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Expression of DcPRP1 Is Linked to Carrot Storage Root Formation and Is Induced by Wounding and Auxin Treatment[1]

William Ebener, Thomas J. Fowler, Hideki Suzuki, Jonathan Shaver, and Mary L. Tierney*

Agronomy Department, Ohio Agricultural Research and Development Center and Biotechnology Center, Ohio State University, Columbus, Ohio 43210

A carrot (*Daucus carota*, L.) genomic clone (DcPRP1) was isolated on the basis of its homology to previously described cDNAs encoding a wound-inducible, proline-rich cell wall protein. DNA sequence analysis showed that DcPRP1 contains a single open-reading frame encoding a 235-amino acid protein that is colinear with that predicted from the cDNA sequence with the exception of four amino acids at the N terminus and a 60-nucleotide insertion present within the genomic clone. Genomic Southern hybridization analysis showed that the cloned sequence hybridized with a single restriction enzyme fragment using several restriction enzymes. Primer extension and northern hybridization analysis indicated that the expression of DcPRP1 is developmentally regulated and linked to the formation of storage roots, where this gene is expressed at high levels after wounding. The level of DcPRP1 mRNA was greatest in tissue immediately adjacent to the wound site. Treatment of unwounded carrot storage roots with 10 μM 2,4-dichlorophenoxyacetic acid, indoleacetic acid, or naphthalene-1-acetic acid also resulted in the accumulation of DcPRP1 transcripts to a level equal to that seen in wounded tissue.

PRPs are one class of structural cell wall proteins that are thought to participate in the formation of cell type-specific matrices in the plant cell wall. PRPs were first identified as wound-inducible transcripts encoding a cell wall protein (p33) in carrot storage roots (Chen and Varner, 1985; Tierney et al., 1988) and are basic proteins composed mainly of Pro, Hyp, Val, Tyr, Lys, Thr, His, and Glu (Averyhart-Fullard et al., 1988; Kleis-San Francisco and Tierney, 1990; Lindstrom and Vodkin, 1991). Amino acid and DNA sequence analysis indicates that PRPs consist of a signal sequence that is cleaved during secretion of the protein into the cell wall, followed by tandem repetitions of the sequence Pro-Pro-Val-X-Lys (Pro-Hyp-Val-X-Lys in the native protein sequence), where X is often Tyr, His, or Glu (Chen and Varner, 1985; Franssen et al., 1987; Hong et al., 1987; Hong et al., 1990; Datta and Marcus, 1990; Kleis-San Francisco and Tierney, 1990; Lindstrom and Vodkin, 1991; Sheng et al., 1991). Some PRPs (SbPRP1, 2, and 3) are composed only of tandem repeats of Pro-Pro-Val-X-Lys, whereas others (carrot p33 and PvPRP1) contain unique regions of sequence interspersed between these repetitive elements.

Members of the PRP gene family have been shown to be developmentally regulated. For example, they are expressed during the early stages of pea and soybean root nodule formation (Govers et al., 1986; Franssen et al., 1987; Van de Wiel et al., 1990) and during seedling, leaf, and seed development in soybean (Tierney et al., 1988; Hong et al., 1989; Kleis-San Francisco and Tierney, 1990; Lindstrom and Vodkin, 1991; Wyatt et al., 1992; Suzuki et al., 1993a). In many cases, expression is limited to specific cell types or organs. In soybean, where PRPs have been best characterized, SbPRP1 is expressed in epidermis and vascular tissue of dark-grown hypocotyl, in the root tip epidermis of light-grown plants, and in the seed coat. SbPRP2 is expressed in the cortex of germinating cotyledons, in the vasculature of hypocotyls and leaves, and in the aleurone of the seed coat. SbPRP3 is expressed in the endodermis, cortex, and pith parenchyma of dark-grown hypocotyls and in the epidermis of the leaf. The expression of structural cell wall proteins in specific cell types is not an exclusive feature of PRPs. For example, extensin has been reported to be localized in vascular elements and the endodermis of lateral roots (Keller and Lamb, 1989; Shirsat et al., 1991), and at least one glycine-rich cell wall protein is specifically localized in vascular elements (Keller et al., 1988; Keller et al., 1989).

