MOLECULAR CHARACTERIZATION OF GLYCOPROTEINS SECRETED BY CARROT SUSPENSION CELLS

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Fred van Engelen

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Stellingen

1. Onderzoekers die verwijzen naar het werk van Steward als een van de eerste waarnemingen van somatische embryogenese, hanteren een onjuiste definitie van embryogenese.

   Steward FC et al. (1958) Am. J. Bot. 45:705-708
   Dudits D et al. (1991) J. Cell Sci. 99:475-484
   Sung ZR et al. (1984) Plant Mol. Biol. Reporter 2:3-14
   Komamine A et al. (1990) In: Progress in Plant Cellular and Molecular Biology
   (Kluwer), pp. 504-513

2. In tegenstelling tot hetgeen zij beweren, laten Cooper en Varner niet zien dat isodityrosine gevormd wordt in de celwand tijdens het onoplosbaar worden van hydroxyproline-rijke eiwitten.

   Cooper JB & Varner JE (1983) Biochem. Biophys. Res. Comm. 112:161-167

3. Het aanduiden van verschillende peroxidases als "iso-enzymen" op basis van de omzetting van hetzelfde niet-natuurlijke substraat is voorbarig en misleidend.

   The Dictionary of Cell Biology (Academic Press) 1989, p. 121
   Lamport DTA & Alizadeh H (1989) 5th Cell wall Meeting, Edinburgh, abstract 15
   Cordewener J et al. (1991) Planta 184:478-486

4. Het waargenomen verschil tussen haver en soja in het effect van calciumchelatie op de extensibiliteit van de celwand moet niet gezien worden als tegenstrijdigheid, maar als aanwijzing voor een fundamenteel verschil tussen gramineeën en dicotylen in de regulatie van extensibiliteit.

   Virk SS & Cleland RE (1988) Planta 176:60-67
   Rayle DL (1989) Planta 178:92-95
   Roberts K (1989) Curr. Opin. Cell Biol. 1:1020-1027

5. De conclusies van Moore en medewerkers met betrekking tot de localisatie van rhamnogalacturonan I worden ondergraven door de sterke kruisreactie van het gebruikte antiserum met polygalacturonzuur.

   Moore PJ et al. (1986) Plant Physiol. 82:787-794
   Moore PJ & Staehelin LA (1988) Planta 174:433-445

6. In hun beschrijving van een 'single-chain' antilichaam tegen fytochroom gaan Owen et al. eraan voorbij dat zij cDNA hebben geïsoleerd van een geheel nieuw type lichte keten.

Owen et al. (1992) Bio/technology 10:790-794

7. Voor een efficiënte wetenschapsbeoefening is het afleren van 'wishful thinking' minstens zo belangrijk als het aanleren van feitenkennis.

Benveniste J (1988) Nature 334: 291
Maddox J et al. (1988) Nature 334:287-290
Buck et al. (1990) Science 248: 208
Fleischmann M et al. (1989) J. Electroanalyt. Chem. 263:187-188

8. Politieke partijen die beweren zowel economische groei als bescherming van het leefmilieu na te streven, maken zich schuldig aan kiezersbedrog.

9. Gezien de dramatische uitwerking van AIDS op de samenleving, dienen condooms gratis verkrijgbaar te zijn dan wel volledig door ziektekostenverzekeraars vergoed te worden.

10. Een beter milieu begint niet bij jezelf.

11. De huidige regelgeving ten aanzien van genetische modificatie van planten staat in een dusdanige verhouding tot de geschatte risico's voor de mens, dat de overheid, indien zij consequent is, onder andere rookartikelen en gemotoriseerd verkeer zou moeten verbieden.

12. Deze stelling is onjuist.

Stellingen behorende bij het proefschrift
"Molecular characterization of glycoproteins secreted by carrot suspension cells"
door Fred van Engelen, te verdedigen op 20 januari 1993

Contents

Scope

Investigations into the molecular mechanisms that underly plant embryogenesis suffer from the limited accessibility of the developing zygotic embryo, which is enveloped by the ovary in the flower and, later on, by the developing seed. The formation of embryos from cultured somatic cells, referred to as somatic embryogenesis, provides an alternative means to study embryo development. In carrot cell suspension cultures, somatic embryogenesis is highly efficient and embryos at different developmental stages can be easily obtained in high quantities. For this reason, carrot cell suspension cultures are often used as an alternative to investigate plant embryogenesis.

The growth medium of plant cell cultures can be regarded as a large extension of the intercellular space; soluble secreted molecules that inhabit the apoplast *in planta* will accumulate in the medium of suspension-cultured cells. Cultured carrot cells secrete many proteins, most of which are glycosylated, into the growth medium. A correlation was found between somatic embryogenesis and the presence or absence of some of these secreted proteins. Evidence was obtained that one or more secreted glycoproteins are actually essential for somatic embryo formation.

Apart from embryogenic cells, carrot cell suspensions contain non-embryogenic cells that contribute to the spectrum of proteins in the culture medium. These proteins may interfere with somatic embryo development or with studies that further analyse the role of extracellular proteins in somatic embryogenesis.

The aim of the study presented in this thesis was to increase our knowledge of the nature and action of glycoproteins secreted by carrot suspension cells and of the cell type-specific expression of the encoding genes both in suspension cultures and *in planta*.

In chapter 1 a brief introduction in zygotic and somatic embryogenesisis is presented, and the current evidence for the involvement of secreted glycoproteins in carrot somatic embryogenesis is summarized. Chapters 2, 3 and 4 describe the molecular characterization of the carrot secreted glycoproteins EP1 and EP4. Antisera specific for EP1 and EP4 and cDNA clones encoding these proteins were employed to study their occurrence in cell suspensions and in seedlings. The results show that the morphological cell-type heterogeneity in carrot cell suspension cultures is parallelled by differences in secreted proteins and suggest that this heterogeneity reflects the different cell types of the explant. Both the EP1 and EP4 proteins were purified from the culture medium and tested for their effect on somatic embryo formation. These experiments revealed neither a promoting nor an inhibiting role for EP1 or EP4 in somatic embryogenesis. In chapter 5 the current knowledge on the structure and function of the plant primary cell wall is reviewed. In this context, the involvement of secreted glycoproteins in somatic embryogenesis and the possible functions of EP1 and EP4 are discussed.

Chapter 1

General introduction

Zygotic embryogenesis

The complex process of sexual reproduction in plants commences with the transition of an indeterminate shoot apical meristem into a determinate flower meristem that produces the reproductive cells (8). After fertilization of the egg cell in the embryo sac, the first asymmetric division of the zygote yields a basal cell that will produce the so-called suspensor and an apical cell that develops into the embryo (figure 1.1a,b). After the eight-celled stage, anticlinal divisions of the surface cells mark the formation of the embryo protoderm (figure 1.1c). At the heart stage (figure 1.1d) the vascular initials, that will connect the root and shoot apices, are formed and cotyledon development starts adjacent to the future shoot apical meristem. The torpedo stage is characterized by cell expansion in addition to cell division and results in elongation of the embryo along the root-shoot axis. During the cotyledon stage the cotyledons expand and are filled with storage material. Simultaneous with the fertilization of the egg cell, the nucleus of a second sperm cell fuses with the diploid central cell nucleus. Proliferation of this cell results in the triploid endosperm that surrounds the developing embryo and enlarges the embryo sac (figure 1.1b-d). The endosperm is then either degraded and totally absorbed by the developing embryo cotyledons, or persists during seed maturation. Finally, the complete seed is dehydrated and after dispersal and germination will generate a seedling (24,38).
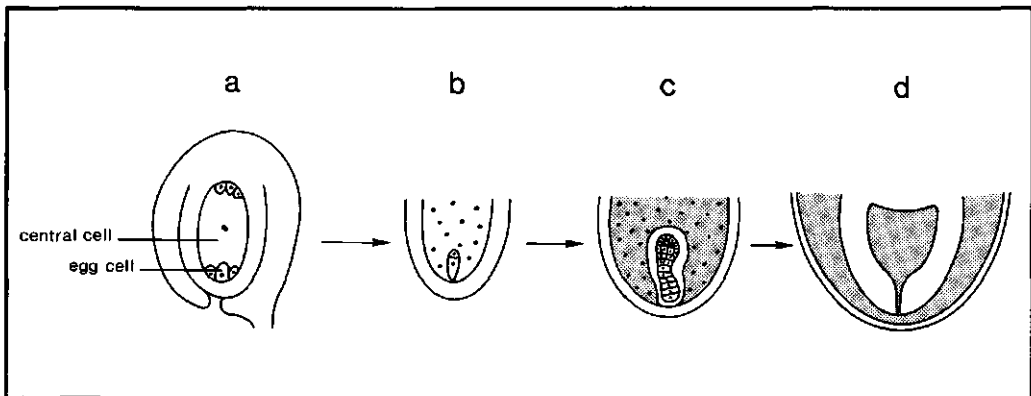
Figure 1.1. Angiosperm zygotic embryo development (24,38). The embryo sac is enveloped by the nucellus and integuments of the ovule. It consists of seven cells: the egg cell, two synergids adjacent to it, three opposite antipodal cells and a large diploid central cell (a). Both the egg cell and the central cell are fertilized. The central cell undergoes a series of nuclear divisions to produce the endosperm storage tissue; the zygote divides asymmetrically (b). The larger basal cell divides several times and forms the suspensor and the smaller apical cell develops into the embryo proper (c, the heart stage is shown in d). Simultaneously, the endosperm matures by septation and synthesis of storage compounds. In carrot, the endosperm region surrounding the embryo appears less solid, suggesting lysis of endosperm cells in this area (c,d).

13

Somatic embryogenesis

The complexity of plant sexual reproduction suggests that embryogenesis is the prerogative of a unique, specialized cell, the egg cell, which only expresses its full developmental potential after the trigger of fertilization by a second specialized cell, the sperm cell, in a carefully controlled micro-environment inside the ovule. This is not true, however, because there are several examples of embryo development from somatic cells of differentiated organs. Formation of embryos has been observed on the epidermis of *in vitro* grown *Ranunculus sceleratus* plantlets (27) and on the tips of mature *Malaxis paludosa* leaves (43). The most widespread example of non-sexual embryogenesis was first described by Reinert in 1959 (35). He showed that carrot cells growing in a cell suspension culture can generate viable embryos after only a few simple culture manipulations (35). These "somatic" embryos are able to develop into fertile plants. Since its intial discovery, somatic embryogenesis has been demonstrated in a still-growing list of plant species and with a variety of differentiated organs as the source of cells (1,46). Carrot and alfalfa have proven to be particularly amenable to experimentation (6,9,26,33), while other species, like *Arabidopsis*, have remained refractory to attempts to achieve high-frequency somatic embryogenesis.

Like their zygotic counterparts, carrot somatic embryos have a unicellular origin (2,33). In figure 1.2 a schematic representation of the formation of somatic carrot cells able to completely recapitulate development is shown. The strategy most often employed is to expose excised plant tissue (explant) to a high concentration of auxin. After a period of proliferation of the released explant cells in the presence of auxin, two main cell types can be distinguished. The first of these is non-embryogenic and highly vacuolated, (figure 1.2d'-g'), whereas the second cell type is embryogenic, small (10 to 15μm), spherical, and highly cytoplasmic (figure 1.2c-f). Either cell type can occur as single cells or in clusters. The origin of the small, embryogenic cells is not well understood; they probably arise from non-embryogenic vacuolated cells by a series of cell divisions (figure 1.2c). While interchange of non-embryogenic and embryogenic cells during culture is likely, this has not yet been demonstrated conclusively. After the embryogenic induction phase (figure 1.2a-f) somatic embryos can develop from clusters of embryogenic cells, referred to as proembryogenic masses (17), at a lowered cell density and in the absence of any added growth regulators (figure 1.2g,h). Somatic embryos closely resemble their zygotic counterparts in appearance. The only morphological differences between zygotic and somatic embryos are delayed cotyledon development and the presence of a parenchymatous remainder of the proembryogenic mass instead of a suspensor in somatic embryos. Embryo development from single embryogenic cells initially demands a low concentration of auxin to generate a proembryogenic mass and subsequently proceeds as described above (26,33).
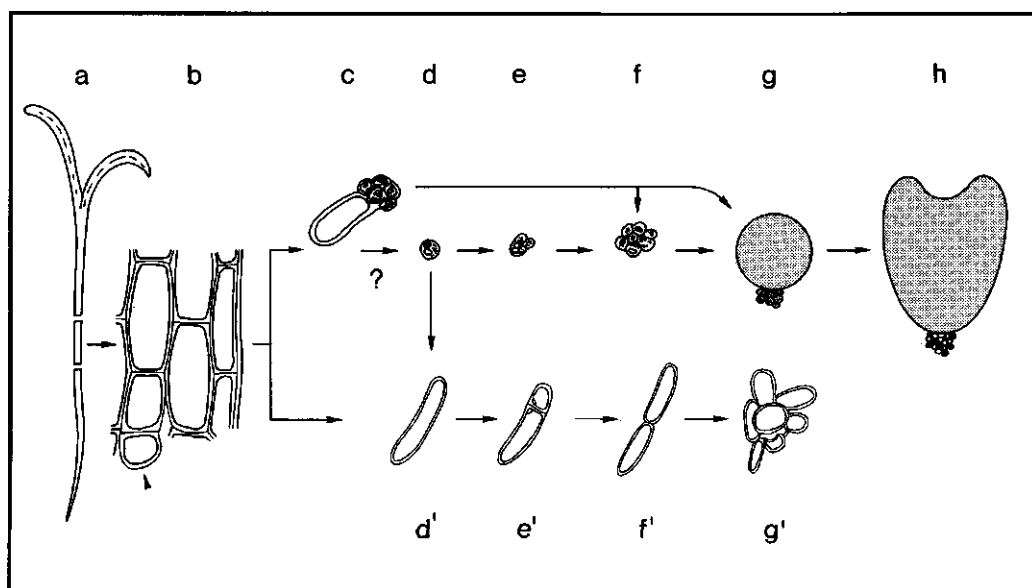
Figure 1.2. Formation of carrot somatic embryos from suspension cells (2,7,20,26). Excised pieces of seedling roots (a,b) are immersed in liquid medium with auxin. Cell divisions become visible in the explant tissue (arrowhead in b) and result in the release of non-embryogenic, vacuolated single cells (c,d'). Some of these cells divide asymmetrically to produce clusters of small embryogenic cells (c). From these clusters small isodiametric cells may be released (d), that are capable of development into embryogenic clusters (e,f). Somatic embryos can develop from a surface cell of embryogenic cell clusters after culture dilution in medium without growth regulators (g,h). Cell division of vacuolated cells without restriction of expansion is shown in d'-g'. This pathway predominates in cultures that have lost their embryogenic capacity but also occurs in embryogenic cultures.

The possibility to generate large numbers of plant somatic embryos in liquid media has been used to investigate early events in plant embryogenesis (9). A number of genes has been cloned with varying degrees of specificity in expression in zygotic and somatic embryos (18,40), and in some cases it has been established that the expression pattern is identical in somatic and zygotic embryos (34). Therefore, it seems reasonable to assume that information obtained on genes that are expressed during somatic embryogenesis is also applicable to zygotic embryogenesis.

Mutants disturbed in zygotic embryo development that are either lethal (10) or have altered patterns of embryo development (31) as well as somatic cell mutants with altered or arrested embryo phenotypes have been described (44), but in most cases the genes affected have not yet been identified. So, although plant embryogenesis is morphologically well described (24,31,38), our understanding of the molecular events that generate the plant embryo is still very limited.

Origin and diversity of secreted molecules in plant cell culture conditioned media

Plant cell culture conditioned medium, i.e. culture medium in which cells have been growing for several days or weeks, contains a complex array of mainly cell wall derived molecules that include polysaccharides, proteoglycans and proteins, among which a large number of enzymes (36,45). The enzymes fall in two main classes, oxidoreductases and hydrolases (11). Classic examples of the first group are peroxidases, implicated in oxidative coupling of phenolic wall components, but also capable of *in vitro* degradation of auxins (19). Examples of the second group are glycosidases and endoglycanases. Many of these enzymes have as potential substrates carbohydrate polymers of the primary cell wall and the middle lamella (see chapter 5). In intact plants these enzymes reside in the appropriate wall compartment where they are kept in place by ionic interactions or diffuse within a limited area (13). From studies into cell wall structure and function, the cell wall has emerged as a tensile, yet highly flexible matrix that is put together from precursor molecules outside the cell it encloses (see chapter 5). Many of its components are at least partially soluble, which accounts for their presence in conditioned culture medium (36).

In carrot embryogenic cultures the small embryogenic cells (figure 2d-f) coexist with the large non-embryogenic cells (figure 2d'-g'); both populations are stably maintained over many subcultures. An important question, therefore, is whether the presence of embryogenic cells is reflected by specific components secreted into the medium. It has indeed been demonstrated that particular proteins are only produced by specific cell types. Two carrot glycoproteins, EP1 and EP4, are only produced by non-embryogenic cells (chapters 2 and 4), while another secreted protein, EP2, is only produced by proembryogenic masses and somatic embryos (39). The EP2 mRNA encodes a lipid transfer protein and is found in the protoderm of both somatic and zygotic embryos, which indicates that proteins secreted into the culture medium of embryogenic somatic cells may also be functional during zygotic embryogenesis (see 5.3.1.). Extracellular proteins of which the secretion ceases upon initiation of embryogenesis have also been found (6,16). Thus, a more general picture is emerging in which a number of secreted proteins is either associated with small embryogenic cells (figure 2c-f), or with non-embryogenic, expanding cells (figure 2d'-g').

Individual secreted proteins control somatic embryogenesis

In the initial experiments demonstrating embryogenesis in carrot cell cultures the culture medium was supplemented with the liquid endosperm of coconuts (35,41). It was reasoned that coconut milk contained special "embryo factors" that were essential to induce somatic cells to become embryogenic (38,41). Further studies showed that simple, fully synthetic culture media containing auxin, cytokinin and ammonium ions allowed the acquisition of embryogenic potential and subsequent somatic embryo development. Since auxins and cytokinins are plant growth regulators that are involved in almost all aspects of plant development, these results indicate that embryo development does not depend on unique compounds produced by the endosperm. If any specific extracellular "embryo factor" is necessary for embryogenesis, it has to be synthesized by the embryogenic cells themselves (20).

As early as in 1966, it was noted by Halperin that "conditioning of the medium by means of cell death, leakage or active secretion may release unknown substances essential to embryo development" (21). Over the years, many observations have indicated that cultured plant cells condition their medium with components that influence cell proliferation and somatic embryo development in a specific way. Dilution of an embryo culture to a very low cell density resulted in reduced numbers of embryos and retarded embryo development (22,23), while the addition of conditioned medium from an embryo culture could partially reverse these effects (23). These findings have been applied in the widespread use of feeder layers to improve growth of cultured plant cells. A conditioning mechanism was even detected at the level of individually cultured single cells. In an elegant series of experiments it was observed that co-culture of one type of regenerating *Brassica* protoplasts increased the division rate in a second type. This effect could be mimicked by medium in which protoplasts had been pre-cultured (37).

Extracellular factors have also been shown to act earlier in the developmental sequence of somatic embryogenesis, i.e. during the establishment of an embryogenic suspension culture. A population of carrot cells newly released from the original somatic tissue (figure 2a,b) became embryogenic much faster in cell-free conditioned medium of an established embryogenic cell line (7). Several other cases of secreted substances that positively affect cell culture have been reported (3,25,47). From these studies it can be concluded that cultured plant cells respond to soluble, secreted components that not only affect cell division but also development.

Despite the dramatic effects of medium conditioning, only few attempts have been made to identify the responsible molecules. Purified low-Mr (< 5 kDa) medium compounds were shown to stimulate cell division in carrot suspension cells (3) or in cultured barley microspores (25). The factor that speeds up the acquisition of embryogenic potential in carrot cell suspensions may be a polypeptide, since it was heat-labile and had an Mr higher than 5 kDa (7). Heat-lability was also observed for a second conditioning factor enhancing barley

17

microspore callus growth (48). In all of these cases, further characterization of the responsible molecules has not been undertaken.

In carrot somatic embryo development, secreted glycoproteins appear to have an important function. A correlation was observed between embryo formation and the presence or absence of a set of medium proteins, several of which were glycosylated (6,16). Three non-embryogenic carrot cell lines showed an aberrant extracellular protein pattern when compared to embryogenic cell lines. Furthermore, the initial stages of embryogenesis in some non-embryogenic lines could be restored by the addition of medium proteins from a wild-type embryo culture (6).

The first assay system that provided direct evidence that secreted glycoproteins are involved in somatic embryo development employed the fungal antibiotic tunicamycin, which, at a low concentration, inhibits somatic embryogenesis but does not affect unorganized proliferation of carrot cells (29). Morphologically, tunicamycin causes expansion of small cells in proembryogenic masses (figure 1.3). Biochemically, it prevents the attachment of high-mannose oligosaccharides to asparagin residues of proteins passing through the endoplasmatic reticulum, and results in decreased glycosylation of secreted proteins. Rescue of embryo development was observed when concentrated conditioned medium from an untreated culture was added together with the tunicamycin. The tunicamycin-complementing effect was heat-labile and protease-sensitive, pointing to secreted glycoproteins as the causative factor (6). On the basis of this complementation assay a single glycoprotein could be purified from conditioned medium that was able to rescue embryogenesis in tunicamycin-inhibited embryo cultures. This glycoprotein was identified as a cationic peroxidase, of which the catalytic properties were indispensable for its embryo-rescue activity (4).

Figure 1.3. Schematic representation of the effect of tunicamycin on an embryogenic cell cluster (4). Surface cells from which the somatic embryos normally develop expand due to turgor pressure. This expansion can be prevented by the addition of purified secreted peroxidase to the culture medium together with the tunicamycin.
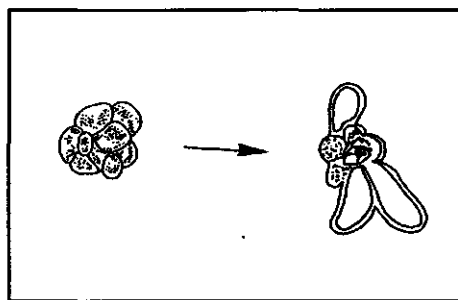
Analysis of the carrot temperature-sensitive mutant cell line ts11 has provided further evidence that extracellular proteins can promote embryogenesis. Somatic embryos from the ts11 cell line develop normally at 24°C. At 32°C they do not progress beyond the globular stage and abnormal development ensues, unless the medium is supplemented with medium conditioned by wild-type embryos (30). The effect is protease-sensitive and, like tunicamycin-arrested embryos, ts11 embryos respond to the addition of a single purified secreted

glycoprotein. In this case, the active protein has chitinase activity (5). Thus, the availability of bioassay systems has allowed the identification of two secreted enzymes that are indispensable for correct somatic embryo development.

Conditioned medium proteins are not only secreted by somatic embryos or embryogenic cells, but also by the non-embryogenic cells that are found in carrot cultures (figure 1.2). These proteins may obscure studies into the role of secreted proteins in somatic embryogenesis, because changes in protein secretion during embryogenesis are not necessarily related to this process. Moreover, the proteins that are secreted by non-embryogenic cells may have adverse effects on somatic embryo development (17). Hence, it is imperative to identify these proteins and to determine the cell type they originate from. In addition, these investigations could provide an answer to the question whether the morphological diversity of cell types is reflected by the proteins they secrete and would give an indication of the degree of cell-type heterogeneity in cell suspensions (see chapter 5). Furthermore, if we can identify cell types by means of the proteins they secrete, it may be possible to follow the developmental changes that different cell types undergo in suspension cultures. Viewed in this way, carrot cell suspensions may be an alternative handle to learn more about the biochemistry of the different cell types of the intact plant.

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Chapter 2

Heterogeneity and cell type-specific localization of a cell wall glycoprotein from carrot suspension cells

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Summary

EP1, an extracellular protein from carrot (*Daucus carota*) cell suspensions, has been partially characterized by means of an antiserum and a cDNA clone. In both embryo and suspension cultures different molecular weight EP1 proteins were detected, some of which (31, 32, 52 and 54 kD) were bound to the cell wall and released into the medium, whereas others (49, 60 and 62 kD) were more firmly bound to the cell wall and could be extracted with a salt solution. Immunoprecipitation of *in vitro* translation products revealed a single primary translation product of 45 kD, suggesting that EP1 heterogeneity is due to differential posttranslational modification. In seedlings organ-specific modification of EP1 proteins was observed, a phenomenon which did not persist in suspension cultures initiated from different seedling organs. In culture EP1 proteins were only found to be associated with vacuolated, non-embryogenic cells, and on these cells they were localized in loosely attached, pectin-containing cell wall material. Purified 52/54 kD EP1 proteins did not alleviate the inhibitory effect of the glycosylation inhibitor tunicamycin on somatic embryogenesis.

