THE CARROT EXTRACELLULAR LIPID TRANSFER PROTEIN EP2

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Stellingen

1. De conclusie dat niet-specifieke lipide transporteiwitten een intracellulaire functie hebben bij de membraansynthese berust op waarnemingsfouten en *wishful thinking*.

   Tchang *et al.* (1988). J. Biol. Chem. 263, 16849-16855.
   Arondel en Kader (1990). Experientia 46, 579-585.
   Dit proefschrift.

2. Het negatieve effect dat te sterke verdunning van embryoculturen heeft op somatische embryogenese kan deels verklaard worden door het optreden van apoptose.

   Halperin (1967). Exp. Cell Res. 48, 170-173.
   Hari (1980). Z. Pflanzenphysiol. 96, 227-231.
   Douglas (1994). New Scientist 1926, 31-34.

3. De conclusie van Hanson *et al*. dat het Zm13-gen specifiek in pollen tot expressie komt is, gelet op het geringe aantal RNA-preparaten dat gebruikt is en de keuze van het uitgangsmateriaal, voorbarig.

   Hanson *et al.* (1989). Plant Cell 1, 173-179.

4. Aangezien er in de levende cel geen sprake is van een evenwichtssituatie, is de door Stryer gebruikte evenwichtsthermodynamica voor de beschrijving van celfysiologische processen niet geldig.

   Stryer. Biochemistry, 3rd edition, 1988.

5. Het onvoorspelbare verschil in de mate waarin verschillende DNA-sequenties, die dezelfde *primer*sequenties bezitten, geamplificeerd worden in een *polymerase chain reaction* (PCR) maakt dat in twijfel moet worden getrokken of het amplificeren van cDNA-sequenties met behulp van PCR wel geschikt is voor differentiële screening van cDNA-banken.

6. Het formuleren en aanscherpen van een politieke opinie is een dynamisch proces, dat sterk gebaat is bij het lezen van kranten die een met de eigen overtuiging strijdig politiek standpunt weergeven.

7. De bezorgdheid die onlangs door een van de leden van het Britse Hogerhuis werd geuit bij het aanvoeren van argumenten tegen het verlagen van de *gay age of consent* (de minimumleeftijd waarop seksuele omgang tussen mannen wettelijk is toegestaan) van 21 tot 18 jaar, dat het aannemen van een homoseksuele leefstijl de gemiddelde levensduur verkort tot 47 jaar, had geloofwaardiger geklonken als tegelijkertijd was gepleit voor een verhoging van de minimumleeftijd van 21 tot 47 jaar.

8. Gelet op het relatief grote aantal mensen dat nooit gebruik maakt van openbare bibliotheken, is het te verwachten dat veel van de informatie beschikbaar via de zogenaamde *information superhighway* nooit benut gaat worden.

9. Goed beheer van informatie is net zo belangrijk als het genereren ervan.

10. Het tegenvallen van vakantiefoto's is vaak niet zozeer te wijten aan onvermogen van de makers, maar meer aan gebrek aan geduld en verbeelding, hetgeen aangeeft dat vakanties niet altijd hun beoogde effect hebben.

11. Ook zonder het nemen van effectieve tegenmaatregelen zal het gat in de ozonlaag verdwijnen.

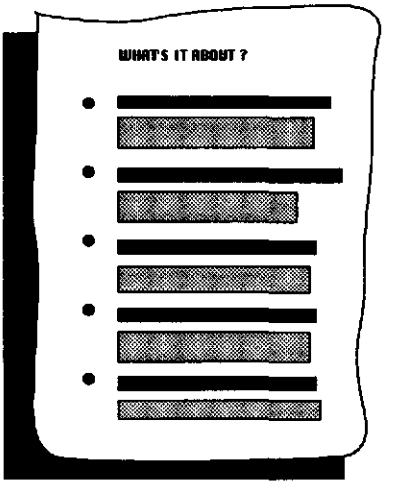
12. Met de ontwikkeling van een mondiaal bruikbare gebarentaal zijn niet alleen doven en slechthorenden gebaat.

Stellingen behorende bij het proefschrift:
"The carrot extracellular lipid transfer protein EP2,"
te verdedigen door Peter Sterk op 14 december 1994.

aan mijn ouders

Contents

Scope



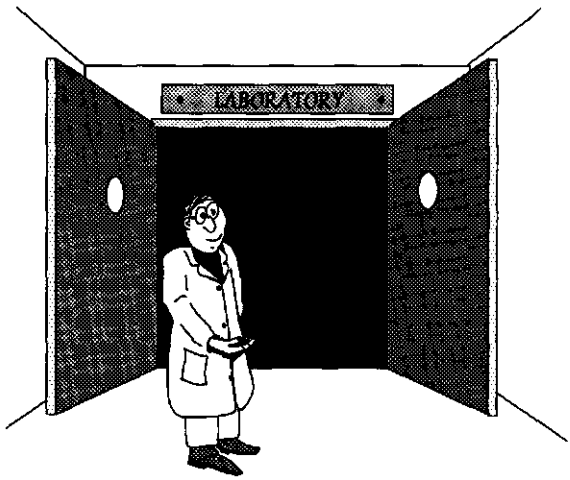
In many plant species embryos can develop from cultured somatic cells in a process referred to as somatic embryogenesis. Apart from their cellular origin somatic embryos develop through the same characteristic morphological stages, *i.e.* globular, heart and torpedo stages, observed during zygotic embryogenesis. Somatic embryos of different developmental stages can easily be obtained in large quantities, one of the main reasons for the frequent use of somatic embryogenesis as an alternative system to study plant embryogenesis.

Despite the wealth of information available on both zygotic and somatic embryo development with respect to their morphology and histology, insight in the molecular events that take place during embryogenesis is still poor. The study presented in this thesis is based on the observation that the presence of a small number of (glyco)proteins secreted into media of cultured carrot cells parallels the appearance of actual somatic embryos in these cultures. The aim of this study was to clone genes encoding these proteins and to study their expression and function in relation to embryogenesis. The approach taken was to screen an expression library with antisera raised against proteins secreted by carrot cells. This screening resulted in four cDNA clones which, upon detailed analysis of the expression patterns of the corresponding genes and identification of the respective encoded proteins by immunological means, were shown to represent genes encoding proteins secreted by distinct cell types.

In chapter 1 an introduction to both somatic and zygotic embryogenesis is presented, as well as an overview and classification of genes and proteins expressed in embryos that have been identified in other studies, based on their temporal expression patterns during embryo development. Chapter 2 describes the cloning of four cDNAs representing the carrot genes Extracellular Protein (EP)1, EP2, EP5 and EP6. Based on the observed close correlation of EP2 gene expression with embryo development, this gene was chosen for further study as described in chapter 3. *In situ* hybridization experiments revealed EP2 expression already in the 60-celled zygotic embryo, and in proembryogenic masses, the precursors of somatic embryos, and in somatic embryos. Based on its sequence homology with other extracellular non-specific lipid transfer proteins, the expression of the EP2 gene in protodermal and epidermal cells in embryos and plants, and the known coincidence of a cuticle on those cells, a role for the EP2 encoded protein was proposed in the transport of monomeric cutin precursors to sites of cutin synthesis. To support this hypothesis, the affinity of the purified EP2 protein for known cutin precursors was demonstrated in chapter 4. In chapter 5 the existing approaches to study early plant embryo development are discussed and compared with the results presented here.

CHAPTER 1

General introduction



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Peter Sterk and Sacco C. de Vries
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K. Redenbaugh, ed (Boca Raton, FL, USA: CRC Press), pp.115-131 (1993)

Introduction

There is a wide interest in the regeneration of plants by artificial means for propagation purposes. Artificial regeneration can be achieved via organogenesis whereby initiation of callus from explants on solid media is followed by hormonal induction of shoots and roots. Eventually plantlets develop which can be transferred to soil. Usually more efficient is propagation via somatic embryogenesis whereby callus is first initiated from explants, either on solid or in liquid media, followed by establishment of a growing cell suspension culture. Embryogenic cells or cell clusters can subsequently form in suspension culture. These cells or cell clusters can further develop into plants via somatic embryos.

Somatic embryogenesis has been reported for such a large number of taxa that incorporate many species over wide-ranging dicotyledonous and monocotyledonous families as the *Apiaceae, Solanaceae, Fabaceae, Rubiaceae, Rutaceae, Vitaceae, Poaceae,* and *Liliaceae,* that embryo formation from somatic cells can be assumed to be a general phenomenon in higher plants (Bornman, 1993). However, the main obstacles still to be overcome are the frequently encountered rather low frequency of embryogenesis and the formation of many aberrant embryos, that results in a high level of morphological variation in the populations of plants regenerated. In order to obtain large numbers of somatic embryos able to develop into uniform fertile plants, it is of utmost importance to establish a sound basis of the molecular and physiological processes that underlie the formation of plant somatic embryos. Although at the morphological and ultrastructural level both zygotic (Miller and Wetmore, 1945; Schultz and Jensen, 1968) and somatic (Halperin, 1966; Backs-Hüsemann and Reinert, 1970; Nomura and Komamine, 1985) embryogenesis have been well characterized, at the molecular level zygotic as well as somatic embryogenesis remain poorly understood. Likewise, the molecular mechanisms that allow some non-embryogenic somatic cells grown in suspension cultures in the presence of auxins like 2,4-D to acquire embryogenic potential, *i.e.* acquire the capacity to form somatic embryos, are also largely unknown.

Far from offering a complete overview of all studies that have been concerned with plant embryogenesis, this chapter will deal with several recent studies of genes and their encoded proteins that are thought to be involved in plant somatic embryogenesis. Because in many aspects, somatic embryogenesis can be regarded as a recapitulation of zygotic embryogenesis, studies of several genes expressed during zygotic embryogenesis will be discussed, with an emphasis on those cases where the expression of a particular gene has been investigated in somatic as well as zygotic embryogenesis. In particular, recent findings will be discussed that have indicated the importance of secreted proteins in somatic embryogenesis, and that form the basis for the work described in this thesis.

In order to correlate the studies on genes expressed during embryo development with morphological and physiological landmarks in the formation of both zygotic and somatic embryogenesis, a brief descriptive summary of plant embryogenesis and the cell types involved will be presented first.

Zygotic embryogenesis

Zygotic embryogenesis takes place inside the embryo sac in the ovule and commences with the fusion of the haploid egg cell nucleus with one haploid sperm cell nucleus. The resulting unicellular diploid zygote divides usually but not always asynchronously into a large vacuolated cell that gives rise to the suspensor, thought to promote continued growth of the embryo proper (reviewed by Yeung and Meinke, 1993), 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 in dicotyledonous plants. In *Capsella*, already after the eight-celled pre-globular stage zygotic embryo, anticlinal divisions of the surface cells result in a dermal cell layer, the protoderm, that marks the beginning of visible tissue differentiation of the embryo proper (Schultz and Jensen, 1968). During the globular stage the longitudinal embryo axis becomes apparent by the formation of the primary root and shoot apical meristem initials. These initials are connected by a column of procambium cells, from which eventually the vascular system will derive (Miller and Wetmore, 1945; Steeves and Sussex, 1989). The formation of both cotyledons adjacent to the shoot apical meristem results in the characteristic heart stage in dicotyledonous plants. Cell expansion in addition to cell division then results in elongation of the embryo axis and gives the embryo a torpedo-like shape. Subsequently, the cotyledons develop to produce the final phase in embryo development, referred to as the cotyledon stage.

Upon fertilization of the egg cell a second haploid sperm cell nucleus fuses with the nucleus of the diploid central mother cell in the embryo sac. The resulting triploid cell divides and develops into the endosperm that will surround the growing embryo. The endosperm contains nutrients such as proteins, carbohydrates and oil and is in many plants fully absorbed by the developing cotyledons, which then become filled with storage compounds such as storage proteins. In other plants, notably the monocotyledonous species, the endosperm persists until seed maturation.

After cessation of all major metabolic processes and dehydration the entire seed including the cotyledon stage embryo becomes dormant. Under appropriate conditions the seed germinates to produce a seedling able to develop into a mature plant. Upon formation of the reproductive cells in the flowers, the sporophytic life-cycle of the plant is completed (Goldberg *et al.*, 1989).

Somatic embryogenesis

Plant embryos can also develop from non-gametophytic somatic cells in liquid culture media and follow in principle the same developmental stages, *i.e.* globular, heart and torpedo stages, of embryo morphology. Apart from their cellular origin, somatic embryos differ from their zygotic counterparts by the frequently noted absence of a suspensor, suppressed cotyledon development and lack of a dormancy period. They develop directly into plantlets, which can after transfer to soil mature, flower and set seed. Somatic embryogenesis has first been observed in carrot (Reinert, 1959) and has subsequently been demonstrated in a wide range of

species (Ammirato, 1983). Although it has been observed that carrot somatic embryos can develop directly from single suspension cells (Backs-Hüsemann and Reinert, 1970; Nomura and Komamine, 1985), they usually form on the surface of clusters, designated proembryogenic masses, that consist of small, tightly adhering cytoplasmic cells (Halperin, 1966). Proembryogenic masses are present as a subpopulation, together with non-embryogenic more vacuolated cells, in suspension cultures containing the synthetic auxin 2,4-dichlorophenoxy acetic acid (2,4-D). This auxin is used to reinitiate division of explant cells and to obtain sustained division in the derived suspension culture. In addition, in responsive plant species, 2,4-D is required to induce embryogenic capacity in some suspension cells. This second action of 2,4-D is most elegantly demonstrated in freshly isolated leaf mesophyll protoplasts as well as suspension cultures of alfalfa, maintained in the presence of naphthalene acetic acid (NAA). A brief exposure to a high concentration of 2,4-D in both systems is sufficient to induce embryogenic potential in the previously non-embryogenic cells (Bögre *et al.*, 1990; Dudits *et al.*, 1991). From a different angle LoSchiavo *et al.*, (1991) recently reported that non-embryogenic carrot suspension cells are capable of increasing their level of auxin binding sites in response to increased exogenous auxin. Once cells present in the same culture had become embryogenic they did not show this response, unless cultured for prolonged time in the presence of auxin.

Acquisition of 2,4-D induced embryogenic potential in carrot has been shown to involve extensive alterations in the pattern of methylation of genomic DNA (Okkels *et al.*, 1988; LoSchiavo *et al.*, 1989). Both cellular and molecular evidence points to a decrease in the duration of the cell cycle as one of the earliest effects of 2,4-D in the formation of embryogenic cells in tissue culture (Bögre *et al.*, 1990). As 2,4-D causes cells to proliferate in an unorganized manner, it is also a potent inhibitor of further somatic embryo development after the early globular stage. For that reason, suspension cultures are usually subcultured in hormone-free medium during somatic embryo development. It must be noted, though, that in carrot not the removal of 2,4-D, but the change to an approximately 100-fold lower cell density actually triggers somatic embryo development. Further development of the embryo, however, is effectively inhibited by 2,4-D (Sung and Okimoto, 1981).

Although 2,4-D is widely used as an inducer of embryogenic potential, it is by no means unique in this property. Several other components such as thidiazuron (Saxena *et al.*, 1992), other potent auxins such as 2,4,5-T as well as shifts in pH (Smith and Krikorian, 1990) and electrical fields (Dijak *et al*, 1986) have been employed to induce embryogenic potential. It therefore emerges that no single specific compound can be pointed out as being responsible for altering the developmental fate of somatic plant cells.

A special case of *in vitro* (essentially non-somatic) embryogenesis is represented by the ability of immature pollen to respond to inducers such as heat shock in *Brassica* (Pechan and Keller, 1988) or starvation as in tobacco (Žárský *et al.*, 1992) to divert from pollen development into haploid embryos. In these systems, there is mounting evidence that cell cycle derepression is highly correlated with the induction of embryogenesis.

Plant embryogenesis and gene expression

Studies on gene expression during embryogenesis have revealed several classes of genes that can be distinguished according to their expression in time and in space as well as to the function of their products. As a number of these genes mark events or developmental stages in embryogenesis, an attempt is made in this chapter to classify genes expressed during both zygotic and somatic embryogenesis in relation to their temporal expression patterns, in order to make a contribution to a clearer understanding of the events that take place during embryogenesis on a molecular level. This classification is largely based on those described by Dure (1985) and Goldberg *et al.* (1989) for genes expressed during zygotic embryogenesis. Genes will be grouped in one of six classes, described as follows:

0. Class 0 consists of genes whose expression is absent or strongly reduced notably during early embryogenesis.
1. Class 1 consists of constitutively expressed genes, which have functions required during normal plant growth and are thus found throughout the entire plant.
2. Class 2 are embryo-specific genes, of which the expression is restricted to the embryo proper and ceases prior to or at germination.
3. Class 3 are genes expressed during early embryogenesis, *i.e.* until cotyledonary stage.
4. Class 4 consists of seed protein genes, expressed during the expansion phase of the cotyledons and maturation of the seed.
5. Class 5 are genes expressed throughout the embryo during late embryogenesis until seed maturation is complete or until early germination.

In the next two paragraphs a number of genes will be discussed that have been studied initially in either zygotic or somatic embryogenesis in relation to their classification as embryo genes. Most of these genes have been identified on the basis of differential library screening. In most cases, there is no direct evidence that these genes are indeed indispensable for embryogenesis.

Genes expressed during zygotic embryogenesis

Two-dimensional gel analysis of proteins synthesized during rapeseed (Crouch, 1982) and cotton (Dure *et al.*, 1989) zygotic embryogenesis showed that only relatively few (detectable) proteins could be marked as actually embryo-specific. cDNA complexity analysis of soybean embryo mRNA (Goldberg *et al.*, 1981), have revealed that approximately 30,000 genes are expressed in cotyledon stage embryos. Among these, genes that encode seed proteins, which include storage proteins, lectins and protease inhibitors, predominate. All of these genes belong to class 4 genes, and are expressed to very high levels during embryo development. The majority of dicotyledonous storage proteins are truly embryo-specific, and accumulate in the developing cotyledons shortly after cessation of cell division in these organs. Storage proteins are deposited into specialized protein bodies derived

from the vacuoles. Occasionally, expression of storage protein genes has also been observed to a much lower level in the embryo axis. In contrast to the mainly cotyledon-specific expression of storage protein genes in dicotyledonous plants, most monocotyledonous storage protein genes are only expressed in the endosperm cells.

Storage proteins can be divided into several classes, based on their solubility in water, salt, aqueous alcohol and acid or alkali solutions. In most dicotyledonous plants the major storage proteins are the salt-soluble globulins, whereas the alcohol-soluble prolamines and acid- or base-soluble glutelins predominate in monocotyledonous plants (Higgins, 1984; Dure, 1989). The main function of storage proteins is to serve as the initial carbon and nitrogen source for the developing seedling, though many of the storage proteins may have derived originally from common cellular enzyme ancestors (Andrews *et al.*, 1988). They are usually encoded by complex multigene families, of which the different members often exhibit striking differences in their expression pattern, both in time and in position in the developing embryo (Perez-Grau and Goldberg, 1989). This indicates that different regulatory elements must exist that confer cell-specificity as well as temporal programming in the expression of these genes. Indeed, in the promoter regions of seed protein genes such regulatory sequences have been identified (Colot *et al.*, 1987; Maier *et al.*, 1987; Chen *et al.*, 1988; Maier *et al.*, 1988; Bustos *et al.*, 1991; reviewed by Thomas, 1993). Currently, much effort is being put into the identification of the genes that encode embryo-specific transcription factors that interact with these regulatory elements (Lessard *et al.*, 1991; Kawagoe and Murai, 1992; Schmidt *et al.*, 1992; Pysh *et al.*, 1993).

During late embryogenesis the level of abscisic acid (ABA) rises inside the seed and this rise is concomitant with the onset or enhancement of expression of many ABA-inducible class 5 genes, among which late embryogenesis abundant (LEA) protein genes predominate. Many of these *lea* genes have been characterized in different plant species (Litts *et al.*, 1987; Baker *et al.*, 1988; Hong *et al.*, 1988; Mundy and Chua, 1988; Harada *et al.*, 1989; Wurtele *et al.*, 1993). Most of the *lea* genes can also be induced in other plant parts by exogenous ABA-treatment or the application of various stresses, such as salt or cold treatment and desiccation. The possibility to induce *lea* genes in non-embryo cells indicates that the expression of these genes is not embryo-specific, and that the expression of these genes may reflect a common response of plant cells to stress conditions. The accumulation of LEA proteins in embryos most probably contributes to the acquisition of desiccation tolerance. Based on their sequences three classes of LEA proteins can be distinguished. The first class of LEA proteins has been predicted to adopt several amphiphilic α-helices within the protein sequences. One face of such a helix is predominantly hydrophobic, whilst the opposite face is mainly hydrophilic. Such structures offer the possibility to simultaneously interact with hydrophobic surfaces, *e.g.* membranes, and hydrophilic components of the cytosol, thereby enabling the stabilisation of cellular membranes in the dehydrated state of the desiccated seed (Dure *et al.*, 1989). Members of the second class of LEA proteins are highly hydrophilic and consequently have a surprisingly high capacity for binding water (McCubbin *et al.*, 1985). This feature and their predicted random coil configuration have led to the hypothesis that they act as a 'molecular sponge' which enables them to stabilize other macromolecules by introducing 'shared water' through the formation of a protein-water-protein matrix (Lane, 1991). A

third class comprises enzymes contributing to the synthesis of cryopreservative carbohydrates. One such enzyme was discovered in barley zygotic embryos. Desiccation tolerance was reported to be already acquired between 12 and 18 days after anthesis, *i.e.* roughly between heart and torpedo stage, which was concomitant with the onset of expression of several genes. One of these genes was shown to encode an aldose reductase related protein postulated to play a role in the regulation of osmolarity of the embryo cells. Expression of this gene was enhanced by ABA and ceased during seed germination (Bartels *et al.*, 1991), so this gene would be one of the first class 2 embryo genes identified in zygotic embryos that has been reported so far.