In addition to their developmental regulation, several PRPs have been implicated in plant defense reactions. PRP expression in carrot (Chen and Varner, 1985), bean (Sheng et al., 1991), and soybean (Kleis-San Francisco and Tierney, 1990) is wound inducible. Also, cell cultures of soybean have been found to accumulate PRPs in response to methyl jasmonate (Creelman et al., 1992).

To better understand PRP structure, function, and regulation, we have isolated and sequenced a carrot (*Daucus carota*, L.) genomic clone (DcPRP1) that shows significant sequence homology with several previously isolated carrot PRP cDNAs. This gene is single copy within the carrot genome and encodes a wound-inducible PRP in carrot storage roots. The expression of this gene appears to be linked to the onset of secondary root growth. In addition, DcPRP1 mRNA rap-

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* Corresponding author; fax 1–614–292–5379.

Abbreviations: NAA, naphthalene-1-acetic acid; PRP, proline-rich proteins; SSC, standard sodium citrate.

idly accumulates in developing storage roots after wounding, where induction of these mRNAs is localized adjacent to the wound site. We also show that auxin application can induce DcPRP1 mRNA in unwounded carrot storage roots.

MATERIALS AND METHODS

Plant Material

All experiments were conducted using carrots (*Daucus carota*, L.) var Nantes planted from seed in 8- or 12-inch plastic pots containing Metro Mix 510 media (Grace/Sierra; Milpitas, CA) and grown in the greenhouse under a 16-h light:8-h dark cycle. Plant material was harvested at indicated times for RNA and DNA isolation. Carrot roots were wounded by cutting tissue into 2- to 3-mm cross-sections and placing them in a water vapor-saturated chamber at room temperature for the period indicated before the isolation of RNA. For hormone treatments, carrot storage roots were sprayed with a solution containing 10 μM 2,4-D, IAA, or NAA and incubated in a water vapor-saturated chamber in the dark for 24 h. In experiments in which wounding and auxin treatments were directly compared, the outer region of the carrot storage root (3–5 mm) was isolated after wounding or auxin treatment and used for RNA isolation.

Genomic Southern Analysis

Total DNA isolated from mature carrot storage roots was digested to completion with either *Bgl*II, *Hin*dIII, or *Sal*I and subjected to electrophoresis in 0.8% Tris-acetic acid-EDTA-agarose gels, followed by transfer to nitrocellulose (Sambrook et al., 1989). The filter-bound DNA was then hybridized at 65°C with ^{32}P-labeled pDc16, a carrot PRP cDNA (Chen and Varner, 1985), in 6× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate [pH 7]), 5× Denhardt's solution (1× Denhardt's solution is 0.02% [w/v] each of BSA, Ficoll, and PVP), 0.5% (w/v) SDS, and 100 μg/mL of sheared salmon sperm DNA. The filter was washed twice in 0.2× SSC and 0.5% SDS at 65°C and exposed to x-ray film at −80°C with two intensifying screens.

Construction of Carrot Genomic Library

Mature storage roots were harvested and used to isolate total DNA (Junghans and Metzlaff, 1990) that was further purified using CsCl gradient centrifugation (Sambrook et al., 1989). DNA was then cut to completion with *Bgl*II and size fractionated on a 6 to 23% sucrose gradient (Davis and Pearson, 1978). Sucrose gradient fractions containing the 9.0-kb restriction enzyme fragment homologous to pDc16 were identified using Southern hybridization, and appropriate fractions were pooled. The DNA was ligated to *Bam*HI-digested λ EMBL3 arms (Promega) and packaged according to the manufacturer's specifications. Recombinant clones that cross-hybridized with pDc16 were isolated using plaque-filter Southern hybridization (Sambrook et al., 1989).

