Chitinases and arabinogalactan proteins in somatic embryogenesis

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen,
dr. C.M. Karssen,
in het openbaar te verdedigen
op woensdag 3 juni 1998
des namiddags te half twee in de Aula.

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

van Hengel, Arjon J.

Chitinases and arabinogalactan proteins in somatic embryogenesis.
Arjon J. van Hengel. [S.l: s.n.]
Thesis Wageningen. -With ref.- With summary in Dutch.
ISBN 90-5485-873-7
Subject headings: somatic embryogenesis / chitinases / arabinogalactan proteins

The investigations described in this thesis were carried out at the department of Molecular Biology, Agricultural University Wageningen, The Netherlands, and were financially supported by a grant from the Dutch Organisation for Scientific Research (NWO).

Stellingen

1) N-acetylglucosamine komt voor in arabinogalactaneiwitten (AGPs)
(dit proefschrift)

2) Een voorbehandeling met chitinase vergroot het vermogen van arabinogalactaneiwitten (AGPs) om de vorming van somatische embryo's te bevorderen.
(dit proefschrift)

3) Bij het analyseren van de gevolgen van het toevoegen van (β-D-Glc)$_3$ Yariv phenylglucosiden aan plantencellen dient rekening te worden gehouden met de biofysische effecten op de celwand.

Serpe, M.D. & Nothnagel, E.A. *Planta* **193**, 542-550 (1994).
Willats, W.G.T. & Knox, J.P. *Plant Journal* **9**, 919-925 (1996).
Serpe, M.D. & Nothnagel, E.A. *Plant Physiol.* **112**, 1261-1271 (1996).

4) De strikte patroonvorming bij embryo- en wortelontwikkeling van onder gestandaardiseerde laboratorium omstandigheden gekweekte *Arabidopsis thaliana* verhult de natuurlijke plasticiteit van deze ontwikkelingsprocessen.

Dolan, L. et al. *Development* **119**, 71-84 (1993).
Jürgens, G. *Cell* **81**, 467-470 (1995).

5) Het vermelden van gegevens over de halfwaardetijd van arabinogalactaneiwitten (AGPs) is voorbarig zolang de structuur en de exacte functie van deze moleculen niet bekend zijn.

Kreuger, M. & Van Holst, G.-J. *Plant Molecular Biology* **30**, 1077-1086 (1996).
Takeuchi, Y. & Komamine, A. *Physiol. Plant.* **50**, 113-118 (1980).
Gibeaut, D. & Carpita, N. *Plant Physiol.* **97**, 551-561 (1991).

6) De Joegoslavische grondwet, die in 1974 onder invloed van Josip Broz Tito tot stand is gekomen, bood geen ruimte voor nationalistische stromingen en kon daarmee Joegoslavië als eenheidsstaat zo lang mogelijk in staat houden.

7) In tegenstelling tot wat zij beweren laten McCabe et al. (1997) niet zien dat cellen waarvan het cytoplasma en de celwand gepolariseerd zijn, cellen zijn die zich kunnen ontwikkelen tot somatische embryo's.

McCabe, P.F. et al. *Plant Cell* **9**, 2225-2241 (1997).

8) De door de producenten van softdrinks uitgeoefende druk op het Congres van de Verenigde Staten om een handelsboycot van Sudan te voorkomen en op die manier de invoer van arabische gom veilig te stellen, laat zien dat AGPs behalve somatische embryogenese ook de internationale politiek kunnen beïnvloeden.

International Herald Tribune (17 october 1997).

9) De politieke keus voor Sarajevo, als lokatie voor het hoofdkwartier van de VN vredesmacht UNPROFOR, heeft het goed functioneren van UNPROFOR in hoge mate gehinderd.

10) Het voorkomen van een tweede golfoorlog door bemiddeling van de Verenigde Naties is de grootste overwinning die deze volkerenorganisatie ooit behaald heeft.

Contents

Scope

In vitro cultured carrot suspension cells can function as starting material for the generation of somatic embryos. Compounds secreted by suspension cells can influence the process of somatic embryogenesis. One class of such compounds, the secreted EP3 endochitinases, was found to lift the developmental arrest of somatic embryos formed in the temperature sensitive carrot cell line *ts11*, when cultured at restrictive temperatures. In addition, this chitinase was able to increase the number of *ts11* somatic embryos as observed at the restrictive temperatures. The bacterial lipochitooligosaccharide (LCO) NodRlv-V(Ac, C18:4) was able to mimick the effects of EP3 endochitinases on *ts11* somatic embryogenesis. The major goal of the study presented in this thesis was to identify plant produced compounds that contain an endochitinase cleavage site. Such compounds could then lead to the discovery of possible plant LCO analogues.

In Chapter 1 an introduction concerning the roles and effects of compounds that influence embryogenesis is given. Besides a description of zygotic and somatic embryogenesis, this overview presents a number of classical and non-classical growth regulators that function in embryogenesis. It is discussed what role carbohydrates and proteoglycans can play in the initiation of embryogenesis.

In Chapter 2 experiments are presented that identify suspension cells and plant tissues expressing *EP3* endochitinase genes. In addition, the localisation of EP3 proteins in suspension cells and in seeds was determined. *EP3* genes were only expressed in cells that were in the vicinity of zygotic or somatic embryos, but not in embryos themselves. The localisation of the secreted EP3 enzymes in the culture medium and in the endosperm suggested a "nursing" function for EP3 endochitinases during embryogenesis.

In Chapter 3 the production of catalytically active carrot and *Arabidopsis* EP3 endochitinases in insect cells infected with recombinant baculoviruses is reported. Characterization of individual carrot EP3 endochitinase isozymes showed that all baculovirus produced carrot chitinases are able to cleave chitin as well as chitosan. Furthermore, the occurrence of the *Arabidopsis* chitinase AtEP3 in the medium of embryogenic *Arabidopsis* suspension cultures and its absence in non-embryogenic cultures identified AtEP3 as a good marker for the capability of such cultures to form somatic embryos.

In Chapter 4 evidence is presented for the occurrence of N-acetylglucosamine (GlcNAc) in arabinogalactan proteins (AGPs) present in the medium of carrot embryogenic suspension cultures. AGPs isolated from immature carrot seeds, were found to contain endochitinase cleavage sites. The identification of these cleavage sites and the differences in the total population of AGPs isolated from carrot seeds at different stages of seed development suggested a substantial amount of processing of AGPs in carrot seeds.

In Chapter 5 experiments are described that demonstrate that both EP3 endochitinases as well as AGPs can promote the formation of protoplast derived wild type somatic embryos. Pre-treatment

of immature seed AGPs with chitinases before addition to carrot protoplasts was shown to increase the embryo-promoting effect.

In chapter 6 the role of GlcNAc containing molecules in plant and animal development is discussed, with a special emphasis on the role of GlcNAc containing AGPs as the natural substrate for endochitinases in plant embryogenesis.

Chapter 1

Plant growth regulators in embryogenesis

Arjon J. van Hengel and Sacco C. de Vries

(Mechanism of action of plant hormones, Eds: K Palme, R. Walden and J. Schell, in press)

Introduction

Traditional growth regulators such as auxins and cytokinins have always played an important role in studies of embryogenesis *in vitro* (Reinert, 1959). This was based in part on earlier work in which it was demonstrated that auxin could induce de novo meristem formation in tissue culture, a process referred to as organogenesis (Skoog and Miller, 1957). The role of exogeneously added auxins, and to a lesser extent cytokinins is therefore well-accepted in the formation of somatic embryos, and is thought to be essential in the reinitiation of cell division in explant tissues. It is less clear what their role is in zygotic embryogenesis. It is for instance not known at what moment in embryo development for the first time gradients of auxin and cytokinin are established and whether they have an instructive role in the establishment of embryo pattern elements. Only recently experiments have been performed that suggest that disruption of endogeneous gradients of auxins by various means indeed changes the outcome of the formation of the embryo pattern (Fisher et al., 1996; Liu et al., 1993). Another emerging area of research is the investigation into the role that a rapidly increasing array of diverse molecules such as chitinases, lipochitooligosaccharides or LCOs (De Jong et al., 1993), and arabinogalactan proteins or AGPs (Kreuger and Van Holst, 1993) play in somatic embryogenesis. These studies have largely been performed in *in vitro* systems and have pointed to the existence of non-traditional growth regulators.

Genetic analysis of plant zygotic embryogenesis has sofar revealed few clues as to the exact role of plant growth regulators in early embryo development. However, it does appear that cell to cell communication is one crucial mechanism employed to set up patterning and to ensure cell differentiation in embryogenesis (Jürgens, 1995; Laux and Jürgens, 1997). Such a mechanism of course requires long, short and intermediate range signalling molecules, some of which may be identical to the traditional growth regulators, while others may be identified as non-traditional ones or even as yet completely unknown molecules.

In this chapter we will focus on studies that show a potential role of growth regulators in early embryogenesis and will attempt to identify essential developmental events in the early embryo, possibly under control of growth regulators. Later steps in seed development and embryogenesis, especially those during the onset of desication that are under control of growth regulators such as ABA and GA, are not discussed here.

Embryogenesis in dicots and monocots

The zygotic embryo begins life as a fertilized egg cell, the zygote (Fig 1A). Concomittant, the second fertilization event results in the triploid endosperm, by fusion of the two polar nuclei of the central cell (Fig 1A) with the second sperm nucleus. The endosperm develops into a tissue that consists of only a

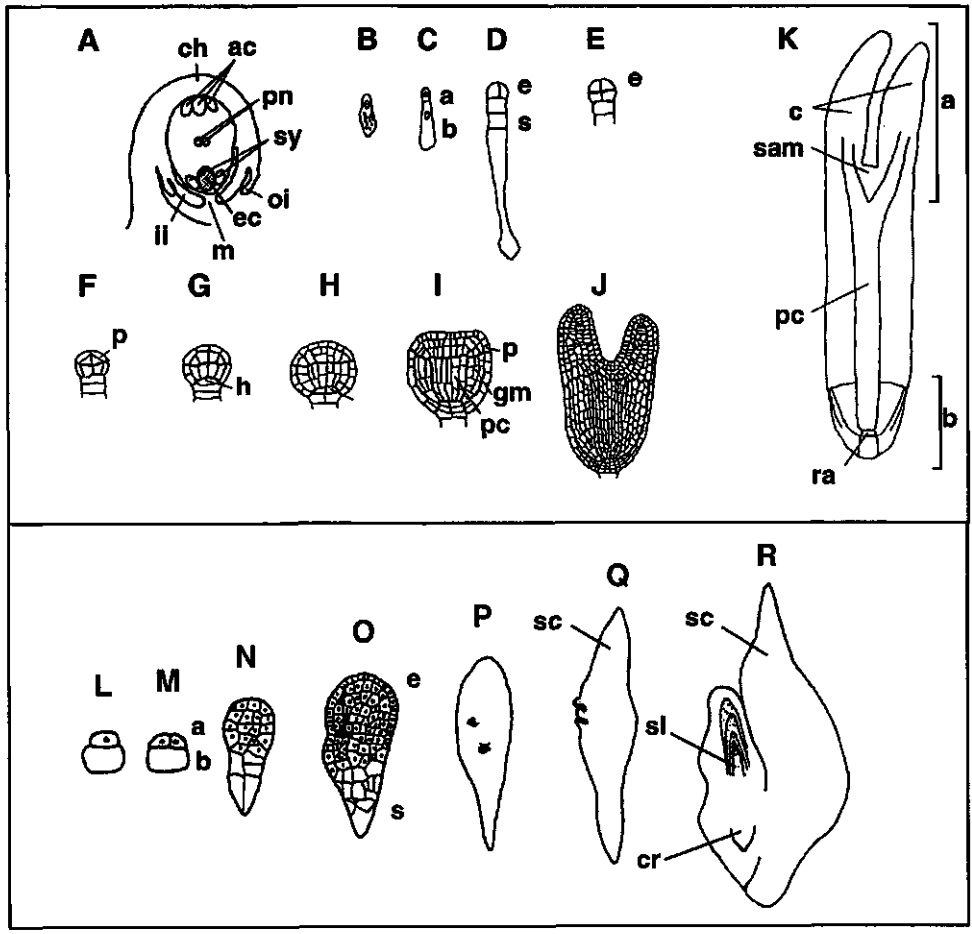

Figure 1. Embryonic development of dicotyledonous (A through K) and monocotyledonous plants (L through R). **A.** Ovule. The egg cell (ec) and synergids (sy) are located at the micropylar end (m) of the ovule, and the antipodal cells (ac) are at the chalazal end (ch). ii, inner integuments; oi, outer integuments; pn, polar nuclei. **B.** Zygote. **C.** The zygote has undergone the first division resulting in a smaller apical (a) cell and a larger basal (b) cell. **D.** Two celled embryo proper (e) and the suspensor (s). **E.** Octant stage embryo. **F.** 16-celled embryo where the protoderm (p) becomes apparent. **G.** Early globular stage embryo. The topmost cell of the suspensor has divided to produce the hypophysis (h). **H.** Mid globular stage embryo. **I.** Transition stage embryo. The cotyledons are about to emerge, the developing procambium (pc) and the ground meristem become visible. **J.** Heart stage embryo. The cotyledonary lobes are enlarging and the bilateral symmetry has clearly become obvious. **K.** Torpedo stage embryo. The morphological organization of the embryo has completed and all embryonic elements have become visible, the cotyledons (c) and the shoot apical meristem (sam) in the apical part, and the root apex (ra) in the basal part. **L.** The zygote has undergone the first division resulting in a 2-celled embryo. **M.** Three celled embryo with smaller apical cells and a larger basal cell. **N.** Club shaped embryo. **O.** Transition stage embryo, containing an embryo proper region with a protodermal cell layer, and a suspensor region. **P.** The subdistal region of the embryo starts to divide actively and the periferal shoot meristem becomes visible. **Q.** The root meristem begins to form internally and the region above the shoot meristem has expanded to form the scutellum (sc). **R.** The morphological organization of the embryo has completed and all embryonic elements have become visible with the shoot apical meristem with leaf primordia (sl) and the coleorhiza including the embryonic root (cr).
Figure adapted from West and Harada (1993), Tykarska (1976,1979) and Lindsey and Topping (1993).

few differentiated cell types and it is known to provide nutrients to the developing embryo and/or the germinating seedling (for review see Lopes and Larkins, 1993).

The zygote elongates before the first division in the future apical-basal axis of the embryo (Fig 1B). This coincides with a reorganization of the previously randomly orientated microtubules, that now become aligned perpendicular to this axis (Webb and Gunning, 1991). The first division of the zygote is usually an unequal transversal division that results in a larger basal cell, oriented towards the micropylar end, and a smaller apical cell, oriented towards the chalazal end of the embryo sac (Fig 1C). The basal cell will give rise to the extra-embryonic suspensor, whereas the apical cell will give rise to the embryo proper. In the model plant *Arabidopsis* the uppermost cell of the suspensor (Fig 1G) contributes to a part of the embryonic root meristem and root cap and thus to the embryo (Scheres et al., 1994). Many differences have been observed between dicot species concerning the contribution of the apical and basal cell derivatives to different parts of the embryo proper (for review see Mordhorst et al., in press).