Employing a completely different strategy that makes use of monoclonal antibodies raised against plasma membrane components, Pennell and Roberts (1990) described an epitope recognized by the monoclonal antibody MAC207, which was present on a class of plasma membrane arabinogalactan proteins of pea that was absent from cells giving rise to the reproductive flower organs. The reproductive cells as well as the young globular zygotic embryo were devoid of this epitope, that reappeared in late, cotyledon-stage embryos. Genes that are responsible for the creation of this epitope might therefore be classified as the here introduced class 0 genes, of which the expression is absent or strongly reduced in early embryogenesis. Within the developmental window, marked by the absence of the MAC207 epitope, another epitope present in arabinogalactan proteins and recognized by the monoclonal antibody JIM8 was found to be restricted to the zygote and the suspensor cells of the preglobular embryo. JIM8 was also found to be transiently expressed in several cell types of the anther including the sperm cells, and in the ovule in the nucellus, the synergid cells and the egg cell, but not in the central cell (Pennell *et al.*, 1991). The genes involved in creation of this epitope would be more difficult to classify. The observed expression of JIM8 illustrates the complexity of the events taking place during the last phases of reproductive cell formation, the actual fertilization and the first phases of zygotic embryo formation.

So far, only very few genes have been described in zygotic embryos that would fall into class 2 or 3, representing genes expressed preferentially in the early stages of embryogenesis. This lack of information has been mainly due to the difficulties encountered in obtaining sufficient early stage zygotic embryos for analysis, but should be rapidly overcome by the introduction of polymerase chain reaction (PCR) based amplification techniques to clone genes from minute amounts of starting material.

An approach that lacks many of the inherent pitfalls associated with establishing the biological importance of genes obtained via differential screening is the use of a genetic dissection of a developmental process by the generation of mutants. This approach, pioneered in *Arabidopsis* by Meinke (1986) builds upon a long tradition of generating embryo mutants in maize and pea. More recently, Jürgens *et al.* (1991) and Mayer *et al.* (1991; 1993) have provided the first comprehensive model of pattern formation in plant zygotic embryogenesis, based on an extensive collection of pattern mutants in *Arabidopsis* (see Chapter 5). With the rapidly developing techniques to clone genes by virtue of their map position or on the basis of T-DNA insertions or transposable element insertions, recovery and analysis of the mutated genes can be expected in the very near future.

Genes expressed during somatic embryogenesis

The possibility to generate large amounts of somatic embryos of the same developmental stage has made somatic embryogenesis an attractive system to identify genes specifically expressed during the early stages of embryo development. Several genes and proteins have been characterized, so far mainly in carrot, that indeed exhibit a stage-specific expression pattern in the early stages of somatic embryogenesis. In some cases, but not in all, the described expression pattern during somatic embryo development has made it possible to fit these genes into the classification system established originally for zygotic embryogenesis.

The carrot Dc8 gene (Choi *et al.*, 1987; Borkird *et al.*, 1988; Franz *et al.*, 1989) was originally identified by screening of a λgt11 expression library with an antiserum raised against an extract of carrot somatic embryos and purified through differential immunoadsorption techniques. It was found to be expressed at low level in proembryogenic masses, and at higher level in all subsequent stages of somatic embryos. The Dc8 gene was also expressed in zygotic embryos, as well as in the endosperm at a low level, but not in seedlings and mature plants. Employing immunocytochemistry it was found that up to torpedo stage the Dc8 protein was found in vacuoles, vesicles, the lumen of the rough endoplasmic reticulum and Golgi bodies. In late torpedo-stage zygotic embryos the Dc8 protein was also found in the cell walls, and in endosperm cells in the middle lamellae. Although the function of the Dc8 protein is not known, the cDNA deduced protein sequence shows some structural similarity with the previously described class 1 LEA proteins. Based on this structural similarity, the Dc8 gene would therefore be a class 5 embryo gene. Based on the expression pattern during somatic embryogenesis, however, it may also have a function required in earlier stages of somatic embryo development and may therefore be a class 2 gene.

Northern hybridization analysis showed that the expression of another carrot gene, Dc59, isolated as described for Dc8, was regulated in a similar fashion as that of Dc8, as expression increased shortly after onset of somatic embryogenesis, and was expressed throughout somatic embryo development. The gene was also expressed in zygotic embryos, but not in seedlings and mature plant parts (Borkird *et al.*, 1988). The encoded protein showed high homology to the L3 membrane-associated protein of lipid bodies from maize (Hatzopoulos *et al.*, 1990), postulated to play a role in stabilizing the structure of the lipid body. The level of Dc59 gene expression was, like many *lea* genes, regulated by ABA. Therefore, Dc59 may represent a class 5 gene, of which the expression is enhanced during late torpedo and cotyledonary stage embryogenesis. However, as noted for the Dc8 gene, expression of Dc59 also occurs already at much earlier stages in somatic embryo development.

Wilde *et al.* (1988) identified a cDNA clone, Dc3, by differential screening of a carrot somatic embryo cDNA library against polyadenylated RNA isolated from embryogenic high-density carrot cell suspensions. The Dc3 gene was expressed in proembryogenic masses as well as in somatic embryos (Wilde *et al.*, 1988; De Vries *et al.*, 1988b). Like Dc8, Dc3 is related to class 5 *lea* genes (Dure *et al.*, 1989) that encode proteins accumulating in zygotic embryo tissues during seed maturation and desiccation. Similar to *lea* genes, the expression of the Dc3 gene can be enhanced in mature plant parts by ABA, salt stress or desiccation (Vivekananda *et al.*, 1992).

Aleith and Richter (1991) have reported the cloning of several cDNAs corresponding to carrot mRNAs that accumulated transiently upon initiation of somatic embryogenesis, roughly during the globular stage. One of these clones, Dc7.1, encoded a glycine-rich protein and one, Dc2.15, a protein with a proline-rich domain. Their function during embryogenesis is unknown, but based on their sequences they probably encode cell wall proteins. Because the expression of Dc7.1 and Dc2.15 during zygotic embryogenesis has not yet been reported, these genes are at present difficult to classify. If expression of these genes in somatic embryos is similar in zygotic embryos, these genes would be examples of class 2 or 3 genes.

A few genes have been isolated by virtue of their enhanced translation during somatic embryogenesis, which include the β-subunit of ATP synthase (ATP-2; Zimmerman, 1993) and translation elongation factor EF1-α (Kawahara *et al.*, 1992; Zimmerman, 1993). Expression of the latter was shown to be enhanced in globular somatic embryos (Kawahara *et al.*, 1992). The enhanced translation of these genes may reflect an increase in metabolic activity during embryogenesis which requires an enhanced generation of energy and synthesis of protein. Arguably, they fall into class 1, although their enhanced translation during somatic embryogenesis might suggest they are class 2 or 3 genes.

Employing specific monoclonal antibodies against carrot proteins obtained from rapidly proliferating cultured cells, Smith *et al.* (1988) identified a 45 kD nuclear protein found in the nucleoli of somatic as well as zygotic embryo cells and also in cells of the shoot apical meristem of seedlings. Different transcripts of the alfalfa *cdc2* protein kinase gene were found to be present in a fluctuating pattern during the induction of embryogenic potential and subsequent embryo development, postulated to reflect changes in the duration of the cell cycle during early embryogenesis. At plant level these transcripts were found predominantly in shoots (Hirt *et al.*, 1991). Genes involved in the control of cell division are clearly of utmost importance in plant embryogenesis and would all fall into the group of class 1 genes, representing cellular functions necessary throughout plant development.

In pea, two proteins of 45 and 70 kD respectively, have been found that were present in a callus line able to form somatic embryos, but absent in a line without regenerative capacity (Stirn and Jacobsen, 1987). The encoding genes would therefore be either class 2 or 3 genes. Kiyosue *et al.* (1991) have described a 31 kD protein ECP1 that was preferentially localized in peripheral cells of carrot proembryogenic masses in the presence of 2,4-D. Upon initiation of somatic embryogenesis, the protein disappeared in the course of embryo development. Surprisingly, in zygotic embryogenesis it remained present throughout the embryo up to cotyledon stage, suggesting that the encoding gene represents a class 3 gene. The deduced ECP1 protein sequence was shown to be highly homologous with a cotton LEA protein. As for many *lea* genes, the level of ECP1 gene expression in somatic embryos was sensitive to ABA treatment (Kiyosue *et al.*, 1992b). Another ABA-regulable gene, ECP40, was shown to be expressed in a similar fashion as ECP31 in embryogenic somatic cells, somatic embryos and zygotic embryos (Kiyosue *et al.*, 1992a).

A number of immunological studies have been carried out aimed at the determination of cell recognition patterns of monoclonal antibodies which were generated after immunization of rats with preparations containing carrot arabino-

galactan proteins (Knox *et al.*, 1989). Stacey *et al.* (1990) described the recognition of a plasma-membrane-associated arabinogalactan-protein epitope by a monoclonal antibody, JIM4. This epitope was present in carrot suspension cultures on the surface of some cells of proembryogenic masses, and became more abundant on developing somatic embryos. As this epitope was also found in the carrot root apex (Knox *et al.*, 1989), the genes involved in the creation of this epitope can be classified as class 1 genes. Another carbohydrate cell wall epitope, recognized by the monoclonal antibody JIM8 described in the previous section, was shown to be present on three types of single cells and one type of cell cluster in embryogenic suspension cultures, however, only a small number of JIM8-reactive cells were able to develop into somatic embryos resulting in the immediate dissipation of the epitope. Through these observations, the JIM8 cell wall epitope was postulated to mark a transitional state during the formation of embryogenic cells in suspension cultures (Pennell *et al.*, 1992). Given the complex expression pattern of the JIM8 plasma membrane epitope in reproductive development and the probability that the plasma membrane epitope and the JIM8 cell wall epitope as detected in cultured cells belong to different molecules, it is not possible to classify JIM8.

From the genes and proteins described above it can be concluded that the study of somatic embryogenesis has indeed shown the existence of class 2 and 3 genes, expressed in early stages of somatic embryo development. It is also clear that in several cases analysis of gene expression during zygotic embryo development reveals differences in temporal expression patterns. For example in the case of the *lea* genes, it seems that the expression during somatic embryogenesis generally occurs at earlier developmental stages such as proembryogenic masses and globular embryos when compared with the predominantly torpedo and cotyledon stage specific expression during zygotic embryogenesis. Unfortunately, this means that the temporal expression pattern observed during somatic embryogenesis cannot automatically be used for a rigid gene classification. However, the spatial expression pattern of the soybean seed protein Kunitz trypsin inhibitor and β-conglycinin genes as analyzed by *in situ* localization of the corresponding mRNAs, has revealed a spatially identical pattern during zygotic as well as somatic embryogenesis (Perez-Grau and Goldberg, 1989). Therefore, although differences in the timing of the onset of expression of particular genes have been observed when comparing zygotic and somatic embryogenesis, the cell type specificity of expression is likely to be retained in somatic embryogenesis.

Attempts to dissect the process of somatic embryogenesis by somatic cell genetic means has also been undertaken, mainly in temperature-sensitive mutant cell lines of carrot (Breton and Sung, 1982; LoSchiavo *et al.*, 1988; Schnall *et al.*, 1988). Due to the inherent difficulties to identify precisely the embryo stages affected and the variation always observed in tissue culture systems this has not yet yielded the desired insight into somatic embryo devel-opment as hoped for originally. The best-studied example of a somatic cell variant obtained this way is the temperature-sensitive mutant carrot cell line ts11 (LoSchiavo *et al.*, 1990). In this cell line embryo development is blocked at the glo-bular stage, and a secreted acidic endochitinase has been identified that was able to overcome the arrest after addition to the culture medium (De Jong *et al.*, 1992).

Role of secreted proteins in somatic embryogenesis

Diversity of proteins secreted into tissue culture media

Suspension culture cells secrete factors into the culture medium, a process referred to as medium conditioning. Several observations have suggested that factors present in the conditioned medium are of importance to somatic embryo development. Promoting effects of conditioned medium on the initiation of somatic embryogenesis (Hari, 1980; Smith and Sung, 1985), as well as on the acquisition of embryogenic potential (De Vries *et al.*, 1988b) have been observed. Major changes were found to occur in the extracellular, in contrast to the intercellular, protein pattern upon initiation of somatic embryogenesis in carrot suspension cultures (De Vries *et al.*, 1988a). In addition, a small number of proteins were detected in the medium of embryogenic cell lines that were absent or present in a lower concentration in cultures unable to produce somatic embryos. This suggested that the presence of these proteins is correlated with somatic embryo development.

Several different proteins have been reported to be secreted into plant tissue culture media. Among those are proteins with enzymatic activity: peroxidases (Van Huystee, 1987), phosphatases (Giarocchi *et al.*, 1984), proteases (Wink, 1984), a protease inhibitor (Carlberg *et al.*, 1987) and a glycosyltransferase (Quentmeier *et al.*, 1987). The presence of other proteins was shown to be dependent on culturing conditions: two carrot glycoproteins, a 57 kD protein secreted by non-embryogenic as well as embryogenic cells in a possibly auxin-dependent fashion, a 65 kD protein secreted by embryogenic cells in the absence of 2,4-D (Satoh *et al*, 1986; Satoh and Fujii, 1988), and glycoproteins of 53-57 kD that disappear from the culture medium upon initiation of embryogenesis in Citrus cultures (Gavish *et al.*, 1991). Other proteins were found to be present regardless of culturing conditions. Van Engelen *et al.* (1991) described a series of post-translationally modified carrot proteins, designated EP1. A 52/54 kD EP1 protein doublet was found in conditioned media of embryogenic as well as non-embryogenic cultures, while an additional doublet of 31/32 kD EP1 proteins was found in suspensions cultured at high cell density. These proteins and an additional three EP1 proteins of 49, 61 and 62 kD were detected in cell walls of non-embryogenic, vacuolated suspension cells. The EP1 gene is only expressed in non-embryogenic suspension cells and not in proembryogenic masses and in somatic embryos, at least up to the torpedo stage. Expression of the EP1 gene is also absent in zygotic embryos and is resumed sometime after germination in seedlings (Van Engelen *et al.*, 1991). Based on this pattern of expression, the EP1 gene would fall into the previously proposed additional category of class 0 genes, of which the expression is absent or reduced during embryo development. In contrast, another secreted carrot protein, designated EP2, was found to be secreted only by embryogenic cells and somatic as well as zygotic embryos (see chapters 3 and 4). Based on its expression throughout the plant life cycle (chapter 3) EP2 can clearly be classified as a class 1 gene.

A number of proteins secreted into tissue culture media have been identified that appear to reflect the developmental state of the cells from which they originate (Van Engelen and De Vries, 1992) and, like several of the genes expressed in embryos and described in this section, appear to be well correlated

with the absence or presence of embryogenic potential. However, for most of these proteins it has not yet been shown whether they are actually decisive to achieve successful somatic embryogenesis.

Two secreted carrot proteins are essential for somatic embryogenesis

Functional evidence for the role of secreted proteins in carrot somatic embryogenesis has been obtained from two experimental systems. The first makes use of the finding that the glycosylation inhibitor tunicamycin at low concentration is able to arrest somatic embryogenesis, but not unorganized proliferation of cells (LoSchiavo *et al.*, 1986). In tunicamycin-treated embryogenic cell clusters the most dramatic change in morphology is the expansion of peripheral cells of the proembryogenic masses, which effectively stops embryo formation from these cells. Restoration of somatic embryogenesis was subsequently observed after addition of a mixture of secreted glycoproteins isolated from the conditioned medium of an untreated embryogenic suspension culture (De Vries *et al.*, 1988a). The secreted protein responsible for the tunicamycin-complementing effect was purified and identified as a cationic peroxidase. In addition, it was shown that the peroxidase enzyme activity was required for the complementing activity (Cordewener *et al.*, 1991).

Employing a different assay system, it was shown that addition of extracellular proteins isolated from embryogenic carrot embryo cultures could rescue embryogenesis in a temperature sensitive variant carrot cell line, ts11. This line is unable to form heart-stage somatic embryos at the restrictive temperature of 32°C (LoSchiavo *et al.*, 1985). Purification of the protein, designated EP3, that complements ts11 has revealed a single 32 kD secreted glycoprotein to be responsible. Based on peptide sequencing and biochemical characterization, this protein was identified as a glycosylated acidic endochitinase (De Jong *et al.*, 1992).

These experiments have shown that two secreted glycoproteins are essential for the correct development of embryos in carrot cell cultures. However, culture media abound with many as yet unidentified proteins, for which no assay system has been developed to establish a possible function during embryogenesis. The study of secreted glycoproteins is further complicated by the fact that in embryogenic suspension cultures the majority of the cells is non-embryogenic, and therefore, only a small fraction of the cell population will, under the appropriate conditions, develop into embryos. Yet, both embryogenic and non-embryogenic cells contribute to the pool of proteins present in culture media (Van Engelen and De Vries, 1992). Some of these proteins are apparently involved in embryogenesis, while others may not be necessary or may even inhibit embryogenesis (Gavish *et al.*, 1992). In this thesis an alternative approach to study proteins in embryogenic carrot culture media is presented, by initially characterizing their encoding genes, before addressing their possible role in somatic embryogenesis.

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CHAPTER 2

cDNA cloning of mRNAs encoding proteins secreted into carrot cell culture media

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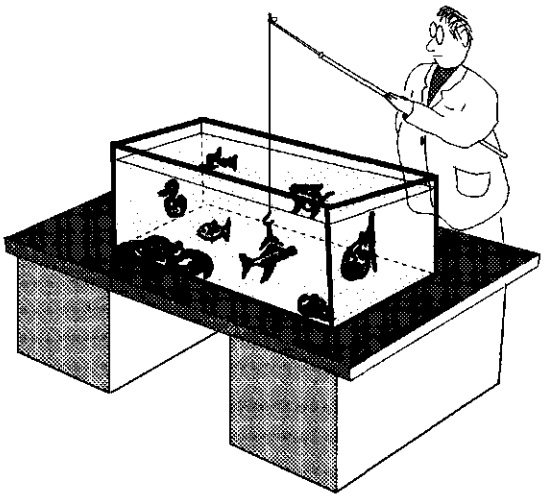

Abstract

Four different cDNA clones, designated extracellular protein (EP)1, EP2, EP5, and EP6 were obtained by expression screening of a carrot embryo cDNA library in phage λgt11 with polyclonal antisera raised against an unfractionated mixture of secreted proteins. Northern hybridization analysis showed that the EP1, EP5, and EP6 genes were expressed in both embryogenic and non-embryogenic cell cultures, regardless of the differentiation state. In contrast, the EP2 gene was expressed in embryogenic cell cultures only. In carrot plants expression of the EP1 gene was found in all tissues investigated. Expression of the EP5 and EP6 genes was also found throughout the plant, but enhanced in the root apical meristematic region and in the inflorescence. EP2 gene expression was only detected in the shoot apical meristematic region, the inflorescence and the immature seed. The EP5 and EP6 encoded peptides did not reveal significant homology to previously reported proteins. The presence of a signal peptide suggests that EP5 may be located in the extracellular matrix. The EP2 cDNA shared homology with plant non-specific lipid transfer protein genes, while the EP1 encoded polypeptide sequence showed weak homology to S-locus glycoproteins and S-locus related glycoproteins from *Brassica*. Employing antisera raised against the β-galactosidase fusion proteins of the EP1 and EP2 clones identified the encoded proteins as a 52/54 kD secreted glycoprotein and a 10 kD secreted protein, respectively.

Introduction

Embryogenic carrot cell suspension cultures are usually maintained as high-density cell suspensions (here referred to as S-cultures) in the presence of the synthetic auxin 2,4-dichlorophenoxy acetic acid (2,4-D). After simple culture manipulations–dilution in hormone-free medium–somatic embryos develop from clusters of tightly-adhering cytoplasmic embryogenic cells designated proembryogenic masses (Halperin, 1966) in embryo (E-) cultures. Dilution in medium containing 2,4-D blocks the formation of embryos at globular stage and results in a culture in which cells proliferate in a seemingly unorganized fashion. Hence, these cultures are referred to as proliferating (P-) cultures (De Vries *et al.*, 1988a).

Employing ^{35}S-methionine pulse labeling it was shown that embryogenic suspension cultures secrete a small number of proteins that were not secreted by suspension cultures that have lost their embryogenic potential, either by prolonged culturing or by mutagenesis. Formation of somatic embryos was found to be concomitant with the secretion of a characteristic set of proteins into the embryo culture medium within two days after culture initiation. These proteins were absent in proliferating cultures in which 2,4-D prevented normal embryo development. The general view that emerged from these studies was that the presence of embryogenic cells and somatic embryos among non-embryogenic cells was reflected in the type of secreted proteins found (De Vries *et al.*, 1988a). To extend and confirm these observations, we set out to clone a number of the mRNAs encoding proteins secreted into the media of embryogenic and somatic embryo containing cultures.

The approach taken was to raise antisera against all proteins secreted into embryogenic suspension cultures and somatic embryo cultures and to use these antisera to screen a λgt11 carrot somatic embryo expression library. Hybridization of positive clones to Northern blots containing total RNA isolated from embryogenic and non-embryogenic cell cultures, as well as total RNA from somatic embryos was employed to determine a possible relationship of the corresponding genes to embryogenesis by analyzing their expression patterns. In addition, Northern blot analysis of total RNA extracted from various parts of carrot plants was employed to determine the expression of the genes encoding secreted proteins throughout the plant's life cycle. The clones were further characterized by determining their DNA nucleotide sequence. The use of a λgt11 library to obtain clones allowed for direct expression of these clones as fusion proteins with β-galactosidase without further subcloning. Antisera were raised against the purified β-galactosidase fusion proteins. These antisera were subsequently used to identify proteins corresponding to the λgt11 clones on Western blots containing total secreted medium proteins. In view of the fact that many secreted medium proteins were shown to be glycosylated, an additional advantage of antisera raised against fusion proteins produced in *Escherischia coli* was that the antisera were only directed against the polypeptide parts of the corresponding carrot glycoproteins and would therefore be expected to be more specific when compared to antisera raised against native secreted medium proteins. Finally, purification of the proteins identified this way from carrot suspension cell media can be performed to study their biochemical properties.