Introduction

Embryogenic carrot suspension cultures grown at a high cell density in the presence of 2,4-D contain a variety of cells, ranging from different kinds of single cells through small cell clusters to large cell clumps. Among these are meristematic cell clusters, referred to as proembryogenic masses (11), which are able to develop somatic embryos upon dilution in auxin-free medium. Embryo cultures are initiated from a culture fraction enriched for these proembryogenic masses, providing a system of high frequency somatic embryogenesis that is widely used as a model system to study plant embryogenesis (20).

Three observations suggest that proteins secreted into the culture medium play an important role in somatic embryo development. First, a number of embryogenic cell lines all secrete a characteristic set of proteins into the medium, whereas the extracellular protein patterns of non-embryogenic mutant cell lines deviate from this characteristic pattern (2). Second, the acquisition of embryogenic potential in a newly initiated culture has been shown to be accelerated by the addition of high-molecular weight, heat-labile components from an established embryogenic cell line (3). Third, inhibition of somatic embryogenesis with the glycosylation inhibitor tunicamycin can be complemented by the simultaneous addition of extracellular proteins from an uninhibited embryo culture (2).

To obtain more information about the nature of cell suspension extracellular proteins we screened a carrot λgt11 cDNA expression library with an antiserum raised against total embryo medium proteins. In this chapter we describe the initial characterization of one of the clones

obtained, termed EP1 (Extracellular Protein 1), employing an antiserum raised against the EP1-encoded fusion protein. We show that the EP1 mRNA codes for a small number of secreted glycoproteins that are associated with cell walls of vacuolated cells in culture and that the observed heterogeneity of these proteins appears to arise from different posttranslational modifications.

Results

Cell suspensions contain multiple, differentially modified EP1 protein forms.

Screening of a λgt11 cDNA library with an antiserum raised against total embryo culture medium proteins yielded several positive cDNA clones. Rabbits were immunized with β-galactosidase fusion proteins prepared from lysogens of positive phages to identify the corresponding medium protein. The serum raised against the fusion protein encoded by clone EP1 recognized a doublet with an M_r of 52,000 and 54,000 on a protein gel blot of conditioned medium proteins from both high-density suspension and embryo cultures (figure 2.1A). These proteins correspond to the 52/54 kD proteins that were previously observed in carrot culture media (2,3). An additional doublet of 31/32 kD was recognized in the medium of cultures with a high cell density, regardless of the presence of 2,4-D (figure 2.1A).

The effect of cell density on the appearance of these proteins was confirmed by a serial dilution of proliferating cell suspensions, which showed an increasing amount of 31/32 kD proteins in register with the increase in initial cell density from 1.10^4 to 1.10^7 cells/ml (figure 2.1B). To test whether EP1 proteins are cell wall proteins that become leached from cell walls by the culture medium, we extracted living suspension cells with a calcium chloride solution. With this method ionically bound cell wall proteins can be isolated without contaminating cytoplasmic proteins (21). Immunoblotting showed that the medium EP1 proteins of 31/32 and 52/54 kD are indeed present in the cell wall (figure 2.1C). Repeated washing of the cells with fresh culture medium before calcium extraction removed these proteins from the wall (data not shown), indicating that the binding of these proteins to the cell wall is weak. Three additional EP1 polypeptides of 49, 60 and 62 kD that were not found in the medium, were also detected in the cell wall, but could not be rinsed from the wall with culture medium.

Since plant cells are known to secrete β-galactosidase (19), the complex pattern of EP1 proteins could have resulted from antibodies directed against the β-galactosidase part of the EP1 fusion protein. This possibility was ruled out by a control antiserum raised against *E. coli* β-galactosidase, which did not recognize any medium or cell wall proteins (figure 2.1D). Furthermore, it is important to stress that the anti-EP1 serum was raised against a protein that was synthesized in *E. coli* and, thus, does not contain antibodies directed against glycoprotein oligosaccharide side chains.

Figure 2.1. Analysis of EP1 proteins in conditioned media and cell walls

(A,B) SDS-PAGE immunoblots of conditioned medium proteins from carrot cell suspensions, 7 days after transfer to fresh culture medium. The blot was developed with the anti-EP1 serum. Initial cell densities (cells/ml) and the presence (+) or absence (-) of 2 μM 2,4-D are listed across the top. Lanes marked E and S below the figure correspond to conditions used for embryo and suspension culture respectively. Lanes contain equal amounts of protein.

(C) Occurrence of EP1 proteins in cell walls. Anti-EP1 immunoblots of cell wall proteins extracted with calcium chloride from cells in a 7-day old suspension (S) or embryo (E) culture. Lanes contain 5 μg of cell wall protein. Sizes are indicated in kilodalton.

(D) Immunoblot of medium (M) and cell wall (W) proteins from a high-density suspension culture, developed with anti-β-galactosidase control serum.
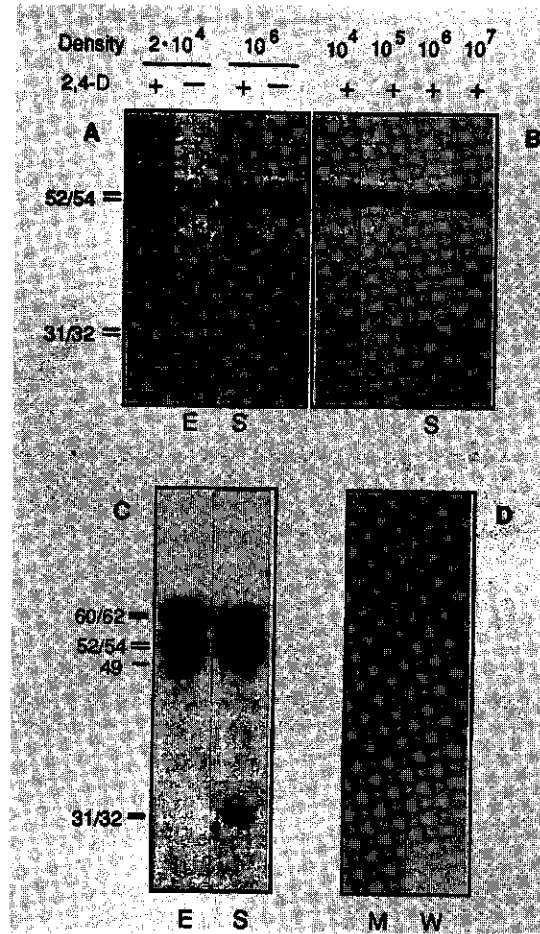
The occurrence of a set of seven anti-EP1-reactive proteins of different sizes raised the question whether these proteins are derived from different mRNAs or arise through posttranslational modification. Low-stringency hybridization of RNA from high-density suspension cells with the EP1 cDNA revealed only a single mRNA band of 1.6 kb (figure 2.2A). In addition, when ^{35}S-labeled *in vitro* translation products of polyA$^+$-RNA from high-density suspension cells were immunoprecipitated with the anti-EP1 serum, only one polypeptide band of 45 kD was detected (figure 2.2B). The anti-β-galactosidase control serum did not recognize any polypeptides (figure 2.2B). Glycosylation of the 49, 52/54 and 62 kD proteins was demonstrated by immunoprecipitation of cell wall proteins from a high-density suspension culture pulse-labeled with N-acetyl-D-[1-^{14}C]glucosamine (figure 2.2C). The

31/32 and 60 kD proteins were not labeled and may not have accumulated above the detection level during the 18 hr labeling period. After [^{35}S]-methionine pulse-labeling the same pattern was observed, which remained unaltered after chase periods of 6 to 90 hours (not shown). The addition of tunicamycin, an inhibitor of N-glycosylation, resulted in the synthesis of only one medium form of 46 kD (figure 2.2D), suggesting that the size difference between the 52 and 54 kD EP1 proteins is caused by differences in N-glycosylation. Deglycosylation of purified 52/54 kD proteins with trifluoromethanesulfonic acid (5) yielded a single broad band that was too diffuse to confirm this observation (not shown). Taken together these results provide strong evidence that the size heterogeneity of EP1 proteins arises from different posttranslational modifications of a single precursor polypeptide.
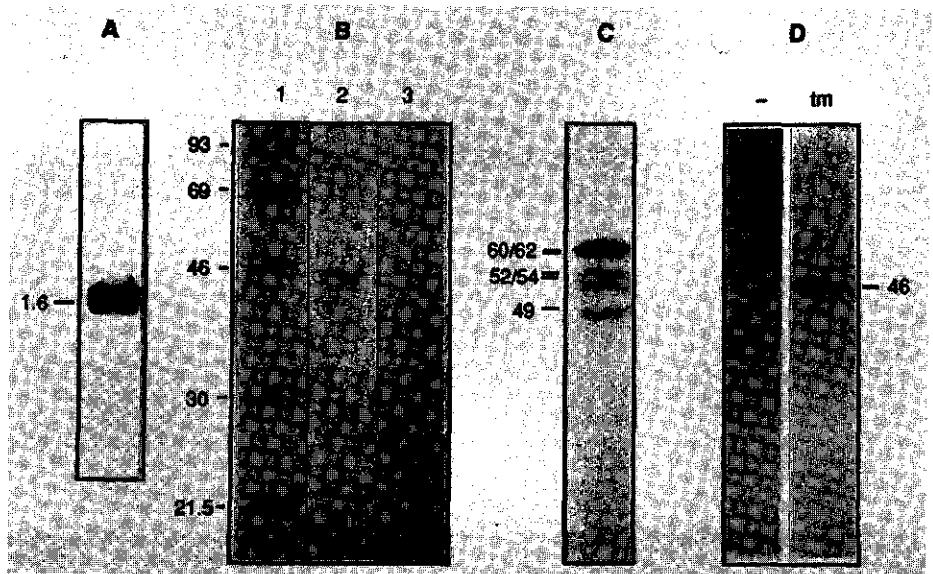
Figure 2.2.
(A) RNA gel blot analysis of 10 μg of total RNA from high-density suspension cells, hybridized with the EP1 cDNA insert. Final washing was in 2 x SSC at 50°C.
(B) Immunoprecipitation of ^{35}S-labeled *in vitro* translation products from polyA$^+$-RNA isolated from high-density suspension cells. Lane 1, protein size markers; lane 2, anti-EP1 serum; lane 3, anti-ß-galactosidase serum.
(C) Glycosylation of EP1 proteins. Suspension cells were grown in medium containing N-acetyl-D-[1-^{14}C]glucosamine. Cell wall proteins were immunoprecipitated with the anti-EP1 serum, separated by SDS-PAGE and autoradiographed.
(D) Immunoprecipitation of ^{35}S-labeled EP1 medium proteins from an embryo culture grown in the absence (-) or presence (tm) of 4 μg/ml tunicamycin.
RNA size is given in kilobases, protein sizes in kilodalton.

Conservation of EP1 epitopes

Total protein extracts from suspension cells of three other members of the *Umbelliferae*, fennel, caraway and parsley, contained proteins with an M_r between 50,000 and 65,000 that were recognized to different extents by the anti-EP1 serum (figure 2.3). The similarity of the carrot and parsley patterns was striking and suggests that between these species not only the recognized epitope but also a major part of the modification of the protein is conserved. Apparently, the EP1-homologous proteins in these species are more strongly bound to the cell wall, because they were not found in the medium (figure 2.3). Homologous proteins were also detected in media from suspension cultures of barley (K. Nielsen, Risø, Roskilde, Denmark, personal communication) as well as in *Citrus* embryo cultures, but not in non-differentiating *Citrus* cultures (R. Fluhr, Weizmann Institute, Rehovot, Israel, personal communication). No anti-EP1-reactive proteins were found in a tomato suspension culture (figure 2.3, lanes 1,6).
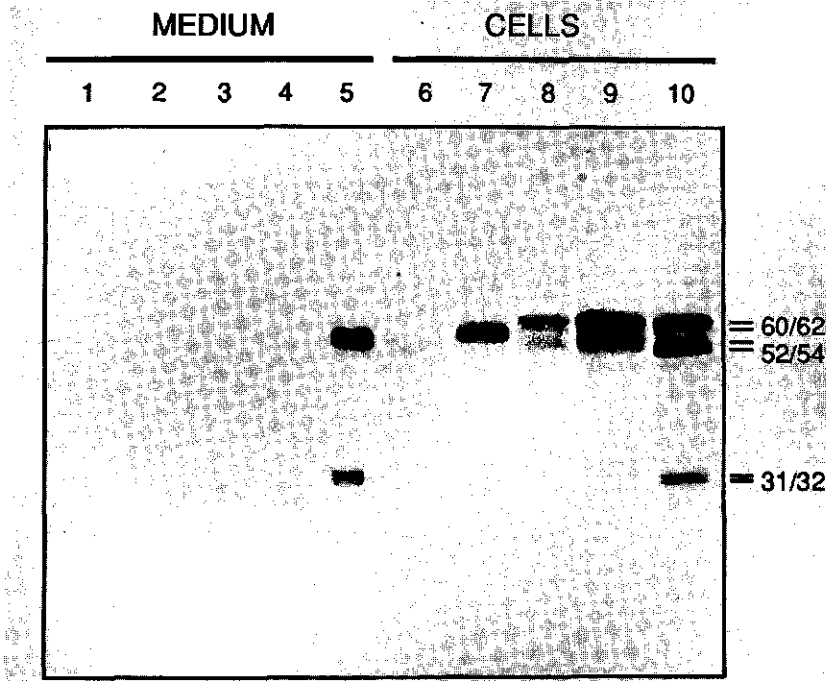

Figure 2.3. Conservation of EP1 epitopes.
SDS-PAGE immunoblot of medium (lanes 1-5) or cellular (lanes 6-10) proteins isolated from high density suspension cultures of tomato (lane 1,6), fennel (lane 2,7), caraway (lane 3,8), parsley (lane 4,9) or carrot (lane 5,10), developed with the anti-EP1 serum. Protein sizes are given in kilodalton.

Expression and modification of EP1 in seedlings.

Figure 2.4 shows protein and RNA gel blot analyses of EP1 expression in carrot seeds and different organs of four week-old seedlings. The highest level of EP1 proteins was detected in basal hypocotyl and root parts. Apical parts of roots and hypocotyls as well as cotyledons and leaves contained lower amounts of EP1 proteins. A similar distribution of EP1 expression was observed on the RNA blots, though it was less pronounced. In endosperm only a very low level of EP1 mRNA was detected, while EP1 proteins could not be identified either in endosperm or in manually isolated zygotic embryos.

Striking differences in EP1 protein patterns were observed in the different seedling organs (figure 2.4A). Roots contained 52/54 and 60/62 kD doublets, but not the 31/32 and 49 kD proteins present in suspension cultures. The latter proteins were not found in any other parts of the seedlings. In hypocotyls and cotyledons only the 60 and 62 kD proteins co-existed with a low amount of a 34 kD polypeptide which itself was neither encountered in roots nor in cultured cells. Leaves did not contain any of the EP1 proteins observed in suspension cultures, but only produced the 34 kD polypeptide, irrespective of the degree of leaf expansion.
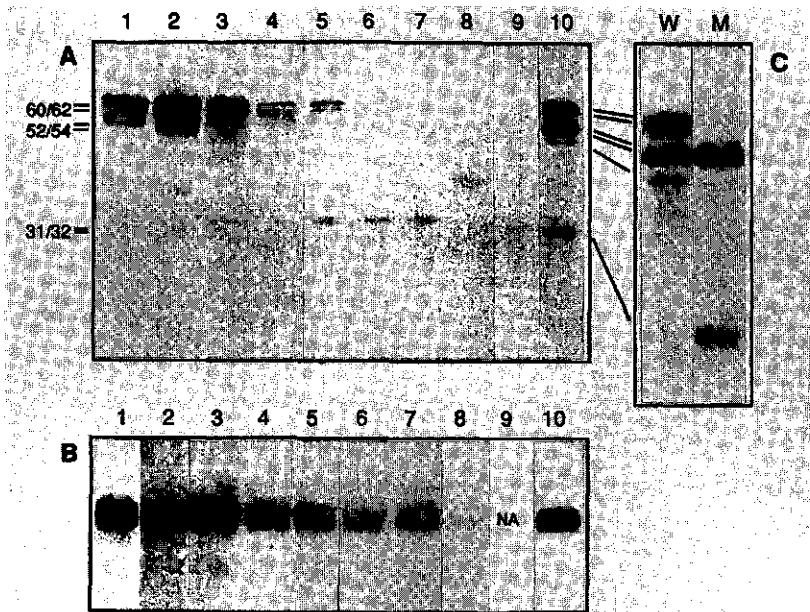
Figure 2.4. Expression and modification of EP1 in seedlings and suspension cultures.
(A) Protein gel blot of 25 µg of total protein isolated from seedling root tips (lane 1), basal part of roots (lane 2), hypocotyls (lane 3), shoot apices (lane 4), cotyledons (lane 5), young leaves (lane 6), expanded leaves (lane 7), endosperm (lane 8), seed embryos (lane 9). For comparison suspension cells are included in lane 10. The blot was developed with the anti-EP1 serum. Protein sizes are in kilodalton. NA = not analysed.
(B) RNA gel blot of 10 µg of total RNA from different seedling tissues and suspension cultures as indicated in (A), hybridized with the EP1 cDNA insert. Final washing was in 0.5 x SSC at 50°C.
(C) Modification of EP1 proteins in a leaf-derived suspension culture. Anti-EP1 immunoblot of proteins from a suspension culture initiated from seedling primary leaf. W, cell wall proteins; M, medium proteins.

The pattern of EP1 proteins in cell suspensions is not related to the source of the explant.

The similarity of the EP1 protein pattern in roots and root-derived suspension cultures suggests that the EP1-producing cells of the explant persist in tissue culture and maintain, at least in part, the original modification pattern. Since in leaves none of the EP1 forms characteristic of cell suspensions are present, a suspension culture was initiated from primary leaf to see whether the subsequent suspension culture had the EP1 modification pattern of the explant. Except for a relatively low level of 31/32 kD proteins in the cell wall, the pattern of EP1 medium and cell wall proteins in the leaf-derived embryogenic culture was indistinguishable from that seen in a root-derived culture (Figure 2.4C). This indicates that the observed modification of EP1 proteins in carrot cell suspensions is not inherited from the source of the explant but appears to be characteristic of suspension culture growth.

Effect of 52/54 kD EP1 proteins on tunicamycin-inhibited and uninhibited somatic embryogenesis

Previously, we have postulated that the 52/54 kD medium proteins may be responsible for the complementation with extracellular glycoproteins of tunicamycin-inhibited somatic embryogenesis (2). Therefore, the 52/54 kD EP1 proteins were purified and tested at different concentrations in tunicamycin-inhibited cultures (Table 2.1). No significant tunicamycin-complementing activity was observed with purified 52/54 kD proteins, indicating that, by themselves, these proteins are not sufficient to prevent proembryogenic mass disruption as observed with total unfractionated medium proteins. The addition of purified 52/54 kD EP1 proteins to the medium of uninhibited cultures had no effect on embryo development and on the morphology of non-embryogenic cells (results not shown).

Table 2.1. Effect of purified 52/54 kD EP1 medium proteins on inhibition of somatic
embryogenesis by tunicamycin.

additions	somatic embryos [a]
none	100 ± 5
tm [b]	7 ± 3
tm + total medium concentrate	64 ± 7
tm + purified 52/54 kD EP1 proteins	11 ± 3 [c]

[a] mean of three assays $\pm$ SE. The number of somatic embryos in control cultures was set at 100%.

[b] tm, 0.75 µg/ml tunicamycin.

[c] highest value of four different concentrations.

31

EP1 proteins are associated with cell walls of non-embryogenic vacuolated cells in culture.

The finding that EP1 proteins are not present in zygotic embryos (figure 2.4A) prompted us to investigate the distribution of EP1 proteins between different cell types in embryo and suspension cultures. Both in suspension and in embryo cultures (figure 2.5A) EP1 cell wall proteins were associated only with the vacuolated single cell population and not with somatic embryos, proembryogenic masses or large callus clumps. Also the EP1 mRNA was only present in single cells of an embryo culture (figure 2.5B).

Figure 2.5. Distribution of EP1 expression in cell suspensions.
(A) Protein gel blot. Seven day suspension (lanes 1 to 3) or embryo (lanes 4 and 5) cultures were fractionated by sieving and Percoll gradient centrifugation or manual selection. Total protein was isolated from different fractions, subjected to SDS-PAGE and immunoblotted with the anti-EP1 serum. Lane 1: <50 μm, <20% Percoll, vacuolated single cells. Lane 2: 50 to 125 μm, dense cell clusters were picked up manually. Lane 3: >170 μm, >20% Percoll, large dense cell clumps. Lane 4: <125 μm, <20% Percoll, vacuolated single cells. Lane 5: >125 μm, 30-40% Percoll, globular to torpedo-stage embryos.
(B) RNA gel blot of total RNA isolated from embryo culture single cells (<50 μm, lane 1) and somatic embryos (lane 2), hybridized with EP1 cDNA insert. Final washing was in 0.5 x SSC at 50°C.
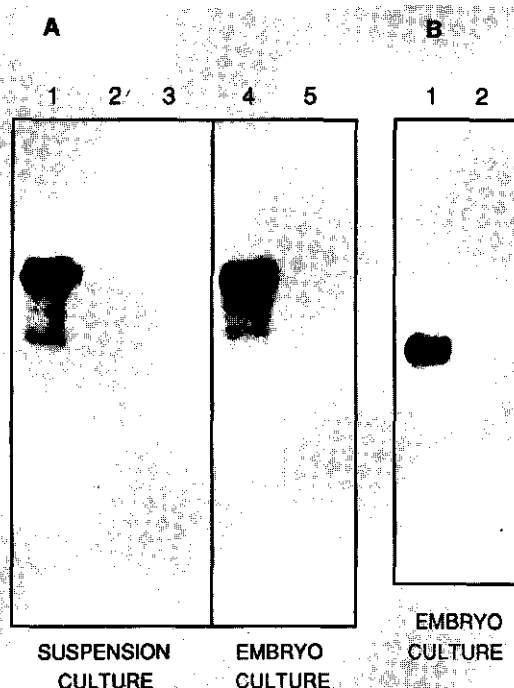
To analyse the cells containing the EP1 proteins in more detail, intact suspension cells were subjected to anti-EP1 immunofluorescence. The anti-EP1 serum labeled cell wall material which was present mostly as diffuse patches on the surface of vacuolated single cells (figure 2.6A,B). Strongly reactive patches of this material that were completely detached from the cells were also frequently observed (figure 2.6C). The appearance of this material was not a fixation or labeling artefact, since identical structures were observed attached to (figure 2.6D) or

32

detached from cells before fixation. The amount of EP1-positive cell surface material in a high density suspension culture decreased with age of the culture, while the amount of free wall material increased, suggesting that this material detaches from the cells. Non-specific labeling with the anti-β-galactosidase control serum was negligible (figure 2.6E). To investigate the nature of the EP1-reactive cell wall material, cultures were immunolabeled with the monoclonal antibody JIM5, which is specific for un-esterified pectins (13). The JIM5 antibody was found to label structures similar to those labeled by the EP1 serum (compare fig 2.6C and F), leading us to conclude that EP1 proteins are bound to pectinaceous cell wall material which detaches from cells growing in liquid culture.
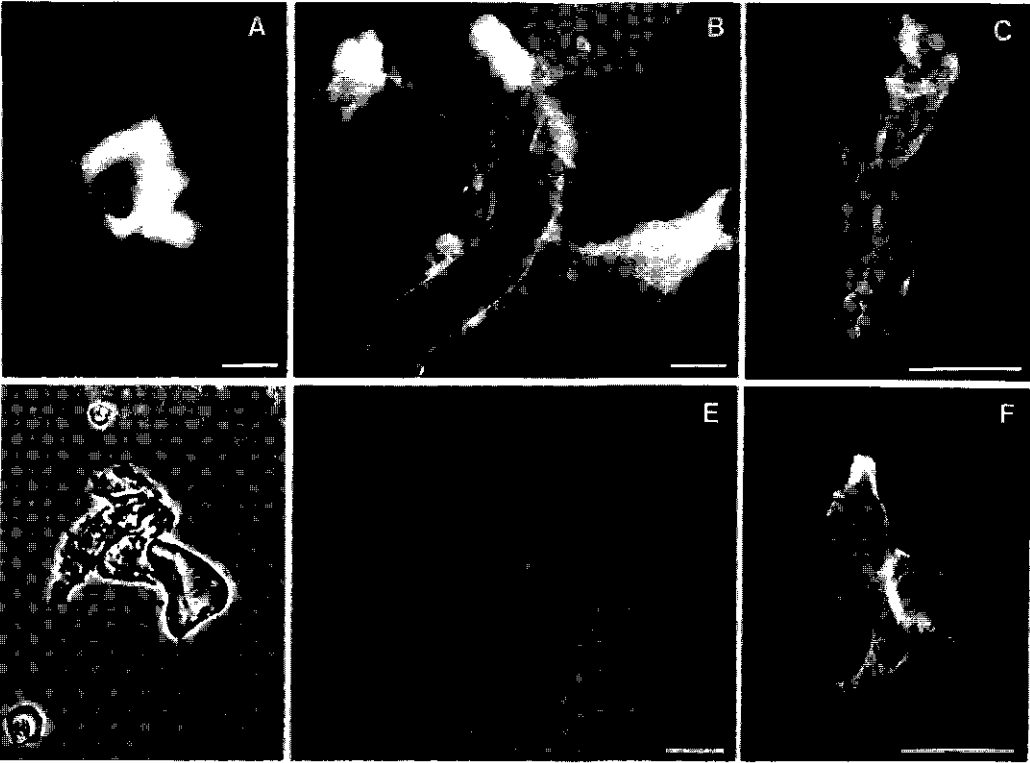
Figure 2.6. Immunolocalization of EP1 proteins and pectins in suspension cells.
Fixed intact cells were treated with anti-EP1 serum (A to C), anti-β-galactosidase control serum (E) or pectin-specific JIM5 monoclonal antibody (F). A and B show EP1-reactive material on the cell surface and in C this material is detached from the cell. Free wall material also labels with JIM5 antibody (F). D is a phase contrast image of a cell that has not undergone any fixation or antibody treatment. Bar = 50µm

Discussion

In this chapter we describe the initial characterization of EP1, a set of cell wall glycoproteins occurring in carrot cell suspensions as well as in seedlings. In high-density suspension cells seven EP1 proteins of different sizes occur, while the cells in these cultures contain mRNA encoding a single-size *in vitro* translation product. These observations imply that EP1 size heterogeneity is introduced posttranslationally by differential modification.