The establishment of two primary meristems in the embryo, the root and the shoot meristem, are of utmost importance in shaping postembryonic development and thus for generating the adult plant (Steeves and Sussex, 1989). Pattern formation is intensively studied by mutational analysis in *Arabidopsis*. The initiation of the two apical meristems at distal positions in the embryo (Barton and Poethig, 1993; Dolan et al., 1993) can be viewed as being part of an apical-basal pattern along the main body axis and a radial pattern perpendicular to this axis (Jürgens, 1995). Both patterns become visible at very early stages in embryo development. Apical-basal pattern formation is visible as an unequal distribution of cellular constituents in the zygote and appears fixed by the first asymmetric division of the zygote, that results in two cells of unequal size and different fates in most dicot species (Fig 1C). Radial pattern formation commences slightly later, in the octant stage embryo, where the outer cell layer (the protoderm) becomes distinct from the inner cells (Fig1E-F). Upon the transition into a heart stage embryo, the radial arrangement gives way to a bilateral symmetry by the formation of both cotyledon primordia. Pattern formation reaches completion in the late heart stage embryo (Fig 1J) with the visible presence of both apical meristems. After that, the body plan of the eventual dicot seedling is essentially finished (Jürgens and Mayer, 1994).

Controlled cell divisions in the early embryo are of utmost importance for generating the body organization that is apparent in the mature embryo (Jürgens, 1995). Another factor that influences the body organization and the shape of the embryo is the control of cell expansion. Recently it has been shown that expansins, extracellular proteins that control tissue expansion, can regulate plant development. Fleming et al. (1997) have reported that beads loaded with purified expansin, placed on the apical meristem of tomato plants induce tissue expansion and can induce leaf formation. These results indicate that also biophysical forces have a role in plant development, so the control of cell expansion might also be an important factor in embryogenesis.

It is less clear from mutational analysis whether the controlled cell divisions and subsequent expansion is actually instrumental in pattern formation. In mutant *fass* embryos the initial cell

divisions are not as regular as in the wild type embryo, so that the characteristic radial organization is not apparent. Despite this all pattern elements develop and the mutant seedlings display all tissues found in wild type (Torres Ruiz and Jürgens, 1994), which seems to confirm observations made in many other plant species (see maize later in this section) that considerable randomness in early embryo cell divisions is apparent (reviewed by Mordhorst et al., in press) Based on these and other observations Jürgens (1995) suggested that the apical-basal pattern elements are established by cellular interactions in a position-dependent manner. The observed variability in early divisions shows that such cellular interactions can establish the embryo pattern independent of the pattern of cell division. Cellular interactions may also play an important role in maintaining and (possibly) initiating cell identity (Van den Berg et al., 1995) and tissue-specific gene expression. Further indications for cell-to-cell communication as a means of generating apical-basal pattern elements comes from clonal analysis with marker genes that show clone barriers to be variable sometimes to run across specific seedling structures, such as the cotyledons or the root meristem (Dolan et al., 1994; Scheres et al., 1994). In *Arabidopsis* several screens have yielded mutants that are arrested in different stages of embryo development (Meinke and Sussex, 1979), that show changes in the apical-basal or radial body pattern (Barton and Poethig, 1993; Jürgens et al., 1991; Mayer et al., 1991; McConnell and Barton, 1995; Scheres et al., 1995) or that show specific expression of marker genes in the embryo (Vroemen et al., 1998)

Several of the genes that upon mutation result in an embryo phenotype and that have been cloned cause rather severe phenotypes with considerable alterations at the cellular level. An example of such a gene is KNOLLE, encoding a syntaxin responsible for cytokinesis-specific vesicle fusion (Lukowitz et al., 1996). The *knolle* mutant was originally described as having a defect in the radial pattern (Mayer et al., 1991). Whether severe embryo or seedling phenotypes are the result of mutations in very early acting genes instructive in pattern formation remains uncertain (for review see Mordhorst et al., in press). The only clue from mutational analysis that cell-cell signalling events are required for shoot apical meristem formation during embryogenesis comes from the cloning of the CLAVATA 1 gene, postulated to be involved in maintaining the number of cycling and undifferentiated cells in the shoot apical meristem and encoding a transmembrane leucin-rich-repeat type receptor kinase (Clark et al., 1997). The ligand that activates this receptor is not known.

In monocots maize is one of the model species for studying embryogenesis, and it is of interest to compare maize embryo development with that of the stereotype dicot embryo development because of the striking differences between the two. In maize fertillization results in a zygote that after the first two divisions gives rise to a 3-celled embryo that contains two small apical cells and one larger basal cell (Fig 1M). In contrast to Arabidopsis, the subsequent divisions are irregular (Randolph, 1936). In the transition stage the embryo has a club-shaped morphology, the emerging 'embryo proper' region is distinct from the 'suspensor region' since the latter does not contain a protodermal cell layer (Randolph, 1936) (Fig 1O). The periferal shoot meristem becomes visible 6 to 7 days after fertilization (Fig 1P-Q), when the subdistal region begins to divide actively on the side facing away

from the endosperm (Van Lammeren, 1986). Later on, the root meristem is initiated internally. The embryonic meristems are initiatied at a later stage in embryogenesis then in the dicot Arabidopsis, while also the initiation of 3 to 5 leaf primordia during embryogenesis (Fig 1R) are characteristic for monocot embryogenesis.

Besides zygotic embryogenesis other modes of embryogenesis exist in plants. Apomixis is an *in vivo* occurring process, that gives rise to embryos of gametophytic or somatic origin with maternal genetic properties (for reviews see Koltunow, 1993; Sharma and Thorpe, 1995). Given the variability in the cells that initiate apomictic embryos, it is likely that signalling systems are employed, but sofar no clues are available to what the nature of such signals is. One possibility is that they belong to the group of classical growth regulators such as gibberellic acid, that was shown to promote parthenocarpy in Arabidopsis (Chaudhury et al., 1994; Vivian-Smith et al., 1997).

Zygotic embryogenesis can be mimicked *in vitro* by the fusion of isolated gametes (for review see Kranz and Dresselhaus, 1996) Zygotic embryogenesis after *in vitro* fertilization is comparable to the *in vivo* situation. Interestingly *in vitro* fertilization requires only both gametes, whereas further embryo development requires 'feeder' cells, usually in the form of an embryogenic suspension culture, suggesting that those 'feeder' cells fulfill an nursing function in early embryogenesis. It is not known whether this nursing function is a phenomenon restricted to the *in vitro* system or whether zygotic embryos also require such a nursing activity provided by the surrounding tissues.

Androgenesis and gynogenesis are processes in wich gametes without fertilization can give rise to embryos (for reviews see Ferrie et al., 1995; Reynolds, 1997). The exact nature of mechanisms that lead to the aquisition of embryogenic potential in microspores have not been elucidated yet. Development of microspore embryos is dependent on stress conditions such as nitrogen starvation or heat treatment (Custers et al., 1994; Kyo and Harado, 1986; Touraev et al., 1996) rather then through the application of growth regulators.

Somatic embryogenesis is the process by which somatic cells develop into plants via the same characteristic morphological stages, and with a comparable timing of development as can be observed in zygotic embryogenesis. Since the initial description in carrot almost 40 years ago (Reinert, 1959; Steward et al., 1958), in this species somatic embryogenesis has been used to study early regulatory events and to identify a variety of molecules including many synthetic plant growth regulators that promote somatic embryogenesis. More recently, also plant-produced molecules are being analysed for effects on the initiation and maturation of somatic embryos. These studies are of course possible because of the excellent accessibility of somatic embryos when compared to zygotic ones.

While the exogeneous application of strong synthetic auxins as 2,4-D is almost universally used to generate embryogenic suspension cultures it is far from clear how embryogenic cells are actually initiated. Petiole (Ammirato, 1985) and hypocotyl explants (Kamada and Harada, 1979) require culturing in the presence of auxin before they produce cells that become competent to form somatic embryos. Even single cells from established suspension cultures (Nomura and Komamine, 1985)

require auxin before they become embryogenic. Auxin causes reinitiation of cell division in explants and proliferation of explant cells once released. Carrot hypocotyl explants can give rise to cells that are capable to generate somatic embryos after exposure to 2,4-D for at least 72 hrs. The only cells that respond to 2,4-D by proliferation are cells of the stelar cylinder, thus the embryogenic cells must derive from these proliferating cells (Guzzo et al., 1994). However, the number of proliferating cells exceeds by far the number of embryogenic cells, as determined by video recording of the development of many thousands of individual cells (Schmidt et al., 1997). This indicates that after auxin treatment only a small subset of the cell population becomes embryogenic, which was previously found to hold true for suspension cultured cells as well (De Vries et al., 1988; Toonen et al., 1994). These experiments also provide evidence that reinitiation of cell division and subsequent proliferation is only one step amongst many in the pathway that leads to embryogenic cell formation. There appears to be only a specific subset of proliferating cells, those that express the Somatic Embryogenesis Receptor Kinase (SERK), that can follow the developmental pathway that leads to embryogenesis (Schmidt et al., 1997).

Most of the alternative systems for embryogenesis exhibit variability in the early division patterns in the embryo, even when the zygotic embryos of the same species exhibited regularity. It appears therefore that despite such variability in early division patterns between species, all dicot embryos develop through the characteristic globular, heart and torpedo stages. In all monocot embryos the globular stage is followed by a transition stage, the initiation of the periferal shoot meristem, and subsequently the formation of an internally located root meristem. In the following section we will discuss studies that demonstrate a role of growth regulators during the initiation of embryogenesis and in later stages of embryo pattern formation during which for instance the apical meristems are established.

Classical plant growth regulators in embryogenesis

In carrot somatic embryogenesis an initially high concentration of either endogenous free IAA or an exogenous auxin is required. Subsequently, the free auxin concentration must fall below a certain maximum in order to allow the organized development of bipolar embryos to occur. Ribnicky et al. (1996) have shown that exogenous auxins have profound effects on endogenous auxin metabolism, and it is therefore likely that removal of exogenous auxins is needed to lower the internal levels sufficiently for internal gradients to appear. Michalczuk et al. (1992) and Cooke and Cohen (1993) assumed that in somatic embryos that contain between 20 and 30 ng g^{-1} fresh weight of total IAA, the free IAA concentration is low enough to allow the establishment of endogenous auxin gradients. In developing seeds very high levels of auxin can be measured, suggesting a requirement for auxin in and around the developing zygotic embryo (Michalczuk et al., 1992). *In vitro* fertilization initially requires externally applied auxins for embryo formation, so the situation in seeds can apparently be

mimicked by application of exogenous auxins. It is assumed, but not proven, that auxins are required to sustain the initial phase of rapid cell divisions in the embryo. There is increasing evidence that they also play a role in later stages in embryo development when pattern formation and cell differentiation occur.

Plant growth regulators exist in low concentrations in cells and therefore it is difficult to localise their site of production and to determine their precise sites of action during embryogenesis (Liu et al., 1993). Two approaches are followed to study the role of endogenous growth regulators during zygotic embryogenesis. In the first one mutants are investigated that show an embryo phenotype in addition to an altered transport or altered level of plant growth regulators. The second approach makes use of growth regulator transport inhibitors applied to excised zygotic embryos. Although both approaches have yielded valuable insight into the role of plant growth regulators in embryogenesis, they suffer from experimental restrictions. Few mutant phenotypes are amenable to measurement of growth regulators due to early arrests and limited availability of tissue for analysis, while excision of zygotic embryos is restricted to later stages of development. It is hoped that increasingly more efficient and reproducible *in vitro* systems will help to overcome these limitations. Determination of specific effects of any particular growth regulator is however often hampered by the fact that one growth regulator appears to be able to modify the synthesis of other classes of growth regulators, either directly or indirectly (Gaspar et al., 1996).

In embryos of the *Arabidopsis* mutant *fass* the initial embryonic divisions are aberrant, but despite this, all pattern elements are developed (Torres Ruiz and Jürgens, 1994). Seedlings of this mutant have been shown to contain 2.5 times higher levels of free auxin, which might in turn cause the higher levels of ethylene that were also observed (Fisher et al., 1996). Furthermore it was shown that the *fass* phenotype can be mimicked when wild type heart-shaped embryos are cultured in the presence of NAA. The *fass* gene product is thought to function as a negative regulator of free auxin formation, or as a positive regulator of auxin conjugation (Fisher et al., 1996).

Another indication for the importance of auxin during embryogenesis comes from studies on auxin transport in the *Arabidopsis* mutant *monopteros*. The *monopteros* gene is important for the apical-basal pattern formation during embryogenesis, since mutant embryos fail to produce hypocotyl and radicle (Berleth and Jürgens, 1993). In adult plants of this mutant the presence of abnormal flowers, reduced or absent veins in leaf laminae, and unoriented, improperly aligned or isolated vessel elements, reveals post-embryonic functions of the *monopteros* gene (Przemeck et al., 1996). In *monopteros* plants, polar auxin transport in the influorescence axis was reduced, and it was proposed that the *monopteros* gene product canalizes a root-to-shoot signal flux in which the polar auxin flux might play a role.

Pin1-1 is an *Arabidopsis* mutant that has several structural abnormalities in the influorescence axis, flowers and leaves (Goto et al., 1987; Haughn and Sommerville, 1988). Analysis of this mutant has shown that the polar auxin transport was decreased to only 14% of wild type (Okada et al., 1991). Culturing wild type plants in the presence of auxin polar transport inhibitors like 9-hydroxyfluorene-

9-carboxylic acid (HFCA), N-(1-naphthyl)phthalamic acid (NPA), or 2,3,5-triiodobenzoic acid (TIBA) resulted in plants that resembled the phenotypes of the *pin1* mutants, indicating that the major genetic defect of the mutation is related to the auxin polar transport system(s). The *pin1-1* phenotype is not restricted to the adult plant since selfing of *pin1-1* heterozygous plants resulted in an offspring containing embryos with a fused, cylindrical cotyledon (Liu et al., 1993). This suggested that polar auxin transport is essential for the establishment of bilateral symmetry during plant embryogenesis. Other indications for the correctness of this hypothesis are also reported by Liu et al. (1993). Globular and heart-shaped zygotic embryos of Indian musterd were dissected and cultured *in vitro*, where embryogenesis proceeded as in the *in vivo* situation. Culturing these embryos in the presence of the auxin transport inhibitors HFCA, TIBA, or *trans*-cinnamic acid resulted in embryos with a phenotype resembling that of the *pin1-1* mutant. The recently described *cuc* mutants (Aida et al., 1997), that are characterized by the observation that the *cuc1 cuc2* double mutant fail to organize a shoot apical meristem, exhibit a phenotype that is similar to the *pin1-1* phenotype. This indicates that either the developmental pathway of organizing the shoot apical meristem in the embryo, in which the *cuc* genes are involved, is also modulated by polar auxin transport, or that it is difficult to conclude much from the *pin1-1* phenotype. Partially or completely fused cotyledons have also been observed in embryos of the *emb30(gnom)* mutant (Mayer et al., 1993; Meinke, 1985), and therefore Liu et al. suggested that the *emb30* gene product might play a role in auxin polar transport. Cloning of the *emb30* gene revealed that it encodes a protein that has similarity to yeast *SEC* proteins (Busch et al., 1996; Shevell et al., 1994)(Shevell et al., 1994). The exact function of the *EMB30* protein is still unknown, but Shevell et al. (1994) speculated that *EMB30* functions in secretion and therefore might affect the transport of auxin, or, alternatively, might affect the synthesis and secretion of glycoproteins. Mutants of the *amp/pt* type exhibit an enlarged shoot apical meristem that gives rise to a polycotyledon phenotype in the seedling. *amp* plants were shown to have a variety of defects and to contain elevated levels of cytokinin (Chaudhury et al., 1993). Whether this elevated level of cytokinin is directly responsible for the polycotyledon phenotype is not established, so it is not clear whether a balance of auxin and cytokinin is involved in cotyledon primordium formation.

