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An mRNA Putatively Coding for an O-Methyltransferase Accumulates Preferentially in Maize Roots and Is Located Predominantly in the Region of the Endodermis[1]

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ZRP4, a 1.4-kb mRNA that preferentially accumulates in roots of young *Zea mays* L. plants, was identified by isolation of the corresponding cDNA clone. Genomic Southern analysis indicates that the *zrp4* gene is represented once in the corn genome. The deduced ZRP4 polypeptide of 39,558 D is rich in leucine, serine, and alanine. Comparison of the deduced ZRP4 polypeptide sequence to polypeptide sequences of previously cloned plant and animal genes indicates that ZRP4 may be an O-methyltransferase. The ZRP4 mRNA preferentially accumulates in young roots and can be detected only at low levels in leaf, stem, and other shoot organs. ZRP4 mRNA accumulation is developmentally regulated within the root, with very low levels of accumulation in the meristematic region, higher levels in the regions of cell elongation, highest levels in the region of cell maturation, and low levels in the mature regions of the root. ZRP4 mRNA is predominantly located in the endodermis, with lower levels in the exodermis. An intriguing possibility is that the ZRP4 mRNA may code for an O-methyltransferase involved in suberin biosynthesis.

The root is a specialized organ that functions in anchorage, absorption, transport, synthesis of plant hormones, and storage. The development of roots differs in certain features from that of aerial shoots. The root apical meristem is covered by a cap of mature tissue, making it subterminal rather than terminal, like the shoot apical meristem. Additionally, the root apical meristem does not produce lateral appendages, as does the shoot apical meristem (Steeves and Sussex, 1989). Instead, secondary roots initiate internally in the pericycle and penetrate through the surrounding tissues.

The vascular tissues of the root are organized into a cylinder surrounded by a specialized layer of cells, the endodermis. The endodermis is characterized by the presence of a Casparian strip. The Casparian strip is a band-like region of the primary cell wall that is impregnated with suberin, a complex polymer composed of varying amounts of aliphatic and aromatic domains (Kolattukudy, 1987; Garbow et al., 1989). The aliphatic domains of suberin originate from fatty acids and are structurally similar to cutin; the aromatic domains originate from phenylpropanoid precursors and are structurally similar to lignin (Kolattukudy, 1987; Garbow et al., 1989). The endodermal cells are compactly arranged, and their protoplasts are attached to the Casparian strip; thus, the protoplasm of the endodermis mediates the transport of dissolved substances between the cortex and vascular tissues (Raven et al., 1992). In some plant species, including maize (*Zea mays*), an exodermis with a Casparian strip is also formed in more mature regions of the root. The exodermis is located immediately internal to the epidermis; eventually the epidermis sloughs away, and the exodermis functions as the barrier between the roots and the soil (Raven et al., 1992)

Plant development and function are coordinately regulated through precise control of gene expression; a given population of mRNAs provides a template that is instrumental in determining the form and function of each cell in the plant body. The study of the regulation of tissue- and cell-type preferential mRNA accumulation is a means to elucidate the genetic and molecular basis of plant development and function. Because of the important function and unique developmental characteristics of the root, recent investigations have focused on differential gene expression in this organ (Evans et al., 1988; Keller and Lamb, 1989; Montoliu et al., 1989; Conkling et al., 1990; Lerner and Raikhel, 1990; Mclean et al., 1990; Schiefelbein and Benfey, 1991; Yamamoto et al., 1991; John et al., 1992).

To begin an investigation of the molecular mechanisms of maize root development and function, we identified genes expressed preferentially in maize roots (John et al., 1992). Here, we report the characterization of pZRP4, a cDNA clone corresponding to an mRNA that preferentially accumulates in the cells of the endodermis of the young maize root and that may encode an *O*-methyltransferase involved in suberin biosynthesis.

MATERIALS AND METHODS

Plant Growth Conditions and Harvesting of Tissue

To provide plants from 3 to 9 d old, seeds of maize (*Zea mays* L. cv NKH31) were allowed to imbibe and were grown

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on germination paper. Approximately 50 seeds were planted on germination paper, which was subsequently rolled and placed inside a polyethylene basket containing 1 L of distilled water. Seedlings were grown at 30°C under a 16-h light/8-h dark cycle in a growth chamber. For collection of root segments, roots were placed on ice-cold glass plates and 1-cm long segments were excised with a razor blade and kept at 4°C until harvesting was completed. Shoots were also harvested in some cases. After harvest, tissues were frozen in liquid nitrogen and then stored at −80°C.

To obtain organs from more mature maize plants, plants were grown under standard greenhouse conditions for 3 weeks or were grown in the field until anthesis (John et al., 1992). Various plant organs were harvested, frozen in liquid nitrogen immediately, and stored at −80°C. Nearly complete root systems, including young prop roots, were harvested from greenhouse-grown plants. Because of adhering soil, only the mature regions of prop roots could be harvested from the field-grown plants.

RNA Isolation

Total RNA was isolated from 1-cm segments of roots from germination-paper-grown maize plants according to the procedures described by Chomczynski and Sacchi (1987). Total RNA was isolated from roots and other organs of greenhouse-grown and field-grown plants according to the procedures described by Dean et al. (1985), with modifications as described by Edwards and Colbert (1990). Poly(U)-Sephadex columns were used to purify poly(A)$^+$ RNA from total RNA as described by Murray et al. (1981) and modified by Lissemore et al. (1987).