N-glycosylation is likely to be one mechanism responsible for EP1 heterogeneity, since a single 46 kD medium protein instead of the normal 52/54 kD doublet occurs in the medium in the presence of tunicamycin, an inhibitor that prevents the addition of glycans to asparagin residues (6). The wall-bound forms may be glycosylated differently from the 52/54 kD glycoproteins, which could be the basis of their enhanced wall-binding (7). The sizes of the high-M_r EP1 glycoproteins are probably overestimations, since it has been found that glycosylated proteins migrate more slowly in SDS-PAGE than unglycosylated proteins of the same mass (15). Thus, only a small number of oligosaccharide side chains could be sufficient to increase the apparent M_r to 52, 54, 60 or 62 kD. If we assume signal peptide cleavage from the 45 kD primary translation product to a polypeptide of 41-43 kD, the 46 kD protein produced in the presence of tunicamycin may contain modifications other than N-glycosylation. We have not identified the nature of these modifications yet. Because of the size of the 31/32 kD doublet, about 10 kD smaller than the putative 41-43 kD precursor, we assume that proteolytic processing is responsible for the formation of these proteins. However, [^{35}S]-methionine pulse-chase labeling did not reveal processing of high-M_r EP1 proteins to lower molecular mass forms.

Although we detect only one EP1 band after *in vitro* translation, we cannot exclude the possibility that this band consists of allelic isoforms that differ in their ability to undergo glycosylation or proteolytic cleavage. This could only explain part of the complex EP1 pattern, as there appears to be only one EP1 gene per haploid genome (F. van Engelen *et al.*, unpublished data).

In seedlings the EP1 modification pattern is organ-specific. Thus, in addition to organ- and tissue-specific gene expression, another level of regulation appears to exist in normal plant development that involves organ-specific modifications. Although we do not know the effect of modification on EP1 protein function, these observations do show that care must be taken in the interpretation of gene expression data from RNA gel blots only.

With a few exceptions (17), organ-specific phenotypes are not inherited by cell cultures initiated from an organ explant. EP1 protein modification appears to conform to this rule, since roots and leaves, which show different EP1 modification patterns, give rise to the same modification pattern in suspension culture. As suggested above, the formation of the low-M_r EP1 proteins probably depends on extracellular protease activity. This activity may be high in

leaves, but is substantially diluted in culture medium. Thus, the observed changes in modification pattern may not reflect changes in gene expression, but represent a cell suspension artefact.

Satoh and Fujii (22) have described the purification of GP57, a 57 kD extracellular protein from carrot suspensions which is glycosylated and exists in two forms which are secreted by non-embryogenic cells, suggesting homology or identity with EP1. This was not the case, as the anti-EP1 antiserum did not react with purified GP57 and a comparison of EP1 and GP57 sequence data did not reveal homology between these proteins (S. Satoh, personal communication).

We have only few clues to the biochemical function of the EP1 proteins. Its conservation in both dicots and monocots suggests that it is a biologically important molecule. The partial EP1 amino acid sequence deduced from the cDNA sequence has not revealed homology with known proteins (chapter 3). The EP1 proteins are abundantly present in cell walls of vacuolated cells in culture and in basal parts of seedling roots and hypocotyls, but their level is reduced in both apices of seedlings. This would favour a role in cell elongation, which is thought to be regulated by formation and cleavage of bonds between cell wall polymers (9). Obviously, cell expansion is restricted in proembryogenic masses and early stages of developing embryos, which could explain the absence of cell wall EP1 proteins from these structures. Nevertheless, they are immersed in medium containing the 52/54 (and 31/32) kD proteins, which apparently does not interfere with embryo development. Are these medium EP1 proteins inactivated by modification or is a proper substrate required for EP1 protein function? Such questions can only be resolved after identification of the biochemical function of these proteins.

Materials and methods

Plant material and culture conditions

Embryogenic high-density suspension cultures of carrot (*Daucus carota*, cell line "10" (2)) were initiated from cut seedling roots (3), maintained in liquid B5 medium (10) containing 2,4-D and subcultured with 14-day intervals at an initial cell density of 1.10^6 cells/ml. Seven days after subculturing embryo cultures were initiated by inoculating a culture fraction enriched for proembryogenic masses at a density of 2.10^4 cells/ml in B5 medium. Inhibition/complementation assays with tunicamycin were performed as described previously (2). High-density suspension cultures from fennel (*Foeniculum vulgare*), parsley (*Petroselinum crispum*) and caraway (*Carum carvi*) were initiated from seeds obtained locally and maintained under the same conditions as the carrot "10" cell line. Tomato (*Lycopersicon esculentum*) suspensions were cultured in R3B medium (18). Carrot seedlings were grown from 'Flakkese' SG766 Trophy seeds and dissected when the first leaves had fully expanded. Zygotic embryos were obtained by gently squeezing embryos from seeds after imbibing for two hours. Pericarps and seedcoats were removed from the remaining seeds and the endosperms collected. Tissues were immediately frozen and stored at -80°C.

Protein isolation and immunoblot analysis

Medium protein samples were prepared by passing conditioned medium through a 0.22-μm filter and concentrating by pressure dialysis (2). Cell wall proteins were isolated from living cells by washing once with fresh culture medium followed by incubation in 0.1 M $CaCl_2$ on ice for 20 min. Cells were removed by centrifugation, the solution was passed through a 0.22-μm filter and mixed with two volumes of ethanol. Precipitated proteins were collected by centrifugation, vacuum-dried and dissolved by boiling in SDS-PAGE sample buffer (14) for five minutes. The viability of $CaCl_2$-extracted cells was determined by Lysamine Green staining and found to be similar to non-treated cells, indicating that contamination of cell wall protein preparations with cytoplasmic proteins was negligible. For the isolation of total cellular protein, frozen plant tissue was ground in SDS-PAGE sample buffer, boiled for five minutes and centrifuged to remove insoluble material. The anti-EP1 serum was obtained by immunizing a rabbit with the EP1-encoded β-galactosidase fusion protein isolated by preparative SDS-PAGE from *E. coli* Y1089 lysogenized with λgt11-EP1 phage (12). A control serum was raised against β-galactosidase isolated from a wild-type λgt11 lysogen. Immunoblot analysis was carried out as described by Burnette (1), using 12.5% polyacrylamide gels and antisera in a 2000- to 3000-fold dilution.

In vivo labeling, in vitro translation and immunoprecipitation

Proteins were pulse-labeled in vivo by growing cells for 18 hours in the presence of N-acetyl-D-[1-^{14}C]glucosamine (Amersham) or [^{35}S]-L-methionine (NEN). In one experiment tunicamycin (Calbiochem) was added one hour before pulse-labeling. In pulse-chase experiments cold L-methionine to a concentration of 10 mM was added after 16 hours pulse-labeling. Medium samples were taken after 0, 6 or 90 hours and immunoprecipitated (see below). For in vitro translations 0.5 μg of polyA$^+$-RNA was incubated for 30 min at 30°C in 10 μL reticulocyte lysate (Green Hectares) with [^{35}S]methionine. Labeled proteins were adjusted to 0.5 x SDS-PAGE sample buffer, boiled for 1 min. and centrifuged (5 min, 14,000g). Immunoprecipitation of the supernatant was then carried out as described by Franssen et al. (8).

cDNA library screening, RNA isolation and Northern blotting

The λgt11 cDNA library was prepared with RNA from an unfractionated embryo culture and was kindly provided by Dr. T.L. Thomas (A&M University, College Station, Texas, USA). This library was screened with an antiserum raised against concentrated embryo culture medium according to the protocol described by Huynh et al. (12). Alkaline phosphatase-conjugated goat-anti-rabbit IgG was used to detect positive plaques. Total RNA was isolated as described by de Vries et al. (4). PolyA$^+$-RNA was selected by oligo(dT)cellulose chromatography according to standard procedures (16). For RNA gel blot analysis, RNA was denatured with DMSO/glyoxal, electrophoresed on agarose gels and transferred to Gene Screen membranes (NEN). Hybridization of the filters was carried out according to the manufacturer's instructions.

Immunofluorescence labeling

Cells were washed with fresh medium and fixed in 96% ethanol for 2 min., followed by 2% (w/v) paraformaldehyde in 10 mM sodium phosphate for 10 min. Fixed cells were washed once with 10 mM Tris-HCl, 150 mM NaCl, pH 7.5 (TBS), blocked for 1 hour with 2% (v/v) calf serum in TBS and incubated for 2 hours in blocking solution containing antiserum (1:500) or JIM5 monoclonal antibody supernatant (1:50, (13)). Subsequently, the cells were washed with TBS, incubated for 30 min. in blocking solution with fluorescein isothiocyanate-conjugated second antibody (1:50), washed twice with TBS and mounted for microscopy.

36

Purification of medium EP1 proteins

Conditioned medium from a 10 day embryo culture was filtered through a 0.2 μm filter. The filtrate was adjusted to pH 8.5 with solid Tris, added to DEAE-sepharose FF (Pharmacia) and incubated for one hour at 4°C. All buffers used in subsequent steps contained 25 mM Tris-HCl pH 8.5. Bound proteins were eluted with 0.2 M KCl, diluted, applied to a DEAE-sepharose FF column and eluted with a gradient from 0 to 250 mM KCl. Final purification was done by Mono Q anion exchange FPLC (Pharmacia) with the same KCl gradient.

Acknowledgments

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Chapter 3

The carrot secreted glycoprotein gene EP1 is expressed in the epidermis and has sequence homology to *Brassica* S-locus glycoproteins

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Summary

Non-embryogenic carrot suspension cells secrete the EP1 glycoprotein. A nearly full-length cDNA clone encoding EP1 was isolated and sequenced. The correspondence between the EP1 cDNA clone and the EP1 glycoprotein was confirmed by protein sequencing of the N-terminus and several glycopeptides of EP1. The EP1 sequence revealed a region of homology with *Brassica* S-locus glycoprotein genes, an *Arabidopsis* S-like gene and putative S-like receptor protein kinases from maize and *Arabidopsis*. EP1 gene expression, analysed by *in situ* mRNA localization, was detected in cells located at the surface of the seedling: in the epidermis of the root, the hypocotyl and the cotyledons, in the root cap, and in a crescent of cells in the apical dome of the shoot. In developing seeds, expression was most pronounced in both the inner and outer integument epidermis.

Introduction

In the past decades it has become recognized that the plant cell wall plays a pivotal role in the development of the plant body (21). Plant cells are thought to regulate their expansion by adjusting the mechanical properties of their walls (7). Controlled, directional cell expansion is one of the mechanisms of plant morphogenesis, together with regulation of the rate and plane of cell division. In addition, plant cell wall fragments, termed oligosaccharins, are able to direct plant growth and development (4). These processes are accompanied by chemical modification of the cell wall and changes in its composition, which can be effected by the secretion of specific cell wall-modifying enzymes or wall constituents. Hence, it can be expected that different cell types or cells at different stages of development each secrete a characteristic set of cell wall proteins.

Embryogenic carrot cell suspensions consist of a mixture of different cell types that proliferate in a synthetic growth medium (20). These cells can be roughly divided into two classes: 1) small, cytoplasmic cells from which somatic embryos can develop and 2) large, vacuolated, non-embryogenic cells. We have previously described a carrot glycoprotein, EP1, that was found to be secreted by expanded, non-embryogenic cells only. This finding, combined with a higher level of EP1 in basal parts of seedling hypocotyls and roots as compared to apical parts, led us to suggest a possible involvement of EP1 in cell elongation (chapter 2).

To obtain additional clues to the function of EP1 we have determined the sequence of the EP1 cDNA and found a region sharing homology with *Brassica* S-locus glycoproteins. In addition, we studied the expression of the EP1 gene in seedlings and developing seeds in more detail by *in situ* hybridization. EP1 is revealed to be expressed in cells at the plant surface: the

epidermis of the root, hypocotyl and cotyledon, the root cap and a patch of cells on the shoot apical dome. In developing seeds, the EP1 mRNA was detected in the epidermis of the inner and outer integument and in the fruit wall epidermis. These observations are discussed in view of a possible biological function of EP1 proteins.

Results

EP1 cDNA sequence

The size of the original λgt11 EP1 cDNA insert was only 220 base pairs, while the EP1 mRNA was estimated to have a size of 1600 nucleotides (chapter 2). To obtain EP1 cDNA clones with full-length inserts, we screened a λZAPII cDNA library constructed with polyA$^+$ RNA from carrot embryo and suspension cells, with the 220 base pair fragment as probe. The largest EP1 cDNA clone contained an insert of 1500 base pairs; this insert was sequenced (figure 3.1A). The nucleotide sequence and the deduced amino acid sequence are presented in figure 3.1B. The EP1 insert contained a large open reading frame of 1152 nucleotides starting at nucleotide 1. By direct sequencing of glycopeptides derived from the 52/54 kD medium EP1 proteins (16, and A. Sturm, unpublished data) it was confirmed that this reading frame encodes the EP1 protein (figure 3.2A). Three peptide sequences, one of which was the N-terminus of EP1 (peptide I), were found to be homologous to different parts of the deduced amino acid sequence. The homology of these three peptides with the EP1 sequences varied between 60 and 80%; a fourth peptide had no significant homology with any part of the protein sequence encoded by EP1. These differences are probably due to the fact that EP1 proteins from a wild carrot variety were used for protein sequencing, while mRNA was obtained from a domesticated carrot variety.

A hydropathy plot of the deduced protein sequence (figure 3.2B) revealed the presence of a hydrophobic domain at the N-terminus, which we assume to be the signal peptide for secretion of the EP1 protein. The weight matrix method of Von Heijne (22) predicts that the signal peptide would be cleaved off between threonine at position 19 and leucine at position 20. The sequence of the N-terminus of purified secreted 52 and 54 kD EP1 proteins (figure 3.2A) demonstrated that this is indeed the cleavage site used *in vivo*. Six potential sites for glycosylation of asparagine residues were present in the EP1 sequence at amino acid positions 24, 62, 98, 225, 230 and 269. The sequences of glycopeptides I and II (figure 3.2A) provided evidence that at least the asparagine residues 24 and 269 are glycosylated *in vivo*. Another conspicuous feature of the EP1 sequence was the occurrence of 7 cysteine residues in the C-terminal 60 amino acids. Since EP1 is secreted, it is likely that disulfide bridges are formed in its C-terminal region. The 3'-untranslated region contained two potential polyadenylation signals at nucleotide positions 1405 and 1483.

A

B

```
      P  L  T  L  T  I  L  L  F  F  I  Q  R  I  D  F  C  H  T  L  V  P  A  N
CCCCTCACCTTGACAATCTTACTTTTCTTCATCCAAAGAATTGATTTTTGTCATACACTTGTTCCGGCAAAT  72

    E  T  F  K  F  V  N  E  G  E  L  G  Q  Y  I  S  E  Y  F  G  D  Y  R  P
GAAACCTTCAAGTTCGTCAATGAAGGAGAACTAGGCCAGTACATCAGTGAATACTTCGGCGATTACCGACCT  144

    L  D  P  F  T  S  P  F  Q  L  C  F  Y  N  Q  T  P  T  A  F  T  L  A  L
CTCGACCCCTTCACCTCCCCATTCCAGCTTTGCTTCTACAACCAAACCCCCACTGCCTTCACACTCGCCCTA  216

    R  M  G  L  R  R  T  E  S  L  M  R  W  V  W  E  A  N  R  G  N  P  V  D
CGCATGGGCCTGAGGGCGCACCGAGTCTCTCATGCGCTGGGTCTGGGAAGCCAACCGGGGCAACCCGGTCGAC  288

    E  N  A  T  L  T  F  G  P  D  G  N  L  V  L  A  R  S  N  G  Q  V  A  W
GAAAACGCCACGCTCACATTCGGCCCAGACGGGAACCTCGTGCTGGCCCGGTCCAACGGCCAGGTCGCATGG  360

    Q  T  S  T  A  N  K  G  V  V  G  L  K  I  L  P  N  G  N  M  V  L  Y  D
CAAACATCTACAGCCAACAAAGGTGTCGTGGGCCTCAAGATTCTCCCTAATGGTAACATGGTGCTTTACGAC  432

    S  K  G  K  F  L  W  Q  S  F  D  T  P  T  D  T  L  L  V  G  Q  S  L  K
TCCAAAGGCAAATTCTTATGGCAAAGCTTCGACACGCCCACCGACACGCTCCTAGTGGGCCAGTCACTCAAG  504

    M  G  A  V  T  K  L  V  S  R  A  S  P  G  E  N  V  N  G  P  Y  S  L  V
ATGGGCGCTGTTACAAAGCTCGTGAGCCGGGCTTCTCCGGGTGAGAACGTGAATGGGCCGTACAGTCTCGTG  576

    M  E  P  K  G  L  H  L  Y  Y  K  P  T  T  S  P  K  P  I  R  Y  Y  S  F
ATGGAGCCCAAAGGGCTGCACTTGTACTACAAGCCCACCACCTCTCCCAAGCCCATCCGGTACTACTCTTTC  648

    S  L  F  T  K  L  N  K  N  E  S  L  Q  N  V  T  F  E  F  E  N  E  N  D
TCCTTGTTCACCAAACTCAACAAGAACGAGAGCCTACAGAATGTTACATTTGAGTTTGAAAATGAGAATGAC  720

    Q  G  F  A  F  L  L  S  L  K  Y  G  T  S  N  S  L  G  G  A  S  I  L  N
CAGGGCTTTGCTTTTTTATTGAGCTTGAAATACGGAACCTCGAACTCATTGGGTGGGGCCTCCATACTCAAT  792

    R  I  K  Y  N  T  T  L  S  F  L  R  L  E  I  D  G  N  V  K  I  Y  T  Y
AGAATCAAGTACAATACCACATTGTCGTTTCTCAGATTAGAGATCGATGGGAATGTCAAGATTTATACTTAT  864

    N  D  K  V  D  Y  G  A  W  E  V  T  Y  T  L  F  L  K  A  P  P  P  L  F
AATGATAAAGTGGATTATGGGGCGTGGGAAGTGACTTATACTTTGTTTCTAAAAGCCCCTCCTCCTTTGTTC  936

    Q  V  S  L  A  A  T  E  S  E  S  S  E  C  Q  L  P  K  K  C  G  N  F  G
CAAGTAAGCCTAGCTGCCACGGAGTCCGAATCCTCTGAATGTCAATTACCCAAGAAGTGTGGTAACTTTGGG  1008

    L  C  E  E  S  Q  C  V  G  C  P  T  S  S  G  P  V  L  A  W  S  K  T  C
CTTTGTGAGGAGAGCCAATGCGTGGGCTGCCCAACCTCTAGTGGGCCGGTTCTTGCTTGGAGCAAGACTTGC  1080

    E  P  P  K  L  S  S  C  G  P  K  D  F  H  Y  N  K  L  G  G  W  I  T  T
GAGCCCCCAAAATTATCATCTTGCGGGCCAAAAGATTTTCACTATAATAAGCTTGGGGGGTGGATCACTACA  1152

  *
TGATAAAGTACACAAGCGGGGATGGGCCGGTGAAAGATGGTGATTGTGGGGTGAAATGTACAGGAGACTGTA  1224

AGTGTTTGGGGTATTTTTATAACCAGGACACGTCGAGATGTTGGATAGCGTATGATCTCAGTACTCTCACCA  1296

GAGTGGTCAATGCCACGCACTTGGCCTACATTAAGGTGCCCAATAAGTGATGCCTTGTTTGGACCTGGCTAT  1368

ATTTAAAGCTTGCTGCTCTCGAGTTTTTCAGCCGACAATAATGAATGTGGCATAGCTGTTGTGACACGTTAT  1440

ATACCTATAATATCTTGTGACAAGCTACTCACCGAAATAAAGAACGGTTTTTGTGTGATGTGT  1503
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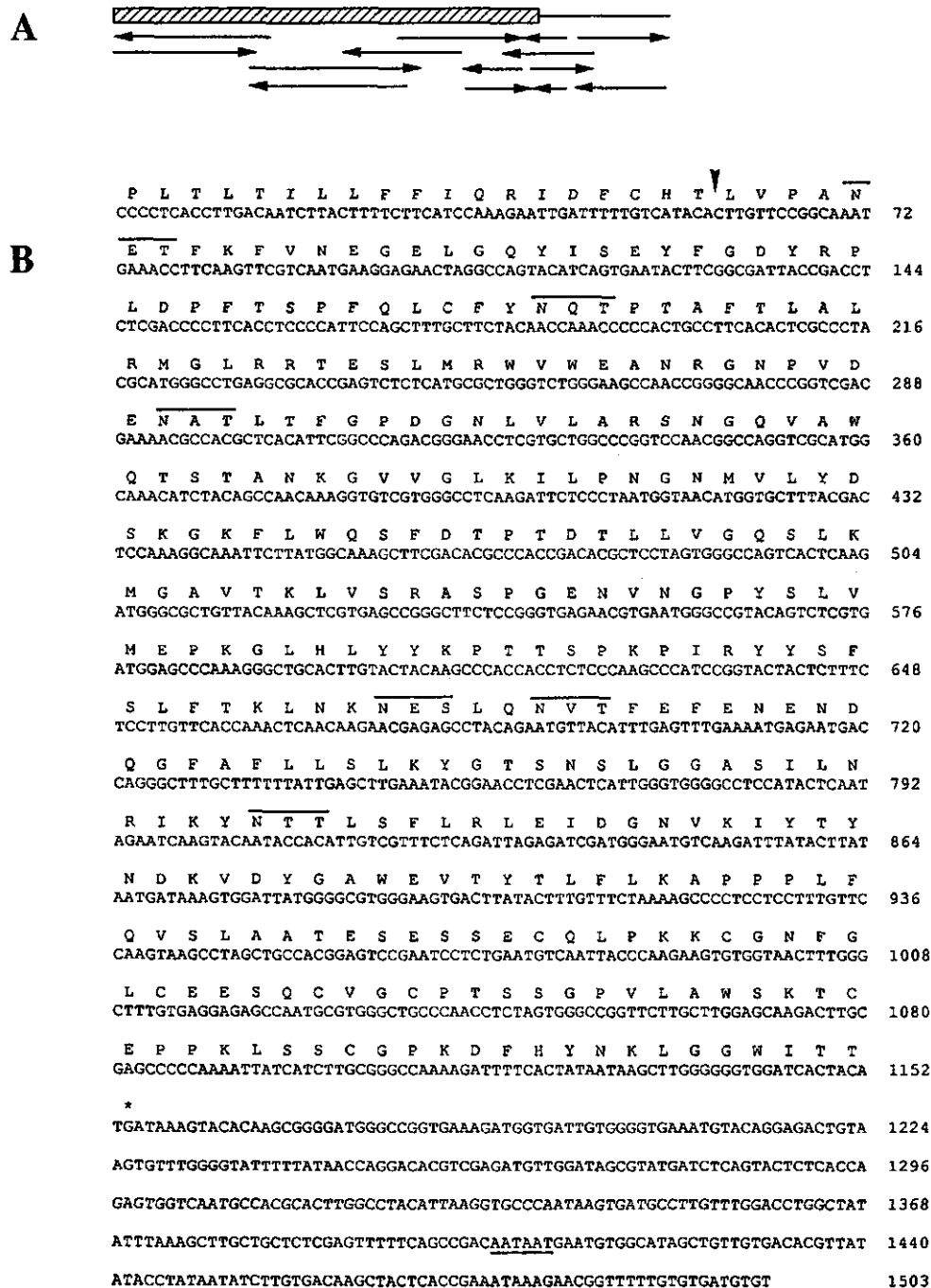
Figure 3.1. EP1 cDNA sequence

A. Sequencing strategy of the EP1 cDNA clone. The coding region is shown as a shaded box.

B. Nucleotide and deduced amino acid sequence of EP1 cDNA. The arrowhead marks the predicted signal peptide cleavage site. Potential N-glycosylation sites are indicated with horizontal bars over the amino acid sequence, potential polyadenylation signals are underlined.

45

A