Besides the influence of polar auxin transport inhibitors on dicot embryo development, the effect of auxins and polar auxin transport inhibitors has also been investigated in monocot embryogenesis (Fischer and Neuhaus, 1996). In the *in vitro* development of wheat zygotic embryos the unilateral formation of the scutellum was blocked when the embryos were cultured in the presence of 2,4-D, 2,4,5-T or IAA. The effect of the auxins depended on the developmental stage of the isolated embryos. Addition of auxin to symmetrical globular embryos resulted in growth, but not in cell differentiation or meristem formation. Addition to transition stage embryos resulted in a normal embryo morphology, but sectioning revealed a loss in cellular organization in the treated embryos. Blocking auxin transport by culturing wheat zygotic embryos in the presence of TIBA did not prevent scutellum formation, but the relative position of the shoot apical meristem in comparison to the scutellum was altered and no root meristems were formed. Based on these obsevations it was

proposed that the non-homogeneous distribution of auxin within the embryo proper at the globular stage or 'auxin gradients' are instrumental in the establishment of embryo polarity (Fischer and Neuhaus, 1996).

Non-classical growth regulators in embryogenesis

In several *in vitro* systems it has been found that proteins secreted into the culture medium can be beneficial or detrimental for embryogenic cell formation and also for progression of embryogenesis (De Vries et al., 1988; Maës et al., 1997). The addition of extracellular proteins isolated from embryogenic carrot cultures could rescue embryogenesis in the temperature sensitive mutant carrot cell line *ts11* (Lo Schiavo et al., 1990). *ts11* is likely to be impaired in its secretion of extracellular proteins and polysaccharides (Baldan et al., 1997), amongst which the extracellular protein 3 (EP3) that caused the complementation has been purified and turned out to be an endochitinase (De Jong et al., 1992; De Jong et al., 1995). The acidic endochitinase EP3 was found to be a member of a small family of highly homologous class IV chitinase genes (Kragh et al., 1996). *EP3* gene expression in suspension cultures was notably found in non-embryogenic cells and not in somatic embryos, while in plants expression was seen in the integuments of developing seeds, in the endosperm of mature seeds, but not in zygotic embryos (van Hengel et al., in press). The fact that EP3 has a promotive role in ts11 somatic embryogenesis, while it is produced predominantly by non-embryogenic cells points to the existence of protein-mediated cell to cell communication in the process of somatic embryogenesis.

The effect of EP3, lifting the arrest in somatic embryo development of ts11, could be mimicked by chitin-containing bacterial signal molecules, the so-called lipochitooligosaccharides (LCOs) or Nod factors (De Jong et al., 1993). These results were originally interpreted to reflect the involvement of EP3 chitinases in the generation of plant analogues of LCOs. Staehelin et al. (1994) have shown that modifications in *Rhizobium meliloti* LCOs influence their stability against hydrolysis by root chitinases and they propose that the activity of LCOs may be partly determined by the action of plant chitinases. These data suggested that plants employ extracellular chitinases to influence a signalling pathway, involving bacterial LCOs, that eventually leads to the formation of root nodules. Chitin fragments consisting of four or more N-acetylglucosamine residues are perceived by tomato cells (Felix et al., 1993). Subnanomolar concentrations of these chitin fragments can induce an extracellular alkalinization and cause changes in protein phosphorylation in suspension cultured tomato cells. Thus, N-acetylglucosamine-containing LCOs trigger root nodule formation and are also able to affect ts11 somatic embryogenesis, while chitin fragments are able to activate a signal transduction pathway that might be part of the plants' biochemical defence response upon pathogen attack. Enzymes that form and degrade biologically active oligosaccharides (termed oligosaccharins; Darvill et al., 1992) are almost certainly largely responsible for when and where oligosaccharides are

active in plant tissues (Albersheim et al., 1994). Endochitinases might therefore be part of such a regulatory mechanism involving chitin fragments and LCOs, both of which are oligosaccharides involved in cell signalling (for review see Spaink, 1996). Another indication that plant development is influenced by plant produced molecules that contain chitin-like fragments comes from tobacco plants that were transformed with the *Rhizobium nodA* and *nodB* genes and that showed severe effects in their development (Schmidt et al., 1993).

Oligosaccharins can act as antagonists of auxin, which has been shown by increasing the auxin concentration in a system where oligogalacturonides normally can inhibit the auxin-induced formation of roots on tobacco leaf explants (Bellincampi et al., 1996; Bellincampi et al., 1993). A functional relationship between LCOs and classical growth regulators was also observed by Röhrig et al. (1995) who have shown that LCOs at femtomolar concentrations confer the ability of tobacco protoplasts to divide in the presence of high auxin concentrations. Recently it was reported that another kind of signalling molecules is involved in the division of protoplasts in this system, since the secreted peptide ENOD40 is also able to alter the response of tobacco protoplasts to auxin (Van De Sande et al., 1996). It appears therefore that several recently described unusual plant growth regulators are developmentally important and may operate through altering the response of plant cells to auxin.

Chitin-based oligosaccharides may also be involved in animal development. In *Xenopus* the *DG42* gene is found to be expressed only between the late midblastula and neurulation stages of embryonic development (Rosa et al., 1988). The gene has some similarity with fungal chitin synthases and an even stronger homology with the *Rhizobium nodC* gene (Bulawa and Wasco, 1991). The DG42 protein has been shown to catalyze the synthesis of short chitin oligosaccharides *in vitro* (Semino and Robbins, 1995), suggesting a role in development for molecules that contain short arrays of N-acetylglucosamine. Homologs of *DG42* are present in zebrafish and mouse (Semino et al., 1996). In zebrafish, recent evidence was obtained that suggests that blocking the DG42-like activity by microinjection of anti DG42 antibodies during zebrafish embryogenesis resulted in defects in trunk and tail development (Bakkers et al., 1997).

While the above-cited examples all involve small diffusable molecules that are likely to belong to intermediate and long range signalling systems, short distance signal molecules must also exist in plants. Evidence for this comes from laser ablation studies in Arabidopsis roots where it was shown that cells can acquire the fate of their neighbouring cells in a directional fashion (Van den Berg et al., 1995). The identity of such compounds is not known, but in other systems it has been suggested that such close-range signalling involves cell wall determinants. The marine brown algae *Fucus* and *Pelvetia* provide model systems to study the initiation and maintainance of polarity in embryogenesis. The *Fucus* zygote establishes a stable polar axis within 12 h after fertilization. External gradients like unilateral light can impose a polar axis and the first cell division results in an apical thallus cell and a basal rhizoid cell. Berger et al. (1994) have shown that at the two cell stage, cell walls derived from either the rhizoid or the thallus cells have the potential to confer and maintain rhizoid and thallus properties on *Fucus* protoplasts and in this way impose a polar axis. These results suggest that cell

walls can contain positional information which can be perceived by the protoplasts' cytoplasm and/or nucleus and thus influence its developmental fate. The establishment of the polar axis is most likely a result of targetted secretion, since polar secretion of Golgi-derived material at the fixed site of polar growth is essential for growth and differentiation of the rhizoid as well as for the first plane of cell division (Shaw and Quatrano, 1996). Which wall components are causative factors for the signalling that influences cell fate in *Fucus* embryogenesis remains to be elucidated, although it is tempting to speculate that oligosaccharides or proteoglycans are involved in this process.

Similar studies have not been performed in higher plant zygotes, but oligosaccharides are known that can function as developmental markers. Several monoclonal antibodies to extracellular matrix polysaccharides, extensins and arabinogalactan proteins (AGPs) of higher plants have been generated and were shown to recognize developmentally regulated cell surface epitopes (for review see Knox, 1997). The differentiation of *Zinnia elegans* mesophyll cells into tracheary elements provides a semi-synchronous system for studying *in vitro* cell differentiation (Fukuda, 1992; Fukuda and Komamine, 1980). Stacey et al. (1995), using monoclonal antibodies, have shown that changes in the secretion of three classes of cell wall molecules occur during differentiation. The precise timing of the appearance and disappearance of epitopes, that are present on either pectic polysaccharides, xyloglucans or arabinogalactan proteins (AGPs), provides a series of carbohydrate cell-surface markers for cell state, particularly correlated with the time of determination of the tracheary element fate.

Arabinogalactan proteins

AGPs are proteoglycans with poly- and oligosaccharide units covalently linked to a central protein core, and are part of the extracellular matrix of most plants (for reviews see Du et al., 1996; Kreuger and Van Holst, 1996). The AGP epitopes that are recognized by the monoclonal antibodies JIM4 and JIM13, are expressed in specific parts of the carrot root apex. Based upon this, a function for AGPs in determining plant cell fate has been postulated (Knox et al., 1991; Knox et al., 1989). A correlation between cell fate and the expression of a specific set of AGPs has also been found in maize coleoptiles that are commited to undergo programmed cell death (Schindler et al., 1995).

Several experiments in different systems suggest that AGPs are not only a consequence of cell differentiation, but may also play a more instructive role in development. The ß-glycosyl Yariv reagent, with which AGPs interact specifically, when bound to cell wall AGPs of rose suspension cells inhibited growth in a reversible fashion (Serpe and Nothnagel, 1994). In suspension cultured carrot cells that have been induced to elongate rather than proliferate, addition of the Yariv reagent resulted in an inhibition of cell elongation. Due to the application of Yariv reagent to *Arabidopsis* seedlings a reduction of root growth as a result of reduced cell elongation was observed (Willats and Knox, 1996). The addition of AGPs, containing the ZUM18 epitope, that were isolated from mature dry carrot seeds was reported to increase the number of embryogenic cell clusters in carrot suspension

cells (Kreuger and Van Holst, 1995). Toonen et al. (1997) have shown that AGPs can overcome the negative effect that the removal of particular cell populations has on the number of somatic embryos produced. These results suggest that AGPs, in addition to the EP3 chitinases may represent a second example of molecules that are involved in cell-cell communication in suspension cultures. No effect was seen by Toonen et al.(1997) on the number of somatic embryos produced by non-fractionated suspension cultures. In the conifer Norway spruce (*Picea abies*) addition of AGPs, isolated from seed extracts, can influence somatic embryo morphology by enlarging the embryonic region that is composed of a mixture of meristematic and expanded cells (Egertsdotter and Von Arnold, 1995). Taken together, these data suggest that certain AGPs can be classified as non-classical plant growth regulators that may have a function in plant development. Whether they are involved in specific processes such as cell differentiation or affect more general functions such as division and expansion remains to be elucidated.

The suggested function(s) of AGPs involve cell-cell signalling and cell-matrix interactions. Based upon their biochemical characteristics AGPs have the potential for two types of interactions: macromolecule-macromolecule or macromolecule-small-ligand (Gane et al., 1995). A signalling function of AGPs could be envisaged by means of such interactions or, alternatively, AGPs can function as the locked-up form of signalling molecules (Bacic et al., 1988) and thus may require extracellular processing for the release of the active forms of the signal molecules.

Cell-cell communication and 'nursing' during plant embryogenesis

While the phenomenon that somatic embryogenesis indeed occurs in plants is often used as an argument against the role of a maternal contribution to plant embryogenesis, several lines of evidence suggest an intimate relation between the developing embryo and its surroundings. In zygotic embryogenesis the embryo is surrounded by the maternal integuments and the endosperm. Little is known about the precise developmental relationship between these three partners, but there is growing evidence that regulatory communication systems during seed development exist (for review see Lopes and Larkins, 1993). Recently Colombo et al. (1997) have provided molecular evidence for maternal factors that control seed and endosperm development. The *floral binding protein7* (*fbp7*) and *fbp11* are genes belonging to the MADS box family of transcription factors. Both genes are expressed in the integuments after fertilization has taken place. Plants in which both genes are downregulated by cosuppression showed a shrunken seed phenotype while mature seeds with a strongly reduced amount of endosperm were produced. The interaction between embryo, endosperm and maternal sporophytic tissue is also studied in the *fertilization independent endosperm* (*fie*) (Ohad et al., 1996) or *fertilization independent seed* (*fis*) (Chaudhury et al., 1997) mutants. The female gametophytic *fie* mutation allows replication of the central cell nucleus and endosperm development without fertilization. *FIE/fie* seed coat and fruit undergo fertilization-independent differentiation, which shows

that the *fie* female gametophyte produces signals that activate sporophytic fruit and seed coat development. Furthermore, embryos that maternally inherit the mutant *fie* allele are aborted, while *fie* does not affect the male gametophyte (Ohad et al., 1996). Also in *fis* mutants certain aspects of seed development are uncoupled from the event of double fertilization, resulting in partial development of the seed without pollination. In addition, pollination of *fis* plants with wild type pollen resulted in *fis* seeds that were found to contain embryos arrested at the torpedo stage. Thus, it appears that the genetic programs of embryo, endosperm and seed are distinct, yet point to a variety of interactions at different time points.