Construction and Screening of Root cDNA Library

A corn root cDNA library was constructed from poly(A)$^+$ RNA isolated from 9-d-old corn roots and screened with ^{32}P-labeled first-strand cDNAs derived from 9-d-old corn shoot and root poly(A)$^+$ RNA (John et al., 1992).

DNA Sequence Analysis

Seven subclones were generated from pZRP4, and three oligonucleotide primers were synthesized to facilitate sequencing of pZRP4 cDNA (Fig. 1B). Each strand of the pZRP4 subclone inserts was sequenced at least twice. Sequencing was done by the Iowa State University Nucleic Acid Facility, according to the dideoxynucleotide chain termination method (Sanger et al., 1977) with double-stranded DNA templates (Chen and Seeburg, 1985), using Applied Biosystems model 373A DNA sequencer, version 1.0.2. DNA sequence data were analyzed using the University of Wisconsin Genetics Computer Group package (Devereux et al., 1984).

Genomic Southern Blot Analysis

Genomic DNA from maize leaves was isolated as described by Saghai-Maroof et al. (1984). The *Bam*HI fragment of pZRP4 containing the cDNA insert was isolated using Gene Clean (Bio 101) and labeled with ^{32}P by random hexanucleotide priming under the conditions specified by the manufacturer (Amersham). DNA gel blot analysis was carried out according to previously published procedures (Sambrook et al., 1989).

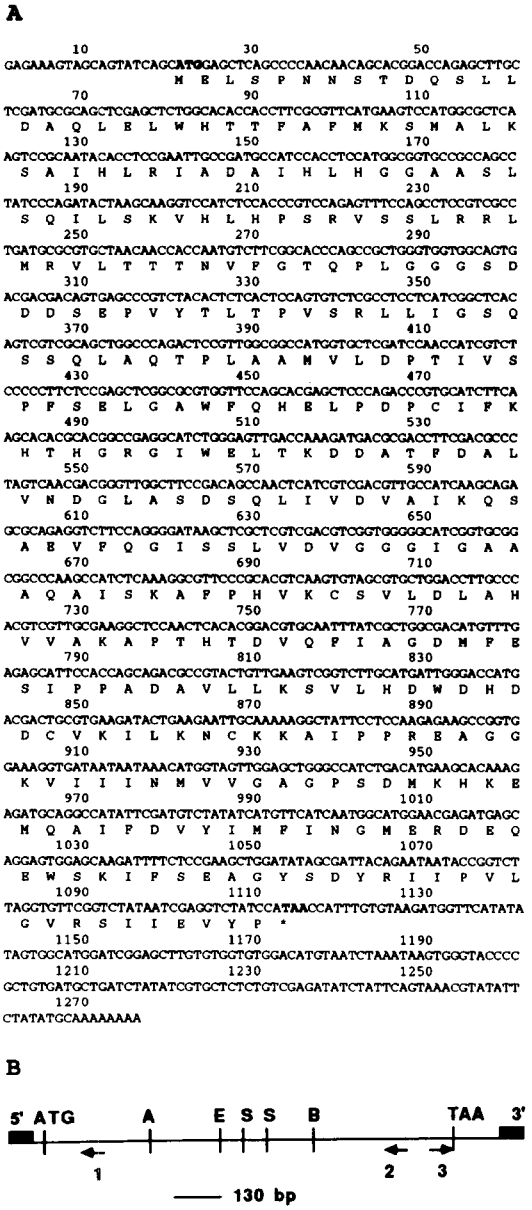

Figure 1. The nucleotide and predicted amino acid sequence of the pZRP4 cDNA. A, The predicted amino acid sequence corresponding to the open reading frame is shown beneath the nucleotide sequence. Bold letters in the nucleotide sequence indicate start and stop codons. B, The restriction enzyme sites used in subcloning pZRP4: *Acc*I (A), *Eag*I (E), *Sal*I (S), *Bsm*I (B). The oligonucleotide primers used to sequence pZRP4: 1 (5′-CCGCCAG CCTATCCCAG-3′), 2 (5′-TGCTCATCTCGTTCCATGC-3′), and 3 (5′-CGGTCT TAGGTGTTCGG-3′).

RNA Gel Blot Analysis

RNA gel blot analyses, hybridization, and washing conditions were as described in Cotton et al. (1990), except that

the hybridization solution contained 300 mM NaCl for the analysis of root segments (see Fig. 6 below) and young roots and leaves (see Fig. 5A below). In addition, in the experiment involving young roots and leaves, the final wash was at 75°C in a 0.1% (w/v) SDS solution. RNA size standards were from BRL. Antisense ZRP4 RNA probe was produced by digesting pBluescript II KS$^+$, containing the ZRP4 cDNA insert, with *Eco*RI, followed by transcription with T3 RNA polymerase. The antisense probe from pZRP3.21 was synthesized as described in John et al. (1992). Liquid scintillation spectrometry was used to quantify probe hybridization as described by Cotton et al. (1990).

