Pumpkin phloem exudate contains two abundant phloem proteins: PP1 is a 96-kD protein that forms polymeric filaments in vivo, and PP2 is a 48-kD dimeric lectin. Polyclonal antibodies raised against pumpkin phloem exudate were used to isolate several cDNAs corresponding to PP1 and PP2. RNA gel blot analysis indicated that PP1 is encoded by an mRNA of ~2500 nucleotides, whereas PP2 subunits are encoded by an mRNA of 1000 nucleotides. Sequence analysis of PP2 cDNAs revealed a 654-bp open reading frame encoding a 218-amino acid polypeptide; this polypeptide had the carbohydrate binding characteristics of a PP2 subunit. The PP2 mRNA was localized within the phloem of pumpkin hypocotyl cross-sections based on in situ hybridization of a digoxigenin-labeled antisense probe. PP2 mRNA was found within the companion cells in both the bicolateral vascular bundles and the extrafascicular phloem network.

INTRODUCTION

Protein inclusions have been observed in phloem tissue of dicotyledonous plants for many years using light microscopy (reviews by Cronshaw and Sabnis, 1990; Evert, 1990). The term P-protein (phloem protein) was introduced to describe these proteinaceous filaments and aggregations observed in sieve elements by transmission electron microscopy (Cronshaw and Esau, 1967; Esau and Cronshaw, 1967). These proteins are a major component of the cytoplasmic contents of sieve elements; they are synthesized very early in phloem ontogeny and persist in senescent sieve elements. Although P-proteins are a major component of sieve elements, P-protein bodies have also been observed in companion cells and phloem parenchyma cells of developing cucurbit phloem tissue (Cronshaw and Esau, 1968a, 1968b).

Phloem exudates from Cucurbita species contain high concentrations of P-protein filaments and have been a useful source for the biochemical analysis of P-proteins. Phloem exudates from pumpkin contain two abundant proteins with similar properties (Kollmann et al., 1970; Beyenbach et al., 1974; Read and Northcote, 1983). The two proteins, PP1 (phloem protein 1) and PP2 (phloem protein 2), are basic polypeptides that have similar amino acid compositions and are components of phloem filaments (Beyenbach et al., 1974; Weber et al., 1974). The PP1 monomer is a 96-kD protein that cross-links with other PP1 monomers by disulfide linkage, forming soluble polymers in vivo (Walker, 1972; Beyenbach et al., 1974; Sabnis and Hart, 1979; Read and Northcote, 1983). Upon oxidation, PP1 polymers cross-link to form an insoluble matrix or gel (Walker, 1972; Read and Northcote, 1983). PP1 appears to be the primary structural protein involved in the formation of slime plugs that are seen at sieve plates in electron micrographs of disrupted vascular tissues (Walker and Thaine, 1971). PP2 is a 48-kD dimeric lectin that preferentially binds oligomers of N-acetyl-D-glucosamine (Beyenbach et al., 1974; Sabnis and Hart, 1978; Allen, 1979; Read and Northcote, 1983). Unlike PP1, purified PP2 is soluble when exposed to either atmospheric oxygen or oxidizing agents. Within the phloem filaments, PP2 is covalently linked to PP1 by disulfide bridges (Kleinig et al., 1975; Read and Northcote, 1983).

Many unresolved questions exist regarding the function of P-proteins and the physiological relationships between phloem cell types. An intriguing relationship exists between the sieve elements and their respective companion cells. The mature sieve element–companion cell complex contains two ontogenetically related cell types that are morphologically and physiologically distinct, yet appear to be functionally interactive. Within the two cell types, P-proteins have been observed in developing companion cells and sieve elements as well as mature sieve elements. Because the nucleus and ribosomes degenerate during the maturation of the sieve element, P-protein synthesis could occur either in the immature sieve elements, companion cells, or both. Resolution of these questions requires more understanding of the cell specificity and developmental timing of P-protein synthesis.

