Molecular analysis of the root cap from *Zea mays*

（トウモロコシ根冠の分子生物学的解析）

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INTRODUCTION

The roots of all vascular plants have at their apices a distinct tissue known as the cap. The root cap covers the root apical meristem during all developmental stages of vascular plants but is absent in nonvascular plants such as liverworts and mosses. The root cap is proposed to perceive gravity and to protect the root apical meristem (Sievers and Braun, 1996).

The root cap consists of several anatomically distinct regions. The maize root cap (Fig. 1) is generally divided into three regions; the calyptrogen, the columella root cap, and the lateral root cap. The calyptrogen faces the distal end of the quiecent center of the root apical meristem, is composed of approximately four cell layers, and serves as a root cap meristem. The columella cells are generated by periclinal cell division from the centeral region of the calyptrogen. Sedimented large amyloplasts containing well developed starch granules are characteristic of the columella cells. These amyloplasts function as statoliths in root gravitropism (Sievers and Braun, 1996). The lateral root cap surrounds the columella root cap. In maize root with closed-type construction, the lateral cap cells originate from calyptrogen (Barlow, 1996). However, in *Arabidopsis thaliana* roots, which have an open-type construction, there is no discrete boundary between the root proper and the cap, and the lateral root cap cells are derived from the same initials as the root epidermal cells (Dolan et al., 1993). The lateral root cap cells are rich in the hypertrophied dictyosome cisternae that form large secretory vesicles (Mollenhauer et al., 1961). These cisternae are proposed to reflect the massive secretion of mucilage from the lateral root cap into the rhizosphere, because the vesicle content was observed to be deposited between the plasma membrane and the outer tangential walls of the lateral cap cells and be correlated biochemically and physiologically with mucilage which adheres to the root tip (Morré et al., 1967).

The root mucilage typically covers the root apex, is amorphous and uneven gel, and ranges in thickness from 50 μm to 1mm. The mucilage is secreted largely from the root cap, but the root epidermis is also covered by a thin film of mucilage which is histochemically distinct from the cap-derived mucilage (Greaves and Darbyshire, 1972; Clarke et al., 1979;

Foster, 1982; Vermeer and McCully, 1982). The matrix of maize mucilage mainly consists of polysaccharides with a high content (20-30%) of fucose (Harris and Northcote, 1970). A small amount of protein was detected in mucilage isolated from the maize root tip, but the protein component has yet to be characterized (Chaboud, 1983).

Continuous renewal and sloughing-off of cells is never seen in most other plant tissues than the root cap. Active cell division of the cap meristem pushes continuously the cap cells toward the root cap periphery and finally into the external root environment. In general, only several days are required for a single cap cell to proceed from the meristem to detachment (1 day in *Zea mays*, 5-6 days in oat, or 6-9 days in *Convolvulus* [Barlow, 1975; Harkes 1973]).

The objective of this study is an understanding of the functions of the root cap at the molecular level. The first chapter describes the isolation of the genes that are specifically or predominantly expressed in maize root cap, and the characterization of one such gene, *zmGRP4 (Zea mays gricine-rich protein 4)*. The second chapter describes the characterization of two homologous genes, *zmRCP1* and *zmRCP2 (Zea mays root-cap periphery gene)*, both of which are expressed specifically in the peripheral one to three cells of the root cap.

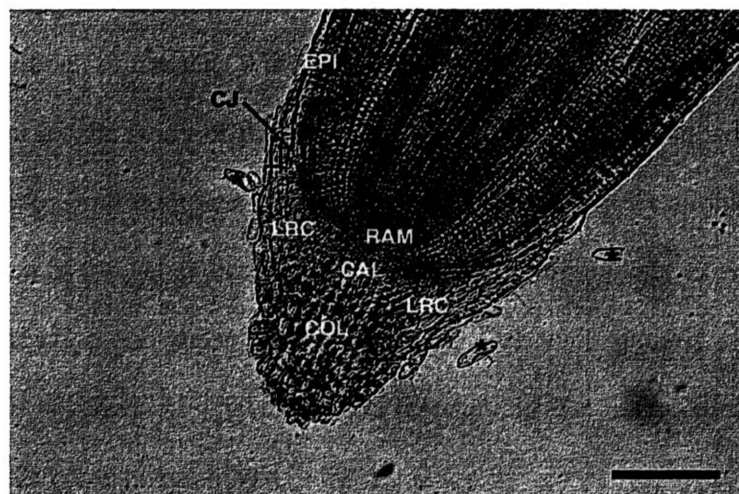

Figure 1 Median longitudinal section of the primary root apex of *Zea mays*, an apex with a "closed" construction. RAM, root apical meristem; CJ, cap junction; CAL, calyptrogen; COL, columella root cap; LRC, lateral root cap; EPI, epidermis. Asterisks show sloughed-off cap cells. Bar indicates 200 μm.

Chapter I A maize glycine-rich protein is synthesized in the lateral root cap and accumulates in the mucilage

Different characters of cells result from distinct patterns of gene expression. Therefore, isolation of the genes that are specifically expressed in one type of cells but not expressed in another type of cells is one good approach to understand the differences of characters between two distinct types of cells at the molecular level. This approach is generally known as differential screening and has been improved by several techniques, cDNA subtraction (Kavathas et al., 1984, Wang and Brown, 1991), differential display (Liang et al., 1992), or micro array (Schena et al., 1995, Desprez, 1998). By characterizing differentially expressed genes, we would understand various relationships between different patterns of gene expression and distinct characters of cells.

The most important process of differntial sceening is a preparation of pure distinct types of cells which have only little different characters. In addition, cDNA subtraction is a good method for an effective isolation of differentially expressed genes at low level from extremly small quantities of tissues (Wang and Brown, 1991). The primary root of *Zea mays* is a suitable material for the study of the root cap. The root apex with closed-type construction is sufficiently large to allow complete removal of cap or mRNA purification. In this chapter, I describe the isolation of the genes that are specifically or predominantly expressed in maize root cap by cDNA subtraction and differential screening. One such gene is *zmGRP4* (*Zea mays glycine-rich protein 4*). *ZmGRP4* is expressed strongly in the lateral root cap, and its gene product is secreted into and accumulates in the mucilage.

MATERIALS AND METHODS

Plant Material

Zea mays L. cv. Merit was supplied by the Asgrow Seed Company (Kalamazoo, USA). Seeds were imbibed with tap water for 72 h in the dark at 30°C. After imbibition, seeds were germinated on paper towels saturated with tap water for 1-2 d in the dark at 30°C. When the primary roots were 2-3 cm long, the cap and selected portions of the root were removed by surgical knife under a magnifying glass, and immediately frozen in liquid nitrogen. The tip region used in this study was apical the 5 mm of the root, and included the root apical meristem and the whole root cap. The region of the root proper was the region between 1 and 3 cm from the distal end of the root.

Maize plantlets were grown under 18-h light/6-h dark or 24-h dark conditions at 30°C on layered wet paper towels in plastic pots.

RNA Isolation, cDNA Synthesis, and Subtractive Hybridization

Poly $(A)^+$ RNA was extracted directly from the root cap and the root proper of maize primary roots using Dynabeads oligo (dT_{25}) (DYNAL, Oslo, Norway). Several hundred nanograms of poly $(A)^+$ RNA were used to construct double-stranded cDNA using a cDNA synthesis kit (Pharmacia).

Subtractive hybridization was done essentially as described by Wang and Brown (1991) and Hashimoto et al. (1993). Briefly, the double-stranded cDNAs were fragmented by *Alu* I and *Rsa* I, and ligated to a PCR linker. cDNA fragments of 0.2 to 2.0 kb were amplified by PCR. The cDNA fragments from the root proper were then biotinylated with Photoprobe biotin (Vector Laboratories, Burlingame, USA) and used as the driver DNA.

One subtraction cycle consisted of five steps: hybridization of the excess driver DNA to the tracer DNA from root cap for 20 h at 68°C; removal of non-hybridizing driver DNA by binding to streptavidin and extraction with organic solvent; another hybridization of the excess driver DNA to the remaining tracer DNA once again for 2 h at 68°C; removal of driver DNA as

above; and finally PCR-amplification of the tracer DNA. This subtraction cycle was repeated twice to produce subtracted root-cap cDNA fragments. Subtracted root-proper cDNA fragments were also generated in the same way, except that cDNA fragments from the root cap and the root proper were respectively used as the driver DNA and the tracer DNA.

Screening of Differentially Expressed cDNAs

The subtracted root-cap cDNA fragments were digested with *Eco*RI which cleaved the PCR linker, and inserted into the *Eco*RI site of pBluescript II SK(-) (Stratagene). These plasmids were introduced into the bacterial strain DH5α to construct a root-cap cDNA library, and 386 independent colonies were grown overnight in LB medium containing 50 $\mu g\ mL^{-1}$ ampicillin at 37°C. From each culture, an aliquot of 50 μl was blotted in duplicate onto a Hybond N^+ membrane (Amersham) using a Filtration Manifold System (GIBCO BRL). After denaturation and neutralization, the duplicate filters were hybridized at 42°C for 16 h with either a ^{32}P-labeled subtracted root-cap cDNA probe or a ^{32}P-labeled non-subtracted root proper cDNA probe, in a hybridization buffer containing 50% formamide, 10% dextran sulfate, 1% SDS, 5x SSPE (1x SSPE: 180 mM NaCl, 1 mM EDTA, and 10 mM Na_2HPO_4, pH 7.5), 5x Denhardt's solution (1x Denhardt's solution: 0.02% [w/v] BSA, 0.02% [w/v] Ficoll, 0.02% [w/v] polyvinylpyrrolidone), and 100 $\mu g\ mL^{-1}$ salmon testis DNA, and washed at 65°C in 0.1x SSPE and 0.1% SDS. A total of 72 positive cDNA clones which hybridized only to the root-cap cDNA probe were obtained.