As discussed in the previous section, there is ample evidence for a contribution of both classical and 'novel' secreted signalling molecules to *in vitro* embryo development. An extreme case was recently reported for wheat androgenesis, where it was shown that co-cultivation with dissected ovaries was absolutely necessary for the induction of embryogenesis (Puolimatka et al., 1996). Together with the requirement for co-cultivation with 'feeder cells' used in several systems including *in vitro* fertilization, and the role of non-embryogenic cells in somatic embryogenesis, it becomes clear that embryogenesis in plants relies heavily on the presence of surrounding tissues and cells. An attractive question that can now be pursued is whether the function of integuments and the endosperm is mimicked *in vitro* by respectively 'feeder cells' and non-embryogenic cells. The precise nature of these signals, their site of synthesis, extracellular and intracellular transduction pathways and the target processes they are involved in are virtually unknown at present. However, many different candidates such as auxins, chitin-based oligosaccharides, AGPs and other cell wall components exist. Regarding the transduction of signals during plant embryogenesis, it is of interest that recently the SERK gene, coding for a leucin-rich repeat containing transmembrane receptor-like kinase was shown to be expressed in both somatic and zygotic embryos (Schmidt et al., 1997). In cells derived from hypocotyl explants the *SERK* gene is transiently expressed in a small population of enlarging single cells during the initiation of embryogenic cultures. *SERK* expression in somatic embryos ceased after the globular stage. In plants, expression of the *SERK* gene could only be detected transiently in the zygotic embryo up to the early globular stage. Schmidt et al. (1997) postulate that the *SERK* receptor-like kinase is a candidate for a protein with an important function in the communication between the early embryo and the surrounding cells, *in vitro* as well as *in vivo*. Identification of additional components of the SERK-mediated signal transduction chain might help to further elucidate such mechanisms of communication.

A surprising observation was recently made on the possible role of oligosaccharides in pattern formation in *Drosophila* , a process known to require the regulated expression of a small forest of signalling molecules. Fringe (FNG), is a secreted *Drosophila* protein that has a key role in dorsal-ventral aspects of wing formation (Irvine and Wieschaus, 1994), and based on homology searches Yuan et al. (1997) have recently suggested that FNG-like signalling molecules may be glycosyltransferases. The expression of glycosyltransferases was shown to increase during mouse embryonic development (Cho et al., 1996) and the extracellular carbohydrate moieties change during

development (Masteller et al., 1995). Furthermore, numerous distinct ESTs from multicellular organisms including *Arabidopsis,* suggest a vast superfamily of glycosyltransferases that might belong to a system of posttranslational modification independent from the Golgi apparatus (interestingly no match was found in yeast or other completely sequenced unicellular organisms). Therefore Yuan et al. (1997) state that the carbohydrate status of the cell during development might be a function of neighboring cells and not only of its own expression set of glycosyltransferases. When this holds true, oligosaccharides might be signalling molecules that are important in embryonic development, and their biological activity is likely to be regulated by enzymes that are produced by the same cells or by neighboring cells.

Concluding remarks

In this chapter we have pointed out experimental studies that place auxin in a central position in the initiation and maintenance of cell divisions in the early plant embryo. Certain aspects in embryo pattern formation, such as the shift from radial to bilateral symmetry are clearly disturbed both by the presence of auxin transport inhibitors and by the presence of high exogeneous auxin levels. Screens that have been performed in order to obtain embryo mutants did sofar not yet result in mutants that are primarily affected in auxin biosynthesis, or mutant phenotypes that can be rescued by the addition of auxins.

We have also described cases where it was shown that classes of 'novel' signal molecules exist that either alone or in combination with classical growth regulators are involved in embryo development. However, the precise mechanisms of such interactions remain to be elucidated in order to determine which plant growth regulators or combinations of growth regulators are decisive for both the initiation and the subsequent development of plant embryos. Likely candidates for such 'novel' growth regulators are oligosaccharides and proteoglycans. Currently these molecules and their biological effects are topics of intense research employing experimental systems in which such molecules can be tested for their effect on plant embryogenesis. The importance and possible necessity of signalling between the developing embryo and the surrounding cells has also emerged from genetic studies aimed at understanding the relation between the zygotic embryo, the endosperm and the maternal integuments. A combination of such genetic backgrounds and *in vitro* systems more amenable to biochemical analysis is expected to provide new insights into the role of growth regulators in plant embryogenesis.

Chapter 2

Expression pattern of the carrot *EP3 endochitinase* genes in suspension cultures and in developing seeds

Carrot EP3 class IV endochitinases were previously identified based on their ability to rescue somatic embryos of the temperature sensitive cell line *ts11*. Employing whole mount in situ hybridisation it was found that a subset of the morphologically distinguishable cell types in embryogenic and non-embryogenic suspension cultures, including *ts11*, express *EP3* genes. No expression was found in somatic embryos. In carrot plants *EP3* genes are expressed in the inner integument cells of young fruits and in a specific subset of cells, located in the middle of the endosperm of mature seeds. No expression was found in zygotic embryos. These results support the hypothesis that the EP3 endochitinase has a "nursing" function during zygotic embryogenesis, and that this function can be mimicked by suspension cells during somatic embryogenesis.

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(Plant Physiology, in press)

Introduction

Carrot cell cultures secrete many different proteins into the medium, a process that contributes to the conditioning of the medium. Conditioned media are reported to have a promoting effect on the initiation of somatic embryogenesis (Hari, 1980; Smith and Sung, 1985). The initiation of somatic embryogenesis results in major changes of the extracellular protein pattern, in contrast to the pattern of intracellular proteins (De Vries et al., 1988). Some of the secreted proteins are thought to be related to the formation of embryogenic cells and somatic embryos. In order to investigate whether a causal relationship between certain secreted proteins and embryogenic potential exists, secreted proteins that were differentially expressed under different culture conditions were analysed and their localisation was studied. The extracellular protein EP1 is only secreted by non-embryogenic cells (Van Engelen et al., 1991), whereas another extracellular protein, EP2, identified as a lipid transfer protein is only secreted by embryogenic cells and somatic embryos (Sterk et al., 1991). These results showed that different cell types contribute to the total pattern of proteins secreted into the culture medium (Van Engelen and De Vries, 1993). Another way of studying a causal relationship between secreted proteins and embryogenic potential has led to the identification of the extracellular protein 3 (EP3), identified as a chitinase. EP3 was originally purified as a protein capable of rescuing somatic embryos in the mutant carrot cell line *ts11* at the non permissive temperature (De Jong et al., 1992). The acidic endochitinase EP3 was found to be a member of a small family of class IV chitinase genes (Kragh et al., 1996). Those highly homologous isoenzymes are encoded by at least 4 *EP3* genes. Two of these proteins, EP3-1 and EP3-3, were purified and shown to have subtly different effects on the formation of somatic embryos in newly initiated *ts11* embryo cultures (Kragh et al., 1996).

Since the effect of the chitinases was mimicked by *Rhizobium*-produced Nod factors (De Jong et al., 1993), it was proposed that the chitinases are involved in the generation of signal molecules essential for embryogenesis in *ts11* (De Jong et al., 1993). The EP3 proteins produced by *ts11* at the permissive as well as at the non-permissive temperature did not show any difference in biochemical characteristics compared to the ones produced in wild-type cultures and they were also capable of rescuing *ts11* somatic embryos. It was also shown that the sensitivity of *ts11* to chitinases coincided with a transient decrease in the amount present of this otherwise functional set of proteins (De Jong et al., 1995).

The roots of leguminous plants are known to produce chitinases and during the interaction with *Rhizobium* these plant produced chitinases have been suggested to control the biological activity of Nod factors by cleaving and inactivating them. In this way, chitinases are proposed to have the potential to control plant morphogenesis and cell division (Staehelin et al., 1994).

Many chitinase genes are induced upon infection, wounding and treatment with elicitors. Infection can lead to a 600-fold induction of chitinase activity (Métraux and Boller, 1986). The molecular mechanisms and signaling pathways, responsible for this induction remain largely unclear (Graham

and Sticklen, 1994). The response of a plant to infection involves nonspecific responses, because challenge by different pathogens can induce production of the same set of PR proteins (Meins and Ahl, 1989). The induction is not only restricted to the infection zone, since uninfected areas of infected leaves and even uninfected second leaves show an increase in chitinase activity (Métraux and Boller, 1986).

In the carrot system we aimed to establish the following. First, to determine which cells in wild-type and *ts11* suspension cultures express the *EP3* genes. And second, to determine whether, and where the same chitinases are expressed during zygotic embryogenesis. The results show that the carrot *EP3 class IV endochitinases* are expressed in a subset of, most likely, non embryogenic suspension cells, and in carrot plants in integument cells and in the endosperm during zygotic embryogenesis.

Results

Cell specific expression of the *EP3* genes and localisation of the encoded proteins in suspension cultures

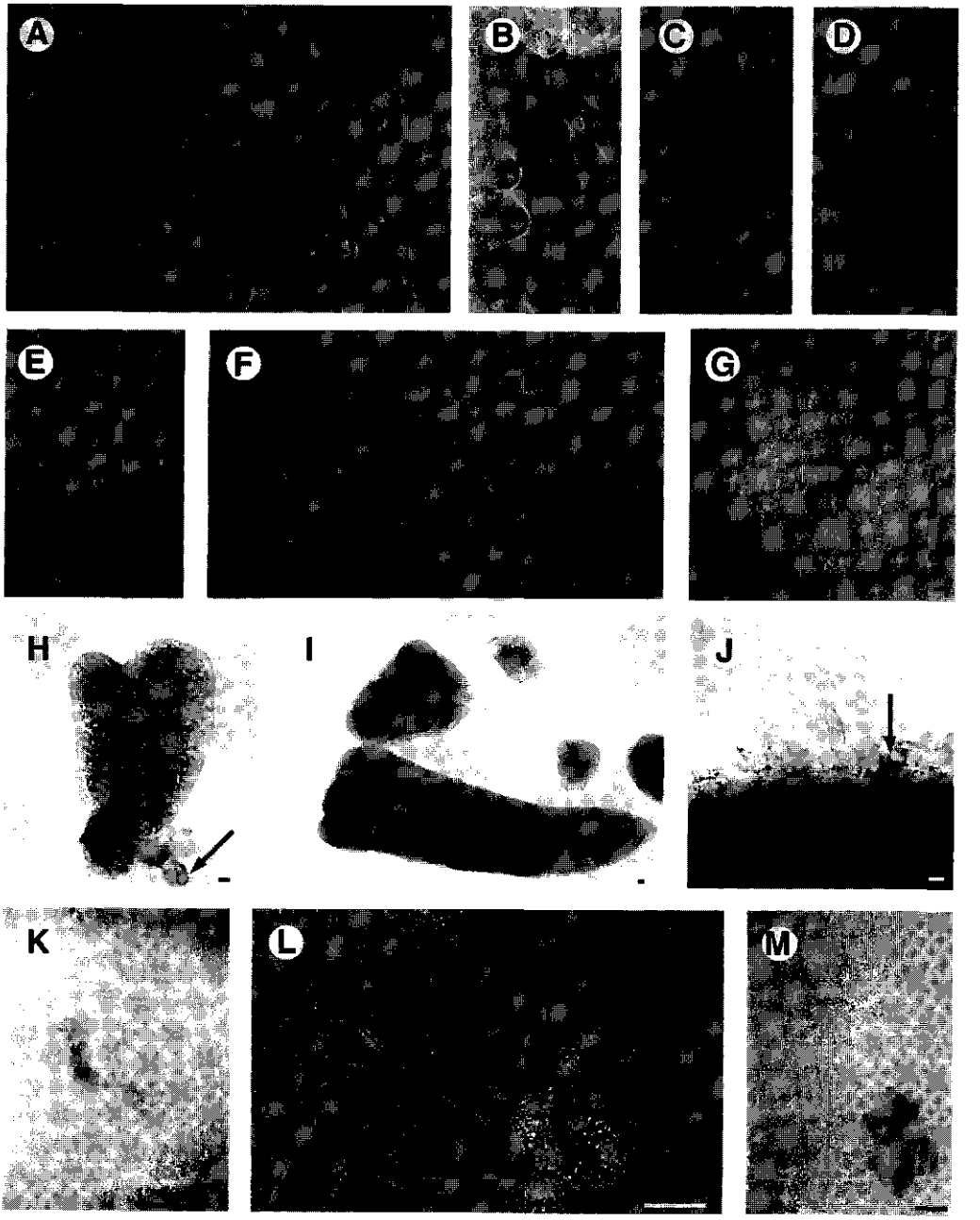
To identify the suspension cells that express the *EP3* genes, whole mount in situ mRNA localisation was employed on entire, immobilized suspension cultures. Several cell lines, differing in embryogenic potential, were used to obtain a reliable indication of cell specificity in *EP3* expression. It was not possible to distinguish between the different members of the family of *EP3* genes, because of their very high homology (Kragh et al., 1996). But since the probe that was used has only a low homology to class I chitinases and spans class IV chitinase specific deletions no class I chitinases could be detected.

Figure 1A shows that there was no staining above background in the majority of the cells, the *EP3* mRNAs could only be detected in a subpopulation of the total embryogenic cell culture. Counting the number of stained cells in several cultures revealed that between 4-6% of the total number of cells in an embryogenic culture express the *EP3* genes. Staining was detected in morphologically different cells (Fig. 1A, B, C, D and E). The highest concentration of *EP3* mRNAs, in both embryogenic as well as in non-embryogenic cultures, was found in single cells that were elongated and often strongly curved or coiled (Fig. 1B). Staining was also often seen in small clusters of cells (Fig. 1E), consisting at the most of about 30 cells. Cells that were loosely attached to large clusters, present in embryogenic cultures, occasionally showed intense staining. Figure 1F shows such a large cluster with several *EP3* expressing cells loosely attached to it. Based on this expression pattern we conclude that *EP3* gene expression does not have a correlation with embryogenic capacity, because *EP3* expressing cells occur in embryogenic and in non-embryogenic cultures (data not shown) and the number of *EP3* expressing cells is higher than the number of single embryonic cells in a comparable

culture (Toonen et al., 1994). In figure 1H a single *EP3* expressing cell (arrow) is attached to a heart stage somatic embryo completely devoid of purple staining. Whole mount in situs on globular, heart or torpedo shaped somatic embryos did not reveal *EP3* mRNAs (Fig. 1I). In order to determine when the first *EP3* expressing cells appeared during embryogenic cell formation hypocotyl explants were used (Guzzo et al., 1994). Hypocotyl explants were treated with 2,4-D for a period of ten days, during which cell division was reinitiated in cells of the vascular tissue. These divisions generated a mass of rapidly proliferating cells. Only after this period a very small number of cells at the periphery of the proliferating mass was found to contain *EP3* mRNA (Fig. 1J). This is three days later as the appearance of the first cells that are competent to form somatic embryos in this system (Schmidt et al., 1997). Subsequently the hypocotyls were transferred to hormone free medium and after another five days some of the peripheral cells started to enlarge. The *EP3* positive cells were in some cases elongated. A population of single cells, released from these explants after 20 days, contained elongated, curved cells as the only cells that stained positively (Fig. 1K). In all experiments sense controls were included, none of which ever resulted in cell-specific staining (see for an example Fig. 1G). Using several other probes it has been shown that cell-specific expression patterns are obtained by employing this method of in situ hybridisation (Schmidt et al., 1996).

