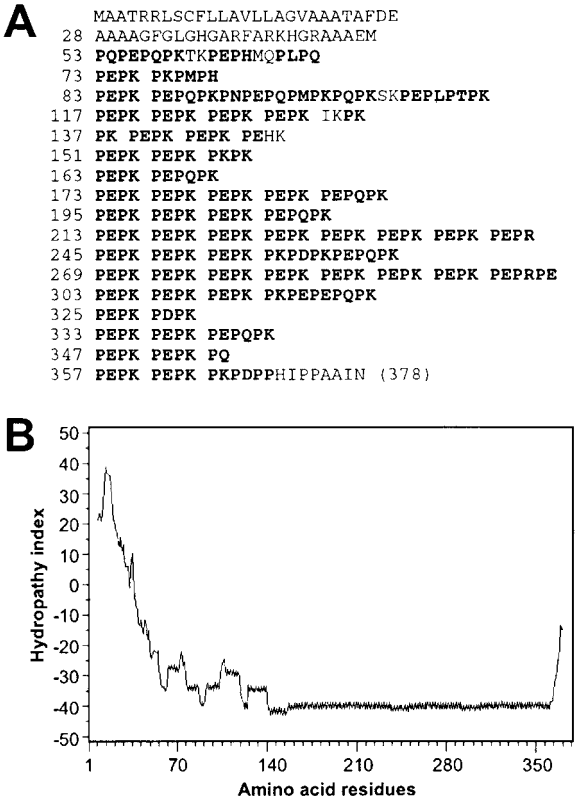

Figure 1. Amino acid sequence of the *Zea mays* ZmPRP. A. Sequence of ZmPRP displayed showing the different domains of the proteins and in particular the PEPK repetitive motif (in bold face). B. Hydropathy profile of ZmPRP protein.

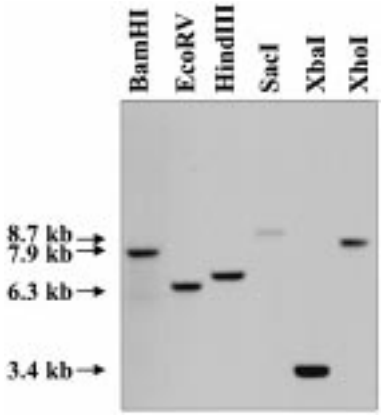

Figure 2. Maize genomic DNA Southern blot analysis. Genomic DNA from maize line W64A (10 μg per lane) was digested by *Bam*HI, *Eco*RV, *Hin*dIII, *Sac*I, *Xba*I, *Xho*I, separated on a 0.8% agarose gel, blotted and probed with the *ZmPRP* cDNA. The size of hybridizing bands is indicated in kb.

mRNA accumulation of ZmPRP *in different tissues of the maize plant*

To determine whether the presence of *ZmPRP* mRNA is representative of cells undergoing maturation, we first investigated the *ZmPRP* mRNA localization in 9-day old maize plants. RNA blot analyses of different parts of the root showed that the *ZmPRP* mRNA is barely detectable in the meristematic region (Figure 3A), whereas its accumulation increases along the radicular system. *ZmPRP* mRNA is also highly abundant in aerial parts of the maize seedling, in the hypocotyl, the coleoptile and especially in the coleoptile node (Figure 3A). Finally, mRNA corresponding to ZmPRP was found neither in immature embryos nor in the anther, although it is highly expressed in the ovary.

Secondly, we compared the pattern of *ZmPRP* mRNA accumulation with that of the *OMT* gene coding for caffeic acid *O*-methyltransferase, an enzyme involved in the lignin biosynthesis pathway whose gene expression in the root is correlated to maturing tissues [8]. We found that *ZmPRP* mRNA accumulation is very similar to that of the *OMT* gene in the aerial parts of the seedling (Figure 3A). In the root, the general pattern of mRNA accumulation of the two probes is also very similar although there seems to be less accumulation of *ZmPRP* mRNA.

We also compared these results with the accumulation of the maize *HRGP* mRNA [27] and we observed a clear difference of gene expression between the two cell wall protein families in the root (Figure 3A). Whereas the *ZmPRP* mRNA is undetectable in the root tip and accumulates in the cells of mature regions, the level of *HRGP* mRNA is high in the root tip and decreases progressively from the elongation zone to the upper part of the root. It has been previously shown that in different regions of the root where cells are undergoing division, the accumulation of *HRGP* mRNA follows the accumulation of histone *H4* mRNA [24].

