Localization of soluble and insoluble fractions of hydroxyproline-rich glycoproteins during maize kernel development

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Summary

Monoclonal antibodies have been developed against PC-1 protein, the major hydroxyproline-rich glycoprotein (HRGP) from maize pericarp, and have been used as probes in an indirect alkaline phosphatase antibody assay to localize this protein in tissue of three maize varieties. Kernels were sampled several days after pollination (d.a.p.). HRGPs were assayed by reacting antibodies with tissue prints of kernels and embedded tissue samples. Tissue prints show that soluble HRGPs are present in small quantities even at early stages of kernel development. The greatest accumulation of PC-1 in the pericarp is at the top of the kernel. As the kernels mature, HRGPs begin to accumulate in the developing embryo. Total HRGPs in embedded tissue begin to accumulate at 10 d.a.p. and increase quite dramatically at 20–30 d.a.p. Popcorn kernels lose antigenic reactivity at 40 d.a.p., while sweet corn kernels continue to show strong reactivity.

Key words: HRGP, maize, pericarp.

Introduction

The primary cell wall is a dynamic structure, varying with physiological fluctuations caused by environmental stimuli, tissue differentiation and maturation (Showalter and Varner, 1988; Tierney et al. 1988). Hydroxyproline-rich glycoproteins (HRGPs) have been studied extensively in dicots and have been identified as one of the main protein components of the cell wall, conferring structural support and protection, and accumulating following infection or physical wounding (Cassab and Varner, 1988; Cooper et al. 1987; Corbin et al. 1987; Esquerre-Tugaye et al. 1979; Hood et al. 1988; Mazau and Esquerre-Tugaye, 1986; Roby et al. 1985; Stiefel et al. 1988; Varner and Lin, 1989).

Dicot HRGPs are high molecular weight glycoproteins, 86,000–92,000 (Stuart and Varner, 1980). Tri- and tetra-arabinose chains linked to the hydroxyproline residues of the protein account for one half to two thirds of the total molecular weight (van Holst and Varner, 1984). Galactose bound to serine accounts for approximately 3% of the sugar residues (van Holst and Varner, 1984). The primary structure of the protein is typically composed of a high number of repetitive units of (Ser-Hyp4–5) with large quantities of valine, threonine, tyrosine and lysine (Averyhart-Fullard et al. 1988; Chen and Varner, 1986; Stuart and Varner, 1980). The repetitive nature and the high proportion of proline and hydroxyproline give these proteins a rod-like shape (van Holst and Varner, 1984). Soluble and insoluble fractions of HRGPs have been identified in several tissues, and though no in vivo evidence has been found for the chemical structure of insoluble HRGP, isodityrosine (IDT) bonds have been observed to increase in parallel with the insolubilization of HRGPs (Cooper and Varner, 1983) and to form the intramolecular links: 0.5 IDT, lysine, 0.5 IDT (Epstein and Lamport, 1984). These bonds may be involved in converting soluble hydroxyproline-rich proteins to insoluble ones while forming a complex rigid cell wall structure (Everdeen et al. 1988).

Maize HRGPs are smaller than the dicot proteins analyzed to date. A molecular weight of 72,000 has been reported, though the authors emphasized that this was most probably an overestimate of the true weight due to the influence of glycosylation (Kieliszewski and Lamport, 1987). PC-1 a hydroxyproline-rich glycoprotein isolated from corn pericarp, has an estimated molecular weight of 65,000 (Hood et al. 1988). The maize protein is rich in proline, lysine and serine, but threonine and hydroxyproline are the most abundant amino acids. The monocot protein, like the dicot protein, appears to have a repetitive nature and a rod-like shape. Arabinose is the only sugar bound to the maize protein, which is a characteristic difference from the dicot glycoprotein. In addition, most (50%) of the hydroxyproline residues in the maize protein are non-glycosylated; the primary glycosylation unit being a tri-arabinoside (25%); (Kieliszewski and Lamport, 1987).

Though small quantities of HRGPs have been found in most plant tissues, they frequently occur in abundance in specialized tissues. Stafstrom and Staehelin (1988) used polyclonal antibodies to localize extensin-1 to the cell wall but not to the middle lamella of phloem-parenchyma cells of the carrot root. Cassab and Varner (1987) localized developmentally regulated soybean HRGPs to hour-glass and palisade cells of the seed coat, the hilum and the vascular tissue. These observations suggest that the primary function of HRGPs is to lend support and strength in a structural and protective way. Their presence in small quantities even in young tissues suggests they may play a role in plant growth and architecture (Cooper et al. 1987;
The objectives of this research were to study the developmental regulation, tissue and cellular location, and tissue specificity and variate variation of PC-1 within maize kernels.

