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Cell-Specific Expression of the Carrot EP2 Lipid Transfer Protein Gene

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A cDNA corresponding to a 10-kD protein, designated extracellular protein 2 (EP2), that is secreted by embryogenic cell cultures of carrot was obtained by expression screening. The derived protein sequence and antisera against heterologous plant lipid transfer proteins identified the EP2 protein as a lipid transfer protein. Protein gel blot analysis showed that the EP2 protein is present in cell walls and conditioned medium of cell cultures. RNA gel blot analysis revealed that the EP2 gene is expressed in embryogenic cell cultures, the shoot apex of seedlings, developing flowers, and maturing seeds. In situ hybridization showed expression of the EP2 gene in protoderm cells of somatic and zygotic embryos and transient expression in epidermis cells of leaf primordia and all flower organs. In the shoot apical meristem, expression is found in the tunica and lateral zone. In maturing seeds, the EP2 gene is expressed in the outer epidermis of the integument, the seed coat, and the pericarp epidermis, as well as transiently in between both mericarps. Based on the extracellular location of the EP2 protein and the expression pattern of the encoding gene, we propose a role for plant lipid transfer proteins in the transport of cutin monomers through the extracellular matrix to sites of cutin synthesis.

INTRODUCTION

Zygotic embryogenesis in plants takes place in the ovule after the fusion of the haploid egg cell nucleus with one haploid sperm cell nucleus to produce the unicellular zygote. A second sperm cell nucleus fuses with the diploid central cell nucleus of the embryo sac to develop into the triploid endosperm. The first asynchronous division of the zygote sets off a large vacuolated cell that gives rise to the suspensor and a small cytoplasmic cell that develops into the embryo proper. Embryo development proceeds through a series of further divisions that result in the characteristic globular, heart, torpedo, and cotyledonary stages during which both primary apical meristems and the embryo polarity axis are formed. The endosperm is either totally absorbed by the developing embryo or is retained in the desiccated seed, which is the final developmental stage of the ovule (Steeves and Sussex, 1989).

In many plant species, an alternative developmental pathway can be initiated from nongametic somatic cells in tissue culture. After appropriate manipulations, nonsexual somatic embryos can be formed that closely resemble their zygotic counterpart in morphology. Somatic embryogenesis has first been described in carrot (Reinert, 1958; Steward et al., 1958). Like zygotic embryos, somatic embryos also have an ultimate single cell origin (Backs-Hüsemann and Reinert, 1970; Nomura and Komamine, 1985), but in practice usually develop from multicellular precursor structures designated proembryogenic masses (Halperin, 1966) that are characteristic for embryogenic carrot cultures (De Vries et al., 1988b). The true morphological identity of proembryogenic masses is not known; they probably represent somatic embryos arrested before the globular stage, rather than a specific precursor cell type (Borkird et al., 1988; De Vries et al., 1988b). Both zygotic embryo development (Raghavan, 1986) and somatic embryo development from suspension cells (Halperin, 1966; Backs-Hüsemann and Reinert, 1970) have been well characterized morphologically. At the molecular level, however, embryo development is not well understood. Two-dimensional gel analysis of carrot somatic embryo proteins (Sung and Okimoto, 1981, 1983; Choi and Sung, 1984), in vitro translation products of carrot embryo mRNA (De Vries et al., 1988b), two-dimensional gel analysis of proteins synthesized during cotton (Dure et al., 1981) and rapeseed (Crouch, 1982) zygotic embryogenesis, as well as cDNA complexity analysis of soybean embryo mRNA (Goldberg et al., 1981) have only revealed a small number of changes in gene expression during embryo axis development. So far, only very few genes have been described that are expressed during the early,

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preglobular stages of somatic embryo development (Aleith and Richter, 1991).

The carrot system has been used by a number of groups to identify genes that are expressed during plant embryogenesis (Choi et al., 1984; Thomas and Wilde, 1985, 1987; Borkird et al., 1988). Some of these were also found to be expressed during zygotic embryogenesis (Borkird et al., 1988). Analysis of gene expression in different tissues formed during zygotic embryo development has been restricted to abundantly expressed seed protein genes (Perez-Grau and Goldberg, 1989). Several of these genes were shown to be expressed during somatic embryogenesis as well. Taken together, these data suggest that apart from the morphological similarities, the molecular events during zygotic embryogenesis are reproduced to a substantial degree during somatic embryogenesis (Crouch, 1982; Goldberg et al., 1989).

We have previously reported that upon initiation of somatic embryogenesis in carrot suspension cultures, a small number of proteins are secreted into the medium within 2 days after embryo initiation. These proteins are either absent or present in reduced concentrations in cultures unable to produce somatic embryos (De Vries et al., 1988a). This suggests that these proteins are correlated with an early, preglobular stage of somatic embryo development. To identify these proteins and to analyze the expression pattern of the encoding genes, an antiserum that recognized the embryo medium proteins was used to select corresponding cDNAs from an expression library. The cDNA-derived protein sequence of one of these clones, designated extracellular protein 2 (EP2), shows homology to plant lipid transfer proteins. Expression of the lipid transfer protein gene is already notable after five to six cell divisions in zygotic embryogenesis, as well as in the precursor cell clusters from which somatic embryos derive in culture. This makes the carrot lipid transfer protein gene one of the earliest molecular markers of both somatic and zygotic plant embryogenesis so far described. Analysis of the expression pattern of the gene by in situ hybridization together with the extracellular localization of the encoded protein allows us to propose a role for the EP2 protein in the transport of fatty acids to the developing cuticle deposited on the outer surface of epidermal cells.

RESULTS

The Carrot EP2 Gene Encodes a Secreted Plant Lipid Transfer Protein

A λgt11 cDNA library representing the mRNA population of globular, heart, and torpedo stage carrot somatic embryos was screened with an antiserum raised against all proteins secreted into the medium of somatic embryo cultures. Several clones were obtained that hybridized

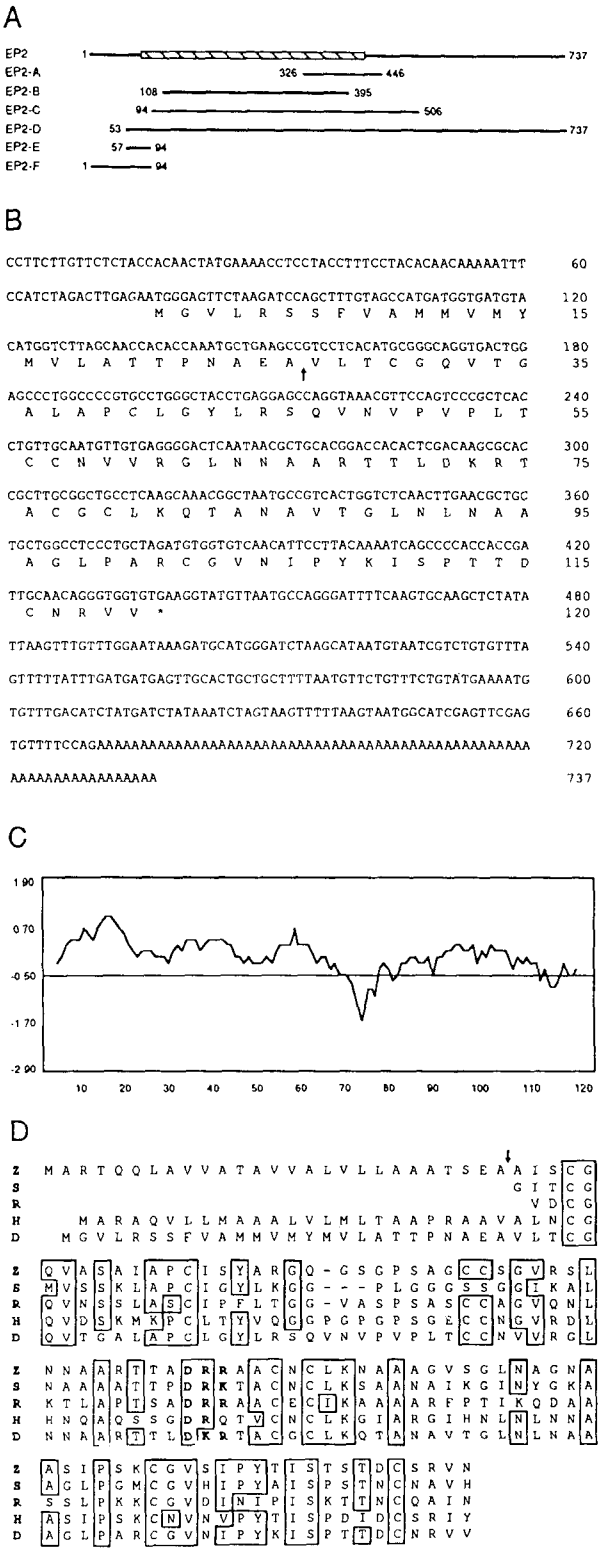


Figure 1. Analysis of cDNA and Deduced Amino Acid Sequence of Clone EP2.

more strongly with embryo RNA than with RNA from proliferating cells (data not shown). One of these clones, designated EP2-A, recognized an mRNA of 750 nucleotides. Screening of a λZAPII cDNA library with this clone yielded three more clones, designated EP2-B, EP2-C, and EP2-D, of a total of 150,000 recombinant phages. Sequence analysis showed that EP2-D contained the EP2-A, EP2-B, and EP2-C sequences and the complete 3′ noncoding region of the corresponding EP2 mRNA, as depicted in Figure 1A. To obtain clones spanning the 5′ end, an oligonucleotide was synthesized complementary to nucleotides 3 to 32 of clone EP2-C, which was used in combination with the M13 −20 sequence primer to amplify 5′ EP2 sequences from the λZAPII cDNA library employing the polymerase chain reaction (PCR). The amplified sequences were subcloned to give clones EP2-E and EP2-F (Figure 1A).