In Situ Hybridizations

In situ hybridization studies with paraffin-embedded sections were carried out as described by Ausubel et al. (1989), with modifications as described by John et al. (1992). ^{35}S-Labeled RNA probes were synthesized from pZRP4.22, a subclone consisting of the 460 nucleotides of the 3′ end of pZRP4. Slides with hybridized tissue sections were coated with nuclear track emulsion (Kodak NTB 2), exposed for 12 h to 4 d, and developed. Photographs were taken with a Leitz microscope under bright-field and dark-field illumination.

RESULTS

Isolation of pZRP4 and Estimation of the Copy Number per Genome

Three ZRP (*Zea* Root Preferential) cDNA clones (pZRP2, 3, and 4) were isolated by differentially screening a corn root cDNA library constructed from poly(A)$^+$ RNA isolated from 9-d-old maize roots (John et al., 1992). Probes used to screen the library were ^{32}P-labeled, first-strand cDNAs prepared from poly(A)$^+$ RNA isolated from 9-d-old shoots and roots. The pZRP4 clone hybridized to the root cDNA probe, but when an equivalent amount of shoot cDNA probe was used there was no detectable hybridization.

To investigate the number of ZRP4 genes in the corn genome, a ^{32}P-labeled ZRP4 DNA probe was hybridized to total corn genomic DNA digested to completion with *Hin*dIII, *Eco*RI, and *Bam*HI restriction enzymes (Fig. 2). A single band was observed in each lane, indicating that the *zrp4* gene is present at low copy number or as a single copy in the corn genome. Restriction fragment-length polymorphism mapping indicated that the *zrp4* gene is located on maize chromosome 4 (D. Mead, personal communication).

Analysis of pZRP4 cDNA Sequence

The pZRP4 sequence is 1268 nucleotides long excluding the poly(A)$^+$ tail (Fig. 1A); thus it appears to be near the length of the 1.4-kb mRNA detected on RNA gel blots (see Fig. 4A below). The positions of restriction enzyme sites and oligonucleotide primers used in sequencing are shown in Figure 1B. The putative 5′ nontranslated region is 20 nucleotides long, and the predicted 3′ nontranslated region is 153 nucleotides long. Within the cDNA sequence, there is a large open reading frame encoding a putative polypeptide of 365

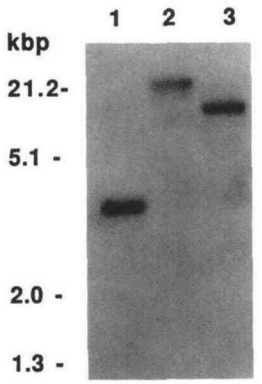

Figure 2. Genomic Southern blot analysis of *zrp4*. Maize genomic DNA (approximately 10 μg) was digested with *Bam*HI (lane 1), *Eco*RI (lane 2), or *Hin*dIII (lane 3), fractionated by agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with the ^{32}P-labeled cDNA insert of pZRP4. *Eco*RI/*Hin*dIII-digested λ DNA fragments were used as size markers.

amino acids with a predicted molecular mass of 39.5 kD. The most prevalent amino acids are Ser, Ala, and Leu, each of which comprises about 9% of the amino acid mol fraction. The predicted ZRP4 polypeptide has 50% similarity (30% identity) over the entire amino acid sequence with five *O*-methyltransferases found in plants (Fig. 3, Table I). The amino acid identity is highest in the carboxyl half of the protein, particularly in the three regions previously shown to be conserved in proteins from plants, animals, and microorganisms requiring *S*-adenosyl-L-Met as a substrate (Ingrosso et al., 1989; Bugos et al., 1991). Comparisons of the amino acid sequences suggest that ZRP4 is distinct from the *O*-methyltransferases previously isolated (Table I).

Accumulation of ZRP4 mRNA in Plant Organs

ZRP4 mRNA accumulation was investigated in more detail by using RNA gel blot analysis to measure the levels of ZRP4 mRNA in various maize organs (Fig. 4). Total RNA (Fig. 4A, lanes 1–5) and poly(A)$^+$ RNA (Fig. 4A, lanes 6–8) isolated from roots and leaves at various stages of development were hybridized to a ZRP4 ^{32}P-labeled antisense RNA probe. The analysis revealed that an mRNA of approximately 1.4 kb preferentially accumulated in the roots. In total RNA, the ZRP4 mRNA was most abundant in 3-d-old roots, but was also present at high levels in 3-week-old whole root systems and prop roots. ZRP4 mRNA could be detected in poly(A)$^+$ RNA from 9-d-old light- or dark-grown shoots, and we presume that ZRP4 mRNA would be detectable in the poly(A)$^+$ RNA of 5-d- and 3-week-old leaves as well. Quantification of the ZRP4 mRNA in the poly(A)$^+$ RNA samples revealed that the ZRP4 mRNA detected in the root (Fig. 4A, lane 6) was about 10 times more abundant than in either light- or dark-grown shoots (Fig. 4A, lanes 7 and 8). The analysis of ZRP4 mRNA accumulation was expanded to include various organs isolated from maturing field-grown plants (Fig. 4B). Lower levels of the 1.4-kb ZRP4 mRNA were detected in all organs, including the mature prop roots (Fig. 4B, lane 2, detectable with longer exposure). The data show