We are interested in understanding the developmental regulation of P-protein gene expression in pumpkin to ultimately address questions about P-protein synthesis, accumulation, interactions, and function in phloem tissue. We raised...
polyclonal antibodies against the total phloem exudate from pumpkin seedlings and used them to isolate cDNAs corresponding to P-proteins. One group of cDNAs contained an open reading frame (ORF) that encoded a polypeptide with the physical and functional characteristics of PP2. Several partial cDNA clones were also isolated that have features corresponding to PP1 mRNA. In situ hybridization of antisense probes localized the PP2 mRNA within the companion cells of phloem tissue in pumpkin hypocotyls.

RESULTS

Isolation of P-Protein cDNAs

To obtain cDNA clones corresponding to PP1, PP2, and additional pumpkin P-proteins, we raised polyclonal antibodies in chickens against total reduced proteins from pumpkin phloem exudate. A complex antiserum was obtained that reacts with many of the phloem exudate proteins resolved by SDS-PAGE, as shown in Figure 1 (lane 2 of SDS-PAGE and lane 1 of the immunoblot). To determine if the antiserum was specific for phloem exudate proteins, we tested it for cross-reactivity with proteins isolated from pumpkin callus tissue (Figure 1, lane 3 of SDS-PAGE and lane 2 of the immunoblot). Although callus tissue contains a large number of abundant proteins, the antiserum cross-reacted with only a single protein band. This protein has the mobility of PP1 and may reflect PP1 synthesis in differentiating phloem cells within the callus, because P-proteins have previously been observed in differentiating sieve elements in squash callus tissue (Lackney, 1991). The absence of cross-reactivity between the antiserum and proteins from callus tissue reflects its specificity for the phloem exudate proteins. The preimmune serum did not cross-react with proteins from pumpkin phloem exudate or pumpkin callus.

To identify mRNAs corresponding to phloem proteins, an expression cDNA library was constructed with poly(A)⁺ RNA isolated from pumpkin seedlings. This library was screened with the phloem protein antiserum and 22 immunopositive plaques were obtained; the 10 most immunoreactive phages were selected for further analysis. To determine if these 10 clones represented unique or related sequences, we excised the pBluescript SK- plasmid containing each cDNA from...
the λ ZAP clone, purified the DNA, and performed a cross-
hybridization analysis. The results of these experiments showed
that the 10 cDNAs represented two groups of closely related
or identical sequences (data not shown). Clones with the largest
cDNA inserts were selected for detailed analysis and were
designated cPC7 (1.38 kb) and cPC13/20 (cPC13 is 980 bp and
cPC20 is 792 bp).

Identification of the Proteins Encoded by
Phloem cDNAs

To determine the type of P-protein encoded by each clone, the
β-galactosidase fusion proteins were used to affinity purify spe-
cific antibodies from the complex antiserum. Each of the
purified antibodies was reacted with a protein blot of total
phloem exudate. Figure 2 shows the result of one such analy-
sis; cPC7 encodes a polypeptide that is immunologically
related to PP1 (lane 7), whereas both cPC13 and cPC20 en-
code a polypeptide that is immunologically related to PP2
(lanes 13 and 20).

As seen in Figure 3, RNA gel blot hybridization of total and
poly(A)* RNA from pumpkin seedlings with cPC7 and cPC13

showed that the corresponding mRNAs are abundant and dis-
tinct species. cPC13 hybridized to a single mRNA of ~1000
nucleotides, which agrees with previous measurements of the
mRNA encoding PP2 (Sham and Northcote, 1987). In contrast,
cPC7 hybridized to an mRNA of ~2500 nucleotides corre-
sponding to the calculated size of an mRNA encoding PP1.
This clone encodes ~1380 nucleotides and is not a full-length
copy of the PP1 mRNA.