From clones EP2-D and EP2-F, the full-length cDNA clone EP2 was constructed (Figure 1A) with a length of 737 bp. A single open reading frame was found that encodes a protein with a calculated molecular mass of 12.5 kD (Figure 1B). The protein is predominantly hydrophobic with only two hydrophilic regions, a stretch of charged amino acids found at positions 71–73, and a polar C terminus (Figure 1C). Because this protein is expected to be secreted, it most likely contains a signal peptide that should be cleaved after the valine residue at position 26, according to the rules described by Von Heijne (1983).

Figure 1. (continued).

(A) Schematic representation of the EP2 cDNA clones A–F. EP2-A was obtained by expression screening and had a length of 121 bp. Rescreening of the λgt11 and λZAPII libraries with EP2-A yielded clones EP2-B, EP2-C, and EP2-D of 288, 413, and 685 bp, respectively. Clones EP2-E and EP2-F were obtained by PCR amplification of DNA fragments present in the λZAPII library and were 38 and 95 bp, respectively. The full-length EP2 cDNA clone was constructed from clones EP2-D and EP2-F. The hatched box represents the coding region.

(B) Nucleotide sequence of the EP2 cDNA and its deduced protein sequence. The termination codon is marked with an asterisk. The putative signal peptide cleavage site is marked with an arrow.

(C) Hydropathy of the 120 amino acid EP2 protein, determined according to Kyte and Doolittle (1982). The horizontal scale indicates the number of amino acid residues and the vertical scale the average free energy in kcal per mole per amino acid for transfer from a hydrophobic to a hydrophilic environment.

(D) Homology among five plant lipid transfer proteins. Boxes indicate homologous amino acids in at least four of the sequences. (Putative) signal peptide cleavage sites are marked with an arrow. All sequences were derived from protein sequencing, except for the carrot sequence. The N-terminal signal sequences were deduced from the corresponding cDNA clones. The central charged amino acids are in bold face. Z, maize; S, spinach; R, castor bean; H, barley; D, carrot.

This results in a protein with a calculated molecular mass of 9.7 kD and a predicted isoelectric point of 8.86. Although many extracellular proteins are glycosylated, the absence of the consensus sequences Asn-X-Ser or Asn-X-Thr indicates that the EP2 protein is not *N*-glycosylated. Comparison of the EP2 cDNA and amino acid sequences with sequences found in the EMBL DNA sequence and the NBRF and Swiss-Prot protein sequence libraries revealed high sequence homologies with phospholipid transfer proteins from maize (54%; Tchang et al., 1988) and barley (45%; Mundy and Rogers, 1986; Bernhard and Somerville, 1989), respectively. Homology was also found with protein sequences from spinach (49%; Bouillon et al., 1987) and castor bean (Takishima et al., 1986) phospholipid transfer proteins (calculation of percentages based on amino acid sequences as shown in Figure 1D). Due to their aspecific lipid binding properties, these proteins have recently been reclassified as nonspecific lipid transfer proteins (Kader, 1990). The predicted existence of a signal peptide was supported by the fact that all homologous N-terminal protein sequences of the mature protein commenced at the predicted cleavage site of the EP2 protein. In addition, the homologous cDNAs from maize (Tchang et al., 1988) and spinach (Bernhard et al., 1991) have a 27 amino acid signal peptide ending precisely at the equivalent position of the predicted EP2 cleavage site. All protein sequences show conservation of six to eight cysteines as well as two or three charged amino acids present at positions 45 to 47, as counted from the presumed signal sequence cleavage site. All proteins have a similar size, between 8.8 and 9.7 kD, and isoelectric points between 8.8 and 10.5.

To confirm the assumption that the EP2-encoded protein is secreted into the medium of embryo cultures, an antiserum was raised against the purified β-galactosidase-EP2A fusion protein. This antiserum reacted with a 10-kD protein in media of somatic embryo cultures as well as in the cell walls of somatic embryos, as shown in Figure 2. No signal above background with a 10-kD protein or its 12.5-kD precursor was obtained in intracellular proteins, indicating that the majority of the EP2 protein is extracellular. In media, a possibly dimeric form of the same protein was also recognized. An antiserum raised against a lipid transfer protein from maize also recognized a 10-kD secreted carrot protein as well as the β-galactosidase-EP2-A fusion protein. This heterologous antiserum also failed to react with intracellular embryo proteins (data not shown). Results obtained with an antiserum raised against a spinach lipid transfer protein were identical to those of the maize antiserum (data not shown).

Experiments applying fluorescent phospholipid analogs (Somerharju et al., 1990) showed that the purified EP2 protein is also capable of binding and transferring phospholipids (P. Sterk and S.C. De Vries, unpublished results).

Based on the high protein sequence homology, the immunological cross-reactivity of heterologous maize and spinach antisera with the β-galactosidase-EP2A fusion

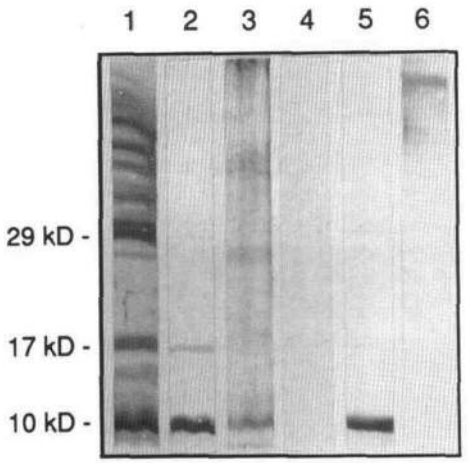


Figure 2. Identification of the EP2 Protein.

Lane 1, reaction of the antiserum raised against proteins secreted into carrot embryo culture medium with these proteins. This serum was used to screen a λgt11 somatic embryo cDNA library. Lane 2, an antiserum raised against the β-galactosidase-EP2-A fusion protein recognizes a 10- and an 18-kD protein in embryo culture media. Lane 3, the anti-β-galactosidase-EP2-A antiserum recognizes a 10-kD cell wall protein extracted with 100 mM $CaCl_2$ from somatic embryos. Lane 4, intracellular proteins from $CaCl_2$-extracted somatic embryos were not recognized by the anti-β-galactosidase-EP2-A antiserum. Lane 5, an antiserum raised against purified maize lipid transfer protein also recognizes the 10-kD extracellular protein. Lane 6, the maize lipid transfer protein antiserum recognizes the β-galactosidase-EP2-A fusion protein. For lanes 1 to 5, 5 μg of protein was loaded in each gel slot. Two micrograms of purified fusion protein was loaded in lane 6.

protein, and the phospholipid transfer activity of the EP2 protein, we concluded that the carrot EP2 gene encodes a secreted lipid transfer protein.

The EP2 Gene Is a Marker for Embryogenic Potential of Carrot Cultures and Is Expressed in the Shoot Apex and Flowers of Carrot Plants

The expression pattern of the EP2 gene was examined by RNA gel blot analysis of total RNA isolated from embryogenic and nonembryogenic suspension cell cultures as well as from plants with EP2-C as a probe, as depicted in Figure 3.

Embryogenic high-density cell suspensions cultures were maintained in B5 medium containing 0.44 mg/L of the synthetic auxin 2,4-D and contained clusters of small cytoplasmic cells designated proembryogenic masses. Somatic embryos developed on the surface of these proembryogenic masses after 200-fold dilution of a suspension culture enriched for proembryogenic masses in 2,4-D-free medium. Embryo development was blocked before globular stage in proembryogenic mass-enriched cultures diluted in the presence of 2,4-D, which were designated proliferating cultures. The EP2 gene was found to be expressed in embryogenic cultures, regardless of the cell density and the presence of 2,4-D, but not in nonembryogenic cultures. In addition, the EP2 gene was highly expressed in manually purified somatic embryos (Figure 3A). No EP2 expression was found in the nonembryogenic or weakly embryogenic cultures FG10, α5II, and the temperature-sensitive (ts) ts11, whereas embryogenic

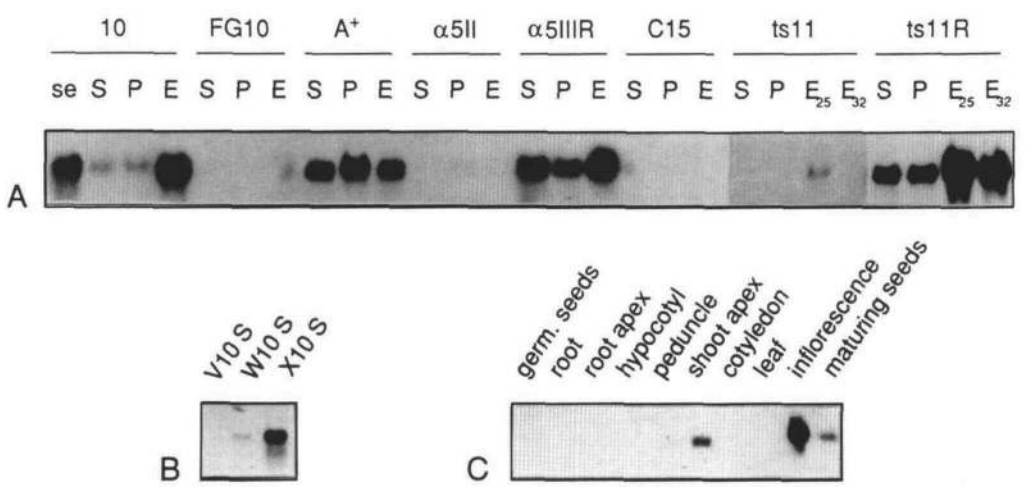


Figure 3. RNA Gel Blot Analysis of EP2 in Tissue Cultures and Plants.