RNA Blot Analysis

Total RNA isolated from carrot leaves, vegetative roots (DeVries et al., 1988), and storage roots (Haffner et al., 1978) was subjected to electrophoresis in 1.2% agarose gels containing formaldehyde and transferred to nitrocellulose (Sambrook et al., 1989). The filter-bound RNA was hybridized with ^{32}P-labeled pDc16 in 50% formamide, 5× SSC, 5× Denhardt's solution, 0.5% (w/v) SDS, and 100 μg/mL of sheared salmon sperm DNA at 58°C. The filter was washed twice in 0.5× SSC and 0.1% SDS at 65°C and exposed to x-ray film at −80°C with two intensifying screens.

DNA Sequencing

Dideoxynucleotide chain termination sequencing was done using double-stranded plasmid DNA and the double-stranded cycle-sequencing system (BRL) according to the manufacturer's specifications.

Primer Extension

Oligonucleotides used for primer extension experiments were synthesized using an Applied Biosystems DNA synthesizer. Primer extension reactions included 30 μg of total RNA isolated from vegetative roots, storage roots, or leaves. The RNA was mixed with 0.1 pmol of ^{32}P-end-labeled primer and heat denatured for 10 min at 68°C in a solution containing 80% (v/v) formamide, 40 mM Pipes (pH 6.4), 400 mM NaCl, and 1 mM EDTA in a final volume of 30 μL. The RNA/primer mixture was annealed at 30°C for 16 h, precipitated with ethanol, and resuspended in 25 μL of 0.6 mM each of dCTP, dGTP, dTTP, and dATP, 40 units of RNasin (Promega), 20 units of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim), 100 mM Tris-HCl (pH 8.3), 140 mM KCl, 10 mM DTT, and 10 mM $MgCl_2$. The primer was extended for 90 min at 42°C, and the reaction was terminated by the addition of EDTA to a final concentration of 20 mM. The RNA was removed by incubating the reaction mixture with 1 μg of RNase A (Boehringer Mannheim) for 30 min at 37°C, and a portion of the synthesized cDNA was analyzed on a 6% polyacrylamide/8 M urea sequencing gel.

RESULTS

Isolation and DNA Sequence of DcPRP1, a Carrot Genomic Clone Encoding a Wound-Induced PRP

Partial length cDNAs encoding a wound-induced PRP cell wall protein were previously identified from RNA isolated from wounded carrot storage roots (Chen and Varner, 1985; Tierney et al., 1988). These PRP cDNAs hybridized to a 1.1-kb RNA, the protein product of which was localized to the cell wall (Tierney et al., 1988). We determined the number of sequences homologous to the carrot PRP cDNAs in the carrot genome using genomic Southern hybridization. A single restriction enzyme fragment hybridized to pDc16, a carrot PRP cDNA, in each of three restriction digestions of total carrot leaf DNA (Fig. 1). This analysis indicated that a 9.0-kb *Bgl*II restriction enzyme fragment was likely to contain the entire PRP gene sequence including flanking DNA. A partial carrot genomic library was made using size-fractionated *Bgl*II-digested total DNA that was cloned into the *Bam*HI site of λ EMBL3. The partial genomic library was screened with ^{32}P-labeled pDc16, and 12 positive clones were recovered. Four

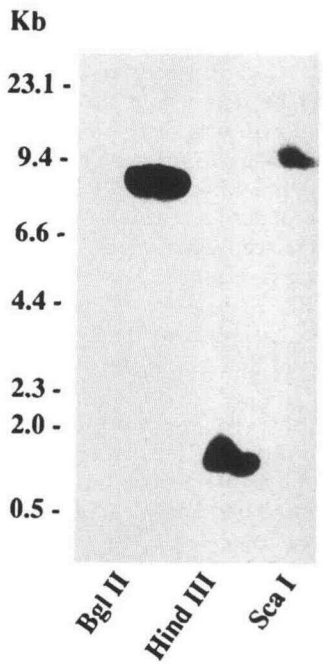

Figure 1. DcPRP1 is a single-copy gene in carrot. Total carrot genomic DNA was isolated and 10 μg of DNA was digested with either *Bgl*II, *Hin*dIII, or *Sca*I. The DNA was size separated using Tris-acetic acid-EDTA-agarose gel electrophoresis, transferred to nitrocellulose, and probed with pDc16, a cDNA clone encoding a carrot wound-inducible PRP.

