

Expression of a Maize Cell Wall Hydroxyproline-Rich Glycoprotein Gene in Early Leaf and Root Vascular Differentiation

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The spatial pattern of expression for a maize gene encoding a hydroxyproline-rich glycoprotein (HRGP) was determined by *in situ* hybridization. During normal development of roots and leaves, the expression of the gene was transient and particularly high in regions initiating vascular elements and associated sclerenchyma. Its expression was also associated with the differentiation of vascular elements in a variety of other tissues. The gene encoded an HRGP that had been extracted from the cell walls of maize suspension culture cells and several other embryonic and post-embryonic tissues. The gene was present in one or two copies in different varieties of maize and in the related monocots teosinte and sorghum. A single gene was cloned from maize using a previously characterized HRGP cDNA clone [Stiefel et al. (1988). *Plant Mol. Biol.* 11, 483–493]. In addition to the coding sequences for the HRGP and an N-terminal signal sequence, the gene contained a single intron in the nontranslated 3' end.

INTRODUCTION

The specialization of cell wall architecture is an important feature of the functional differentiation of plant cells (Varner and Lin, 1989). For example, the rigidity of sclerenchyma cells, the pressure-resistant nature of xylem cells, and the gas impermeability of photosynthetic bundle sheath cells all rely on the properties of specialized walls that each of these cells deposits during differentiation. The wall of each distinct cell type appears to have a characteristic combination and spatial organization of polysaccharides, structural proteins, and other wall components, often with unique patterns of cross-links and other modifications (Fry, 1986; Cassab and Varner, 1988). These structural specializations are accomplished in part through the differential expression of genes encoding wall structural proteins and synthetic and modification enzymes.

The cell-specific expression of several genes encoding specialized wall components has been described in dicots. A gene encoding phenylalanine ammonia-lyase, the enzyme catalyzing the first step in the synthesis of lignin monomers, is expressed at developing vascular centers, coincident with the differentiation of lignified xylem elements (Bevan et al., 1989; Liang et al., 1989). Similarly, a gene encoding a member of the wall glycine-rich protein

(GRP) class that is accumulated in lignified secondary walls of xylem elements is expressed specifically in differentiating protoxylem cells (Keller et al., 1989a). The genes for several members of another class of wall-associated proteins, the hydroxyproline-rich glycoproteins (HRGPs), have been shown to have distinct cellular patterns of expression.

The most studied of the HRGPs are the dicot extensins (Cassab and Varner, 1988; Showalter and Varner, 1989). Extensins contain a characteristic repeat of the pentapeptide Ser-Pro₄, in which proline residues are hydroxylated and glycosylated. Extensins become insoluble with time and contribute to the mechanical strength of the wall, probably via cross-linking to other monomers and to other wall components (Fry, 1986). Extensins have been localized in dicots to the walls of several cells in which strength is a key property, including seed coat sclerenchyma and cotyledon vascular elements (Cassab and Varner, 1987). Individual extensin genes are expressed in tomato and tobacco with temporal and spatial patterns that suggest a high degree of developmental control (Showalter et al., 1985; Memelink et al., 1987). Extensin accumulation can also be induced by fungal infection and by wounding, although distinct extensin genes may be induced in each case (Showalter et al., 1985; Corbin et al., 1987). Recently,

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a distinct tobacco HRGP was described that is specifically synthesized and accumulated at sites of lateral root initiation (Keller and Lamb, 1989).

Here we describe the expression pattern of a gene encoding a maize HRGP. We reported previously the isolation of a cDNA encoding a protein consisting of 13 repeats of a proline- and threonine-rich peptide (Stiefel et al., 1988). This protein contains only a single copy of the Ser-Pro₄ motif repeated in extensins. The deduced protein probably corresponds to the hydroxyproline- and threonine-rich glycoproteins extracted from maize pericarp (Hood et al., 1988) and cell walls of suspension culture cells and various seedling and embryo tissues (Kieliszewski and Lampert, 1987; Kieliszewski et al., 1990). The correspondence of these protein preparations to the protein predicted by cDNA sequencing was recently confirmed by peptide sequencing (Kieliszewski et al., 1990). The gene encoding this protein is present in one or two copies in the genomes of maize, teosinte, and sorghum. The maize transcript is interrupted by a single intron in the 3' untranslated region. We show that the gene is expressed at sites of early vascular differentiation in embryos, coleoptiles, leaves, hypocotyl, and both primary and lateral roots, as well as at much lower levels throughout the developing plant.