In contrast to the results observed in the root, the expression of *ZmPRP*, *OMT* and *HRGP* genes in the maize seed (Figure 3B) is similar for all three genes. The corresponding mRNAs are detected at a very high level only in the pericarp, and not in the embryo, the endosperm or the aleurone tissues. A faint signal is obtained for *HRGP* in the embryo at 20 days after pollination (Figure 3B), probably due to the synthesis of this mRNA in the embryo axis and not in the scutellum as has been previously shown [29].

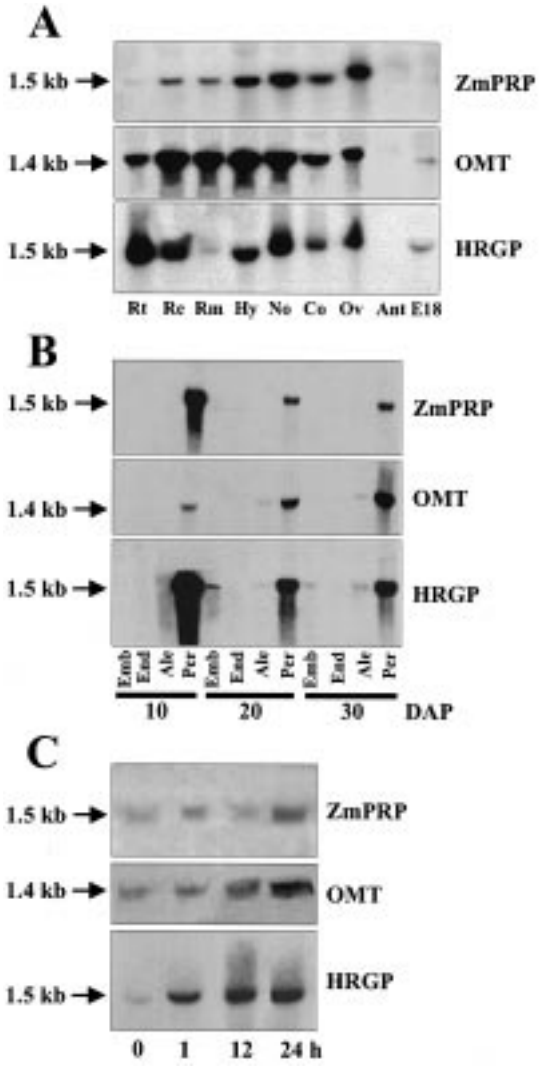

Figure 3. RNA gel blot analysis of the expression of the *ZmPRP* gene in maize. A. Accumulation of *ZmPRP* mRNA in 9-day old maize plants. RNA blot analysis was carried out with 10 μg of total RNA extracted from root tip (Rt), root elongation zone (Re), root maturing region (Rm), hypocotyl (Hy), coleoptile node (No), coleoptile (Co), pistillate spikelet with ovary at anthesis time (Ov), anther (Ant) and immature embryos at 18 DAP (days after pollination) (E18). RNAs were treated as described in Materials and methods and hybridized with the full-length *ZmPRP*, *OMT* or *HRGP* cDNAs as probes. The same filter was used for all the probes. B. Accumulation of *ZmPRP* mRNA accumulation in maize seeds. The embryo (Emb), endosperm (End), aleurone (Ale) and pericarp (Per) were excised from seeds harvested after 10, 20 or 30 DAP. The RNA extracted from these tissues were treated and hybridized successively with the *ZmPRP*, *OMT* and *HRGP* cDNA probes as described in Figure 3A (C). Induction of mRNA accumulation by wounding. Coleoptiles of 3-day old plantlets were slightly wounded by a blade razor longitudinally and the mRNA accumulation of the three probes analysed at different times (0, 1, 12 and 24 h).

A feature that makes *ZmPRP* similar to both *HRGP* and *OMT* is that the *ZmPRP* gene also seems to respond to wounding. Like both *OMT* [5] and *HRGP* [3], whose mRNA levels increase after mechanical coleoptile wounding, a change in mRNA accumulation is also observed when using the *ZmPRP* probe (Figure 3C). The level of induction is nevertheless lower for *ZmPRP* than for the other two genes. In fact, quantitation of the results shown in Figure 3C indicates that *ZmPRP* is increased 2.5 times, while *OMT* and *HRGP* increase 3.8 and 4.0 times, respectively, 24 h after wounding.

These results indicate that, in the root system, *ZmPRP* mRNA is accumulated in a way parallel to the one of mRNA corresponding to the OMT enzyme involved in the lignin biosynthesis pathway, but unlike the *HRGP* mRNA which codes for another cell wall protein. On the other hand, the three mRNAs follow the same pattern of accumulation in aerial parts and in organs where both primary and secondary cell walls are abundant in tissues such as the pericarp and the coleoptile node.