of these were chosen for further characterization. Figure 2 shows a partial restriction map of one of the λ clones (Dc1–1) that contained several diagnostic restriction enzyme sites predicted from the genomic Southern analysis and the PRP cDNA sequence (Chen and Varner, 1985). Specifically, Dc1–1 contains an internal 1.6-kb *Hin*dIII fragment, which cross-hybridizes with pDc16 and is predicted from genomic Southern analysis, and a *Sca*I site predicted from the partial length cDNA sequence (Fig. 2).

The 1.6-kb *Hin*dIII restriction enzyme fragment was subcloned into pUC19 and sequenced (Fig. 3). The carrot PRP gene (DcPRP1) located within this restriction fragment contained an open reading frame encoding a 235-amino acid PRP. The sequence of DcPRP1 is colinear with the partial-length PRP cDNAs throughout the coding sequence and through much of the 3′-untranslated region of the gene (Chen and Varner, 1985) with the exception of four amino acids at the N terminus and a 60-nucleotide insert within the genomic coding sequence at position 444 to 503 (Fig. 3). The four N-terminal amino acids complete the sequence of the open reading frame predicted from the partial length PRP cDNAs by providing an N-terminal methionine residue. The 60-nucleotide insertion maintains the open reading frame of the encoded protein and adds four repetitive pentapeptide units to the protein sequence. Directly upstream of the transcription initiation sites (see below) are a putative TATA element and several potential CAAT boxes (Fig. 3). Several

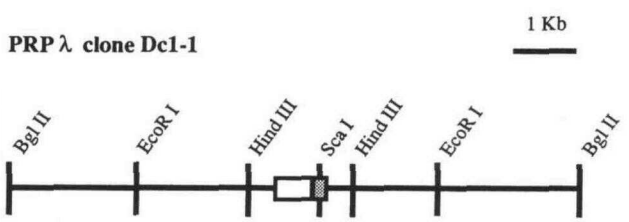

Figure 2. Partial restriction enzyme map of DcPRP1. A partial restriction enzyme map of λ clone Dc1–1, containing a 9-kb *Bgl*II insert, is shown indicating the region within the *Hin*dIII restriction enzyme fragment that contains the PRP open reading frame (open box) and 3′-untranslated region (shaded box), based on homology to the p33 cDNAs (Chen and Varner, 1985). The positions of the *Eco*RI restriction fragments, relative to the terminal *Bgl*II sites, have not been determined.