The results of the immunoblot and RNA gel blot analyses
suggested that CPC13/20 encoded PP2. Nucleotide sequence
analysis of CPC13/20 revealed an ORF of 654 nucleotides. The
ORF encodes an entire PP2 subunit; the deduced polypep-
tide of 218 amino acids shown in Figure 4 has a calculated
molecular mass of 24,478 D. As shown in Table 1, this poly-
peptide is rich in glycine (10.1%), leucine (10.1%), and lysine
(9.6%). The amidated and acidic amino acids account for 20.6%
(A Sax 10.1%, Glx 10.5%) of the total amino acid content. As-
paragine is more prevalent than glutamine (Asn 6.0%, Gin
3.2%), whereas glutamic acid is more abundant than aspar-
atic acid (Glu 7.3%, Asp 4.1%). The polypeptide has six cysteine
and no threonine residues. The six cysteine codons of
CPC13/20 agree with the six cysteine residues identified within
fully reduced PP2 polypeptides by biochemical methods
(Beyenbach et al., 1974; Read and Northcote, 1983). The ab-
sence of threonine codons concurs with the amino acid
analysis of the squash phloem lectin reported by Alien (1979),
but contrasts with the analysis reported by Beyenbach et al.
(1974) who corrected the serine and threonine values for decom-
position. We also compared the amino acid composition of
the deduced polypeptide with experimentally determined
amino acid compositions of phloem lectins from pumpkin
(Beyenbach et al., 1974) and squash (Alien, 1979). All three
have similar amino acid compositions (Table 1).

To demonstrate carbohydrate binding activity of the poly-
peptide encoded by cPC13/20, we inserted the entire ORF,
including the translation termination codon, into a T7
Table 1. Deduced and Observed PP2 Amino Acid Compositions of cPC13/20 and Previously Published Biochemical Quantitations

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a aa, amino acids.
b Published values were adjusted to reflect a deduced polypeptide of 218 amino acids.
c Allen and Beyenbach et al. reported values for Asx and Glx.

expression vector, pRSETB, and synthesized a fusion protein in Escherichia coli. Synthesis of the fusion protein was initiated from the pRSETB vector sequence, which increased the molecular size of the fusion protein by 3740 D. Following induction, the fusion protein accumulated to high levels in the bacteria, as shown in Figure 5A. The fusion protein was affinity purified to homogeneity from the crude bacterial lysate by batch adsorption to ovomucoid-acryl beads. Elution from the affinity matrix with 1 mM tris(N-acetyl-D-glucosamine) resulted in a quantitative recovery of the protein (Figure 5B). The pRSET vector also encodes a metal binding protein domain that is expressed as the N-terminal portion of the recombinant protein. A fusion protein encoded by the chloramphenicol acetyltransferase (CAT) gene in pRSET also contains the metal binding domain. However, the CAT fusion protein did not bind to the ovomucoid affinity matrix, demonstrating that binding was specific to the polypeptide region of the fusion protein encoded by cPC13/20 and not the metal binding domain.

In Situ Hybridization of PP2 mRNA

Previous studies have shown that the phloem lectin occurs in both sieve elements and companion cells (Smith et al., 1987). To obtain further evidence that the cDNA clone we isolated corresponded to a phloem-specific protein and to determine its site of synthesis, we localized PP2 mRNA by in situ hybridization. Cross-sections of pumpkin hypocotyl tissue were incubated with in vitro--synthesized transcripts labeled with digoxigenin-11-UTP. The use of this nonisotopic labeling method allowed us to achieve high spatial resolution of the signal with retention of tissue morphology. Figure 6 shows the complex phloem anatomy of the pumpkin seedling hypocotyl. The Cucurbitaceae is one of several plant families that have bicollateral vascular bundles composed of internal and external phloem (fascicular phloem). A second feature that adds to the complexity of cucurbit phloem anatomy is the extrafascicular phloem, which occurs in strands within the cortex and in arcs bordering both sides of the bundle (Crafts, 1932; Blyth, 1958). In addition to the primary phloem, secondary phloem within the vascular bundle is derived from a vascular cambium.

PP2 antisense transcripts hybridized to mRNA within the phloem of hypocotyl tissues and are visible as a blue-purple precipitant after the alkaline phosphatase reaction, as seen in Figure 7. PP2 transcripts occurred in both the bundle and extrafascicular phloem tissue, although labeling of individual sections was variable. In certain sections, label was only detected in the extrafascicular arc of the phloem (Figure 7A), whereas, in others, signal appeared in both the bundle phloem and the extrafascicular phloem (Figure 7B). In addition, the extrafascicular phloem strands within the cortex were often labeled (Figures 7C and 7D).