```
52/54   V V P A N A T F K Y T N E G E L G E Y I V E Y
        | | | |   | | |     | | | | | |   | |   | |   (I)
EP1     L V P A N E T F K F V N E G E L G Q Y I S E Y
```

```
52/54   K Y N T T Y S M L R
        | | | | |   |   | |   (II)
EP1     K Y N T T L S F L R
```

```
52/54   L G L L T N G N L V
        |       |   | | |   |   (III)
EP1     L K I L P N G N M V
```
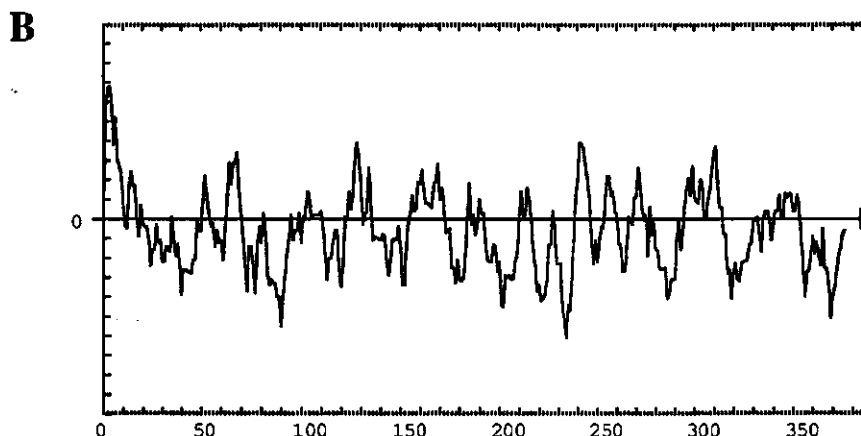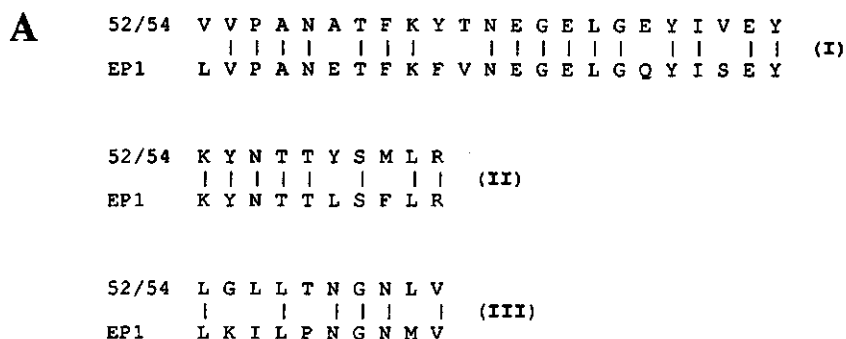
B

Figure 3.2.

A. Comparison of amino acid sequences as determined by glycopeptide sequencing of the 52/54 kD EP1 proteins ("52/54") or deduced from the EP1 cDNA sequence ("EP1"). Peptide I consists of 23 amino acids and is the N-terminus of the mature 52/54 kD proteins. Peptides II and three each consist of 10 amino acids. The numbers on the left side of the EP1 sequences indicate the amino acid position in the EP1 sequence.

B. Hydrophobicity plot of the deduced EP1 protein sequence. Positive values mark hydrophobic stretches.

The amino-terminal sequence of the protein predicted by the EP1 cDNA did not contain a methionine residue; the first in-frame methionine occured at amino acid position 74. Thus, the EP1 cDNA insert did not represent the full coding sequence. The calculated Mr of the protein encoded by the large open reading frame of EP1 was 43 kD. Since the primary translation product had been estimated to have an Mr of 45 kD (chapter 2), at most 10 to 20 amino acids are missing from the N-terminus. The complete signal peptide would then have a size of 30 to 40 amino acids, which is relatively large in comparison with known signal peptides (23).

EP1 shares homology with self-incompatibility glycoproteins

The EP1 cDNA sequence was compared with sequences deposited in the EMBL (version 30.0) and Swiss-Prot (version 21.0) databases. Significant homology was found with all known sequences of *Brassica* self-incompatibility-associated glycoproteins (S-locus glycoproteins or SLG), with *Brassica* S-locus-related (SLR) glycoproteins (11), and with AtS1, an S-like protein from *Arabidopsis* (6). In addition, EP1 was found to be homologous to the S-like domain, but not the kinase or membrane-spanning domains, of putative receptor protein kinases from *Brassica*(14), *Arabidopsis* (17) and maize (ZmPK1, ref. 24). Figure 3.3 shows the optimal alignment of the N-terminal half of four (predicted) amino acid sequences. The homology was limited to a region of EP1 from amino acid 85 to 180, where it amounted to 48% identity to AtS1(6), and 43% to SLG29 (18) and ZmPK1 (24).

In situ *localization of EP1 mRNA*

EP1 mRNA was localized by *in situ* hybridization on sections from a 14 day old carrot seedling and from developing carrot seeds (figure 3.4). In the shoot, EP1 expression was found in the epidermis (figure 3.4A and B). The labeling intensity was highest in the region of the apex and slightly decreased in the direction of both the hypocotyl and the cotyledons. In the leaf primordia no EP1 mRNA could be detected. In the shoot apical dome, a stretch of cells in the outer cell layer of the initiation zone and peripheral zone expressed EP1 (figure 3.4C and D). Scrutiny of serial sections suggested that this small region of EP1 expression was crescent-shaped and located off the centre of the dome towards one of the adjacent leaf primordia. No signal above background was observed on similar sections hybridized with EP1 sense RNA (figure 3.4E and F). Higher magnifications of the cotyledon (figure 3.4G to I) revealed that most of the signal was located over the cytoplasm adjacent to the outer cell walls of the epidermal cells. This subcellular distribution of EP1 mRNA is probably due to the asymmetrical localization of vacuole, nucleus and cytoplasm that is often observed in epidermal cells (8,10).

In the root tip (figure 3.4K and L), EP1 expression was detected in the epidermis as well. Also the root cap contained some EP1 mRNA. Distal from the root tip, EP1 expression remained fairly constant along a length of about 6 mm (figure 3.4M,N, and data not shown). Because the EP1 mRNA could still be detected at a similar level close to the root-hypocotyl transition (figure 3.4O and P), we assume that the EP1 gene is expressed at an almost constant level in epidermal cells all along the root and hypocotyl.

In the developing seed EP1 mRNA was abundant in both the inner and outer epidermis of the integument and, to a lesser extent, in the fruit wall epidermis (figure 3.4Q and R).

```
    Ep1    .........  PLTLTILLFF  IQRIDFCHTL  VPANETFKFV  NEGELGQYIS  EYFGDYRPLD
    Ats1   MRGVTPMYY   HSYTFFFFFF  VVLLALFLHV  FSINT.....  ....LSSTET  LTISSNRTIV
    SLG29  MKGVGKPYE   NSHTSFLLVF  FV.LTLFSPA  FSINT.....  ....LSSIES  LKISNSRTLV
    ZmPK1  MPRPLAALL   .STACILSFF  IALFPRAASS  RDILP.....  ....LGSSLV  VESYESSTLQ

    50  PFTSPFQLCF  YNQTPTA...  ..FTLALRMG  ..LRRTESLM  RWVWEANRGN  PVDEN.ATLT
        SPGNIFELGF  FKTTTSSRNG  DHWYLGIWYK  SI.....SER  TYVWVANRTN  PLSKSIGTLK
        SPGNVLELGF  FRTPSSSR..  ..WYLGMWYK  KL.....SER  TYVWVANRDN  PLSCSIGTLK
        SSDGTSSSGF  YEVYTHA...  ..FTFSVWYS  KTEAPAANNK  TIVWSANPDR  PVHARRSALT

   102  FGPDGNLVLA  RSNGQVAW..  .QTSTANKGV  VGLKILPNGN  MVLYDSKGK.  ...FLWQSFD
        I.SYANLVLL  DHSGTLVWST  NLTRT.VKSP  VVAELLDNGN  FVLRDSKGNY  QNRFLWQSFD
        I.SNMNLVLL  DHSNKSLWST  NHTRGNERSP  VVAELLANGN  FVLRDSNKND  RSGFLWQSFD
        LQKDGNMVLT  DYDGAAVWRA  D..GNNFTGV  QRARLLDTGN  LMIEDSGGNT  ....VWQSFD