Results

Expression screening of a λgt11 carrot embryo cDNA library

Two polyclonal antisera were raised against a mixture of proteins isolated from 7-day-old high-density embryogenic suspension (antiserum αS) or embryo culture (antiserum αE) media of line "10," respectively. As expected, these antisera reacted with many medium proteins after Western blotting (Figure 1A). Since many of these proteins were shown to be glycosylated (De Vries *et al.*, 1988a), the question arose whether the pattern observed in Figure 1A was due to aspecific reactions with sugar side chains present on many glycoproteins, or whether these antisera also recognized protein epitopes, essential for their use as probes for expression screening. To investigate this, blotted medium proteins were treated with sodium metaperiodate, a compound reported to open the non-reducing ends of carbohydrates, thereby destroying carbohydrate epitopes (Woodward *et al.*, 1985). The resulting blots were incubated with the antisera. A control blot containing ^{35}S-labeled medium proteins was autoradiographed before and after incubation with periodate to check whether this treatment would remove proteins from the blot. A second control blot was used to test the extent of sugar epitope removal using concanavalin A binding. Sodium periodate treatment did indeed destroy sugar epitopes, though not completely as judged by concanavalin A binding (Figure 1B). No protein was removed from the blots (Figure 1C). The number of bands

recognized by either antiserum after periodate treatment was significantly reduced (Figure 1D), confirming that both antisera recognized many carbohydrate epitopes. Based on the periodate-resistant reaction with several secreted proteins, we concluded that both antisera did recognize protein epitopes and could therefore be used as probes to screen a λgt11 expression library. Out of the nine clones obtained, four clones did not cross-hybridize and were designated EP1, EP2, EP5, and EP6. A λZAPII cDNA library was screened with ^{32}P-labeled EP1, EP2 and EP5 to obtain longer clones. A 1739-base pair cDNA was obtained with EP5 and was designated EP5B. The results for EP1 have been published elsewhere (Van Engelen *et al.*, 1993) and the results for EP2 are presented in Chapter 3. In Table 1 the lengths of the cDNA clones, their corresponding mRNA lengths as determined by Northern blot analysis, the antisera with which they were found, as well as their abundance in the cDNA libraries are given.

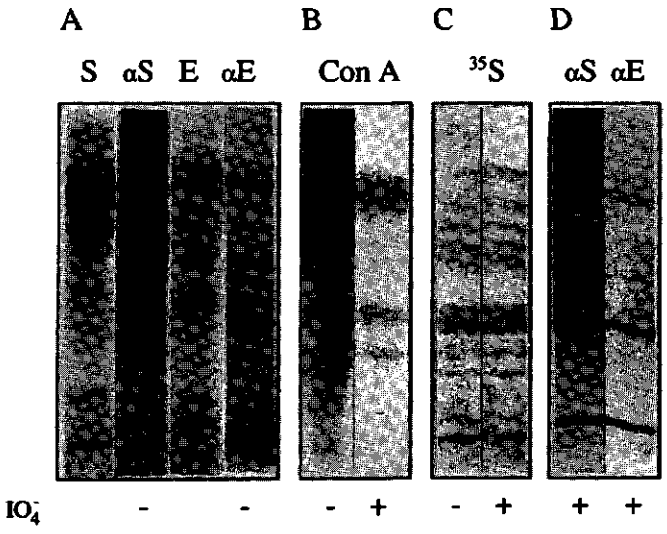

Figure 1. Characterization of antisera raised against carrot medium proteins.

(**A**) Coomassie Brilliant Blue stained high-density cell culture medium proteins, and their recognition by antiserum αS raised against those proteins (lanes S and αS, respectively), and Coomassie Brilliant Blue stained embryo culture medium proteins, and their recognition by antiserum αE raised against those proteins (lanes E and αE, respectively).
(**B**) Deglycosylation of medium proteins visualized with a concanavilin A staining assay. Residual staining in the right lane indicates that deglycosylation was not complete.
(**C**) Fluorographs of ^{35}S-labeled Western blotted medium proteins before and after periodate treatment. Periodate treatment did not result in removal of protein from blots, as judged by equal intensities of corresponding bands in both lanes.
(**D**) Reduction of bands recognized with antisera αS and αE after periodate treatment as compared to (**A**).

Clone	Antiserum	Insert size (bp)	mRNA size (nt)	Frequency in library
EP1	αS/αE	249	1600	4 in $2x10^5$
EP2	αS/αE	121	750	1 in 10^6
EP5	αE	537	2000	1 in 10^6
EP5B	---	1707	2000	6 in 10^6
EP6	αS	248	3400	3 in 10^6

Table 1. Clones obtained from cDNA library screening.

Expression screening of the λgt11 carrot somatic embryo cDNA library with antisera raised against high-density suspension culture medium proteins (αS) and against embryo culture medium proteins (αE) yielded four cDNA clones designated EP1, EP2, EP5, and EP6. Their lengths and their corresponding mRNA sizes are given. In addition, their approximate representation in the λgt11 library is indicated. Because only one in six clones corresponding to a particular mRNA will be in the correct reading frame to allow expression as a correct fusion protein, the actual representation of an mRNA in the library is expected to be six time higher. Clone EP5B was obtained from a λZAPII cDNA library screening with ^{32}P-labeled EP5 as probe.

Expression patterns of four genes encoding secreted proteins in carrot cell cultures and plants

To investigate whether the genes corresponding to the cDNA clones obtained were related with embryogenesis, we studied their expression in both embryogenic and non-embryogenic cell cultures employing Northern hybridization analysis. Suspension cell cultures were grown in the presence of 2,4-D at a high cell density from 10^6 to 10^7 cells/ml (S-cultures). After enrichment for proembryogenic masses by sieving, cultures were diluted to $2x10^4$ cells/ml in either the presence of 2,4-D, to yield low density proliferating (P-) cultures, or in basal medium without growth regulators to yield embryo (E-) cultures (De Vries *et al.*, 1988a). Figure 2A shows that the level of expression of the EP1, EP5, and EP6 genes was rather constant in embryogenic and non-embryogenic S-, P-, and E-cultures, in other words independent of embryogenic potential or the presence of somatic embryos. Comparison of the expression in cells growing under different conditions, *i.e.* as high density suspension S-cultures, low density proliferating P-cultures, or embryo E-cultures, showed that none of these genes is clearly regulated by 2,4-D. In contrast, expression of the EP2 gene was restricted to embryogenic cultures, both in the presence and absence of 2,4-D. The EP2, EP5, and EP6 genes were found to be expressed in somatic embryos whereas EP1 was not. It therefore appears that the four genes analyzed represent three different classes of secreted proteins. The EP5 and EP6 genes are expressed in various cell types present in all cultures investigated, including somatic embryo cells. The EP1 gene clearly differs from

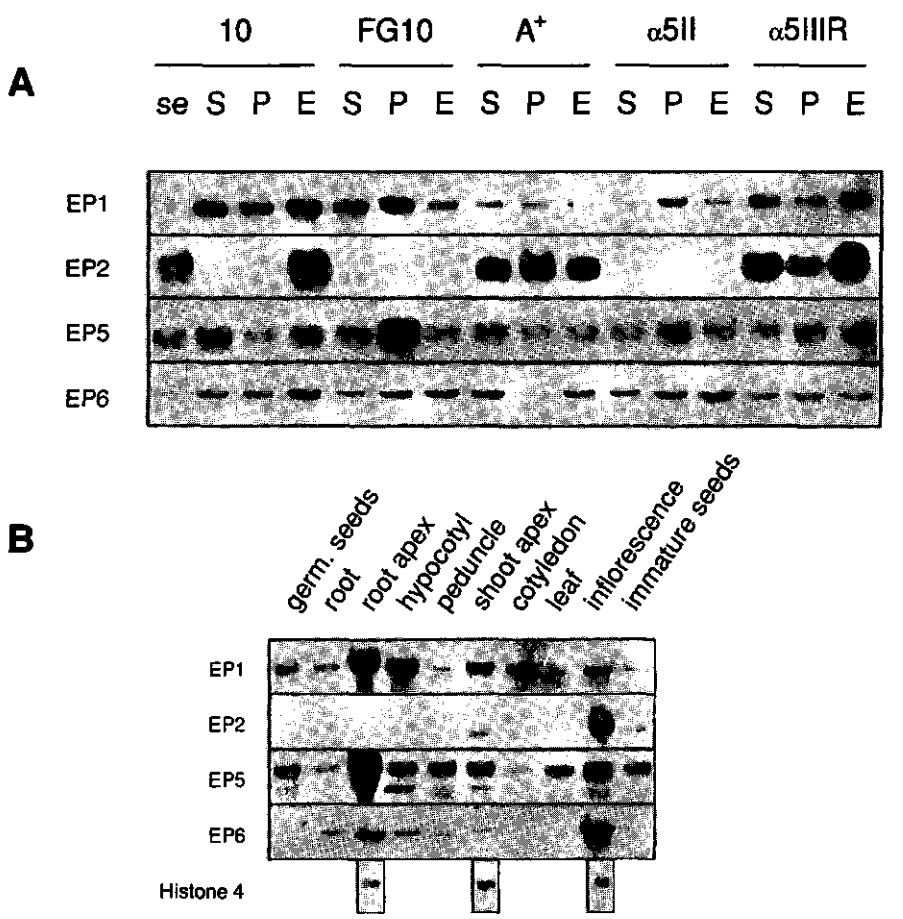

Figure 2. Northern blot analysis of EP1, EP2, EP5 and EP6 in cell suspension cultures and plants.

For each lane, 15 μg of total RNA was loaded on gel. Blotting, hybridization and washing steps were as described in Methods. The blots described in (**A**) were washed more stringently (0.2xSSC, 60°C) than those described in (**B**; 2xSSC, 60°C). Films were exposed with an intensifying screen for 3 days.

(**A**) Expression 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 embryogenic cell lines 10, A+, and α5III, and non-embryogenic cell lines FG10 and α5II. The EP1, EP5, and EP6 genes are expressed in all cultures, whereas the EP2 gene is expressed in embryogenic cultures only. All genes except EP1 are expressed in somatic embryos.

(**B**) Expression in carrot plants. Expression of the EP1, EP5, and the EP6 genes was found throughout the whole carrot plant. EP5 and EP6 gene expression was enhanced in the root apex and the immature inflorescence. EP2 gene expression was found in the shoot apex of seedlings, in the immature inflorescence and in immature seeds. Hybridization with a soybean histone 4 probe showed that the level of cell division is approximately equal in the root apex, the shoot apex and in the immature inflorescence. The weaker lower EP5 band may be due to the presence of a related mRNA, and disappears under more stringent washing conditions (**A**).

these based on the absence of signal in somatic embryos, and has been shown to be expressed by non-embryogenic cells (Van Engelen *et al.*, 1991). The third class is represented by the EP2 gene and appears to be expressed in embryogenic cells and embryos only.

Since the cultures used in these experiments were initiated from hypocotyl explants, it was of interest to determine whether the EP1, EP2, EP5, and EP6 genes were expressed in hypocotyls, or whether their expression was restricted to cell cultures. At the same time, expression of these genes in various other carrot plant parts was investigated, including those parts in which zygotic embryogenesis takes or has taken place. Total RNA was isolated from 7 to 14-day-old carrot seedlings dissected into the root apex (1-2 mm), root without apex, hypocotyl, shoot apex (1-2 mm), and cotyledons. From available adult plants total RNA was extracted from various parts including the peduncle, immature inflorescence, and green immature seeds, as well as from mature seeds imbibed for 48 hours. As shown in Figure 2B expression of the EP1, EP5, and EP6 genes was found in all tissues examined, but not at the same level. The EP1 gene was highly expressed in the root apex and hypocotyl of seedlings, and to a lesser extent in the shoot apex, the cotyledons and the inflorescence. Very little EP1 gene expression was observed in the peduncle and immature seeds. The expression of the EP5 and EP6 genes was enhanced in the root apex and the immature inflorescence. EP6 gene expression was most pronounced in the immature inflorescence, whereas EP5 gene expression was highest in the root apex. Expression of the EP2 gene was restricted to the shoot apex, the inflorescence and immature seeds. We therefore conclude that the EP1, EP5, and EP6 genes may represent genes of which the expression is retained upon initiation of cell culture from hypocotyl-derived explants. In contrast, the EP2 gene is not expressed in hypocotyls, but is apparently switched on when a cell suspension cultures acquires embryogenic potential.

To investigate a possible relationship of the EP genes with cell division, blots containing equal amounts of total RNA extracted from several plant tissues active in division–the root apex, shoot apex and immature inflorescence–were hybridized with a histone 4 probe from soybean (Franssen, unpublished results) as histone 4 gene expression is known to take place during the S-phase of the cell cycle. Figure 2B shows that the level of histone 4 gene expression was approximately equal in the immature inflorescence, in the root, and in the shoot apex, suggesting that the amount of dividing cells contributing to the RNA preparations is about the same in those tissues. In contrast, none of the EP genes was expressed at the same level in the immature inflorescence, the root and the shoot apex. It is therefore not likely that these genes are directly involved in cell division.

Identification of the proteins encoded by the EP1 and EP2 genes

To identify the proteins encoded by the EP1, EP2, EP5, and EP6 genes, antisera were raised against the corresponding β-galactosidase fusion proteins isolated from lysogenic *E. coli* Y1089 cells containing recombinant λgt11 genomes. It appeared not possible to express the EP5 cDNA clone as a stable fusion protein with β-galactosidase. Fusion proteins of the clones EP1, EP2, and EP6 were isolated using a protocol optimized for the purification of the 115 kD (monomeric) β-galactosidase protein, under the assumption that fusion of a relatively small

additional polypeptide would not significantly alter the behaviour of the protein during purification. By this method, purification of milligram quantities of purified fusion protein was possible. Figure 3 shows the purification of β-galactosidase from Y1089 cell lysates. Most β-galactosidase activity was eluted from a DEAE-Sephacel column with a linear NaCl gradient (Figure 3A). After pooling fractions with β-galactosidase activity and precipitation with 60% ammonium sulphate, Sephacryl S300 HR gel filtration, separation on preparative SDS-PAGE followed by electro-elution from these gels, the protein was purified to apparent homogeneity. Polyclonal antisera were raised against the fusion proteins. Figure 4 shows the reaction of two of those antisera with secreted medium proteins. The EP1 antiserum recognizes a protein doublet of 52/54 kD, and the EP2 antiserum a 10 kD protein. Both the EP1 and EP2 proteins were present in media of high-density suspension cultures, proliferating cultures and embryo cultures of cell line "10." It was not possible to detect a specific protein with the antiserum against the EP6 fusion protein (data not shown). The identity of the encoded EP6 protein remains therefore unresolved.

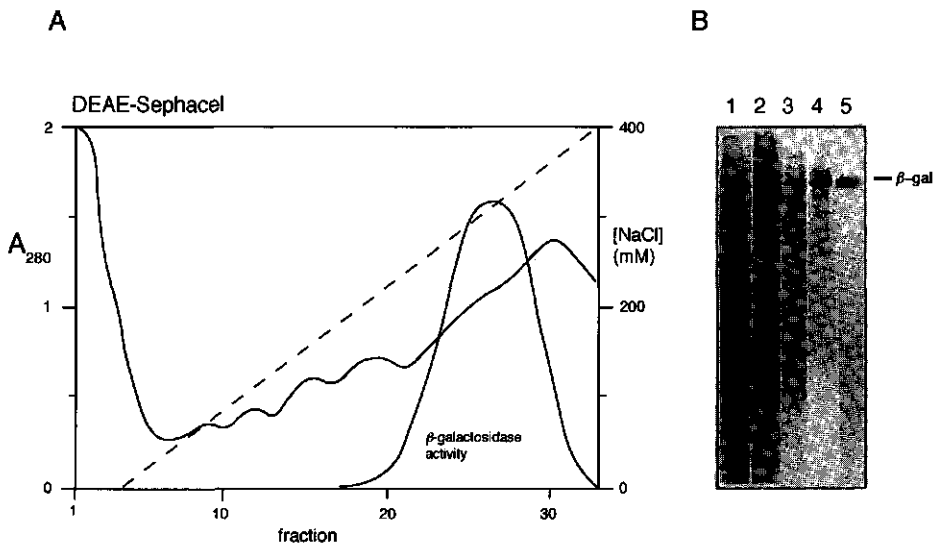

Figure 3. Purification of β-galactosidase and fusion proteins.

(A) DEAE-Sephacel column chromatography of a cell-free extract containing β-galactosidase. Elution of protein from the column was performed with a 0-400 mM NaCl gradient and was monitored by measuring the absorbance at 280 nm. The elution profile shown was similar to those obtained with β-galactosidase fusion proteins.

(B) Purification to homogeneity of β-galactosidase. Lane 1, cell-free extract. The following lanes show further purification after various steps. Lane 2, DEAE-Sephacel; lane 3, 60% ammonium sulphate precipitation; lane 4, Sephacryl S300 HR gel filtration; lane 5, electro-elution of SDS-polyacrylamide gel excised β-galactosidase band.

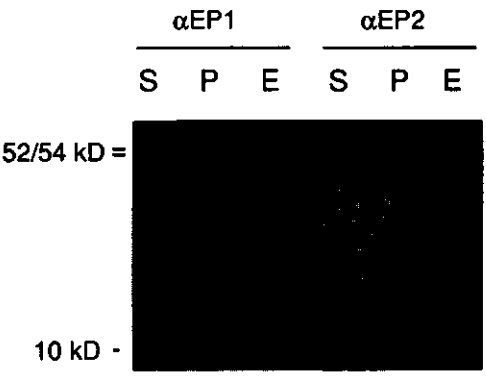

Figure 4. Identification of EP1 and EP2 related carrot extracellular proteins.

Antisera raised against the β-galactosidase-EP1 and the β-galactosidase-EP2 fusion proteins recognize a 52/54 kD protein doublet and a 10 kD protein in media of high-density suspension cultures (S), proliferating cultures (P), and embryo cultures (E), respectively.

DNA and deduced amino acid sequences of the EP1, EP2, EP5 and EP6 cDNA clones

In order to determine whether the four cDNA clones had sequence homology with previously reported genes, all clones were sequenced. In Figure 5 the DNA and the deduced protein sequences of the EP1, EP2, EP5B (and the original EP5) and EP6 cDNA clones are presented. The complete sequence of the EP1 cDNA has been published elsewhere (Van Engelen *et al.*, 1993), while the complete sequence of the EP2 cDNA is presented in Chapter 3. Comparison of the cDNA as well as the deduced amino acid sequences with sequences deposited in the current versions of the GenBank and EMBL DNA sequence and the Swiss-Prot protein sequence libraries revealed no significant homologies with the EP5/EP5B and EP6 sequences. Although the EP5B cDNA sequence does not contain a poly(A) tail, the length of this cDNA approximates the estimated mRNA length (see Table 1), and is therefore nearly full-length. If the ATG codon at position 9-11 represents the translation start site, a signal peptide based on the deduced amino acid sequence of the *N*-terminus and a cleavage site can be predicted between amino acids 16 and 17 (Von Heijne, 1983) as indicated in Figure 5. The EP5 protein sequence contains five potential *N*-glycosylation sites (see Figure 5) and could therefore be a glycoprotein. Interestingly, the EP5B encoded peptide has a relatively high glycine (14%) and proline (8%) content, but the absence of repetitive proline patterns observed in structural cell wall proteins like the extensins (Tierney and Varner, 1987) and proline-rich proteins (Chen and Varner, 1985), glycine patterns in structural glycine-rich proteins (DeOlveira *et al.*, 1990) or glycine/proline patterns in collagens in vertebrates (Linsenmayer, 1981), suggests that EP5 may be present in the extracellular matrix of the cell as a non-structural glycoprotein .

The EP1 encoded polypeptide sequence showed weak but significant homology to S-locus glycoprotein and S-locus related glycoprotein sequences from *Brassica*, the AtS1 protein from *Arabidopsis*, as well as the S-like domains of putative receptor protein kinases from *Brassica*, *Arabidopsis*, and maize (Van Engelen *et al.*, 1993). *Brassica* S-like genes are expressed in the papillar cells of the stigma and pollen and play a role in self-incompatibility, the inhibition of germination of pollen ending up on stigmas of the same plant. As far as known,

carrot does not possess a genetic self-incompatibility system, hence EP1 is not involved in self-incompatibility (Van Engelen *et al.*, 1993).

The EP2 cDNA was found to share high sequence homology with several non-specific lipid transfer proteins, including the ones identified in maize (Tchang *et al.*, 1988) and barley (Mundy and Rogers, 1986; Bernhard and Somerville, 1989). In Chapter 3 and 4 more detailed studies of the EP2 gene and a possible role of the EP2 protein are presented.