Accumulation of ZmPRP *mRNA in specific cell types as observed by* in situ *hybridization*

In situ hybridization experiments were performed using a probe derived from the *ZmPRP* cDNA to observe the specific cell type accumulation of *ZmPRP* mRNA. Two regions of the plant were analysed because they correspond to the organs exhibiting the highest *ZmPRP* mRNA accumulation levels: the maturing region of the root and the hypocotyl of the seedling (Figure 4). In the maturing root section (Figure 4, A–D), the digoxygenin-labelled antisense probe is localized in the vascular cells present in the central cylinder but not in the phloem cells. A diffuse background can also be seen in scattered cells in the cortex. In lateral roots, strong signal is detected around the protoxylem cells, especially in those where the characteristic spiral of tracheids can be seen (Figure 4F). Control sections with a sense probe show the absence of hybridization in these sections (Figure 4E).

Sections from the hypocotyl were also studied using the antisense probe of the *ZmPRP* cDNA (Figure 4G to K). The pattern obtained in this tissue is similar to the one observed in the root. The signal can be detected in the central vascular cylinder around xylem vessels but not in phloem groups of cells. In this part of the plant, a clear signal can also be observed in the epidermis.

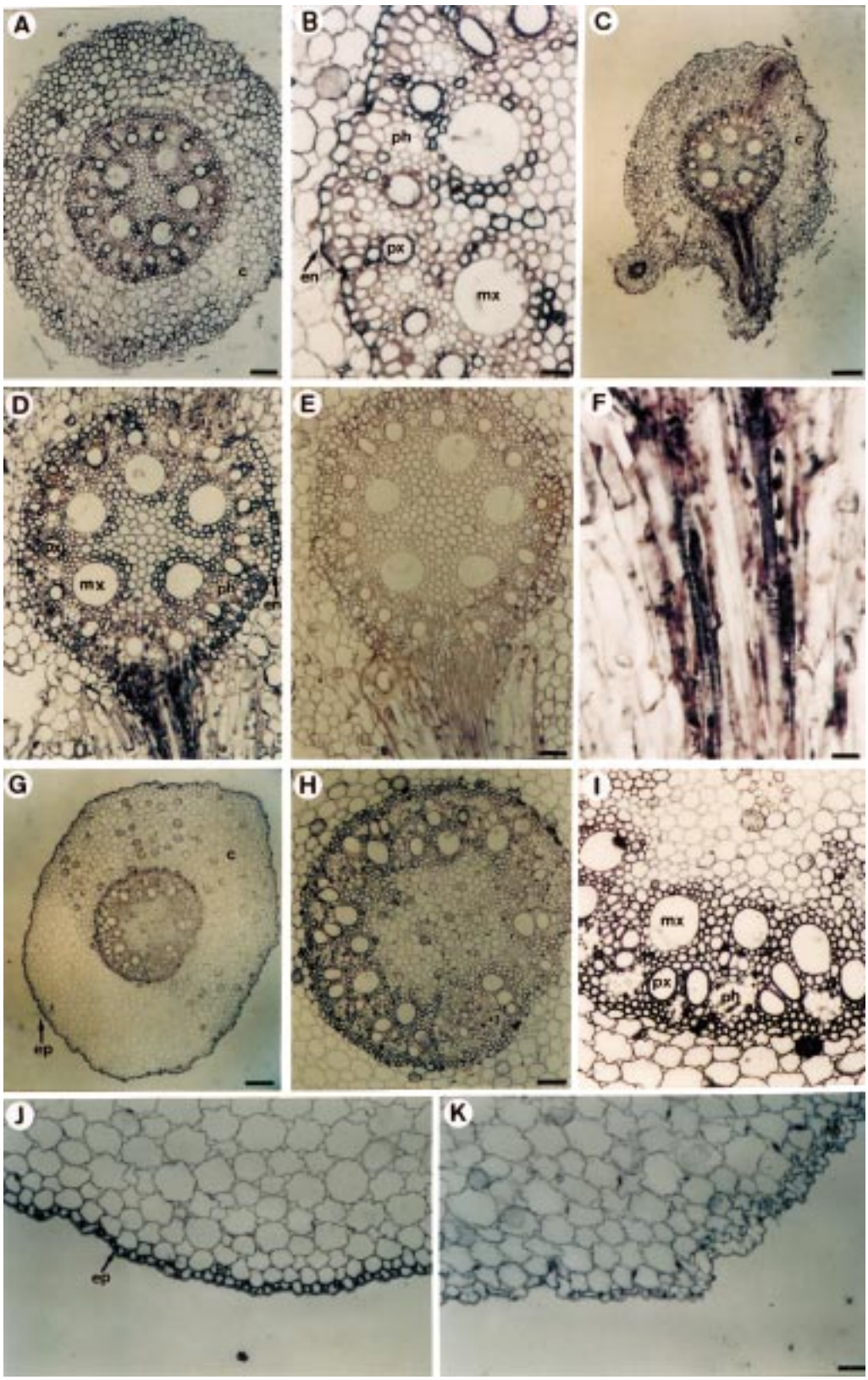

Figure 4. Cell-specific localization of *ZmPRP* mRNA in maize root and hypocotyl. *In situ* hybridizations of *ZmPRP* transcripts were performed as described [22] using digoxygenin-labelled *ZmPRP* antisense (A–D, F–J) or sense (E–K) probes. A, Cross section of the 9-day old maturing root; B, close-up of A showing xylem cells in the cortex; C, cross section of the root with lateral roots; D, close-up of the vascular cylinder shown in C with lateral root labelled with antisense probe; E, lateral root hybridized with the sense probe as a control; F, close-up of C showing the lateral root in a longitudinal section; G, cross section of the 9-day old hypocotyl; H, close-up of the vascular cylinder of G; I, close-up of a defined portion of the vascular region; J, close-up of the hypocotyl epidermis hybridized with the antisense probe; K, with the sense probe as a control. c, cortex; en, endodermis; ep, epidermis; mx, metaxylem; ph, phloem; px, protoxylem.

Discussion

The complete sequence of a proline-rich protein from maize, ZmPRP, has been deduced from cDNA clones identified by its expression in the maturing region of the developing root. The protein sequence has an initial hydrophobic sequence that is present in proteins secreted to the cell wall. The mature protein has a highly repetitive proline-rich sequence. In fact, 88% of the mature protein is composed of a Pro-X sequence, where X is alternatingly either Lys or Glu in most cases. This is a particular feature of this protein in comparison to other proline-rich proteins and it results in a highly hydrophilic polypeptide. The property of proline residues to disrupt secondary structures suggests an extended fibre-like structure that may interact