Previously it was found that *EP3* encoded chitinases occur in the medium (De Jong et al., 1992). To investigate whether all of these chitinases are secreted in the medium, or whether a part remains in the cell walls of the suspension cells that produce them, and whether they are present in somatic embryos, immersion immunofluorescence was employed (Van Engelen et al., 1991). Since EP3 chitinases are secreted proteins and contain signal sequences for secretion (Kragh et al., 1996) we assume that the EP3 chitinases, as shown in figure 1L, are some component of the extracellular matrix. In non-embryogenic suspension cultures the EP3 chitinases were detected in approximately equal amounts at the surface of all cells (data not shown). In embryogenic suspension cultures a relatively high amount of the protein could be detected on the surface of a subset of cells. The chitinase was identified on single cells as well as on small clusters (Fig. 1L). On the surface of somatic embryos the protein could not be detected, but cells that were loosely connected with somatic embryos often contained EP3 (data not shown). This pattern of localisation was also observed in suspension cultures of the mutant cell line *ts11* (Fig. 1M), that is also capable of producing EP3 proteins (De Jong et al., 1995).

Figure 1. Whole mount in situ hybridisation and immunolocalisation of *EP3* in suspension cultures.
Plant material was analysed by light microscopy (coupled to Nomarski optics for fig. A to E and M) and photographed. In fig. A to K gene expression is visible as a purple precipitate in individual cells (the use of Nomarski optics resulted in a change from purple to brown). Bar: 50 μm.
A. to D. Morphologically different cells in the embryogenic cell culture Ar. **E. to F.** Cell clusters present in the embryogenic cell culture Ar. **G.** An embryogenic cell culture hybridized with a sense probe of the *EP3*. **H. and I.** Somatic embryos in various stages of development. **J.** Cells present at the periphery of the proliferated cell mass on a section of a hypocotyl explant that was cultured in the presence of 2,4-D for 10 days. **K.** An elongated curved single cell released from a hypocotyl explant that was cultured in the presence of 2,4-D for 20 days. **L.** An embryogenic cell suspension. The presence of the protein is indicated by the green fluorescent signal. **M.** A cell cluster in a suspension of the mutant cell line *ts11*. The presence of the protein is indicated by a purple precipitate.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The localisation of the EP3 proteins therefore appears to correspond to the pattern of expression of their encoding genes. This suggests that EP3 proteins are also retained in the cell walls of the cells that produce the proteins, even though a substantial amount is secreted into the medium.
Since the production of many chitinases can be induced by pathogens and elicitors, and in carrot suspension cultures many chitinases are induced after treatment with fungal wall components (Kurosaki et al., 1990), suspension cultured cells were exposed to chitosan as an elicitor, or to *Phytophtora infestans*, or *Botrytis cinerea*. The media of those cultures was isolated and the amount of EP3 produced was determined with Western blots. Scanning of the optical density of the bands revealed that the constitutive production of the protein was not affected by the presence of the fungi used, nor of the elicitor employed (Fig 2).

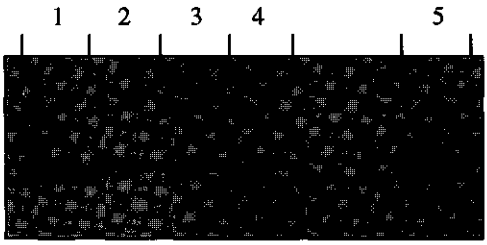


Figure 2. Western blot of EP3 chitinases in media of cultures treated with fungi and elicitors.
Lane 1 Medium of a suspension culture grown in the presence of 0.002 mg/ml chitosan. Lane 2 Medium of a suspension culture grown in the presenceof 0.02 mg/ml chitosan. Lane 3 Medium of a suspension culture grown in the presence of *Phytophthora infestans*. Lane 4 Medium of a suspension culture grown in the presence of *Botrytis cinerea*. Lane 5 Medium of a control suspension culture. Proteins were separated by denaturing SDS-PAGE.

Expression of the EP3 isoenzymes in suspension cultures

With the methods employed so far it was not possible to discriminate between different EP3 isoenzymes or the mRNAs belonging to them. By using native PAGE, 5 different EP3 chitinase isoenzymes have been detected in the medium of suspension cultured cells of different cell lines (Kragh et al., 1996). Given the subtle differences of the isoenzymes EP3-1 and EP3-3 in their biological effect on *ts11* embryo formation, it was of interest to determine whether the different isoenzymes were produced by the same or by different cell types. No correlation was found between the embryogenic capacity of a given cell line, and the EP3 isoenzymes produced (Fig 3A). However, fractionation of suspension cultures showed that different cell types produced different relative amounts of isoenzymes. A population of cells < 50 µm consisted almost solely of single cells. A Western blot of the medium proteins showed that only the isoenzymes EP3-1 and EP3-3 were produced by this cell population (Fig 3B). Cells that were aggregated in large clusters did not pass the 125 µm sieve. This fraction produced the isoenzymes EP3-1, EP3-3, EP3-4 and EP3-5. Density fractionation of a 50 - 125 µm cell fraction on a Percoll block gradient resulted in 4 subfractions of cells with different densities. The subfractions with relatively high densities contained more clusters compared to the low density subfractions, which contained mainly single cells. After culturing for

another 5 days the original difference in cell types was retained. In all cell populations comparable amounts of EP3 proteins were produced. However, a shift in the relative amounts of the different isoenzymes was observed. The pattern of isoenzymes produced by the cells with the lowest density resembled that of the cell population that passed through the 50 µm sieve. A relative increase of the isoenzymes EP3-2 and EP3-5 was observed in cell populations with higher densities. This is in agreement with the pattern of isoenzymes produced by other cell lines as illustrated in Figure 3A. Cell lines that produce isoenzymes EP3-2 and EP3-5 always contain clusters of small, usually cytoplasm-rich cells. Presumably EP3 chitinase isoenzymes 2 and 5 are produced by these cell clusters, whereas single cells are preferentially responsible for the production of EP3 chitinase isoenzymes 1 and 3.

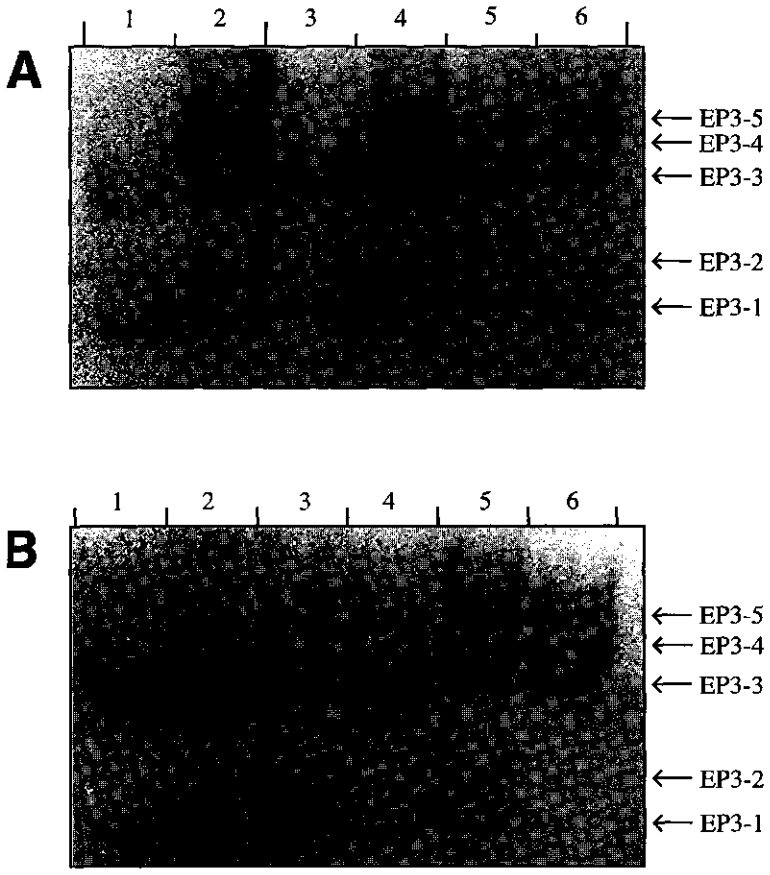


Figure 3. Western blots of EP3 chitinases in media of non-embryogenic, embryogenic and density fractionated embryogenic cultures.
A. Detection of EP3 isoenzymes in the medium of several different suspension cultures. Lane 1 and 2, non-embryogenic suspension cultures Lanes 3, 4, 5 and 6, embryogenic suspension cultures **B.** Detection of EP3 isoenzymes in the medium of a Percoll fractionated suspension culture. Lane 1, cells < 50 µm; lane 2, cells recovered from 0-10% Percoll; lane 3, cells recovered from 10-20% Percoll; lane 4, cells recovered from 20-30% Percoll; lane 5, cells recovered from 30-40% Percoll; lane 6 cells >125 µm. Proteins were separated by non-denaturing PAGE.

Localisation of *EP3* expression in plants

The more sensitive method of RT-PCR was used instead of Northern blot analysis to study *EP3* gene expression. The primers used allowed to discriminate between EP3 and chitinases of class I, since the downstream primer only has homology with class VI chitinases and the primers span a region that contains deletions that are characteristic for class VI chitinases. However, discrimination of the different *EP3 isoenzymes* was not allowed, due to their very high homology (Kragh et al., 1996).
In leaves, stems, storage roots as well as normal roots (Fig. 4A lanes 1-4) hardly any expression of *EP3* could be detected. In developing seeds, at 3, 7, 10 and 20 DAP, *EP3* mRNA was found (lanes 6-9). Only very low levels of *EP3* gene expression occurred prior to pollination (lane 5). The highest expression was found in seeds 10 DAP, which roughly corresponds to the early globular stage embryo. In mature dry seeds the amount of *EP3* mRNA had decreased (lane 10), while during germination the concentration of *EP3* mRNA is hardly detectable (lane 11-12). The presence of *EP3* mRNA in developing seeds of 3, 7, 10 and 20 DAP could be confirmed by hybridizing a Northern blot with the end-labeled downstream primer that had been designed for the the RT-PCR (data not shown). The length of the *EP3* transcripts were estimated to be around 950 nucleotides.

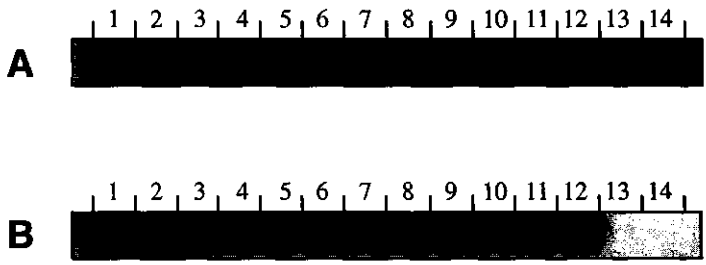
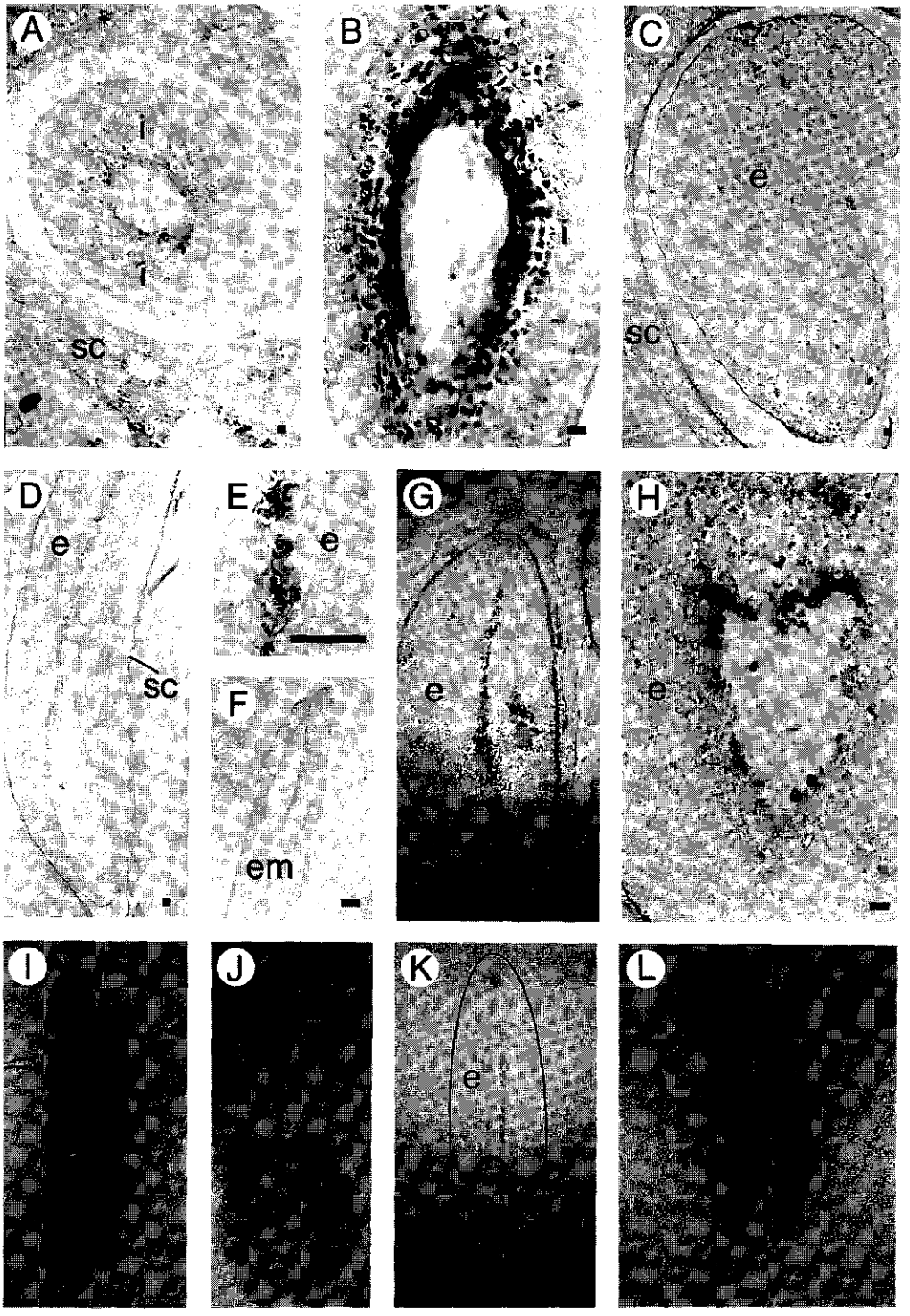


Figure 4. Expression of *EP3* genes determined by RT-PCR.
A: *EP3* Lane 1, leaf; lane 2, stem; lane 3,storage root; lane 4, root; lane 5, flower: lane 6, fruits harvested 3 DAP; lane 7, 7 DAP; lane 8, 10 DAP; lane 9, 20 DAP; lane 10, mature seed; lane 11 seed 12 hrs after imbibition; lane 12, seed 60 hrs after imbibition; lane 13, genomic DNA; lane 14, water control. **B**: *Ubiquitin* control

Figure 5. In situ hybridisation on sectioned carrot seeds and immunolocalisation of the EP3 in tissue prints.
Plant material was analysed by light microscopy (coupled to Nomarski optics for fig. A to E) and photographed.
In the in situ hybridisations (A to F), the presence of *EP3* gene expression is visible as a purple precipitate in individual cells. Bar: 50 µm.
A. Cross section of a fruit, 7 DAP. i, integuments surrounding the developing embryo and endosperm; sc, seed coat. **B.** Longitudinal section of a fruit, 7 DAP. **C.** Cross section of a fruit 20 DAP. e, endosperm. **D.** Longitudinal section of a mature seed. **E.** Longitudinal section of the central region of a mature seed. **F.** Zygotic embryo (em) in a longitudinal section of a mature seed. **G.** Transversal section of a seed that has been imbibed in water for 60 hrs. **H.** Transversal section of a seed that has been imbibed in water for 60 hrs.
In the immunolocalisations, the presence of EP3 proteins is visible as a dark precipitate. Bar: 100 µm.
I. Control tissue print of a mature seed stained with amido black. **J.** Immunolocalisation of EP3 on a tissue print of a fruit containing two developing seeds 20 DAP. **K.** Immunolocalisation of EP3 on a tissue print of a mature seed (a drawing of the printed seed is superimposed on the picture).
L. Immunolocalisation of EP3 on a tissue print of a seed that has been imbibed in water for 60 hrs.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In situ mRNA localisation using sections of different plant tissues, such as flowers, roots, root tips, hypocotyls, cotyledons, shoot apices and shoot meristems confirmed that *EP3* mRNA was not detectable in these tissues (data not shown). Only in developing and mature seeds *EP3* mRNAs could be found. In early stages of seed development, approximately 5-6 DAP, *EP3* mRNA was detected in the inner integument cells, lining the surface of the embryo sac in which the zygote or the early embryo is located (Fig 5A and B).