RESULTS

Maize HRGP Is Encoded by a Gene with a 3' Intron

We previously described the isolation of a cDNA encoding a maize HRGP and showed that the corresponding mRNA is enriched in tissues with mitotic activity (Stiefel et al., 1988) and in wounded tissues (Ludevid et al., 1990). The extensins, a group of HRGP proteins that have been found in differentiating and wounded tissues of several dicot species (Showalter and Varner, 1989), are encoded by small gene families. We performed genomic blot analysis to determine the number of sequences in the maize genome and in those of the monocots teosinte and sorghum. Genomic DNA of maize, teosinte, and sorghum was digested with a variety of restriction enzymes, blotted to nylon membrane, and probed with the maize HRGP cDNA (pMC56), all as described in Methods. Figures 1A and 1B show that the HRGP gene is present in one or two copies in the genomes of four different maize inbred lines. Using the same cDNA probe, the gene has been mapped to a single locus on maize chromosome 2 by RFLP analysis (locus UMC 145, C. Guillon and D. Hoisington, personal communication). Consistent with this, independent cDNAs from plumule (leaf) and root RNA were found to have identical sequences in noncoding regions, suggesting that these different tissues contain transcripts from a single gene (V. Stiefel, unpublished results). Figure 1B shows

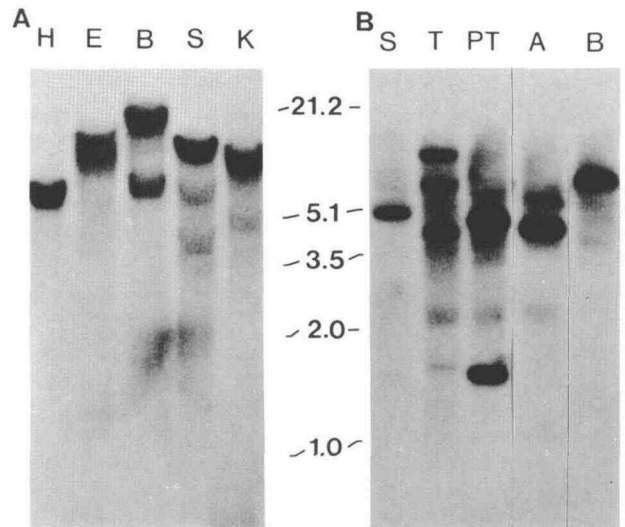


Figure 1. Genomic DNA Blot Analysis of Sequences Homologous to the HRGP cDNA in Maize and Related Species.

(A) DNA (10 μ g) from the maize W64A variety was digested with HindIII (H), EcoRI (E), BamHI (B), SacI (S), and KpnI (K).

(B) DNA (10 μ g) from different sources was digested with SacI. Samples are from sorghum (S), teosinte (T), and the maize varieties Palomero Toluqueno (PT), A188 (A), and Black Mexican Sweet (B). In both cases HRGP cDNA (probe MC56 from Stiefel et al., 1988) was used as a probe. Position of size markers (in kb) is shown.

that the teosinte and sorghum genomes contain similar sequences, also in relatively low copy number.

We cloned a genomic copy of the sequences represented in the HRGP cDNA, as described in Methods. Several overlapping clones were isolated from a maize inbred AC1503 library in λ EMBL3, and one was chosen for sequence analysis. The DNA sequence of 1.8 kb including the HRGP gene is shown in Figure 2. A TATA box and polyadenylation signal are found at expected locations in 5' and 3' regions flanking the HRGP coding region at -112 bp and +1492 bp relative to the initial ATG. Upstream of the TATA box, several short repetitive sequences and polypyrimidine-polypurine stretches are found.

Comparison of the HRGP genomic sequence with the previously determined cDNA sequence (Stiefel et al., 1988) revealed some minor sequence differences in the 3' untranslated region, probably due to the difference in maize inbred varieties used for cDNA and genomic cloning (W64A versus AC1503). Comparison of sequences in the coding region indicated that the characterized maize cDNA represents an mRNA from this gene. In addition, the genomic HRGP clone includes a 166-bp sequence (underlined in Figure 2) delimited by consensus sequences for intron splice junctions. An extensin gene from carrot has this