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          1                                                  50
Popome    MGSTGETQMT PTQVSDEEAh L..FAMQLAS ASVLPMILKt AIELDLLEIM
Ptomt1    MGSTGETQMT PTQVSDEEAh L..FAMQLAS ASVLPMILKt AIELDLLEIM
Comt1     MGSTGETQiT PThISDEEAn L..FAMQLAS ASVLPMILKS AlELDLLEIi
Mzeomth   MGSTag...d vaaVvDEEAc M..YAMQLAS sSILPMtLKn AIELgLLEVL
Imt1      MttytngnyT qpktlDkDeq LagLAvtLAn AaaFPMILKS AfELkiLDIF
Zrp4      ...melspnn sTdqSllDAq LelWhttFA. .fmksMaLKS AIhLriaDai
          51                                                 100
Popome    AKAGPG..AF LSTSEIAShL PTKNPD...A PVMLDRiLRL LASYSIL.TC
Ptomt1    AKAGPG..AF LSTSEIAShL PTKNPD...A PVMLDRiLRL LASYSIL.TC
Comt1     AKAGPG..Aq iSpiEIASqL PTtNPD...A PVMLDRMLRL LAcYiIL.TC
Mzeomth   qKeagGgkAa LapeEVvarM PaapsDpaaA aaMvDRMLRL LASYdVv.rC
Imt1      sKAGeG..vF vSTSEIASqi gaKNPn...A PVLLDRMLRL LAShSVL.TC
Zrp4      hlhGgaa... .SlSqIlSkv hlhpsrvss. ...LrRLMRv LtttnVFgTq
          101                                                150
Popome    SLKDhPDGKV ERLYGLAPVC KFL.TKNEDG VSVSPLcLMN QDKVLMESWY
Ptomt1    SLKDlPDGKV ERLYGLAPVC KFL.TKNEDG VSVSPLcLMN QDKVLMESWY
Comt1     SvrtqqDGKV qRLYGLAtVa KYL.vKNEDG VSISaLnLMN QDKVLMESWY
Mzeomth   qMeD.rDGry ERrYsaAPVC KWL.TpNEDG VSmaaLaLMN QDKVLMESWY
Imt1      kLqkGegGs. qRvYGpAPlC nYL.asNDgq gSlgPLlvLh hDKVMMESWF
Zrp4      pLggGsDdds EpvYtLtPVs rLLigsqssq laqtPLaaMv lDptivspFs
          151                                                200
Popome    YLK...DAIL DGGIPFNKAY GMTAFEYHGT DPRFNKVFNK GMSDHSTItM
Ptomt1    YLK...DAIL DGGIPFNKAY GMTAFEYHGT DPRFNKVFNK GMSDHSTItM
Comt1     hLK...DAVL DGGIPFNKAY GMTAFEYHGT DPRFNKVFNK GMSDHSTItM
Mzeomth   YLK...DAVL DGGIPFNKAY GMTAFEYHGT DaRFNrVFNe GMknHSvIit
Imt1      hLn...DyIL EGGVPFkrAh GMiqFDYtGT DeRFNhVFNq GMahHtilvM
Zrp4      eLgawfqheL pdpciFkhth GrgiWEltkd DatFdalvNd GLasdSqliv
          201                                                250
Popome    K.KILETYkG FEGltSLVDV GGGtGAVVNT IVSKYPsIKG INFDLPHVIE
Ptomt1    K.KILETYkG FEGltSLVDV GGGtGAVVNT IVSKYPsIKG INFDLPHVIE
Comt1     K.KILETYtG FEGlkSLVDV GGGtGAVINT IVSKYPtIKG INFDLPHVIE
Mzeomth   K.KILDfYtG FEGvstLVDV GGGvGAtlha ItSrhPHIsG VNFDLPHVIs
Imt1      K.KlLDnYnG FndvkvLVDV GGniGvnVsm IVaKhtHIKG INYDLPHVIa
Zrp4      dvaIkqsaev FqGisSLVDV GGGiGAaaga IskaFPHVKc svLDLaHVVa
          251                                                300
Popome    DAPSYPGVEH VGGDMFVSVP KADAVFMKWI cHDWSDaHCL KFLKNCYDAL
Ptomt1    DAPSYPGVEH VGGDMFVSVP KADAVFMKWI cHDWSDaHCL KFLKNCYDAL
Comt1     DAPSYPGVEH VGGDMFVSIP KADAVFMKWI cHDWSDeHCL KFLKNCYEAL
Mzeomth   EAPpFPGVrH VGGDMFaSVP agDAILMKWI lHDWSDaHCa tLLKNCYDAL
Imt1      DAPSYPGVEH VGGnMFeSIP qADAIFMKWV lHDWSDeHCv KiLnkCYEsL
Zrp4      kAPthtdVqf IaGDMFeSIP pADAVLLKsV lHDWdhddCv KiLKNCkkAi
          301                                                350
Popome    P...ENGKVI lVECILPVAP DtSLATKGVV HIDVIMLAHN PGGKERTEKE
Ptomt1    P...ENGKVI lVECILPVAP DtSLATKGVV HVDVIMLAHN PGGKERTEKE
Comt1     P...DNGKVI vaECILPVAP DsSLATKGVV HIDVIMLAHN PGGKERTqKE
Mzeomth   P...ENGKVI vVECVLPVnt EatpkaqGVf HVDmIMLAHN PGGKERyErE
Imt1      a...kgGKII lVEsliPViP EdnLeshmVf slDchtLvHN qGGKERskeD
Zrp4      PpreagGKVI iInmVvgagP sdmkhkemqa ifDVyiMfiN ..GmERdEqE
          351                                                381
Popome    FEgLAKGAGF qGFEVmCCAF NThVIELrKn .
Ptomt1    FEgLAKGAGF qGFEVmCCAF NThVIEFrKK a
Comt1     FEdLAKGAGF qGFkVhCnAF NTYImEFlKK v
Mzeomth   FreLAKGAGF sGFkatYiya NaWaIEFiK. .
Imt1      FEaLAsktGF stvDViCCAY dTWVmELyKK .
Zrp4      WskifseAGY sdYrIipvlg vrsIIEvyp. .
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Figure 3. *Amino acid sequence alignment of the ZRP4 polypeptide with O-methyltransferases from other plant species. The ZRP4 amino acid sequence was aligned with O-methyltransferases from cottonwood (Popome; Dumas et al., GenBank accession No. M73431), aspen (Ptomt1; Bugos et al., 1991), alfalfa (Comt1; Gowri et al., 1991), maize (Mzeomth; Collazo et al., 1992), and ice plant (Imt1; Vernon and Bohnert, 1992) using the University of Wisconsin Genetics Computer Group program PRETTY. Identical amino acids are represented with bold uppercase letters, consensus and similar amino acids with uppercase letters, and nonconserved amino acids with lowercase letters. The underlined regions represent amino acids conserved in enzymes that require S-adenosyl-L-Met as a substrate (Bugos et al., 1991).*