The nonisotopic labeling method can detect transcripts within individual cell types using light microscopy. We identified sieve
In Situ Localization of PP2 mRNA

Figure 5. Carbohydrate Binding Activity of the Fusion ProteinEncoded by cPC13.

The ORF encoded by cPC13 was amplified by PCR and inserted into the protein expression vector pRSETB (Invitrogen).

(A) A Coomassie-stained SDS-polyacrylamide gel of the E. coli crude lysate at 0, 1, 2, 3, 4, and 5 hr following the induction of the fusion protein. The letter M designates molecular size markers. In descending order, the molecular size markers are 106, 80, 49.5, 32.5, 27.5, and 18.5 kD. Synthesis of the fusion protein increases the molecular size of PP2 by 3.74 kD.

(B) A silver-stained SDS-polyacrylamide gel of PP2 and PP2 fusion protein affinity purification by ovomucoid-acryl beads. In separate reactions, total crude phloem exudate from pumpkin seedlings (lane 1) and crude E. coli lysate containing the PP2 fusion protein (lane 3) were adsorbed onto ovomucoid-acryl beads. Purified PP2 (lane 2) and fusion PP2 (lane 4) were eluted from the matrix with 1 mM tri(N-acetyl-D-glucosamine). Crude E. coli lysate containing the CAT fusion protein (lane 5) did not bind to the affinity matrix (lane 6). The letter M indicates molecular size markers. In descending order, the molecular size markers are 106, 80, 49.5, 32.5, 27.5, and 18.5 kD.

DISCUSSION

Although several hypotheses have been proposed regarding the role of P-proteins in phloem cells, the function of these proteins is unknown. Early proposals that P-protein filaments play an active physical role in translocation, or that P-proteins may be degradation products formed during the maturation of sieve elements, have generally been discarded (Cronshaw, 1975). Current hypotheses suggest that the lectin (PP2) binds to glycoconjugates of either the sieve element reticulum or the plasma membrane, thereby anchoring the P-protein filaments (Sabnis and Hart, 1978; Smith et al., 1987). The loss of hydrostatic pressure that occurs upon phloem wounding disrupts the lectin–carbohydrate interaction releasing the filaments from their parietal positions into the assimilate stream. The filaments could block the flow of assimilates at the sieve plate by forming slime plugs and oxidize at the wound surface to seal the wound (Sabnis and Hart, 1979). A variation of this hypothesis suggests that PP1 filaments form a gel creating a physical...
Figure 7. In Situ Localization of PP2 mRNA in Pumpkin Hypocotyl Cross-Sections.
barrier and that the associated PP2 lectins bind fungi and bacteria as a biochemical barrier to microbial ingress into the wounded tissue (Read and Northcote, 1983). There is little experimental evidence to either support or reject these hypotheses, because assessing the function of these proteins is technically complex and the tools to perform these analyses have not been available. The molecular probes we have developed will allow us to investigate the roles of these proteins in the phloem.

The analysis of the protein encoded by cPC13/20 is consistent with the conclusion that these clones encode the phloem lectin. Assuming this is true, one would predict that the corresponding mRNA transcripts would originate from either sieve elements, companion cells, or both. The use of nonisotopic labeling methods for in situ localization of mRNA allowed us to localize PP2 mRNA, with a high degree of resolution, within the companion cells in seedling hypocotyls. The localization of this mRNA parallels the pattern of PP2 distribution in bundle and extrafascicular phloem previously reported by Smith et al. (1987). They found that polyclonal lectin antibodies bound predominantly to the P-protein of the cortical extrafascicular phloem and in the arcs of extrafascicular phloem located on either side of vascular bundles. Our localization of PP2 mRNA in extrafascicular phloem clearly mimics the localization of the protein in both the extrafascicular arcs and the cortical strands. In addition, Smith et al. (1987) reported that the fascicular phloem of mature stems stained lightly, while the fascicular phloem of older stems stained strongly. We also detected transcripts in the fascicular phloem, although less often than in the extrafascicular phloem.