A

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CGAGTCTCTCATGCGCTGGGTCTGGGAAGCCAACCGGGGCAACCCGGTCGACGAAAACGC     60
  E  S  L  M  R  W  V  W  E  A  N  R  G  N  P  V  D  E  N  A     20

CACGCTCACATTCGGCCCAGACGGGAACCTCGTGCTGGCCCGGTCCAACGGCCAGGTCGC    120
  T  L  T  F  G  P  D  G  N  L  V  L  A  R  S  N  G  Q  V  A     40

ATGGCAAACATCTACAGCCAACAAAGGTGTCGTGGGCCTCAAGATTCTCCCTAATGGTAA    180
  W  Q  T  S  T  A  N  K  G  V  V  G  L  K  I  L  P  N  G  N     60

CATGGTGCTTTACGACTCCAAAGGCAAATTCTTATGGCAAAGCTTCGACACGCCCACCGA    240
  M  V  L  Y  D  S  K  G  K  F  L  W  Q  S  F  D  T  P  T  D     80

CACGCTCCT                                                        249
  T  L  L                                                         83
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B

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CTAATGCCGTCACTGGTCTCAACTTGAACGCTGCTGCTGGCCTCCCTGCTAGATGTGGTG     60
   N  A  V  T  G  L  N  L  N  A  A  A  G  L  P  A  R  C  G      19

TCAACATTCCTTACAAAATCAGCCCCACCACCGATTGCAACAGGGTGGTGTGAAGGTATG    120
V  N  I  P  Y  K  I  S  P  T  T  D  C  N  R  V  V  *            36

T                                                                121
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C

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CCGTGCACATGGTTCGGGGTTCTCCCCCTATTGCATCGCCACCAGGTGCTGCAGCTTCGA     60
         M  V  R  G  S  P  P  I  A  S  P  P  G  A  A  A  S       17
                                                      ↑

GTGTTGGAAATCCAAATGCCACTACCAATGCTACCCAGCCTGTTAATCCTATTGATGGAG    120
S  V  G  N  P  N  A  T  T  N  A  T  Q  P  V  N  P  I  D  G      37

GGGGTTTAGGAGGTGCTGGTCTTGGGGCATCACTGTTAAACGGTCTTGGTGGTTTTGGTG    180
G  G  L  G  G  A  G  L  G  A  S  L  L  N  G  L  G  G  F  G      57

GGCTTGGCTTAGGTGCAGGCAGTGGTACTGGTGCTCAGGGCTTATTTGGAGCTGGACTTC    240
G  L  G  L  G  A  G  S  G  T  G  A  Q  G  L  F  G  A  G  L      77
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CAGAGTTTGAACAAGTTCAACAGCAACTTGCTCAAAATCCCAACATGATGCAGGATATGA   300
P  E  F  E  Q  V  Q  Q  Q  L  A  Q  N  P  N  M  M  Q  D  M      97

TGAATACACCAGCCATTCAGAACTTAATGAATAACCCAGAGATAATGCGCACGTTGATTA   360
M  N  T  P  A  I  Q  N  L  M  N  N  P  E  I  M  R  T  L  I     117

TGAGCAACCCCCAAATGCGTGATATCATTGATCGGAACCCGGAACTTGCGCATGTTCTTA   420
M  S  N  P  Q  M  R  D  I  I  D  R  N  P  E  L  A  H  V  L     137

ATGATCCTGGCATTCTTAGGCAGACATTGGAGGCTGCTAGAAACCCTGAACTCATGCGTG   480
N  D  P  G  I  L  R  Q  T  L  E  A  A  R  N  P  E  L  M  R     157

AGATGATGCGCAATACTGATAGGGCAATGAGTAACATTGAATCATCTCCTGAGGGTTTTA   540
E  M  M  R  N  T  D  R  A  M  S  N  I  E  S  S  P  E  G  F     177

ACATGCTTAGGCGCATGTACGAAAATGTTCAGGAACCTTTCCTGAATGCTACAACAATGG   600
N  M  L  R  R  M  Y  E  N  V  Q  E  P  F  L  N  A  T  T  M     197

GTGCAGGGGCTGGAAACGATTTAGGATCAAATCCATTTGCCGCGCTTCTAGGAAATCAGG   660
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GTGGTGGTAACCAAGGCACAAATGCCTCTAGCGACACTTTGAACACCGGTTCTGAAGCAG   720
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GTTCCCAAACAAATACTGCTGCAAGGTCAAATCCAACAGGAGATGCAAGGGGAGCAGGAA   840
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CATCAGCGATGAACCAACTTCTGCAAAATCCAGGTGTTTCACAGATGATGCAGAGTCTGC   960
P  S  A  M  N  Q  L  L  Q  N  P  G  V  S  Q  M  M  Q  S  L     317

TTTCAAACCCACAATACATGAATCAGTTTCTTGGAACTAATCCTCAACTCCAGAGTATGT  1020
L  S  N  P  Q  Y  M  N  Q  F  L  G  T  N  P  Q  L  Q  S  M     337

TTGACATGAACCCTCAATTAAGAGAAATGATGCAGAATCCAGAAATCATTCGTCAGCTTA  1080
F  D  M  N  P  Q  L  R  E  M  M  Q  N  P  E  I  I  R  Q  L     357

CGTCTCCAGAAATGATGCAGCGGATGTCGACATTTCATCAGGCACTTCTTTCTCAGCAGA  1140
T  S  P  E  M  M  Q  R  M  S  T  F  H  Q  A  L  L  S  Q  Q     377

ACAGACCACAAACAACTCAGGAACCAGCTCAAACTGGTGGGACTACAGGTGCAGGAGCAC  1200
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AAAACAACATGGGACTGGATATGTTGATGAATATGTTTAGTGGACTTGGTGGACTTGGAG  1260
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Q  L  S  Q  L  Q  E  M  G  F  F  D  T  Q  E  N  I  R  A  L     457

GTGCTACCTCAGGGAATGTGCATGCAGCAGTGGAGAGACTTTTAGGCAACCTCGGGCAGT  1440
R  A  T  S  G  N  V  H  A  A  V  E  R  L  L  G  N  L  G  Q     477

AGCACCATTTCTGTTTCCTCGGAAACTTGGTGGTGTGTAAACTTAATCAAGTAATACAAG  1500
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D

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GCAAGCTGCCGAGAGTGATACCCGTATTGAGCGATCAGTGAGGGAACATTCATCTCTCAT     60
  Q  A  A  E  S  D  T  R  I  E  R  S  V  R  E  H  S  S  L  M     20

GTCAATACTTCATCGACATCCAATAGAGTCTGTCCTTCCAACCTTAGGGAGGCCAATCAT    120
  S  I  L  D  R  H  P  I  E  S  V  L  P  T  L  G  R  P  I  M     40

GTCTTTAGACGCCAATGAAGATGCAATATTGGGGGCTTTGAAACAGAGCTTGAGGCAATT    180
  S  L  D  A  N  E  D  A  I  L  G  A  L  K  Q  S  L  R  Q  L     60

GGAAACTCTGGGAGCTCAAAGGGCAGGTCTTGAAGACATGCTTAAAGAAATGAAACGAAA    240
  E  T  L  G  A  Q  R  A  G  L  E  D  M  L  K  E  M  K  R  K     80