   155  TPTDTLLL...  .VGQSLKMGA  VTKLVSRASP  GENVNGPYSL  VMEPKGLHLY  YKPTTSPKPI
        YPVDTLLPEM  KIGRDLKTG.  HEFLSSWRSP  YDPSSGDFS.  ..........  FKL.GTQGLP
        YPTDTLLPEM  KLGYDLRTGL  NRFLTSWRSS  DDPSSGDFS.  ..........  YKL.QTRRLP
        SPTDTFLPT.  ....QLITAA  TRLVPTTQS.  ..RSPGNYI.  ..........  FRFSDLSVLS
```

Figure 3.3. Sequence homology of EP1.

Alignment of EP1 cDNA sequence with *Arabidopsis* Ats1, *Brassica* S$_{29}$ S-locus glycoprotein (SLG29) and maize ZmPK1 putative receptor protein kinase sequences. Amino acids that are shared between EP1 and two or three other sequences are boxed. Only the amino terminal region is shown. Gaps were introduced for optimal alignment.

Figure 3.4. (opposite page) Localization of EP1 mRNA in sections of seedlings and developing seeds.

Unless indicated, hybridization was performed with antisense RNA. Silver grains are visible as bright white dots in dark-field and epipolarization images. A,C,E,G,I,K,M,O and Q are bright-field photographs.

A. Longitudinal section through the apical region of the shoot. cot, cotyledon; lp, leaf primordium, hyp, hypocotyl.
B. Dark-field photograph of A. Bar = 200 μm.
C. Median longitudinal section of the shoot apical meristem. lp, leaf primordium; iz, initiation zone; pz, peripheral zone; cz, central zone; ax, leaf axil.
D. Epipolarization photograph of C. Bar = 20 μm
E. Longitudinal section through the apical region of the shoot, hybridized with EP1 sense RNA.
F. Dark-field photograph of E. Bar = 200 μm.
G. Longitudinal section through the cotyledon.
H. Dark-field photograph of G. Bar = 100 μm
I. Longitudinal section through the cotyledon epidermis and subepidermal cell layer. ep, epidermis.
J. Epipolarization photograph of I. Bar = 20 μm
K. Longitudinal section through the root tip. rc, root cap.
L. Epipolarization photograph of K. Bar = 100 μm
M. Longitudinal section through the root two to five mm distal from the tip. The arrow indicates a site where, due to the curvature of the root, the plane of section was through the epidermis.
N. Dark-field photograph of M. Bar = 200 μm.
O. Longitudinal section through the root close to the transition from the root to the hypocotyl.
P. Dark-field photograph of O. Bar = 100 μm
Q. Transverse section through a young developing fruit prior to dehiscence. The section was cut at a distance 1/3 of length of the fruit from the basis. es=embryo sac, int=integument, fw=fruit wall, fun=funiculus, loc=locule.
R. Dark-field photograph of Q. Bar = 100 μm

48
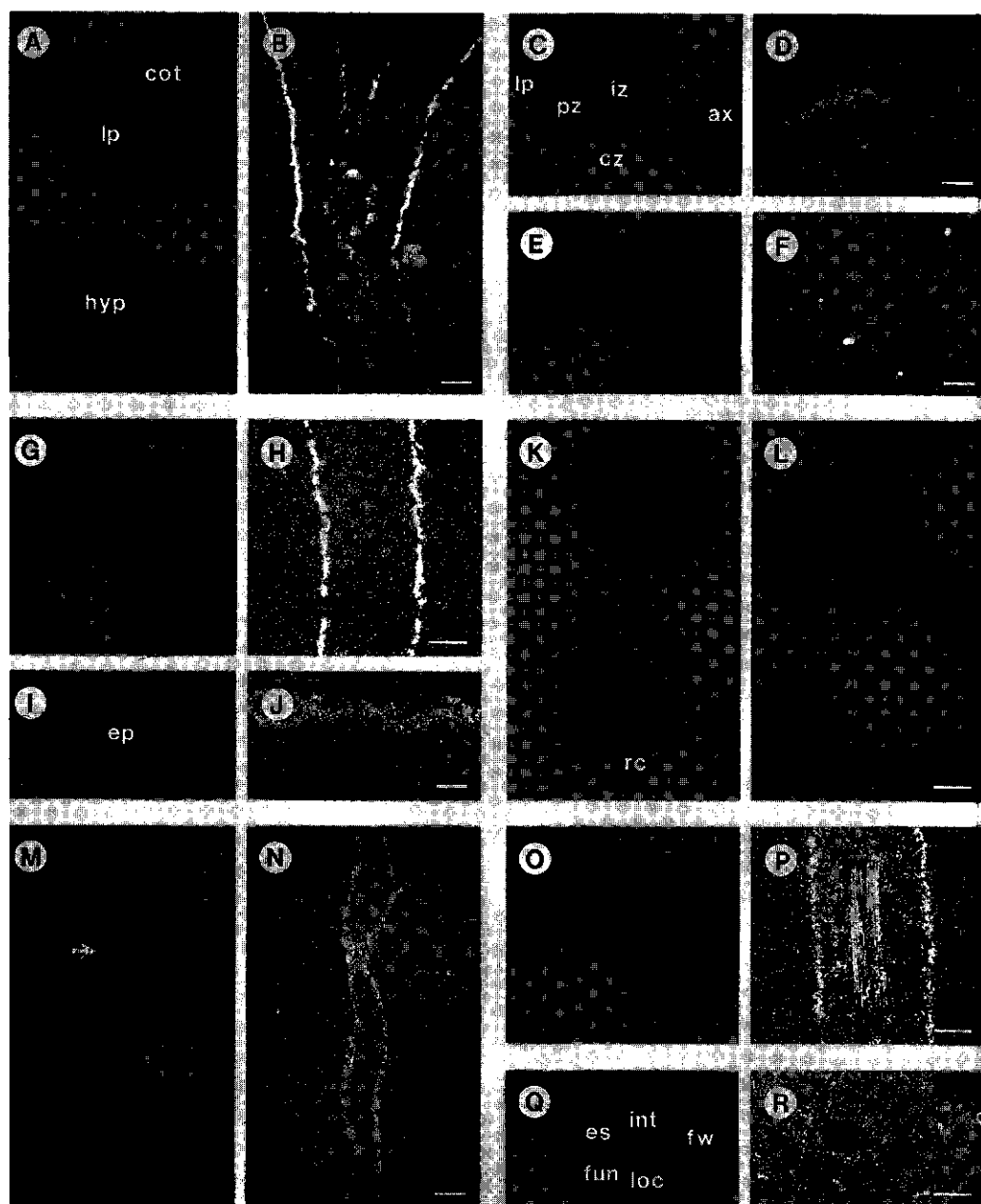
Discussion

To further characterize the carrot secreted glycoprotein EP1, we have determined the EP1 cDNA sequence and localized its mRNA in seedlings and developing seeds. The localization revealed four sites of EP1 gene expression: 1) the epidermis of the seedling root, hypocotyl and cotyledon, 2) the root cap, 3) a crescent of cells at the surface of the shoot apical dome, and 4) the outer cell layers of the integument and the fruit wall of the developing seed. The fact that EP1 is also expressed in a subset of non-embryogenic suspension-cultured cells (chapter 2) strongly suggests that this cell population is derived from the epidermis of the explant and has retained at least one of the epidermis-specific features.

The expression pattern of EP1 in seedlings appears to be complementary to the expression pattern previously described for the carrot EP2 lipid transfer protein gene (15). The EP2 gene is expressed in protodermal and epidermal cells that are devoid of EP1 mRNA and *vice versa*. This mutually exclusive expression pattern is also found in cell suspensions. Here, embryogenic cell clusters and somatic embryos express EP2, but not EP1, while non-embryogenic cells express EP1, but not EP2 ((15) and chapter 2). The only exceptions to this complementary expression pattern are the peripheral zone of the shoot apical meristem and the outer integument epidermis, where both genes are expressed.

What do the observed epidermal localization of EP1 mRNA and the sequence homology with SLG and S-like genes tell us about a possible function of EP1? We have previously proposed a possible involvement of EP1 in cell elongation, based on its localization on the surface of expanded suspension cells and its enhanced expression in root and hypocotyl basal regions as compared to apices (chapter 2). Although many studies support the contention that the control of auxin-induced shoot elongation resides in the epidermis (1,9), EP1 expression along the growth axis does not parallel the region of elongation. Moreover, recent experiments have suggested that epidermal and outer cortical cells of the pea epicotyl are equally responsive to auxin (12). Thus, following the conclusions from that work, in order to be part of a general mechanism of auxin-induced elongation, EP1 would have to be expressed in cortical cell layers as well. Root elongation has been much less studied, but evidence has been presented that it is controlled by inner rather than outer cell layers (2). Taken together, the *in situ* hybridization data presented in this paper do not lend support to a role for EP1 in cell elongation.

One of the properties of the epidermis is the closely packed arrangement of cells (10). On the one hand, this makes the epidermis a barrier that is difficult to penetrate for pathogens. On the other hand, tight cell adhesion in the epidermis is essential to maintain the integrity of the primary plant body, by resisting the forces exerted by internal tissue layers. Cell-to-cell contact may be mediated by xyloglucans that interconnect cellulose microfibrils of adjacent primary cell walls or by the pectins that constitute the middle lamella (7). The binding of some forms of the EP1 protein to the pectinaceous material that loosely envelops expanded carrot suspension cells

(chapter 2), and that probably represents the remnant of the middle lamella, may indicate that EP1 enhances the adhesive properties of the epidermal middle lamellae. The most likely candidate for such a function is pectin methyl esterase, which uncovers polygalacturonate blocks that are able to form strong gels in the presence of calcium (21). However, the 52/54 kD EP1 proteins neither have demonstrable pectin esterase activity (results not shown) nor do they enhance cell adhesion observably in cell suspensions (chapter 2). Moreover, the high expression of EP1 in single, i.e. non-adhesive, suspension cells and the lack of EP1 expression in embryogenic cell clusters or somatic embryos, which do show tight cell adherence, contradicts the idea of enhancement of cell adhesion by EP1.

The homology of EP1 with SLG's and S-like genes sheds a different light on EP1 function. The *Brassica* SLG's are expressed in the papillar cells of the stigma and in pollen and mediate recognition of "self" pollen by the pistil, inhibiting pollen germination by an as yet unknown biochemical mechanism (11). The identification of a putative receptor protein kinase gene at the S-locus that includes an S-like domain has led to the hypothesis that the self-incompatibility signal is transduced through binding of the SLG to its cognate S-like receptor (14). EP1 is not involved in self-incompatibility, because carrot does not possess a genetic self-incompatibility system. Other S-like genes, the SLR genes (11), are also expressed in mature stigmatic papillae, but are not genetically linked to self-incompatibility and show a high conservation between different *Brassica* species. These observations suggest that SLR proteins have a different role in pollination. The expression pattern of EP1 in seedlings rules out a specific function of EP1 in pollination. In this respect, EP1 could be more related to genes isolated from the self-compatible species *Arabidopsis* and maize encoding S-like putative receptor kinases, which are expressed in leaves (17) or in shoots and roots (24), respectively.

Beside sequence homology, EP1 shares other characteristics with the SLG and SLR proteins: they all have a size of 50-60 kD, are heavily glycosylated in the N-terminal two-third of the protein, and contain a C-terminal domain that is cysteine-rich (11). Thus, the overall structure of these proteins seems to be similar. The region that is conserved between EP1 and S-like proteins may confer to the proteins the property of binding to a homologous domain. The observation that the 52/54 kD EP1 proteins are present in suspension culture media as a dimer (J. Cordewener, unpublished data) demonstrates that EP1 indeed exhibits homophilic binding. This leads us to speculate that, by analogy with the SLG's, isolated EP1 subunits may be able to bind to an EP1-like receptor. However, dimerization of EP1 monomers before secretion would preclude them from binding to this receptor. Hence, EP1 would have to be secreted as a pro-protein that can only bind to its receptor or to another EP1 monomer upon modification after secretion. Candidates for this pro-protein are the wall-bound 60/62kD EP1 proteins observed previously (chapter 2). We have no indications yet that an EP1-like receptor exists in carrot.

SLG's may act through limiting water transport across the stigma surface, thereby inhibiting the hydration and subsequent germination of pollen grains (11). Because prevention of water loss is also an important function of the epidermis, EP1 may be involved in the limitation of water flow through the outer epidermal cell wall. It may do so by direct modification of wall structure, like impregnation of the wall with hydrophobic cutin, or, by analogy with the proposed mode of SLG action, act as a signal that instructs the protoplast of epidermal cells to restrict water transport across the outer cell wall. In cell suspensions, such a function of EP1 is probably disturbed and is also clearly superfluous.

The EP1 gene is expressed in the epidermis. Hence, the existence of a family of S-like genes with members in both self-compatible and self-incompatible plant species suggests that the genes involved in pollen-stigma interactions may have evolved from a common epidermal ancestor of sofar unknown function. It would therefore be of interest to our thinking about the mode of action of S-like proteins to determine whether EP1-homologous S-like genes are expressed in the epidermis of *Brassica* plants.

Materials and methods

Plant material
Carrot (*Daucus carota*) seedlings used for initiation of suspension cultures (5) and *in situ* hybridization experiments were grown for 14 days from "Flakkese" SG766 Trophy seeds (Zaadunie, Enkhuizen, The Netherlands). The wild carrot cell line W001C was used for peptide sequencing.

DNA methods
A λZAPII (Stratagene) cDNA library was synthesized with poly(A)[+]-RNA from both suspension and embryo culture cells. To isolate a full-length EP1 cDNA clone this library was screened with ^{32}P-labeled EP1 insert DNA from the original λgt11 cDNA clone (chapter 2) using standard procedures (13). DNA sequencing was performed in the Advanced DNA Technologies Laboratory at Texas A&M University by the dideoxy chain termination method (13), using Sequenase 2.0 and protocols supplied by United States Biochemical. Overlapping fragments of both strands were sequenced. DNA sequences were analysed using the GCG sequence analysis software (version 7.1).

Protein sequencing
This part of the work was carried out at the Friedrich Miescher Institute, Basel, Switzerland. Purification of the medium 52/54 kD EP1 proteins from the wild carrot cell line W001C, isolation of glycopeptides from these proteins, and sequencing of intact proteins and glycopeptides were done as described by Sturm (16).

In situ *hybridization*
In situ hybridization was carried out according to the protocol of Cox & Goldberg (3). Briefly, tissues were embedded in agarose to facilitate handling (15), fixed in paraformaldehyde/glutaraldehyde and dehydrated with a graded ethanol series. Ethanol was replaced by xylene, and tissues were embedded in parafin. 7 μm sections were cut and mounted on polylysine-coated slides. The sections were then hybridized at 42°C with ^{35}S-labeled EP1

sense or antisense RNA probes. A subclone of EP1, covering 650 base pairs at the 5' end was used for the synthesis of RNA probes. Film emulsions were exposed for three weeks.

Acknowledgments

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Chapter 4

Purification, immunological characterization and cDNA cloning of a 47 kDa glycoprotein secreted by carrot suspension cells

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Sacco de Vries & Ab van Kammen

Summary

A 47kD glycoprotein, termed EP4, has been purified from carrot cell suspension culture medium. A specific antiserum raised against EP4 recognized a second protein of 45kD that was ionically bound to the cell wall. EP4 was detected in culture media from both embryogenic and non-embryogenic cell lines and was found to be secreted by a specific subset of non-embryogenic cells. Secretion of the 47kD glycoprotein by embryogenic cells was not evident. The 45kD cell wall-bound EP4 protein was specific for non-embryogenic cells and was shown by immunolocalization to occur in the wall of grouped cells, with the highest levels in the walls separating adjacent cells. In seedlings, EP4 proteins were mainly found in roots. EP4 cDNA was cloned by screening of a library with an oligonucleotide derived from an EP4 peptide sequence. The nucleotide sequence did not reveal homology with known proteins.

Introduction

Molecular studies of plant embryogenesis are hampered by the poor accessibility of embryos developing inside the ovule. To overcome this problem, many researchers have resorted to the alternative of somatic embryogenesis in cell suspension cultures (6). An important finding in the studies of somatic embryo development is that it is highly dependent on components that are secreted into the culture medium (9,10,21). Secreted glycoproteins have been described that are able to restore embryo development blocked by either a glycosylation inhibitor (3,5) or a mutation (4,11).

A role for extracellular proteins in developmental processes may be explained by considering the importance of the structure they are part of: the cell wall and the middle lamella, collectively called the "plant extracellular matrix" (13,21). It has been argued that early stages of embryogenesis are dependent on specific properties of the extracellular matrix that are required to prevent cell expansion and to provide tight cell-to-cell contact (8). Later on in development, cells are allowed to undergo controlled directional expansion, eventually shaping the plant body. Secreted proteins are thought to act in these processes by modifying the polymers that make up the extracellular matrix, thereby changing its viscoelastic properties.

Beside embryogenic cells, which are small, spherical and rich in cytoplasm, carrot cultures contain non-embryogenic cells that are vacuolated and expanded. This population of non-embryogenic cells still exhibits a large morphological heterogeneity; cells differ in shape and degree of expansion and may be present as single cells or as loose cell clusters. The biochemical basis of the morphological differences between cell types is not known, but we may expect that such differences are paralleled by differences in secreted cell wall proteins. In

previous studies on the nature and occurrence of secreted proteins from carrot cell suspensions, glycoproteins have been identified that are specific for either embryogenic cells (EP2 (19)) or non-embryogenic cells (EP1 (chapter 2)). Here, we describe the characterization and cDNA cloning of the carrot glycoprotein EP4 (Extracellular Protein 4) that, like the EP1 protein, is secreted by non-embryogenic cells. EP4, however, is secreted by a subpopulation of non-embryogenic cells that differs from the one that produces EP1.

Results

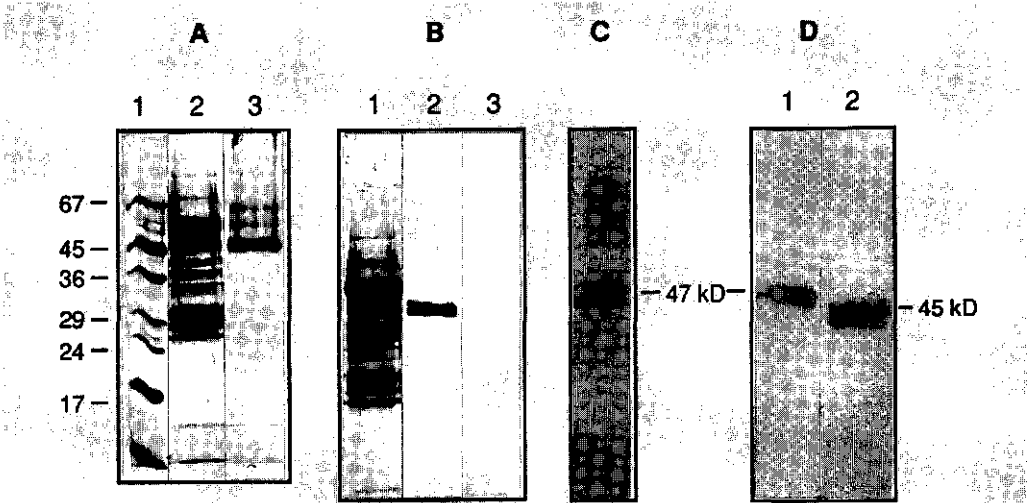*Purification and serological characterization of the 47kD EP4 protein*

Three rounds of anion exchange chromatography of medium proteins from carrot embryo cultures yielded several protein fractions (4). Initial experiments with these different protein fractions suggested that a 47kD glycoprotein could increase the frequency of embryo initiation in the mutant cell line ts11 (11) about tenfold (Lo Schiavo and De Jong, unpublished observations). Based on these results, the 47 kD glycoprotein, which we designate EP4, was further characterized. For this purpose, the 47kD EP4 protein was isolated from FPLC fractions by preparative SDS-PAGE (figure 4.1A) and this preparation was used to raise a mouse antiserum. The anti-EP4 serum reacted strongly with a single protein band of 47kD on a gel blot of embryo culture medium proteins (figure 4.1B). Pre-immune serum was entirely unreactive, indicating a high specificity of the anti-EP4 serum for the antigen. This was a remarkable finding, since Concanavalin A affinity blotting had shown that the 47kD protein is glycosylated (figure 4.1C). This observation rendered it likely that an antiserum raised against the 47kD glycoprotein would cross-react with other glycosylated medium proteins. Apparently, either the oligosaccharide side chains are not very antigenic in mice or they are unique for the 47kD protein.

Previous studies on a different secreted carrot glycoprotein, EP1, had shown that different molecular mass forms of EP1 protein were found in the cell wall. We tested whether this was also the case for EP4. Cell wall proteins, extracted from intact cells with 0.1 M $CaCl_2$, were indeed found to contain a protein reacting with the anti-EP4 serum (figure 4.1D). The estimated Mr of this protein was 45kD, 2kD less than the medium form of EP4.

*EP4 proteins are secreted by a specific type of non-embryogenic cells*

To examine a possible correlation between the occurrence of the 47 kD protein and embryogenic potential, immunoblots were made with medium proteins from different embryogenic and non-embryogenic high-density suspension cultures (figure 4.2). Anti-EP4-

reactive proteins were detected in all cell lines. The position of the bands slightly varied between different cell lines and in a few cases a doublet was observed, but no correlation between the amount or position of EP4 protein and embryogenic potential was apparent.

Figure 4.1. Purification and serological characterization EP4 protein.
A. Ion exchange chromatography of embryo culture medium, monitored by SDS-PAGE. Lane 1, Molecular mass markers. Lane 2, medium proteins eluted from DEAE Sepharose with 25mM Tris-HCl pH 8.5, 0.2M KCl. Lane 3, 47 kD EP4 protein eluting from MonoQ FPLC column at 175mM KCl, 25mM Tris-HCl pH 8.5.
B. Immunoblotting of embryo culture medium proteins with anti-EP4 serum. Lane 1, silver stained SDS-PA gel of the embryo culture medium proteins used for blotting. Lane 2, protein detected by anti-EP4 serum. Lane 3, protein detected by pre-immune serum.
C. Glycosylation of 47 kD protein demonstrated by concanavaline A staining. Protein gel blot of purified 47kD protein, incubated with concanavaline A and developed with peroxidase.
D. A 45 kD form of EP4 is associated with the cell wall. Anti-EP4 immunoblot of embryo culture medium proteins (lane 1) or cell wall proteins (lane 2)

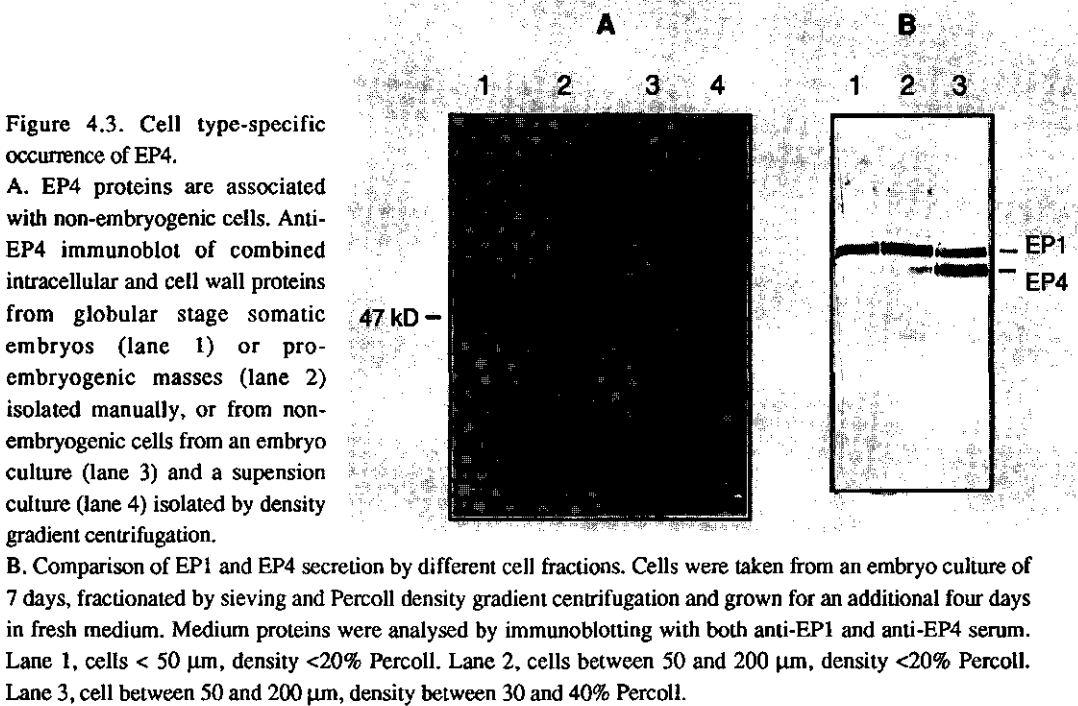Figure 4.2. Detection of EP4 in embryogenic and non-embryogenic cell lines.
Anti-EP4 immunoblot of medium proteins from high density suspension cultures. Cell lines 10 and A+ are embryogenic, FG10, a5II, C15 are non-embryogenic (5).

61
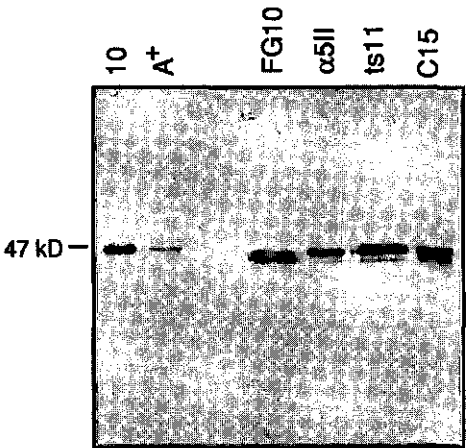
The presence of EP4 glycoprotein in media from cultures that do not produce embryos or that do not even contain embryogenic cells (figure 4.2) suggested that the 47kD EP4 protein is secreted by non-embryogenic cells. This could be demonstrated by Percoll density gradient fractionation of suspension and embryo cultures. The 47kD protein was readily detected in fractions consisting of vacuolated, non-embryogenic cells (data not shown). An answer to the question whether somatic embryos or proembryogenic masses also secrete this protein was much more difficult to obtain unequivocally. The 47kD EP4 protein was detected in media conditioned by proembryogenic masses or globular embryos that had been isolated by Percoll gradient centrifugation. However, this observation should be interpreted with caution. First, contamination with non-embryogenic cells is almost unavoidable because of their tendency to stick to the proembryogenic masses or embryos. Second, somatic embryos are still attached to the parenchymatous remainder of the proembryogenic mass from which they originated and proembryogenic masses themselves may also contain some vacuolated cells. Third, because vacuolated cells appear to secrete much larger amounts of medium proteins than embryos or proembryogenic masses (results not shown), even a slight contamination with vacuolated cells will contribute considerably to the secreted protein profile of cultured somatic embryos. Hence, EP4 protein may be detected in the medium from any cell fraction, if not highly purified. By manual isolation and selection of somatic embryos and proembryogenic masses contamination with vacuolated cells could be precluded, but the yield of medium proteins was too low for gel blot analysis. However, detection of cell wall EP4 proteins was possible in these pure cell fractions. Wall-bound proteins were isolated together with intracellular proteins and the latter were used as an internal standard to determine the amount of protein to be loaded in each gel lane. Immunoblotting with the anti-EP4 serum revealed that only non-embryogenic cells contained the wall-bound 45kD form of EP4, while the manually isolated embryos and proembryogenic masses did not (figure 4.3A). Therefore, we conclude that 1) both the 45kD medium form and the 47 kD wall-bound form of EP4 are secreted by vacuolated, non-embryogenic cells, 2) proembryogenic masses or embryos do not carry the 45kD protein in their walls and 3) secretion of the 47kD medium form by proembryogenic masses or embryos is possible, but unlikely.

A suspension culture was fractionated to examine whether EP4 and the previously described EP1 are produced by the same non-embryogenic cell type. By sieving through 200 μm and 50 μm meshes and by Percoll block gradient centrifugation the following cell fractions were obtained: 1) cells smaller than 50 μm, density lower than 20% Percoll, 2) cells between 50 and 200 μm, density lower than 20% Percoll, and 3) cells between 50 and 200 μm, density of 30 to 40% Percoll. Fraction 1 consisted mainly of small, single, vacuolated cells. The other two cell fractions were a mixture of different cell types. Proteins secreted by the different fractions were tested on the same protein gel blot with both EP1 and EP4 antiserum (figure 4.3B). The distribution of EP1 and EP4 proteins between the fractions was clearly different:

cells smaller than 50 µm produced EP1, but no EP4, while cells between 50 and 200 µm and with a density of 30 to 40% Percoll secreted significantly less EP1 and a high amount of EP4. Thus, it appears that the cell populations secreting EP1 and EP4 proteins are not identical.

Figure 4.3. Cell type-specific occurrence of EP4.
A. EP4 proteins are associated with non-embryogenic cells. Anti-EP4 immunoblot of combined intracellular and cell wall proteins from globular stage somatic embryos (lane 1) or pro-embryogenic masses (lane 2) isolated manually, or from non-embryogenic cells from an embryo culture (lane 3) and a supension culture (lane 4) isolated by density gradient centrifugation.
B. Comparison of EP1 and EP4 secretion by different cell fractions. Cells were taken from an embryo culture of 7 days, fractionated by sieving and Percoll density gradient centrifugation and grown for an additional four days in fresh medium. Medium proteins were analysed by immunoblotting with both anti-EP1 and anti-EP4 serum. Lane 1, cells < 50 µm, density <20% Percoll. Lane 2, cells between 50 and 200 µm, density <20% Percoll. Lane 3, cell between 50 and 200 µm, density between 30 and 40% Percoll.

Immersion immunofluorescence localization of EP4 cell wall proteins on intact suspension cells revealed specific labeling of the cell walls of relatively small, vacuolated cells in small clusters (figure 4.4A,B). The highest intensity of fluorescence was observed in the walls separating adjacent cells. EP4 labeling was not observed on single cells, which was in line with the lack of secretion of 47 kD EP4 protein in the fraction of cells smaller than 50 µm consisting of single cells (figure 4.3B). Preimmune serum showed a low level of background labeling only (figure 4.4C).

Enzyme assays

Purified 47 kD EP4 protein was assayed for a number of enzymes known to occur in the apoplast (7). No activity was observed for endo-trans-xyloglucanase (SC Fry, personal communication), α- and β-glucosidase, α- and β-galactosidase, β-xylosidase, β-glucuronidase, α-mannosidase, protease, peroxidase, polyamine oxidase, phosphatase, non-specific esterase and pectinesterase (results not shown).
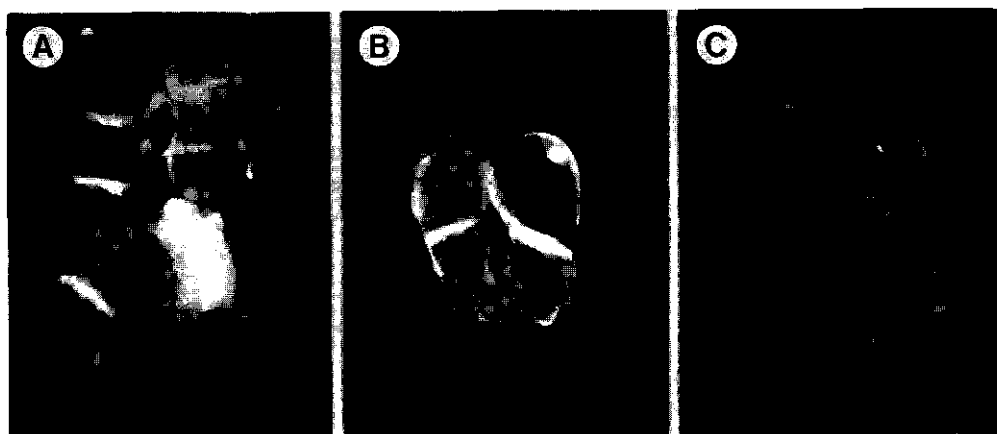
Figure 4.4. Immersion immunofluorescence localization of EP4 wall-bound protein on intact suspension cells. a,b. Anti-EP4 serum. c, preimmune serum.

Occurrence of EP4 in seedlings

Organ-specific expression of EP4 in carrot seedlings was analysed by means of immunoblotting. The highest level of EP4 protein was found in roots (figure 4.5). The amounts of EP4 in cotyledons, petioles and hypocotyls were significantly lower than in roots, while in leaves no EP4 protein was detectable. Qualitative differences were also observed: only in roots and hypocotyls a 45kD protein band was visible in addition to the 47kD band.
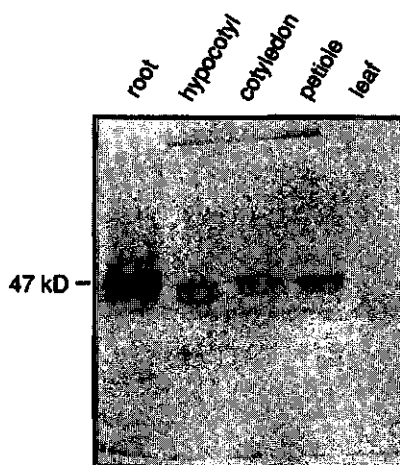
Figure 4.5. Occurrence of EP4 in seedling organs.
Anti-EP4 immunoblot of total proteins from different carrot seedling organs.

Cloning of EP4 cDNA

To further characterize the EP4 glycoprotein, we made an attempt to clone EP4 cDNA. Screening of a λZAPII cDNA expression library with the anti-EP4 serum did not yield positive clones. Therefore, oligonucleotide screening of the same library was performed. To this end, tryptic peptides of the 47kD protein were isolated and sequenced. Five peptide sequences were obtained, only one of which was sufficiently long and unambiguous to derive an oligonucleotide sequence (figure 4.6A). The following rules of thumb were applied in the design of the probe: 1) two bases without degeneracy at both ends, 2) degeneracies of three or four bases were replaced by inosine, and 3) in case of twofold degeneracies both possibilities were incorporated. The resulting mixture comprised 32 different oligonucleotides, each consisting of 32 bases and containing 6 inosines (figure 4.6B). The melting temperature of hybrids between an oligonucleotide and EP4 cDNA was calculated to be 70°C on average. Screening of 60,000 phage plaques yielded two positive clones: EP4a and EP4b, with an insert length of about 170 and 780 base pairs, respectively.