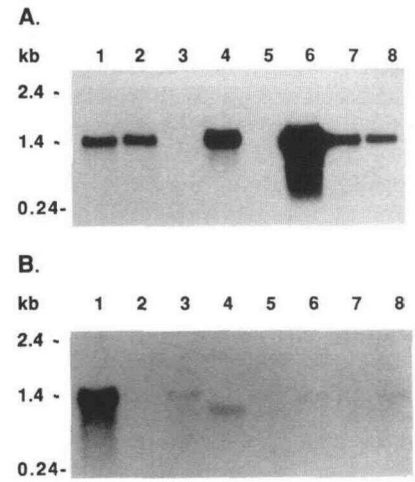

Figure 4. Abundance of ZRP4 mRNA in various maize organs at different developmental stages. Total or poly(A)$^+$ RNA was fractionated by electrophoresis in a 3% formaldehyde/1% agarose gel. After electrophoresis, the RNA samples were transferred to a nylon membrane and hybridized with ^{32}P-labeled antisense ZRP4 RNA probe. A, Total RNAs were isolated from 3-week-old greenhouse-grown plants: entire root system (lane 1), prop roots (lane 2), and leaves (lane 3). Total RNAs were also isolated from 3-d-old roots (lane 4) and 5-d-old leaves (lane 5). Poly(A)$^+$ RNAs were isolated from 9-d-old roots (lane 6), 9-d-old light-grown shoots (lane 7), and etiolated shoots (lane 8). One-microgram samples of poly(A)$^+$ RNA and 10-μg samples of total RNA were analyzed. B, Total RNAs (10 μg) from roots of 3-week-old greenhouse-grown plants (lane 1) and from other organs harvested from field-grown plants at pollination: root (lane 2), stem (lane 3), leaf (lane 4), ear at pollination (lane 5), ears at 10 d after pollination (lane 6), silk (lane 7), and tassel (lane 8).

that the ZRP4 mRNA does not accumulate exclusively in the root, and is present at a much lower level in the mature regions of the root than during earlier stages of root development.

A second band, of about 1 kb, was apparent in total RNA from the leaf (Fig. 4B, lane 4). This band was initially observed in blot analysis of the total RNA isolated from leaves that was used to produce Figure 4A, but when the blot was washed under more stringent conditions (75°C) the band was removed. In addition, the extra band was not detected in the

Table I. *Percent amino acid identity and similarity[a] among plant O-methyltransferases*

	Caffeic Acid/5-Hydroxyferulic O-Methyltransferases				Other O-Methyltransferases	
	Popome	Ptomt1	Comt1	Mzeomth	Imt1	ZRP4
Popome[b]	100	99 (99)	85 (93)	64 (78)	53 (71)	30 (52)
Ptomt1		100	85 (93)	65 (78)	53 (71)	30 (51)
Comt1			100	62 (77)	52 (69)	30 (52)
Mzeomth				100	44 (65)	29 (54)
Imt1					100	31 (53)
ZRP4						100

[a] Percent amino acid similarity values are in parentheses. [b] Abbreviations and references are as described in Figure 3.

shoot poly(A)$^+$ RNA (Fig. 4A, lanes 7 and 8). Thus, we conclude that the 1-kb band observed in leaves (Fig. 4B, lane 4) was due to spurious hybridization to an abundant non-polyadenylated RNA, rather than to a slightly smaller version of the ZRP4 mRNA.