Since the highest amount of *EP3* mRNAs was found 10 DAP, and the in situ hybridisations showed their presence in the integument cells, we conclude that the EP3 chitinases are produced by the integument cells shortly before they are degraded. At 20 DAP the degradation of the integuments is almost completed (data not shown) and in the developing endosperm at 20 DAP, *EP3* mRNA could not be detected (Fig 5C). During the period that the number of integument cells declines and the volume of endosperm cells increases, until 35 days after anthesis a decline of *EP3* mRNAs was observed. In the endosperm of mature seeds *EP3* mRNA was restricted to a narrow zone of endosperm cells starting at the cavity in which the embryo is located up to almost the opposite end of the endosperm (Fig 5D and E). Transverse sections showed that the *EP3* expressing zone has a width of 2-3 cells and is located in the middle of the endosperm. In zygotic embryos no *EP3* mRNA could be detected (Fig. 5F). During seed development cellularisation of the endosperm starts with the outer layers of endosperm and is completed in the middle of the endosperm, which implies that *EP3* genes are expressed in a place where the cellularisation of the endosperm has been completed. The cells that contained the *EP3* mRNAs also have protein bodies, which could be made visible using Nomarski optics (data not shown).

When carrot seeds are imbibed, germination is initiated, the embryo starts to develop and the central cavity is enlarged because of degradation of the endosperm. The cells in which *EP3* mRNA is seen are thought to be the first ones to be degraded. The expression of the *EP3* genes then shifts outward to the next cell layer that now lines the central cavity as shown by in situ localisation of *EP3* mRNA in germinating seeds (Fig 5G and H). Because EP3 chitinases are secreted proteins it was of interest to localise these enzymes during the development of seeds and in germinating seeds. For this purpose tissue printing of cut seeds onto nitrocellulose was applied. Protein transfer was ensured by staining with amido black, revealing a protein distribution pattern in which the major seed tissues could be clearly observed (Fig 5I). In the tissue prints the EP3 chitinase protein was localised using EP3 antibodies. Those antibodies strongly react with all EP3 class IV chitinases, but have a weak affinity for the carrot 34 kD class I chitinase (Kragh et al., 1996). Control experiments were performed using preimmune serum or antibodies raised against the related barley class I chitinase K (a-ChitK) that only recognises the 34 kD class I chitinase and not the EP3 chitinases. No seed proteins present on the tissue prints were recognized by these sera (data not shown). Since the EP3 antiserum does not recognize any additional chitinases, we conclude that the tissue prints almost exclusively show EP3 chitinases. The EP3 protein was uniformly spread in the integuments at 10 DAP and in the developing endosperm at 20 DAP (Fig. 5J). The presence of the chitinase proteins in the developing seeds is

therefore only partially in agreement with the *EP3* gene expression data. This points to transport of the EP3 proteins from the maternal integument tissues towards the endosperm. However we cannot completely rule out that a very low level of gene expression occurs in the peripheral endosperm cells. In mature seeds the protein was restricted to the inner cell layer of the cavity that surrounds the embryo and to a zone of cells that starts at this cavity and ends almost at the opposite side of the endosperm (Fig 5K), which corresponds precisely with the expression pattern of the *EP3* genes as determined by in situ hybridisation(Fig 5D). In imbibed seeds the protein was again found to be spread uniformly in the endosperm. This pattern was observed in seeds imbibed for 16 hrs as well as in seeds imbibed for 60 hrs (Fig 5L). In the 60 hrs imbibed seeds enlargement of the embryo and commencing of the degradation of the endosperm were already visible. Seeds imbibed in water containing 1mM cycloheximide did not show any enlargement of the embryo, nor endosperm degradation. Tissue prints of the cycloheximide-treated seeds showed that the localisation of the EP3 chitinase protein was the same as in the seeds imbibed in water only (data not shown). This suggests that there is no *de novo* synthesis of EP3 in the outer layers of the endosperm upon imbition, but that the water take up is responsible for the observed outward spread which results in a uniformly presence of EP3 protein in the endosperm.

The *EP3* genes are expressed in the maternal integument cells. The EP3 chitinases however, were detected in the developing endosperm, at a time that *EP3* chitinase gene products cannot be detected in the endosperm and as a consequence they are of maternal origin. In mature seeds the integuments are completely degraded and then the EP3 proteins are produced in a subset of the endosperm cells. During germination the secreted EP3 chitinases diffuse in the water that is present in the cell walls to the outer region of the endosperm and around the embryo, in contrast to the inwards directed transport that occurs in the developing seeds.

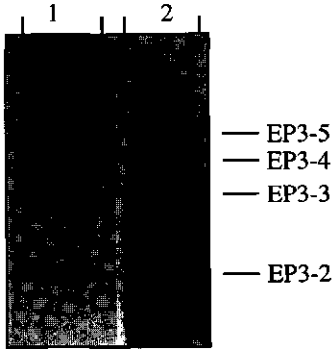

Figure 6. Western blot of EP3 chitinases in the medium of a suspension culture and in mature seeds.
Lane 1, Control suspension culture; lane 2, Seed Proteins were separated by non-denaturing PAGE.

A Western blot of partially purified seed proteins showed that at least isoenzymes EP3-2, EP3-4 and EP3-5 were present in dry mature seeds (Fig 6). In seeds isoenzyme EP3-1 could not be detected due to its low affinity for the DEAE-Sepharose FF resin that was used in the purification method, and isoenzyme EP3-3 is obscured by a smear. The chitinase activity was confirmed by using glycol-chitin

overlay gels, which showed that isoenzymes EP3-2 to EP3-5 were all capable of degrading chitin (data not shown). These data show that, except for isoenzyme EP3-1, all EP3 isoenzymes found in suspension cultures are present in developing seeds. It was striking that the relative amounts of EP3 isoenzymes in suspension cultures were quite different from those in seeds (Fig 3).

Discussion

In this study the carrot suspension cells that produce EP3 carrot chitinases were identified. Employing whole mount in situ hybridisation, it was found that a subset of cells present in an embryogenic culture express *EP3* genes. On the basis of the number, cell type and presence of *EP3* expressing cells in embryogenic and non-embryogenic cultures, there was no correlation with the ability to produce somatic embryos and the expression of *EP3*. No expression in somatic embryos was found. The occurrence of the EP3 chitinase protein in the walls of suspension cultured cells corresponds to that of the *EP3* mRNA expression in the cells.

In plants, *EP3* genes are expressed in the inner integuments, while the EP3 chitinase proteins are found in the endosperm. Later, cells in the centre of the endosperm express the *EP3* genes and this is likely to be responsible for the EP3 chitinase proteins present during imbition and germination. No expression was found in zygotic embryos. Together with the absense of a pathogen- or elicitor-induced response in *EP3* gene expression the results support the earlier conclusion that the carrot EP3 class IV chitinases are primarily involved in embryogenesis (De Jong et al., 1992).

The EP3 chitinases that are produced by single cells were identified as isoenzymes EP3-1 and EP3-3, whereas isoenzymes EP3-2 and EP3-5 were predominantly produced by clusters. Purified EP3-1 and EP3-3 chitinases are both able to rescue the carrot somatic embryo variant *ts11* (Kragh et al., 1996). Other EP3 isoenzymes have not been tested for their capability of rescuing *ts11*. The arrest in somatic embryo development in this carrot variant *ts11* has been shown to be due to a transient reduction in secreted EP3 (De Jong et al., 1995). On the basis of the expression pattern of the *EP3* genes it is likely that cells that produce EP3 do not develop into embryos themselves. Thus, it appears that EP3 chitinases, or products of their enzymatic activity, diffuse via the conditioned medium to cells that are able to form somatic embryos and in this way play their "nursing" role in the process of somatic embryogenesis.

Since *EP3* expression was also found in developing carrot seeds, it could be expected that the chitinases play a similar role in zygotic embryogenesis. In carrot, flowers are pollinated directly after anthesis, and endosperm development starts soon after fertilisation. Division of the primary endosperm nucleus leads to a large number of endosperm nuclei before the first division of the zygote has taken place. These nuclei are located in the upper part of the embryo sac, around the zygote, and at the periphery of the embryo sac (Borthwick, 1931). Cell wall formation in the carrot endosperm

starts 7 days after anthesis (Gray et al., 1984), at about the time that the embryo is in the two cell stage (Borthwick, 1931), and is complete 21 days after anthesis. The integuments that surround the embryo sac start to break down 6 -7 DAP, at the moment that the endosperm becomes cellular (Lackie and Yeung, 1996 and Yeung personal communication). Maximum endosperm volume is reached about 35 days after anthesis. Mature seeds can be harvested about 80 days after anthesis. The endosperm of mature carrot seeds consists of two different cell types, peripheral cell layers containing calcium oxalate crystals and more centrally located cells containing protein bodies with globular inclusions (Menon and Dave, 1988). The outermost cell layer of the endosperm has a high content of ER, whereas the central cells contain large protein bodies and lipid droplets, or only lipid droplets. The endosperm cells that directly surround the embryo have very thick cell walls, contain lipid droplets and have a large vacuole (Timmers, 1993).

The EP3 chitinase proteins found in the endosperm of 20 DAP seeds are most likely produced by integument cells. This indicates a maternal contribution to the proteins that are present in the endosperm.

Recent evidence for a role of maternal tissues in endosperm formation comes from the analysis of gametophytic mutations in *Arabidopsis*. In this species, *fie* (fertilisation-independent endosperm) mutations are known (Ohad et al., 1996) that are female gametophytic and specifically affect endosperm formation. These data demonstrate a maternal component in endosperm formation. *FIE / fie* integuments that surround a mutant *fie* female gametophyte are degraded during development and give rise to the seed coat in the absense of fertilisation. This implies that *Arabidopsis* integument and seed coat development are initiated in response to a signal produced by the female gametophyte (Ohad et al., 1996).

In mature, dry seeds the *EP3* mRNAs are located in the middle of the endosperm. In several other plant species, chitinases have been found in seeds. For example in cucumber an endochitinase was found that is only present in mature seeds and in seeds during the early stages of germination (up to 2 days) (Majeau et al., 1990). In barley, a class I (Chi26) and a probable class II chitinase were found primarily in endosperm and aleurone tissues (Swegle et al., 1992). Chitinase mRNAs accumulated in barley endosperm and aleurone during seed development and were present from 15 days after anthesis until the end of germination (Leah et al., 1991; Swegle et al., 1992). For Chi26 an enhancer sequence has been identified that directs the aleurone specific expression of this gene (Leah et al., 1994). Cells of the starchy endosperm of cereals are dead at maturity, and incapable of synthetic processes (Bewley and Black, 1994), so the synthetic processes in the aleurone layer may resemble those that take place in the endosperm in species that do not contain aleurone tissue, but rather live endosperm cells. In maize two 28-kD chitinases have been found that show high levels of expression in imbibed seeds. These chitinases were reported as class I chitinases, but based on current definitions they should be reclassified as class IV chitinases (Huynh et al., 1992). Our results show that the localisation of EP3 producing cells is restricted to the inner tissues of both the young fruits and the mature seeds. This pattern of localisation suggests that the EP3 chitinases do not primarily

function as enzymes that protect seeds against pathogens, as is commonly assumed for seed chitinases (Graham and Sticklen, 1994).

EP3 mRNAs are found in integuments and in endosperm cells that surround the central space in which the embryo is located. Both tissues are destined for degradation. In barley, apoptosis in the aleurone has been demonstrated during seed germination (Wang et al., 1996), and fungal infection has been shown to induce apoptosis (Wang et al., 1996). Since pathogen induced chitinases are also induced prior to cell death (Kurosaki et al., 1987), this may suggest a more general role for chitinases in apoptosis.

The *EP3* gene expression pattern in seeds supports the hypothesis that chitinases are not only essential for the *ts11* mutant, but play a more general role in plant embryogenesis. Another, indirect indication for the importance of chitinases during seed development comes from a recent study of tobacco plants with low chitinase levels because of silencing caused by transgene expression of a tobacco class I chitinase. The silenced chitinases were transiently reset to a high expressing state 8-11 DAP (Kunz et al., 1996). An explanation for the resetting of the silenced chitinases is not given by Kunz and coworkers, but it cannot be excluded that the requirement for chitinases during this period in seed development is a driving force for the resetting.

The finding that besides the EP3 chitinases *Rhizobium* produced Nod factors are capable of rescuing embryo formation in *ts11* (De Jong et al., 1993) has led to the hypothesis that the presence of EP3s may result in the generation of N-acetylglucosamine containing molecules that have a positive effect upon the development of somatic embryos, although the correlation between EP3s and the *Rhizobium* Nod factors remains to be elucidated (De Jong et al., 1995).

Several examples in different systems appear to support the hypothesis that N-acetylglucosamine containing oligosaccharides are important in development. During *Xenopus* embryonic development the DG42 gene is found to be expressed only between the late midblastula and neurulation stages (Rosa et al., 1988). This gene has some similarity with fungal chitin synthases and an even stronger homology with the *Rhizobium* NodC gene (Bulawa and Wasco, 1991). The DG42 protein has been shown to catalyze the synthesis of short chitin oligosaccharides in vitro. The array of chitin oligosaccharides formed bears a striking resemblance to the oligosaccharides produced by NodC (Semino and Robbins, 1995). Homologs of this *Xenopus* DG42 are present in zebrafish and mouse (Semino et al., 1996).