We detected PP2 mRNA only in the companion cells, whereas PP2 protein was immunocytochemically localized in both the companion cells and sieve elements (Smith et al., 1987). Both of these results are consistent with our current knowledge of sieve element and companion cell contents. Gietl and Ziegler (1979) found no evidence of mRNA or other components of the translational machinery in exudates from mature sieve elements. Because the nucleus and ribosomes degenerate during sieve element maturation, it is not surprising that we did not detect PP2 mRNA in these cells.

Based on the abundance of P-protein in the mature sieve elements, it is easy to envision transcription and protein synthesis within the immature sieve elements. P-protein bodies have been observed in immature sieve elements and companion cells of cucurbits (Cronshaw and Esau, 1968a).

The perplexing issue is the accumulation of protein and mRNA within mature companion cells. The presence of PP2 mRNA and protein in mature companion cells suggests that protein synthesis may be active within a cell type where P-proteins are not expected to accumulate. Smith et al. (1987) speculated that PP2 could be synthesized in the companion cells and subsequently transported into the sieve elements via plasmodesmata. Our results support this hypothesis. However, the localization of PP2 mRNA in seedling hypocotyl tissue was done at a single stage of development where the primary phloem tissue was mature. Thus, we cannot eliminate the possibility that PP2 synthesis occurs in both cell types during early stages of cell differentiation. We are currently using in situ hybridization and immunocytochemical techniques to examine the temporal and spatial pattern of PP2 gene expression during phloem differentiation.

Biochemical analyses of PP2 have shown that this protein is a dimer and has carbohydrate binding characteristics typical of plant lectins. Read and Northcote (1983) showed that PP2 is composed of two subunits, α (M, 26,500) and β (M, 25,000), joined by disulfide linkages between cysteine residues. However, separation of the subunits could only be observed when iodoacetamide-treated PP2 dimers were fully reduced and separated on SDS–polyacrylamide gels at pH 9.2. Comparing the deduced and experimentally determined amino acid content of PP2 suggests that the two subunits are similar. Whether the two subunits are encoded by different genes or if their differences in mobility by SDS-PAGE are due to transcriptional processing or post-translational modification of a single gene product is unknown. In addition, little is known about the specific protein–carbohydrate interactions that occur between PP2 and the chitin ligand. A conserved 43-amino acid domain that is typical of most chitin binding proteins (Chrispeels and Raikhel, 1991) could not be identified in PP2; thus, PP2 appears to be structurally distinct from other chitin binding lectins.

METHODS

Materials

Restriction endonucleases were from Bethesda Research Laboratories. α[32P]-dATP or dCTP (3000 Ci/mmol) and α[35S]-dATP (1000 to 1500 Ci/mmol) were from DuPont-New England Nuclear. Oligonucleo-
otides were synthesized by the University of Arizona Macromolecular Structure Facility, Tucson. Random primer radioactive labeling kits were from Ambion (Austin, TX). Polymerase chain reaction (PCR) reagent kits were from Perkin Elmer Cetus. Nitrocellulose and Nytran membranes were from Schleicher & Schuell.

Isolation of Pumpkin Phloem Exudate Proteins and Callus Proteins

Pumpkin (Cucurbita maxima Duch. cv Big Max) seedlings were grown at 25°C with a 16-hr photoperiod. At 10 to 12 days after germination, seedling hypocotyls were cut and microf acr quantities of phloem exudate were collected in microtubes. The exudate was diluted 1:4 in a deated extraction buffer consisting of 0.1 M Tris, pH 8.2, 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and either 100 mM 2-mercaptoethanol or 20 mM DTT (Read and Northcote, 1983). Pumpkin callus cultures were initiated from surface-sterilized leaf tissue on a medium consisting of MS salts (Murashige and Skoog, 1962), 3% sucrose, 0.4 mg L-1 thiamine, 100 mg L-1 myo-inositol, 0.5 mM kinetin, 13.6 mM 2,4-D, pH 5.6, and 0.6% agar. After 4 weeks, the callus was transferred to a similar medium containing 5 mM benzyladenine and 0.5 mM 2,4-D (Kim et al., 1988). These cultures were grown under constant illumination at 22°C. Pumpkin callus tissue (0.5 g) was homogenized in 500 μL of extraction buffer containing 100 mM 2-mercaptoethanol. The cellular debris was pelleted by centrifugation at 10,000 × g for 5 min, and the supernatant was collected.