with itself and/or with other cell wall components through ionic interactions. It is interesting to note that in animal systems, repetitive proline-rich proteins can have an adhesive function [1, 11], through modifications that convert the protein into a polyphenolic polymer [11]. However, the presence of alternating basic and acidic amino acid residues separated by proline is a specific feature of ZmPRP among proline-rich proteins described so far in both animal and plant systems and may result in a highly insoluble protein.

The accumulation of *ZmPRP* mRNA as seen both by northern blot analysis and *in situ* hybridization occurs in the vascular organs of the plant, mainly in cells related to xylem development, and in the epidermis. It is especially interesting that there is a simultaneous accumulation of *ZmPRP* and *OMT* mRNAs in root, coleoptile and seed, while *HRGP* mRNA has a different pattern. This is particularly evident when different regions of the root are analysed. Also of interest is that *ZmPRP* mRNA is absent in the phloem. These data suggest that while HRGP is involved in primary cell wall biosynthesis, the ZmPRP protein is involved in the formation of the secondary cell wall in maize.

The most similar sequence to *ZmPRP* is a cDNA sequence described in wheat [25]. The amino acid sequence of the homologous proteins in the two species is very similar, the wheat protein being also repetitive and containing the PEPK repetitive sequence, although the wheat protein is more hydrophobic because methionine residues are interspersed in the sequence. The main difference is that the wheat *PRP* mRNA accumulates in growing tissues of the plant [25] and not in the maturing regions, as occurs in maize (our data). Although this may be due to differences in the type of analyses carried out in the two systems, intrinsic variations in the control of gene expression may also be involved. In the case of HRGP, it has been reported that the mechanisms controlling gene expression in rice and maize may be intrinsically different [12]. This argument and differences in sequence distinguish ZmPRP from other PRPs, in particular those described in soybean, although also in this case the function of the protein is unknown.

The similarity between the *ZmPRP* mRNA accumulation with the expression of genes coding for enzymes involved in the lignin biosynthesis pathway suggests a role for ZmPRP in the process of secondary cell wall formation. As previously mentioned, proteins having a proline-rich repetitive sequence have been described in animal systems related to the biosynthesis of polyphenol adhesive polymers [11]. In plants, ZmPRP is the first proline-rich protein that has been reported to exhibit a pattern of mRNA accumulation related to secondary cell wall formation. The most similar proteins in this respect are glycine-rich proteins deduced from cDNAs described in bean [19]. The fibre-like structure of ZmPRP indicates the possible role of this protein in the correct deposition of the lignin polymers in the secondary cell wall in maize.

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