cDNA sequence of EP4

An open reading frame of 245 amino acids was found in the EP4B sequence starting at nucleotide 57 and extending to the 3' end of the cDNA, with the first in-frame methionine at amino acid position 20 (figure 4.6B). This open reading frame contained the peptide sequence, including amino acids that had not been used to construct the oligonucleotide, which confirmed that the reading frame encoded the EP4 protein. Only one amino acid difference was found: the deduced EP4B amino acid sequence contained an isoleucine instead of valine. A hydrophobic domain, which is likely to be the signal peptide of the secreted EP4 protein, was present at the N-terminus of the open reading frame. According to the weight matrix method of Von Heijne (23), this signal peptide may be cleaved after the alanine at position 17 or the serine at position 21. Two putative N-glycosylation sites were found at asparagine at position 150 and 193. EP4B encoded a protein with a calculated Mr of 26.5 kD. From the size of the primary translation product of 45 kD (see below) it can be concluded that EP4B encodes only the N-terminal two-third of the EP4 protein. EP4B completely contained EP4A, but they differed by four bases. These differences did not affect the amino acid sequence. The EP4 sequence was compared with sequences in EMBL (version 30.0) and Swiss-Prot (version 21.0) databases, but did not share significant homology with known protein or nucleic acid sequences.

A

Gly/Asp Leu Asn Pro Val Asn Leu Asp Ile Gln Val Ala Gln Phe(?)

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        AAT CCI GTI AAT TTI GAT ATI CAA GTI GCI CA
         C           C C     C       G
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B

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                                                         *
    A  G  N  I  K  I  C  S  P  T  L  D  K  P  *  R  S  *  S  M  G  R  F  S   5
GCTGGAAATATCAAAATCTGCTCACCGACACTTGATAAACCTTAAAGATCTTGAAGCATGGGGAGATTTTCA  72
                                    ↓              ↓
    V  F  S  V  F  L  V  S  I  L  V  A  H  C  T  S  Q  V  S  A  A  A  A  D  29
GTATTCTCTGTATTTTTAGTTTCTATTCTCGTTGCTCATTGTACTAGTCAGGTTTCTGCAGCTGCAGCAGAT 144

    T  C  K  F  P  A  I  F  N  F  G  D  A  N  S  D  T  G  A  F  A  A  W  F  53
ACTTGTAAATTTCCAGCAATTTTCAACTTCGGAGATGCGAACTCCGACACTGGTGCATTTGCTGCTTGGTTT 216

    F  G  N  P  P  F  F  G  Q  S  F  F  G  G  S  A  G  R  V  S  D  G  R  L  77
TTCGGCAACCCGCCTTTCTTCGGTCAGTCCTTCTTTGGAGGATCAGCAGGAAGAGTGTCTGATGGACGCCTC 288

    L  I  D  F  M  A  T  K  L  G  L  P  F  L  H  P  Y  M  D  S  L  G  A  D 101
TTGATCGACTTCATGGCAACTAAATTAGGATTACCATTCCTACATCCATACATGGATTCTTTGGGTGCTGAC 360

    F  A  H  G  A  N  F  A  E  I  L  S  T  I  A  L  P  P  A  N  N  I  I  P 125
TTCGCTCATGGTGCCAACTTTGCGGAAATCTTATCCACCATTGCACTTCCCCCGGCCAACAATATCATTCCC 432
                                                            EP4A→|G
    G  V  R  P  P  R  G  L  N  P  (I) N  L  D  I  Q  V  A  Q  F  A  Q  F  I 149
GGAGTTAGACCACCCCGGGGACTTAATCCCATCAATCTCGACATTCAAGTGGCTCAGTTCGCACAATTCATA 504
    G  C
    N  R  S  Q  T  I  R  Q  R  G  G  V  F  K  K  F  M  P  K  A  K  Y  F  S 173
AACCGATCTCAGACTATCAGGCAACGAGGAGGAGTTTTCAAGAAGTTCATGCCTAAGGCGAAATATTTTTCA 576
                                                        T
    Q  A  L  Y  T  I  D  M  G  Q  I  D  I  T  Q  L  F  L  N  N  K  T  D  E 197
CAAGCTTTGTATACTATTGATATGGGCCAAATTGATATCACCCAATTGTTCTTGAACAATAAGACCGACGAA 648
                        |←EP4A
    E  I  K  A  A  V  P  A  L  I  A  S  L  S  S  N  I  K  I  I  Y  S  L  G 221
GAAATTAAAGCTGCTGTGCCAGCCTTGATAGCTAGCTTGTCATCGAATATTAAGATTATCTATAGTCTGGGA 720

    A  R  S  F  W  I  H  N  L  G  P  N  G  C  L  P  I  L  L  T  L  A  P  E 245
GCTAGATCATTCTGGATCCATAACCTCGGACCCAACGGATGTCTTCCAATTCTTTTGACACTGGCTCCGGAA 792
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Figure 4.6. Derivation of EP4 oligonucleotide probe and EP4 cDNA sequence.

A. Tryptic peptide sequence and composition of derived oligonucleotide. I = inosine.

B. cDNA sequence of EP4A and B. The EP4B sequence is completely shown. The location of the EP4A sequence is indicated below EP4B; only the nucleotides that differ from EP4B are given. The sequence homologous to the peptide used to make the oligonucleotide is underlined. The isoleucine residue that replaces the valine of the peptide sequence is circled. The putative starting methionine is marked with an asterisk. Vertical arrows indicate possible signal peptide cleavage sites. Potential N-glycosylation sites are indicated with a horizontal bar over the amino acid sequence.

Additional evidence for the correspondence between EP4 protein and cDNA

Because the EP4B sequence was incomplete and contained one amino acid different from the 47kD tryptic peptide sequence, additional evidence was sought to confirm that the EP4 cDNA clones correspond to the EP4 protein. Therefore, the size of the EP4 primary translation product was determined. The mRNA selected by the EP4B plasmid was translated *in vitro* and the labeled product analysed by SDS-PAGE. The EP4B hybrid-selected protein had a size of 45 kD (figure 4.7A). Assuming signal peptide cleavage and glycosylation of the EP4 primary translation product, this size is within the range expected for a 47 kD mature protein.
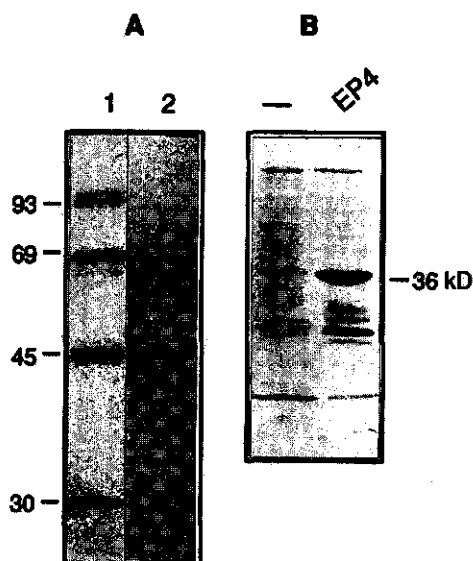

Figure 4.7. Correspondence between EP4 cDNA and protein.
A. *In vitro* translation of mRNA selected by denatured EP4b plasmid. Marker protein sizes are indicated in kilodalton.
B. Recognition of EP4 cDNA-encoded fusion protein by anti-EP4 serum. Anti-EP4 immunoblot of total protein produced by *E. coli* containing pGEX plasmid or pGEX-EP4 plasmid.

Further information was obtained from EP4B fusion protein expression. A 335 base pair SmaI-EcoRI fragment from the 3'-end of EP4B was cloned into the pGEX-1N expression vector (16). While the pGEX-1n encoded a 26 kD glutathione S-transferase, the pGEX-EP4 recombinant plasmid produced a fusion protein of 36 kD (figure 4.7B), which was 2 kD smaller than the 38 kD expected on the basis of the derived EP4 amino acid sequence. Immunoblotting showed that the serum raised against the 47kD EP4 glycoprotein from the culture medium recognized the glutathione S-transferase-EP4 fusion protein, but not glutathione S-transferase alone (figure 4.7B). These results supported the conclusion that the EP4 cDNA clones correspond to the EP4 protein.

Discussion

In this chapter, the purification and characterization of a carrot cell suspension extracellular glycoprotein, termed EP4, is described. The cell type-specific secretion of EP4 has been examined, the protein has been immunolocalized on suspension cells and an EP4 cDNA clone has been isolated and sequenced.

Carrot embryogenic cell suspensions are composed of cell types that differ in size, shape and degree of clustering and that follow different developmental routes (18,21). From studies of individual extracellular proteins the picture is emerging that these differences are correlated with the specific proteins that the cells secrete. This is in line with the generally held view that the extracellular matrix and its associated proteins play an important part in plant development (8,22). The small and cytoplasmic embryogenic cells are specifically marked by the expression of the secreted lipid transfer protein gene EP2 (19), while vacuolated, non-embryogenic cells secrete EP1 (chapter 2) and EP4 proteins. Immunolocalization of the wall-bound forms of EP1 and EP4 has revealed that each may be attributed to a different subpopulation of non-embryogenic cells. While EP1 is found in a loosely attached layer on the surface of single, sometimes highly expanded cells (chapter 2), EP4 appears to occur in the wall of clustered cells of moderate size, with the highest levels in the walls that separate neighbouring cells. Close inspection of these walls and the clusters that contain them suggests that they are cross-walls formed by cell division in expanded cells. Cross-wall formation in expanded cells has been described for suspension culture cells (18), but also occurs *in planta*, as for instance in suspensor development (17). Since EP4 protein is not found in the walls of proembryogenic masses or somatic embryos, which show a meristematic type of division, EP4 function appears to be restricted to cell division in expanded, non-embryogenic suspension cells. If this hypothesis is correct, the enhanced level of EP4 in roots indicates that division of elongated cells apparently occurs more frequently in roots than in seedling hypocotyls, cotyledons or leaves.

Since expression of the EP1 gene in a seedling is found exclusively in the epidermis, it is likely that suspension cells that show EP1 expression are of epidermal origin (chapter 3). The localization of the EP4 glycoprotein in the cell walls of a different population of non-embryogenic cells may then be explained in two ways. EP4-positive cells could either be derived from a different cell type expressing EP4 in the original explant, or they might represent a distinct developmental phase of the epidermal cells that is induced by culturing of the cells. *In situ* localization of the EP4 mRNA or protein in seedlings should allow us to decide between these possibilities.

In cell suspensions, EP4 protein is found in two different forms: a 47 kD medium protein and a 45 kD protein ionically bound to the cell wall. The difference in size and wall binding between the 45 and the 47 kD proteins may be due to differences in glycosylation or proteolytic

processing. A difference in glycosylation may expose the positively charged residues adjacent to glycosylation sites (arginine at position 151 and lysine at position 193) resulting in an increased affinity of the 45 kD protein for the negatively charged cell wall. Proteolytic cleavage of the 47 kD EP4 protein could remove a negatively charged peptide or induce a conformational change, thereby allowing the 45 kD form to bind to the wall. We have previously reported the existence of wall-bound and free forms of the EP1 secreted glycoprotein (chapter 2). EP1 heterogeneity was found to be due to different posttranslational modifications of a single polypeptide core. The same may be true for EP4, since translation of mRNA selected by the EP4b cDNA yielded only a single product of 45kD.

On the basis of the absence in the deduced EP4 protein sequence of repetitive motifs, as found in structural cell wall proteins, and the large number of enzymes known to inhabit the apoplast (7), we assume that the EP4 glycoprotein is an enzyme. However, we could neither detect enzyme activity in several assays for secreted enzymes nor find homology between EP4 and sequences deposited in databases. Unfortunately, we have not been able to reproduce the initial experiments showing a stimulation of the frequency of embryo formation in the ts11 cell line. Addition of the purified 47kD EP4 glycoprotein to wild-type embryo cultures had no effect on somatic embryo development, even when the amount added was 50 to 100 times the amount normally present in the culture medium. If the binding to the cell wall of the 45kD form of EP4 is interpreted to reflect a higher affinity for its putative substrate in the wall, it might be of interest to purify the 45kD cell wall form of EP4 and to examine its effect on different kinds of suspension cells.

In conclusion, although the functional significance of the EP4 glycoprotein remains to be established, the presence of this protein does appear to be cell-specific. The availability of different probes, such as EP1, EP2 and EP4, each of which identifies a specific cell type in embryogenic suspension cultures, may greatly facilitate an investigation into the fate of individual suspension cells that either acquire or lose embryogenic potential (21).

Materials and methods

Plant material and cell cultures
   Carrot seedlings were grown from "Flakkese" SG766 Trophy seeds, kindly donated by Zaadunie, Enkhuizen, The Netherlands. Embryogenic suspension cultures were initiated from cut seedling roots and propagated in 2,4-D-containing B5 medium as described previously (5). For the initiation of embryo cultures, suspension cells were sieved through nylon meshes and the fraction between 50 and 125 µm, enriched for proembryogenic masses, was diluted to 20,000 cells per ml in 2,4-D-free B5 medium. For density gradient fractionation, cells were layered on Percoll (Pharmacia LKB) block gradients (20-30-40-50%) and centrifuged at 175g for 15 minutes.

Protein purification, immunological techniques and silver staining

The 47kD EP4 glycoprotein was purified from conditioned embryo culture media by three-step DEAE anion exchange chromatography in 25 mM Tris-HCl (pH 8.5) as described by De Jong et al. (4). The 47kD EP4 protein eluted from the final FPLC MonoQ column (LKB-Pharmacia) at 175mM KCl. For anti-EP4 serum production in balb/c mice, the 47 kD glycoprotein was further purified from FPLC fractions by preparative SDS-PAGE, followed by brief staining with Coomassie Brilliant Blue and excision of the protein band from the gel. The gel strip was ground in PBS and the mice were immunized by three intraperitoneal injections of 2 to 5 µg of protein at 4-week intervals. Antisera were assayed in dilutions of 1:3000 to 1:5000 on nitrocellulose blots from 12.5% SDS-polyacrylamide mini gels (20), using alkaline phosphatase-conjugated goat-anti-mouse IgG for detection as described by Sterk et al. (19). Protein samples for SDS-PAGE were prepared as described previously (20). Silver staining of protein gels was performed essentially as described by Blum et al. (2), except that formaldehyde was omitted from the fixing solution and ethanol was used instead of methanol. Localization of EP4 protein on whole cells by immersion immunofluorescence was performed as described previously (20)

Protein sequencing, oligonucleotide synthesis and labeling

For protein sequencing, the 47 kD EP4 protein was further purified from contaminating proteins by two-dimensional PAGE and blotted onto PVDF membranes. The 47 kD protein spot was visualized with amido black and cut from the filter. *In situ* tryptic digestions of the 47 kD protein and amino acid sequence determination of the tryptic peptides were performed at the Laboratory of Physiological Chemistry, University of Gent, Gent, Belgium, as described by Bauw et al. (1).

Oligonucleotides were synthesized using β-cyanoethyl phosphramidites with a Cyclone DNA synthesizer (Bioresearch Inc.). Oligonucleotides were labeled with T4-polynucleotide kinase and $[\gamma\text{-}^{32}P]$dATP in 50mM Tris, pH 7.6, 10mM MgCl$_2$, 5mM DTT, 0.1mM spermidine and 0.1mM EDTA. Unincorporated $[\gamma\text{-}^{32}P]$dATP was removed by ethanol precipitation in the presence of 1M ammonium acetate (12).

cDNA library screening, subcloning and DNA sequencing.

A λZAPII cDNA library (Stratagene) was prepared against poly(A)$^+$-RNA from suspension culture cells (sieved < 170µm) and embryo culture cells (sieved < 300µm) using oligo(dT) and random primers. Nitrocellulose replica's of phage plaque DNA were prepared by *in situ* amplification (14) to enhance hybridization signals. This amplification step was found to be essential for the detection of positive plaques. After prehybridization at 40°C in 6xSSC, 5xDenhardt solution (12), 0.5% SDS and 200µg/ml denatured salmon sperm DNA the filters were hybridized for 16 hours at 40°C in the same solution containing labeled oligonucleotide. Then the filters were washed twice in 6xSSC, 0.1% SDS at 40°C and autoradiographed. Positive plaques were purified by a second screening. The Bluescript plasmid containg the insert was excised in vivo from the λZAPII phage exactly as described in the manufacturer's protocol. Briefly, XL1-Blue cells were co-infected with λZAPII phage and R408 helper phage. After heating, the supernatant was plated with XL1-Blue cells on medium containing 50µg/ml ampicillin to select for plasmid-containing bacteria. Inserts were further subcloned in Bluescript plasmids using standard procedures (14). Plasmid DNA was denatured by alkali and sequenced with the dideoxy chain termination method using standard methods. Sequencing errors were eliminated by sequencing both strands of overlapping clones.

Hybrid selected translation

EP4 mRNA was selected with 20µg of denatured EP4b plasmid immobilized on a nitrocellulose filter following the method described by Maniatis (12), except that hybridization was done in 50%(v/v) formamide, 0.1% SDS, 0.6M NaCl, 4mM EDTA, 80mM Tris-HCl, pH 7.8 at 50°C for three hours, followed by three hours at 40°C. Selected mRNA was translated in a rabbit reticulocyte lysate (Green Hectares) containing [^{35}S]methionine.

Fusion protein expression

A SmaI-EcoRI fragment of the EP4B cDNA was cloned into plasmid pGEX-1N (Amrad), which allows high level expression of an *E. coli* fusion protein with the C-terminus of *Schistosoma japonicum* glutathione S-transferase protein (16). For fusion protein production cells were grown in LB medium (12), induced with 0.1mM IPTG and harvested after three to four hours.

Enzyme assays

Enzyme activities of purified EP4 medium glycoprotein were assayed under the following conditions: α–galactosidase, β–galactosidase, α–mannosidase, α–glucosidase, β–glucosidase, β–glucuronidase and β–xylosidase: 0.1 M sodium acetate, pH 5.2, 30°C, substrate: *o*-nitrophenyl glycosides of the corresponding sugars (Serva). Peroxidase: according to Cordewener et al. (3). Polyamine oxidase: substrate: spermidine; H_2O_2 production was monitored with a peroxidase assay (3). Esterase: 0.1 M sodium acetate, pH 5.2, 30°C, substrate: *o*-nitrophenyl acetate. Pectinesterase: substrate: esterified pectin, pH change monitored with methylred. Protease: 15 mM citrate, 10 mM phosphate, pH 4.6, 35°C, substrate: Azocoll (Sigma) or Azocaseine (Serva). Phosphatase: 0.1 M sodium acetate pH 5.2 or 0.1 M Tris pH 9, 30°C, substrate *o*-nitrophenylphosphate.

Acknowledgments

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Chapter 5

General discussion

Every plant cell is enveloped by a cell wall. Two general types of cell walls can be discerned in higher plants. Primary cell walls are thin (about 100 nm) and are deposited by dividing and expanding cells. As growth ceases and cells acquire specialized functions, they may form a more rigid secondary wall. The plant primary cell wall is now generally believed to play an important part in the early phases of plant growth and development. This primary wall is faced with the complex task of providing a strong skeleton for the organism as a whole and protecting it from environmental influences, such as attack by pathogens or drought, while at the same time it has to retain the plasticity to accomodate controlled cell extension in response to turgor pressure. The latter process is crucial for plant morphogenesis, since, due to the very presence of the wall, morphogenesis is only determined by regulation of the rate and plane of cell division and the degree and direction of expansion of the cells after division. For this purpose, plants have evolved an intricate wall structure composed of many different polymers. Regulation of wall properties can be achieved by secretion of new wall constituents or enzymes that modify the polymers that are already present. Numerous activities of enzymes that have cell wall polymers as their potential substrates have indeed been detected in the cell wall (35). This chapter will provide an overview of the constituents and assembly of the plant primary cell wall, as well as the biochemical processes that are thought to take place in it. In this background, the possible functions of proteins secreted into plant cell culture media, with special focus on somatic embryogenesis, are discussed. Finally, some more general questions regarding the control of extracellular protein function and interactions between different cell types in embryogenic plant cell cultures are addressed.

5.1. Components of the primary cell wall

Any attempt to unravel the roles of the primary cell wall, and the proteins that are associated with it, in plant developmental processes must set out with a description of the structure and properties of its constituents. The primary cell wall has a water content of 70 to 80%, and its solids are composed - on the average - of 90% polysaccharides and 10% proteins (66). Both of these may be further subdivided in classes of polymers with widely differing properties. This complex array of cell wall polymers is described below. The focus will be on dicotyledonous (dicot) species with occasional reference to *Gramineae* (grasses, cereals), the best studied monocotyledonous (monocot) family.

5.1.1. Polysaccharides

Cell wall polysaccharides can be classified into three groups: pectins, hemicelluloses and cellulose. This classification has been based on procedures used to sequentially extract polysaccharides from the wall. The different components of the fractions obtained have been found to share certain properties and the classical terminology has been maintained. Except for cellulose and callose, cell wall polysaccharides are synthesized in the endomembrane system and subsequently secreted; cellulose and callose are polymerized by plasma membrane-localized enzymes (68). Most of the following information has been extracted from a number of review articles (21,22,35,38,39,40,41,48,50,62,66,77,97). For further details, I refer to the papers cited therein.

Pectins

Pectins are an extremely complex mixture of polysaccharides that make up 15-35% of the dicot primary cell wall (dry weight) and are the major constituents of the middle lamella. Middle lamella pectins differ from cell wall pectins in composition (80). Most, but not all (80), pectic polysaccharides can be extracted with hot water, ammonium oxalate, weak acid or chelating agents. The majority of the polysaccharides obtained are rich in galacturonic acid, but some are largely composed of galactose and/or arabinose. Since some of the extraction procedures are likely to cleave covalent bonds (80), the different polymer types described below are currently thought to be domains of larger molecules *in muro* (48). Pectins extracted by mild procedures are 250 to 500nm long (62). Graminaeceous monocots contain very low levels of pectin (~3%). Other monocots are closer to dicots or have intermediate amounts of pectin (49). The pectins fall into four classes:

1. Homogalacturonan

Homogalacturonan is an unbranched chain of α-(1-4)-linked galacturonic acid with occasional rhamnose residues that give rise to kinks in the molecule. At the time of secretion a large proportion of the carboxyl groups is methyl-esterified and these are distributed in blocks along the polysaccharide chain (95). In some pectins hydroxyl groups of galacturonic acid residues are acetylated.

2. Rhamnogalacturonan I

Rhamnogalacturonan I (RGI) consists of 1000-2000 alternating residues of galacturonic acid and rhamnose. About 50% of the rhamnose units bear side chains of 1 to more than 15 arabinose and/or galactose residues in many (up to 30) different branched structures (66). In

78

some *Chenopodiaceae* (spinach and beet) the phenolic groups ferulate and *p*-coumarate have been found to be attached to terminal arabinose or galactose (38).

3. Rhamnogalacturonan II

The structure of rhamnogalacturonan II (RGII) is quite different from RGI. In sycamore it is a small, highly branched molecule consisting of only about 60 residues with 12 different sugars (38), among which many exotic ones like 2-O-methylglucose, 2-O-methylxylose, apiose, 3-C-carboxy-5-deoxyxylose and 3-deoxy-manno-octulosonic acid. It has a galacturonic acid-rich backbone, with several copies of two different heptasaccharides and some smaller oligosaccharides as side chains. Because RGII is solubilized from the wall with an endopolygalacturonase it is believed to be covalently linked to wall polymers through homogalacturonan stretches.

4. Neutral pectins

This class comprises arabinans, galactans and arabinogalactans with a composition that is similar to the side chains of RGI. Because pectins are susceptible to degradation, it has been argued that the separate occurrence of these neutral polysaccharides an artefact of the isolation procedure (80).

Hemicelluloses

After extraction of pectins the hemicelluloses can be obtained by treatment of the walls with alkali. Again different polysaccharides are found, most of which are capable of hydrogen-bonding to cellulose. Hemicelluloses have been "defined" as "non-cellulosic wall polysaccharides other than pectin" (38). The major hemicellulose of dicots is xyloglucan, while in *Gramineae* arabinoxylan and mixed-linkage β-glucan are abundant.

1. Xyloglucan

The backbone of xyloglucan is linear and consists of 300 to 3000 β-1,4-linked residues of glucose (40), giving rise to a molecule of 150 to 1500 nm. Sixty to 75% of the glucose units, usually groups of three consecutive residues, have α-xylose linked to position 6. In turn, 10-20% of the xyloses are β–linked to galactose or to galactose plus an α-linked fucose. The molecule can be cleaved by cellulase at unsubstituted glucosyl residues. The side chains probably fold around the backbone, which results in a string-shaped molecule of 0.1 to 1.5 μm long. Xyloglucan is able to associate with cellulose microfibrils (see 5.2.1.) by means of hydrogen bonds.

2. Arabinans/galactans/arabinogalactans (AG)

The hemicellulosic AG has a composition similar to the AG side chains of rhamnogalacturonan I, but the chains probably consist of up to 6000 sugar residues (92).

3. Xylans

Xylans have a backbone of β-1,4-linked xylose which may carry many susbstitutions: 1) acetyl ester groups on many of the xyloses, 2) oligosaccharide side chains containing arabinose (*Gramineae*: arabinoxylan) and/or galactose, xylose and (4-metyl-)glucuronic acid (pea: glucuronoxylan), 3) feruloyl ester groups on terminal arabinose residues (*Gramineae*). Like xyloglucan the xylans can hydrogen bond to cellulose, but the affinity decreases with the number of carbohydrate side chains.

4. Mixed-linkage β-glucan

Mixed-linkage β-glucan consists entirely of β-glucose residues, of which 30% are 1,3-linked and 70% 1,4-linked. The 1,4 linkages commonly occur as blocks of two or three residues, with an occasional stretch of 10, separated by single 1,3-linked glucoses. The molecule is very flexible, except for the longer 1,4-linked regions that are able to hydrogen bond to cellulose. The 1,4 linkages may be cleaved by cellulase.

5. Callose

Callose is an unbranched chain of β-1,3-linked glucose units. It is present at a low level around the plasmodesmata of the primary walls of healthy cells (69), but it accumulates upon wounding.