Chitinases like EP3 might be involved in either releasing N-acetylglucosamine containing signal molecules, or modifying those signal molecules, because of their specific localisation, as shown in our work, and the finding that chitinases are capable of hydrolysing Nod factors (Staehelin et al., 1994). Labeling studies indicate that plants may produce molecules that are analogous to Nod factors (Spaink et al., 1993). Regarding the biological function of the EP3 chitinases in plant embryogenesis we propose that they are involved in reinitiating cell division in embryogenic cells and embryos as part of a "nursing cell" system. This hypothesis is based on the following observations: 1, the EP3 chitinases promote embryogenic cell formation and the number of somatic embryos as well as their

progression in development when added to *ts11* cultures (De Jong et al., 1992), 2, the expression of *EP3* genes in cells that do not develop into embryos in culture and an absence of expression in somatic embryos, 3, the expression of *EP3* genes in maternal tissue and subsequent secretion of the encoded proteins, resulting in the presence of EP3 in the extracellular matrix of the endosperm surrounding globular-stage zygotic embryos and 4, the absence of expression in zygotic embryos and expression in endosperm cells prior to and during germination. The role of EP3 could either be direct, but more likely indirect through release of an N-acetylglucosamine containing signal molecule.

Materials and methods

Plant material and culture conditions

Daucus carota cell suspension cultures of cv. Autumn king / Trophy as well as *ts11*were initiated and maintained as described before (De Vries et al., 1988). One week old suspension cultures were used for gene expression analysis and protein localisation experiments. Hypocotyl sections were prepared as described by Guzzo et al. (1994). The hypocotyls of one week old plantlets were divided in segments of 3-5 mm and incubated for ten days in B5 medium with 2 µM 2,4-D and then returned to B5 medium without 2,4-D.

One week old cell suspension cultures of an embryogenic cell line were grown in the presence of *Phytophthora infestans* or *Botrytis cinerea*, or supplemented with 0.002 or 0.02 mg. ml^{-1} chitosan. After 2 days of incubation the cells and fungi were removed by filtration and the resulting cell-free conditioned media were assayed for the presence of EP3 by means of Western blotting as described by Sterk et al. (1991).

Cell suspension fractionation analysis

Suspension cultures were sieved to obtain cells and cell clusters with a size between 50 and 125 µm. The cells were loaded on a discontinuous gradient of 10, 20, 30 and 40% Percoll (Van Engelen et al., 1995). After centrifugation the cells at the interfaces between the different densities of Percoll were recovered with the use of a syringe, washed in B5 medium containing 2 µM 2,4-D and allowed to grow in B5 medium with 2 µM 2,4-D. After 5 days the medium proteins were analysed.

Partial purification of seed proteins

Dry mature seeds were ground in liquid nitrogen with pestle and mortar. 10 ml 25 mM Tris-HCl pH 8.5 was added to 2 g of ground seeds. After incubation for 15 min on a rotary shaker at 4°C the resulting slurry was filtrated through Whatman filter paper and supplemented with 0.5 ml of DEAE-Sepharose FF resin, equilibrated in 25 mM Tris-HCl pH 8.5. After incubation on a rotary shaker for 1 hr at 4°C a column was poured of the DEAE-Sepharose FF resin. This column was washed 2 times with 25 mM Tris-HCl pH 8.5 and the bound proteins were eluted with 0.2 M KCl in 25 mM Tris-HCl pH 8.5. The protein sample was desalted by means of dialysis.

Chitinase activity determination

Chitinase activity was determined essentially as described by Trudel and Asselin (1989).

In situ hybridisation

For whole mount in situ hybridisations one week old suspension cultures, grown in B5 medium in the presence of 2 µM 2,4-dichlorophenoxyaceticacid (2,4-D), were washed in B5 medium and sieved through nylon sieves with pore sizes of 125 or 30 µm mesh and allowed to grow for another week in the absence of 2,4-D. The cells were concentrated by centrifugation and immobilized onto poly-L-lysine coated glass slides by mixing 0.5 ml cell suspension with an equal volume of fixation buffer (130 mM NaCl, 10 mM $NaPO_4$ buffer pH 6.4, 0.1% Tween 20 (PBT), 70 mM EGTA, 4% paraformaldehyde, 0.25% glutaraldehyde and 10% DMSO). For fixation the slides were placed on a heated plate (30°C) for 30 min. followed by methanol and ethanol washes. Postfixations were performed after xylene and proteinase K treatments. Fixed cells and embryos were prehybridized in a solution containing PBT, 50% deionized formamide, 0.33M NaCl and 50 µg/ml heparin. Single-stranded RNA probes of 230 nucleotides in length were transcribed from pAJ41, a plasmid containing the NruI-StyI fragment from *EP3B* cDNA (Kragh et al., 1996). Digoxygenin sense and antisense probes were synthesized (Boehringer Mannheim). For each slide 100 ng of RNA probe was denatured in a solution containing 15 µg yeast tRNA and 50 µg polyA RNA. This was mixed with prehybridisation solution and applied to the slides. The hybridisation took place for 16 hours at 42°C. The slides were washed, and incubated with anti-digoxigenin FAB alkaline phosphatase, diluted 1:2500 in PBT, containing 50 µg BSA and 25 µg plant protein extract, to reduce non specific staining. The non bound antibodies were washed away by several washes with PBT and the cells were stained in 100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl (pH 9.5) and 0.1% Tween 20

supplemented with 9 µl/ml NBT and 7 µl/ml BCIP. Slides were mounted in PBS and 0.8% glutaraldehyde and analysed using Nomarski optics.
In situ hybridisation on sections was essentially carried out according to the protocol of Cox and Goldberg (1988) except for the use of digoxygenin labeled RNA probes.

Immunolocalisation

Immunofluorescence labeling on suspension cells was carried out as described previously (Van Engelen et al., 1991). The anti-EP3 serum was obtained by immunizing a rabbit with the purified protein (De Jong et al., 1995). Both fluorescein isothiocyanate- and alkaline phosphatase-conjugated antibodies were used. For the experiments in which alkaline phosphatase-conjugated antibodies were used the staining was performed as described above. In control experiments preimmune serum was used.

Tissue printing

Fresh plant material was cut and pressed onto polyvinylidene fluoride (PVDF) membranes (Millipore). These membranes were washed in methanol and incubated in PBS. They were either directly stained in an amido black solution (Gershoni and Palade, 1982), or they were treated as immunoblots and incubated with rabbit EP3 polyclonal antibodies.

RT-PCR

Total RNA was isolated from different plant tissues as previously described (De Vries et al., 1982). From each sample 2 µg were used for reverse transcription. A mixture of RNA, 20 units RNA guard, 1 mM dNTPs, 5 mM $MgCl_2$, 1x RT buffer (10 mM Tris pH 8.8, 50 mM KCl, 0.1% Triton X-100) and 50 ng oligo dT_{12-18} in a volume of 20 µl was incubated at 83°C for 3 min. Subsequently incubated at 42°C for 10 min after which 4 units of AMV reverse transcriptase was added and the incubation was continued for an hour. The samples were denaturated at 95°C for 5 min and diluted to a final volume of 100 µl. A PCR reaction for the amplification of the ubiquitin cDNA was carried out, using 5 µl from the RT mixture, 1x Taq polymerase buffer (Boehringer Mannheim), 100 µM dNTPs, 1 unit Taq polymerase, 100 ng downstream ubiquitin primer (5' TATGGATCCACCACCACG$^{G}/_{A}$AGACGGAG 3') and 100 ng upstream ubiquitin primer (5' TAGAAGCTTATGCAGAT$^{C}/_{T}$TTTGTGAAGAC 3') (Horvath et al., 1993), in a total volume of 50 µl. After denaturation for 30 sec at 94°C the samples were submitted to either 15, 20 or 30 cycles of:

30 sec 94°C, 30 sec 48°C and 2 min 72°C. The PCR products were run on a 1% agarose gel and blotted on Nitran plus membranes (Schleicher & Schuell). For Southern blot analyses a full length ubiquitin probe was used. The concentration of cDNA was standardized by comparing the signals on the Southern. Equal amounts of cDNA were used for a control PCR reaction with ubiquitin primers, and one with *EP3* primers. Both PCR reactions were done simultaneously, using the same PCR conditions. In database searches the downstream primer (5' ATGGCACGGATGGTTGCCCCGAAACCTTG 3') for *EP3* showed homology only to class IV chitinases. So amplification of class I chitinase cDNAs cannot occur, despite their high homology to *EP3s*. By using this primer and an upstream primer (5' GTATTTTGGCCGCGGCCCTCTTCAGC 3') a PCR product with a length of 201 nucleotides was amplified. The probe used for the detection of the *EP3* PCR product was the 230 nucleotides insert from pAJ-41. All Southerns were washed 3 times in 0.1% SSC, 1% SDS at 65°C, before exposing to X-ray film.

Chapter 3

Production of carrot EP3 and *Arabidopsis* class IV endochitinases in baculovirus-infected insect cells

Catalytically active carrot and *Arabidopsis* EP3 endochitinases can be produced by insect cells infected with recombinant baculoviruses. Carrot EP3 endochitinases, produced in the baculovirus expression system, possess both chitinase and chitosanase activity. A comparison of the specific chitinase activity of four different carrot EP3 isozymes revealed differences in catalytic activity that seem to result from minor differences in the aminoacid composition of the highly homologous EP3 isozymes. The *Arabidopsis* EP3 endochitinase (AtEP3) has a higher specific chitinase activity than individual carrot EP3 isozymes. AtEP3 can be detected in the medium of embryogenic *Arabidopsis* cell suspension, but is nearly absent in non-embryogenic cultures. In such circumstance AtEP3 is a good marker for embryogenic *Arabidopsis* cultures.

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Introduction

EP3 endochitinases promote somatic embryogenesis in the carrot cell variant *ts11*. In this cell line two effects of addition of the EP3 endochitinases were noted: 1.) the arrest at the globular embryo stage was overcome (De Jong et al., 1992), and 2.) the number of *ts11* embryos formed increased (De Jong et al., 1993). In view of the observation that five different carrot EP3 isozymes could be identified, it seems possible that each effect was the result of a different individual isozyme. The isozymes EP3-1 and EP3-3 were purified to homogeneity from the medium of wild type suspension cultures. Each enzyme was able to increase the number of embryos formed in *ts11*, but EP3-3 was about two times more effective. However, only isozyme EP3-3 was able to lift the arrest at the globular stage and produced later stages of *ts11* somatic embryos (Kragh et al., 1996). Full length cDNAs corresponding to EP3-1, EP3-3 and two other isozymes, EP3-2 and EP3B, were obtained. The sequences of the cDNA clones indicated that the EP3 endochitinases are members of a small highly homologous family of acidic class IV chitinases (Kragh et al., 1996). The deduced amino acid sequences of EP3-1 and EP3-3 did not give a clue for explaning the observed difference in *ts11* rescue activity.

In this chapter we describe the production of individual recombinant EP3 isozymes using the baculovirus expression system in insect cells and demonstrate that the proteins are catalytically active. In that way we obtained sufficient amounts of each individual chitinase for biochemical analysis, and we were able to compare the *ts11* rescue activity of individual enzymes.

Besides the class IV EP3 endochitinases, a related carrot class I endochitinase was found to be effective in *ts11* rescue, whereas a carrot putative class II chitinase and a barley class IV chitinase were not effective (Kragh et al., 1996). These results suggested plant and enzyme specificity of chitinases able to rescue *ts11*. In *Arabidopsis*, a single related class IV endochitinase was identified in the culture medium and it was suggested that this AtEP3 is encoded by only one *AtEP3* gene in the *Arabidopsis* genome (Passarinho et al., in preparation). Therefore, using the AtEP3 protein, produced in insect cells or purified from embryogenic *Arabidopsis* cell culture medium, might provide additional clues on the characteristics and specificity of chitinases capable of *ts11* embryo rescue.

Results

Production of plant chitinases

Insect cells were infected with recombinant baculoviruses, containing a cDNA insert encoding either one of the carrot EP3 isozymes EP3-1, EP3-2, EP3-3, EP3B, or the *Arabidopsis* class IV

endochitinase AtEP3. Infection resulted in the presence of EP3 chitinases in both the insect cells and in the insect cell culture medium. In uninfected or wild type baculovirus infected cells or media no proteins were recognised by EP3 and chit4 antisera (data not shown). In the media only one major protein of around 30 kD was detected by the carrot EP3 antiserum. After electrophoresis of cellular proteins several proteins with molecular weights lower than 30 kD were recognised by the chitinase antisera, suggesting that inside the insect cells the plant chitinases are subject to degradation or incomplete synthesis. Therefore, the medium was used for purification of the chitinases.
The chitinases were purified using affinity columns containing bound EP3 or chit4 antisera for carrot and *Arabidopsis* chitinases respectively. The affinity columns had a fairly low capacity and therefore the chitinases were also purified by means of preparative electrophoresis. The purity of the chitinases was analysed using PAGE followed by silver staining and western blotting (data not shown). Both purification methods yielded pure chitinase preparations as judged by the above described methods. All plant chitinases produced in the baculovirus expression system were capable of degrading tritiated chitin and thus are catalytically active chitinases.

Catalytic activity of insect cell produced chitinases

The availability of the four purified EP3-1, EP3-2, EP3-3 and EP3B chitinase isozymes allowed a comparison of their individual catalytic activities. Comparison of the predicted amino acid composition of the four carrot EP3 isozymes (Kragh et al., 1996), showed EP3-1 and EP3-2 to be most homologous in amino acid sequence (98.5% identity). EP3-3 and EP3B are 97.5% identical, but have less homology (around 90%) with EP3-1 and EP3-2. Because EP3-3, in contrast to EP3-1, was able to lift the embryo arrest in *ts11* cell suspensions, it was of interest to determine whether there is also a difference in catalytic activity.
In Figure 1A the specific chitinase activity of all four carrot EP3 endochitinases in a pH range between 4.0 and 6.5 is compared. EP3-1 and EP3-2 do not have a clear pH optimum within the pH range that was used. The highest specific chitinase activity measured for isozyme EP3-1 was ~ 150 nmol / min / mg at pH 6.5, whereas for EP3-2 it was ~ 600 nmol / min / mg at pH 4.0. The optimal pH for isozyme EP3-3 was pH 5.0 when it had a specific activity of ~ 3200 nmol / min / mg. EP3B had optimal activity at the same pH and showed a specific activity of ~ 2000 nmol / min / mg. Thus, the predicted differences in amino acid composition between both groups of highly homologous proteins is found again as differences in specific chitinase activity and pH optimum.
Some chitinases are known to possess both chitinase and chitosanase activity (Ohtakara et al., 1990). Figure 1B shows that all four EP3 chitinase isozymes were capable to degrade polymeric glucosamine or chitosan. In contrast to the chitinase activity, no pH optimum for chitosanase activity was detected for either of the isozymes. EP3-3 had the highest chitosanase activity, just as it had the highest chitinase activity. The chitosanase activity of the isozymes EP3-3, EP3B and EP3-2 was, however,

7-8 times lower in comparison to their chitinase activity tested with chitin (Fig 1A). EP3-1, that had the lowest chitinase activity, could degrade chitosan and chitin with equal efficiency. Together, these results show that the four different EP3 isozymes have slightly different biochemical properties.