GGATGATA                                                         248
  D  D                                                            82
```

Figure 5. DNA and deduced protein sequences of clones EP1, EP2, EP5, and EP6.

DNA and deduced protein sequences of the clones obtained by λgt11 expression library screening: (**A**) EP1, (**B**) EP2, (C) EP5B, and (D) EP6. The sequence of the original EP5 cDNA starts at base 844 and extends to base 1380 of the nearly full-length cDNA clone EP5B, which was obtained after screening of a λZAPII cDNA library with radiolabeled EP5 as probe. Full-length cDNA clones were also obtained for EP2 (Chapter 3) and EP1 (Van Engelen *et al.*, 1993). An asterisk (*) indicates a translation stop codon. Underlined amino acid triplets are potential *N*-glycosylation sites. The arrow indicates the predicted signal peptide cleavage site in the EP5 peptide sequence (C).

Discussion

Expression screening with an antiserum raised against a mixture of glycoproteins secreted by embryogenic carrot cells has yielded four different cDNA clones. Northern analysis with all four clones revealed distinct patterns of expression, not only in cell suspension cultures, but also in seedlings and mature plants. For two of those the corresponding secreted proteins were identified immunologically with antisera raised against β-galactosidase fusion proteins.

In conditioned media approximately 50 protein bands can be resolved by mono-dimensional SDS-PAGE (De Vries *et al.*, 1988a). Because the primary antisera used for library screening reacted with many of these proteins on Western blots after periodate treatment, it is surprising that only four different clones were obtained. The number of plaques screened exceeded several times the

estimated complexity of the carrot mRNA population, and the fact that several cross-reacting clones were obtained indicated that the screening itself was done on a sufficiently large number of phages. Several explanations may account for the small number of clones obtained. Firstly, the screening of the λgt11 library depended on expression of carrot cDNAs as non-glycosylated fusion proteins with β-galactosidase and the recognition of these fusion proteins by two antisera raised against carrot culture medium proteins. As these antisera had been raised mainly against glycoproteins, they might only have recognized a small proportion of the expressed cDNAs corresponding to genes encoding extracellular proteins. Secondly, the antibody titer for a given protein reflects its antigenic properties, which depends on amino acid sequence as well as structure. In the case of glycoproteins, protein epitopes may be shielded by sugar chains and hence not be sufficiently exposed. Most antibodies may therefore not recognize protein epitopes, but sugar epitopes instead. Thirdly, several protein bands observed in ^{35}S-labeled protein patterns may actually be derivatives of the same protein through *e.g.* post-translational modifications, as evidenced by the five different protein bands of the EP1 protein observed by Van Engelen *et al.* (1991). The actual number of unique extracellular proteins might therefore be lower than assumed previously. Fourthly, the antisera used for screening were raised against proteins accumulated in the medium after several days. This may have resulted in overrepresentation of some and underrepresentation of other proteins in comparison to their mRNA abundance. Fifthly, proteins foreign to *E. coli* may be unstable in this host. It is assumed that short cDNA inserts produce more stable fusion proteins (Tamkun *et al.*, 1986). A number of epitopes may therefore not have been present. Sixthly, the λgt11 library used here mostly contained inserts smaller than 500 base pairs, but as the library was constructed with cDNA synthesized on oligo(dT)-primed poly(A)$^+$ RNA, it was likely to be biased towards 3' sequences. Therefore, *N*-terminal epitopes of secreted carrot proteins would be underrepresented. To illustrate the inherent difficulties of expression screening, subsequent expression screening experiments with antisera raised against SDS-polyacrylamide gel-purified secreted proteins of 27, 29, 38 and 46 kD failed to yield positive clones, even though these antisera gave very strong and reproducible signals on Western blots (P. Sterk and H. Booij, unpublished results).

A preliminary characterization of the EP1, EP2, EP5, and EP6 clones was aimed to provide an answer to two questions concerning the suitability of our approach to clone cDNAs corresponding to genes encoding extracellular proteins and the possible involvement of these genes and proteins in carrot somatic embryogenesis. Firstly, it had to be established that the corresponding proteins were indeed secreted ones. Reaction of antisera raised against the β-galactosidase fusion proteins with EP1 and EP2 with a 52/54 kD protein doublet and a 10 kD protein, respectively, confirmed that this was indeed the case. None of these proteins could be detected intracellularly (Van Engelen *et al.*, 1991; Chapter 3). It was not possible to identify the EP5 and EP6 proteins in a similar fashion. However, the deduced EP5 cDNA sequence appeared to contain a signal sequence indicating that EP5 is also secreted into the extracellular matrix. We conclude that the screening strategy employed to isolate cDNA clones encoding secreted proteins was successful. Secondly, expression analysis of the corresponding genes had to provide an indication whether their regulation was correlated with somatic embryo development. Clearly, the results shown provide evidence for the existence

of cell-specific expression of genes encoding secreted proteins in tissue culture. Two of these, EP5 and EP6, appeared to be expressed by various cell types such as non-embryogenic cells, embryogenic cells and somatic embryos. Expression of one of these genes, EP1, appeared to be absent in somatic embryos and was indeed restricted to non-embryogenic vacuolated cells either single or clustered (Van Engelen *et al.*, 1991). Although the expression pattern of the EP5 and EP6 genes appeared similar in cell culture, subtle differences in levels of expression in different parts of the plant may indicate that they originate from specific as yet unidentified subsets of cells in culture. The expression of the EP2 gene was in contrast to the other genes restricted to embryogenic cells and somatic embryos. Whether EP2 plays a crucial role in somatic embryogenesis cannot be concluded from the results here presented.

In conclusion, we have obtained four cDNA clones encoding extracellular proteins secreted into carrot cell culture media and identified in two cases the corresponding proteins by immunological means. These results have shown that the strategy employed here has been successful in generating clones corresponding to secreted proteins. The finding that at least three different patterns of gene expression exist in embryogenic tissue cultures, suggests that the total pattern of proteins detected is the sum of all different cell types. Therefore, changes in the abundance of a particular protein, in relation to a change in culture conditions cannot be interpreted directly as a reflection of altered gene expression; it can also be interpreted to reflect the loss or decrease of the subpopulation of cells that produce that particular protein. Only one of the clones, EP2, represents a gene clearly correlated with embryogenic potential and somatic embryo development, and was therefore chosen for further study (Chapter 3 and 4)..

Methods

Plant materials

Cultures of *Daucus carota* L. cv Trophy (Zaadunie B.V., Enkhuizen, The Netherlands), designated as cultivar "10" (embryogenic) and FG10 (non-embryogenic), and cultures of *D. carota* L. cv San Valery, designated A$^+$, α5II (non-embryogenic; Vergara *et al.*, 1982) and α5IIIR (a revertant of α5II; embryogenic), were maintained as described by De Vries *et al.* (1988a). Carrot seedlings were grown in vermiculite at 25°C for 7-14 days. Mature greenhouse plants were kindly provided by Zaadunie B.V.

Medium protein isolation and production of antisera against medium proteins

Medium protein samples were prepared by passing conditioned medium through a 0.22-μm filter and subsequent concentration by either pressure dialysis (De Vries *et al.*, 1988a), or precipitation by adding 5 volumes of cold 100% acetone, followed by centrifugation at 10,000*g* for 10 minutes, and a 70% ethanol washing step, after which the protein pellet was vacuum-dried and redissolved in PBS (10 mM NaH_2PO_4/Na_2HPO_4, pH 7.5, 150 mM NaCl). Partial purification of secreted proteins was done by electroeluting proteins from gel slices excised from 12.5%

preparative SDS-polyacrylamide gels. Antisera were raised against denatured extracellular proteins. Rabbits were injected subcutaneously with approximately 50 μg protein in Freund's complete adjuvant and subsequently in Freund's incomplete adjuvant at four weeks intervals. Blood was collected 7 to 10 days after each injection.

Protein deglycosylation and immunoblot analysis

Extracellular proteins were separated on 12.5-15% SDS-polyacrylamide gels as described by Laemmli (1970) and transferred to nitrocellulose membranes (Towbin *et al.* 1979). To reduce aspecific cross-reaction with sugar groups blots were incubated in 10% (w/v) sodium meta-periodate for 15 minutes, transferred to 0.1 N hydrochloric acid, neutralized after 5 minutes in 250 mM Tris.HCl, pH 7.5, and subsequently washed twice in 50 mM Tris.HCl, pH 7.5. The extent of deglycosylation was tested with a concanavalin A binding assay. Therefore, periodate-treated blots were incubated with PBS + 0.4% gelatin for one hour, subsequently with PBS + 10 μM $MgCl_2$ + 10 μg $CaCl_2$ + 0.5% triton X100 for one hour, and washed 5x5 minutes in the same buffer. The blot was transferred to 100 ml Tris.HCl, pH 7.5, containing 50 mg 4-chloro-1-naphthol and 30 μl 30% H_2O_2 and incubated for 5-15 minutes in the dark. Glycoproteins containing mannosyl and glycosyl groups stain purple. The reaction was stopped by rinsing the blot with distilled water. The blot was dried in the dark and subsequently photographed. All steps were carried out at room temperature. Immunological detection of proteins was carried out essentially according to Burnette (1981). Goat anti-rabbit IgG-alkaline phosphatase conjugate with Nitro Blue Tetrazolium (NBT; Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma) as color substrates was used to detect proteins that reacted with the antisera.

Library screening

A λgt11 cDNA library, prepared from poly(A)$^+$ RNA isolated from an unfractionated embryo culture of *D. carota* L. cv. Northrup King, was kindly provided by dr. T.L. Thomas (Texas A&M University, College Station, Texas). This library was screened with antisera raised against extracellular proteins essentially according to Huynh *et al.* (1985), with the exception that alkaline phosphatase-conjugated goat-anti-rabbit IgG and the color substrates NBT and BCIP were used to detect positive plaques. A λZAPII cDNA library (Stratagene) prepared from poly(A)$^+$ RNA isolated from mixed embryogenic suspension and somatic embryo cultures of cultivar Trophy was screened for longer clones with radiolabeled DNA probes using standard procedures (Maniatis *et al.*, 1982).

Purification of β-galactosidase fusion proteins

Lysogenic *E. coli* Y1089 bacteria were generated as described by Huynh *et al.* (1985). Fusion proteins were purified according to a protocol optimized for the purification of β-galactosidase from an *E. coli* Y1089 lysogen containing the non-recombinant λgt11 genome. The lysogen was grown in one litre of LB medium to OD_{600}=0.5, incubated at 42°C for 30 minutes to induce replication of the phage genome. Induction of expression of the *lac*Z gene and subsequent production of β-galactosidase was achieved by the addition of IPTG to 2 mM and incubation at 37°C for 1.5 hours. After addition of PMSF to 1 mM a cell-free extract was prepared by spinning

the culture for 5 minutes at 5000*g* at room temperature, resuspending the pellet in ice-cold 40 ml DEAE-Sephacel column buffer (10 mM Tris.HCl (pH 7.5); 2 mM $MgCl_2$; 0.2 mM PMSF; 0.2 mM DTT), freezing in liquid nitrogen and subsequent thawing, sonication for 2 minutes and centrifugation for 10 minutes at 13,000*g* at 4°C to remove debris. The supernatant was loaded onto a DEAE-Sephacel (Pharmacia) column (15x80 mm), after which protein was eluted with DEAE-Sephacel column buffer containing 200 mM NaCl and next with 100 ml of column buffer with a linear 200 to 400 mM NaCl gradient. Five ml fractions were collected and assayed for β-galactosidase activity using a kinetic assay with ONPG as substrate. Fractions with β-galactosidase-activity were pooled. Further purification was achieved by selectively precipitating proteins with 60% ammonium sulphate. After centrifugation at 13,000*g* for 30 minutes at 4°C, protein pellets were resuspended in minimal amounts of Sephacryl column buffer (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 assayed for β-galactosidase-activity. Active fractions were loaded onto a Sephacryl S300 HR (Pharmacia) column (26x800 mm) and protein was eluted with Sephacryl column buffer with a flow rate of 2.5 ml/minute. Five ml fractions were collected, activity-containing fractions were pooled and protein was precipitated by adding ammonium sulphate to 60% saturation. After centrifugation the pellet was resuspended in a solution containing 10 mM Tris.HCl (pH 8.0), 0.2 mM PMSF and 0.2 mM DTT and desalted on a Biogel P6PG (BioRad) column (10x70 mm). The protein containing fraction was subjected to preparative 7.5% SDS-PAGE, the gel was subsequently stained with Coomassie Brilliant Blue, the β-galactosidase band excised and eluted in a Biotrap device (Schleicher and Schuell). Protein was precipitated with 5 volumes of acetone, spun down, dried and dissolved in PBS.

Northern blot analysis

Total RNA was isolated from cultured cells and plant tissues as described by De Vries *et al.* (1988b). Glyoxylated RNA samples were electrophoresed on 1.5% agarose gels and transferred to GeneScreen membranes (New England Nuclear) according to the manufacturer's instructions. Hybridization with ^{32}P-labeled cDNA clones was carried out in 5xSSC, 2xDenhardt's, 1% SDS, 100 μg/ml denatured salmon sperm DNA, 50% formamide for 16 hours at 42°C. Blots were washed twice either 2xSSC, 0.5% SDS or in 0.5xSSC, 0.5% SDS at 60°C for 30 minutes and exposed to Kodak X-Omat AR film with intensifying screen.

DNA sequence analysis

Sequencing of both strands of cDNA inserts was done using the double stranded dideoxy chain termination method using the large fragment of *E. coli* DNA polymerase I (Korneluk *et al.*, 1985) or Sequenase (United States Biochemical Corporation). The EP cDNA sequences and the deduced protein sequences were compared with sequences deposited in the GenBank (version 79.0), EMBL (version 36.0) and Swiss-Prot (version 26.0) databases using the FASTA program (Pierson and Lipman, 1988) on a VAX/VMS computer.

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CHAPTER 3

Cell-specific expression of the carrot EP2 lipid transfer protein

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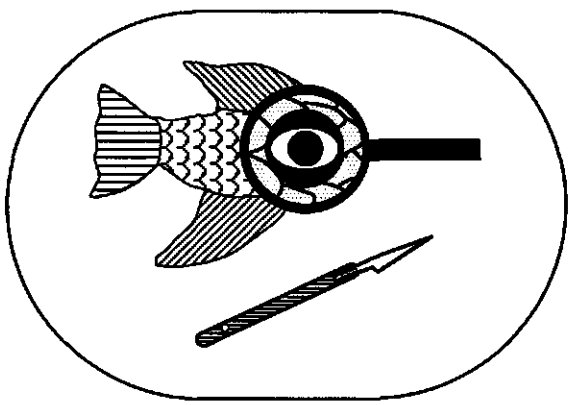

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Abstract

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 immature seeds. *In situ* hybridization revealed 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 immature 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, 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 (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

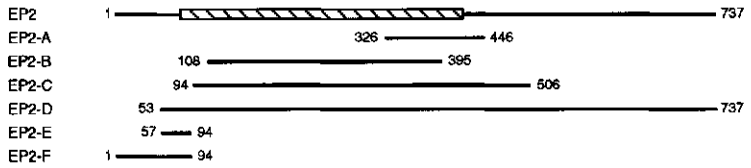
A λgt11 cDNA library representing the mRNA population of globular, heart, and torpedo stage carrot somatic embryos was screened with an antiserum raised

against all proteins secreted into the medium of somatic embryo cultures. Several clones were obtained that hybridized 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 weight 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 is likely to contain a signal peptide that should be cleaved off after the valine residue at position 26, according to the rules described by Von Heijne (1983). 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 sequence with sequences deposited in the current versions of 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 these percentages is based on amino acid sequences as shown in Figure 1D). Due to their aspecific lipid binding properties, these proteins have recently been reclassified as non-specific 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

A



B

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                  M  G  V  L  R  S  S  F  V  A  M  M  V  M  Y      15

CATGGTCTTAGCAACCACACCAAATGCTGAAGCCGTCCTCACATGCGGGCAGGTGACTGG     180
  M  V  L  A  T  T  P  N  A  E  A  V  L  T  C  G  Q  V  T  G      35

AGCCCTGGCCCCGTGCCTGGGCTACCTGAGGAGCCAGGTAAACGTTCCAGTCCCGCTCAC     240
  A  L  A  P  C  L  G  Y  L  R  S  Q  V  N  V  P  V  P  L  T      55

CTGTTGCAATGTTGTGAGGGGACTCAATAACGCTGCACGGACCACACTCGACAAGCGCAC     300
  C  C  N  V  V  R  G  L  N  N  A  A  R  T  T  L  D  K  R  T      75

CGCTTGCGGCTGCCTCAAGCAAACGGCTAATGCCGTCACTGGTCTCAACTTGAACGCTGC     360
  A  C  G  C  L  K  Q  T  A  N  A  V  T  G  L  N  L  N  A  A      95

TGCTGGCCTCCCTGCTAGATGTGGTGTCAACATTCCTTACAAAATCAGCCCCACCACCGA     420
  A  G  L  P  A  R  C  G  V  N  I  P  Y  K  I  S  P  T  T  D     115

TTGCAACAGGGTGGTGTGAAGGTATGTTAATGCCAGGGATTTTCAAGTGCAAGCTCTATA     480
  C  N  R  V  V  *                                               120

TTAAGTTTGTTTGGAATAAAGATGCATGGGATCTAAGCATAATGTAATCGTCTGTGTTTA     540

GTTTTTATTTGATGATGAGTTGCACTGCTGCTTTTAATGTTCTGTTTCTGTATGAAAATG     600

TGTTTGACATCTATGATCTATAAATCTAGTAAGTTTTTAAGTAATGGCATCGAGTTCGAG     660

TGTTTTCCAGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA     720

AAAAAAAAAAAAAAAA                                                737
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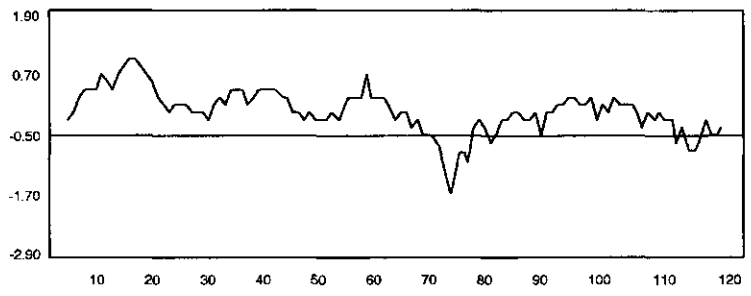
C



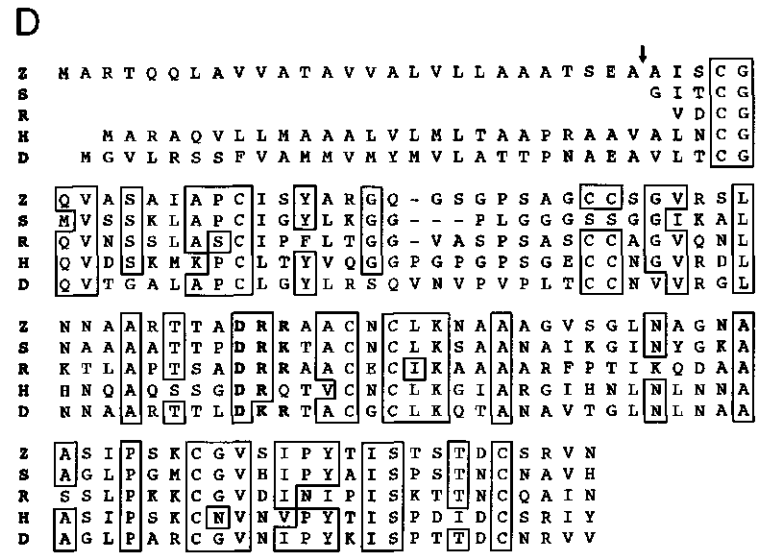


Figure 1. Analysis of cDNA and deduced amino acid sequence of clone EP2.

(A) Schematic representation of the EP2 cDNA clones A-F. EP2-A was obtained by expression screening and had a size of 121 bp. Rescreening of the λgt11 and λZAP II 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 asterix. The putative signal peptide cleavage site is marked with 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 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.

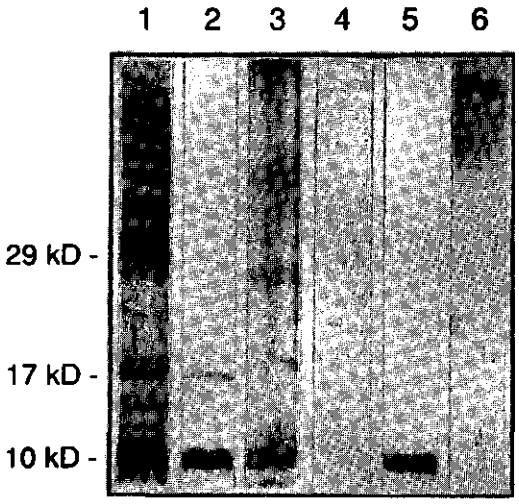
or its 12.5-kD precursor was obtained, indicating that the majority of the EP2 protein is extracellular. In media, a possibly dimeric form of the same protein was also recognized. An antiserum raised against a lipid transfer protein from maize also recognized a 10-kD secreted carrot protein as well as 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 against a spinach lipid transfer protein were identical to those of the maize antiserum (data not shown).

Experiments applying fluorescent phospholipid analogues (Somerharju *et al.*, 1990) showed that the purified EP2 protein is also capable of binding and transferring phospholipids (see Chapter 4).

Based on the high protein sequence homology, the immunological cross-reactivity of heterologous maize and spinach antisera with the β-galactosidase-EP2-A fusion protein and the phospholipid transfer activity of the EP2 protein, we conclude that the carrot EP2 gene encodes a secreted lipid transfer protein.

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 expression 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 serum 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 antiserum against β-galactosidase-EP2-A. 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.

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 non-embryogenic suspension cell cultures as well as from plants with EP2-C as probe as depicted in Figure 3.

Embryogenic high-density cell suspensions cultures are maintained in B5 medium containing 0.44 mg/l of the synthetic auxin 2,4-D, and contain clusters of small cytoplasmic cells designated proembryogenic masses. Somatic embryos develop 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 is blocked before globular stage in proembryogenic mass-enriched cultures diluted in the presence of 2,4-D, which are 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 non-embryogenic cultures. In addition, the EP2 gene was highly expressed in manually purified somatic embryos (Figure 3A). No EP2 expression was found in the non-embryogenic or weakly embryogenic cultures FG10, α5II, and ts11, while

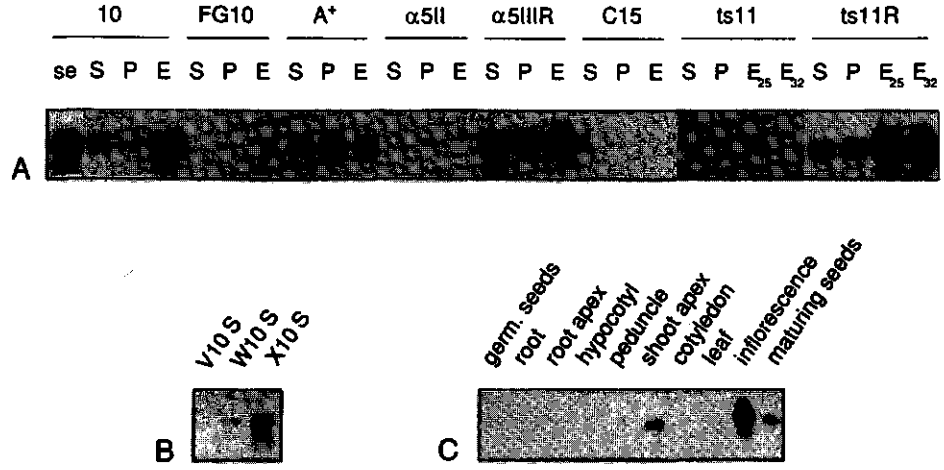


Figure 3. RNA gel blot analysis of EP2 in tissue cultures and plants.

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.

(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 non-embryogenic 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 non-embryogenic; 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 immature 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 immature seeds.

embryogenic 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 to embryogenic "10" lines, resulting in a stronger hybridization signal with RNA from the A^+ and α5IIIR lines. Taken together, we conclude 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 immature seeds. No expression was found in seeds imbibed for 48 hours. 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 soybean 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 (see Chapter 2), 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 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.

The EP2 gene is expressed in protoderm cells of somatic and zygotic embryos

To elucidate the pattern of EP2 gene expression in more detail, we employed *in situ* localization of the EP2 mRNAs in sectioned tissue culture and intact plant material. Hybridization of sectioned somatic embryos with EP2-C anti-sense 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

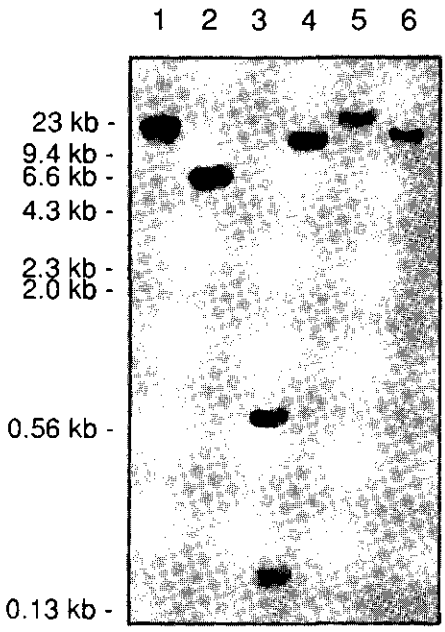


Figure 4. Determination of the number of EP2-related genes per carrot genome.

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.

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 (ts) variant cell line, ts11 embryo development is arrested at the non-permissive 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 non-embryogenic 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 post-fertilization 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 anti-sense 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 conclude that the expression of the EP2 gene is a very early event in zygotic embryogenesis. In addition, a very strong signal is observed in the cell layers that will 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. This pattern persists through 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.

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 anti-sense 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 carried out 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 has developed 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, non-embryogenic 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.

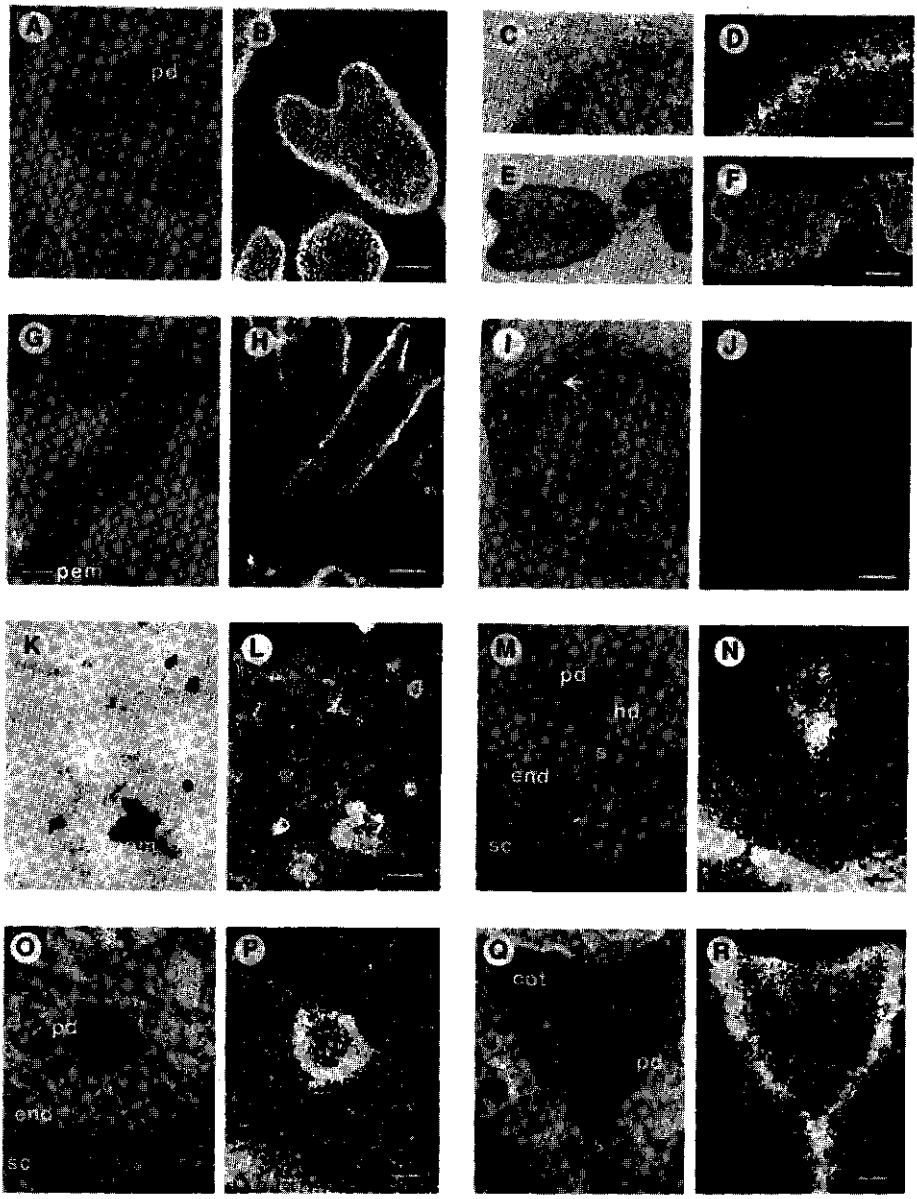
(**N**) Dark-field photograph of (**M**). Bar=20 μm.