Distribution of ZRP4 mRNA within Roots

RNA gel blots were used to analyze the distribution of ZRP4 mRNA in roots from 4-d-old plants (Fig. 5). Total RNA was isolated from the 1st cm (including the root tip) and from each successive cm of the root and was probed with the ZRP4 ^{32}P-labeled antisense RNA (Fig. 5A). The ZRP4 mRNA accumulation was lowest in the 1st cm. However, longer exposure times allowed detection of ZRP4 mRNA in the root tip (data not shown). The abundance of ZRP4 mRNA increased between the 2nd and 3rd cm, after which the level remained constant. As a control, the same blot was stripped and reprobed with ZRP3.21 ^{32}P-labeled antisense RNA (Fig. 5B). ZRP3 mRNA has previously been shown to accumulate to the highest level near the root tip (John et al., 1992).

The cellular localization of ZRP4 mRNA in 9-d-old maize seedlings was determined using in situ hybridization (Fig. 6). ^{35}S-Labeled pZRP4.22 antisense RNA probes were hybridized to cross-sections of root taken at approximately the 4th cm from the root tip (Fig. 6, A-C). In this region of the root, the cells have ceased to divide and elongate and are in the process of maturation (Erickson and Sax, 1956). Secondary walls have not yet formed and the endodermis has not yet synthesized its Casparian strip. At this developmental stage, the ZRP4 mRNA accumulated predominantly in the cells of the endodermis (Fig. 6, B and C). A control hybridization using ^{35}S-labeled sense ZRP4.22 RNA probe showed little detectable hybridization (Fig. 6A). Figure 6, D to G, shows in situ hybridization to a later stage of root development, approximately 10 cm from the root tip, in which maturation of the endodermal cells is more complete, an exodermal layer has been initiated, and the thick secondary cell walls of the mature exodermis and xylem have been deposited. In this more mature region, the ZRP4 mRNA accumulated in the cells of both the endodermis and the exodermis (Fig. 6, E and F). A control hybridization shows little detectable signal (Fig. 6, D and G). The cell walls of the xylem and outer cortical cells appear bright under dark-field illumination in both the sense and antisense hybridizations (cf. Fig. 6, F and G). However, bright silver grains are evident only in the endodermis and exodermis of sections probed with antisense ZRP4.22 RNA.

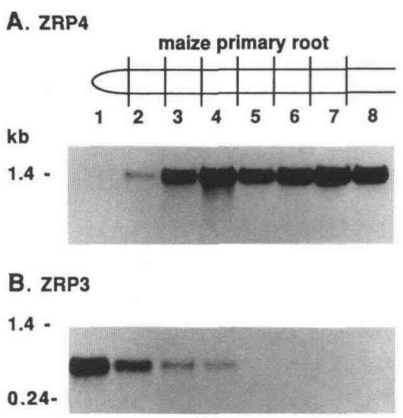

Figure 5. Longitudinal distribution of ZRP4 and ZRP3 mRNAs throughout the maize primary root. A, Dissection of a 4-d-old primary root. Roots were dissected as shown and total RNA was isolated from samples representing each root segment. Total RNA (5 μg) from the root tip (lane 1) and from cm 1 to 2 (lane 2), 2 to 3 (lane 3), 3 to 4 (lane 4), 4 to 5 (lane 5), 5 to 6 (lane 6), 6 to 7 (lane 7), and 7 to 9 (lane 8) was probed for ZRP4 mRNA. B, As a control, the blot from panel A was stripped and reprobed with a pZRP 3.21 antisense RNA probe (John et al., 1992).

DISCUSSION

RNA gel blot analyses indicate that the longitudinal distribution of ZRP4 mRNA in the root is developmentally regulated. In 4-d-old roots, ZRP4 mRNA accumulation is lowest in the region of the apical meristem, increases to a maximum level between the 2nd and 3rd cm from the root tip, and remains at this level throughout the remaining 5 cm of the 4-d-old root. ZRP4 mRNA also accumulates to relatively high levels in 3-week-old roots. In contrast, the mature portions of the roots of field-grown maize showed only very low levels of ZRP4 mRNA. Together, these data indicate that ZRP4 mRNA accumulation is low in the root apical meristem and increases in the developing root until a certain stage is reached; then the level remains constant through more mature stages in development, followed by a decline in ZRP4 mRNA level at later stages of development. The precise stage of root development during which the abundance of ZRP4 mRNA begins to decline has not yet been determined. It is plausible that this stage could define a change in function of the endodermis in which the ZRP4 protein is no longer required, or a point at which sufficient levels of ZRP4 protein have accumulated. ZRP4 mRNA also accumulates to low levels in aerial organs of the maize plant.