Materials and methods

Plant growth and sampling

One sweet corn, Zea mays L. (Golden x Bantam-GXB), and two popcorns (Japanese Hulless (JHL) and South American Yellow (SAY)) were planted, 3 seeds per 12 inch pot, in soil-less medium in the greenhouse. After germination plants were thinned to one per pot. Plants were fertilized weekly with concentrated Peter's Fertilizer (20:20:20) and grown at 29–30°C. Several days prior to pollen receptivity and maturation, ear bags were placed on female flowers. Pollen was collected by placing tassel bags on male flowers the preceding evening. Ears were self-pollinated and marked as to date of pollination. Kernels were sampled at 0, 5, 10, 20, 30 and 40 days after pollination (d.a.p.).

Tissue prints

Tissue prints of kernels from these three varieties and at six developmental times were made using the procedures of Cassab and Varner (1987) as the samples were collected. Six replicates of each series were printed. Prior to printing, nitrocellulose membranes were wet once in 0.2 M CaCl₂ and dried at room temperature on blotting paper. Two freshly cut kernel faces from these three varieties and at six developmental times were dabbed on Kimwipes to remove ethanol, and left or overnight each in 25%, 50%, 75% and 90% ethanol (50:5:10:35, by vol. 95% ethanol/glacial acetic acid/40% formaldehyde/water) and stored at room temperature. Just prior to fixation and embedding, embryo.

Embending

Kernels at 0, 5, 10, 20, 30 and 40 d.a.p. were fixed in FAA fixative (50:5:10:35, by vol. 95% ethanol/glacial acetic acid/40% formaldehyde/water) and stored at room temperature. Just prior to infiltration and embedding, sample kernels were passed through an ethanol series to dehydrate the tissue. Samples were shaken at room temperature for one hour each in 25%, 50%, 75% and 90% ethanol, and 1 h or overnight each in 95% and 100% ethanol. Infiltration was with 2 ml glycol methacrylate JB-4 resin (Polysciences) per 1–2 kernels shaken 6 h at room temperature, then placed overnight in a 4°C refrigerator. Embedding was done the next day by mixing fresh JB-4 solution with polymerization solution and casting tissue in small plastic molds. Sections (3 µm) were cut on a microtome using handmade glass knives. Sections were floated on water, warmed to attach to slides, and stored dry in a slide box until immunocytolocalization of PC-1.

Protein purification and monoclonal antibody production

PC-1 protein from Golden x Bantam pericarp was isolated as described by Hoed et al. (1988) and purified on a cationic neutral gel (Thomas and Hodes, 1981). The band of PC-1 protein was cut from the polyacrylamide gel, pulverized, and injected into mice. Spleen cells from the injected mice were removed and fused with a compatible mouse myeloma cell line, Ag8, and grown on the HAT selective medium of RPMI1640 with 15% fetal bovine serum (Harlow and Lane, 1988). Only products of the cell fusion survived and endosperm has become dry and the proteins do not print or stain. JHL (40 d.a.p.) embryos stained clearly, while the other two varieties did not.

Immunocytolocalization of PC-1 in embedded tissue

Slides with embedded tissue were treated for 5 min with 4 M NaOH to etch glycol methacrylate and make antigenic sites available. Sodium hydroxide was neutralized and the reaction stopped by washing three times for 10 min each in double-distilled water. Slides were then washed three times in Tris-buffered saline with Tween (TBST: 10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20), shaking at slow speed for 10 min each. Slides were shaken during all subsequent steps and washes were for 10 min each. Normal goat serum (3%, Sigma Chemical Co.) diluted in TBST was used overnight to block non-specific background. Following three washes in TBST, slides were reacted with a dilution (for details see figure legends) of primary antibody in TBST for 45–60 min. Secondary antibody (Sigma Chemical Company, St Louis, MO) was anti-mouse polyclonal immunoglobulin conjugated to alkaline phosphatase. After washing three times with TBST, a dilution of secondary antibody was applied and left to react for 60–90 min. After three washes in TBST, and two washes in alkaline phosphatase (AP) buffer (AP: 100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂), slides were immersed in color reaction: 66 µl nitro blue tetrazolium (NBT), 33 µl bromo-chloro-indolyl phosphate (BCIP) per 10 ml AP buffer. Slides were monitored under the microscope and removed when the staining reaction was complete, usually after several hours. Slides were washed in water, allowed to air dry and photographed under normal light-microscopic conditions using an Olympus microscope and 160 ASA Ektachrome Tungsten color slide film.