To group the positive clones, the 72 recombinant bacterial cultures containing positive cDNA clones were blotted onto a Hybond N^+ membrane, and processed as described above. One cDNA clone which had hybridized specifically and strongly to the root cap-cDNA probe was chosen, labeled with ^{32}P, and hybridized to the membrane. Positive clones were regarded as members of the same group. Next, another strongly and specifically hybridizing cDNA clone other than the members of this group was chosen, and processed as above. A total of four hybridizations was done, resulting in four independent groups and 29 remaining cDNA clones. Representative clones from the four groups and the extra 29 cDNA clones were partially sequenced by DNA sequencer (model 373A, Perkin-Elmer), using M13 reverse and

universal primers. The sequence analysis classified the root cap-positive cDNA clones into 23 groups.

Subtracted cDNA fragments from root cap, non-subtracted cDNA fragments from root cap, and non-subtracted cDNA fragments from root proper were blotted in amounts of 0.05, 0.5, and 5 μg per slot onto a Hybond N^+ membrane, as described above. Representative cDNA fragments from the 23 groups were used as probes for hybridization. Ten cDNA fragments hybridized to the subtracted and non-subtracted root-cap cDNA pools or the subtracted root-cap cDNA pool, but not to the root-proper cDNA pool, and were hereafter referred to be as root cap-specific. The other 13 clones either hybridized to the root-proper cDNA pool, or did not hybridize to any cDNA pools.

A cDNA library of maize primary root-tip region from within 1 mm of the distal tip end was made in λZAPII (Stratagene) (Matsuyama et al., unpublished). A total of 4x 10^6 recombinants were independently screened with the ten root cap-specific cDNA fragments as probes. Hybridization and other procedures were done as described above. Positive plaques were identified with three of the ten root cap-specific probes. In this chapter, one cDNA representing 6 phage clones was analyzed. These positive recombinant phages were converted to pBluescript SK(-) plasmids by *in vivo* excision using the manufacturer's protocol (Stratagene). Both DNA strands of the longest insert of the 6 clones were sequenced. DNA and predicted amino acid sequences were analyzed with GeneWorks software (IntelliGenetics, Campbell, CA).

Genomic DNA Hybridization Analysis

Total genomic DNA was isolated from 3-day-old etiolated maize seedlings by CTAB extraction (Murray and Thompson, 1980). Genomic DNA (30 μg) was digested with restriction enzymes, electrophoresed on a 1% agarose gel, and blotted onto a Hybond N^+ membrane. The membrane was hybridized to the full-length *zmGRP4* cDNA probe and washed under the conditions described above.

Northern Hybridization and RT-PCR Analysis

Total RNA was isolated from several tissues, including root tip, root proper, young leaf from 2-week-old plants, and shoots from 3-day-old light-grown and etiolated plants, using phenol:chloroform extraction and LiCl precipitation (Mohnen et al., 1985). Poly $(A)^+$ RNA was purified from total RNA using Oligotex-dT30 Super (Roche). Poly $(A)^+$ RNA (1.5 μg per lane) was electrophoresed on a 1.2% formaldehyde agarose gel, blotted onto a Hybond N^+ membrane, and hybridized to the full-length *zmGRP4* cDNA probe under the conditions described above. After stripping the probe from the membrane by incubating at 67°C in a buffer containing 50% formamide, 10 mM Tris-HCl and 10 mM EDTA, pH 8.0, the ^{32}P-labeled *Pst*I-*Sac*I fragment of a ubiquitin cDNA (Christensen and Quail, 1989) was hybridized to the same membrane.

For RT-PCR analysis, total RNA was isolated from approximately 100 mg of root tip, root proper, shoot and etiolated shoot using RNeasy Plant Mini Kit (QIAGEN), and used to construct first-strand cDNA using the SUPERSCRIPT preamplification system (GIBCO BRL). PCR primers for *zmGRP4* were 5'-TTGTATCTCACAATGGCAGGC and 5'-GCGTTGGAATTCCAAGAACC (see Fig. 2), and PCR primers for maize *α-tubulin1* (Montoliu et al., 1989) were 5'-CTTGATCGCATCAGGAAGC and 5'-TCAGCAGAGATGACTGGAGC. PCR amplification was carried out for 18 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 30 sec, and elongation at 72°C for 2 min. Amplified *zmGRP4* fragments were electrophoresed on a 1.2% agarose gel, blotted onto a Hybond N^+ membrane, and hybridized to the full-length *zmGRP4* cDNA probe as described above. Representative amplified DNA fragments were partially sequenced to confirm their identity.

In situ Hybridization Analysis

Maize primary root tips were fixed in 3% paraformaldehyde and 2% glutaraldehyde for 12 h at 4°C. After dehydration in a graded ethanol series and clearing in a graded xylene series, samples were embedded in wax (Histoprep 580; WAKO, Osaka, Japan) and sectioned at 10 μm by using a rotary microtome. Digoxigenin-labeled antisense and sense RNA probes were

prepared from a 3'-untranslated region of *zmGRP4* (see Fig. 2) using a DIG RNA Labeling Kit (Boehringer Mannheim). Samples were incubated with the RNA probes at 50°C for 16 h, treated with RNase A (2.5 μg mL^{-1} in 0.5 M NaCl, 10 mM Tris-HCl and 1 mM EDTA, pH 7.5) at 37°C for 30 min, and washed with several changes of 2x SSC (1x SSC: 150 mM NaCl and 15 mM $Na_3C_6H_5O_7$) and once with 0.1x SSC at 50°C. Signals were detected by DIG Nucleic Acid Detection Kit (Boehringer Mannheim). The color reaction was stopped with 10 mM Tris-HCl and 1 mM EDTA, pH 8.0. Sections were passed through an ethanol series and mounted for microscopic observation.

zmGRP4 Antiserum

A portion of the *zmGRP4* cDNA encoding the carboxyl-terminal amino acid residues from 132 to 192 was subcloned into the *Eco*RI site of pET-32b(+) (Novagen), which would then express a fusion protein consisting of the N-terminal of thioredoxin and the of C-terminal zmGRP4. This plasmid was introduced into the BL21 (DE3) bacterial strain (Novagen), and the expression of the fusion protein was induced by 1 mM isopropyl ß-D-thiogalactopyranoside at 37°C for 3 h in LB medium. Bacterial cells were harvested by centrifugation, suspended in 50 mM potassium phosphate buffer, pH 8.0 containing 1% Triton X-100 and 1 μg mL^{-1} lysozyme, incubated at 30°C for 15 min, and then ruptured by three cycles of freeze/thaw treatment and sonication for 1 min. After centrifugation of the homogenate, the fusion protein in the supernatant was separated by preparative SDS-PAGE using a PrepCell (model 491, BIO-RAD). The eluate fractions containing the fusion protein were concentrated with a YM-10 membrane filter (Amicon). The buffer of the concentrated protein solution was exchanged by using a PD-10 column (Pharmacia) equilibrated with buffer A (20 mM Tris-HCl and 10% glycerol, pH 7.0). The above solution was loaded onto a Mono-Q FPLC column (Pharmacia) previously equilibrated with buffer A, and eluted with a linear gradient of KCl from 0 to 0.5 M in buffer A. The fractions containing the fusion protein were concentrated with a YM-10 filter and desalted using a PD-10 column. The fusion protein had an approximate purity of 99.9% as determined by CBB-staining after SDS-PAGE, and was used to raise antiserum in mice.

The reactivity of the antiserum against zmGRP4 was confirmed as follows. A

*Bam*HI-*Sma*I fragment of the *zmGRP4* cDNA encoding the carboxyl-terminal amino acid residues from 137 to 192 was subcloned into pGEX-2T (Pharmacia). The resultant pGEX-zmGRP4 plasmid or pGEX-2T was introduced into the BL21 (DE3) bacterial strain, and the expression of either a chimeric protein consisting of zmGRP4 and glutathione S-transferase (GST), or GST alone, respectively, was induced at 37°C for 3 h with 1 mM isopropyl ß-D-thiogalactopyranoside. Approximately 100 ng of total protein extracts from these bacterial cells was separated on a 15% SDS-polyacrylamide gel and transferred to an Immobilon PVDF membrane (Millipore). The membrane was blocked at room temperature in buffer B (100 mM Tris-HCl and 150 mM NaCl, pH 7.5) containing 5% skim milk powder for 1 h. The antiserum was diluted 1:5000 in buffer B and incubated with the membranes at room temperature for 1 h. After washing the antiserum several times with buffer B containing 0.2% Tween 20, the following procedures, including secondary antibody treatment and immunodetection, were performed by using an ECL Plus kit (Amersham) according to the manufacturer's instructions. The antiserum reacted strongly with the fusion protein consisting of zmGRP4 and GST, but not with GST alone.

Immunohistochemical Analysis

Fixed sections were prepared as described for in situ hybridization, and blocked at room temperature in buffer B containing 0.2% Tween 20 and 3% BSA for 1 h. Anti-zmGRP4 serum or pre-immune mouse serum was diluted 1:300 in blocking buffer, and incubated with the sections at room temperature for 1 h. After washing the primary antibody several times in buffer B containing 0.2% Tween 20, anti-mouse IgG conjugated with alkaline phosphatase (Kirkegaard & Perry Laboratories Inc., Gaitherburg, USA) was diluted 1:1000 in buffer B, and incubated with the sections at room temperature for 1 h. After briefly washing the slides with buffer B, immunodetection was performed as described for in situ hybridization analysis.

Immunoblot Analysis

Approximately 100 maize root tips were frozen with liquid nitrogen and ground to a fine powder with a pestle. For some sample preparations, root mucilage was gently wiped

from the root tip with a paper towel. The pulverized root cells were extracted with buffer (50 mM Tris-HCl, 3 mM EDTA, 3 mM DTT, and 3 mM phenylmethylsulfonyl fluoride), and the suspension was centrifuged at 15,000x g for 5 min. The supernatant was referred to as the soluble fraction. The pellet was resuspended in sample buffer (125 mM Tris-HCl, pH 6.8, containing 1% SDS), and used as the insoluble fraction. A total protein fraction was prepared by directly extracting the pulverized cells with sample buffer. Protein concentration was determined using the BCA protein assay reagent (PIERCE).