The amino acid sequence of the deduced ZRP4 polypeptide shares identity with the functional domains of *O*-methyltransferases from a variety of organisms. *O*-Methyltransferases are required for the biosynthesis of a variety of plant products. Compounds that are formed by *O*-methylation using *S*-adenosyl-Met as the methyl donor include the three phenylpropanoid precursors of lignin biosynthesis (Griesbach, 1981), a variety of *O*-methylated phenylpropanoids and other phenolic derivatives (e.g. the furanocoumarins, bergapten, isopimpinellin, and xanthotoxin) (Hauffe et al., 1986; Hahlbrock and Scheel, 1989), *O*-methylated isoflavonoids and flavonoids, ononitol (Vernon and Bohnert, 1992), possibly the secondarily methylated lignins (Goodwin and Mercer, 1988), possibly furocoumarins (Goodwin and Mercer, 1988), possibly lignans (the biosynthesis of which is not understood, but which contain *O*-methylated phenylpropanoids) (Goodwin and Mercer, 1988), and the phenylpropanoid precursors of suberin (Kolattukudy, 1987; Hahlbrock and Scheel, 1989).

O-Methyltransferase clones have been isolated from aspen, alfalfa, ice plant, and maize. The aspen and alfalfa cDNAs have been identified as caffeic acid/5-hydroxyferulic acid *O*-

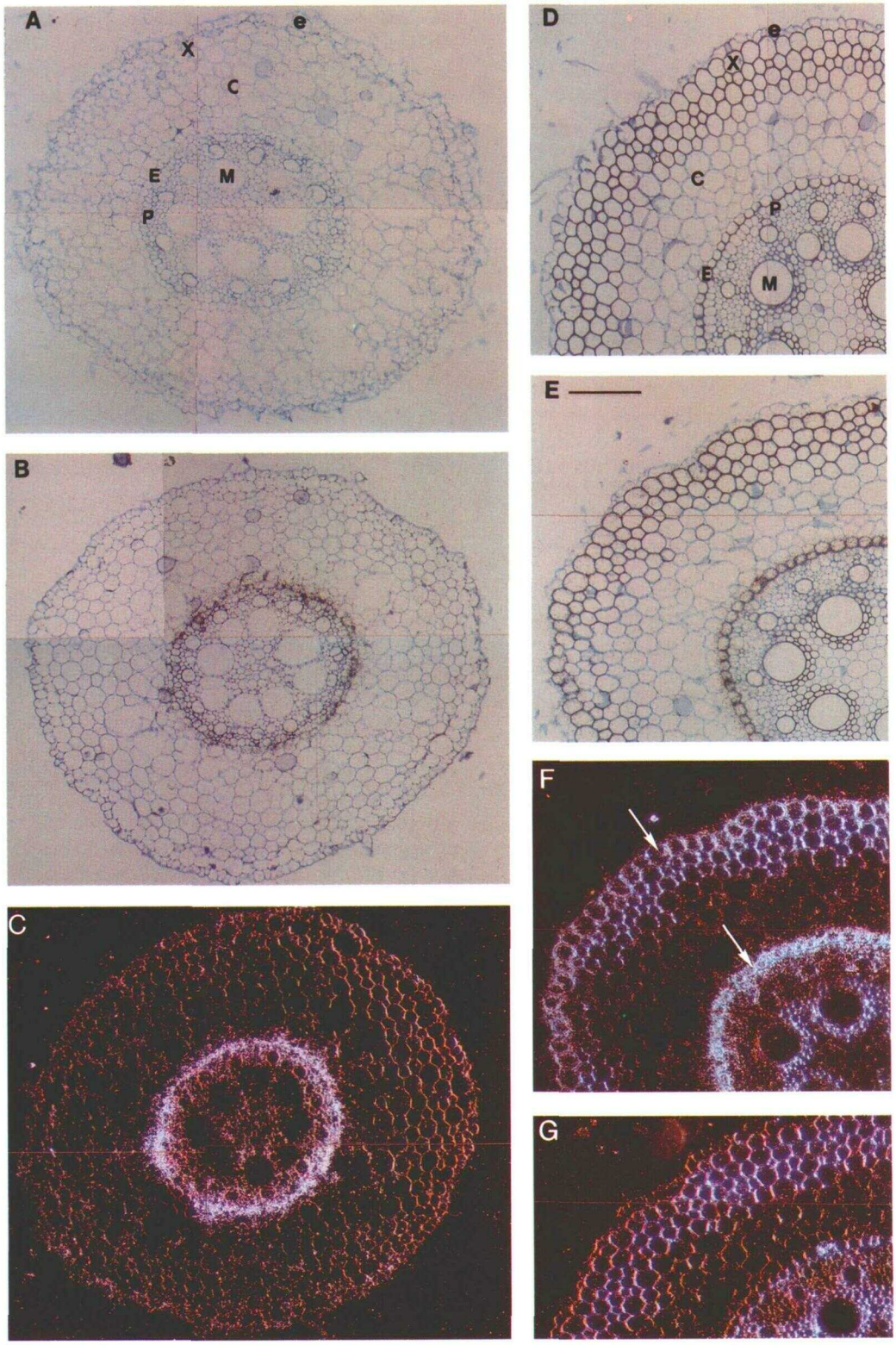

Figure 6. (Legend appears on facing page.)

methyltransferases, a bispecific enzyme involved in lignin biosynthesis (Bugos et al., 1991; Gowri et al., 1991). This *O*-methyltransferase catalyzes the *S*-adenosyl-Met-dependent *O*-methylation of 3,4-dihydroxycinnamic acid (caffeic acid) to form the 3-methoxy, 4-hydroxycinnamic acid (ferulic acid) and also *O*-methylation of 5-hydroxyferulic acid to form 3,5 dimethoxy, 4-hydroxycinnamic acid (sinapic acid). The mRNA coding for caffeic/5-hydroxyferulic *O*-methyltransferase and the enzyme itself are localized in xylem tissue (Bugos et al., 1991); this would be expected for a protein involved in lignin biosynthesis. The Mzeomth cDNA from maize (Collazo et al., 1992) may be the homolog to the aspen and alfalfa lignin caffeic/5-hydroxyferulic *O*-methyltransferases, because it has 77% amino acid similarity to these sequences (as compared with the 50% similarity of ZRP4) (Table I).