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                agggcatcggaggeccccacccccacctt -121
cctccggtatataagcagtgggcaggtgagcgtctctcctcagaccaccactgcgccat -61
tggccagctagagccaaccagaagagcttgcagttactgagagtgtgtgagagagagg -1
ATGGGTGGCAGCGGCAGGGCTGCTCTGCTGCTGCGCCCTGGTGGTGGCCGTTAGCCTG 60
M G G S G R A A L L L A L V V V A V S L 20
GCCGTGGAGATCCAGCGCCAGCCGGGTACGGTTACGGCGGGGTACACCCGACGCGCG 120
A V E I Q A D A G Y G Y G G G Y T P T P 40
ACGCGCGCCACCCGACCCCGAAGCCGAGAACGCCCCCAACGAGGGCCGAAGCCGGAC 180
T P A T P T P P K P E K P P T K G P K P P D 60
AAGCCGCCAAGGAGCAGCGCCCAAGCCGAGAACGCCCAAGGAGCACAAGCCGAGC 240
K P P K E H G P K P E K P P K E H K P T 80
CCGCCCCGTACACCCCGACCCCAAAACCCAGCCGCGGACGTACACTCCACCCCGAGC 300
P P T Y T P P S P P K P P T P P T Y T P T P L 100
CCCCCAAGCGCAGCCACACATACACTCCCGCCCTACGCCCAAAACCCACACCA 360
P P K P T P P T Y T P A P T P H K P T P 120
AAACCCACTCCCACTCCTCCGAGCTACACCCGACCCCAAGCCTCCGACACCTAAGCCG 420
K P T P T P P K P P P T P P T P P T P P 140
ACCCCGCCGAGCTACACTCAAGCCCAAAACCTCCGACGCCCAAGCCGACCCCAAGCAG 480
T P P T Y T P S P K P P T P K P T P P T 160
TACACCCCTTCCCCAAGCCTCCGACACCTAAGCCGACCCCGCCTACGTACACTCCAAG 540
Y T P S P K P P P K P P T P P T P P T Y T P 180
CCTAAGCCACCGGCTACCAAGCCTCCACGCCCAAGCCGACCCGCAACGTACACCCCT 600
P K P P A T K P P T P K P T P P T Y T P 200
TCGCCAAAGCCTCCGACACCAAGCCGACCCCGGACGTACACCCCTTCTCCCAAGCCT 660
S P K P P T P P P K P P T P P T P P T P P P 220
CGAGCCCAAGCCGACCCCGGACGTACACTCAAGCCCAAGCCTCCACACACCCG 720
P T P K P T P P P T Y T P S P K P P T H P 240
ACGCCAAGCCGACCCCGGACGTACACCCCTTCCCCAAGCCTCCGACGCCCAAGCCG 780
T P K P T P P T Y T P S P K P P T P P T P K P 260
ACCCACCGAGCTACACCCCTTCCCCAAGCCTCCGACACCAAGCCGACCCCAAGCAG 840
T P P T Y T P S P K P P T P K P T P P T 280
TACACCCCTTCCCCAAGCCTCCGACACCAAGCCGACCCCGGACGTACACTCCACA 900
Y T P S P K P P P T P P P T P P T Y T P T 300
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P K P P A T K P P T Y T P T P P V S H T 320
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P S P P P P Y Y 328
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tgggcaaatatgcaaggaaaaaggctctcactctactgtacacttactacc 1553

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Figure 2. Nucleotide Sequence of the Maize HRGP Gene and the Corresponding Protein Sequence.

Nucleotides are numbered from the A of the start codon for translation (+1). Putative TATA box and polyadenylation signals are boxed. The intron in the 3' untranslated end is underlined. The deduced amino acid sequence for HRGP is depicted as single letter code. The site of processing of the mature protein between Ala 26 and Asp 27 is indicated by a vertical arrow.

same unusual feature of an intron in the 3' untranslated region of the transcript (Chen and Varner, 1985).

The protein predicted by the genomic sequence was identical to that predicted by the cDNA, with the additional

N-terminal amino acids that were missing in the (incomplete) cDNA. Figure 3 shows that the predicted protein begins with a hydrophobic stretch having features typical of a signal peptide. As confirmed by N-terminal amino acid sequencing (see below), the mature protein sequence begins with the aspartic acid residue in position 27, followed by a short stretch (8 residues) of glycine and tyrosine residues. This is followed by a proline-rich region that includes the repeated peptide Gly-Pro-Lys-Pro-(Asp/Glu)-Lys-Pro-Pro-Lys-Glu-His. Finally, the proline-, threonine-rich region that corresponds to the sequenced cDNA begins. The repeated hydrophilic/hydrophobic character of the unmodified mature protein is very apparent in this hydropathy plot.

We identified the N-terminal amino acid of the mature protein by N-terminal amino acid sequencing of the protein encoded by the maize HRGP gene. A cell wall protein with characteristics of the protein predicted by the above sequence has been isolated from maize pericarp (Hood et al., 1988), suspension culture cells (Kieliszewski and Lamport, 1987), and several other embryo and seedling tissues (Kieliszewski et al., 1990). The amino acid composition of these proteins matches that predicted from the HRGP DNA sequence (Table 1), and Kieliszewski et al. (1990) confirm this with partial amino acid sequencing. We used a similar purification scheme (see Methods) to isolate the corresponding protein fraction from maize coleoptiles. This protein has the amino acid composition (Table 1) and repeated chymotryptic cleavage pattern (data not shown) expected for the encoded HRGP. As reported by Kieliszewski et al. (1990), the extracted protein migrates as a diffuse band at approximately 70 kD on SDS polyacrylamide gels, presumably due to its extensive modification and high proline content. We determined the N-terminal