Protein preparations (10 μg per lane) were separated on a 15% SDS-polyacrylamide gel. The remaining steps were performed as described above, except that 3% BSA was substituted for 5% skim milk powder in the blocking buffer.

RESULTS

Isolation of a maize root cap-specific glycine-rich protein cDNA

The root cap and the root proper are sharply delineated in the maize primary root, which has a closed-type construction. This anatomical feature is used to facilitate excision of maize root cap tissues from the root proper using a surgical knife (Barlow, 1975). I collected approximately five hundred root caps, and extracted poly $(A)^+$ RNA directly from the root cap and also from the root proper. The cDNAs specifically present in the root cap were enriched by subtracting the root-proper cDNA fragment pool from the root-cap cDNA fragment pool (see Materials and Methods). Subsequently, the subtracted root-cap cDNA fragment library was duplicated and hybridized independently with the above root-proper cDNA fragment pool or the root-cap cDNA fragment pool as probes. This differential screening recovered 72 cDNA fragments that hybridized specifically to the root-cap cDNA fragment pool, and these clones were classified into 23 groups by cross-hybridization and partial DNA sequencing. Further slot-blot hybridization with the above two probes confirmed that 10 cDNA groups were specific to the root cap. Representative cDNA fragments from these 10 clones were used as probes to screen a maize root-tip cDNA library, and three distinct cDNA clones were obtained.

Figure 2 shows the nucleotide and deduced amino acid sequences of one of the three

cDNA clones. The cDNA was 821-bp long and contained an open reading frame encoding a 16.9-kD polypeptide of 192 amino acids. The predicted protein had a hydrophobic putative signal peptide with a potential cleavage site between 22 and 23 amino acid residues (von Heijne, 1985), and was a member of the cell wall glycine-rich proteins (GRPs; Showalter, 1993). We hereafter refer to this protein as *Zea mays* glycine-rich protein 4 (zmGRP4). zmGRP4, excluding the putative signal peptide, was rich in glycine (40%), serine (19%), asparagine (7%), alanine (7%), and tyrosine (6%). The high content of Gly, Ser and Ala of zmGRP4 is consistent with the general characteristics of glycine-rich cell wall proteins.

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  1  TCTACTTGTATCTCACAATGGCAGGCACAAAGCTAATATCACTT
                        M  A  G  T  K  L  I  S  L      9
 45  GGGCTCCTTGTTCTCATAGGCATAGGACTAGCCAATGCTGTGAGG
     G  L  L  V  L  I  G  I  G  L  A  N  A  V  R     24
 90  GTGGCTAGATACTCTAGTGCTGATGGGACTGGCACAGGAGAGGGA
     V  A  R  Y  S  S  A  D  G  T  G  T  G  E  G     39
135  CAGGGTGGTGGATACGTGAATGGTGGGGGATCAGGATCTGGGTCC
     Q  G  G  G  Y  V  N  G  G  G  S  G  S  G  S     54
180  GGCACCGGATCTGGTGATAGTAGTCCTTATGGTGTCCATACAAGT
     G  T  G  S  G  D  S  S  P  Y  G  V  H  T  S     69
225  GCTGGAGGGGGTGGTGCAGGTGGTGGAGCTAGCCAAAACGGTGGG
     A  G  G  G  G  A  G  G  G  A  S  Q  N  G  G     84
270  TCTGGATATGGTTCAGGATCAGGGTCAGGGTCAGGGTCAAGTACG
     S  G  Y  G  S  G  S  G  S  G  S  G  S  S  T     99
315  TACAGTCAGGGAGGGTATTATTCAGGTTATGGAGAATCTTCTAAT
     Y  S  Q  G  G  Y  Y  S  G  Y  G  E  S  S  N    114
360  GCTGGTGGTACTGGTGGGGGTGGGGGTGGAGGACAAGCTGGAGGT
     A  G  G  T  G  G  G  G  G  G  G  Q  A  G  G    129
405  TCTTGGAATTCCAACGCTCAAGGATCCGGTAGTGGCACCGGTTCT
     S  W  N  S  N  A  Q  G  S  G  S  G  T  G  S    144
450  GGTTCTAGCTATGCTAACAGGAATTGGGATGGATCAAGTGGAGGT
     G  S  S  Y  A  N  R  N  W  D  G  S  S  G  G    159
495  GCAAATGCTAATGGCAATGGTGGTGGCACGGGAAATAGTCAAAAT
     A  N  A  N  G  N  G  G  G  T  G  N  S  Q  N    174
540  GGTGGGGGCGGCGGTGGTTCAGGTGATGGGTCTGGATATGGCAAT
     G  G  G  G  G  G  S  G  D  G  S  G  Y  G  N    189
585  GCCTACCCATGAACTTGATATCAGTTAATCTAATGTTGGGCCCGC
     A  Y  P  *                                     192
630  CATCCATCCTTGTTTGAATTTGTTGGATTATTATTTTCCTTGGCT
675  CTTTGTCGTGGTTTTGGTAGAACCGAATTAATAAAGGGTCCAACT
720  ATCTTGTAAGGTCCAACGTATAAGATGATGTTGTAACAGTGTCTG
765  TTAATGAAAACTATATATCAACAAATTTCTTATGTTTAAAAAAAA
810  AAAAAAAAAAA 821
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Figure 2 Nucleotide and deduced amino acid sequences of *zmGRP4*. A putative signal peptide is double-underlined. The sequence between the vertical arrowheads was used as antisense and sense probes for *in situ* hybridization analysis. The two arrows indicate the positions of PCR primers used for RT-PCR analysis. The stop codon is shown by an asterisk.

Genomic DNA blot hybridization analysis was done with a full-length *zmGRP4* cDNA probe at high stringency conditions (Fig. 3). *Bam*HI and *Eco*RI digest the *zmGRP4* cDNA once, while *Bgl*II and *Xho*I do not digest the cDNA. Two to three strong bands, and two to four weak bands were detected when the maize genome was digested with *Bam*HI, *Bgl*II, *Eco*RI, or *Xho*I. Therefore, a small number of genes homologous to *zmGRP4* are likely to exist in maize. In support of this, the amino acid sequence of another maize *GRP* cDNA (accession number AF031083) and zmGRP4 share an 82% identical region of approximately 90 amino acid residues (data not shown). At the nucleotide sequence level, this region is 83% identical between these two *GRPs*.

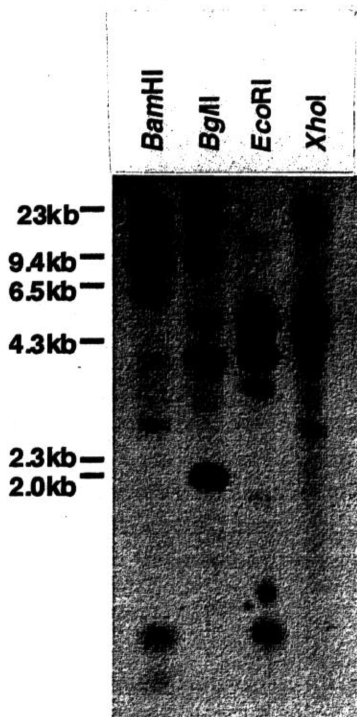

Figure 3 Genomic DNA hybridization analysis of *zmGRP4*. Full-length *zmGRP4* cDNA was used as a probe. Maize genomic DNA (20 mg) was digested with *Bam*HI, *Bgl*II, *Eco*RI, or *Xho*I. The position of molecular markers is shown on the left.

zmGRP4 is expressed strongly in the lateral root cap and weakly in the epidermis of the root proper

RNA blot hybridization analysis with the full length cDNA of *zmGRP4* as the probe detected *zmGRP4* expression in root tip, but not root proper, etiolated shoot, shoot, and mature leaf (Fig. 4A). Since RNA blot hybridization may not detect low levels of *zmGRP4* expression and may detect expression of *zmGRP4*-related gene(s) as well, RT-PCR was done

to specifically amplify *zmGRP4* RNA (Fig. 4B). Expression of *zmGRP4* was detected in root tip and root proper, but not in shoot and etiolated shoot. Although quantitative analysis is often difficult with RT-PCR, repeated RT-PCR analyses in which different amplification cycles were used (data not shown) confirmed that *zmGRP4* is expressed more strongly in root tip than root proper. Amplification of maize α-tubulin RNA indicated that approximately equal amounts of cDNA were used for each tissue sample.

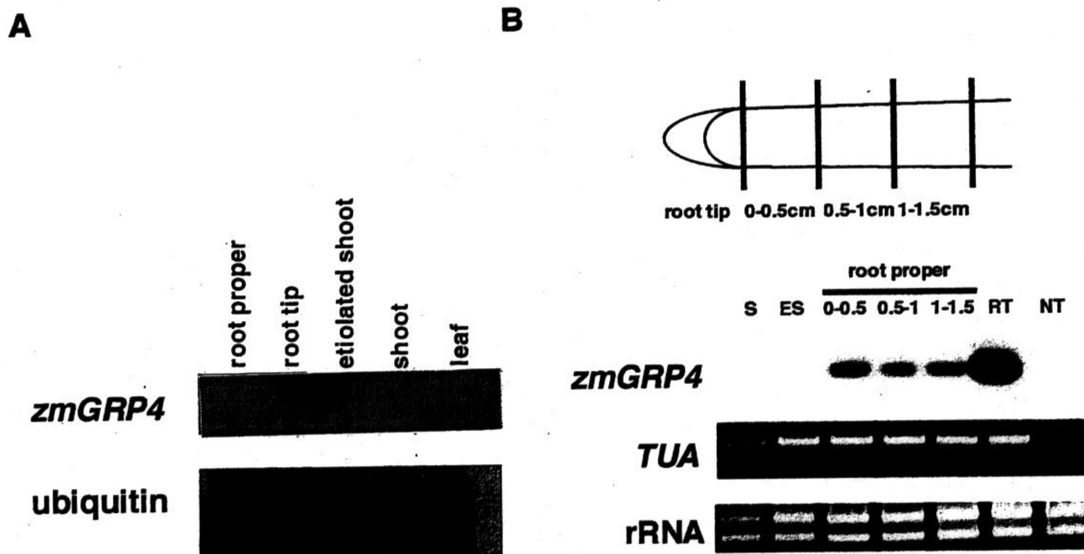

Figure 4 Expression of *zmGRP4* in maize tissues. A, Northern hybridization analysis. Poly(A)$^+$RNA (1.5 μg) was isolated from 2-3 cm long root, 3-day-old etiolated shoot, 3-day-old shoot and leaf of 2-week-old plants. A maize ubiquitin probe served as a control to estimate the relative loading of RNA in each lane. B, RT-PCR analysis. Shoot (S), etiolated shoot (ES), root proper (0-0.5cm, 0.5-1.0cm and 1.0-1.5cm regions distal from the excision site), root tip (RT) and a negative control without reverse transcription (NT). A maize α-tublin gene (*TUA*) served as a positive control.