Cellulose

The remaining 20-30% of the cell wall after pectin and hemicellulose extraction consists of cellulose. Like mixed-linkage glucan and callose, it is a linear chain of β-glucose, but it differs in having only 1-4-linkages. The cellulose chains of primary walls contain either less than 500 or between 2500 and 4500 glucose residues. The conformation of cellulose allows numerous hydrogen bonds to be formed between adjacent molecules. About 150 cellulose molecules thus aggregate to form very strong, crystalline microfibrils of 0.5 to 3 mm long.

5.1.2. Proteins

Structural proteins

Extensin

Extensins (12,19,38,94) are characterized by the repetitive sequence Ser-Pro-Pro-Pro-Pro, of which most of the proline residues are hydroxylated and bear tri- and tetra-arabinoside side chains. Thus, mature carrot extensin has a carbohydrate content of 65% and has a molecular mass of 86 kDa. Extensins are often referred to as hydroxyproline-rich glycoproteins (HRGP). Interspersed between the hydroxyproline repeats are short sequences rich in tyrosine, lysine, valine and histidine, which also show some repetition and give the molecule a large positive charge at physiological pH. Electron microscopy has shown that the molecule has the structure of a fibrous rod, with occasional kinks. These kinks are thought to be due to intramolecular cross-links between tyrosine residues occurring in the sequence Tyr-Lys-Tyr-Lys (28). Different forms of extensin, encoded by a multigene family, can be found in a single species; at least four genes occur in tomato (19). A gene encoding an extensin-like protein has been isolated from maize (89). It differs from dicot extensins in being threonine-rich and having a different repeat structure.

Proline-rich proteins (PRPs)

PRPs, or genes and cDNAs encoding them, have been isolated from soybean (2,33,47) and carrot (15). They are composed of the repetitive motif Pro-Pro-X-Y-Z, where X-Y-Z may be Val-Tyr/Glu-Lys or His-Glu-Lys. Other amino acid combinations also occur. In one of the soybean PRPs only half of the prolines is hydroxylated and not or scarcely glycosylated. Taken together with the sequence data, this suggests that PRPs are not related to extensin. A sugar beet wall protein, however, has sequence homology to the PRPs but has all other characteristics of a typical extensin (59). This indicates that the PRPs and extensins may be at opposite ends of a spectrum of (hydroxy)proline-rich cell wall proteins.

Glycine-rich proteins (GRPs)

Genes encoding glycine-rich proteins have been isolated from *Petunia*, bean and *Arabidopsis* (17,26,51,96). Their sequences were found to be similar and highly repetitive: $[Gly-Gly-Gly-X]_n$. The proposed protein structure consists of eight antiparallel strands forming a sheet, with all non-glycine residues (leucine, valine, alanine) extending to one side of the molecule, which would permit hydrophobic interactions.

Arabinogalactan proteins (AGPs)

The AGPs are a heterogeneous family of proteoglycans which have in common that they contain 90-98% carbohydrate consisting mainly of arabinose and galactose in varying branched structures (32). In addition, the protein core is rich in hydroxyproline and in serine, alanine and glycine.

Enzymes

The structural components of the cell wall are subject to the action of numerous enzymes, most of which appear to have a catabolic function. Some enzymes can freely diffuse in the apoplast, others are bound to the wall through ionic interactions or covalent bonds. Known cell wall enzyme activities have been listed by Fry (35,38). Therefore, only a few examples of each class of enzymes are given below.

Glycosidases

Glycosidases release monosaccharides from the non-reducing end of a polysaccharide. These enzymes usually have a high specificity for the released sugar and its linkage type, but their activity is much less dependent on the polymer from which the sugar is cleaved. Examples are β-glucosidase, α-galacturonidase and α-mannosidase.

Endoglycanases

These enzymes catalyse the hydrolysis of carbohydrate linkages in mid-chain. Examples are endopolygalacturonase, cellulase and endo-β-1,3-glucanase. As for glycosidases, the cleavage sites of endoglycanases may occur in different kinds of substrates.

Transglycosylases

An enzyme is said to have transglycosylase activity if after cleavage it transfers the released (chain of) sugar residue(s) to a different chain, thereby conserving the total number of glycosidic bonds. An endo-trans-xyloglucanase activity has recently been demonstrated *in vivo* (83).

Other hydrolases

A variety of esterase, protease and phosphatase activities has been detected in the wall. Some esterases may act on pectins, cleaving methyl-, acetyl- or feruloyl-ester bonds. Proteases may degrade any of the wall proteins, but no specific cases have been described yet. Extracellular substrates for phosphatase are not known.

Peroxidases

Peroxidases are heme-containing glycoproteins that can catalyse intra- or intermolecular oxidative cross-linking reactions like the polymerisation of lignin (14) or the coupling of tyrosine residues of extensin and ferulate side chains of pectins and xylans (31,34,36). In addition, they are capable of oxidation of auxins. A large number of different peroxidases is found that differ in their isoelectric point, substrate preference (*in vitro*) and/or organ-specific localization (45).

Oxidases

Substrates for oxidases are ascorbate, polyphenols, indoleacetic acid and polyamines. The oxidation of polyamines generates H_2O_2, which has been inferred to be a mechanism to control the activity of peroxidases (1).

Malate dehydrogenase

Malate dehydrogenase activity results in the formation of reduced NAD. The oxidation of reduced NAD by peroxidase results in the formation of H_2O_2. This cycling of NAD in the wall may be used as an alternative mechanism to generate the H_2O_2 required for peroxidase activity.

Other proteins

Cell walls may contain proteins that are difficult to classify as "enzyme" or "structural protein". Cell walls of the endosperm and leaves of cereals contain high levels of thionins (6,42). These small polypeptides have an antimicrobial activity possibly due to an increase in membrane permeability, which results in leakage of ions, protein and nucleotides. Thionins may also be involved in redox reactions in the wall. Furthermore they have been shown to inhibit α-amylase and papain.

Lectins have been extracted from the cell walls of members of the *Leguminosae* and *Gramineae* (30). A lectin has been broadly defined as a carbohydrate-binding protein other than an antibody or an enzyme (3), but it is unclear whether they serve a structural function. It is not known whether similar extracellular lectins exist in other plant families. AGPs (see above) are able to bind to artificial carbohydrate antigens and may therefore also be regarded as lectins (32). However, binding to other cell wall components has not yet been reported.

5.2. Construction of the primary cell wall

In contrast to the structure of many of the cell wall's building blocks, knowledge of the assembly of these components into an ordered functional structure is very incomplete. The wall polymers may be held together by a variety of intermolecular bridges, such as hydrogen bonds, ionic bonds and covalent bonds, thus giving rise to a viscoelastic gel. It is still unclear which of the cross-links are involved in the regulation of wall mechanics. In recent dicot wall models (77,92,97) the presence of three separate polymer systems has been proposed: a cellulose/xyloglucan network, an extensin network and a pectin network. These networks appear to be assembled into a functional wall structure through non-covalent interactions and physical entanglement (92).

5.2.1. Cellulose/xyloglucan network

There is growing evidence that a cellulose/xyloglucan network is a central factor in the regulation of cell expansion (39). NMR and binding studies (46,61) have suggested that in the wall the cellulose microfibrils are coated with xyloglucan through hydrogen bonds. Because of their size and flexibility, individual xyloglucan molecules may bind to, and thereby cross-link, different microfibrils, which results in a network. Electron microscopy studies support this configuration (63). With such a network, expanding cells encounter resistance from xyloglucan molecules that have become stretched taut between different cellulose microfibrils. Continued expansion would then require release of hydrogen bonds or enzymatic cleavage of the stretched xyloglucan chains, because the cellulose microfibrils are not extensible. A model has been proposed for auxin-induced wall loosening in stem and coleoptile growth involving a cellulase that hydrolyzes xyloglucan rather than cellulose *in vivo* (39). According to this model, auxin increases the endo-xyloglucanase activity by *de novo* synthesis of cellulase or by proton secretion which activates the enzyme. Undesired weakening of the cell wall by xyloglucan cleavage may be prevented if the enzyme also has a trans-xyloglucanase activity that reforms bonds between different segments. Such an activity has indeed been found in expanding suspension cell walls (83).

Other enzymes that may augment the susceptibility of xyloglucan to enzymatic cleavage are glycosidases able to remove side chains from the xyloglucan backbone, viz. α-fucosidase, β-galactosidase and α-xylosidase.

In graminaeceous monocots the role of xyloglucan is probably fulfilled by mixed-linkage β-glucan.

5.2.2. Extensin network

Extensin is secreted as a soluble protein but gradually becomes inextractable from the wall, which indicates that the protein is somehow coupled to the wall. That this coupling takes the form of a covalently cross-linked network has been inferred from several observations. First, treatment of complete cell walls with hydrogen fluoride dissolves all carbohydrate, but an insoluble structure consisting of cross-linked proteins, including extensin, remains (67). Second, the tyrosine dimer isodityrosine has been found in the wall (34) and in purified tomato extensin (28). The presence of isodityrosine is no direct proof for intermolecular cross-linking, because at least some of the bridges are intramolecular (28). Third, covalently bound extensin can be solubilized by an agent that can specifically cleave isodityrosine (5). Fourth, wall-derived extensin multimers have been visualized (19). Peroxidase is thought to be the cross-linking agent and peroxidases indeed form extensin dimers *in vitro* (18,31), though in solution the cross-link formed is dityrosine, not isodityrosine (18). Peroxidase may exhibit the isodityrosine-synthesizing activity only under suitable conditions *in muro*.

It is a long-standing hypothesis that extensin network formation is a major determinant of cell expansion (58), but this is far from proven. In pea epicotyls, extensin accumulation coincided with the cessation of growth (79), which is in agreement with a role in extension. However, other localization studies argue against a role in expansion. In soybean, high levels of extensin mRNAs and soluble protein were detected in meristematic cells of seedling root and hypocotyl apices and cambium cells of older stems and petioles. Insolubilization of extensins, however, occurred clearly before elongation stopped (100). The high level of extensin deposited in soybean seed coats (13) or secreted in response to wounding and infection (29, 90) point to a strengthening and protective function unrelated to extensibility. Furthermore, since hypocotyl elongation appears to be regulated at the level of the epidermis (10,11, however see 76), the observed secretion of extensin throughout the shoot apex (100) would be superfluous. At present, the function of extensin in plant cell wall architecture remains an open question.

5.2.3. Pectin network

Different kinds of cross-links may give rise to a strong pectin gel. Well-established is the formation of calcium ion bridges between stretches of un-esterified homogalacturonan (48). The strength of this type of pectin gel can be regulated by the level of calcium, the pH, or the action of pectin methyl esterase. This apoplastic enzyme is thought to remove methyl groups from esterified pectin blocks delimited by rhamnose residues, permitting strong calcium pectate gel formation. Immunolocalization has shown that unesterified homogalacturonan occurs in the

middle lamella of the root cortex, with high concentrations around cell junctions at intercellular spaces (54). This suggests that calcium pectate gels constitute the "glue" between cells. Unesterified polygalacturonate is also present close to the plasma membrane (54). Since this is thought to be the load-bearing region of the primary cell wall (91), it is possible that calcium-pectin gels also influence wall extensibility. This is supported by the wall-loosening effect of calcium chelation on soybean hypocotyls (98). Other proposed pectin cross-links include ester or secondary amide linkages to other wall polysaccharides and peroxidase-catalyzed coupling of phenolic goups (36). The stiffening effect of cross-link formation may be counteracted by esterases that cleave off the phenolic groups, or by endopolygalacturonase, which cuts homogalacturonan stretches.

The existence of an independent cross-linked pectin network that restricts cell expansion is supported by the behaviour of tomato cells adapted to growth on an inhibitor of cellulose synthesis (81). Such cells lack a cellulose/xyloglucan network and appear to consist primarily of pectin with phenolic cross-links. Despite the aberrant walls of these cells, cell division and controlled extension is possible.

A distinct role of pectins in the cell wall may be in the determination of sieving properties. Permeation and electron microscopy studies both indicate that the size of wall pores increases when pectins are removed from the wall (4,63). Thus, pectins may be essential to prevent leakage of larger molecules from the cell wall or to keep these molecules from reaching the plasma membrane.

5.2.4. Additional wall polymers

AGPs

It has been demonstrated with monoclonal antibodies that a family of plasma membrane AGPs is localized at the outer face of the protoplast and the inner 20-30 nm of the cell wall (53,72). This observation led to the speculation that AGPs may anchor the primary cell wall to the plasma membrane by binding to wall polysaccharides with their lectin domain. There appear to be subtle differences in AGP structure between tissues or regions of developing organs, as indicated by the differential expression AGP epitopes recognized by monoclonal antibodies (53,55,73,74,84). The bearing of these patterns on morphogenesis and differentiation is unknown. AGPs appear to be rapidly turned over by extracellular hydrolytic enzymes (32,43,74).

GRPs

Though GRPs are deposited in primary cell walls, their function may be related to secondary wall formation in specific cell types. GRP expression has been localized in lignified cells and cells that are going to be lignified (51,52,99,100). This has led to the view that GRPs, by means of their tyrosine side chains, constitute an initiation site for the deposition of lignin.

5.2.5. A model for the primary cell wall

Recently, a model for the primary cell wall has been presented in which the wall polymers are associated mostly non-covalently (92). Although the model is based on experiments with pea, it may apply to other dicots as well. In this model, arabinan/galactan forms a loosely bound layer around xyloglucan-coated microfibrils and interacts with the side chains of pectic rhamnogalacturonan blocks. Positively charged extensin molecules may align with stretches of negatively charged homogalacturonan (92). Although not mentioned in the model, it is likely that the polysaccharide side chains of plasma membrane arabinogalactan proteins extend some distance into the cell wall and possibly interact with the arabinan/galactan.
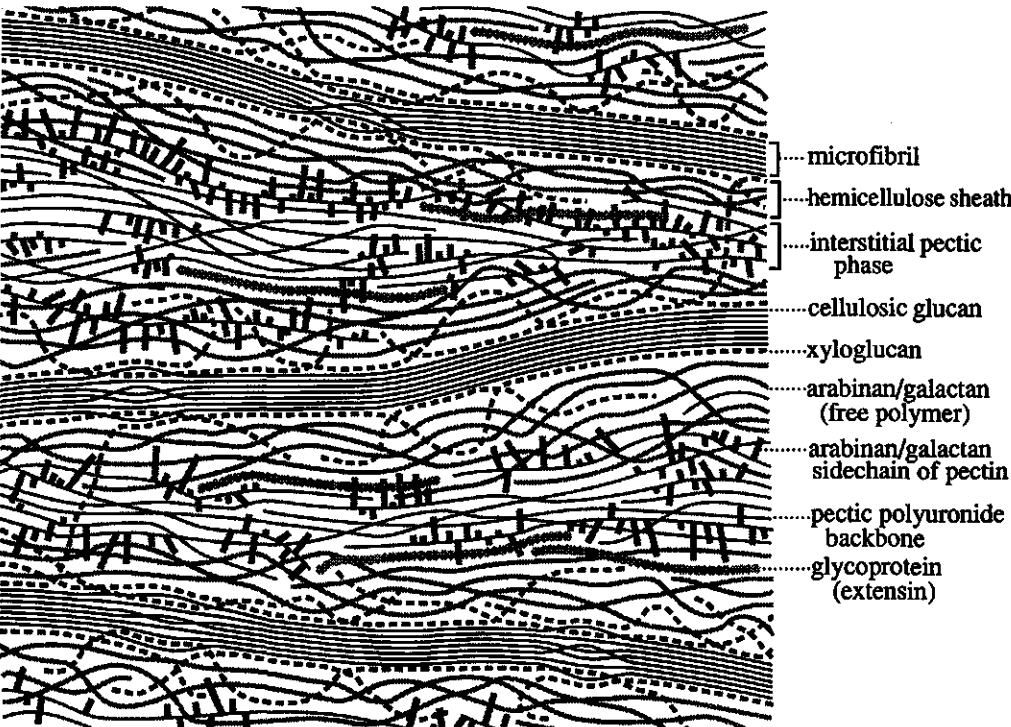

Figure 5.1. A model of the pea primary cell wall (ref. 92, courtesy of Dr. P.M. Ray)

Together with the physical and biochemical properties of the wall polymers described above, this model leads to the following picture of primary cell wall functioning. Deposition of cellulose microfibrils in the aqueous environment of the primary cell wall does not, by itself, result in a strong network. Therefore, the fibrils have to be cross-linked with xyloglucan. This structure is strong, yet flexible enough to permit growth, because xyloglucan can be enzymatically cleaved and the resulting ends rejoined to other xyloglucan molecules. To keep the microfibrils apart, they are embedded in a viscous gel of pectin and arabinan/galactan, in which they can slide past and away from each other. The viscosity of this gel could attenuate the motility of the microfibrils and may be adjusted by enzymatic degradation of polysaccharides or by changes in non-covalent cross-links between these polysaccharides. Finally, extensin forms an independent network that may make the wall tough, i.e. resistant to piercing, without affecting extension.

The available evidence, then, suggests that the primary cell wall loosening essential for growth may be effected by the, possibly concerted, action of several mechanisms: cleavage of covalent bonds, exchange of bonds between existing and newly secreted polymers, and reduction of the strength or number of non-covalent bonds.

5.2.6. Cell wall fragments

The structural complexity of pectins and, to a lesser extent, hemicelluloses makes it unlikely that these molecules serve only a structural purpose. Indeed, it has been discovered that the cell wall is a source of chemical messages: the oligosaccharins. A range of effects on plant growth and development has been reported for these cell wall-derived oligosaccharides.

Xyloglucan fragments produced by cellulase digestion inhibited auxin-induced stem elongation (64) or mimicked the auxin effect (65), depending on the specific oligosaccharin structure and the concentration applied. The growth-promoting effect appears to be the result of enhancement of cellulase-catalyzed mid-chain hydrolysis of load-bearing xyloglucan molecules (65). Acid treatment of sycamore cell walls released a pectic oligosaccharide that inhibits flowering and promotes vegetative growth in *Lemna gibba* (44). Other pectic wall fragments have been shown to induce the formation of flowers on tobacco thin-cell-layer explants or to inhibit root formation on these explants under root-inducing conditions (27). Oligogalacturonides are known to elicit the synthesis of phytoalexins (70) and have also been reported to affect auxin-induced growth (8). The effects of pectic oligosaccharides may be due to the presence of oxidized oligogalacturonides, which have been shown to promote the oxidation of the auxin indoleacetic acid by peroxidase (75). Since auxins are involved in many aspects of differentiation and growth (23), their oxidation might account for the observed effects.

Though many of the wall fragments have been generated *in vitro*, oligosaccharins may also occur *in vivo*, as has been shown for the xyloglucan fragment with anti-auxin activity (37). The enzymes required to release the fragments may be secreted by invading pathogens or by the plant cells themselves. These enzymes include endoxyloglucanase (cellulase) and endopolygalacturonase, but enzymes cleaving RGI or RGII as well as glycosidases that modify the released fragments can also be envisaged.

5.3. Plant cell cultures

Whereas the cell wall model described in section 5.2 suggests an important function for extracellular matrix proteins in plant development, studies that directly demonstrate the role of an individual cell wall protein in a specific developmental process are rare. In part, this is due to the lack of plants mutated in one of their extracellular protein genes. More important, probably, is the difficulty of designing experiments that directly assay the effect of secreted proteins on the intact cell wall *in planta*. Thus, simple and accessible experimental systems are needed to study the role of secreted proteins. Somatic embryogenesis in cell suspension cultures represents such a system. Because the culture medium is continuous with the liquid phase of the cell wall, secreted soluble components that are associated with developmental changes diffuse from the cell wall into the medium. This renders conditioned culture medium a source of biologically interesting molecules and also allows these molecules to be directly tested for their activity in somatic embryo development by addition to the culture medium.

### 5.3.1. Functions of secreted proteins in somatic embryogenesis

How can we explain a role of secreted proteins in somatic embryogenesis? Embryos invariantly consist of small, cytoplasmic cells that are closely packed together. Thus, the morphology of embryos suggests that in order to embark on an embryogenic pathway cultured plant cells have to fulfill at least two requirements: 1) to prevent expansion after division and 2) to make daughter cells adhere tightly (41).

Limitation of cell expansion
The transition of expanded, non-dividing somatic cells into embryogenic cells requires initiation of cell division and a mechanism that, at least temporarily, prevents turgor-driven cell expansion after division in the hypotonic culture medium. This requirement is obvious in the formation of the cells of the proembryogenic mass and its 'type 1' single progenitor cell (56),

which are always small and highly cytoplasmic, but is also necessary for somatic as well as zygotic embryos up to approximately globular stage (figure 5.2). In general, young organized structures like embryos and organ primordia are found to consist of small cells (86) suggesting that the prevention of cell expansion is essential during early stages of morphogenesis. Although the turgor pressure in relation to somatic embryogenesis has not been investigated, a large body of evidence indicates that plant cell expansion is controlled by the extensibility of the cell wall (41,91). As described in chapter 1, a peroxidase has been found to be responsible for countering the inhibition of somatic embryogenesis by tunicamycin (20). Peroxidases form a well-studied group of plant enzymes that have been proposed to restrict cell expansion by cross-linking of phenolic side-chains of xylans or pectins in the cell wall (36,41,45). The observed morphological effect of tunicamycin on carrot somatic embryo formation, which is the gradual disruption of the proembryogenic mass due to expansion of the surface cells, could then be counteracted by the cross-linking activity of the added peroxidase. Although it remains to be demonstrated that this peroxidase indeed acts on cell wall polymers of the proembryogenic mass, the protein appears to fulfill the first criterion for maintenance of embryogenic cells, that is to restrict cell size after division.

Figure 5.2. The formation of proembryogenic masses and somatic embryos requires restriction of expansion after cell division.
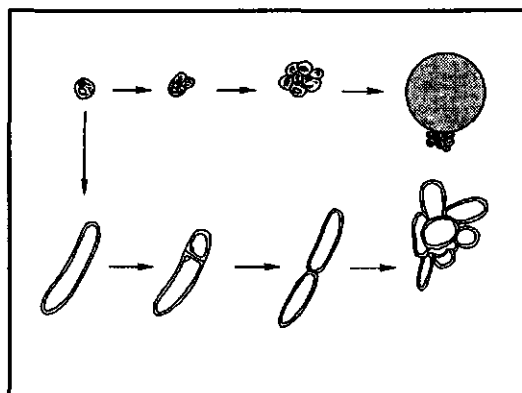
An alternative mechanism to limit expansion may be the deposition of a hydrophobic layer around somatic embryos and proembryogenic masses. The synthesis of a water-repellent "cuticle" on the outer cell wall of proembryogenic masses and embryos might impede water influx, which would prevent expansion. Although direct evidence that somatic embryos are coated with a cuticle is lacking, micrographs of carrot (78) and *Citrus* (9) zygotic embryos reveal a lipophilic wall modification of protodermal cells. Further support for the presence of a cuticle on the surface of zygotic as well as somatic embryos has come from an investigation of the expression of the carrot EP2 gene (87). EP2 encodes a secreted lipid transfer protein and has been posulated to transport cutin monomers to the cell surface. In carrot cell cultures, the EP2 gene was exclusively expressed in peripheral cells of proembryogenic masses and in the

protoderm of somatic embryos. This early and highly specific expression qualified EP2 as a marker for embryogenic potential. Also in zygotic embryogenesis, EP2 expression was already detected in a 60-celled embryo and continued in the protoderm of subsequent embryo stages. Taken together, these observations indicate that the deposition of a layer of hydrophobic material is an important early event in the development of both somatic and zygotic embryos.

A mechanism that limits cell expansion may also be required at later stages of somatic embryo development, as indicated by the complementation of arrested globular embryos of ts11c with extracellular proteins. Morphologically, complementation appears to prevent the formation of an aberrant, irregular protodermal layer, consisting of enlarged, vacuolated cells (25). The ts11c embryo-rescuing protein has also been purified and found to exhibit chitinase activity (25).

Tight cell adhesion

A second condition for embryogenic development appears to be the tight contact between neighbouring cells (41). The coherency of proembryogenic masses and somatic embryos is much tighter than that of unorganized clumps of cells and this characteristic would predict altered properties of the polymers that constitute the junctions between cells: the middle lamella and the outer layers of the primary cell wall. Because cell separation can be brought about by pectin degrading enzymes and chelating agents, the middle lamella pectins are generally believed to stick plant cells together. Hence, enzymes that enhance pectin gelling, such as pectin methyl esterase or enzymes that can covalently cross-link pectic polysaccharides, are candidates for extracellular proteins with a promotive role in embryogenesis. However, there is no evidence to support this suggestion.

5.3.2. Non-embryogenic cells: functions of EP1 and EP4 proteins

EP1

From the description of the cell wall in sections 5.1 and 5.2 it has become clear that the small embryogenic cells and expanded non-embryogenic cells differ in their extracellular matrix biochemistry. EP1 proteins are extracellular matrix proteins. Unlike the structural cell wall proteins (HRGP's, GRP's and PRP's), the predicted EP1 amino acid sequence does not contain repetitive sequence motifs. This suggests a non-structural role for EP1. The epidermis-specific localization of EP1 mRNA indicates that in order to find a function for EP1 we have to search among epidermal extracellular matrix proteins.

91