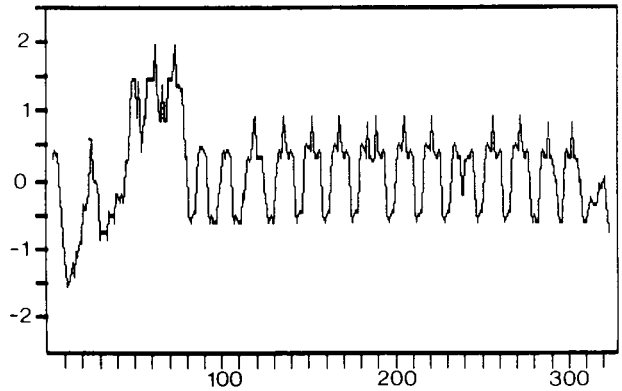


Figure 3. Hydropathy Profile of the Amino Acid Sequence Deduced from the Genomic Clone of Maize HRGP.

The mean hydropathy of a window of 6 consecutive residues (as described by Hopp and Wood, 1981) is plotted against the amino acid number.

Table 1. Amino Acid Composition (in mol %) of Maize HRGPs from Different Sources

	E ^a	P ^b	PC-1 ^c	THRGP ^d
HP ^e		24.7	21.9	24.8
P	45.4	11.7	13.5	14.5
T	23.8	24.0	17.5	25.3
K	12.2	15.0	11.3	13.5
Y	6.6	4.6	4.6	3.9
S	4.0	4.7	5.5	7.3
G	2.3	5.1	7.1	2.4
A	1.7	5.7	5.2	1.7
E	1.3	2.3	2.5	2.3
H	1.7	1.8	3.6	2.4
D	0.7	2.4	—	0.7
V	0.3	0.9	2.7	0.7
Others	—	1.0	4.6	0.5

^a Expected amino acid composition deduced from the nucleotide sequence of the HRGP gene. Amino acids corresponding to signal peptide (residues 1 to 26) were not computed.

^b HRGP protein purified from maize coleoptiles as described in Methods.

^c HRGP protein purified from maize pericarps (Hood et al., 1988).

^d HRGP protein from Black Mexican Sweet suspension cultures (Kieliszewski and Lamport, 1987).

^e HP, hydroxyproline.

sequence of the extracted protein by Edman degradation with a gas phase sequencer. Sequencing cycles 4, 6, and 10 gave tyrosine residues followed by the sequence Thr-Pro-Thr-Pro-X-Pro-Ala. This sequence appears at amino acids 37 to 43 of the genomic sequence open reading frame. Residues at positions 1 to 3, 5, and 7 to 9 (corresponding to glycine) relative to the N terminus were not clearly assigned due to a high background. From the amino acid sequence predicted by the genomic clone, this indicates that amino acid 1 is aspartate. The predicted hydrophobic sequence prior to this aspartate corresponds to a signal peptide that is probably processed at the Ala-Asp junction between positions 26 and 27 of the predicted protein. This would make the mol wt of the processed protein (without modifications) 31,729 D.

HRGP Transcripts Are Localized at Sites of Vascular Differentiation

Our previous work suggests that mRNA for the maize HRGP and extractable HRGP are enriched in a variety of tissues coincident with mitotic activity during normal development or after wounding (Stiefel et al., 1988; Ludevid et al., 1990). HRGP mRNA abundance decreases dramatically in mature tissue. To localize more precisely the accumulation of HRGP mRNA, we used cDNA pMC56 as a probe for in situ hybridizations with developing and mature tissues of the shoot and root systems. Figures 4 to 7 show that mRNA was localized to regions of vascular

differentiation in hypocotyl and mesocotyl regions of the embryo axis (1 day post-germination), the first node of the developing shoot, leaf primordia in the plumule (6 days post-germination), and sheaths of more mature developing leaves. The resolution of the hybridization technique does not permit the assignment of signals over specific vascular elements, although signals are strongest over xylem elements and surrounding sclerenchyma. In each case, the most abundant signal appeared at a morphological stage in which xylem tracheary element differentiation is in progress.