The 3'-untranslated region of the *zmGRP4* cDNA was used as a probe for in situ hybridization analysis to study the detailed expression pattern of *zmGRP4* in maize primary root. The antisense probe detected strong *zmGRP4* expression in lateral root cap cells, and rather weak expression in epidermal cells of the root proper (Fig. 5A). The sense probe did not detect any hybridization signals (Fig. 5B). Peripheral cells that had been or were being detached from the lateral root cap showed little *zmGRP4* expression (Fig. 5C, asterisks), while weak *zmGRP4* expression extended to several peripheral cells toward the central region of the root cap (Fig. 5D). *zmGRP4* expression in epidermal cells of the root proper terminated in the region where *zmGRP4* expression in the lateral root cap ended (Fig. 5C, arrow).

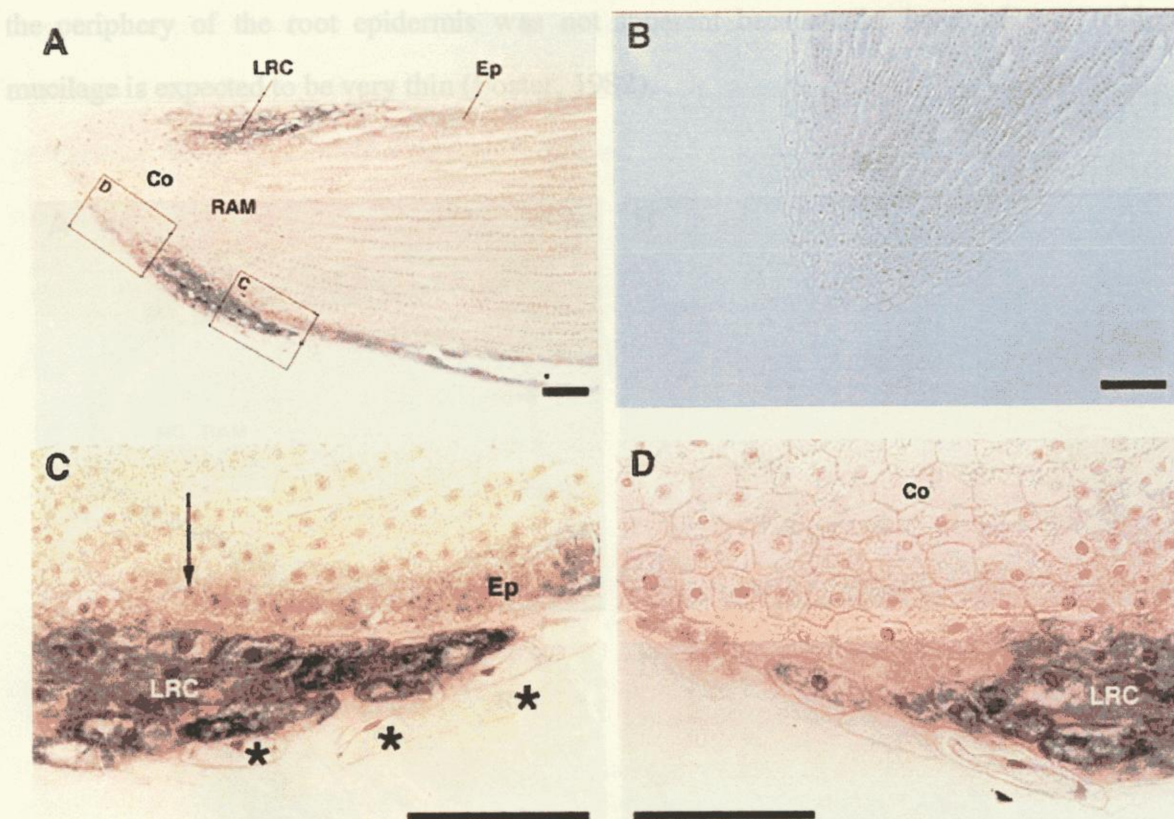

Figure 5 In situ hybridization analysis of *zmGRP4*. Longitudinal sections of maize primary root tip were hybridized with antisense (A, C, and D) or sense (B) digoxigenin-labelled *zmGRP4*-specific probes. Photographs C and D were enlarged from the rectangles in A. An arrow shows the end of *zmGRP4* expression in the epidermis, while asterisks indicate sloughed-off cap cells. Co, columella; LRC, lateral root cap; Ep, epidermis; RAM, root apical meristem. Scale bars indicate 100 μm.

zmGRP4 accumulates in root mucilage

A polyclonal antibody was raised against a truncated zmGRP4 protein that contained amino acid residues 132 to 192. This carboxy-terminal region of zmGRP4 includes amino acid stretches of low glycine abundance and is expected to be specific for zmGRP4. The closest homolog of zmGRP, encoded by a maize expressed sequence tag (AF031083), is 58% identical in this region (data not shown).

Immunohistochemical analysis using this antiserum showed that zmGRP4 is present specifically in the mucilage that covers the root tip (Fig. 6A). A pre-immune mouse antiserum detected no signals (Fig. 6B). Longer exposure detected a relatively small amount of zmGRP4 in the lateral root cap cells (Fig. 6C). A weak signal was also observed in epidermal cells of the root proper in the distal 1 cm of the root tip (Fig. 6D). The presence of mucilage at

the periphery of the root epidermis was not apparent because the layer of root epidermal mucilage is expected to be very thin (Foster, 1982).

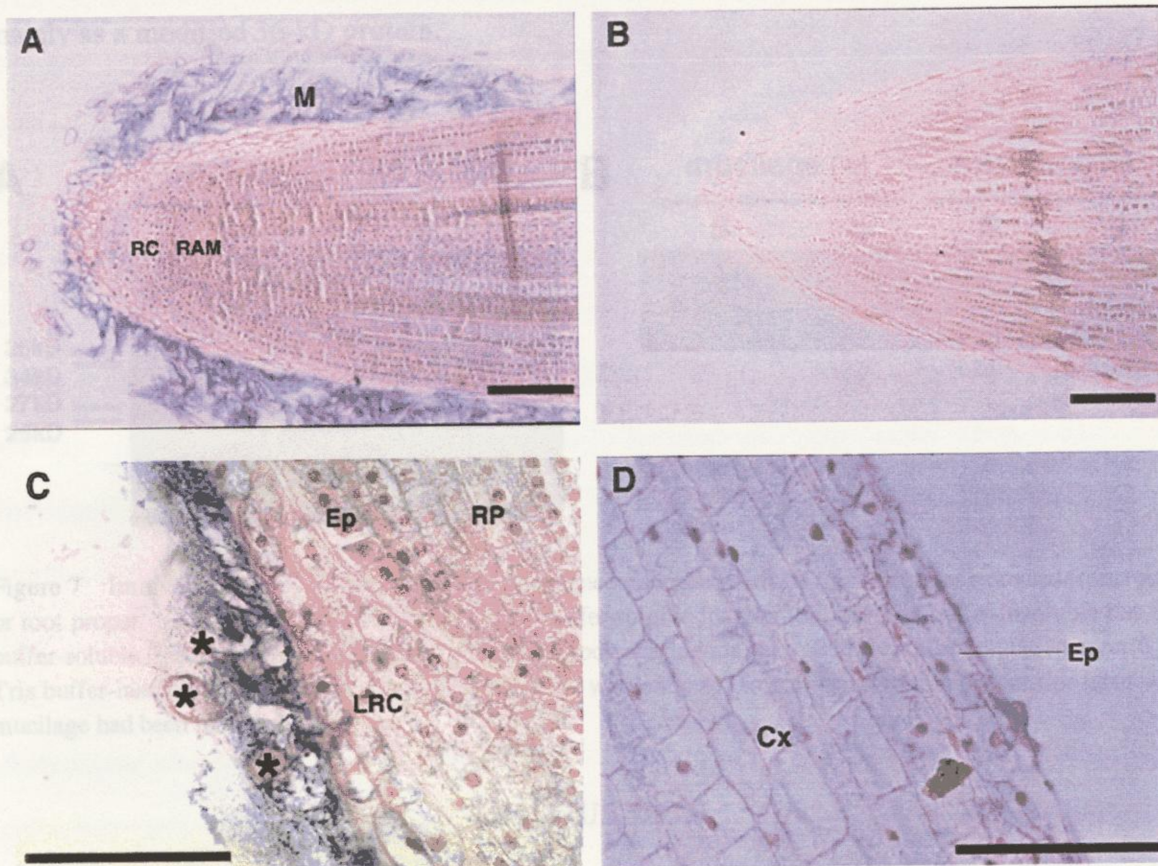

Figure 6 Immunolocalization of zmGRP4. Longitudinal sections of maize primary root tip were incubated with anti-zmGRP4 serum (A, C, and D) or pre-immune serum (B). The lateral root cap region adjacent to the root proper is shown with higher magnification in C, while the root proper region 1 cm distal to the cap is shown in D. Alkaline-phosphatase reactions were done for 1 h in A and B, and for 3 h in C and D. Asterisks indicate sloughed-off cap cells. RC, root cap; RAM, root apical meristem; M, mucilage; RP, root proper; LRC, lateral root cap; Cx, cortex; Ep, epidermis. Scale bars indicate 100 μm.

zmGRP4 may be post-translationally modified

Immunoblot analysis using the anti-zmGRP4 serum revealed that zmGRP4 exists in the maize root as a major band with an apparent molecular weight of 36 kD, and a minor band with an apparent molecular weight of 34 kD, in the insoluble fraction which was extracted with SDS-containing buffer (Fig. 7A). Extraction with Tris buffer without SDS did not recover any zmGRP4 protein. The 36-kD form was much more abundant in the root tip than in the root proper. A few faint bands of 27 kD and 25 kD were also detected in the insoluble fraction from root tip. Since the deduced molecular weight of the mature zmGRP4 is 14.4 kD, post-

translational modifications of zmGRP4 in maize root are suspected. Manual removal of root mucilage from the root tip markedly reduced the abundance of the 36-kD zmGRP4 in the preparation (Fig. 7B). This strongly suggests that zmGRP4 accumulates in root mucilage mainly as a modified 36-kD protein.