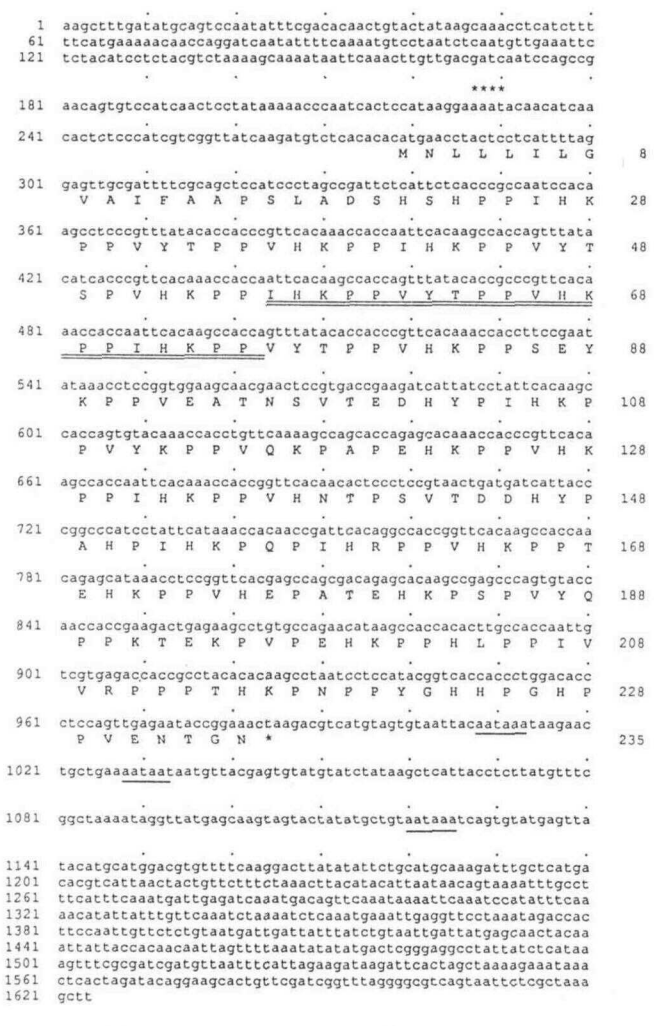

Figure 3. DNA sequence of DcPRP1. The DNA sequence of the 1.6-kb *Hin*dIII restriction enzyme fragment contained within λ clone Dc1–1 was subcloned into pUC19 and sequenced. This clone contains a single open-reading frame that is colinear with carrot PRP cDNAs (Chen and Varner, 1985) with the exception of four N-terminal amino acids and a 60-nucleotide insertion. Transcription initiation sites are indicated by ****. The double solid line indicates the 60-nucleotide insertion, and the single solid lines designate possible polyadenylation signals.

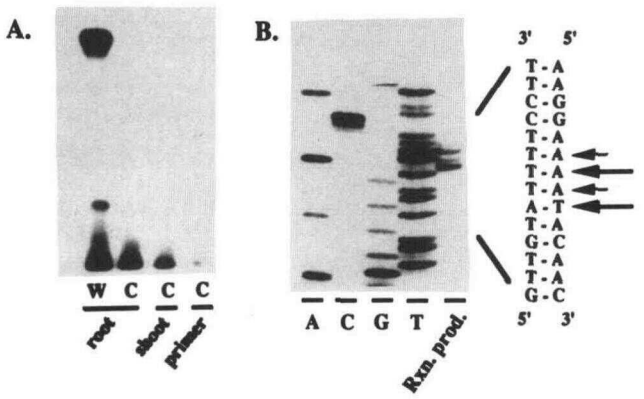

Figure 4. DcPRP1 encodes a wound-induced transcript in carrot storage roots. A, Primer spanning nucleotides 324 to 347 (Fig. 3) encoding a unique sequence within the PRP open-reading frame was hybridized with 30 μg of total RNA from wounded (W) storage roots, unwounded (C) storage roots, or unwounded leaves (shoot) and used for primer extension analysis. Primer (C) represents primer alone (no RNA). B, Primer extension was used to map the transcription start sites for DcPRP1, using RNA from wounded carrot storage roots. The products obtained corresponded to positions 227 to 230 (Fig. 3). Arrows indicate major and minor reaction products.

potential polyadenylation sites are located downstream of the termination codon (Fig. 3).

DcPRP1 Encodes a Transcript that Accumulates in Wounded Storage Roots

Because of the differences in sequence between DcPRP1 and the previously isolated carrot PRP cDNAs, it was necessary to demonstrate that this genomic clone encoded a transcript that was differentially expressed after wounding. To do this, we used primer extension of RNA from unwounded and wounded carrot storage roots with a primer that spanned a unique region of nucleotide sequence within the carrot PRP1 gene (positions 324–347 in Fig. 3). Primer extension of RNA from wounded carrot roots yielded a product of approximately 120 nucleotides (Fig. 4A) that could also be faintly detected in unwounded carrot roots after a longer exposure (data not shown). No primer extension product was detected when leaf RNA was used (Fig. 4A). These data indicate that DcPRP1 encodes a wound-inducible transcript in carrot storage roots. Primer extension analysis was also used to identify four transcription initiation sites, at positions 227 to 230, in wounded storage roots (Fig. 4B).