HRGP mRNA accumulated transiently in regions undergoing vascularization in developing primary and lateral roots. Figure 8 shows an oblique section through the tip region of a primary root. Hybridization was strongest in regions of differentiating metaxylem and protoxylem elements. The region of hybridization was always localized in a differentiating zone near the tip and was absent in more distant (more mature) regions. Figure 9 shows the highly localized accumulation of mRNA at sites of lateral root initiation, just behind the advancing lateral tip. Hybridization appeared to be most intense in regions of vascular differentiation and formed a continuous connection from the primary root vascular cylinder to the new lateral root

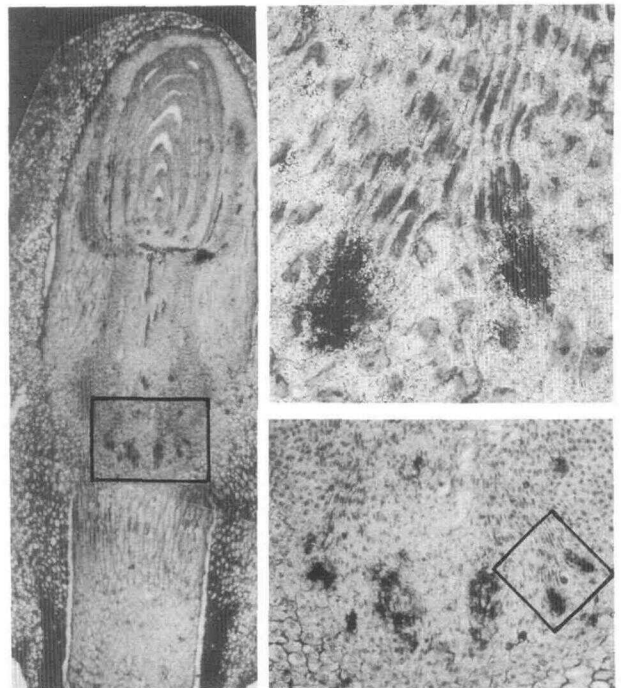


Figure 4. Localization of HRGP Transcripts in the Maize Embryo Axis.

Sections of 1 day post-germination embryos were hybridized with the HRGP probe as described in Methods.

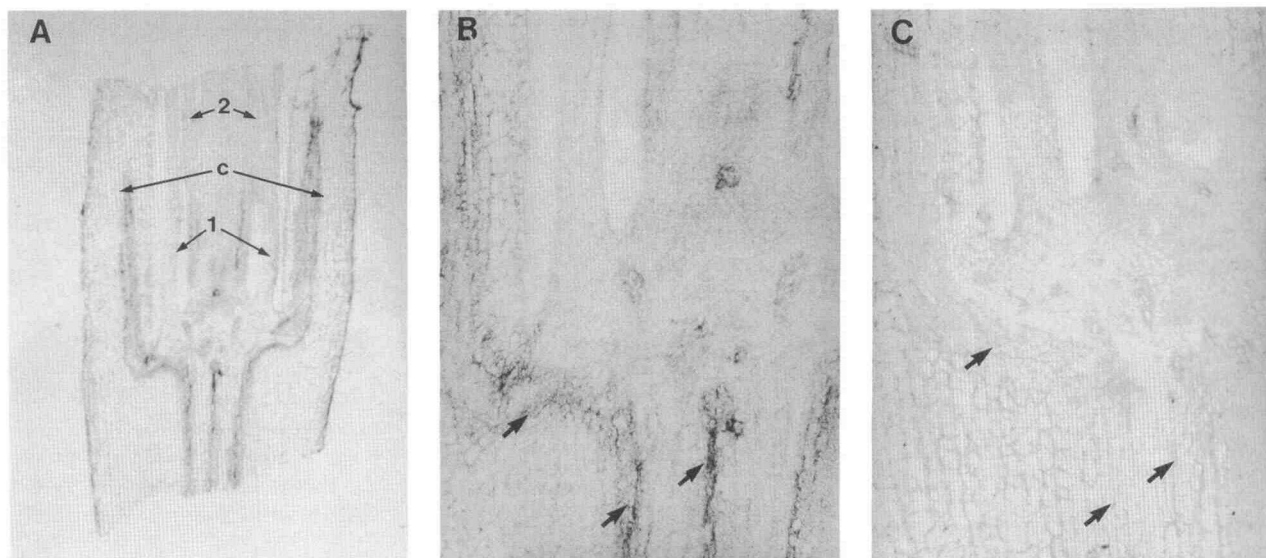


Figure 5. Localization of HRGP Transcripts in the Region of the Coleoptilar Node.

Longitudinal sections of 7 day post-germination plants were hybridized with the HRGP probe.

(A) Coleoptilar node region. c, coleoptile; 1, first leaf; 2, second leaf.

(B) Enlargement of same section, hybridized with antisense strand (signal) probe. Arrows indicate signal over coleoptilar vein and central vein in axis.

(C) Adjacent section hybridized with sense strand (control) probe. Arrows indicate absence of signal at same locations.

tip. This cross-section also shows that no significant hybridization occurred over the now mature meta- and protoxylem elements of the primary root.

DISCUSSION

We have determined the DNA sequence and expression pattern for a maize gene encoding a highly repetitive proline- and threonine-rich protein. The deduced amino acid sequence suggests that it corresponds to an HRGP that can be extracted from cell walls of several maize tissues (Kieliszewski and Lampert, 1987; Hood et al., 1988; Kieliszewski et al., 1990).