(**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.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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

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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 anti-sense 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 prior to dehiscence. The section was cut at a distance one third of the length of the fruit from the basis. The arrowhead points towards 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.

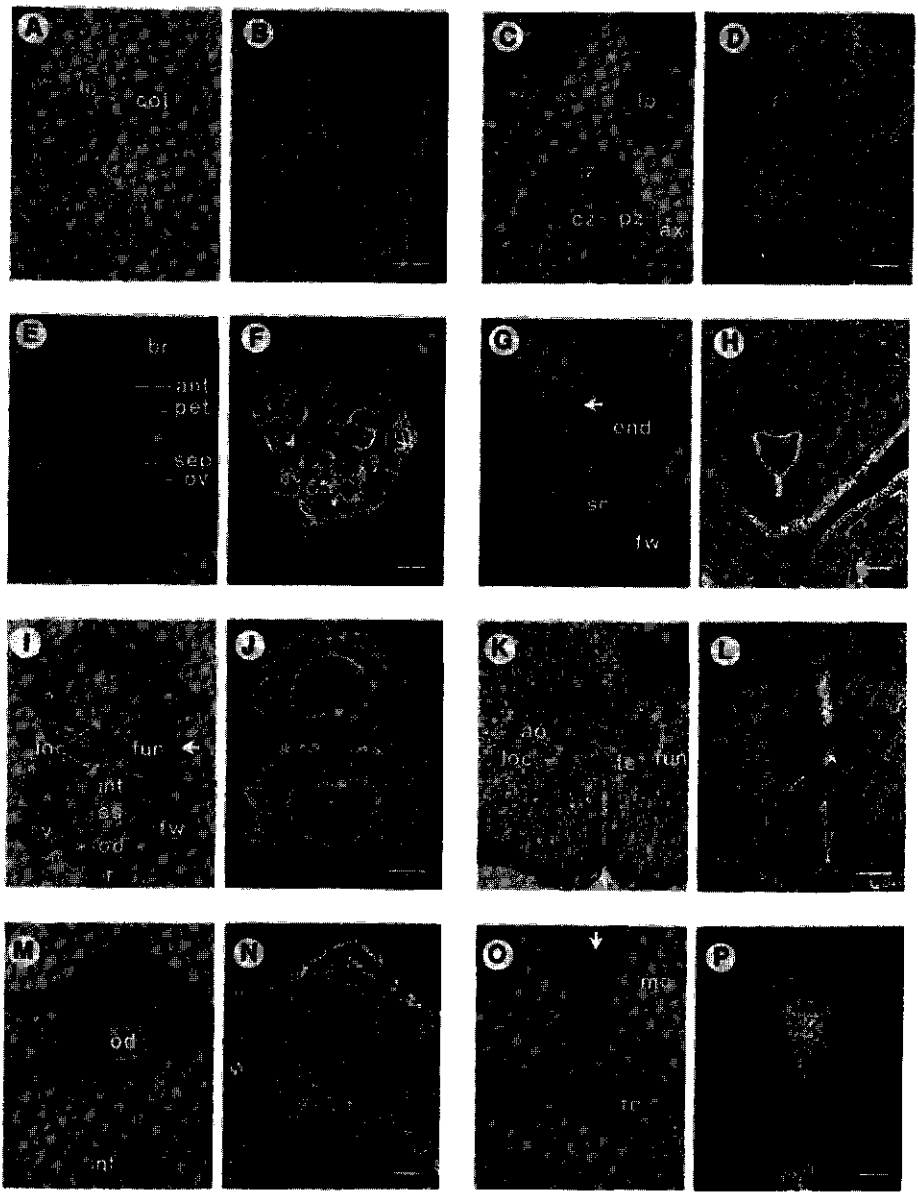
(**L**) Dark-field photograph of (**K**). Bar=20 μm.

(**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.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protein encoded by EP2 is only essential in early stages of leaf and cotyledon development. When viewed at 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 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 at 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, while 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 EP2 gene expression 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. At 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; Chapter 2 and this chapter) 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 non-embryogenic 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, 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, while expression ceases upon maturation of stamen, petals, and sepals. In the ovary, expression continues in the integument epidermis, again a in 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 non-specific 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, while 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 oleyl-coenzyme A (Rickers *et al.*, 1984, 1985; Kader, 1990). Therefore, we conclude that the EP2 protein is a secreted protein, which 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 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 would therefore 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 non-specific 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.

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 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* L. cv Trophy (Zaadunie B.V., Enkhuizen, The Netherlands), designated as cultivar "10" (embryogenic) and FG10 (non-embryogenic), and cultures of *D. carota* L. cv San Valery, designated A^+, α5II (non-embryogenic; Vergara *et al.*, 1982), α5IIIR (a revertant of α5II; embryogenic), c15 (non-embryogenic; 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 7-14 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* L. cv. 'Northrup King,' was kindly provided by Dr. T.L. Thomas (Texas A&M University, College Station, Texas, USA). 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 *D. carota* L. cv Trophy 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 pfu of the library were extracted once an equal volume of with phenol/chloroform (1:1). Phage DNA was precipitated from the aqueous phase by adding an equal volume of 2-propanol, pelleted, washed with 0.5 ml 70% ethanol. The DNA was dried and redissolved in 1x 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 *Taq* polymerase (Perkin-Elmer Cetus Instruments) 50 cycles (1 min 92°C; 1 min 60°C; 3 min 72°) 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 *E. coli* Y1089 as described by Huynh *et al.* (1985). The protein was purified from 1-litre cultures 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 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 sulphate (60% saturation). After centrifugation at 13,000*g* for 30 min, the protein pellet was dissolved in 2-4 ml 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 sulphate, 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 Biogel P6PG (BioRad) column and subjected to preparative 7.5% SDS-PAGE. After staining with Coomassie Brilliant Blue R250, 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 (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) 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, College Station, Texas, USA, 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 using the Staden program on a micro VAX/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 appears in the EMBL, GenBank and DDBJ nucleotide sequence databases under 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 (New England Nuclear) according to the manufacturer's instructions. Hybridization with ^{32}P-labeled cDNA clones was carried out in 5xSSC (1xSSC is 0.15 M NaCl,

0.015 M sodium citrate), 2xDenhardt's (1xDenhardt'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 hours at 42°C. Blots were washed twice in 0.1 x 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 (New England Nuclear) using 10xSSC. The blot was hybridized with ^{32}P-labeled EP2-C cDNA in 1 M NaCl, 1% SDS, 10% dextran sulphate, and 100 μg denatured salmon sperm DNA at 65°C for 2 days. The blots were washed twice in 5xSSC for 10 min and twice in 5xSSC, 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* prior to fixation and embedding. ^{35}S-labeled antisense and sense (control) RNA probes were transcribed from clone EP2-C using either the T3 of the T7 promoter and hydrolyzed to a size of approximately 0.2 kb. Hybridization was performed for 16 hours under a 42°C, 0.3 M Na^+, 50% formamide criterion. The washing stringency was 4xSSC 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 epi-polarization optics.

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CHAPTER 4

Characterization of the non-specific lipid transfer protein EP2 from carrot (*Daucus carota* L.)

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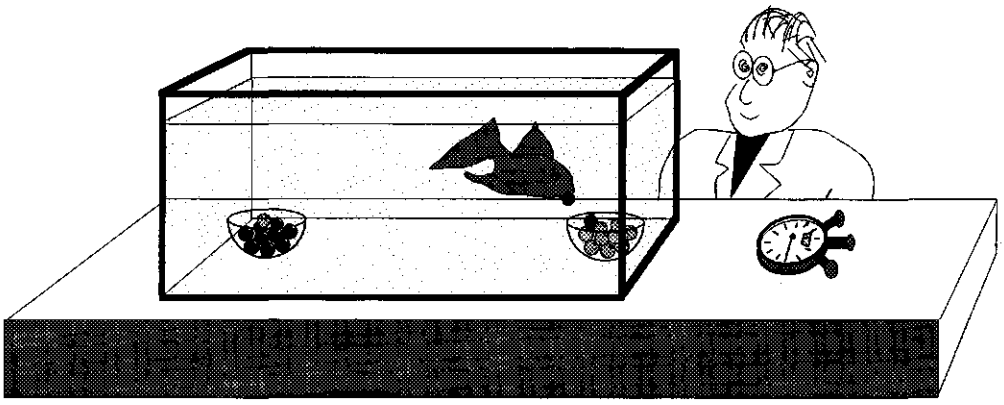

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Abstract

The extracellular protein EP2 was previously identified as a non-specific lipid transfer protein based on its cDNA-derived amino acid sequence. Here, the purification of the EP2 protein from the medium of somatic embryo cultures is described. After two cycles of ion-exchange and gel permeation chromatography, a single silver-stained protein band with an apparent molecular mass of 10 kD was observed on SDS-polyacrylamide gels. This protein band was recognized on Western blots by an antiserum raised against a EP2-β-galactosidase fusion protein. Employing a fluorescent phospholipid analog, it was shown that the purified EP2 protein is capable of binding phospholipids and is able to enhance their transfer between artificial phospholipid membranes. Employing a gel permeation assay, it was demonstrated that the EP2 protein was also capable of binding palmitic and oleic acid as well as oleyl-CoA. Because in plants these fatty acids are used as precursor molecules for cutin, these results are in support of the proposed role of the EP2 protein to transport cutin monomers from their site of synthesis through the cell wall of epidermal cells to sites of cutin polymerization.

Introduction

A cDNA clone corresponding to a 10-kD extracellular protein, secreted into the medium of embryogenic carrot suspension cultures, has been isolated employing expression screening of a carrot somatic embryo cDNA library with antisera raised against a mixture of extracellular proteins (Chapters 2 and 3). The cDNA-derived amino acid sequence of this protein, designated extracellular protein 2 (EP2), showed homology to non-specific lipid transfer proteins (nsLTP) identified in several other plant species. Employing *in situ* mRNA localization the EP2 gene was shown to be expressed in the protoderm of both zygotic and somatic embryos, and in epidermal cells of leaf primordia, flower organs and immature seeds. The expression of the gene in protoderm or epidermal cells and the extracellular location of the protein appear difficult to reconcile with the proposed function of plant nsLTPs in the transfer of phospholipids between intracellular membrane compartments (Arondel and Kader, 1990). Instead, we have proposed a role for the carrot EP2 protein in the secretion of cutin monomers (Chapter 3).

Cutin is a large hydrophobic biopolymer present in the cuticle of aerial plant organs and is composed of esterified hydroxylated fatty acids. Cutin monomers are derived mainly from palmitic and/or oleic acid and are likely to be secreted from their site of synthesis towards the growing cutin polymer in the form of acyl-CoA esters (Kolattukudy, 1981).

In this chapter we report on the purification of the EP2 protein from the medium of carrot somatic embryo cultures. Furthermore, we show that the EP2 protein was able to enhance the transfer of fluorescent phospholipid analogs between artificial phospholipid membranes, and to bind putative cutin monomers, such as palmitic and oleic acid and oleyl-CoA. These results support the hypothesis that the EP2 protein is involved in cutin synthesis.

Results

Isolation and purification of EP2

The purification of EP2 from the concentrated protein fraction isolated from the medium of 12-day-old embryo cultures (ECP) required three steps. After separation of the anionic from the cationic proteins by means of cation-exchange chromatography, the final purification was achieved in two steps by means of FPLC (Figure 1). Aliquots from each of the fractions eluted from the Superdex 75 and Mono S column were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by silver staining (Figure 1B) and immunoblotting (Figure 1D, 1E), using the antiserum raised against EP2-β-galactosidase fusion-protein (Chapter 3). This resulted in a preparation consisting of a single 10 kD protein band on a silver stained SDS-polyacrylamide gel (Figure 1F).

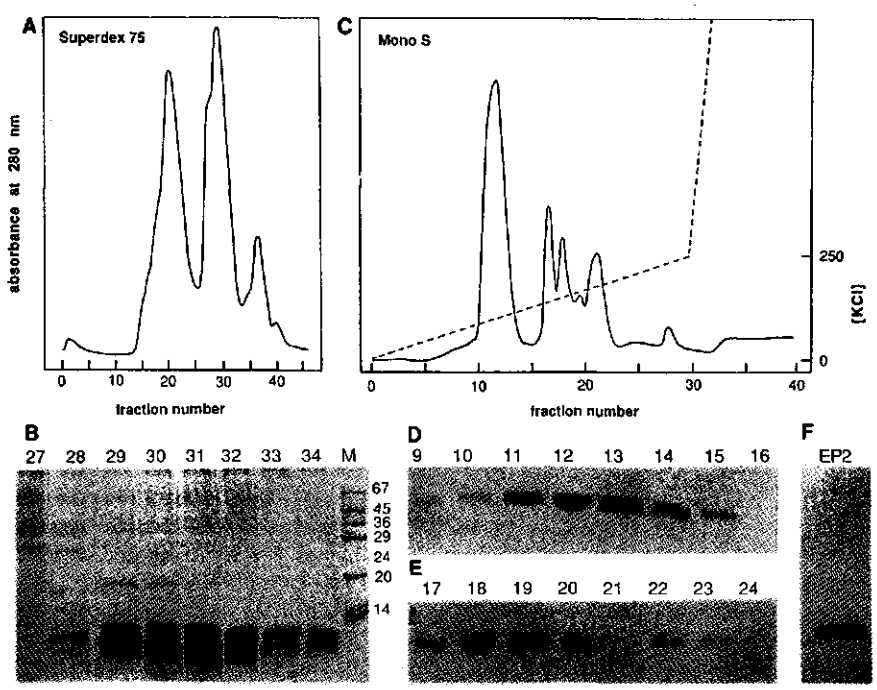

Figure 1. Final steps in the purification of carrot nsLTP EP2.

(**A**) Elution profile of the Superdex 75 gel filtration, and (**B**) analysis of the eluted fractions 27-34 by means of SDS-PAGE and silver staining. (**C**) Elution profile of the Mono S cation-exchange chromatography, and (**C**) and (**D**) analysis of the eluted fractions 9-24 by means of immunoblotting, using an antiserum raised against an EP2-β-galactosidase fusion-protein. (**E**) Silver-stained SDS-polyacrylamide gel of the pooled fractions 10-15, as analyzed in (**C**), showing a single protein band representing the purified EP2 protein. Lane M represents marker proteins with their molecular masses (kD) indicated on the right.

In addition to EP2, the antiserum recognized other small proteins which eluted from the Mono S column (Figure 1C, 1E). It is most likely that these proteins represent modified forms of EP2, since they were not present in the medium of non-embryogenic cell lines (not shown), similarly as reported for the EP2 protein (Chapter 3), although this was not further investigated.

Determination of the final yield of the EP2 protein was complicated by observed discrepancies in determination of the protein concentration. Each of the three methods used gave different results (Table 1), making it difficult to quantitate both purification and yield. The protein-dye binding assay (Bradford, 1976) was used routinely, but later experiments (see following sections) indicated that the BCA method (Smith *et al.*, 1985) may be a more reliable estimate of the amount of purified EP2 protein obtained. The amount of purified EP2 estimated by the absorbance at 280 nm is probably an underestimate, since EP2 contains only two aromatic acid (tyrosine) residues per molecule (Chapter 3). When the amount of EP2 protein was calculated on a basis of the molecular extinction of tyrosine at 280 nm, on the assumption that this is the same for free amino acids and incorporated amino acids, a figure was obtained close to that determined by the BCA method (5.1 mg; see Table 1). Direct comparison of amino acid composition analysis with other protein measurements is in progress.

	Amount of protein (mg)		
Fraction	Bradford	Smith *et al.*	A_{280} nm
ECP	16	n.d.	251
S-Sepharose, flow through	13	n.d.	211
S-Sepharose, salt pulse	6.3	38	45
Superdex 74, fractions 28-34	0.24	9.6	n.d.
Mono S, fractions 10-15 (EP2)	0.16	6.5	2.2

n.d.= not determined.

Table 1. Purification of carrot nsLTP EP2

The amounts of protein obtained during the purification of EP2 were determined by three methods based on different principles. In all methods the amounts of protein given are relative to the BSA standard.

Phospholipid transfer activity of EP2

As shown in Figure 2A, the addition of purified EP2 to 1-palmitoyl-2-[6-(1-pyrenyl) hexanoyl]-*sn*-glycero-3-phosphocholine (Pyr(6)PC) caused a shift in the fluorescence emission spectrum of the phospholipid analog towards that of the monomer

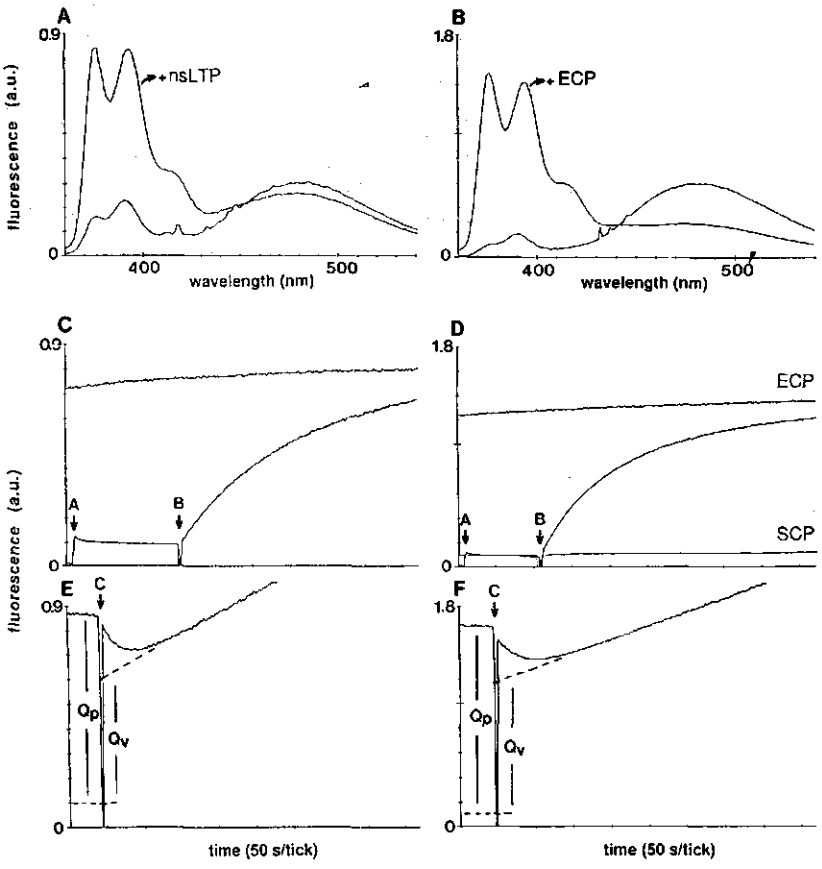

Figure 2. Binding and transfer to acceptor vesicles of the fluorescent phospholipid analog Pyr(6)PC by carrot nsLTP EP2.

(**A**, **B**) Changes in the fluorescence emission spectrum (excitation at 346 nm) of 1 nmol Pyr(6)PC in 2 ml TBS-buffer (20 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 1 mM EDTA and 1 mM EGTA) in the presence of 0.5 μg purified EP2 (**A**) or 50 μg unfractionated embryo culture medium proteins (ECP) (**B**). (**C**, **D**) Pyr(6)PC binding by EP2 (**C**) or ECP (**D**). Fluorescence emission of Pyr(6)PC was measured at 378 nm. Arrow A indicates the injection of 1.8 nmol Pyr(6)PC and 0.2 nmol *N*-[2,4,6-trinitrophenyl]-phosphatidyl-ethanolamine (TNP-PE, a fluorescence quencher) in 20 μl ethanol into a cuvette containing 1.88 ml TBS-buffer. Arrow B indicates the addition of 0.25 μg EP2, 3 μg ECP, or 3 μg of unfractionated suspension culture medium proteins (SCP). (**E**, **F**) Transfer of Pyr(6)PC to acceptor vesicles by EP2 (**E**) or ECP (**F**). Arrow C indicates the addition of 100 nmol acceptor vesicles (phosphatidylcholine : phosphatic acid = 95 : 5 mol%). The fluorescence quantum yield of Pyr(6)PC in EP2 relative to that in acceptor vesicles is calculated by dividing distance Q_p by Q_v. In all instances the amounts of protein in the assay were based on determinations according to Bradford (1976; cf. Table 1).

(emission maxima at 378 and 495 nm). This indicated that EP2 is capable of binding Pyr(6)PC in its monomeric form.

An increase in the fluorescence of Pyr(6)PC monomers was also observed upon the addition of EP2 to quenched donor vesicles containing Pyr(6)PC (Figure 2B). After equilibrium was reached, the addition of the acceptor vesicles caused the fluorescence to initially decrease, and then, after going through a minimum, to increase with time (Figure 2C). The initial decrease in the fluorescence is probably caused by the rapid transfer of Pyr(6)PC bound to EP2 to the acceptor vesicles. The fluorescence quantum yield of Pyr(6)PC bound to EP2 was approximately 1.4 times higher than when the phospholipid analog was present in the acceptor vesicles. This value is only slightly smaller than the 1.75 times increase in the quantum yield obtained for pyrenyl-labeled lipids in nsLTP from bovine liver (Gadella *et al.*, 1991).

A more quantitative analysis with respect to the phospholipid transfer assay was hampered by the uncertainty in the determination of the amount of EP2 protein. By callibrating the Pyr(6)PC fluorescence in acceptor vesicles, the use of the 1.4 increase in the quantum yield of Pyr(6)PC when bound to EP2, and the assumption that EP2 binds a single Pyr(6)PC molecule, it was calculated that 80% of the amount of protein applied to the assay mixture, estimated according to Bradford (1976), would be occupied. This is extremely high, when compared to the 8% estimated for nsLTP from bovine liver in the same assay (Gadella *et al.*, 1991). When the estimation described by Smith *et al.* (1985) was used (see Table 1), it was calculated that about 2% of the EP2 protein molecules would be occupied by a single Pyr(6)PC molecule.

A binding and transfer activity similar to that shown for purified EP2 was observed when unfractionated medium proteins from an embryogenic cell line were used (Figure 2D-2F). Medium proteins from a non-embryogenic cell line, however, failed to bind Pyr(6)PC monomers (Figure 2E). This is in line with the finding that the EP2 gene is not expressed in non-embryogenic cell lines (Chapter 3). In addition, these results render it unlikely that non-specific lipid transfer proteins other than EP2 are secreted into carrot cell culture media.

Fatty acid binding activity of EP2

To test whether EP2 might function as a carrier for cutin monomers, we used a gel filtration assay based on the difference in elution between bound and unbound ^{14}C-labeled fatty acids or acyl-CoA esters. For putative cutin monomers we used palmitic and oleic acid and oleyl-CoA. Figure 3A shows that gel filtration of a mixture containing purified EP2 and oleyl-CoA, incubated for 30 min at room temperature, yielded two peaks. The first peak corresponded to the position at which EP2 was also eluted, whereas the second peak corresponded to the position of free oleyl-CoA (Figure 3B). Unfortunately, the binding of oleyl-CoA to EP2 was lost upon gel electrophoresis, making it impossible to detect the EP2-oleyl-CoA complex by means of autoradiography. By calculating the number of oleyl-CoA molecules which eluted in the first peak, and the assumption that EP2 binds either one or two oleyl-CoA molecules, we came to the conclusion that the estimated amount of EP2 present in the assay mixture (0.5 μg) was far too low, even when all EP2 molecules were involved in the binding. Based on the amount of protein

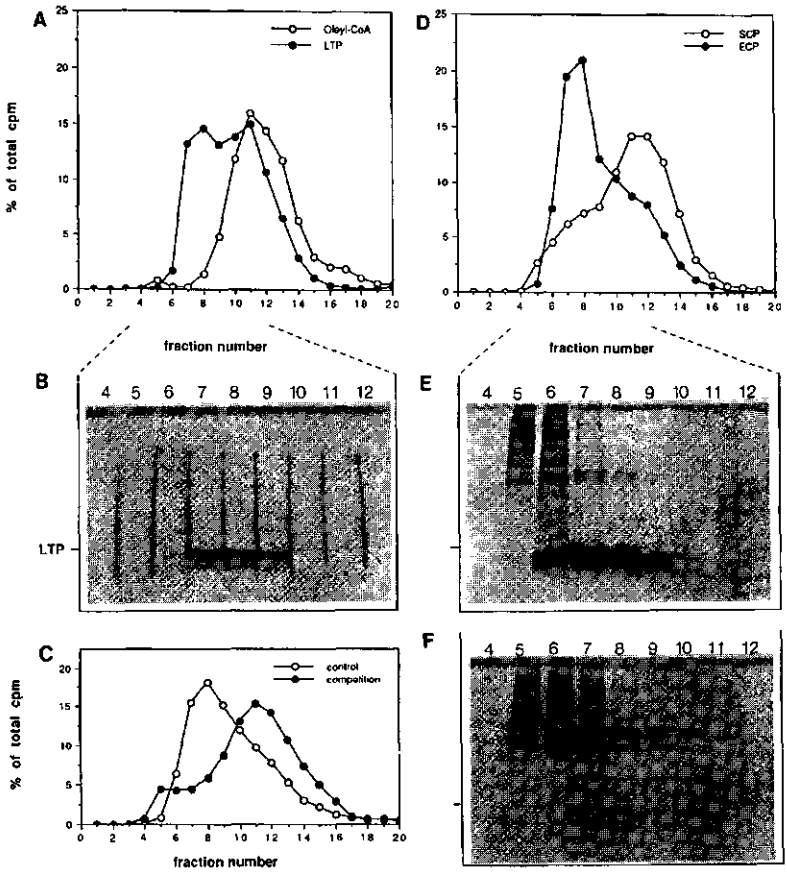

Figure 3. Binding of oleyl-CoA by carrot nsLTP EP2.

^{14}C-labeled oleyl-CoA (2 nmol; 220,000 cpm) dissolved in TBS buffer (20 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 1 mM EDTA and 1 mM EGTA) was incubated with 0.5 μg EP2 protein or 50 μg of proteins from the medium of an embryo culture (ECP) or suspension culture (SCP). The amounts of protein in the assays were based on determinations according to Bradford (1976; cf. Table 1). After incubation for 30 min, the mixtures (100 μl) were applied to 1 ml Sephadex G50 gel filtration columns and eluted with TBS buffer. Fractions (100 μl) were collected, the amount of radioactivity determined, and analysed for the presence of EP2 by SDS-PAGE and silver staining. (**A**) Elution profile of oleyl-CoA alone (o) and after incubation with purified EP2 (•). (**B**) SDS-PAGE analysis of the fractions corresponding to the first radiactive peak in (**A**). Position of EP2 is indicated by LTP. (**C**) Competition experiment in which fractions containing ^{14}C-oleyl-CoA bound to EP2 obtained from a previous binding assay were pooled and applied to a new gel filtration column directly (control; o), or after incubation with 10 a fold excess of unlabeled oleyl-CoA (competition; •). (**D**) Elution profile of oleyl-CoA after incubation with SCP (o) or ECP (•). (**E, F**) SDS-PAGE analysis of the fractions corresponding to the first radioactive peak in (**D**) obtained after elution of the assay-mixture with SCP (**E**) or ECP (**F**). The position of purified EP2 is marked.

estimated by the BCA method (20 μg), approximately 10% of the EP2 protein molecules would have bound a single oleyl-CoA molecule, or half that amount in case two oleyl-CoA molecules bind to the protein.

To test whether the binding of oleyl-CoA to EP2 was specific, a competition experiment was carried out. As shown in Figure 3C unlabeled oleyl-CoA was able to release ^{14}C-labeled oleyl-CoA bound to EP2. A similar result could be obtained by using unlabeled oleic acid (data not shown), indicating that it is the fatty acid part that is responsible for the binding to EP2. The results obtained for palmitic and oleic acid (not shown) were comparable to those shown for oleyl-CoA, except that the recoveries of the radioactivity were lower as compared to oleyl-CoA, for which recoveries were approximately 70%. This was probably caused by a higher aspecific binding of these molecules to the column material and glassware due to their more hydrophobic nature. Interestingly, these losses were always less when EP2 was present (not shown), suggesting that these hydrophobic molecules are kept more easily in solution in an aqueous environment when bound to EP2.

To test medium proteins isolated from an embryogenic cell line for fatty acid binding properties, we carried out an assay similar to the one to measure phospholipid transfer. Medium proteins isolated from a suspension culture appeared to bind less oleyl-CoA than an equal amount of proteins isolated from an embryo culture (Figure 3D). Since EP2 was present at much lower concentrations in suspension culture medium than in embryo culture medium (Figure 3E, 3F), this observation suggests that EP2 is the only secreted protein in carrot cultures capable of binding fatty acids and acyl-CoA esters.

Discussion

As discussed in chapter 3 of this thesis, the EP2 protein might function as a carrier of cutin monomers from their site of synthesis or secretion, towards the growing cutin polymer. This hypothesis was based on three observations, first, the homology of the cDNA-derived amino acid sequence with other plant nsLTPs, second, the extracellular location of the EP2 protein and third, the expression of the EP2 encoding gene in protoderm or epidermal cells involved in cutin synthesis. In this chapter, we have shown that the EP2 protein is indeed an nsLTP capable of enhancing the transfer of phospholipids between membranes *in vitro*. More important with respect to its proposed function are the results indicating that the EP2 protein is capable of binding putative cutin monomers such as palmitic and oleic acid and oleyl-CoA. Although these results are still preliminary and at present lack kinetic analysis, they are in line with the proposed role of EP2 in the transport of cutin monomers.

Plant nsLTPs have been isolated from several sources from different plant species. Based on their ability to transfer phospholipids between various types of membranes *in vitro*, they were initially thought to mediate phospholipid transfer *in vivo* (Arondel and Kader, 1990; Kader, 1990). However, the presence of a signal sequence in many of the identified plant nsLTPs (see Chapter 3 and references cited therein), indicates that these proteins are synthesized on membrane-bound polysomes and are most likely secreted. As this is difficult to reconcile with a function in the cytoplasm, several if not all of these plant nsLTPs may have a function similar to the one proposed for the carrot nsLTP EP2.

Methods

Plant material and culture conditions

Cultures from carrot (*Daucus carota* L.) cv Trophy, Zaadunie B.V., Enkhuizen, The Netherlands) were started and maintained as described by De Vries *et al.* (1988).

Protein isolation and purification