Expression of DcPRP1 Is Linked to Secondary Root Growth

PRP expression was originally observed in carrots as a 1.1-kb transcript that accumulated in storage roots in response to wounding (Chen and Varner, 1985). To further characterize the wound response, RNA was isolated from leaves, an early (seedling) and mature stage of vegetative root growth, and two stages during storage root growth—a very early stage when the epidermal and cortex cells were just beginning to be shed and carotenoids were starting to be expressed, as well as a mature stage of storage root development. Expression of DcPRP1 could not be detected in either control or wounded vegetative roots or in leaves of carrot plants using pDc16 as a probe (Fig. 5). However, a low level of DcPRP1 expression could be detected in unwounded tissue when secondary root growth was first visible, and this expression continued at a low level during storage root development (data not shown). In addition, DcPRP1 was wound inducible at all stages of secondary root growth (Fig. 5). Similar expression patterns were seen when the 3'-untranslated region of DcPRP1 was used as a probe (data not shown). These data indicate that DcPRP1 encodes a proline-rich cell wall protein whose expression is linked to the formation of secondary roots in carrots and that this expression is greatly enhanced by wounding.

Northern hybridization was used to investigate the accumulation pattern of DcPRP1 transcripts at various distances from the site of physical damage. Carrot storage roots were sliced once and incubated for 24 h at room temperature. RNA was then isolated from 2-mm cross-sections of root tissue harvested at 0, 0.5, and 2 cm from the wound site and hybridized with pDc16 (Fig. 6). The accumulation of DcPRP1 mRNA in carrot storage roots after wounding was most abundant in cells near the site of physical damage with a sharp decrease in the amount of wound-inducible PRP mRNA detected at increasing distances (0.5 and 2 cm) from the wound site. However, storage root tissue located 2 cm from the original wound site was capable of accumulating PRP mRNA if freshly sliced and incubated for 24 h before RNA isolation (Fig. 6). Thus, the signal responsible for PRP expression in wounded tissue is restricted, for the most part, to cells directly adjacent to the site of physical damage.

DcPRP1 Transcripts Can Be Induced in Carrot Storage Roots by Auxin

Previous work showed that, although PRP expression in carrot storage roots responded dramatically to wounding, neither ethylene nor an endogenous elicitor fraction from

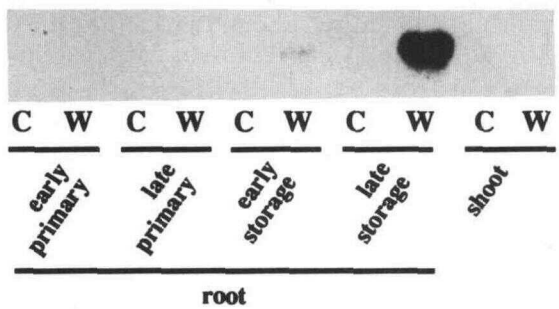

Figure 5. The wound-induced expression of DcPRP1 is linked to storage root formation. Vegetative roots, storage roots, and leaves (shoots) of carrots were wounded by slicing tissue into 1- to 2-mm pieces and incubating them in darkness for 24 h. Total RNA was then isolated from wounded (W) and unwounded (C) vegetative roots at early and late stages of growth, storage roots at early and late stages of development, and leaves (shoot). The RNA was characterized by northern hybridization using 5 μg of total RNA per lane and pDc16 as a probe (Chen and Varner, 1985).