The predicted protein primary structure included several distinct blocks that may have distinct wall structural functions. A hydrophobic signal sequence was located at the N terminus and was absent in the mature protein, presumably due to processing that accompanies its passage through the endoplasmic reticulum to the wall (Gardiner and Chrispeels, 1975; Von Heijne, 1981). The mature protein began with an eight amino acid hydrophobic glycine-tyrosine-rich region. At least one dicot glycine-rich protein is highly specific for tracheary element walls (Keller et al., 1989b). The glycine-rich region was followed by a short stretch of proline-rich sequence with alternating

acidic and basic amino acids, a feature also observed in putative cell wall proteins from dicots (Franssen et al., 1987; Hong et al., 1987). The remaining 248 amino acids

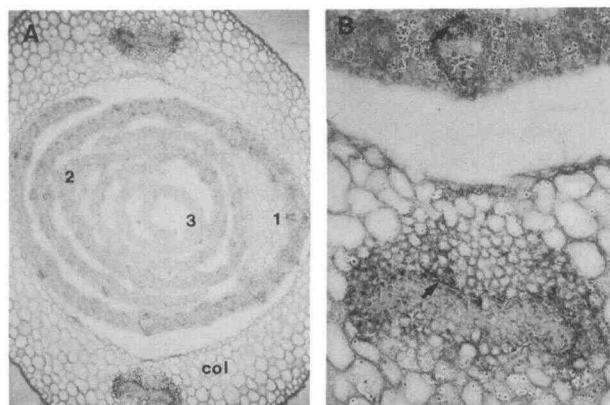


Figure 6. Localization of HRGP Transcripts in Plumules.

Cross-sections through plumules of 6 day post-germination plants were hybridized with the HRGP probe.

(A) Plumule cross-section. col, coleoptile; 1, 2, 3, leaves 1, 2, and 3. Signals are present at coleoptilar veins and at major veins of leaf 1.

(B) Enlargement of region including coleoptilar vein and adjacent leaf 2 vein. Signals over both veins indicated by arrows.

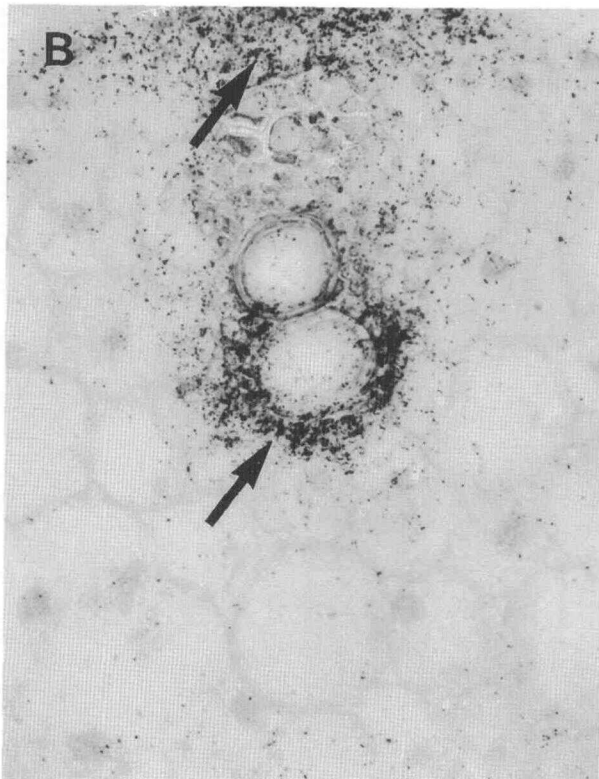
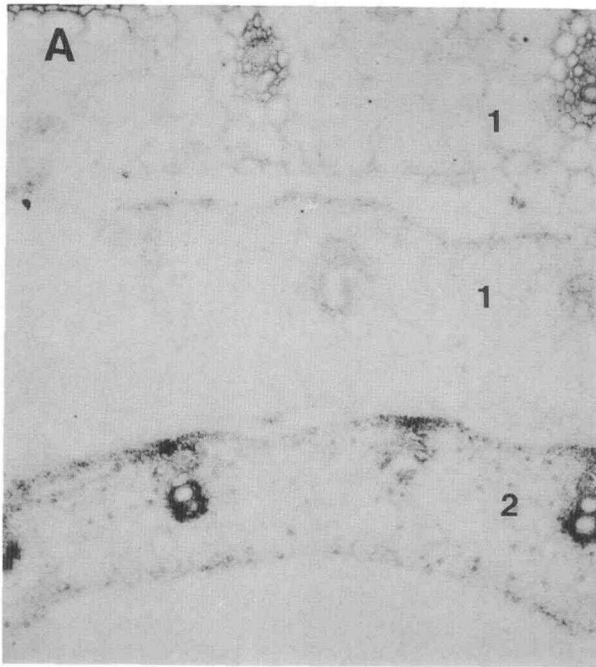


Figure 7. Localization of HRGP Transcripts in Sheaths of Developing Leaves.