For each lane, 15 μg of total RNA was loaded on gel. Blotting, hybridization, and washing steps were as described in Methods. Unless indicated, films were exposed with an intensifying screen for 2 days.

revertants of the latter two, α5IIIR and ts11R, re-expressed EP2 at the wild-type level. Comparison of EP2 expression in three suspension cultures of line "10," which differed in embryogenic potential as judged by the number of proembryogenic masses present, revealed a quantitative correlation between EP2 expression and embryogenic capacity of the culture (Figure 3B). This was further supported by the observation that many more proembryogenic masses were present in the A^+ and α5IIIR lines when compared with embryogenic "10" lines, resulting in a stronger hybridization signal with RNA from the A^+ and α5IIIR lines. Taken together, we concluded that expression of the EP2 gene marks the acquisition of and is a measure for embryogenic potential in carrot suspension cultures.

To determine whether the EP2 gene is also expressed in carrot plants, we used RNA gel blot analysis of total RNA isolated from 10-day-old carrot seedlings dissected into root apex, root without apex, hypocotyl, shoot apex, and cotyledons. EP2 gene expression was detected in the shoot apex only. In floral tissues (Figure 3C), we observed a very high level of expression in the young developing inflorescence and a much lower level in maturing seeds. No expression was found in seeds imbibed for 48 hr. Because the EP2 gene was expressed in meristematic regions of the shoot and the flower, but not in the root, we tested a possible correlation of EP2 expression with rapid cell division by rehybridizing the deprobed blot with a histone 4 probe. Hybridization signals of equal intensity were obtained with RNAs from both root and shoot apices as well as from the developing inflorescence (data not shown), indicating that EP2 function is not directly related to rapid cell division.

The EP2 Gene Is a Single Copy Gene in the Haploid Carrot Genome

The expression pattern of the EP2 gene as determined by RNA gel blot analysis revealed that the gene is not embryo specific, but that EP2 gene expression is also found later in development. This raised the question whether the EP2 gene is a member of a multigene family of which the different members are differentially expressed in time and space. However, as depicted in Figure 4, DNA gel blot analysis only showed one hybridizing genomic DNA fragment when digested with enzymes that do not cut within the EP2 sequence, suggesting that the carrot genome contains a single EP2 gene per haploid genome.

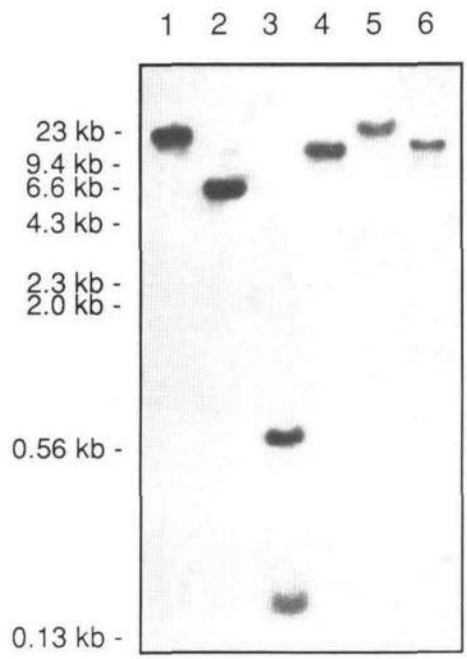


Figure 4. Determination of the Number of EP2-Related Genes per Carrot Genome.

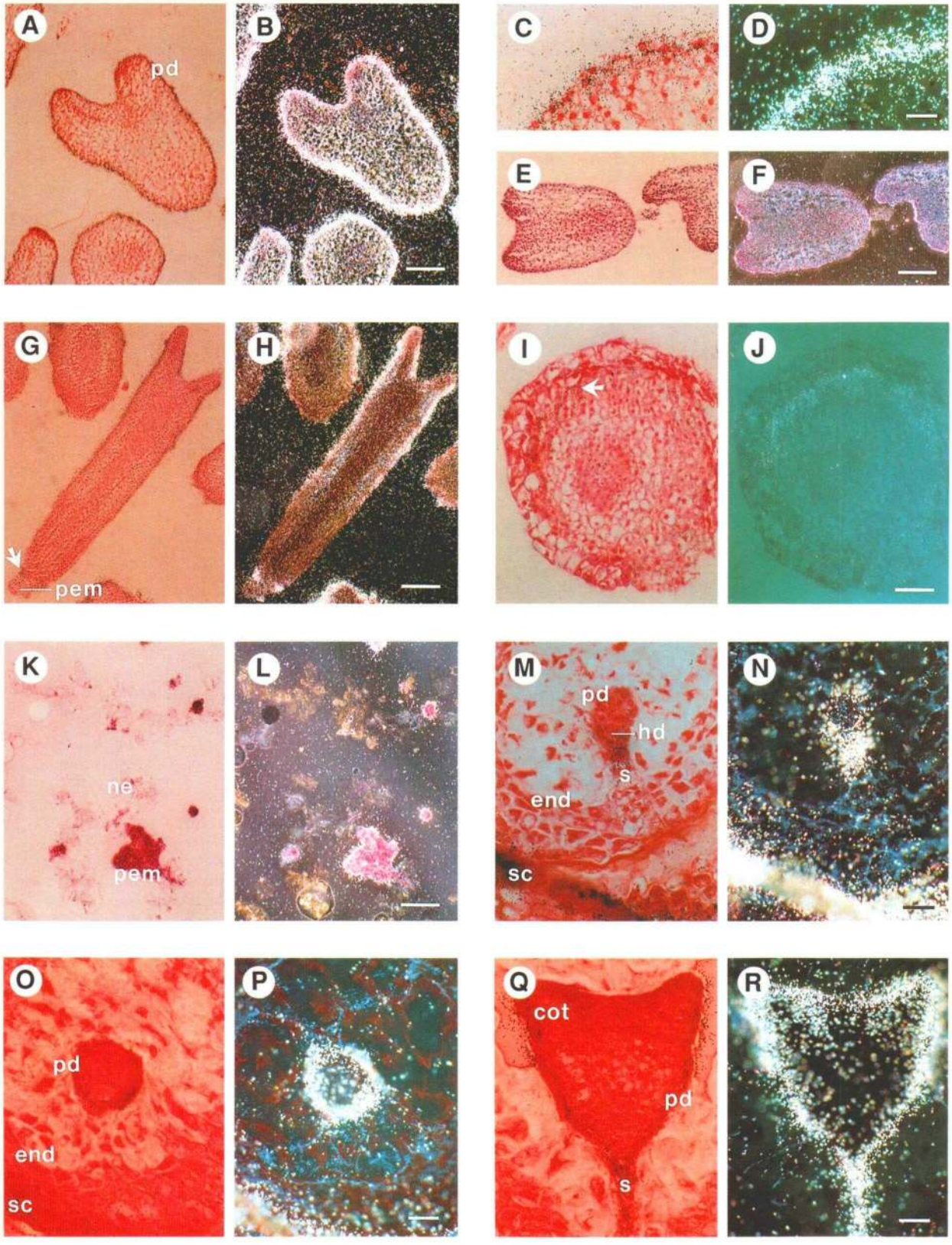
Autoradiogram of EP2-C hybridization to digests of genomic DNA from carrot. Lane 1, EcoRI digest; lane 2, EcoRV digest; lane 3, HincII digest; lane 4, HindIII digest; lane 5, KpnI digest; lane 6, PvuII digest. In each lane except lane 3, due to the presence of a HincII site in the EP2 sequence, single bands were obtained.

Figure 3. (continued).

(A) Expression of EP2 in highly purified somatic embryos (se) of line 10, in high-density suspension cultures (S), and low-density proliferating (P) and embryo (E) cultures of the embryogenic cell lines 10, A^+, and α5III. No expression was observed in the nonembryogenic cell lines FG10, α5II, and C15, even after prolonged exposure (7 days; data not shown). After 7 days of exposure, a very weak signal was found in embryo culture cells of the slightly embryogenic culture ts11 grown at the permissive temperature (25°C), in contrast to those grown at the restrictive temperature (32°C).

(B) EP2 expression is correlated with embryogenic potential of high density suspension cultures. V10 S, 18 months in culture and nonembryogenic; W10 S, 6 months in culture and moderately embryogenic; X10 S, 2 months in culture and highly embryogenic.

(C) Expression of EP2 in carrot plants. Plant parts investigated were germinated seeds (imbibed for 48 hr), the root (without apex), the root apex (approximately 2 mm), the hypocotyl, the peduncle, the shoot apex, the cotyledon, leaf, bud stage inflorescence, and maturing seeds. The roots, shoots, hypocotyl, and the cotyledon were derived from 7-day-old seedlings, and the other parts from mature greenhouse plants. EP2 expression was found in the shoot apex of seedlings, in the young inflorescence, and in maturing seeds.