The EP2 protein was purified from the secreted proteins present in the medium of 12-day-old embryo cultures. Twenty-five litres of medium were subsequently filtered through Whatman 1MM paper and a 0.2 μm PVDF membrane (Millipore, Etten-Leur, The Netherlands). Proteins in the cell-free medium were concentrated approximately 160-fold by pressure dialysis using membranes with a 3 kD cut-off (Amicon, Oosterhout, The Netherlands) and equilibrated with 50 mM MES buffer, pH 5.8. The concentrated proteins, designated ECP, were applied to an S-Sepharose FF (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden) column equilibrated with 50 mM MES buffer, pH 5.8. Retained proteins were eluted using the same buffer containing 1 M KCl. Elution was monitored at 280 nm. Protein fractions were collected, pooled and concentrated by pressure dialysis, and equilibrated in 50 mM potassium phosphate buffer, pH 6.5, containing 150 mM KCl. This cationic protein fraction was size-fractionated on a Superdex 75 HR column (Pharmacia LKB Biotechnology Inc.) equilibrated and eluted with the same buffer by fast protein liquid chromatography (FPLC). Elution was monitored at 280 nm and protein peak fractions were analysed by SDS-PAGE, followed by silver staining and immunoblotting, using an antiserum raised against an EP2-β-galactosidase fusion protein, as described previously (Cordewener *et al.*, 1991; De Jong *et al.*, 1992; Chapter 3). Fractions containing EP2 protein were pooled, concentrated, equilibrated in 50 mM MES buffer, pH 5.8, and applied to a Mono S column (Pharmacia LKB Biotechnology Inc.) for final EP2 purification. Retained proteins were eluted (FPLC) with a linear 0-250 mM KCl gradient and monitored at 280 nm. The fractions containing purified EP2 were pooled and stored at –20°C. Amounts of protein present in the several fractions obtained during the purification of EP2 were determined according to Bradford (1976), Smith *et al.* (1985), or from the absorbance at 280 nm. In each case BSA was used as a standard.

Phospholipid transfer assay

Phospholipid transfer activity of EP2 was tested by applying the fluorescent phospholipid analog 1-palmitoyl-2-[6-(1-pyrenyl) hexanoyl]-*sn*-glycero-3-phosphocholine (Pyr(6)PC) as described by Gadella and Wirtz (1991). Purified EP2 protein, ECP and proteins from non-embryogenic carrot cell line (SCP) medium, were equilibrated in 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA and 1 mM EGTA, previous to the assay.

Fatty acid binding assay

EP2, ECP or SCP, equilibrated in the same buffer as described for the phospholipid transfer assay, were incubated with 2 nmol (220,000 cpm) ^{14}C-labeled palmitic acid, oleic acid, or oleyl-

CoA (Amersham, U.K.). After incubation for 30 min at room temperature, the mixture (100 ml) was applied to a 1 ml Sephadex G50 (Pharmacia LKB Biotechnology Inc.) column in a glass pipette to separate the free from the bound fatty acid or oleyl-CoA molecules. Fractions (100 ml) were collected, monitored by scintillation counting, and analyzed for the presence of EP2 using SDS-PAGE. In a competition experiment the fractions containing ^{14}C-oleyl-CoA bound to purified EP2 were pooled and reapplied to a Sephadex G50 column directly or after incubation for 30 min in the presence of unlabeled oleyl-CoA in a ten-fold excess over the amount of ^{14}C-oleyl-CoA bound to EP2.

Acknowledgments

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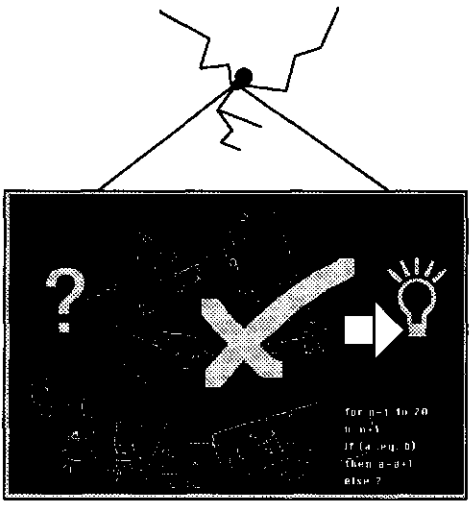
CHAPTER 5

General discussion



Based on the morphologically similar stages observed during zygotic and somatic embryo development, both zygotic and somatic embryogenesis appear to follow a common pathway. However, the respective starting points are obviously quite different: zygotic embryos develop after fertilization of the egg cell, whereas somatic embryos originate from embryogenic somatic cells. Other frequently observed differences are the lack of a suspensor and suppressed cotyledon development in somatic embryos and the absence of a dormancy period during somatic embryogenesis. Even if there are some morphological and physiological differences, somatic embryos are often used as a source of material to clone genes expressed during embryogenesis. As discussed in chapter 1, typical embryo-specific proteins like storage proteins and LEA proteins have been found in both zygotic and somatic embryos, which indicates that at least certain genes expressed during zygotic embryogenesis are also expressed during somatic embryogenesis.

Most genes expressed in somatic embryos are common house-keeping genes that are expressed throughout the plant, and are therefore not embryo-specific. This is illustrated by patterns obtained from two-dimensional gel electrophoresis of cellular proteins (Sung and Okimoto, 1981, 1983; Choi and Sung, 1984) or *in vitro* translated poly(A)$^+$ RNA (De Vries *et al.*, 1988b; Wilde *et al.*, 1988) isolated from somatic embryos, their precursor cells and a variety of somatic plant tissues, which show remarkably few embryo-specific or even embryo-enhanced proteins. Genes detected as being more highly expressed in embryos may mark the beginning of a developmental pathway, *e.g.* the start of tissue differentiation, or the onset of desiccation of the (zygotic) embryo. As described in this thesis the EP2 gene is an example of a gene that is expressed first at an early stage of embryo development, and the expression of the EP2 gene persists in particular tissues throughout the life cycle of the plant. EP2 gene expression was not detected in non-embryogenic cell suspension cultures, but appeared to be correlated with the presence of embryogenic potential. Expression of the EP2 gene was first found in proembryogenic masses, where it appears to mark the initial formation of protoderm cells, cells that are apparently absent in non-embryogenic cell clusters. Although expression of the EP2 gene is by no means restricted to the embryo, and messenger RNA is, in fact, found in epidermal cells of leaf primordia, flower organs and immature seeds, the characterization of the EP2 gene and its encoded protein has pointed out that formation of epidermal cells is a crucial event in the development of plant embryos.

Because different approaches have been used to identify genes involved in embryogenesis, in the first section of this chapter a number of these approaches and their respective advantages and drawbacks will be discussed. The second section will concern the use of secreted proteins as markers for embryogenic cells and embryos in tissue culture systems.

5.1. Strategies for identification of genes expressed in embryos

5.1.1. Differential screening and related strategies

Differential screening of cDNA libraries is frequently used to find genes that are preferentially expressed in a tissue of interest. Populations of messenger RNAs isolated from different tissues are compared, and tissue-specific mRNA sequences represented by cDNA clones can be selected. This technique has at least two limitations. Firstly, it is usually not sensitive enough to detect relatively low-abundant tissue-specific messenger RNAs. Secondly, the milligram amounts of cell material required to isolate sufficient mRNA for cDNA and probe production render this technique less suitable for isolation of cDNA clones from tissues that are only available in small amounts.

To overcome, at least in theory, these two limitations of differential screening, a novel strategy, referred to as differential display of messenger RNA, has been developed (Liang and Pardee, 1992). With this technique, DNA patterns characteristic for messenger RNA populations are generated on sequencing gels by polymerase chain reaction (PCR) amplification of complementary DNA populations in the presence of a pair of specially designed oligonucleotide primers, one binding to the poly(A)-tail of a subpopulation of the messenger RNAs, and the other binding further upstream and, due to its short size and random nucleotide sequence, to again a subpopulation of messenger RNAs. Patterns obtained for different tissues are compared whereby unique bands representing tissue-specific messenger RNAs are selected and characterized. Variation of one or both oligonucleotide primers gives rise to different patterns, thereby increasing the chance to detect tissue-specific messenger RNAs. Since this technique employs PCR, small amounts of tissues are sufficient. Furthermore, PCR seems much less biased towards more abundant templates, hence the suitability of this technique to possibly reveal tissue-specific low-abundance messengers. However, inherent to their low-abundance is the extreme difficulty to further characterize such messengers. It therefore seems that the main advantage of this technique is the small amount of cell material required.

5.1.2 Alternative screening procedures

In cases where proteins have been identified more direct approaches to isolate the corresponding genes or cDNA clones can be adopted. The two most common approaches are (1) screening of libraries with degenerate oligonucleotide probes of which the design is based on known (partial) amino acid sequences, and (2) expression screening of a cDNA library with an antisera raised against proteins of interest. The success of both types of screening depends largely on the specificity of the probes. In case of oligonucleotide probes the specificity is usually dictated by the level of degeneracy. Further complications can arise if the gene of interest is part of a gene family with highly homologous members. In case of antibody probes recognition of the original epitope(s) encoded by the gene of interest may be lost in

a different (usually *E. coli*) background. The screening described in chapter 2 of this thesis, aimed at the cloning of genes encoding secreted proteins, was in fact an adaptation of this kind of expression screening, as antisera raised against a mixture of extracellular proteins rather than a single protein were used. The finding that for two cDNA clones corresponding extracellular proteins, EP1 and EP2, could be identified, shows the potential of such an approach.

Other screening strategies are based on the properties of specific DNA sequences, *e.g.* the binding of proteins to those sequences. In this case, previously identified DNA binding proteins can be used to identify DNA motifs involved in gene regulation. A number of these proteins and DNA motifs that control storage and LEA protein gene regulation during embryogenesis have already been identified in this way (reviewed by Thomas, 1993).

5.1.3 Molecular genetic approaches

A powerful approach to study problems in developmental biology is based on the introduction of mutations in genes and the subsequent identification of a defective phenotype. In the somatic embryo system such studies have been carried out with temperature-sensitive mutant cell lines which undergo normal somatic embryogenesis at the permissive temperature, but are blocked when grown at increased temperature (Sung and Breton, 1982; Schnall *et al.*, 1988; LoSchiavo *et al.*, 1988; De Jong *et al.*, 1992). Because of the usually observed variation in cell cultures, and the accumulation of mutations after prolonged maintenance of cell lines in culture (Widholm, 1984) that may obscure the original mutation, the conditional nature of a temperature-sensitive mutation is a great advantage for the analysis of its effects. However, in a number of cases mutations have been unstable or mutant cell lines have been lost, which highlights the limitations of somatic embryogenesis as a genetic system to study early plant development.

In zygotic embryogenesis, the loss of mutations, even lethal ones, is prevented by maintaining these mutations in heterozygous plants, as long as the mutations are recessive and the plants can be propagated through selfing. In this way, the mutation only affects those zygotes, and/or the plants that subsequently develop from those zygotes, that are homozygous for the mutation. Mutagenization is usually done by treatment with chemical agents or irradiation, or by insertional inactivation of genes by transposons or T-DNA. The advantage of insertional inactivation is that the presence of the transposon or the T-DNA acts as a tag which facilitates the cloning and analysis of the inactivated gene.

A comprehensive study of embryo-lethal mutants in *Arabidopsis* (reviewed by Meinke, 1991) has revealed essentially three classes of mutations, ranging from mutants affected in house-keeping genes such as auxotrophs (Schneider *et al.*, 1989), through mutants that cause blocks occurring at defined times during embryo development, to those that alter the pattern of embryo development. Mutants of the last class may define genes which regulate the establishment of the form in the embryo. Other mutants of this type show aberrations in the establishment of polarity and symmetry in the *Arabidopsis* embryo (Jürgens *et al.*, 1991; Mayer *et al.*, 1991). These mutations appeared not lethal during embryogenesis and were screened for on the basis of aberrant seedling morphology.

Although the characterization of the isolated mutants is at an early stage and the affected genes have only been isolated in a few cases, the approaches used here to study early plant development may prove to be as powerful as they have proven to be in studies of animal development.

5.2. Analysis of genes expressed during embryogenesis: towards an understanding of somatic embryogenesis

In chapter 1 of this thesis a number of proteins and their encoding genes have been discussed that are expressed during the various stages of either zygotic or somatic embryogenesis. Based on the studies of these proteins and genes a number of general observations will be discussed in the next two paragraphs. The remaining paragraphs of this chapter will be dealing with studies concerning the role of secreted proteins during carrot somatic embryogenesis. In 5.2.3. the general views that have emerged from those studies will be discussed. More specifically and relevant to the study presented in this thesis, the function of secreted lipid transfer proteins in embryogenesis and their use as molecular markers to study somatic embryogenesis will be the topics of paragraphs 5.2.4 and 5.2.5.

5.2.1. Few genes and proteins identified in plant embryos are specific for embryogenesis

Only few genes identified in embryos exhibit an absolutely embryo-specific pattern of expression. Most specific are genes that encode storage proteins. Expression of these genes is abundant during relatively late stages, *i.e.* late heart to cotyledon stage, in embryo development and is in most cases restricted to the developing cotyledons. But, many of the *lea* class genes, although mainly expressed during late stages of embryo development and during seed maturation, can often also be expressed in other, non-embryo tissues in response to ABA, salt or desiccation, and are therefore not strictly embryo-specific. Genes that control essential steps in pattern formation in zygotic embryo development have not been isolated by conventional cloning methods and can most likely only be identified by molecular genetic approaches (cf. 5.1.3.; Jürgens *et al.*, 1991).

5.2.2. Gene expression programs during zygotic and somatic embryogenesis are similar but not identical

As mentioned in 5.2.1. few genes have been reported that are expressed in zygotic embryos, but not in other plant tissues. Expression of a number of those, *e.g.* the soybean storage protein genes encoding β-conglycinin and a Kunitz protease inhibitor (Perez-Grau and Goldberg, 1989), has also been observed in somatic embryos. In addition, genes with similar functions such as the *lea* genes have been

identified independently in both zygotic and somatic embryogenesis. Furthermore, *in situ* hybridization analysis of soybean storage protein genes (Perez-Grau and Goldberg, 1989) and the carrot EP2 gene (Chapter 3) has shown that spatial expression patterns of those genes are similar in both somatic and zygotic embryos. It is therefore likely that apart from the similarity in morphology, also the molecular events that underlie zygotic embryogenesis are reproduced to a substantial degree during somatic embryogenesis.

Surprisingly, several class 5 (cf. Chapter 1) *lea* genes, *e.g.* Dc3 (Wilde *et al.*, 1988), Dc8 (Borkird *et al.*, 1988; Franz *et al.*, 1989), ECP31 (Kiyosue *et al.*, 1992b) and ECP40 (Kiyosue *et al.*, 1992a) were found to be already expressed in proembryogenic masses and globular somatic embryos. Since these genes are normally expressed much later in zygotic embryogenesis, their earlier expression may point to a functional necessity of the LEA proteins during very early stages of somatic embryogenesis. The observed differences in temporal expression patterns clearly indicate that gene expression programs during zygotic and somatic embryogenesis are not identical. They have also led to the realization that the proembryogenic masses, rather than representing a special intermediate cell type in somatic embryogenesis, are most likely somatic embryos blocked before globular stage. If so, somatic embryo development may start already during so-called unorganized proliferation in the presence of 2,4-D. If this view is correct, the removal of 2,4-D and the dilution of the culture only serve to remove a block in embryo development rather than initiate somatic embryogenesis.

5.2.3. Functions of secreted proteins in carrot somatic embryogenesis

The observation that the presence of a small number of proteins secreted into carrot cell culture media was correlated with the presence of embryogenic cells and somatic embryos among non-embryogenic cells (De Vries *et al.*, 1988a; 1988b), formed the basis of several studies aimed at the identification of those proteins and their encoding genes. These studies were carried out employing one of two strategies. These strategies were: (i) cDNA cloning of mRNAs encoding extracellular proteins via expression screening of a somatic embryo cDNA library with antisera raised against a mixture of extracellular proteins. This resulted in the cDNA clones EP1 (Chapter 2; Van Engelen *et al.*, 1991), EP2, EP5 and EP6 (Chapter 2); (ii) identification of proteins able to restore arrested somatic embryogenesis in the carrot systems, in which either the glycosylation-inhibitor tunicamycin was used to inhibit somatic embryogenesis, which resulted in the identification of a cationic peroxidase (Cordewener *et al.*, 1991), or in which a temperature-sensitive variant carrot cell line was used, which led to the identification of an acidic endochitinase, EP3 (De Jong *et al.*, 1992).

From gene expression analysis of the cDNA clones found with expression screening it emerged that the EP1 gene was expressed in non-embryogenic cells only (Van Engelen *et al.*, 1991; Van Engelen, 1993) and the EP5 and EP6 genes in both non-embryogenic and embryogenic cells (Chapter 2). The level of expression of these genes was found to be independent of the culturing conditions. In contrast to EP1, EP5 and EP6 expression, EP2 expression was only observed in embryo-

genic suspension cultures, where it was found to be positively correlated with the embryogenic potential of those cultures (Chapter 3).

The cDNA derived protein sequences of EP1 (Van Engelen *et al.*, 1993), EP2 (Chapter 3) and EP5 (Chapter 2) show that these proteins contain a signal peptide and may therefore be secreted proteins. The absence of repetitive amino acid sequence motives commonly found in structural cell wall proteins, suggests that EP1, EP2, EP5 and possibly also EP6 are non-structural proteins present in the extracellular matrix.

Based on sequence homology with *Brassica* S-locus glycoproteins and the observed expression of the EP1 gene in epidermal cells that do not express EP2, with exception of those in the peripheral zone of the shoot apical meristem and the outer integument, Van Engelen *et al.* (1993) have speculated that the EP1 protein might be involved in the limitation of water flow through the outer epidermal cell wall, by either directly modifying the cell wall structure, or indirectly in analogy with the proposed mode of S-locus glycoprotein action, by instructing the protoplast of epidermal cells to restrict the water transport across the outer cell wall.

From sequence homology analysis of the EP2 cDNA and the deduced protein sequence the EP2 protein could be identified as a non-specific lipid transfer protein. Based on the expression pattern of the gene in protoderm cells in suspension cultures and in epidermal cells of the plant known to deposit cuticles, and the affinity of the purified protein for precursor molecules for cutin, a role was proposed in the transport of known cutin monomers through the extracellular matrix to sites of cutin synthesis (Chapters 3 and 4). Although expression of EP2 is not specific for embryogenesis, the EP2 mRNA has been shown to appear very early, during both somatic and zygotic embryogenesis, in proembryogenic masses and in 60-celled globular embryos, respectively. The initial expression of EP2 reflects the start of differentiation of (notably dermal) cells in early embryo development. EP2 could therefore serve as an extremely useful marker for both zygotic and somatic embryogenesis (see also 5.2.5.).

EP5 and EP6 gene expression in suspension cultures was the same in non-embryogenic and embryogenic cultures. In contrast, the expression of these genes appeared to be regulated in the plant, where it was enhanced in the root apex and the immature inflorescence. The DNA and deduced protein sequences revealed no significant homology with other genes and proteins. The functions of the EP5 and EP6 proteins remain therefore unresolved.

The acidic endochitinase EP3 and the cationic peroxidase were first identified on the basis of their ability to restore arrested somatic embryogenesis. The addition of the EP3 protein to the temperature-sensitive cell line ts11 resulted in the appearance of a correctly formed protoderm in globular ts11 embryos grown at the restricted temperature and recovery of EP2 gene expression in the protoderm (De Jong *et al.*, 1994). Interestingly, EP3 is present and fully functional in ts11 cultures at the restricted temperature, but apparently at a suboptimal concentration. Rescue of arrested embryogenesis therefore occurs after addition of EP3 to an optimal concentration (De Jong, 1993). Although the substrate for this chitinase is not known, the observation that a *Rhizobium* nodulation (Nod) factor can mimic ts11 rescue by EP3 may imply that EP3 might generate plant analogs of Nod factors that initiate meristematic cell divisions (De Jong *et al.*, 1993). In a different system, a cationic peroxidase rescued tunicamycin-arrested embryogenic cell clusters. Addition of tunicamycin results in expansion of peripheral cells of the

proembryogenic masses, which effectively stops embryo formation from these cells. The main effect of the addition of the cationic peroxidase appeared to be the prevention of expansion of small peripheral cells of the proembryogenic masses (Cordewener *et al.*, 1991). Considering the fact that a peroxidase enzyme activity could counteract this expansion, and the well-documented ability of wall peroxidases to cross-link a variety of structural components of primary and secondary cell walls, the restriction of cell expansion is considered to be an essential prerequisite in the acquisition and maintenance of embryogenic potential (Fry, 1990; Van Engelen and De Vries, 1992).

Taken together, the view emerges from these complementation studies that the formation of an epidermis and the restriction of cell expansion are crucial events in early embryogenesis whereby extracellular proteins play an important role. The role extracellular lipid transfer proteins like EP2 may play in this context will be discussed in the next paragraph.

5.2.4. Functions of non-specific lipid transfer proteins