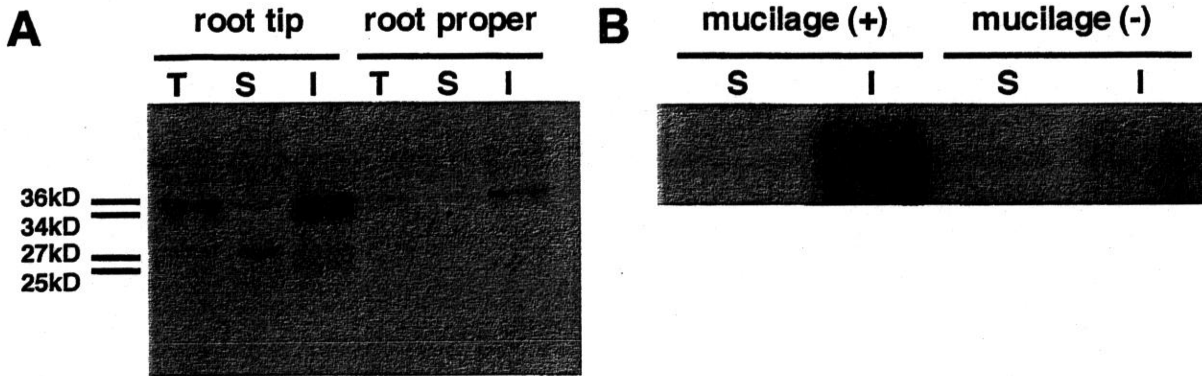

Figure 7 Immunoblot analysis of zmGRP4. Each lane was loaded with 10 μg of proteins extracted from root tip or root proper. A, Total protein fraction (T), Tris buffer-soluble fraction (S), and Tris buffer-insoluble but SDS buffer-soluble fraction (I) were separated on a 15% SDS-polyacrylamide gel. B, Tris buffer-soluble fraction (S) and Tris buffer-insoluble but SDS buffer-soluble fraction (I) were extracted from intact root tips or root tips from which mucilage had been removed.

DISCUSSION

zmGRP4 is a new member of maize GRPs

Many structural cell wall proteins which have putative signal peptides and no catalytic domains have been reported in various plants (Showalter, 1993). These cell wall proteins are characterized by a high abundance of a single amino acid, repetitive sequence motifs, and a tendency to become insolubilized within the cell wall. The three major plant cell wall protein classes include hydroxyproline-rich glycoproteins (HRGPs), proline-rich proteins (PRPs), and GRPs. GRP cDNAs have been isolated from several plants, including three from maize. *zmGRP* is expressed in the epidermal cells of embryo, scutellar tissue and young leaf, and induced by abscisic acid, water stress, and wounding in leaves (Gómez et al., 1988). Since zmGRP does not have an amino-terminal signal peptide, it may be a cytosolic protein. Besides being rich in glycine, zmGRP has a putative RNA-binding motif (Gómez et al., 1988). Therefore, zmGRP and zmGRP4 belong to different subclasses of the GRP family. zmGRP3

(Goodmeier et al., 1998) has an N-terminal signal peptide but shows no significant homology to zmGRP4 except abundant glycine residues. The expression of *zmGRP3* was root-specific, with the highest expression level in the meristematic and elongation regions (Goodmeier et al., 1998). RNA blot analysis of *zmGRP3* indicates that *zmGRP3* and *zmGRP4* are expressed in different regions of the maize root.

zmGRP4 expression in the root

The expression of cell wall proteins depends on cell type, developmental stage, and stress responses (Showalter, 1993). *zmGRP4* is strongly expressed in lateral root cap and weakly in root epidermis, but not in sloughed-off cap cells (Fig. 5). The immunohistochemical localization of zmGRP4 (Fig. 6) strongly indicates that zmGRP4 is synthesized in lateral root cap cells and root epidermal cells, and then secreted into the mucilage. Lateral root cap cells develop considerable hypertrophied golgi cisternae and are the main site of mucilage secretion. Maize root epidermal cells are also reported to contain hypertrophied dictyosome cistanae and release mucilage (Clarke et al., 1979; Foster, 1982). Detached cap cells, however, have dictyosomes that are no longer hypertrophied (Clowes and Juniper, 1968). This close correlation between *zmGRP4* expression and differentiation of secretion machinery suggests that zmGRP4 is secreted via hypertrophied golgi cisternae into the mucilage. Likewise, bean GRP 1.8 was localized to dictyosomes of xylem parenchyma cells, and was suggested to be exported into the walls of neighboring protoxylem vessels (Ryser and Keller, 1992).

Sloughed-off cells did not express *zmGRP4*, while the outermost cap periphery cells did express *zmGRP4* (Fig. 5C, D). A notable switch in gene expression was also reported to occur upon cap border cell differentiation in pea (Brigham et al., 1995).

zmGRP4 is post-translationally modified

Many cell wall proteins are modified post-translationally. For example, proline residues of HRGP are enzymatically converted into hydroxyprolines, which are then glycosylated to various degrees (Cassab, 1998). zmGRP4 mainly existed as a 36-kD protein,

while the deduced molecular weight of mature zmGRP4 is 14.4 kD protein. The high glycine content in GRPs is known to cause aberrant electrophoretic migration on SDS gels. The mature ptGRP1 protein of 25.6 kD, when expressed in *E.coli*, was detected as a 23-kD protein by SDS-PAGE analysis (Condit et al., 1990). Likewise, when zmGRP4 was expressed in *E.coli* as a GST-fusion protein, the recombinant fusion protein was detected approximately 2-kD larger than expected by SDS-PAGE analysis (our unpublished results). However, this aberrant migration alone does not explain the more than 20-kD difference between the expected and observed size of zmGRP4 extracted from maize root tips.

Insolubilization of cell wall proteins has been observed in various developmental or stress-responsive processes (Cassab, 1998). Insolubilization of bean GRP 1.8 occurs during hypocotyl development (Keller et al., 1989). Hydrogen peroxide generated by fungal elicitor- or glutathione- treatment of bean or soybean cells causes oxidative cross-linking, therefore the insolubilization of PRP (Bradley et al., 1992). Recovery of isodityrosine after hydrolysis of cross-linked HRGP indicates that the tyrosine hydroxy groups in HRGP undergo intermolecular condensation via H_2O_2 (Fry, 1986). zmGRP4 contains a relatively high percentage of tyrosine residues. Oxidative cross-linking between zmGRP4s themselves or between zmGRP4 and other proteins via tyrosine residues might result in insolubilization and increased molecular weight of zmGRP4. It should also be noted that PRPs insolubilized by H_2O_2 were not extracted even in SDS-containing buffer (Brisson et al., 1994), and potential cross-linking of xylem GRPs with the aromatic residues of lignin has also been proposed (Showalter, 1993; Cassab, 1998). The absence of lignin and polyphenolics in root mucilage suggests that the cross-linking partners of zmGRP4 may be at least partly different from those of previously reported cell-wall proteins.

Glycosylation is a common post-translational modification found in secreted proteins. There are, however, few reports on the potential glycosylation of GRPs (Showalter, 1993). Exceptions include a 30-kD GRP purified from soybean aleurone layers, which was reported to contain approximately 9% (w/w) sugars, including mannose, arabinose, glucose, xylose, and galactose (Matsui et al., 1995). Purified soybean GRP showed a broad band after SDS-PAGE separation, indicating a microheterogeneity in the sugar component (Matsui et al., 1995).

On the other hand, zmGRP4 extracted from maize root tips migrated as discrete bands on SDS-PAGE (Fig. 7). Since the deduced zmGRP4 amino acid sequence has no canonical *N*-glycosylation sites, the modification could be *O*-glycosylation with homogeneous sugar side chains, if zmGRP4 were to be glycosylated.

Possible functions of zmGRP4 in the root mucilage

Soil and sand sheaths usually cling tightly to the roots of field-grown grasses, such as maize root. The sheath is thought to be formed by the binding of soil particles in mucilage originating from the root (Vermeer and McCully, 1982; and the references therein). Root hairs are probably not primarily responsible for the adhesion of soil aggregates. Mucilage, soil particles, sloughed-off root cap cells, and some soil bacteria together form the rhizosphere, and the chemical and physical properties of the mucilage should be very important in determining the nature of the rhizosphere.

Root mucilage is composed of 99.9% water (Guinel and McGully, 1986). The dry mass of mucilage consists mainly of polysaccharides and polyuronic acids (Jones and Morré, 1967; Floyd and Ohlrogge, 1970; Paull et al. 1975). Although proteins have been detected in maize mucilage (Chabound, 1983), their properties and possible roles have so far attracted little attention. We have shown here that zmGRP4 is a mucilage protein. Other well-characterized GRPs are localized in the vascular system, and in xylem in particular (Ryser and Keller, 1992; and the references therein). Ultrastructural localization, however, has demonstrated that bean GRP 1.8 is localized to unlignified primary walls of protoxylem cells, and a correlation between GRP 1.8 deposition and lignification was evidently lacking in bean hypocotyls (Ryser and Keller, 1992). An apparent positive correlation of GRP deposition with expansive growth, and an inverse correlation with lignification, have been reported for petunia ptGRP1, which is deposited at the cell wall/membrane interface, rather than within the cell wall (Condit, 1993). These GRPs thus may provide elasticity to the stretching wall, or some protective environment to cells under frictional stress. Some GRP sequences are predicted to adopt β-pleated sheets composed of varying numbers of antiparallel strands; such a structure could provide elasticity and tensile strength during vascular development (Showalter, 1993).