The EP2 Gene Is Expressed in Protoderm Cells of Somatic and Zygotic Embryos

To elucidate the pattern of gene expression in more detail, we used in situ localization of the EP2 mRNAs in sectioned tissue culture and intact plant material. Hybridization of sectioned somatic embryos with EP2-C antisense RNA revealed that the EP2 gene is expressed in the periphery of globular and heart stage somatic embryos, as shown in Figures 5A and 5B. At higher magnification, it was evident that the EP2 gene is only expressed in the protoderm cells (Figures 5C and 5D). No signal above background was detected after hybridization with EP2-C sense RNA (Figures 5E and 5F). In torpedo stages of somatic embryos, EP2 expression was gradually reduced in the epidermal cells of the primary root, while expression continued in some surface cells of the still attached proembryogenic mass from which the embryo had developed (arrow in Figure 5G; see also Figure 5H). As can be seen in Figures 6A and 6B, upon continued development of somatic embryos into seedlings, expression of the EP2 gene was further restricted toward the shoot apex (cf. Figure 3C).

In the temperature-sensitive variant cell line, ts11 embryo development is arrested at the nonpermissive temperature of 32°C in late globular stage, but can be rescued by secreted glycoproteins (LoSchiavo et al., 1990). In arrested globular embryos of ts11, EP2 gene expression was slightly more diffuse and about two cell layers inward from the protoderm (Figures 5I and 5J).

The finding that EP2 expression marks the acquisition of embryogenic potential in carrot suspension cultures suggested that the EP2 gene is also expressed in embryogenic cells. In situ localization after sectioning embedded embryogenic suspension culture cells confirmed that the EP2 mRNA is only present in proembryogenic masses and not in nonembryogenic cell clusters (Figures 5K and 5L).

If the EP2 protein is essential for an early phase in somatic embryogenesis, it should also be so for zygotic embryogenesis. Therefore, maturing carrot seeds at different times postfertilization were sectioned. In Figures 5M and 5N, a longitudinal section through an approximately 60-celled zygotic embryo is presented. The endosperm surrounding the developing embryo is already partially degraded. Hybridization with EP2-C antisense RNA revealed high expression in all suspensor cells, the cells derived from the hypophyseal cell, and in the protoderm cells of the embryo proper. At this stage of development, protoderm differentiation has just been initiated. Because the embryo depicted in Figures 5M and 5N has only undergone about five to six cell divisions after fertilization, we concluded that the expression of the EP2 gene is a very early event in zygotic embryogenesis. In addition, a very strong signal was observed in the cell layers that would develop into the seed coat, which emphasizes the fact that EP2 expression is not limited to the embryo. In a later stage of zygotic embryogenesis (Figures 5O and 5P), the protoderm has developed into the embryo epidermis and is marked by a very high expression of the EP2 gene.

Figure 5. Localization of EP2 mRNA during Somatic and Zygotic Embryogenesis.

Plant material was fixed, embedded, and cut into 7-μm sections. Unless indicated, hybridization was performed with ^{35}S-UTP-labeled single-stranded antisense RNA, as outlined in Methods. Film emulsions were exposed for 3 weeks. Sections were photographed by bright-field and dark-field or epipolarization microscopy. Silver grains are visible as bright white dots in dark-field images.

(A) Bright-field photograph of globular and late heart stage somatic embryos. pd, protoderm.

(B) Dark-field photograph of **(A)**. Bar = 100 μm.

(C) Bright-field photograph of the cotyledon of a heart stage somatic embryo.

(D) Dark-field photograph of **(C)**. Bar = 20 μm.

(E) Bright-field photograph of a heart stage somatic embryo. Hybridization was with a ^{35}S-UTP labeled sense probe.

(F) Dark-field photograph of **(E)**. Bar = 100 μm.

(G) Bright-field photograph of a late torpedo stage somatic embryo. The original proembryogenic mass (arrow) from which the embryo develop is still attached to the root apex. pem, proembryogenic mass.

(H) Dark-field photograph of **(G)**. The cortex regions show some autofluorescence due to the dark-field illumination. Bar = 100 μm.

(I) Bright-field photograph of a ts11 mutant somatic embryo arrested at late globular stage. The arrowhead indicates the cell layers in which the EP2 gene is expressed.

(J) Epipolarization micrograph of **(I)**. Exposure was for 6 weeks. Bar = 50 μm.

(K) Bright-field photograph of an embryogenic suspension culture. pem, proembryogenic masses; ne, nonembryogenic cell cluster.

(L) Dark-field photograph of **(K)**. Bar = 100 μm.

(M) Dark-field photograph of a 60-celled globular zygotic embryo. end, endosperm; hd, derivatives of hypophysis; pd, protoderm; s, suspensor; sc, developing seed coat.

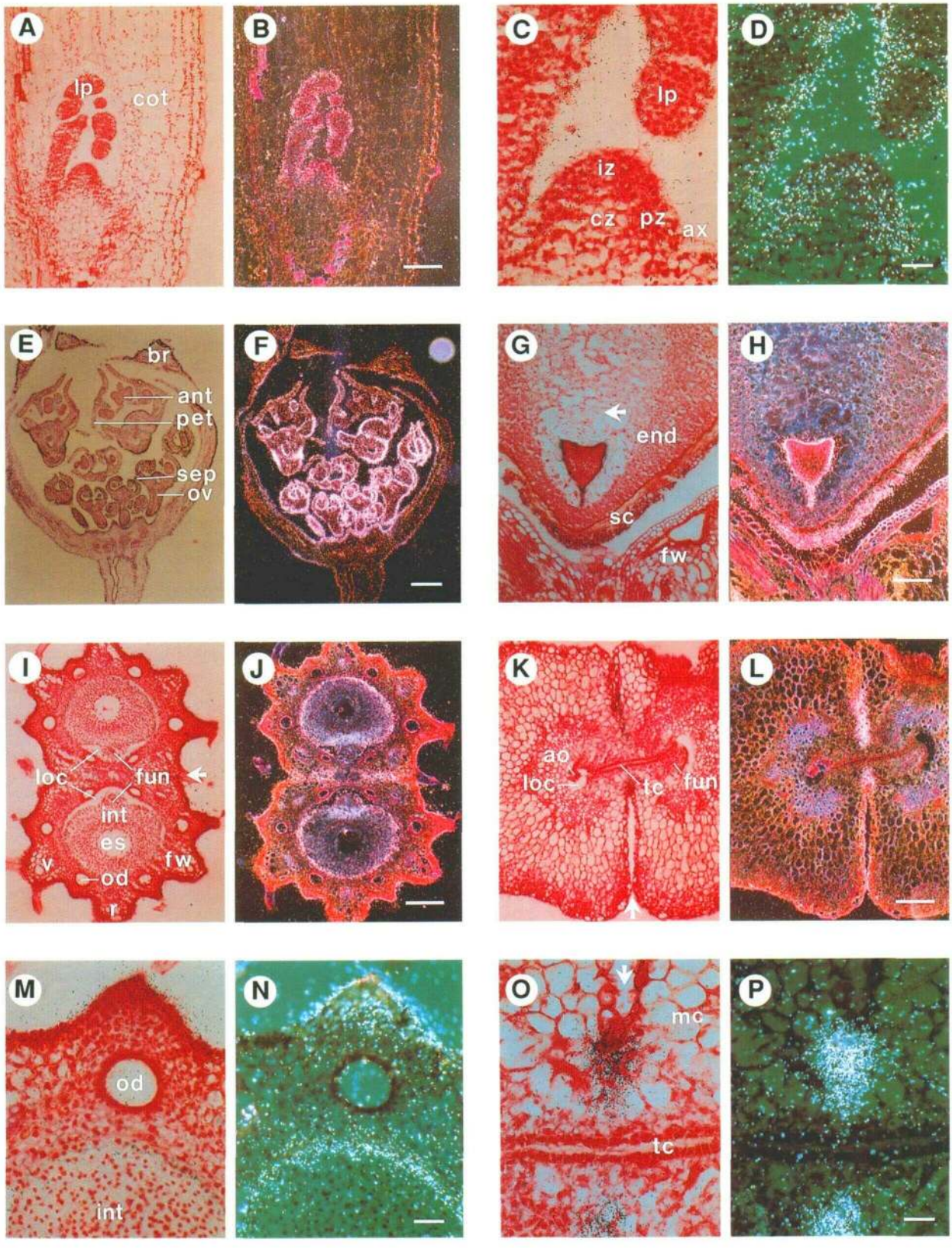
(N) Dark-field photograph of **(M)**. Bar = 20 μm.

(O) Bright-field photograph of a globular zygotic embryo. The suspensor is out of the plane of sectioning. pd, protoderm; end, endosperm; sc, developing seed coat.

(P) Dark-field photograph of **(O)**. Bar = 20 μm.

(Q) Bright-field photograph of a late heart stage zygotic embryo with developing cotyledons. cot, cotyledon; s, suspensor; pd, protoderm.

(R) Dark-field photograph of **(Q)**. Bar = 20 μm.



This pattern persists through the heart stage (Figures 5Q and 5R), where expression continues in the suspensor but is now highest in the epidermal cells of the developing cotyledons. Approaching seed maturation, the expression of the EP2 gene is gradually restricted to the epidermis of the developed cotyledons until it is completely absent when the seed is mature (data not shown). Although there are marked morphological differences between somatic and zygotic embryos, such as the larger size of somatic embryos at a corresponding developmental stage and the suppressed cotyledon development during somatic embryogenesis, the pattern of gradual restriction of EP2 gene expression toward the future shoot apex appears strikingly similar.