Immunocytolocalization of PC-1 on tissue prints

Immunocytolocalization of PC-1 on tissue prints was similar to that for slides. Nitrocellulose membranes were wet with TBST, blocked with 3% BSA in TBST for 2 h, then treated as above with primary and secondary antibody, each diluted 1:10,000. The staining reaction generally was very fast, showing bright purple color after approximately 10 min. Controls were performed on both tissue print and embedded materials to verify the specificity and reactivity of the antibody binding reaction, and to ensure that the pink color was indeed due to the secondary antibody conjugate rather than endogenous alkaline phosphatase activity. The following control treatments were substituted for the primary antibody during the reaction procedure: TBST only, mouse cell growth serum, primary antibody plus PC-1 protein, primary antibody plus CaCl₂ extract from wounded carrot root cell walls, primary antibody plus α-arabinose, and primary antibody plus 5% BSA. A final control with primary antibody but no secondary antibody was performed.

Results

Tissue prints

Cassab and Varner (1987) and McClure and Guilfoyle (1989) have demonstrated that tissue printing is a quick, easy method for assessing developmental regulation of protein accumulation or gene expression, respectively. Tissue prints of maize kernels stained with India ink for total soluble protein as shown in Fig. 1 indicate that total soluble protein accumulates during development, but varies between genotypes tested. The morphology of the kernels can be seen at all stages. At 0–10 d.a.p., soluble proteins are concentrated at the surface showing only ovular tissue and accessory glumes. At 10 and 20 d.a.p., embryos begin to stain and endosperm proteins begin to accumulate. At the later stages of SAY (30 and 40 d.a.p.) and JHL (40 d.a.p.) embryos stained clearly, while the endosperm has become dry and the proteins do not print or stain. JHL shows a steady increase in total soluble proteins over the 40 days tested in both endosperm and embryo.

Five anti-PC-1 specific monoclonal antibodies derived from ascites fluid were tested and showed consistent...
Fig. 1. India Ink stain of kernel tissue prints on nitrocellulose. Kernels from the GXB variety at 0, 5, 10, 20, 30 and 40 d.a.p. were printed onto nitrocellulose as described in Materials and methods. Nitrocellulose was subjected to India Ink staining to highlight total protein. Individual kernel prints were photographed and printed at equal magnification.

results on Western blots and trial tissue prints. The MC-3 antibody diluted 1:10000 was subsequently used for the tissue prints shown here. Several control combinations with antibodies MC-1 or MC-3 were also tested including mouse cell growth serum-Ag8, no primary antibody followed by addition of secondary antibody, and primary antibody followed by no secondary antibody (tissue prints not shown). These treatments showed slight to no purple staining, indicating that there were no detectable endogenous alkaline phosphatases, background or contaminating antigenic activity. Addition of 25 μg purified PC-1 protein to 100 ml of a 1:10000 primary antibody dilution effectively absorbed the antibody and prevented its tissue binding and color reaction. In contrast, addition of 62 μg CaCl2 extract from carrot root cell walls, 15 μg D-arabinose, 5% bovine serum albumin, and 5% ovalbumin to 100 ml of 1:10000 primary antibody dilutions did not absorb the antibody. These treatments produced strong purple staining as though only the primary antibody had been added. Though the carrot and maize HRGPs show some similarity in that they have high levels of hydroxyproline and repetitive amino acid sequences, the fact that none of our five monoclonal antibodies recognized the carrot protein (data not shown) indicates that the corn and carrot proteins are quite distinct. Indeed, if the gene sequence data of Stiefel et al. (1988) and protein sequence data of Kieliszewski et al. (1990) are indicative of the majority of maize cell wall proteins, the carrot and maize proteins are very different. The failure of arabinose to absorb the PC-1 antibody suggests that the sugar is not part of the PC-1 epitope recognized by the antibody; thus our results are not complicated by cross-reaction with other arabinosylated glycoproteins. This is not unexpected, since glycosylation is not as predominant a feature of the maize HRGP as it is of the dicot protein (Kieliszewski and Lamport, 1987).