The EP1 proteins are abundantly secreted by expanded non-embryogenic cells (chapter 2). Based on this observation, the EP1 proteins were initially suspected to be involved in cell expansion. This hypothesis had to be abandoned, however, when *in situ* hybridization revealed an almost uniform expression of the EP1 gene in the root and the shoot epidermis, which argued against a correlation with extension growth (chapter 3).

The epidermis of plants is a highly specialized tissue that serves two major purposes: 1) minimization of water loss to the environment, and 2) protection of the plant body from harmful external influences. For this second role the extracellular matrix structure of epidermal cells has acquired special features which may offer clues to the role of EP1. First, epidermal cells adhere tightly and have no intercellular spaces. Immunolocalization has shown that the cell wall EP1 proteins are bound to a fuzzy, pectin-rich layer on the surface of single non-embryogenic suspension cells (chapter 2), which is most likely the remainder of the middle lamella of these cells. This suggests a possible involvement in pectin-mediated cell adhesion, as outlined in the preceding section. However, the observed cell type-specific EP1 expression in suspension cells and seedlings appear to be contradictory with respect to cell adhesion, since the vacuolated suspension cells that express EP1 do not adhere, while the epidermal cells that express EP1 adhere tightly. Moreover, pectin methyl esterase activity, which has been implicated in cell-to-cell contact, was not found for the 52/54 kD EP1 proteins.

Beside close cell-to-cell contact, epidermal cells are also characterized by a thick outer wall that is covered with a hydrophobic cuticle. The major component of the cuticle, cutin, is synthesized extracellularly from its precursor molecules. This probably requires several enzymatic steps, one of which could be catalyzed by EP1. If this is the function of EP1, the homology of EP1 with self-incompatibility-associated (S-)glycoproteins suggests a similar water-repellent function for the S-glycoproteins, possibly preventing hydration of pollen.

S-glycoproteins have been proposed to act through binding to a homologous S-like receptor that generates a cytoplasmic signal. Hence, EP1 may not be an enzyme at all, but a signal molecule. The most important information an epidermal cell needs is its orientation with respect to the plant surface. It is, therefore, tempting to speculate that an epidermal cell secretes EP1 to determine its position in the tissue. This would require a mechanism to generate a centripetal extracellular EP1 gradient (see 5.3.3.).

EP4

Like EP1, EP4 is an extracellular matrix protein that, on the basis of the absence of repetitive sequence motifs, is likely to be non-structural. EP4 is also secreted by non-embryogenic cells. The cell type, however, that produces the EP4 proteins is less extended and occurs in clusters. In these clusters, the wall-bound form of EP4 is found mainly in the primary walls between adjacent cells, which seem to be formed by internal divisions in

elongated cells (88). This localization may be a clue to the function of EP4, but an answer to the question of what makes these walls different from the walls formed by cell division in meristematic structures is not obvious. Further examination of the cell type that expresses EP4 in roots and additional assays for cell wall enzyme activities of the EP4 proteins may be important steps towards the identification of EP4 function.

5.3.3. Spatial control of extracellular protein activity

In general, tissue-specific gene expression implies the production of a specific gene product that acts locally. Cytoplasmic proteins are easily kept within the cell boundaries, though some of it will 'escape' through plasmodesmata to neighbouring cells. For proteins that are secreted into the extracellular matrix the situation is more difficult as they diffuse more easily through the apoplast to adjacent cells. Thus, it is likely that mechanisms exist to spatially regulate the activity of secreted proteins.

As an illustration of this problem we may consider the epidermis and limit the discussion to enzymes. In epidermal cells, the nucleus and the cytoplasm are localized asymmetrically, which is reflected by the observed polarized localization of the EP1 mRNA (chapter 3). This localization ensures that epidermal wall thickening and cuticle formation occur only in the cell walls at the plant surface, which is evidently vital to the plant. Once secreted, the enzymes that form the epidermal wall should act only locally and have to be limited in their diffusion. An obvious way to achieve this is to bind the proteins to the extracellular matrix. This immobilization of enzymes, however, is only useful if their substrates are diffusible. If the enzyme's substrate is fixed in the wall, as is the case for wall polysaccharides, the enzyme has to be mobile to reach its substrate. More likely than binding, in this case, is degradation of extracellular epidermal enzymes by proteases that are secreted by the outer cell layer of the cortex. Applied to EP1, this system would involve opposite gradients of epidermal EP1 proteins and cortical proteases. To prevent complete breakdown by the proteases, continuous expression of EP1 would be required, which is indeed observed in the root and in the hypocotyl (chapter 3). The observation that EP1 protein accumulation in the basal part of the root and hypocotyl is only moderate (chapter 2), despite a more or less constant level of EP1 expression (chapter 3), also suggests a high turnover rate of EP1 proteins.

If proteolytic breakdown is a general mechanism to control the activity of apoplastic proteins, it is probably highly disturbed in cell suspension cultures. On the one hand secreted proteases, if produced at all under the applied culture conditions, will be highly diluted by the culture medium, allowing accumulation of the target proteins. On the other hand, due to the mixing of different cell types, extracellular enzymes may be exposed to proteases they would never encounter *in planta*. The occurrence of a non-cortical protease in suspension culture

medium may cause the formation of the 31/32 kD EP1 proteins that are not encountered *in planta*. Furthermore, the accumulation of EP1 proteins in a suspension culture derived from leaf tissue which, in itself, appears to degrade its EP1 proteins (chapter 2), may be due to dilution of leaf-specific proteases.

5.3.4. Somatic embryogenesis in cell suspension cultures: development in a hostile environment?

In the preceding chapters the central role of the plant cell wall in morphogenesis has been stressed several times. The view has been put forward that secreted enzymes act as regulators of morphogenesis by changing the chemical structure of cell wall polymers and, consequently, the mechanical properties of the wall as a whole. When this view is applied to somatic embryogenesis in cell suspension cultures, there is enough reason to be surprised that embryos develop at all in this system. In carrot embryogenic cell suspensions, embryogenic cells are outnumbered by far by non-embryogenic cells, which are characterized by extensive expansion and loose attachment to other cells. If these properties of non-embryogenic cells are mediated by secreted enzymes, these enzymes will accumulate in the culture medium, especially in high density cultures. Thus, we might expect from this that conditioned culture medium is essentially hostile towards the development of proembryogenic masses as they are flooded by enzymes promoting cell expansion or loosening cell contacts.

However, experience tells us that suspension culture conditions do permit the development of proembryogenic masses, i.e. under identical growth conditions some cells expand while others do not expand. This may be explained in two ways. First, the enzymes may simply be diluted too much by the culture medium to reach a critical level. The highest enzyme concentration is clearly found in the walls of the non-embryogenic cells themselves, where they must be active, and probably decreases sharply towards the medium. The effect on the cell wall of an embryogenic cell might then eventually be too small to significantly alter its properties. Second, the structure of the wall of an embryogenic cell may *a priori* be such that the enzymes have no access to their substrates. One way to achieve this would be the deposition of a hydrophobic layer on the surface of proembryogenic masses and somatic embryos, which appears to occur in zygotic embryogenesis (see 5.3.1). The low amounts of protein secreted by proembryogenic masses and somatic embryos suggests that this barrier may function both ways, limiting the diffusion of the proteins secreted by these structures. The ability of cultured cells to protect themselves from adverse influences of culture medium components could be an early and crucial step in the formation of somatic embryos.

Similarly, in zygotic embryogenesis, the developing embryo may require an outer layer to protect it against the hydrolytic enzymes degrading the endosperm. In view of this, it is of interest to take into consideration that the two secreted enzymes identified sofar as having promotive effects in carrot somatic embryogenesis are members of a group of proteins that are also induced when plants are challenged by pathogens or injury (7,16,57). In a defense response, these so-called "defense-related" proteins protect the plant by sealing off a wound or a site of pathogen invasion. Thus, the link between the defense response and embryogenesis may be their requirement to form a specialized outer cell layer capable of protection against the environment.

5.3.5. Concluding remarks

In this chapter, extracellular proteins from carrot cell suspensions have been discussed in relation to the structure and function of the plant primary cell wall. It has been shown that the properties of the primary wall may be adjusted through the action of secreted enzymes. This leads us to expect that different cell types will each secrete a characteristic set of proteins. An examination of carrot extracellular proteins and the cell types that produce them appears to corroborate this expectation. EP1 is produced by vacuolated, single, non-embryogenic cells (chapter 2), EP2 by proembryogenic masses and somatic embryos (87) and EP4 by relatively small, vacuolated, non-embryogenic cells in clusters (chapter 4).

The apparent heterogeneity of cell types in carrot cell suspension cultures raises the question of how these different cell types originate. In seedlings, EP1 is specifically expressed in epidermal cells, while in cell suspensions it is specific for a subpopulation of non-embryogenic cells. This strongly suggests that cells expressing EP1 have an epidermal origin and maintain epidermal characteristics in cell culture. This is contrary to the generally held view that the reinitiation of cell division and subsequent unorganized growth behaviour observed in cell suspension cultures involves complete dedifferentiation of the cells. There are other examples of supension cultures that continue to produce proteins specific for the organs they were derived from (20, and references cited therein). Thus, our thinking about the state of cells in suspension cultures should take into account that unorganized growth does not neccessarily imply complete loss of the differentiated state. In view of this, secretion of EP4 may be a property of cells from another tissue of the explant, like the cortex. Division of these cells in culture would then give rise to the EP4-producing cell population. Thus, the cell type heterogeneity in cell cultures might reflect the differences between cells from different tissues of the explant.

Cells in suspension cultures do not get the positional information from adjacent cells that appears to regulate their differentiation *in planta* (24). If a continued supply of appropriate positional information is necessary for stability of the differentiated state of cells, it is likely that the state of suspension-cultured cells will change upon prolonged subculture. As all cells read the same positional information from the homogeneous culture medium, an originally heterogeneous cell suspension will gradually lose cell types. This may include the embryogenic cells, which would account for the progressive loss of embryogenic potential generally observed in carrot cultures.

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Samenvatting

Het leven van hogere planten begint doorgaans met de bevruchting van de eicel in de bloem. De hieruit ontstane zygote deelt zich vele malen en onwikkelt zich tot een embryo, dat ingebed ligt in het beschermende en voedende weefsel van het zaad. Na kieming van het zaad groeit het embryo uit tot een plant. Hoewel de morfologie van de ontwikkeling van embryo's goed beschreven is, is onze kennis over de genen die aan dit proces ten grondslag liggen zeer gering. De gebrekkige toegankelijkheid van het embryo in het zich ontwikkelend zaad geeft helaas maar beperkte mogelijkheden voor het uitbreiden van deze kennis. Embryogenese is echter bij veel planten ook langs niet-geslachtelijke weg buiten de plant mogelijk. Hiertoe wordt een stukje weefsel van een plant gesneden en in een kweekmedium gebracht met een hoge concentratie van de groeiregulator auxine. Cellen aan het wondoppervlak gaan vervolgens delen en de zo ontstane cellen vormen een celsuspensie. Na enige tijd vormen zich in deze celsuspensie klompjes cytoplasma-rijke cellen, aangeduid als pro-embryogene massa's, die na verdunning in auxine-vrij medium uitgroeien tot embryo's. Deze vorming van embryo's langs niet-geslachtelijke weg wordt somatische embryogenese genoemd. Vooral in celsuspensies van *Daucus carota* (Peen) verloopt somatische embryogenese zeer efficient. De grote aantallen somatische embryo's die op deze wijze verkregen kunnen worden en de toegankelijkheid van somatische embryo's maken *Daucus* celsuspensies zeer geschikt als model voor de bestudering van de moleculaire basis van embryogenese.

Verschillende experimenten geven aan dat de ontwikkeling van somatische embryo's onder invloed staat van componenten die door suspensiecellen worden uitgescheiden in het kweekmedium, dat om deze reden "geconditioneerd" wordt genoemd. De aanleiding voor het hier beschreven onderzoek was een serie waarnemingen die aangaven dat met name extracellulaire glycoproteinen van belang zijn voor embryogenese. Zo bleek een hitte-labiele, hoog-moleculaire factor uit geconditioneerd medium in staat de vorming van embryo's te herstellen bij een mutante, niet-embryogene celsuspensie. Een soortgelijk positief effect op embryo ontwikkeling werd gevonden bij een celsuspensie waarin de embryogenese geblokkeerd was als gevolg van de toevoeging van tunicamycine, een remmer van de eiwitglycosylering. Het vaststellen van de identiteit van de eiwitten die deze effecten teweeg brengen, was daarom van groot belang.

Celsuspensies van *Daucus* bevatten naast embryogene cellen ook veel niet-embryogene cellen. Niet-embryogene cellen worden gekenmerkt door de aanwezigheid van een grote vacuole als gevolg van celexpansie. De niet-embryogene celpopulatie is echter niet homogeen; er bestaan verschillen in vorm en in de mate van expansie, en de cellen komen zowel afzonderlijk voor als in groepjes. De eiwitten die door niet-embryogene cellen uitgescheiden worden, kunnen studies naar de rol van extracellulaire eiwitten in de somatische embryogenese

vertroebelen. Zo hoeft een verandering in de concentratie van een mediumeiwit niet noodzakelijkerwijze verband te houden met embryogenese. Daarnaast is het mogelijk dat eiwitten die uitgescheiden worden door niet-embryogene cellen zelfs nadelige effecten hebben op de ontwikkeling van somatische embryos. Kennis van deze eiwitten zou daarom ons begrip van cel-celinteracties in embryogene suspensies kunnen vergroten. Het in dit proefschrift beschreven onderzoek had tot doel inzicht te verkrijgen in de rol van eiwitten die uitgescheiden worden in het kweekmedium van embryogene *Daucus carota* celsuspensies. De experimenten waren erop gericht de identiteit van enkele extracellulaire eiwitten op te helderen en de expressiepatronen van de voor deze eiwitten coderende genen te bepalen.

Hoofdstuk 2 beschrijft de karakterisering van EP1, een van de glycoproteinen die door *Daucus* suspensiecellen worden uitgescheiden. Een cDNA kloon van EP1 werd geisoleerd door een expressiescreening van een cDNA bank. Tegen het door deze kloon geproduceerde fusie-eiwit werd een antiserum opgewekt. Met het anti-EP1 serum werden EP1 eiwitten met verschillende molecuulgewichten gedetecteerd zowel in het medium als in een calciumchloride extract van de celwand van suspensiecellen. Er kon slechts één primair translatieproduct van EP1 worden aangetoond, zodat de heterogeniteit van EP1 het gevolg lijkt van verschillende post-translationele modificaties (glycosylering, proteolytische splitsing). In zaailingen werd EP1 mRNA in alle organen aangetroffen met slechts geringe verschillen in expressieniveau. De heterogeniteit van EP1 op eiwitniveau bleek wèl orgaan-specifiek te zijn. In embryogene suspensies kon EP1 niet aangetoond worden in somatische embryos of in pro-embryogene massa's, maar uitsluitend in afzonderlijke, niet-embryogene cellen. Immunolocalisatie van EP1 op intacte niet-embryogene cellen liet zien dat de celwandvormen van EP1 aan het oppervlak van deze cellen gebonden zijn aan een niet vast aan de celwand hechtende laag pectine-rijk celwandmateriaal, mogelijk een restant van de middenlamel. De EP1 eiwitten van 52 en 54 kD werden voorts gezuiverd uit het medium en getoetst op hun effect op zich ontwikkelende somatische embryo's. Deze bleken ongevoelig te zijn voor toevoeging van hoge concentraties EP1 aan het medium. Daarnaast waren deze EP1 eiwiten ook niet in staat de door tunicamycine veroorzaakte remming van de somatische embryogenese op te heffen.

Door middel van *in situ* hybridisatie met EP1 cDNA is de weefsel-specifieke expressie van EP1 in kiemplanten onderzocht (hoofdstuk 3). EP1 mRNA werd aangetoond in de epidermis van de wortel, het hypocotyl en de cotylen. Ook werd EP1 expressie gevonden in een halve maan-vormige strook cellen in de buitenste laag van het scheutmeristeem en in de buitenste cellen van het wortelmutsje. In ontwikkelend zaad bevatten de binnenste en de buitenste epidermis van het integument eveneens EP1 mRNA. EP1 expressie lijkt dus specifiek te zijn voor epidermale cellen. Bepaling van de basenvolgorde van het EP1 cDNA bracht een zwakke, maar significante homologie aan het licht met S-locus glycoproteinen (SLG's) uit *Brassica*, die betrokken zijn bij zelf-incompatibiliteit (hoofdstuk 3). Tevens werd homologie gevonden met

een *Arabidopsis* SLG-achtig gen en met het SLG-domein van mogelijke receptor proteine kinases uit mais en *Arabidopsis*. De epidermale expressie van EP1 suggereert drie mogelijke epidermis-specifieke functies waar EP1 bij betrokken kan zijn: 1) celstrekking, 2) nauw cel-celcontact en 3) beperking van waterverlies naar de omgeving. De bijna uniforme expressie van EP1 in zich strekkende en niet-strekkende gedeelten van de kiemplant maakt het onwaarschijnlijk dat EP1 betrokken is bij de celstrekking. Ook het bevorderen van cel-celcontacten lijkt niet de functie van EP1, omdat EP1 in celsuspensies juist gemaakt wordt door cellen die niet aan elkaar hechten. Een rol van EP1 bij de beperking van watertransport door de celwand is wèl aannemelijk, omdat dit van belang is bij zowel de normale epidermisfunctie als bij de zelf-incompatibiliteitsreactie. De werking van SLG's resulteert mogelijk in een sterke vermindering van wateropname door pollenkorrels, hetgeen de kieming van het pollen remt. In de epidermis zou EP1 een vergelijkbare functie kunnen vervullen bij het tegengaan van verdamping. Deze functie zou niet van belang zijn bij suspensiecellen die EP1 produceren.

Een tweede uitgescheiden glycoproteine, EP4, wordt beschreven in hoofdstuk 4. Het EP4 eiwit, met een molecuulmassa van 47 kD, werd gezuiverd uit het kweekmedium, waarna er een specifiek antiserum tegen werd opgewekt. Dit anti-EP4 serum herkende een afwijkende vorm van het EP4 eiwit, met een molecuulmassa van 45 kD, in een celwandextract. EP4 kon worden aangetoond in kweekmedia van zowel embryogene als niet-embryogene lijnen. Het 47 kD EP4 eiwit blijkt uitgescheiden te worden in het medium door een subpopulatie van niet-embryogene cellen, die echter afwijkt van de populatie die EP1 produceert. Deze verdeling werd bevestigd door immunolocalisatie van de celwand-gebonden vorm van EP4. Dit eiwit bevindt zich in de wand van groepjes relatief kleine, gevacuoliseerde cellen. De hoeveelheid EP4 is hier het grootst in celwanden tussen twee cellen in, die ontstaan lijken door deling van een reeds gestrekte cel. De functie van EP4 zou in verband kunnen staan met de vorming van dit type dwarswanden. Het 47 kD EP4 eiwit had geen effect op somatische embryogenese als het aan het medium van een embryo cultuur werd toegevoegd. De basenvolgorde van een EP4 cDNA kloon gaf geen homologie te zien met bekende genen.

Het voorkomen van eiwitten in kweekmedia moet gezien worden tegen de achtergrond van de structuur en het functioneren van de primaire celwand (hoofdstuk 5). De primaire celwand is opgebouwd uit verschillende polymeren met een grote structurele diversiteit. Deze componenten vormen een celwand die niet alleen stevigheid en bescherming biedt, maar die tijdens de ontwikkeling ook celexpansie mogelijk maakt. Om aan al deze eisen te voldoen scheidt de plantecel een breed spectrum aan enzymen uit, die de structuur van celwandpolymeren modificeren. Een rol voor extracellulaire eiwitten bij embryogenese wordt dan begrijpelijk als men zich realiseert dat in zich ontwikkelende embryo's de celwanden een belangrijke rol spelen. Deze celwanden moeten expansie van cellen na deling verhinderen en tevens zorgen voor een sterke hechting van cellen aan elkaar. Veel enzymen die in de celwand

aanwezig zijn, worden ook aangetroffen in het medium. Daarom ligt het voor de hand te veronderstellen dat veranderingen in de structuur van de primaire celwand ten grondslag kunnen liggen aan de waargenomen positieve effecten van extracellulaire eiwitten op de ontwikkeling van somatische embryo's.

Ook niet-embryogene cellen scheiden eiwitten uit in het medium, zoals EP1 en EP4. Een aantal hiervan zou voor somatische embryo's nadelige effecten op celstrekking en cel-celcontacten kunnen hebben. De vorming van een beschermende laag op het oppervlak van het embryo zou deze schadelijke invloeden kunnen voorkomen. Deze situatie is vergelijkbaar met die in het zaad, waar het embryo zich moet beschermen tegen de hydrolytische enzymen die het endosperm afbreken tot voedingsstoffen voor het embryo.

Uit de identificatie van de celtypen die extracellulaire eiwitten produceren, komt naar voren dat de morfologische heterogeniteit van cellen in een embryogene suspensie weerspiegeld wordt in de eiwitten die deze cellen uitscheiden in het medium. De specifieke expressie van EP1 in de epidermis van kiemplanten geeft voorts aan dat de suspensiecellen die EP1 uitscheiden waarschijnlijk van de epidermis afkomstig zijn. De heterogeniteit van celsuspensies is dus mogelijk een afspiegeling van de verschillende weefsels van het explantaat waaruit de celsuspensie is gevormd. Hieruit vloeit voort dat cellen in een *Daucus carota* celsuspensie weliswaar ongeorganiseerd, maar niet ongedifferentieerd zijn.

Account

Parts of this thesis have been published before, or will be published, in the following papers:

Van Engelen FA, Sterk P, Booij H, Cordewener JHG, Rook W, Van Kammen A & De Vries SC (1991) Heterogeneity and cell type-specific localization of a cell wall glycoprotein from carrot suspension cells. Plant Physiol. 96:705-712

Van Engelen FA & De Vries SC (1992) Extracellular proteins in plant embryogenesis. Trends Genet. 8:66-70

Van Engelen FA & De Vries SC (1993) Secreted proteins in plant cell cultures. In: Markers of Plant Morphogenesis, KA Roubelakis-Angelakis ed., NATO-ASI series, Plenum, New York, USA, in press.

Van Engelen FA, Hartog MV, Thomas TL, Taylor B, Sturm A, Van Kammen A & De Vries SC. The carrot secreted glycoprotein gene EP1 is expressed in the epidermis and has sequence homology to *Brassica* S-locus glycoproteins. Submitted.

Van Engelen FA, De Jong AJ, Meijer EA, Kuil CW, Meyboom JC, Booij H, Hartog MV, Vandekerckhove J, Van Kammen A & De Vries SC. Purification, immunological characterization and cDNA cloning of a 47 kDa glycoprotein secreted by carrot suspension cells. In preparation.

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

De Vries S, Booij H, Cordewener J, Van Engelen F, Hartog M, Hendriks T, De Jong A, Meijer E, Sterk P & Van Kammen A (1992) Cell wall glycoprotein-encoding genes in somatic and zygotic embryogenesis. In: Reproductive Biology and Plant Breeding, Dattèe Y, Dumas C & Gallais A, eds., Springer Verlag, Berlin, Germany, pp. 261-268

Nawoord

Dit proefschrift bestaat uit 112 pagina's, 3429 regels, 31338 woorden en 204511 lettertekens. Het moge duidelijk zijn dat ik dit ei niet alleen gelegd heb. Ik ben iedereen dankbaar die mij geholpen heeft bij het bedenken, opzetten en uitvoeren van experimenten, het interpreteren van de resultaten, het verwerken van teleurstellingen en het samenvoegen van een brij gegevens tot een leesbaar boekje.

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De steun uit het buitenland, met name van Robert Fluhr, Paul Knox, Fiorella LoSchiavo, Kirsten Nielsen, Shinobu Satoh, Arnd Sturm, Mario Terzi, Terry Thomas en Joël Vandekerckhove is bijzonder waardevol geweest.

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Curriculum vitae

Fred van Engelen werd geboren op 15 juli 1962 te Baarn. Zijn wetenschappelijke opleiding begon op het Alberdingk Thijm College te Hilversum, alwaar hij in 1980 slaagde voor het eindexamen VWO. De studie werd hierna voortgezet aan de Landbouwuniversiteit Wageningen in de studierichting Moleculaire Wetenschappen (biologische orientatie). Het kandidaatsdiploma werd in 1984 behaald (met lof). In de doctoraalfase participeerde hij in onderzoeken van de vakgroepen Dierfysiologie (eiwitmetabolisme van de rat), Celbiologie (bepaling van MHC haplotypen van karpers) en Moleculaire Biologie (wortelknolvorming bij erwt). Tijdens zijn stage aan het Laboratorium voor Farmakologie van de Universitaire Instelling Antwerpen stond het effect van antidepressiva op prikkeloverdracht in de hersenen centraal. In maart 1987 werd het ingenieursdiploma behaald. Van april 1987 tot april 1991 werkte hij aan de vakgroep Moleculaire Biologie van de Landbouwuniversiteit Wageningen aan het onderzoek dat in dit proefschrift beschreven staat. Sinds 1 februari 1992 is hij, in dienst van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek, werkzaam bij het Centrum voor Plantenveredelings- en Reproductie Onderzoek (CPRO-DLO).