The EP2 Gene Is Transiently Expressed in Epidermal Cell Layers of Leaves, Flowers, and Seeds

After germination of carrot seeds and formation of plantlets from somatic embryos, the EP2 gene was only expressed in the shoot apex of the resulting plants (cf. Figure 3C). This finding was confirmed by in situ localization of EP2 mRNAs in the shoot apex (Figures 6A and 6B). In situ hybridization in stems, roots, and leaves confirmed the absence of EP2 mRNAs in these plant organs (data not shown). The now fully expanded and green cotyledons did not express the EP2 gene, in contrast to the epidermal cells of the shoot meristem and the young leaf primordia (Figures 6A and 6B). Apparently, the function of the lipid transfer protein encoded by the EP2 gene is only essential in early stages of leaf and cotyledon development. When viewed with higher magnification, the EP2 mRNAs appeared to be found only in the epidermal cells of the leaf primordia. In the shoot meristem itself, there was also a marked amount of EP2 mRNAs in subepidermal cells of the lateral zone. In the initiation zone, there was a reduced expression similar to the central zone of the shoot meristem. The expression of the EP2 gene in the embryo hypophyseal region and the expression of EP2 in the lateral zone of the shoot meristem were the only exceptions in wild-type plant development where expression of the EP2 gene was not restricted to epidermal cells or cells destined to become epidermal. Expression of the EP2 gene ceased abruptly in the axil (Figures 6C and 6D).

Another example of the gradual or abrupt restriction of EP2 gene expression can be seen in Figures 6E and 6F, where a young umbellet containing numerous flowers of different age is shown. EP2 mRNAs are abundant in the youngest flowers close to the base of the umbellet, and are found in surface cells of all flower organs, the ovary, stamen, petals, and sepals. In the older flowers of the umbellet, expression is already reduced and is restricted mainly to the ovary and anther epidermis. In the epidermis of the bractlets surrounding this group of flowers, the

Figure 6. Localization of EP2 mRNAs in Seedlings and Flowers.

Plant material was fixed, embedded, and cut into 7-μm sections. Hybridization was performed with ^{35}S-UTP-labeled single-stranded antisense RNA as outlined in Methods. Film emulsions were exposed for 3 weeks. Sections were photographed by bright-field and dark-field or epipolarization microscopy. Silver grains are visible as bright white dots in dark-field images.

(A) Bright-field photograph of a longitudinal section through the shoot apex of a 10-day seedling. cot, cotyledonary leaf; lp, leaf primordium.

(B) Dark-field photograph of **(A)**. Bar = 100 μm.

(C) Bright-field photograph of a longitudinal section cut through the medium of the shoot primary meristem. iz, initiation zone; cz, central zone; pz, peripheral zone; lp, leaf primordium; ax, leaf axil.

(D) Dark-field photograph of **(C)**. Bar = 20 μm.

(E) Bright-field photograph of a longitudinal section through a developing umbellet with numerous individual flowers. ov, ovary; ant, anther; sep, sepal; pet, petal; br, bractlet.

(F) Dark-field photograph of **(E)**. Bar = 200 μm.

(G) Bright-field photograph of a longitudinal section through a developing seed containing a heart stage embryo. The arrowheads indicate the cellular endosperm in the process of liquefaction. end, endosperm; sc, seed coat; fw, fruit wall (pericarp).

(H) Dark-field photograph of **(G)**. Bar = 100 μm.

(I) Bright-field photograph of a transverse section through a young developing bicarpellate fruit before dehiscence. The section was cut at a distance 1/3 of the length of the fruit from the basis. The arrowhead points to the region where both mericarps will separate. es, embryo sac; int, integument; fw, fruit wall; od, oil duct; v, vascular bundles; r, rib; fun, funiculus; loc, locule.

(J) Dark-field photograph of **(I)**. Bar = 100 μm.

(K) Bright-field photograph of a transverse section through the same fruit as in **(I)** and **(J)**. The section was cut at the upper part of the fruit, where both mericarps are connected by lateral bundles. The arrow indicates the plane of separation, which is almost complete in the lower part. ao, abortive ovule; fun, funiculus; loc, locule; tc, transverse canal connecting locules.

(L) Dark-field photograph of **(K)**. Bar = 20 μm.

(M) Bright-field photograph of an enlargement of **(I)**, showing a part of the fused integuments and one rib of the mericarp. int, integument; od, oil duct.

(N) Dark-field photograph of **(M)**. Bar = 20 μm.

(O) Bright-field photograph of an enlargement of **(L)**, showing almost complete separation between mericarps. The arrow indicates the plane of separation. mc, mericarp; tc, transverse canal.

(P) Dark-field photograph of **(O)**. Bar = 20 μm.

expression of EP2 has already ceased. During seed maturation, a high amount of EP2 mRNAs is present in the developing seed coat (Figures 6G and 6H). After the seed has matured, expression ceases in the now fully developed seed coat, at approximately the same time when expression in the matured embryo ceases (data not shown).

Because the seed coat is partially derived from the compressed remnants of the single integument (Fahn, 1974; Esau, 1977), we analyzed an earlier stage of seed development to answer whether EP2 expression was detected in the integument as well. In Figures 6I and 6J, a cross-section of a developing seed shortly after fertilization is shown. This stage is approximately equal to that discussed by Borthwick (1931). No endosperm has been formed, but the integument surrounding the embryo sac is fully developed. Expression of the EP2 gene is apparent in the outer epidermis of the integument, but not in the inner epidermis facing the embryo sac. No EP2 mRNA is present in the funiculus, which is the oldest part of the integuments from a developmental viewpoint. Low expression of the EP2 gene is detected in the epidermis between the developing ribs of the pericarp. An unexpectedly high amount of EP2 mRNA is found in the region (arrow in Figure 6I; see also Figure 6J), where both mericarps of the bicarpellate carrot flower will eventually separate. No EP2 mRNA is found in vascular bundles or oil duct walls. Viewed under higher magnification (Figures 6M and 6N), the presence of EP2 mRNA in the epidermis between the ribs as well as in the outer epidermis of the integument is clearly visible. A section through the same flower at the bottom of the stylopodium indicates that separation of the mericarps has already commenced (Figures 6K and 6L). On one side, the mericarps are still attached, whereas on the other side (lower part of Figures 6K and 6L) the separation is already complete. The separation of both mericarps appears to be preceded by a high expression of the EP2 gene in the cell layers that after separation will form the new pericarp epidermis. The clearly visible transverse canal connecting the locules of both mericarps, as well as an abortive ovule and the top of the funiculus, are clearly devoid of EP2 mRNA. Under higher magnification (Figures 6O and 6P), the precise cessation of EP2 expression upon complete separation of the mericarps is emphasized.

Taken together, expression of the EP2 gene appears to be restricted to the epidermis of early developmental stages of a particular tissue or organ, followed by either gradual or abrupt spatial and temporal cessation of expression.

DISCUSSION

As part of our ongoing research effort to investigate the role of secreted proteins in carrot somatic embryogenesis (De Vries et al., 1988a, 1988b; LoSchiavo et al., 1990; Cordewener et al., 1991), we set out to clone genes encoding secreted proteins (Van Engelen et al., 1991; this work; P. Sterk and S.C. De Vries, unpublished observations), employing expression screening of an embryo cDNA library. Among the secreted proteins, we identified a plant lipid transfer protein, EP2, present in cell walls of somatic embryos as well as in the conditioned medium of embryo cultures. RNA gel blot analysis revealed that the lipid transfer protein gene EP2 is expressed in somatic embryos and embryogenic suspension cultures, but not in nonembryogenic cultures. In carrot plants, EP2 gene expression is restricted to the shoot apical meristem, leaf primordia, and flowers, indicating that the EP2 gene is not embryo specific, but reflects a cellular function required in these tissues and apparently also during somatic embryogenesis. Employing in situ localization, it was found that the EP2 gene is expressed in the peripheral cells of both somatic embryos and their precursor cell clusters and in the epidermal cells of the shoot apical meristem and leaf primordia. In the inflorescence, the EP2 gene is transiently expressed in epidermal cells of all flower organs, whereas expression ceases upon maturation of stamen, petals, and sepals. In the ovary, expression continues in the integument epidermis, again in a transient fashion, and reappears when both the inner and outer integument epidermis combine to yield the seed coat. Like somatic embryos, zygotic embryos express the EP2 gene in the protoderm cells and the epidermal cells of the cotyledons. Finally, expression is seen in the epidermis of the pericarp and in the region where both mericarps separate.

The class of plant proteins now referred to as nonspecific lipid transfer proteins were originally identified as phospholipid transfer proteins (Kader, 1990). In several studies, it has been shown that these proteins can mediate in vitro transfer of radiolabeled phospholipids from liposomal donor membranes to mitochondrial acceptor membranes (Douady et al., 1978, 1982; Kader et al., 1984). Based on such studies, it has been suggested that plant lipid transfer proteins recycle phospholipids between organellar and cellular membranes and, therefore, play a major role in replenishment of membranes in cells actively engaged in secretion (Arondel and Kader, 1990), and that they transport phospholipids from their place of synthesis in the endoplasmic reticulum to membranes of organelles. Therefore, the conventional models for explaining the role of lipid transfer proteins assume that these proteins are intracellular and cytosolic or membrane bound. However, the presence of a secretory signal peptide and the absence of an endoplasmic reticulum retention signal (KDEL) at the carboxy terminus of all lipid transfer proteins homologous with EP2 seems difficult to reconcile with an intracellular function. In this respect, it is of interest to note that Mundy and Rogers (1986) already reported that the barley lipid transfer protein is found in aleurone cell culture media, whereas Bernhard et al. (1991) showed that the spinach

lipid transfer protein is efficiently translocated by canine pancreas microsomes. In support of these observations, we did not detect the carrot lipid transfer proteins among intracellular proteins of somatic embryos. Other studies have shown that, apart from phospholipids, plant lipid transfer proteins can also bind galactolipids (Nishida and Yamada, 1986), lipids, and fatty acids, such as oleic acid, linoleic acid, and oleoyl-coenzyme A (Rickers et al., 1984, 1985; Kader, 1990). Therefore, we conclude that the EP2 protein is a secreted protein that apparently is able to transport a variety of apolar molecules out of the cells that express the gene. Because there is only one EP2 gene per haploid carrot genome, the encoded protein is likely to have the same function in all cells that secrete it. However, the question remains which process in embryo, shoot meristem, leaf primordia, flower, and fruit epidermis requires the presence of apolar molecules outside the cell.