Cross-sections through 14 day post-germination shoots at the level of the developing sheaths of leaves 1 and 2 were hybridized with the HRGP probe.

(A) Sheath regions of leaves 1 (1) and 2 (2). Signals are present only over veins of leaf 2.

(B) Enlargement of vein from leaf 2. Signal is apparent over xylem and adjacent sclerenchyma (arrows).

formed blocks of repetitive sequences in which tyrosine residues were regularly located in the most hydrophobic part of the molecule (Stiefel et al., 1988). Tyrosine and threonine residues were predominantly located in PPTY peptides, a motif that is repeated in a tobacco HRGP associated with lateral root initiation (Keller and Lamb, 1989). Tyrosine residues have been proposed as sites of extensin polymerization (Fry, 1986). The hydrophobic environment of regularly spaced tyrosines in this maize HRGP might permit a polymerized hydrophobic surface on one face with a hydrophilic opposite surface, although this pattern would be altered by modifications such as glycosylations, proline hydroxylations, and tyrosine polymerization. Near the C terminus is a single Ser-Pro₄ sequence which is a motif repeated in extensins (Showalter and Varner, 1989).

The localization of the maize HRGP mRNA and the corresponding protein suggests that it plays a role in the early construction of walls surrounding a vascular element or elements common to many organs. We showed that mRNA accumulates transiently at new vascular sites in embryos, leaves, and roots. This pattern is similar to that observed in bean for a glycine-rich protein (GRP), which appears to be a component of xylem walls (Keller et al., 1988). This maize HRGP mRNA is specifically induced at wound sites in young tissues (Ludevid et al., 1990). This pattern may represent the regeneration of vascular elements to circumvent wounded sites, although the rapid time course (mRNA peak at 1 hr to 2 hr) makes it less likely.

The highly localized accumulation of the mRNA at sites

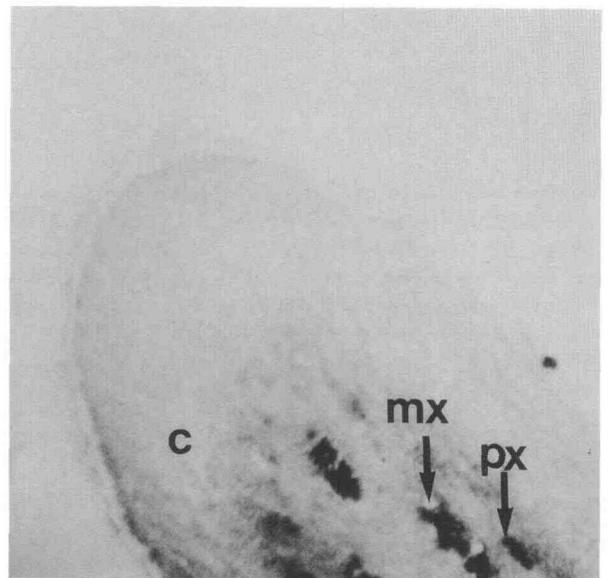


Figure 8. Localization of HRGP Transcripts in the Primary Root.

Oblique sections through tip region of 10 day post-germination root systems were hybridized with the HRGP probe. Arrows indicate hybridization signal over differentiating meta- (mx) and protoxylem (px) elements. c, cortex.