The soil sheath adhering along the entire length of field-grown maize roots is mostly permeated by mucilage which is histochemically similar to that produced by the root cap (Vermeer and McCully, 1982). An experiment designed to measure the penetration resistance showed that maize roots receive much less frictional resistance than metal probes when growing into the soil (Bengough and McKenzie, 1997). One function of root mucilage, working together with sloughing root cap cells, may be to decrease the frictional resistance during growth in the soil, and to protect growing roots from abrasion by soil particles. If zmGRP4 has physical properties similar to other GRPs, it may provide elasticity to the root mucilage, and may complement other mucilage components (e.g., polysaccharides and pectin) for a lubricant function.

Large amounts of fixed carbon are secreted into the rhizosphere from the surface of grass roots (Russell, 1977). The secreted carbon is mostly in the form of sugar, but a wide range of amino acids, organic acids, vitamins and auxins are either released from the roots or synthesized by microorganisms in the root environment (Bar-Yosef, 1996). These organic compounds may support survival and growth of detached cap cells and soil bacteria. Some of the compounds even may be involved in interactions between particular plant genotypes and soil microorganisms. Secreted proteins in the rhizosphere may play similar roles. In this regard, distribution of GRPs in root mucilages of other maize genotypes and other plant species, and the stability of zmGRP4 in the rhizosphere should be interesting to examine in the future.

Chapter II Maize proteins specifically expressed in the outermost cells of root cap

Root cap cells are continuously pushed toward the root cap periphery and finally slough off into the external root environment. These detached cells are found at the root peripherary even at some distance from the root cap (Varmeer and McCully, 1982), are metabolically active and have unique patterns of gene expression (Brigham et al., 1995). Several functions for the sloughed-off cap cells as a root-soil interface have been proposed (Hawes, 1998).

Dynamic morphological changes occur in the peripheral root cap cells during their separation from the root cap proper. Moore and McClelen indicated that the sloughing-off cap cells containig the mucilage between the cell wall and plasmalemma differ morphologically from the sloughed-off cap cells characterized by the highly vacuolate cells (Moore and McClelen, 1983). Recently, the TdT end-labeling (TUNEL) method and the DNA staining with Hoechst 33342 detected a significant loss of DNA as well as fragmented DNA in the outermost one to three cells of onion root cap (Wang et al., 1996). The TUNEL-positive structures in the cap eventually formed apoptotic-like bodies. Therefore, the sloughing root cap cells may undergo programmed cell death as their normal developmental process, such as high vacuolation.

I describe in this chapter the characterization of two related maize cDNAs, *zmRCP1* and *zmRCP2 (Zea mays root-cap periphery gene)*. These genes are expressed in a few outermost cell layers of the peripheral root cap. The peripheral root cap cells are namely the sloghing-off cap cells which may undergo programmed cell death.

MATERIALS AND METHODS

RNA blot and Reverse Transcription (RT)-PCR analyses

Total RNA (10 μg) was separated by electrophoresis on 1.2% formaldehyde agarose gel, and blotted onto a Hybond-N membrane (Amersham). The blots were hybridized either with radiolabeled *ZmRCP1* or *ZmRCP2* cDNA fragments (from 228 to 1235 in the *ZmRCP1* cDNA, and from 166 to 1318 in the *ZmRCP2* cDNA). Hybridization was done as reported (Hashimoto et al. 1998).

For RT-PCR analysis, total RNA was isolated from approximately 100 mg of root tip, root proper, shoot and etiolated shoot using RNeasy Plant Mini Kit (QIAGEN), and used to construct first-strand cDNA using the SUPERSCRIPT preamplification system (GIBCO BRL). PCR primers were 5'-CTACGTCAACAGCCTCAACG and 5'-AGACACACAACGATGGATCG for *zmRCP1*, 5'-GGCAGGTTCACCATCAGG and 5'-TGAATACCACAACCAGTGGC for *zmRCP2*, and 5'-CTTGATCGCATCAGGAAGC and 5'-TCAGCAGAGATGACTGGAGC for maize *α-tubulin1* (Montoliu et al., 1989). PCR amplification was carried out for 18 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 30 sec, and elongation at 72°C for 2 min. Amplified fragments were electrophorased on a 1.2% agarose gel, blotted onto a Hybond N^+ membrane, and hybridized to the full-length *zmRCP1*, *zmRCP2*, and *zmGRP4* (Matsuyama et al., unpublished) cDNA probes. Representative amplified DNA fragments were partially sequenced to confirm their identity.

Rapid Amplification of 5'-cDNA Ends (5'-RACE)

Total RNA was isolated from approximately 500 mg of root tip using RNeasy Plant Mini Kit (QIAGEN), and poly $(A)^+$ RNA was purified from total RNA using an mRNA purification kit (Pharmacia). First-strand cDNA of *zmRCP2* was synthesized from 1 μg of poly $(A)^+$ RNA using a *zmRCP2*-specific primer, 5'-GGAAGTAGAAGGCGTTGCCG, with a 5'-RACE system, version 2.0 (GIBCO BRL). After purification and TdT tailing of the cDNA, dC-tailed cDNA was amplified by PCR using a nested *zmRCP2*-specific primer, 5'-

GTCGGCGTAGCATGACTGG, and an abridged anchor primer supplied by the kit. Amplified fragments were ligated into the pGEM-T vector (Promega). After these plasmids were introduced into the bacterial strain JM109, 7 independent clones were randomly picked up and sequenced.

TdT-mediated dUTP nick end labeling (TUNEL) assay

Section of maize primary root tip was made as described above. Protease treatment, dUTP 3'-end labeling, and color reaction with 3, 3'-diaminobenzidine tetrahydrochloride were done using an Apoptosis in situ detection kit (Wako, Osaka). After washing with distilled-water and counter-staining with 4', 6-diamidino-2-phenylindole dihydrochloride, section was mounted in 50% glycerol for microscopic observation.

Electron microscopy

Root tips were fixed with 5% glutaraldehyde in phosphate buffer (pH 7.4) for 8 h at 4°C. After washing with water, they were dehydrated with acetone at 4°C, and embedded in Spurr resin. Polymerization of the resin was carried out at 60°C. Samples were sectioned with an ultramicrotome, ULTRACUT UCT (LEICA). Sections with 70 nm thickness were attached on Formvar-coated nickel glids. Thin sections were stained with lead citrate and examined under an electron microscope, Hitachi H-7100.

RESULTS

ZmRCP1 and *zmRCP2* are members of a novel protein family conserved in plants

ZmRCP1 and *zmRCP2* are two related maize cDNAs, both of which are expressed specifically in the peripheral one to three cells of the maize root cap. I isolated *zmRCP2* cDNA by a subtractive hybridization/differential screening, described in the previous chapter. *ZmRCP1* cDNA was isolated by my colleague from the same maize root tip cDNA library by a transmembrane-domain trapping screening strategy (Matsuyama et al., unpublished). Figure 8 shows the nucleotide and deduced amino acid sequences of *zmRCP1* (Fig. 8A) and *zmRCP2*

(Fig. 8B). *ZmRCP1* cDNA was 1235-bp long and contained an open reading frame encoding a 37.2-kD polypeptide of 342 amino acids, while *zmRCP2* cDNA was 1318-bp long and contained an open reading frame encoding a 37.1-kD polypeptide of 349 amino acids. Because I could not obtain the first methionine of the deduced amino acid sequence of zmRCP2 by screening the maize root tip cDNA library, the 5'-end nucleotide sequence of *zmRCP2* was determined by 5'-RACE (see Material and methods).

A **B**

Figure 8 Nucleotide and deduced amino acid sequences of *zmRCP1* (A) and *zmRCP2* (B). The sequences between the vertical arrowheads were used as antisense and sense probes for *in situ* hybridization analyses. The arrows indicate the positions of PCR primers used for RT-PCR analysis. The stop codon is shown by an asterisk

Amino acid sequences of zmRCP1 and zmRCP2 are homologous. Comparison of these sequences with sequences in GenBank identified several highly homologous sequences from plants, including a late embryogenesis-abundant (LEA) protein from white spruce, and several proteins encoded in Arabidopsis expressed sequence tags and genomic sequences. Figure 9A shows alignment of zmRCP1, zmRCP2, the white spruce LEA protein, and three

A

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ZmRCP1   MAHSHRSSMSFCLVCIVVMAAAAALLQPASS-QQAVPDIPNLKVGSNDRS TTLKCTNIKSNKTTCSAT-------------------------------- 67
ZmRCP2   MARLGALIPLAIVLLAAVAATA----------APSDRPPKAQGPKPHKEK EKEKPKPMKVKCRPRKLYPYCPGKPME----------------------- 67
L47117   MAANKLMNVVAVAVCVMVMVNAAS--------AVGKAKCTDKWYPRCYGY QYD----------------------------------------------- 45
M4I22    MEVPKSSLLMIIFVVASCFL------------HVKAWHGQTYCGGNATPR CQLRYID------------------------------------------- 49
MUF9.10  MLRVLVTIIVLVSVSSVSAE------------TEKGKKLKKGAYDAASTN YNVLYPLGSGQERVQCLARGSCNQKILT---------------------- 65
MUF9.11  MERRQSYVLVALIGLLLLTDIVIA--------QSDGGKGNGNNGKGNEVQ VDKGKGDNGKSNGNGPKDKEQEKKDKEKAAKDKKEKEKKDKEEKEKKDKE 92

ZmRCP1   -------------------------------------------------- ----------------------CNARCPHK----------CLIQC-PS- 82
ZmRCP2   -------------------------------------------------- ----------------------CPAECSQS----------CYADC-SS- 82
L47117   -------------------------------------------------- ----------------------CPANCPYN----------CDMDC-KT- 60
M4I22    -------------------------------------------------- ----------------------CPEECPTEMFP-NSQNKICWVDCFKPL 71
MUF9.10  -------------------------------------------------- ----------------------CPKECPERKPKMNKKKKACFIDC-SSK 91
MUF9.11  RKEKEKKDKLEKEKKDKERKEKERKEKERKAKEKKDKEESEAAARYRILS PLPTGQEQAMCQGRGSCYYKTLVCPGECPKRKPTKNKNTKGCFIDC-TNK 191