The main components of cuticles are the insoluble polymer cutin (Martin and Juniper, 1970; Kolattukudy, 1981) and associated soluble waxes (Tulloch, 1976). Most cutin monomers belong to a C_{16} or C_{18} family of epoxidated and/or hydroxylated fatty acids. The epoxidation of hydroxylated fatty acids and hydration of epoxidated fatty acids take place, at least partly, after transfer of hydroxylated fatty acids to the wall (Croteau and Kolattukudy, 1974; Kolattukudy, 1981; Lendzian and Schönherr, 1983). The molecular mechanism by which the hydrophobic cutin monomers are transported from their place of synthesis through the aqueous environment of the Golgi complex and across the cell wall is not well understood; in principle, exocytosis of fatty acid-containing vesicles could result in deposition of the cutin monomers in the periplasmic space directly, but this still leaves the aqueous environment of the cell wall to be traversed to reach the site of cutin synthesis in or on the outside of the cell wall. An alternative mechanism, therefore, would be to bind the cutin monomers to an EP2-like carrier protein that could transport these monomers to the outer epidermal wall surface where they can be released and esterified into the growing cuticle. The presence of the EP2 protein in the medium of embryogenic carrot cultures would then reflect the empty lipid carrier, discarded after delivery of the transported lipid. Whether the empty lipid carriers can be used again after endocytosis of the protein is not yet known. Given the fact that the class of extracellular nonspecific lipid transfer proteins like EP2 is capable of transporting the straight-chain fatty acids that serve as unmodified cutin monomers, it appears quite possible that these extracellular lipid transfer proteins provide the material for the synthesis of a cutin-containing cuticle. Conclusive experimentation to establish whether the purified carrot EP2 protein is indeed capable of transporting these cutin monomers and whether the EP2 protein is recycled to increase the efficiency of the lipid export is now in progress.

In this work, we have not directly investigated whether the cells that express the EP2 gene are indeed active in the synthesis of cutin. However, a survey of the known locations of plant cuticle formation reveals that there is indeed a marked coincidence. In all higher land plants, the aerial surfaces are covered with a cutin-containing cuticle, where it serves to protect against uncontrollable water loss (Martin and Juniper, 1970). During leaf development, the composition of the cuticle changes (Heide-Jørgensen, 1991) and may require the introduction of other modified or longer-chain fatty acids, which would account for the absence of EP2 expression in carrot leaves and the developed cotyledons. The maize and spinach lipid transfer proteins, however, have been isolated from leaves, which may reflect differences in cuticle composition between plant species. In several plants, it has been demonstrated that all reproductive organs are covered with a cutin-containing cuticular layer (Martin and Juniper, 1970). The absence of EP2 expression in the root can be explained by the fact that both the root epidermis and endodermis cells have suberized epidermal cell walls. The suberin monomers are ω-hydroxyfatty acids, dicarboxylic acids, long-chain (C_{20} to C_{30}) fatty acids, fatty alcohols, and phenolic components (Kolattukudy, 1981). Due to their structure that differs from the cutin monomers, it is unlikely that they can be transported by EP2-like lipid transport proteins. In many plants, the integuments are covered with a cuticular layer on both sides during ovary development (Bradbury et al., 1956; Martin and Juniper, 1970). Finally, the developing seed coat is also well known to be heavily covered with cuticular layers.

If our hypothesis that the expression of the EP2 gene marks those cells that are actively engaged in the formation of a cuticular layer is correct, it must be considered what its function is in early somatic and zygotic embryo development. Although not discussed by the authors, close examination of the electron micrographs of the classical example of zygotic embryo development in Capsella (Schulz and Jensen, 1968) reveals that the outer cell walls of the entire embryo and suspensor are covered with a thin electron-dense layer that is generally considered to represent lipophilic material. This layer is seen to extend in the cell walls separating the hypophyseal region from the suspensor as well as in cross-walls in the suspensor of an early globular embryo. This pattern coincides precisely with the cells expressing the EP2 gene in a carrot zygotic embryo of the same developmental stage. The presence of a cuticular layer surrounding maize embryos at 8 days after pollination has also been observed (Van Lammeren, 1986). Based on these studies and the presence of lipophilic substances on the surface of carrot proembryogenic masses (P. Sterk and S.C. De Vries, unpublished observations), it appears likely that early plant embryos are indeed covered with a cuticular layer as soon as the protoderm is formed.

The function of this layer may be different from the cuticle in aerial plant parts due to the liquid environment of both somatic and zygotic embryos. It would be more

logical to assume the opposite function, to prevent turgor-driven water uptake in the developing embryos, rather than preventing water loss. This would serve the purpose of maintaining a small cell size, postulated to be necessary for early differentiation of plant cells (Fry, 1990). Given the fact that somatic embryos of carrot consist of more highly vacuolated cells and are on average much larger than their zygotic counterparts of the same developmental stage, this process of protection against water uptake may not be fully functional in somatic embryos. In this respect, it is of interest to note that the osmotic pressure in the endosperm during very early stages of embryo development is quite high and decreases at later stages (Smith, 1973), without increase in the average cell size of the embryo (Pollock and Jensen, 1964), which suggests resistance of the zygotic embryo against variation in osmotic pressure in the endosperm. Clearly, in somatic embryogenesis the environment is always hypotonic and thus a mechanism to prevent cell enlargement would have to be functioning already in proembryogenic masses. It is widely accepted that plant cells can resist cell expansion under hypotonic conditions by cross-linking cell wall polymers (Fry, 1990). An additional mechanism to restrict cell expansion of embryo cells under hypotonic conditions, therefore, would be to coat the entire embryo with a water-repellent layer such as a cuticle and thus slow down turgor-driven water uptake. In this view, it is interesting to note that ts11 arrested globular embryos are thought to be defective in the correct formation of the epidermis (LoSchiavo et al., 1990), which would be in line with the observed EP2 expression in subepidermal cell layers as an attempt of the arrested embryos to form new cell layers with the protective function of the epidermis.

A second, and perhaps more important, function of an embryo cuticle may be inferred from sections of carrot zygotic embryos surrounded by partially liquefied endosperm. To render the nutrients stored there accessible to the growing embryo, the cellular endosperm is dissolved by hydrolytic enzymes. To protect the embryo itself from these enzymes, the formation of a water-repellent coating would clearly be beneficial. This may also explain why the vacuolated suspensor cells also express the EP2 gene. Somatic embryos may exist in a similar situation because the conditioned medium of plant cell cultures abounds with hydrolytic, cell wall-degrading enzymes (Fry, 1985).

METHODS

Plant Materials

Cultures of *Daucus carota* cv Trophy (Zaadunie B.V., Enkhuizen, The Netherlands), designated as cultivar "10" (embryogenic) and FG10 (nonembryogenic), and cultures of *D. carota* cv San Valery, designated A^+, α5II (nonembryogenic; Vergara et al., 1982), α5IIIR (a revertant of α5II; embryogenic), c15 (nonembryogenic; LoSchiavo et al., 1983), ts11 (temperature sensitive: permissive at 25°C, nonpermissive at 32°C; LoSchiavo et al., 1985) and ts11R (a revertant of ts11; embryogenic, LoSchiavo et al., 1990), were maintained as described previously (De Vries et al., 1988a). Carrot seedlings were grown in vermiculite at 25°C for 7 to 14 days. Mature greenhouse plants and flowers at different developmental stages were kindly provided by Zaadunie B.V.

Library Screening

A λgt11 cDNA library, prepared from RNA isolated from all stages of somatic embryos of *D. carota* cv "Northrup King," was kindly provided by Dr. T.L. Thomas (Texas A&M University, College Station, TX). A primary rabbit antiserum was raised against proteins secreted into the embryo culture medium. Expression screening was performed essentially as described by Huynh et al. (1985). Goat anti-rabbit IgG-alkaline phosphatase conjugate with nitro blue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) as substrates was used to detect positive clones. Corresponding cDNA clones were isolated from the λgt11 and a λZAPII cDNA library (Stratagene) prepared from poly(A)$^+$ RNA isolated from mixed embryogenic suspension and somatic embryo cultures of cultivar Trophy by using standard screening procedures (Maniatis et al., 1982). In addition, PCR was used with an EP2-specific 30-mer oligonucleotide primer and the M13 −20 sequence primer (GTAAAACGACGGCCAGT) to amplify EP2 sequences from the λZAPII library upstream the EP2 primer sequence. For this purpose, 10^8 plaque-forming units of the library were extracted once with an equal volume of phenol/chloroform (1:1). Phage DNA was precipitated from the aqueous phase by adding an equal volume of 2-propanol, pelleted, and washed with 0.5 mL of 70% ethanol. The DNA was dried and redissolved in 100 μL of 1× PCR buffer (20 mM Tris-HCl, pH 8.3, 25 mM KCl, 1.5 mM $MgCl_2$, 0.05 μg/mL of Tween 20, 100 μg/mL of gelatin) containing 300 ng of both primers. After addition of 2 units of *Taq* polymerase (Perkin-Elmer Cetus Instruments), 50 cycles (1 min at 92°C, 1 min at 60°C, 3 min at 72°C) were performed. The reaction products were analyzed on 1.5% agarose gels and subcloned in pBluescript SK− (Stratagene) for sequence analysis.