The ice plant Imt1 cDNA that has recently been isolated codes for *myo*-inositol *O*-methyltransferase, the enzyme that catalyzes the first step in the biosynthesis of pinitol, a cyclic sugar alcohol (Vernon and Bohnert, 1992). Pinitol is produced in certain plant species at high levels in response to osmotic stress; the Imt1 mRNA is induced by osmotic stress and is more abundant in stressed leaves than in stressed roots.

The pattern of expression of ZRP4 is quite distinct from that expected for either a caffeic/5-hydroxyferulic *O*-methyltransferase of lignin biosynthesis or *myo*-inositol *O*-methyltransferase. ZRP4 mRNA is not detected in xylem tissue where most lignin biosynthesis occurs, and, in contrast to *myo*-inositol *O*-methyltransferase, ZRP4 mRNA is found at high levels in roots. Furthermore, sequence identity is low between most regions of the ZRP4 polypeptide and the previously described *O*-methyltransferases. We conclude that the ZRP4 protein is probably not involved in the methylation of caffeic and hydroxyferulic acids for lignin biosynthesis or for the biosynthesis of pinitol.

ZRP4 mRNA accumulates to its highest levels in the endodermis and at lower levels in the exodermis during the time when these regions are in the process of forming the Casparian strip. The ZRP4 cDNA sequence displays homology to the functional domains of *O*-methyltransferases. The only *O*-methylated plant metabolite known to accumulate specifically in the endodermis and exodermis at the time of Casparian strip deposition is suberin. The suberin phenylpropanoid subunits are synthesized in the cytoplasm, deposited, and polymerized extracellularly in a manner analogous to lignin (Griesbach, 1981; Kollatukudy, 1987). However, the suberin phenylpropanoid subunits are *p*-coumaryl alcohol and coniferyl alcohol; little or no sinapyl alcohol is utilized (Kollatukudy, 1987). Thus, the *O*-methyltransferase required for biosynthesis of coniferyl alcohol would be a caffeic acid *O*-methyltransferase and, presumably, would be distinct from the bifunctional caffeic acid/5-hydroxyferulic acid *O*-methyltransferase of lignin biosynthesis. When the localization and sequence data are taken together, the function of the ZRP4 protein in the *O*-methylation of suberin phenylpropanoid precursors is an attractive possibility. The presence of suberin in aerial organs of the maize plant (e.g. in the cell walls of bundle sheath cells; Salisbury and Ross, 1992) is consistent with the observation of low levels of ZRP4 mRNA in these organs.

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Figure 6 (on facing page). Localization of ZRP4 mRNA in maize roots by in situ hybridization. A-C, Cross-sections 4 to 5 cm from the root tip; D-G, cross-sections 9 to 10 cm from the root tip. The bar (E) equals 150 μm; all magnifications are the same. A, Control bright-field photograph of a cross-section 4 cm from the root tip hybridized with the ^{35}S-labeled ZRP4.22 sense RNA probe. The position of the epidermis (e), future exodermis (X), cortex (C), endodermis (E), pericycle (P), and a developing metaxylem vessel (M) are indicated. B, Bright-field photograph of a cross-section 4 cm from the root tip hybridized with the ^{35}S-labeled ZRP4.22 antisense RNA probe. The black spots in the region of the endodermis are silver grains and represent ZRP4 mRNA accumulation. C, Dark-field photograph of a cross-section 4 cm from the root tip hybridized with the ^{35}S-labeled ZRP4.22 antisense RNA probe. The white spots in the region of the endodermis are silver grains and represent ZRP4 mRNA accumulation. D, Control bright-field photograph of a cross-section 10 cm from the root tip hybridized with the ^{35}S-labeled ZRP4.22 sense RNA probe. The position of the epidermis (e), exodermis (X), cortex (C), endodermis (E), pericycle (P), and a metaxylem vessel (M) are indicated. E, Bright-field photograph of a cross-section hybridized with the ^{35}S-labeled ZRP4.22 antisense RNA probe. The black spots in the region of the endodermis and exodermis are silver grains and represent ZRP4 mRNA accumulation. F, Dark-field photograph of a cross-section hybridized with the ^{35}S-labeled ZRP4.22 antisense RNA probe. The white spots in the region of the endodermis and exodermis, indicated by arrows, are silver grains and represent ZRP4 mRNA accumulation. G, Control dark-field photograph of a cross-section hybridized with the ^{35}S-labeled ZRP4.22 sense RNA probe.

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