ZmRCP1   CKTFCL-----CDFYPCVSCGDPRFTGADGNNFYFHGKKDRDFCIVSDAA LHINAHFIGKRNP-AMSRDFTWIQALGIRFAHHHL-YVAAQRTPRWDAAA 175
ZmRCP2   CKPVCV-----C--SVPGACGDPRFIGGDGNAFYFHGRRDADFCVLSDRD LHINAHFIGKHGADGMSRDFTWIQAIAVLFDGHEL-YVGARKTAAWDDDV 174
L47117   CKTVCP-----CD-KPCGVCQDPRFIGGDGIMFYFHGKRDQDFCLISDSN LHINAHFIGKRGQ-GMGRDFTWVQSIGVLLEDGRQFYLGAKKVSTWDNSV 153
M4I22    CEAVCRAVKPNCESY-CSICLDPRFIGGDGIVFYFHGKSNEHFSIVSDPD FQINARFTGHRPA-GRTRDFTWIQALCELFNSHKFS-LETTKVATWDSNL 168
MUF9.10  CEVTCKWRKANCNGY-CSLCYDPRFVGGDGVMFYFHGNKDGNFAIVSDEN LQINAHFICTRPA-GRTRDFTWVQAESVMFDSHNLV-TAAKKVASWDDSV 188
MUF9.11  CEATCKWRKTNCNGY-CSLCYDPRFVGGDGVMFYFHGSKGGNFAIVSDNN LQINAHFICTRPV-GRTRDFTWVQALNVMFENHKLVITANR-VNQWDETS 288

ZmRCP1   DHLALALDDEDVDVASLLPRFVGARWSPPTAPALSVTRTARVNTVVVELR CAFRIVASVVPITAEDSRIHNYGVREDDCDTLAHLDLGFKFYDLTDDVHG 275
ZmRCP2   DRMELTLDGEPV---RLLPG-TDAAWTSGAVPALSVTRTSAANGVLVSLD CRFTIRANAVPITEEESRVHRYGVTADD--CLAHLDLAFKFGALTADVHG 268
L47117   DQLTMALNGQTLTL---PPG-EGATW--ATASGLNVTRSDRANEVVVQVE DKLKISARVVPISEEESRVHNYGIIAGE-DCFAHLELSFKFYSLSPNVSG 246
M4I22    DHLKETIIDGQDLII----PQETLSTWY-SSDKDIKIERLTEKNSVIVTIK DKAEIMVNVVPVTKEDDRIHNYKLPVDD--CFAHREVQFKFINLSPKVDG 261
MUF9.10  DSLVVRWNGEEVEV---PTEGEAEWRIDLDEREVIVERTDERNNVRVTVS CIVQIDIQVRPIGKEEDRVHKYQLPKDD--AFAHLETQFKFFNLSDLVEG 283
MUF9.11  DAFTIRYDGELITL--PEDEQSEWREISGQKKDIIIERTDERNSVRVLVS DLVQMDIRVRPIGKEENRVHNYQLPQDD--AFAHLETQFKFLDLSELVEG 384