Antiserum against β-Galactosidase Fusion Protein

Clone EP2-A was expressed as a fusion protein with β-galactosidase in lysogenic *Escherichia coli* Y1089 as described by Huynh et al. (1985). The protein was purified from 1-liter cultures by using a protocol optimized for β-galactosidase. Cells were pelleted, resuspended in 40 mL 10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.2 mM DTT, and lysed by freezing in liquid nitrogen and subsequent thawing. The protein was kept at 4°C from here. Debris was removed by centrifugation at 13,000*g* for 10 min. The protein was eluted from a DEAE-Sephacel (Pharmacia) column with a linear 200 to 400 mM NaCl gradient. Fusion protein was precipitated with ammonium sulfate (60% saturation). After centrifugation at 13,000*g* for 30 min, the protein pellet was dissolved in 2 to 4 mL of 0.2 M NaH_2PO_4/Na_2HPO_4, pH 7.8, 1 mM $MgCl_2$, 0.2 mM PMSF, 0.2 mM DTT and eluted from a Sephacryl S-300 HR (Pharmacia) column with the same buffer. Fusion protein was precipitated with 60% ammonium sulfate, pelleted and dissolved in 10 mM

Tris-HCl, pH 8.0, 0.2 mM PMSF, and 0.2 mM DTT. The solution was desalted on a Bio-Gel P6PG (Bio-Rad) column and subjected to preparative 7.5% SDS-PAGE. After staining with Coomassie Brilliant Blue R-250, the fusion protein was excised, eluted in a Biotrap device (Schleicher and Schuell), and subsequently precipitated with 5 volumes of acetone. The protein was redissolved in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl and used for immunization.

Protein Isolation and Protein Gel Blot Analysis

Medium protein samples, cell wall protein extracts, and intracellular protein samples were prepared as described by Van Engelen et al. (1991). Protein gel blot analysis was carried out essentially as described by Burnette (1981), using 15% SDS-polyacrylamide gels and 1000-fold diluted antisera. Goat anti-rabbit IgG-alkaline phosphatase conjugate with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates was used to detect proteins recognized by the antisera.

DNA Sequencing

Sequencing of both strands of cDNA inserts was done in part at The Advanced DNA Technologies Laboratory, Texas A&M University, by using the double-stranded dideoxy chain termination method (Korneluk et al., 1985) using either the large fragment of *E. coli* DNA polymerase I or *Taq* DNA polymerase. Sequence data were analyzed by using the Staden program on a microVAX/VMS computer. EMBL, NBRF, and Swiss-Prot databases were searched for homology on DNA and protein level with known sequences using the FASTA program (Pierson and Lipman, 1988). The sequence of the carrot lipid transfer protein cDNA has been submitted to EMBL as accession number M64746.

RNA Gel Blot Analysis

Total RNA was isolated from cultured cells and plant tissues as described by De Vries et al. (1988c). Glyoxylated RNA samples were electrophoresed on 1.5% agarose gels and transferred to GeneScreen membranes (Du Pont-New England Nuclear) according to the manufacturer's instructions. Hybridization with ^{32}P-labeled cDNA clones was carried out in 5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 2 × Denhardt's (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, 0.02% BSA), 1% SDS, 100 μg/mL denatured salmon sperm DNA, 50% formamide for 16 hr at 42°C. Blots were washed twice in 0.1 × SSC, 0.5% SDS at 60°C for 30 min and exposed to Kodak X-Omat AR film with an intensifying screen.

Genomic DNA Isolation and DNA Gel Blot Analysis

Genomic DNA was isolated from cultured suspension cells of line "10" initially following the protocol for RNA isolation described by De Vries et al. (1988c). After removal of RNA by LiCl precipitation, DNA was precipitated by adding ethanol to 70% to the supernatant. After centrifugation the DNA pellet was washed twice with 70% ethanol, dried in a vacuum desiccator, and dissolved in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. The DNA was further purified by equilibrium centrifugation in a CsCl-ethidium bromide gradient as described by Maniatis et al. (1982). DNA was digested under standard conditions and electrophoresed on a 0.8% agarose gel. DNA was transferred to GeneScreen-*Plus* membranes using 10 × SSC. The blot was hybridized with ^{32}P-labeled EP2-C cDNA in 1 M NaCl, 1% SDS, 10% dextran sulfate, and 100 μg of denatured salmon sperm DNA at 65°C for 2 days. The blots were washed twice in 5 × SSC for 10 min and twice in 5 × SSC, 0.1% SDS at 65°C, and exposed to Kodak X-Omat AR film.

In Situ Hybridization

In situ hybridizations were performed essentially as described by Cox and Goldberg (1988). To facilitate handling, somatic embryos and suspension cells were transferred to 1% low-melting agarose at 40°C and concentrated by centrifugation for 20 sec at 40*g* before fixation and embedding.

^{35}S-labeled antisense and sense (control) RNA probes were transcribed from clone EP2-C using either the T3 or the T7 promoter and hydrolyzed to a size of approximately 0.2 kb. Hybridization was performed for 16 hr under a 42°C, 0.3 M Na^+, 50% formamide criterion. The washing stringency was 4 × SSC at 37°C. Slides were coated with Kodak NTB2 nuclear emulsion, exposed for 1 to 3 weeks at 4°C, and subsequently developed in Kodak D19 developer and fixed in Kodak Fix. Sections were stained with either safranin red or toluidine blue. Sections were photographed with a Nikon Optiphot-2 microscope equipped with dark-field and epipolarization optics.

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REFERENCES

Aleith, F., and Richter, G. (1991). Gene expression during induction of somatic embryogenesis in carrot cell suspensions. Planta **183,** 17–24.

Arondel, V., and Kader, J.C. (1990). Lipid transfer in plants. Experientia **46,** 579–585.

Backs-Hüsemann, D., and Reinert, J. (1970). Embryo formation by isolated single cells from tissue cultures of *Daucus carota*. Protoplasma **70,** 49–60.

Bernhard, W.R., and Somerville, C.R. (1989). Coidentity of putative amylase inhibitors from barley and finger millet with phospholipid transfer proteins inferred from amino acid sequence homology. Arch. Biochem. Biophys. **269,** 695–697.

Bernhard, W.R., Thoma, S., Botella, J., and Somerville, C.R. (1991). Isolation of a cDNA clone for spinach lipid transfer protein and evidence that the protein is synthesized by the secretory pathway. Plant Physiol. **95,** 164–170.

Borkird, C., Choi, J.H., Jin, Z-H., Franz, G., Hatzopoulos, P., Chorneau, R., Bonas, U., Pelegri, F., and Sung, Z.R. (1988). Developmental regulation of embryonic genes in plants. Proc. Natl. Acad. Sci. USA **85,** 6399–6403.

Borthwick, H.A. (1931). Development of the macrogametophyte and embryo of *Daucus carota*. Bot. Gaz. **2,** 23–44.

Bouillon, P., Drischel, C., Vergnolle, V., Duranton, H., and Kader, J.C. (1987). Complete primary structure of spinach leaf phospholipid transfer protein. Eur. J. Biochem. **166,** 387–391.

Bradbury, D., MacMasters, M.M., and Cull, I.M. (1956). Structure of mature wheat kernel. II. Microscopic structure of pericarp, seed coat, and other coverings of the endosperm and germ of hard red winter sheat. Cereal Chem. **33,** 342–360.

Burnette, W.N. (1981). "Western blotting": Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112,** 195–203.

Choi, J.H., and Sung, Z.R. (1984). Two-dimensional gel analysis of carrot somatic embryogenesis proteins. Plant Mol. Biol. Rep. **2,** 19–25.

Choi, J.H., Liu, L-S., Borkird, C., and Sung, Z.R. (1984). Cloning of genes developmentally regulated during plant embryogenesis. Proc. Natl. Acad. Sci. USA **84,** 1906–1910.

Cordewener, J., Booij, H., Van der Zandt, H., Van Engelen, F., Van Kammen, A., and De Vries, S. (1991). Tunicamycin-inhibited carrot somatic embryogenesis can be restored by secreted cationic peroxidase isoenzymes. Planta **184,** 478–486.

Cox, K.H., and Goldberg, R.B. (1988). Analysis of plant gene expression. In Plant Molecular Biology: A Practical Approach, C.H. Shaw, ed (Oxford, England: IRL Press), pp. 1–34.

Croteau, R., and Kolattukudy, P.E. (1974). Direct evidence for the involvement of epoxide intermediates in the biosynthesis of the C_{18} family of cutin acids. Arch. Biochem. Biophys. **162,** 471–480.

Crouch, M.L. (1982). Non-zygotic embryos of *Brassica napus* contain embryo-specific storage proteins. Planta **156,** 520–524.

De Vries, S.C., Booij, H., Janssen, R., Vogels, R., Saris, L., LoSchiavo, F., Terzi, M., and van Kammen, A. (1988a). Carrot somatic embryogenesis depends on the phytohormone-controlled expression of correctly glycosylated extracellular proteins. Genes Dev. **2,** 462–476.

De Vries, S.C., Booij, H., Meyerink, P., Huisman, G., Wilde, H.D., Thomas, T.L., and Van Kammen, A. (1988b). Acquisition of embryogenic potential in carrot cell-suspension cultures. Planta **176,** 196–204.

De Vries, S.C., Hoge, H., and Bisseling, T. (1988c). Isolation of total and polysomal RNA from plant tissues. In Plant Molecular Biology Manual, B6, S.B. Gelvin, R.A. Schilperoort, and D.P.S. Verma, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 1–13.