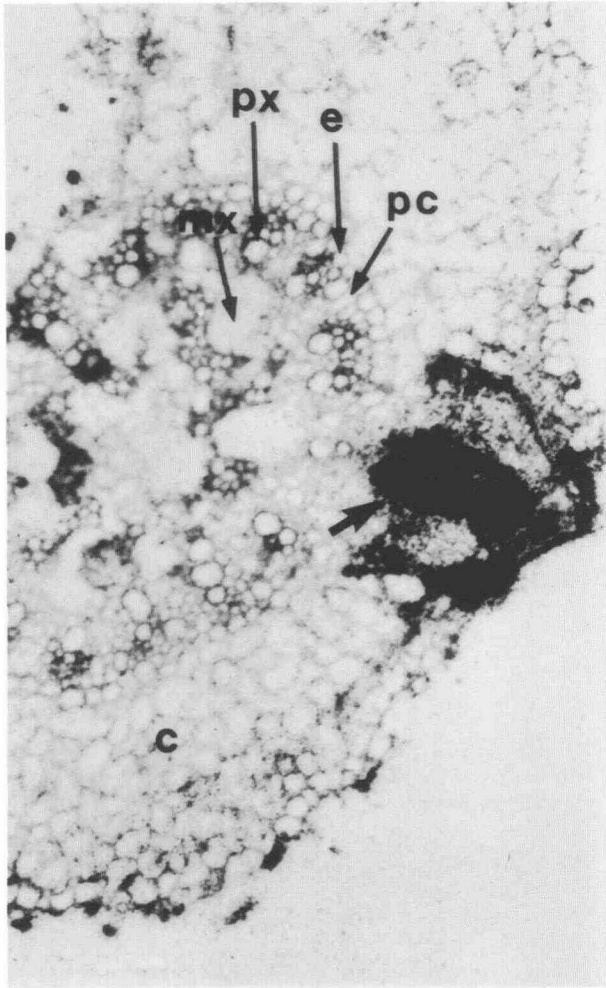


Figure 9. Localization of HRGP Transcripts in Developing Lateral Roots.

Cross-sections through 10 day post-germination root systems were hybridized with the HRGP probe. This section through the primary root is at the level of a newly initiated lateral root. c, cortex; e, endodermis; pc, pericycle; px, protoxylem; mx, metaxylem. No signal is present in primary root. Arrow indicates considerable signal over developing vascular bundle of lateral root.

of lateral root initiation might also be interpreted as being at sites of wounding, as the initiated lateral root penetrates existing tissue. However, our *in situ* hybridization patterns showed that the HRGP mRNA is associated with the developing vascular link of the new lateral root to the primary root, rather than at the "wounding" tip. This pattern is in contrast to the accumulation pattern of a distinct HRGP gene in tobacco, which is expressed only at the tips of new lateral roots and which may have a mechanical

role in penetration of new roots through existing tissue (Keller and Lamb, 1989). However, we also observed accumulation of the maize HRGP in regions associated with xylem differentiation in the primary root, where no wound-like process occurs. The distinct patterns of gene expression and protein accumulation for various dicot and monocot HRGPs suggest that they constitute a family of proteins with diverse roles in plant walls.

METHODS

Genomic Blot Analysis

Genomic DNA was prepared from leaves of maize inbreds W64A, A188, Palomero Toluqueño, and Black Mexican Sweet varieties and from teosinte (*Zea diploperennis*) and sorghum (*Sorghum bicolor*), as described by Burr and Burr (1981). The DNA was digested with restriction enzymes, separated by size in 0.8% agarose gels, and blotted onto nylon membranes as recommended by the manufacturer (Zeta-Probe, Bio-Rad). Hybridization was carried out in 180 mM NaCl, 10 mM sodium phosphate, 10 mM EDTA, 1% SDS, 0.5% nonfat milk, 0.5 mg/mL sonicated salmon sperm DNA at 68°C. The DNA probe was labeled with ^{32}P to a specific activity of 0.5 to 2×10^9 cpm/ μg by random priming (Boehringer Mannheim). Final washes were done in $0.1 \times \text{SSC}$, 0.1% SDS at 65°C, and the membrane was exposed to Kodak XAR5 film with intensifying screens (DuPont Lightning Plus) at -70°C .

Genomic Cloning

A maize inbred AC1503 genomic library (kindly provided by A. Gierl, Köln; Sau3A partial library in λEMBL3) was screened with the insert of the pMC56 HRGP cDNA clone (Stiefel et al., 1988) by standard methods (Sambrook et al., 1989). A 5-kb genomic clone hybridizing with the probe was sequenced using the dideoxy method after subcloning in M13mp18 and 19 (Sanger et al., 1977); 100% of both strands were sequenced over the 5-kb region.

Protein Analysis

Protein for amino acid analysis and N-terminal sequencing was extracted (Mazeau et al., 1982) from the coleoptiles of 6-day-old maize plantlets (inbred W64A) and deglycosylated as previously described (Stiefel et al., 1988). Amino acid analyses of purified HRGP were carried out after acid hydrolysis in a Pico-Taq (Millipore) analyzer according to the manufacturer. N-terminal amino acid sequence was determined in a gas-phase sequencer (model 470A, Applied Biosystems) according to the manufacturer.

In Situ Hybridization

For post-embryonic material, histological techniques and *in situ* hybridization were performed on paraffin-embedded samples as previously described (Langdale et al., 1988). Frozen sections were

used for embryos. Embryos germinated for 48 hr were embedded and frozen in OTC (Tissue Tek) compound on dry ice. Cryostat (Reichert-Jung) sections 8 μm to 10 μm thick were collected on gelatin-subbed slides, dried on a hot plate at 60°C for 1 min, and fixed in a freshly made 4% paraformaldehyde solution in PBS (0.15 M NaCl, 10 mM sodium phosphate, pH 7.4). ^{35}S -labeled riboprobes were synthesized with T3 and T7 RNA polymerases (Bethesda Research Laboratories), using linearized cDNA subclones derived from pMC56 as template.

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