In chapter 3 it was postulated that the secreted non-specific lipid transfer protein (nsLTP) EP2 may be involved in cutin synthesis by transporting cutin monomers through the cell wall. This hypothesis was based on the coincidence of the EP2 expression with known sites of cutin synthesis in plants. In addition, a thin lipophilic layer resembling a cuticle was observed after histochemical staining on proembryogenic masses that express the EP2 gene. More evidence for the deposition of cutin on the embryo protoderm was recently found in fluorescence microscopy studies of *Capsella, Arabidopsis* and *Stellaria* zygotic embryos (Rodkiewicz *et al.*, 1994). In chapter 4, it was shown that the purified EP2 protein was capable of binding several fatty acids known to be precursors of cutin monomers, which supports the proposed role of EP2 to bind and transport cutin monomers. If EP2 is the sole carrier of cutin monomers, it can be calculated that the amount of EP2 needed to form a cuticle around a globular embryo probably exceeds by far the amount of EP2 produced (Hendriks *et al.*, 1994). Therefore either the EP2 protein is rapidly recycled or it is not a general carrier for all cutin monomers. A model for the putative function of plant nsLTPs has been proposed, whereby plant nsLTP molecules are secreted and function as a shuttle for cutin monomers from their site of synthesis through the cell wall of protoderm or epidermis cells to sites of cutin synthesis. In this model the nsLTP molecules are either taken up by receptor-mediated endocytosis and loaded intracellularly by fusion of an endocytic vesicle with cutin monomer-containing vesicles derived from Golgi or endoplasmic reticulum, or nsLTP molecules remain extracellular and are loaded by a membrane-bound cutin monomer carrier. It must be stressed that this model is highly speculative as no evidence to support this model has yet been found (Hendriks *et al.*, 1994).

According to Thoma *et al.* (1994) at least two genes are present in *Arabidopsis* that encode nsLTPs. Examination of the expression pattern of one of these genes, LTP1, by means of β-glucuronidase (GUS) activity analysis in transgenic plants containing LTP1 promoter-GUS fusions, and by *in situ* RNA localization showed that the expression pattern of the LTP1 gene in *Arabidopsis* zygotic embryos and plants is very similar to that of EP2 in carrot, *i.e.*

predominantly in the protoderm and epidermal cells (Thoma *et al.*, 1994). *Arabidopsis* LTP proteins were shown to be present in cell walls of epidermal cells of leaves and stem in *Arabidopsis* (Thoma *et al.*, 1993). Although EP2 was not found in the stem and leaves of carrot, other nsLTPs have been isolated from leaves of spinach (Kader *et al.*, 1984) and *Avena* seedlings (Rickers *et al.*, 1984). The fact that nsLTPs are present in the cell wall of epidermal cells of various plant species suggests that nsLTPs have similar functions in different plants.

If nsLTPs are indeed involved in the formation of a cuticle, why would this function be that important in early embryogenesis? In chapter 3 it was suggested that a cuticular layer may help protodermal cells to maintain their small size by restricting the water flow through the cell wall. In addition, an alternative function may be the protection against hydrolytic enzymes that surround zygotic embryos but also somatic embryos in culture.

Several recent studies report the isolation of genes encoding nsLTPs which are induced by cold temperature treatment, drought stress or ABA application in barley shoot meristems (Hughes *et al.*, 1992) and NaCl, mannitol or ABA treatment in tomato stems (Torres-Schumann, 1992). Antifungal activity was observed *in vitro* for a seed protein homologous to nsLTPs from radish (Terras *et al.*, 1992) as well as *in vitro* growth inhibition of bacterial and fungal pathogens by nsLTPs from barley and maize leaves (Molina *et al.*, 1993). However, the latter studies do not show whether the plants respond to fungal or bacterial pathogens by (enhanced) expression of nsLTPs, hence the significance of these observations remains uncertain until *in vivo* studies have been carried out. How nsLTPs by themselves can exhibit antifungal or antibacterial activity remains unclear. Perhaps, a protection or defence mechanism induced under these conditions, albeit highly speculative at this moment, indeed exists whereby, in analogy to the proposed function of EP2, the plant responds by thickening the cuticle in the affected areas, thereby protecting those areas from transpiration or infection by pathogens.

In addition to protection of underlying tissues, the presence of a cuticle might help protoderm and epidermis cells to establish themselves as dermal cells. If cells communicate with each other through signal molecules present in the extracellular matrix, the presence of a cuticle would effectively block the diffusion or movement of these recognition signals to neighboring cells. In this way, epidermal cells are able to sense not only their position amidst other cells, but also their polarity, as only part of their cell walls is covered with a cuticle. Because the embryo is embedded in the endosperm, it is of crucial importance for the embryo to sense its own boundaries. In this context, if expression of EP2 reflects the presence of a protoderm, the expression of EP2 in rescued ts11 embryos approximately two layers inward from the outer cell layer could indicate that the protoderm is not always the outermost cell layer (cf. Chapter 3; De Jong *et al.*, 1993). It would be interesting to determine whether a cuticle-like structure is present around the cells that express EP2, *e.g.* by means of a histochemical staining, which would suggest that the embryo probably ignores the presence of the cells beyond the protoderm.

5.2.5. The lipid transfer proteins as markers for embryogenesis

If the application of genes expressed in plant embryos as markers to assess the developmental stage of somatic embryos is evaluated, it may be appropriate as well to raise the question whether such markers have any practical use. At first glance the answer to this question should be negative, since once somatic embryos have formed, they are easily recognized by their characteristic shapes, and thus, molecular markers are not necessary for their identification. What would be helpful is if molecular markers would be available that are related to embryogenic potential and could be used to predict embryo formation, before somatic embryos are actually present. Especially when embryogenic cells are initially present in low numbers and have to be selected for in order to obtain reasonable regeneration frequencies, such markers could be very useful. Besides, selection for cell lines with high regenerative potential is still mainly performed by visual inspection of calli and suspension cultures and would greatly benefit from the availability of independent molecular markers to judge the effect of basic tissue culture variables as well as selection strategies. As far as that is concerned, based on the number of embryo genes so far described, it is fortunate that many of these genes are also expressed in their immediate precursor cells present in embryogenic suspension cultures.

In order for a gene to qualify as a good independent marker for embryogenic potential, a number of criteria should be fulfilled: (i) its expression should be sufficiently high to be easily detectable, (ii) it should not exhibit any species specificity, (iii) detection of its expression or the corresponding protein should not require elaborate and time-consuming analysis and (iv) require as little cell material as possible. It is important to note that a good marker does not necessarily have to be embryo-specific in strict sense; genes that are also expressed in the adult plant, such as the class 1 genes, can be very useful as long as their expression predicts the entry into the embryo developmental pathway.

Based on the expression pattern during embryogenesis, a number of the genes and corresponding proteins discussed in chapter 1 of this thesis would make good candidates for use as embryogenic potential markers. Genes like Dc3, Dc8, ECP31 and ECP40 are examples, as they are already expressed in proembryogenic masses (see Chapter 1). If expression is monitored by reverse transcription PCR analysis, very low amounts of starting material are required, though this method might be too laborious, if large numbers of samples are to be monitored. Therefore, an approach that would use immunological techniques would be more favorable. However, the sensitivity of such techniques may be a limiting factor and require too much cell material for analysis.

In the conditioned medium of embryogenic cell suspension cultures some proteins are present that reflect the embryogenic potential of the cells that secrete it. A good example is the carrot lipid transfer protein EP2 presented in this thesis, which is secreted by embryogenic carrot cells only. Once cells have developed into embryos in embryo cultures, the EP2 gene is continuously expressed in the protoderm cells. The observed expression allows for assessment of the embryogenic potential of a culture in two ways: by monitoring the level of expression of the EP2 gene or by quantifying the amount of EP2 protein secreted. As media of embryogenic carrot suspension cultures contain reasonable amounts of the protein, EP2 appears to be a marker that fulfils most, if not all, of the

criteria listed above: the presence of the lipid transfer protein can be determined immunologically with high sensitivity, these proteins are ubiquitous in all plant species studied to date, and immunodetection procedures are very fast, so analysis of a large number of samples can be done within hours. Perhaps the most interesting aspect is that samples of the conditioned medium can be used, thereby minimizing the loss of valuable cell material. In recalcitrant species where the amount of lipid transfer protein may be too low to detect by immunological means, PCR based techniques can be used to at least determine the presence of embryogenic cells and if so, to try and promote their development.

In conclusion, the answer to the question whether molecular markers for somatic embryos are useful, should be affirmative, not only because such markers can be used to measure embryogenic potential as well as mark progression in somatic embryo development, but also because they may eventually aid to achieve more efficient somatic embryogenesis in recalcitrant species. The non-specific lipid transfer protein EP2 not only marks embryogenic precursor cells that are able to undergo somatic embryogenesis, but the occurrence of protoderm cell differentiation as well. Currently nsLTP gene probes are used to study the effects of mutations that affect embryo pattern development in *Arabidopsis* (C. Vroemen, S. Langeveld and S.C. de Vries, personal communication). The availability of an nsLTP marker gene may become apparent in those cases where zygotic embryo mutations cause the absence or disturbance of the protoderm and at the same time aberrant expression patterns of the nsLTP gene. These studies can be extended when more marker genes reflecting other cell functions become available, and will contribute to a better understanding of the molecular mechanisms that underlie the formation of the protoderm in plant embryogenesis.

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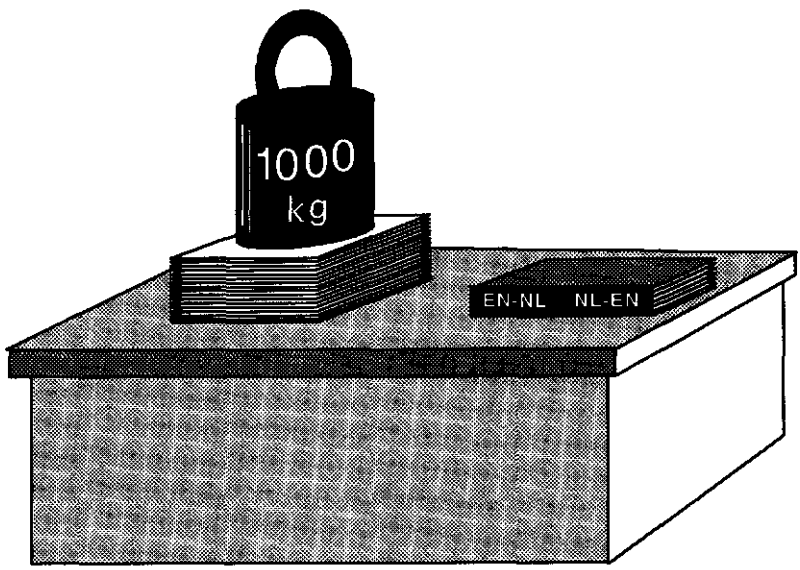
Samenvatting



De geslachtelijke voortplanting van hogere planten vindt plaats in de bloem. Bestuiving van de bloem met stuifmeel, de mannelijke gameten, leidt tot bevruchting van de eicel, die zich in de embryozak in de bloem bevindt. De ontstane zygote deelt zich vele malen tot een embryo en tegelijkertijd ontstaat rondom het embryo het zogenaamde endosperm dat als reservevoedsel dient tijdens de latere ontwikkeling van het embryo en het kiemplantje. De verschillende ontwikkelingsstadia van het embryo worden aangegeven met de vorm van het embryo; vanuit de zygote ontwikkelt zich allereerst een *globulair* embryo, dat uitgroeit tot een *hartvormig* en tenslotte een *torpedovormig* embryo. Het weefsel dat de embryozak omgeeft heeft zich intussen tot een zaadhuid ontwikkeld, die het embryo en het endosperm moet gaan beschermen tijdens de kiemrust, de periode waarin het zaad sterk ontwaterd is en het embryo zich niet verder ontwikkelt (vandaar kiemrust). Het zaad kan nu verspreid worden en ontkiemen zodra de condities gunstig zijn. De hier beschreven ontwikkeling van een plante-embryo, die gepaard gaat met de vorming van een zaad, wordt zygotische embryogenese genoemd.

Naast zygotische embryogenese is bij veel planten ook somatische embryogenese mogelijk, waarbij embryo's via kunstmatige, niet-geslachtelijke weg uit somatische cellen ontstaan. Allereerst wordt fijn gesneden plantmateriaal gekweekt op vast, of in vloeibaar, medium dat auxine bevat. Auxine is een groeiregulator die een sterke deling van cellen teweeg brengt, hetgeen resulteert in het ontstaan van gedifferentiëerd weefsel, callus genaamd. Bij verder kweken van dit callus in vloeibaar medium ontstaat door loslaten van cellen en celklompjes een celsuspensie. Na verloop van tijd ontwikkelt een aantal cellen zich tot proembryogene massa's, bestaande uit kleine, niet of nauwelijks gevacuoliseerde, cytoplasmarijke cellen. Deze proembryogene massa's kunnen zich na verdunning in auxine-vrij voedingsmedium verder ontwikkelen tot somatische embryo's volgens dezelfde karakteristieke ontwikkelingsstadia—de globulaire, hartvormige en torpedostadia—als bij zygotische embryo's. Omdat somatische embryogenese vooral bij de peen (*Daucus carota* L.) zeer efficiënt is, wordt somatische embryogenese bij de peen vaak als modelsysteem voor de bestudering van de vroege ontwikkeling van planten gebruikt.

Het hier beschreven onderzoek is gebaseerd op waarnemingen die suggereren dat in het voedingsmedium uitgescheiden, veelal geglycosyleerde eiwitten een belangrijke rol spelen bij de ontwikkeling van somatische embryo's. Zo wordt een klein aantal karakteristieke extracellulaire (glyco)proteïnen aangetroffen in voedingsmedia waarin zich somatische embryo's ontwikkelen. In dit onderzoek is getracht genen te kloneren en te identificeren die koderen voor deze extracellulaire eiwitten.

In hoofdstuk 1 wordt een overzicht gegeven van in de literatuur beschreven genen en eiwitten die tot expressie komen tijdens zygotische en somatische embryogenese. Aan de hand van hun expressiepatronen is getracht deze genen en

eiwitten op een tijdschaal te plaatsen en op basis daarvan in te delen in groepen, om vervolgens tot een beter moleculair beeld te komen omtrent de ontwikkelingen tijdens embryogenese. Zoals reeds genoemd vertonen de ontwikkeling van zygotische en somatische embryo's morfologisch grote overeenkomst. Het hier gepresenteerde overzicht en de indeling van een aantal genen geeft aan dat dit ook op moleculair niveau het geval is, maar dat er daarnaast verschillen zijn; zo blijken bijvoorbeeld met name *lea*-genen tijdens somatische embryogenese vroeger tot expressie te komen. Dit voorbeeld onderstreept het belang van nauwkeurige vergelijking tussen somatische en zygotische systemen, hetgeen tijdens dit onderzoek dan ook zoveel mogelijk getracht is.

Hoofdstuk 2 beschrijft de isolatie van vier cDNA klonen door het screenen van een expressiebank, gemaakt van boodschapper RNA geïsoleerd uit somatische embryo's, met antisera die opgewekt zijn tegen eiwitten uitgescheiden in media van embryogene suspensies. Na bestudering van de expressie van de overeenkomstige genen koderend voor respectievelijk de extracellulair proteïnen (EP)1, EP2, EP5 en EP6, kon voor een van de genen, het gen voor EP2, een positieve correlatie tussen het embryogeen vermogen van celsuspensies en de expressie van het gen worden vastgesteld. Tevens werd gevonden dat de expressie van dit gen gedurende de ontwikkeling van de plant sterk gereguleerd werd: expressie kon slechts worden aangetoond in de scheutapex, de bloeiwijze en in onrijp zaad en niet in andere delen van de plant. Op basis van deze resultaten werd EP2 uitgekozen voor verdere bestudering.

In hoofdstuk 3 wordt beschreven hoe de expressie van het EP2-gen in meer detail is bestudeerd. Door middel van *in situ* hybridisatie werd aangetoond dat EP2 tijdens somatische embryogenese voor het eerst tot expressie komt in proembryogene massa's. Ook tijdens de verdere ontwikkeling van het embryo tot en met het torpedostadium komt EP2 tot expressie en wel specifiek in het protoderm.

Expressie van het EP2-gen begint zeer vroeg tijdens zygotische embryogenese en kan al worden aangetoond in een 60-cellig zygotisch embryo, en, net als bij het somatische embryo, vindt de expressie van het EP2-gen tijdens de ontwikkeling van het zygotische embryo plaats in het protoderm. EP2 genexpressie werd verder gevonden in, met name, de tunica en de laterale zone van het apicaal scheutmeristeem, in epidermale cellen van de bladprimordia en bloemorganen en de integumenten.

Samenvattend kan gesteld worden dat EP2 tot expressie lijkt te komen in epidermale cellen, en, zoals uit nadere bestudering is gebleken, met name daar waar een cuticula wordt aangelegd.

Verder onderzoek bracht naar voren dat de sequenties van het EP2 cDNA en het daardoor gekodeerde eiwit sterke homologie bleken te vertonen met niet-specifieke lipide transport eiwitten (ns-LTP's), die in staat zijn vetzuren en fosfolipiden te binden. De gekodeerde eiwitsequentie bleek een signaalpeptide te bevatten, hetgeen aangeeft dat EP2 gesecreteerd zou kunnen worden. Met een antiserum dat EP2 herkent werd aangetoond dit inderdaad het geval is. De vaststelling dat een ns-LTP-gen tot expressie komt daar waar een cuticula voorkomt suggereert dat EP2 betrokken zou kunnen zijn bij het transport van apolaire cutinemonomeren door de hydrofiele celwand naar de buitenkant van epidermale cellen waarop een cuticula wordt afgezet. Het is interessant dat EP2

erg vroeg tijdens de ontwikkeling van de plant tot expressie komt. Als het EP2-eiwit inderdaad de hier voorgestelde functie heeft, dan zou een cuticulaire laag rondom plante-embryo's aanwezig moeten zijn. Met behulp van een histochemische kleuring werden daar inderdaad aanwijzingen voor gevonden. Analoog aan de functie van een cuticula in de volwassen plant, zou de embryocuticula enerzijds kunnen dienen voor regulatie van watertransport en vandaar het voorkomen van ongewenste celexpansie, anderzijds zou deze cuticula bescherming kunnen bieden aan het embryo, dat zich immers bevindt in een omgeving met hydrolytische enzymen die het endosperm hydrolyseren, maar het embryo uiteraard intakt moeten laten.

In hoofdstuk 4 wordt beschreven hoe het EP2 eiwit uit media van somatische embryoculturen werd gezuiverd. Tevens werd vastgesteld dat het eiwit in staat was de cutinemonomeren palmitinezuur, oleïnezuur en oleyl-CoA te binden. Deze resultaten ondersteunen de hier gepostuleerde functie van het EP2-eiwit.

In hoofdstuk 5 wordt een overzicht gegeven van een aantal methoden die hier en door anderen gebruikt zijn voor het isoleren van embryo-genen. Tot op heden hebben deze methoden niet geresulteerd in genen die embryogenese van begin af aan reguleren. Toch is tijdens dit onderzoek naar voren gekomen dat een ogenschijnlijk alledaags eiwit als EP2 buitengewoon interessant en belangrijk kan zijn bij de bestudering van de vroege ontwikkeling van planten, niet alleen vanwege zijn hypothetische biologische functie, maar vooral vanwege de correlatie met embryogeen vermogen en embryo-ontwikkeling en zijn algemeen voorkomen in de plantenwereld. Expressie van het EP2-gen is tot nu toe een van de vroegste kenmerken van embryogenese en zou, bijvoorbeeld, gebruikt kunnen worden voor de bepaling van het embryogene vermogen van celculturen van meer recalcitrante plantesoorten.

Voor de bestudering van de moleculaire basis van embryogenese is voor de toekomst de hoop gevestigd op de relatief recente moleculair genetische benaderingen, vooral bij *Arabidopsis,* die zullen kunnen leiden tot het isoleren van genen die de ontwikkeling van het embryo reguleren. De bestudering van embryomutanten die afwijkingen vertonen tijdens verschillende stadia van de embryo-ontwikkeling kan zeer gebaat zijn met de beschikbaarheid van moleculaire merkers. Als zodanig kan EP2 ook hier van nut zijn, omdat expressie van dit gen niet alleen aangeeft dat embryogenese wel of niet plaatsvindt, maar ook of een correcte aanleg van het protoderm en dus correcte celdifferentiatie wel of niet optreedt.

Nawoord

Hoewel volgens het promotiereglement van de Landbouwuniversiteit slechts één naam op de titelpagina van dit proefschrift mag prijken, betekent dat niet dat slechts één persoon al het werk voor dit proefschrift heeft geleverd. In tegendeel, dit proefschrift is tot stand gekomen door de inzet en hulp van velen en ik zou dan ook graag een ieder die een bijdrage heeft geleverd hier van harte willen bedanken. Een aantal mensen wil ik met name noemen:

Sacco de Vries, mijn co-promotor. Jouw gedrevenheid, inzicht en ideeën hebben er in belangrijke mate toe bijgedragen dat dit proefschrift vorm heeft gekregen en is geworden tot een verhaal met een duidelijk begin, ontwikkeling en een conclusie. Jouw nadruk op het gedegen presenteren van onderzoeksresultaten, leidde er vaak toe dat manuscripten zwart van de correcties en op- en aanmerkingen terugkwamen, maar ik heb er veel van geleerd en zal daar zeker nog veel vruchten van plukken.

Ab van Kammen, mijn promotor. Van groot belang is jouw objectieve en kritische kijk geweest."Ik weet wel wat je bedoelt, maar het staat er niet," zei je daar waar ik dacht dat er geen speld meer tussen te krijgen was. En je had gelijk. Na het laatste hoofdstuk was ik gelukkig ook redelijk in staat je handschrift te ontcijferen. Ik moet het hier toch even kwijt: ik wist uiteindelijk wel wat je bedoelde, maar het stond er beslist niet.

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Curriculum vitae

Peter Sterk werd op 25 juli 1963 te Ridderkerk geboren. In 1981 slaagde hij voor het eindexamen VWO aan de Nassau Scholengemeenschap te Breda. In hetzelfde jaar begon hij met de studierichting Moleculaire Wetenschappen (biologische oriëntatie) aan de Landbouwuniversiteit te Wageningen. Hij behaalde het kandidaatsdiploma in 1985. De doctoraalfase omvatte de hoofdvakken Virologie (Prof. Dr. Ir. J.P.H. van der Want) en Moleculaire Biologie (Prof. Dr. A. van Kammen). Zijn stageperiode bracht hij door in de Verenigde Staten op het laboratorium van Dr. T.L. Thomas, Department of Biology, Texas A&M University, College Station, Texas. In november 1987 werd het ingenieurs-diploma behaald. In de periode van drieënhalf jaar die volgde, werkte hij op de vakgroep Moleculaire Biologie van de Landbouwuniversiteit te Wageningen in de groep van Dr. S.C. de Vries aan het onderzoek dat in dit proefschrift beschreven staat en dat mede gefinancierd werd door de Europese Gemeenschap (Grant BAP-0093-NL). Van januari 1992 tot en met augustus 1994 was hij als post-doctoraal medewerker werkzaam in de groep van Dr. P.J. Hussey, Department of Biology, Royal Holloway, University of London te Egham, Surrey, Engeland. Sinds 1 september 1994 is hij als medewerker verbonden aan het European Bioinformatics Institute (EBI), onderdeel van het European Molecular Biology Laboratory (EMBL), te Hinxton, Cambridge, Engeland.