Douady, D., Kader, J.C., and Mazliak, P. (1978). Movement of phospholipids involving plant mitochondria: Regulation by phospholipid-exchange proteins. In Plant Mitochondria, G. Ducet, and C. Lance, eds (Amsterdam: Elsevier Biomedical Press), pp. 357–364.

Douady, D., Grosbois, M., Guerbette, F., and Kader, J.C. (1982). Purification of basic lipid transfer protein from maize seedlings. Biochim. Biophys. Acta **710,** 143–153.

Dure, L., Greenway, S.C., and Galau, G.A. (1981). Developmental biochemistry of cotton seed embryogenesis and germination: Changing mRNA populations as shown by in vitro and in vivo protein synthesis. Biochemistry **20,** 4162–4168.

Esau, K. (1977). Anatomy of Seed Plants. (New York: John Wiley and Sons).

Fahn, A. (1974). Plant Anatomy. (Oxford, England: Pergamon Press).

Fry, S.C. (1985). Primary cell wall metabolism. Oxf. Surv. Plant Mol. Cell Biol. **2,** 1–42.

Fry, S.C. (1990). Roles of the primary cell wall in morphogenesis. In Progress in Plant Cellular and Molecular Biology, H.J.J. Nijkamp, L.H.W. van der Plas, and J. van Aartrijk, eds (Dordrecht, The Netherlands: Kluwer Academic Press), pp. 504–513.

Goldberg, R.B., Hoschek, G., Tam, S.H., Ditta, G.S., and Breidenbach, R.W. (1981). Abundance, diversity and regulation of mRNA sequence sets in soybean embryogenesis. Dev. Biol. **83,** 201–217.

Goldberg, R.B., Barker, S.J., and Perez-Grau, L. (1989). Regulation of gene expression during plant embryogenesis. Cell **56,** 149–160.

Halperin, W. (1966). Alternative morphogenetic events in cell suspensions. Am. J. Bot. **53,** 443–453.

Heide-Jørgensen, H.S. (1991). Cuticle development and ultrastructure: Evidence for a procuticle of high osmium affinity. Planta **183,** 511–519.

Huynh, T.V., Young, R.A., and Davies, R.W. (1985). Construction and screening of cDNA libraries in λgt10 and λgt11. In DNA Cloning: A Practical Guide, Vol. 1, D.M. Glover, ed (Oxford, England: IRL Press), pp. 49–78.

Kader, J.C. (1990). Intracellular transfer of phospholipids, galactolipids, and fatty acids in plant cells. Subcell. Biochem. **16,** 69–111.

Kader, J.C., Julienne, M., and Vergnolle, C. (1984). Purification and characterization of a spinach leaf protein capable of transferring phospholipids from liposomes to mitochondria or chloroplasts. Eur. J. Biochem. **139,** 411–416.

Kolattukudy, P.E. (1981). Structure, biosynthesis, and biodegradation of cutin and suberin. Annu. Rev. Plant Physiol. **32,** 539–567.

Korneluk, R.G., Quan, F., and Gravel, R.A. (1985). Rapid and reliable dideoxy sequencing of double-stranded DNA. Gene **40,** 317–323.

Kyte, J., and Doolittle, R.F. (1982). A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157,** 105–132.

Lendzian, K.J., and Schönherr, J. (1983). In-vivo study of cutin synthesis in leaves of *Clivia miniata* Reg. Planta **158,** 70–75.

LoSchiavo, F., Giovinazzo, G., and Terzi, M. (1983). 8-Azaguanine resistant carrot cell mutants and their use as universal hybridizers. Mol. Gen. Genet. **192,** 326–329.

LoSchiavo, F., Giovinazzo, G., and Terzi, M. (1985). Pattern of polypeptides excreted in the conditioned medium and its alteration in a mutant ts for embryogenesis. In Somatic Embryogenesis, M. Terzi, L. Pitto, and Z.R. Sung, eds (Rome: Incremento Produttività Risorse Agricole), pp. 32–34.

LoSchiavo, F., Giuliano, G., De Vries, S.C., Genga, A., Bollini, R., Pitto, L., Cozzani, F., Nuti-Ronchi, V., and Terzi, M. (1990). A carrot cell variant temperature sensitive for somatic embryogenesis reveals a defect in the glycosylation of extracellular proteins. Mol. Gen. Genet. **223,** 385–393.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular cloning: A Laboratory Manual. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Martin, J.T., and Juniper, B.E. (1970). The Cuticles of Plants. (London: Edward Arnold).

Mundy, J., and Rogers, J.C. (1986). Selective expression of a probable amylase/protease inhibitor in barley aleurone cells: Comparison to the barley amylase/subtilisin inhibitor. Planta **169,** 51–63.

Nishida, I., and Yamada, M. (1986). Semisynthesis of a spin-labeled monogalactosyldiacyl-glycerol and its application in the assay for galactolipid-transfer activity in spinach leaves. Biochim. Biophys. Acta **813,** 298–306.

Nomura, K., and Komamine, A. (1985). Identification and isolation of single cells that produce somatic embryos at a high frequency in carrot suspension cultures. Plant Physiol. **79,** 988–991.

Perez-Grau, L., and Goldberg, R.B. (1989). Soybean seed protein genes are regulated spatially during embryogenesis. Plant Cell **1,** 1095–1109.

Pierson, W.R., and Lipman, D.J. (1988). Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85,** 2444–2448.

Pollock, E.G., and Jensen, W.A. (1964). Cell development during early embryogenesis in *Capsella* and *Gossypium*. Am. J. Bot. **51,** 915–921.

Raghavan, V. (1986). Embryogenesis in Angiosperms: A Developmental and Experimental Study. (Cambridge, England: Cambridge University Press).

Reinert, J. (1958). Morphogenese und ihre Kontrolle an Gewebekulturen aus Karotten. Naturwissenschaften **45,** 344–345.

Rickers, J., Tober, I., and Spener, F. (1984). Purification and binding characteristics of a basic fatty acid binding protein from *Avena sativa* seedlings. Biochim. Biophys. Acta **784,** 313–319.

Rickers, J., Spener, F., and Kader, J.C. (1985). A phospholipid transfer protein that binds long-chain fatty acids. FEBS Lett. **180,** 29–32.

Schultz, R., and Jensen, W.A. (1968). Capsella embryogenesis: The egg, zygote and young embryo. Am. J. Bot. **55,** 881–916.

Smith, J.G. (1973). Embryo development in *Phaseolus vulgaris*. II. Analysis of selected inorganic ions, ammonia, organic acids, amino acids, and sugars in the endosperm liquid. Plant Physiol. **51,** 454–458.

Somerharju, P.J., Van Paridon, P.A., and Wirtz, K.W.A. (1990). Application of fluorescent phospholipid analogues to studies on phospholipid transfer proteins. Subcell. Biochem. **16,** 21–43.

Steeves, T.A., and Sussex, I.M. (1989). Patterns in Plant Development. (Cambridge, England: Cambridge University Press).

Steward, F.C., Mapes, M.O., and Hears, K. (1958). Growth and organized development of cultured cells. II. Organization in cultures grown from freely suspended cells. Am J. Bot. **45,** 705–708.

Sung, Z.R., and Okimoto, R. (1981). Embryonic proteins in somatic embryos of carrot. Proc. Natl. Acad. Sci. USA **78,** 3683–3687.

Sung, Z.R., and Okimoto, R. (1983). Coordinate gene expression during somatic embryogenesis of carrot. Proc. Natl. Acad. Sci. USA **80,** 2661–2665.

Takishima, K., Watanabe, S., Yamada, M., and Mamiya, G. (1986). The amino acid sequence of the non-specific lipid transfer protein from germinated castor bean endosperm. Biochim. Biophys. Acta **870,** 248–250.

Tchang, F., This, P., Stiefel, V., Arondel, V., Morch, M.D., Pages, M., Puigdomenech, P., Grellet, F., Delseny, M., Bouillon, P., Huet, J.C., Guerbette, F., Beauvais-Cante, F., Duranton, H., Pernollet, J.C., and Kader, J.C. (1988). Phospholipid transfer protein: Full-length cDNA and amino acid sequence in maize. J. Biol. Chem. **263,** 16849–16855.

Thomas, T.L., and Wilde, H.D. (1985). Analysis of gene expression in carrot somatic embryos. In Somatic Embryogenesis, M. Terzi, L. Pitto, and Z.R. Sung, eds (Rome: Incremento Produttività Risorse Agricole), pp. 77–85.

Thomas, T.L., and Wilde, H.D. (1987). Analysis of carrot somatic embryo gene expression programs. In Proceedings of the VI International Congress of Plant Cell and Tissue Culture. (New York: Alan R. Liss), pp. 83–93.

Tulloch, A.P. (1976). Chemistry of waxes of higher plants. In Chemistry and Biochemistry of Natural Waxes, P.E. Kolattukudy, ed (Amsterdam: Elsevier), pp. 235–287.

Van Engelen, F.A., Sterk, P., Booij, H., Cordewener, J.H.G., Rook, W., Van Kammen, A., and De Vries, S.C. (1991). Heterogeneity and cell type-specific localization of a cell wall glycoprotein from carrot suspension cells. Plant Physiol. **96,** 705–712.

Van Lammeren, A.A.M. (1986). Developmental morphology and cytology of the young maize embryo (*Zea mays* L.). Acta Bot. Neerl. **35,** 169–188.

Vergara, M.R., Biasini, G., LoSchiavo, F., and Terzi, M. (1982). Isolation and characterization of carrot cell mutants resistant to α-amanitin. Z. Pflanzenphysiol. **107,** 313–319.

Von Heijne, G. (1983). Patterns of amino acids near signal-sequence cleavage sites. Eur. J. Biochem. **133,** 17–21.