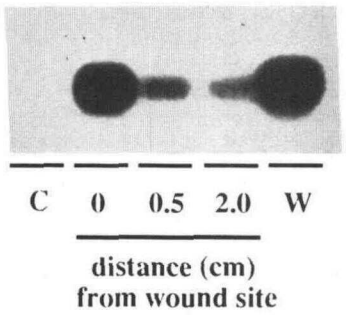

Figure 6. Expression of DcPRP1 mRNA in storage roots is localized near the wound site. Carrot storage roots were harvested and frozen immediately (C) or sliced once and incubated for 24 h after which 2-mm cross-sections of root tissue were harvested at 0, 0.5, and 2.0 cm from the wound site for RNA isolation. The remaining root was sliced and incubated for an additional 24 h, and RNA was isolated from tissue adjacent to the wound (W). The RNA was characterized by northern hybridization using 5 μg of total RNA per lane and pDc16 as a probe (Chen and Varner, 1985).

carrot storage roots appeared to induce these transcripts in unwounded tissue or suspension cultured cells (Tierney et al., 1988). Recently, a region of the *nos* promoter necessary for wound induction was found to be coincident with that necessary for its auxin-induced expression (An et al., 1990). Therefore, we investigated whether the application of auxin would lead to an accumulation of DcPRP1 transcripts in unwounded carrot storage roots. Whole carrot storage roots were harvested and treated with 10 μM 2,4-D, IAA, or NAA. Although no affect of auxin treatment was seen at 3 h, all three auxins were found to increase the accumulation of DcPRP1 mRNA in carrot storage roots after 24 h to a level similar to that found in wounded tissue (Fig. 7). These data suggest that auxin may play a role in the wound-induced expression of DcPRP1 in carrot storage roots.

DISCUSSION

We have isolated and sequenced a carrot genomic clone, DcPRP1, encoding a proline-rich cell wall protein that corresponds closely in sequence with several previously isolated wound-induced PRP cDNAs (Chen and Varner, 1985). The derived amino acid sequence of DcPRP1 is colinear with the previously isolated carrot PRP cDNAs except for four additional amino acids at the N terminus of the protein that provide an initiating Met residue and a 60-nucleotide insertion within the open-reading frame (Chen and Varner, 1985). The additional 60 nucleotides are not bordered by splice sites and do not change the reading frame of the predicted protein but result in the addition of four repetitive units of the sequence Pro-Pro-Val-X-Lys to the predicted PRP protein sequence (Fig. 3). Because genomic Southern analysis indicates that the PRP cDNAs were encoded by a single-copy gene (Fig. 1), this difference in nucleotide sequence between the PRP cDNAs and DcPRP1 is most likely due to variation in gene structure between the cultivars used to make these two libraries.

The expression of DcPRP1 appears to be developmentally regulated during plant development. DcPRP1 begins to be expressed at the earliest visible stages of carrot storage root growth. This is a stage in development that has been characterized in some detail (Esau, 1939, 1940) and results in the sloughing off of epidermal and cortex layers of root and hypocotyl cells along with a proliferation of parenchyma cells and the development of an extensive vascular network. The organ-specific expression of DcPRP1 suggests that this cell wall protein may be important in determining cell wall structure within parenchyma or vascular tissues in storage roots. Similar types of regulation have been observed for other PRPs (Hong et al., 1989; Van de Weil, 1990; Wyatt, 1992), which supports the model that these proteins play unique roles in determining cell type-specific matrices within the cell wall.

The relationship between the developmental regulation of DcPRP1 expression in carrot storage roots and its wound sensitivity is striking. Although DcPRP1 expression could be detected in unwounded carrot storage roots at all stages of development, the accumulation of DcPRP1 transcripts is greatly amplified upon wounding (Figs. 4–6). However, no expression of DcPRP1 could be detected in either unwounded or wounded leaf tissue or vegetative roots, indicating that the developmental regulation of this gene supercedes its wound inducibility in these organs. Recently, the wound-induced pattern of gene expression of two PRP genes from soybean has also been shown to be affected by their developmental regulation (Kleis-San Francisco and Tierney, 1990; Suzuki et al., 1993b), which suggests that the regulatory mechanisms required for the synthesis of PRPs in wounded tissue may require the presence of an already established developmental program.