ZmRCP1   VLGQTYRPDYVNSLNVTSNMPVMGGAPDYLSSDLFSTDCAVARFGGRRHQ QATAANIAMLTDDDDME                                 342
ZmRCP2   VVGQTYRSDYVNRFDVKASMPTMGGDSNYTTSSLFAADCAVARYAPSGGS RDRDDGVAMVSEIAGITCSSGMGGQGVVCKK                   349
L47117   VLGQTYGAEYRSPVKMGVAMPIMGGESNYVTSNLFAADCKVARFASSSDD EYAITSALDCNSGRGSGHGIVSRR                          320
M4I22    ILGRTYRPDFKNPAKPGVVMPVVGGEDSFRTSSLLSHVCKTCLFSEDPAV ASGSVKPKSTYALLDCSRGASSGYGLVCRK                    341
MUF9.10  VLGKTYRPGYVSPVKTGVPMPMMGGEDKYQTPSLFSPLCNVCRFCGKTGP GVAKY                                             338
MUF9.11  VLGKTYRPDYVSSAKTGVPMPVLGGEDKYQTPSLFSPTCRLCRFKPQEES LSADI                                             439
```

B

0 50 100 150 200 250 300 350 400 450

K-D

320- 240- 160- 80- 0- -80- -160- -240- -320-

RPC1

M4I22

MUF9.10

L47117

RPC2

MUF9.11

Figure 9 (A) Alignment of deduced amino acid sequences of zmRCP1, zmRCP2, and other related proteins. Residues are boxed to indicate amino acid identity (in black boxes) or functional conservation (in shaded boxes; L, I, V; A, S, G; F, Y; D, E; and H, K, R). The cDNA sequence of a white spruce homolog was obtained from GenBank (L47117). The sequences of Arabidopsis homologs (M4I22, MUF9.10, and MUF9.11) were deduced from the genomic sequences by predicting the probable splice sites. Predicted signal peptides are boxed. Asterisks indicate conserved Cys residues. (B) Hydrophobicity plots of zmRCP1, zmRCP2, and other related proteins. These plots were generated with Gene Works software and the method of Kyte and Doolittle.

similar proteins encoded in the Arabidopsis genome. All the six proteins had *N*-terminal hydrophobic putative signal peptides (von Heijne, 1985). ZmRCP1 has a putative signal peptide with a potential cleavage site between 31 and 32 amino acid residues, while zmRCP2 has a putative signal peptide with a potential cleavege site between 22 and 23 amino acid residues (Fig. 9A). Although the *N*-terminal regions of the mature proteins are not well conserved, six strictly conserved cysteine residues are present in three pairs of Cys-X-X-X-Cys. The profiles of the hydrophobicity plots of these 6 proteins are also very similar (Fig. 9B), although both zmRCP2 and MUF9.11 have a very hydrophilic region chracterized by the repetitive sequence, Glu (E)-Lys (K), at their N-terminal regions without their .putative signal peptides.

Hybridization of maize genomic blots with *zmRCP1* and *zmRCP2* cDNA probes detected several hybridization signals (especially with the *zmRCP1* probe) on the genomic DNAs independently digested with four different restriction enzymes (Fig. 10), indicating that additional sequences homologous to *zmRCP1* (and *zmRCP2*) may be present in the maize genome.

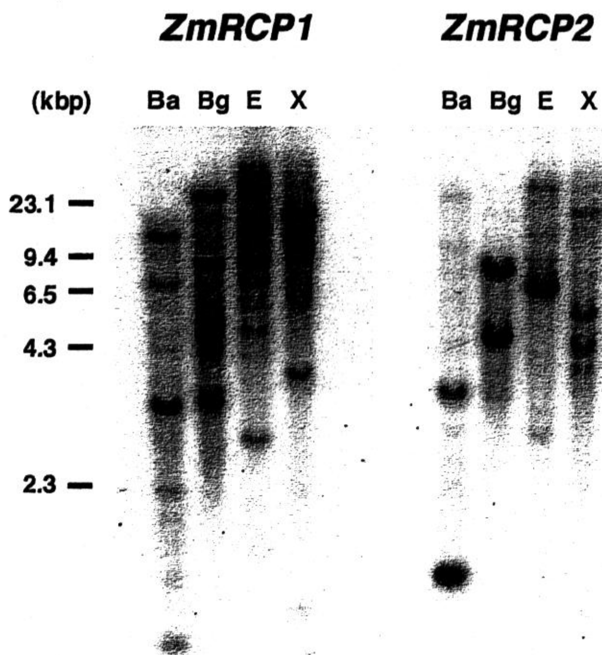

Figure 10 Genomic DNA blot analysis. Maizegenomic DNA was digested with the indicated restriction enzymes, and probed with *zmRCP1* cDNA or *zmRCP2* cDNA. Ba: *Bam* HI, Bg: *Bgl* II, E: *Eco* RI, and X: *Xba* I.

ZmRCP1 and zmRCP2 are specifically expressed in root tip

The expression patterns of *zmRCP1* and *zmRCP2* were studied in various maize tissues (Fig. 11). RNA gel blot analysis showed that both genes are expressed in root tip, but not in root proper, leaf, and stem of maize plantlets (Fig. 11A). Since RNA blot hybridization may not detect low levels of *zmRCP* expression and may detect expression of homologous gene(s) as well, RT-PCR was done to specifically amplify the RNA species from *zmRCP1* and *zmRCP2* (Fig. 11B). As a reference, we also analyzed the expression of *zmGRP4* which is expressed strongly in lateral root cap and weakly in epidermis of maize roots (Matsuyama et al., unpublished). Expression of both *zmRCP1* and *zmRCP2* was detected only in root tip, but not in stem, etiolated shoot, and root proper region excluding root cap. Amplification of maize α-tubulin RNA confirmed that approximately equal amounts of cDNA were used for each tissue sample.

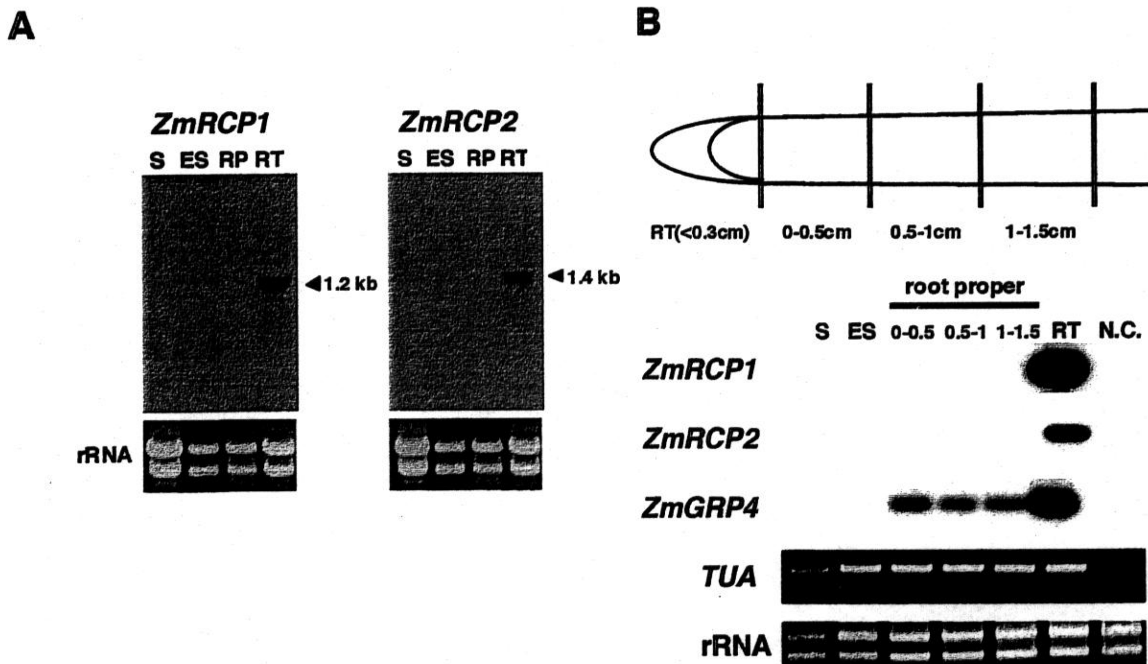

Figure 11 Expression analysis of *zmRCP1* and *zmRCP2*. A, RNA gel blot analysis. RNA gel blots were made from total RNA (10 μg per lane) extracted from shoot (S), etiolated shoot (ES), root proper (RP), and root tip (RT) of maize seedlings, and probed with *zmRCP1* cDNA or *zmRCP2* cDNA. Ethidium bromide staining of rRNA was also shown to indicate equal loading of total RNA samples. B, RT-PCR analysis. Gene-specific PCR primers for *zmRCP1*, *zmRCP2*, *zmGRP4*, and a maize α-tubulin gene (*TUA*) were used to amplify total RNA by RT-PCR from shoot (S), etiolated shoot (ES), three regions of root proper, and root tip (RT) of maize seedlings. Total RNA from root tip was also amplified without a reverse-transcription step as a negative control (N.C.). Amplification products for *zmRCP1*, *zmRCP2*, and *zmGRP4* were blotted on nylon membranes, and probed with corresponding labeled cDNAs. Ethidium bromide staining of rRNA was also shown to indicate equal template amounts used for cDNA synthesis.

ZmRCP1 and _zmRCP2_ are specifically expressed in the peripheral region of root cap

The 3'-untranslated regions of the cDNAs were used as probes for in situ hybridization analysis to study the detailed expression patterns of *zmRCP* genes in maize primary root. The antisense probes detected clear expression of both *zmRCP1* and *zmRCP2* in the outermost one to three cells of the root cap (Fig. 12A and B). The outermost cells of lateral root cap gave stronger hybridization signals than the outermost cells of columella. When peripheral cap cells had detached from root cap and were embedded in mucilage, little *zmRCP* RNA was detected (asterisks in Fig. 12 A and B). The sense probes did not detect any hybridization signals (not shown).

The cap cells in which *zmRCP1* and *zmRCP2* were expressed appear to overlap with the dying cells in which nuclear DNA fragmentation was previously reported in onion root caps (Wang et al. 1996). To detect nuclear fragmentation *in situ* in maize root cap, a TUNEL method was used to end-label the free 3'-OH groups of fragmented DNA. The nuclei of the outermost one or two cells were strongly stained with this method (Fig. 12C). We noticed that a layer of one or two cells covered with blurred gray material separated the TUNEL-positive outermost cells from inner cap cells (double-arrows in Fig. 12C). This separating middle cell layer could be observed, although less clearly, in the tissue sections for in situ hybridization (double arrows in Fig. 12A), and in the root cap sections stained with 4', 6-diamidino-2-phenylindole dihydrochloride or with potassium iodide (data not shown). Analysis with transmission electron microscopy revealed that outermost one or two maize cap cells (arrowhead in Fig. 12D) are elongated and contain large vacuoles and disorganized nuclei surrounded by rough membranes. Intact nucleoli are frequently missing in the nuclei of cap periphery cells. These observations suggest that outermost cells in maize root cap are in the process of developmentally programmed cell death.

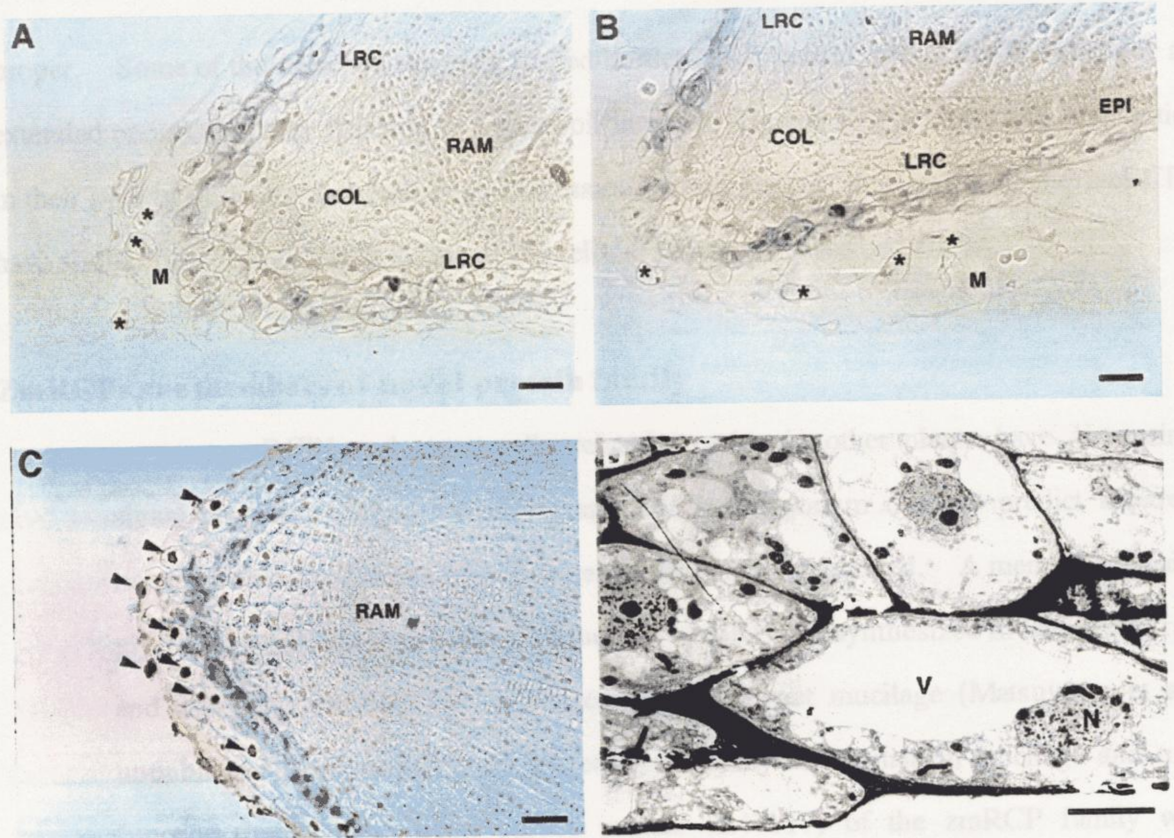

Figure 12 Expression of *zmRCP1* and *zmRCP2* in the root cap periphery cells and their characteristics. A and B, in situ localization of *zmRCP1* (A) and *zmRCP2* (B) transcripts in longitudinal sections of maize primary root cap. RAM, root apical meristem; COL, columella root cap; LRC, lateral root cap; M, mucilage; EPI, epidermis. Asterisks show sloughed-off cap periphery cells. C, in situ detection of apoptosis in maize primary root tip. Nuclei with fragmented DNA were stained by using the TUNEL method (arrows). D, transmission electron microscopic view of outermost periphery cells in maize lateral root cap. The peripheral cell had a large central vacuole (V) and a nucleus of distorted shape (N). Bars in A, B, and C indicate 50 μm, while a bar in D shows 10 μm.

DISCUSSION

ZmRCPs expression in the peripheral root cap

Expression of *zmRCP1* and *zmRCP2* are highly cell-specific; the only cells in which we observed their expression are outermost few cells of maize root cap. These cap periphery cells correspond with PC II cells according to the morphological classification of Moore and McClelen (1983). PC II cells accumulate mucilage between cell walls and plasma membrane, are just being sloughed off into mucilage, and appear to be dying by a process involving fragmentation of nuclear DNA (Wang et al. 1996, this study). Biochemical properties of cell wall in these sloughing-off cells should be modified to facilitate cell separation from the cap

proper. Some of the detached cap cells (called border cells) remain metabolically active for an extended period, and may function as a root-soil interface (Hawes 1998). Striking similarities in their protein structure and their gene expression pattern suggest that zmRCP1 and zmRCP2 have similar function, possibly in one of the cellular processes described above.

ZmRCPs are members of novel protein family

zmRCP1 and structurally related proteins in other plants have *N*-terminal signal peptides. Since the Keyte and Doolittle algorism did not predict apparent transmembrane domains in these proteins, they may be secreted. A member of maize glycine-rich protein with a signal sequence (zmGRP4) is synthesized in lateral root cap, and efficiently secreted into and accumulates in root mucilage (Matsuyama et al., unpublished). If zmRCPs are secreted, they may accumulate in mucilage and may function there. The white spruce protein (L47117) of the zmRCP family was screened from a late-embryogenesis cDNA library but its expression specificity has not been reported. Since Arabidopsis homologs of zmRCPs are identified in expressed sequence tag collections and genomic DNA sequences, their expression patterns are yet to be known. To obtain further insight into the function(s) of zmRCPs, it should be helpful to analyze cell-specific expression patterns of these homologous genes.

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