Photographs of kernel tissue prints of the three maize varieties reacted with primary antibody MC-3 are shown in Fig. 2. At 0 d.a.p. staining is general: the ovule, the tips of the glumes, and the silk scar show staining. At 5 d.a.p. the ovules have enlarged and the pericarp is emphasized. JHL shows most distinctly the concentration of stain at the tip of the ovule. At 10 d.a.p. most of the pericarp stains, but it appears darkest at the tip or silk scar. As the kernels mature to 20 and 30 d.a.p. the intensity of staining moves from the tip of the kernel down the sides toward the base. By 30 and 40 d.a.p. the majority of the staining is associated with the embryo and base of the kernel. Soluble HRGPs appear to be deposited primarily at the tip of the ovule or silk scar at 5 d.a.p., extending through the pericarp to surround the growing embryo at 10 and 20 d.a.p. and becoming progressively less soluble or detectable within the pericarp while accumulating in the developing embryo at 30 and 40 d.a.p. By 40 d.a.p. this HRGP also appears to become insolubilized as staining is less intense and does not cover the whole embryo. South American Yellow popcorn varies slightly from this pattern. Staining is general in both 0 and 5 d.a.p. kernels. At day 10, the silk scar stains intensely in addition to the rest of the pericarp. At 20 d.a.p., HRGPs surround the ovule in the pericarp, and at 30 d.a.p. the basal region and embryo are emphasized. By 40 d.a.p. faint staining is present around the pericarp and embryo but most HRGPs are not detectable. Deposition and possible insolubiliz-
Fig. 2. Antibody reaction with tissue prints of kernels of three maize varieties throughout development. Maize kernels at 0, 5, 10, 20, 30 and 40 days after pollination were printed onto nitrocellulose as described in Materials and methods. Tissue prints were reacted with a monoclonal antibody raised against PC-1 and diluted 1:10000, then treated with a secondary antibody conjugated to alkaline phosphatase diluted 1:10000. Individual kernels were photographed and printed at the same magnification.

Antibody reactions with embedded kernels of SAY, JHL and GXB at 0, 5, 10, 20, 30 and 40 days after pollination. Photographs show pericarp near the top of the developing kernels. Staining is quite faint in the earlier developmental stages of 0 and 5 d.a.p., but that which is present seems to be concentrated at the silk scar or tip of the kernel. Pericarp from JHL and GXB at 10 d.a.p. shows very little staining, while that from SAY at 10 d.a.p. shows light pink staining. The outermost cell layers stain first. As the kernels develop the staining becomes more intense first at the top of the kernel (20 d.a.p.), then gradually extending down the sides of the pericarp toward the base (30 d.a.p.). Pericarp staining is different on opposite sides of the kernel (data not shown). The elongated cells in pericarp next to the embryo shoot tip of SAY at 20 d.a.p. are less intensely stained than those rounder cells opposite the endosperm on the anterior side. Elongated pericarp cells at the top of the kernel are darker than round cells at the base of the kernel. This pattern is also evident at 30 and 40 d.a.p. Staining intensity peaks at 30 d.a.p. for the two popcorns and then declines at 40 d.a.p. GXB shows a different staining pattern, increasing in intensity from 20 to 40 d.a.p.

As shown in Fig. 3B, only with TBST controls (no primary antibody) show slight pink staining of embryonic root and shoot tissue, as well as faint pink intracellular staining, indicating that there is some native alkaline phosphatase activity in situ but it is not significant at the 1:2000 secondary antibody dilution used. Controls of preimmune serum and primary antibody (MC-1) absorbed with PC-1 protein do not show the root and shoot tip staining but do show the faint intercellular staining. Controls using primary antibody MC-1 absorbed with 5% bovine serum albumin, CaCl$_2$ extract from carrot roots, or 15 &micro;g d-arabinose, all show strong staining.

Discussion

We have characterized the accumulation of HRGPs in maize pericarp throughout seed development in three maize varieties. These experiments confirm that PC-1 protein, a hydroxyproline-rich glycoprotein isolated from the pericarp of maize, is developmentally regulated. These results are similar to those found for soybean seed coat, which is another protective tissue that accumulates HRGPs throughout development (Cassab et al. 1985; Cassab and Varner, 1987).

As evidenced by tissue prints, in situ cytolocalization and hydroxyproline assays (K. R. Hood, S. E. Fritz and E. E. Hood, unpublished data), HRGPs are initially soluble and present in low quantities even in very young tissue. As the maize ovule matures HRGPs become increasingly