Affinity Purification of P-Protein Antibodies

Plaque-purified λ ZAP II bacteriophage containing the individual cDNAs cPC7, cPC13, or cPC20 were each grown to confluency on separate plates. The expression of fusion proteins was induced by placing a nitrocellulose filter impregnated with 50 mM isopropyl β-thiogalactoside on each plate at 37°C. The nitrocellulose filters with the bound fusion proteins were washed with TTBS (10 mM Tris, pH 7.4, 140 mM NaCl, 0.15% [v/v] Tween 20), blocked with 1% gelatin in TTBS, and incubated with the complex antiserum (1:2500) diluted in TTBS. After the non-specific antibodies were removed with several washes of 1% Triton X-100 in TTBS, the specific antibodies were eluted from the filters with 0.2 M glycine, pH 2.8, 1 mM EDTA, neutralized with 0.1 volume of 1 M Tris base, and concentrated by precipitation with an equal volume of 4 M (NH4)2SO4. The pellet was resuspended in 1 mL of 10 mM Na2HPO4, pH 7.5, and dialyzed overnight against 10 mM NaH2PO4, pH 7.5, and 0.01% NaN3 at 4°C. Immunoblot analysis and visualization were conducted as described above.

RNA Isolation and Blotting

Total RNA was extracted from hypocotyl tissue of pumpkin seedlings 10 to 12 days after germination using a scaled-down guanidine-HCl isolation method originally described by Sambrook et al. (1989). Poly(A)+ RNA, purified on Hybond mAP paper (Amersham International), was used as the template for cDNA synthesis (Pharmacia cDNA synthesis kit). EcoRI/NotI adapters were ligated to the double-stranded cDNA, and the cDNAs were inserted into the EcoRI-digested bacteriophage λ ZAP II (Stratagene). The bacteriophage were packaged with Gigapack II Gold packaging extract (Stratagene) and grown in XLI-Blue Escherichia coli host cells. The initial cDNA library contained ~5.6 x 10^6 recombinant plaques and was amplified to an approximate titer of 2.3 x 10^7 plaque-forming units per μL. A total of 5 x 10^4 phage clones were grown in XLI-Blue host cells, and β-galactosidase fusion proteins were induced by placing nitrocellulose filters impregnated with 50 mM isopropyl β-thiogalactoside onto the plated bacteriophage. The filters were incubated with the phloem exudate antiserum, and plaques immunoreactive with RAC antibodies conjugated to alkaline phosphatase were visualized by BCIP/NBT color development. Immunopositive plaques were purified, and the pBlue-script SK+ phagemids containing the cDNAs were excised according to the manufacturer's instructions.

Electrophoresis and Blotting of Proteins

Total reduced phloem exudate proteins and reduced soluble callus proteins were separated by SDS-PAGE in a 12.5% acrylamide gel (Laemmli, 1970). Following electrophoresis, the gel was cut and microf acr quantities of phloem exudate were collected in microtubes. The exudate was diluted 1:4 in a deated extraction buffer consisting of 0.1 M Tris, pH 8.2, 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and either 100 mM 2-mercaptoethanol or 20 mM DTT (Read and Northcote, 1983). Pumpkin callus cultures were initiated from surface-sterilized leaf tissue on a medium consisting of MS salts (Murashige and Skoog, 1962), 3% sucrose, 0.4 mg L-1 thiamine, 100 mg L-1 myo-inositol, 0.5 mM kinetin, 13.6 mM 2,4-D, pH 5.6, and 0.6% agar. After 4 weeks, the callus was transferred to a similar medium containing 5 mM benzyladenine and 0.5 mM 2,4-D (Kim et al., 1988). These cultures were grown under constant illumination at 22°C. Pumpkin callus tissue (0.5 g) was homogenized in 500 μL of extraction buffer containing 100 mM 2-mercaptoethanol. The cellular debris was pelleted by centrifugation at 10,000 × g for 5 min, and the supernatant was collected.