The accumulation of DcPRP1 transcripts in storage roots treated with 10 μM 2,4-D, IAA, or NAA to a level equal to that in wounded tissue indicates that auxin may play a role in the wound-induced expression of this gene in carrot. Recently, a number of auxin-regulated genes were described (Hong et al., 1987; Ainley, 1988; McClure et al., 1989; An et al., 1990), and several of these, including the gene for nopa-

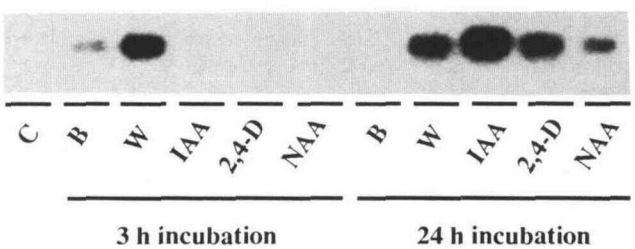

Figure 7. Application of auxins to unwounded carrot storage roots causes an accumulation of DcPRP1 mRNA. Unwounded carrot storage roots were either harvested immediately (C), sprayed with buffer alone (10 mM K_2HPO_4, pH 7.0) (B), or sprayed with solutions containing 10 μM IAA, 2,4-D, or NAA in buffer. Wounded (W) carrot storage roots were incubated in buffer at room temperature on a shaker. All treated samples were incubated in darkness for either 3 or 24 h. RNA was then isolated from each of these samples and characterized by northern hybridization using 5 μg of total RNA per lane and pDc16 as a probe (Chen and Varner, 1985).

line synthase (An et al., 1990) and two soybean PRP genes (Kleis-San Francisco and Tierney, 1990; Suzuki et al., 1993b), are also wound inducible. A region of 5′-flanking sequence within the *nos* promoter has been identified that is necessary for both its auxin regulation and its response to wounding, indicating that auxin may be responsible for the expression of this gene after physical damage (An et al., 1990). It remains to be determined whether a similar DNA-binding sequence is present within the DcPRP1 promoter and, if so, whether this sequence functions in both the auxin-induced and wound-induced expression of DcPRP1 in carrot storage roots.

The accumulation of DcPRP1 transcripts in wounded storage roots is found mostly in cells immediately adjacent to the wound site and appears to be very localized (Fig. 6). This pattern of expression is similar to that observed for genes that are induced by fungal or plant cell wall elicitors, due to the inability of oligosaccharins to move far from the wound site (Ryan and Farmer, 1991). However, PRP transcripts do not accumulate in carrot suspension-culture cells in response to a carrot cell wall elicitor fraction (Tierney et al., 1987) and appear to be down-regulated in bean suspension-culture cells incubated with a fungal elicitor (Sheng et al., 1991). The ability of auxins to induce the accumulation of DcPRP1 in unwounded storage roots (Fig. 7) indicates that a change in the concentration of auxin near the wound site could result in the localized expression of DcPRP1 in wounded tissue. It has been suggested that flavonoids, which are released in cells after wounding, may serve as natural inhibitors of auxin transport (Jacobs and Rubery, 1988), resulting in locally high auxin concentrations. Consistent with this is the observation that enhanced auxin levels (presumably) can induce the expression of a PRP in alfalfa; both naphthylphthalmic acid and 2,3,5-triiodobenzoic acid, synthetic auxin transport inhibitors, have been shown to induce nodulation and the expression of ENOD2 on alfalfa roots (Hirsch et al., 1989, Van de Wiel et al., 1990). Further experiments will be needed to determine the relationship between auxin concentration and the localized expression of DcPRP1 in wounded carrot roots.

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