were carried out. Experiments varying the primary and secondary antibody dilution factors were carried out to optimize the staining reaction. Slides treated with lower dilutions of primary antibody, 1:2000 and 1:5000, and a 1:2000 dilution of secondary antibody stained tissues more brilliantly purple than slides treated with 1:10000 primary antibody and 1:2000 secondary antibody dilutions. Experiments in which the secondary antibody was diluted to 1:10000 while the primary antibody was varied between 1:2500, 1:5000 and 1:10000 did not show any differences between staining intensity, being stained lightly or brown instead of distinctly purple. Non-specific background staining of the tissue increases at the lower secondary antibody dilutions, but is not significant.
Fig. 3. Antibody reaction with embedded sections of kernels from three maize varieties throughout development. (A) Columns A, B and C represent maize varieties GXB, JHL and SAY, respectively. Individual pictures represent pericarp sections from kernels at 0, 5, 10, 20, 30 and 40 d.a.p. in descending order from top to bottom of each column. All photographs were taken at the same magnification and printed at the same enlargement. In all three varieties, the pre-pericarp tissue (i.e. integument) is visible as a wide soft tissue composed of many cell layers. In kernels at 20, 30 and 40 d.a.p. it is composed of the same number of cell layers but has collapsed into a narrow darkly staining tissue.

Primary antibody dilution was 1:2000 and secondary antibody dilution was 1:2000. p, pericarp. Bar, 150 μm.

(B) Controls to test specificity of antibody staining of tissue sections shown in A. All kernel sections are from SAY popcorn at 30 d.a.p.
Primary antibody dilution was 1:2000 and secondary antibody dilution was 1:2000. From left to right on row 1: no primary antibody but normal secondary antibody; preimmune serum in place of primary antibody; primary antibody preabsorbed with PC-1 protein. From left to right on row 2: primary antibody preabsorbed with salt extract of carrot cell walls; primary antibody preabsorbed with D-arabinose; primary antibody preabsorbed with 5% BSA.
abundant in the pericarp and, eventually, in the embryo shoot and root tips. Pericarp does not stain in tissue prints at late stages of popcorn development, although the embryo stains. This indicates that the embryo is producing soluble HRGPs even though the staining is faint in the in situ sections. This difference in staining intensity may be due to the difference in amount of tissue sampled. In situ sections were 3 μm while HRGPs measured from tissue prints may represent soluble proteins from a greater depth. Tissue print data suggest that less protein is being synthesized and secreted as the kernel ages and dries although salt solubility of hydroxyproline from isolated cell walls is not different at any of these stages (K. R. Hood, S. E. Fritz and E. E. Hood, unpublished data). However, insoluble hydroxyproline-antibody. GXB at 40 d.a.p. contrasts fairly strongly with the accumulation and possible insolubilization of these proteins may occur simultaneously. In GXB, staining intensity of the pericarp increases up to 40 d.a.p., while in SAY and JHL staining intensity is maximal at 30 d.a.p. and decreases at 40 d.a.p. Actual hydroxyproline levels as determined by biochemical assays indicate that HRGPs continue to increase or remain the same in the three genotypes at 40 d.a.p. (K. R. Hood, S. E. Fritz and E. E. Hood, unpublished data). However, insoluble hydroxyproline does not increase significantly; thus these HRGPs are presumably not being cross-linked into the wall though they have lost antigenic reactivity (Fig. 3A). The loss of antigenic reactivity could be explained if, between 30 and 40 d.a.p., there is a chemical change in this protein configuration that modifies the epitope that the antibody recognizes. Alternatively, there could be several genes differentially expressed during kernel development that are recognized with different affinities by our monoclonal antibody, but contain the hydroxyproline assayed by biochemical means. The pericarp in later stages is most darkly stained at the top of the kernel or near the silk scar, gradually decreasing in staining intensity at the base of the kernel. There also appear to be differences in deposition and cellular morphology between anterior and posterior sides of the kernel. In the anterior region next to the developing embryo the pericarp cells are large and elongated, while those on the posterior side next to the endosperm are smaller, rounder and more densely stained. At the top of the kernel the pericarp is thin, and cells are elongated and densely stained for HRGPs (data not shown). The biological significance of these observed differences is not known.

The amounts of total and soluble HRGP protein that accumulate in developing embryos have not been determined. However, on the tissue prints, pericarp in kernels at 0–10 d.a.p. and embryo axis in kernels at 20–40 d.a.p. stain at approximately equal intensities. In addition, in situ sections of 0–5 and 10 d.a.p. show faint pink staining in pericarp similar to the staining of embryo axes at 20–40 d.a.p. Therefore, we conclude that these embryos may have similar quantities of HRGPs as 5 d.a.p. pericarps.

Characterization of the process of HRGP accumulation, deposition, and of tissue and varietal specificity, will help elucidate the function of this interesting protein in normal plant development. Subsequently we will determine its possible role in maintaining structure in a perturbed environment, such as during wounding or disease attack.

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