Isolation of Phloem Exudate Antibodies

Antibodies were raised in laying hens against the total phloem exudate isolated from pumpkin seedlings. A 100-μL aliquot of the diluted phloem exudate sample (~750 μg of total protein) was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously. Two subsequent injections with an equal amount of phloem exudate and Freund's incomplete adjuvant were made at 2-week intervals. Eggs were collected 1 week following the final injection, and antibodies were purified from egg yolks as described by Carroll and Stoller (1983) and Song et al. (1985). Antibody preparations were used at dilutions of 1:2500.
DNA Sequence Analysis

Enzymatic sequencing was accomplished by the dideoxy chain termination method according to the instructions accompanying the Sequenase version 2.0 sequencing kits (U.S. Biochemicals). Computer-aided analysis of the nucleotide and amino acid sequences was performed with programs supplied by the Genetics Computer Group (Madison, WI), Microgenius computer program (Beckman Instruments, Inc., Palo Alto, CA), and Genejockey computer program (Biosoft, Cambridge, UK).

Synthesis of PP2 Fusion Proteins

A 678-bp sequence of cPC13 that included the complete PP2 ORF was amplified by PCR. Two 25-bp oligonucleotide primers were designed from the sequence of cPC13: the 5' oligonucleotide contained the translation initiating methionine codon and the 3' oligonucleotide contained the translation stop codon. The ORF was amplified for 30 cycles (95°C for 1 min, 55°C for 1 min, 72°C for 1 min) and ligated in frame into the protein expression vector pRSETB (Invitrogen, San Diego, CA). The construction was sequenced to confirm the maintenance of the reading frame, and fusion protein was synthesized according to the manufacturer's instructions.

Lectin Binding Assay

The lectin binding assay was based on the method described by Read and Northcote (1983). Five hundred milligrams of ovomucoid-acryl beads (Sigma) wasequilibrated in binding buffer (50 mM Tris, pH 8.2, 500 mM NaCl, 3 mM NaN₃, 2 mM EDTA, 0.5% Triton X-100, 20 mM DTT). Twenty micrograms of total phloem protein or 25 µL of the fusion protein lysate in 100 µL of binding buffer was adsorbed to 20 µL of ovomucoid-acryl beads with constant mixing at 4°C for 2 hr. The supernatant was removed, and the affinity matrix was washed three times with 1 mL of the binding buffer. The purified PP2 was eluted from the matrix in 100 µL of 1 mM N₃-N₅-N₇-triacetylchitotriose (Sigma) with constant mixing at 4°C for 30 min. The eluate was removed and the matrix was washed two additional times with 100 µL of 1 mM triacetylchitotriose. The eluate and washes were combined, vacuum dried, and dissolved in 20 µL of distilled water.

In Situ Hybridization

Hypocotyl pieces of pumpkin seedlings 10 to 12 days after germination were fixed at room temperature in 2% glutaraldehyde/50 mM KPO₄, pH 7.0, for 3 hr, then dehydrated and embedded in paraffin. Paraflin blocks were sectioned at 10 µm and mounted on poly-L-lysine-coated slides. Digoxigenin-labeled sense and antisense riboprobes were synthesized by in vitro transcription from pBluescript KS+ (Stratagene) that contained the cDNA template pCPC20 (PP2). Plasmids were linearized and digoxigenin-11-UTP was incorporated with either T3 or T7 polymerase according to the manufacturer's instructions (Boehringer Mannheim). The slides were then washed four times in descending concentrations of SSC (2 x, 1 x, 0.5 x, 0.1 x) with 1 mM DDT for 30 min each. The first three washes were at 37°C, and the final wash was at 42°C. Hybridization of the riboprobes was detected with antidigoxigenin antibodies conjugated to alkaline phosphatase and visualized by color development with BCIP and NBT (Boehringer Mannheim).

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