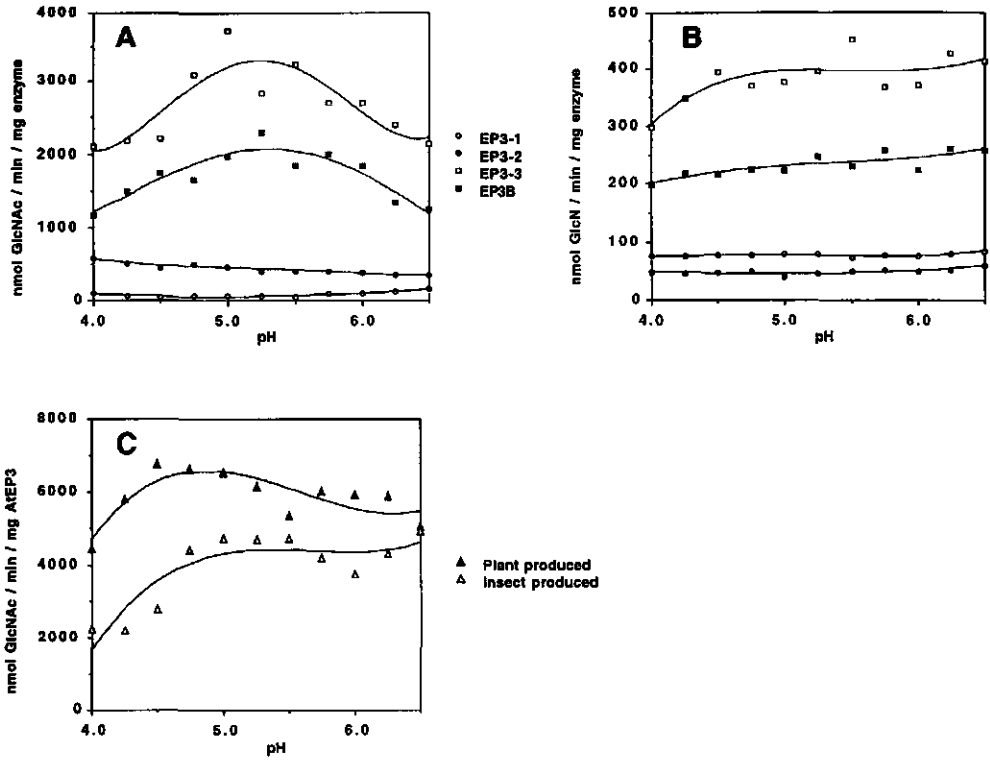

Figure 1. Comparison of the specific activities of EP3 and AtEP3 chitinases.
A. Specific chitinase activity of insect cell produced carrot EP3 endochitinases, expressed as nanomol GlcNAc released during 1 minute, by 1 mg enzyme. Chitinase assays were performed using ^{3}H-chitin, which was incubated with the enzymes for 24 hrs.
B. Specific chitosanase activity of insect cell produced carrot EP3 endochitinases, expressed as nanomol GlcN released during 1 minute, by 1 mg enzyme. Chitosanase assays were performed using solubilised chitosan, which was incubated with the enzymes for 24 hrs.
C. Specific chitinase activity of plant- and insect cell produced AtEP3 endochitinase, expressed as nanomol GlcNAc released during 1 minute, by 1 mg enzyme. Chitinase assays were performed using ^{3}H-chitin, which was incubated with the enzymes for 24 hrs.

Due to the small amounts that were obtained for each of the EP3 isozymes, after purification from the culture medium of suspension cells, it was not possible to compare the specific chitinase activity of EP3 isozymes produced by plant and insect cells. However, such comparison was possible with the EP3 homologue from *Arabidopsis*, AtEP3, since this chitinase is encoded by a single gene (Passarinho et al., in preparation). Moreover, purification of AtEP3 from the culture medium of *Arabidopsis* or insect cells could be facilitated by using antibodies against the AtEP3 chitinase.

Antibodies raised against carrot EP3 protein recognize AtEP3 only very poorly. Fortunately, chit4 antibodies, raised against sugar beet chitinase 4 which do not recognize carrot EP3 (Kragh et al., 1996), were able to recognize AtEP3, and could be used for specific immunological detection and purification of AtEP3. Medium proteins of insect cells infected with baculoviruses containing the *AtEP3* cDNA showed after denaturing PAGE, followed by western blotting and immunological detection, a thick band of 30 kD (Fig 2, lane 1). After immunoaffinity column purification of AtEP3, using chit4 antibodies, a single band of 30 kD was present on immuno blots (Figure 2, lane 2). A similar result was obtained after purification by preparative electrophoresis. Under non-denaturing conditions, also only one protein was recognized by the chit4 antiserum (data not shown). This indicates that the *Arabidopsis* EP3 is a single secreted protein, in contrast to the situation in carrot where at least five EP3 isozymes can be identified (Kragh et al., 1996).

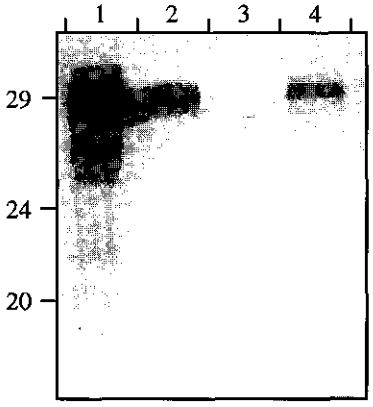

Figure 2. Occurrence of AtEP3 in the medium of *Spodoptera frugiperda* (Sf21) insect cells, 5 days after infection with baculovirus containing AtEP3 cDNA before and after purification, and in the medium of embryogenic and non-embryogenic *pt* suspension cultures. The presence of AtEP3 was determined by SDS-PAGE and immunoblotting using chit4 antiserum. Lane 1, proteins present in the medium of insect cells; lane 2, AtEP3 produced in the baculovirus expression system and purified by means of immunoaffinity column purification; lane 3, non-embryogenic culture; lane 4, embryogenic culture. The position of three molecular weight size markers is indicated at the left.

After purification of AtEP3 from the medium of baculovirus infected insect cells and from the medium of *Arabidopsis* suspension cells the enzyme activity of the purified class IV endochitinases were compared. Figure 1C shows that the chitinase purified from *Arabidopsis* cell cultures had a 1.5 to 2 times higher specific activity then the chitinase from the insect cells. The pH optimum for the chitinases purified from the two different sources was similar. The lower activity of the enzyme from the insect cells might be explained by the presence of not correctly folded or less stable chitinases with a lower enzymatic activity. The specific chitinase activity of the insect cell produced AtEP3 was 4200 nmol / min / mg, slightly higher than that of EP3-3, the carrot EP3 isozyme with the highest specific chitinase activity of ~ 3200 nmol / min / mg (Fig 1A).

AtEP3 is produced in embryogenic suspension cultures

Subsequently, the occurrence of AtEP3 was studied in embryogenic and non-embryogenic *Arabidopsis* cell suspensions. In *Arabidopsis thaliana*, wild type embryogenic and non-embryogenic cell suspension cultures can be obtained from immature zygotic embryos (Mordhorst et al., submitted). Using seedlings of the mutant *primordia timing* (*pt*), both embryogenic and non-embryogenic callus can be obtained from a single seedling. If cultured in the light, embryogenic cell lines consist of compact green aggregates, whereas non-embryogenic cell lines form rough yellowish clusters of cells. Embryogenic cell lines can also be grown in the dark which results in yellowish cultures that remain embryogenic. The occurrence of AtEP3 was studied in different embryogenic and non-embryogenic cultures.

In the medium of an embryogenic *pt* cell suspension culture a single protein of 30 kD was found using the chit4 antiserum (Figure 2, lane 4). In contrast to carrot in which both embryogenic and non-embryogenic cell cultures produce roughly equivalent amounts of EP3 endochitinases (Kragh et al., 1996; van Hengel et al., in press), in *Arabidopsis* the AtEP3 protein was not, or barely, detectable in non-embryogenic *pt* cultures (Figure 2, lane 3). To determine whether the occurrence of the *Arabidopsis* class IV endochitinase was indeed restricted to embryogenic cultures, media of several different cell lines were tested for the presence of AtEP3 chitinase. In carrot, the lipid transfer protein (LTP) is a good marker for embryogenic suspension cultures (Sterk et al., 1991), and the *Arabidopsis* homologue, *AtLTP*, is expressed in both zygotic (Vroemen et al., 1996) and somatic embryos (Mordhorst, personal communication). Therefore, the presence of AtLTP was also determined in the different media.

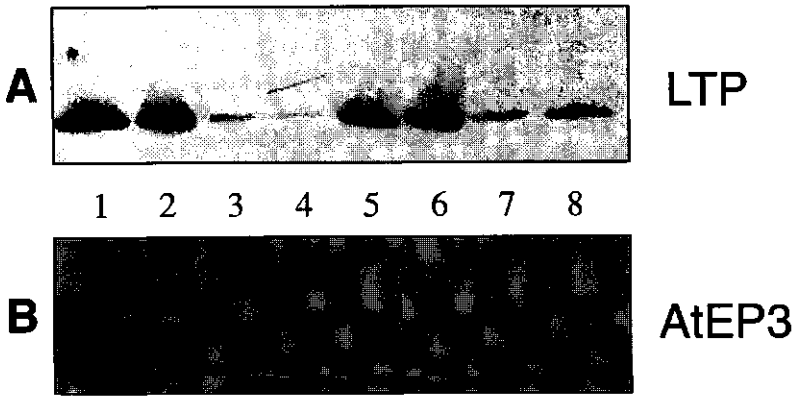

Figure 3. A. Occurrence of LTP and **B.** AtEP3 in the medium of embryogenic and non-embryogenic, wild type and *pt/amp1 Arabidopsis thaliana* suspension cultures grown in the light or in the dark, determined by SDS-PAGE and immunoblotting. Lane 1, embryogenic *pt/amp1* culture grown in the light; lane 2, embryogenic *pt/amp1* culture grown in the dark; lane 3, non-embryogenic *pt/amp1* culture grown in the light; lane 4, non-embryogenic *pt/amp1* culture grown in the dark. Lane 5, wt embryogenic culture grown in the light; lane 6, wt embryogenic culture grown in the dark; lane 7, wt non-embryogenic culture grown in the light; lane 8, wt non-embryogenic culture grown in the dark. For detection of LTP, antibodies raised against *Arabidopsis thaliana* LTP were used (Thoma et al., 1993) and for the detection of AtEP3, chit4 antibodies were used (Mikkelsen et al., 1992).

Figure 3A shows that in embryogenic cultures of both *pt* and wild type *Arabidopsis* large amounts of LTP were present in the medium. Considerably less was found in the media of non-embryogenic cultures. Figure 3B shows that the presence of AtEP3 in the media went together with the occurrence of LTP, but AtEP3 was even more specific for embryogenic cultures. These results indicate that both in wild type and *pt Arabidopsis* suspension cultures, there is a positive correlation between somatic embryogenesis and the production of LTP and AtEP3. Furthermore, the presence of AtEP3 in the medium was not restricted to cultures that contain green cells (Figure 3B) and thus, the production of AtEP3 is not linked to photosynthesis. In embryogenic cultures grown in the dark, less chitinase was produced then in similar cultures grown in the light. Whether this difference was correlated to a difference in embryogenic capacity of dark- and light grown cultures, was not investigated further.

Discussion

Expression of each of the four carrot EP3 isozymes and the single *Arabidopsis* AtEP3 enzyme, in the baculovirus expression system, resulted in the production of recombinant proteins which were secreted by the insect cells into the medium. Apparently the plant EP3 signal sequences are functional in insect cells. The recombinant chitinases were all active enzymes capable of degrading chitin and, to a lesser extent, also chitosan. In addition we have shown that the occurrence of AtEP3 in *Arabidopsis* cell suspension cultures is restricted to embryogenic cell lines.

Previously it was shown that isozymes EP3-1 and EP3-3 that were purified from the medium of carrot suspension cultures differed in their effect upon somatic embryogenesis in *ts11* cultures (Kragh et al., 1996). The addition of either of the two isozymes to *ts11* resulted in an increase in the number of preglobular and globular embryos that were formed, but the EP3-3 isozyme was more effective than EP3-1, and, moreover, it could also rescue the arrest of embryogenesis at the globular stage. This difference in embryo rescue ability was observed over a wide range of chitinase concentrations added to the cultures. Therefore, it is likely that the observed difference is due to an intrinsic difference between EP3-1 and EP3-3 and not to the higher specific chitinase activity of EP3-3.

At the other hand, the higher specific chitinase activity of EP3-3, compared to EP3-1, might explain why similar numbers of preglobuar and globular *ts11* somatic embryos were obtained after addition of 200 ng EP3-3 or 1000 ng EP3-1 respectively (Kragh et al., 1996). Kragh et al. (1996) suggested that the difference between EP3-1 and EP3-3 was not caused by alterations in the catalytic site, but rather resulted from changes in charge caused by substitution of threonine at position 154 and asparagine at position 164 for lysines, which might result in different affinity of the proteins towards chitin. Our results on the comparison of all four different EP3 isozymes show that there is a correlation between the presence of lysine residues at position 154 and 164 in EP3-3 and EP3B and the higher specific chitinase activity of these isozymes in comparison to both EP3-1 and EP3-2 that contain threonine and asparagine at position 154 and 164. Plant chitinases are known to have very

broad pH optima between pH 3-9 (Pantaleone et al., 1992), and this appears to be true for the EP3 isozymes and AtEP3 as well.

Some chitinases are known to possess other enzymatic activities beside their capability to degrade chitin. Some basic class III chitinases were shown to exhibit lysozyme activity (Bernasconi et al., 1987; Tata et al., 1983), whereas some putative class I citrus chitinases were shown to possess chitosanase activity (Osswald et al., 1993). The observation that all EP3 isozymes can degrade chitosan shows that their substrate specificity is not restricted to N-acetylglucosamine polymers, but also includes glucosamine polymers. The specific chitosan activity, however, is only minor compared to the specific chitinase activity, which was also found for citrus chitinases (Osswald et al., 1993). At least one of the EP3 isozymes and a related class I endochitinase are able to rescue *ts11* somatic embryogenesis (Kragh et al., 1996). The biological significance of the other 4 EP3 isozymes in the carrot cultivar Trophy is not clear. It appears that only one endochitinase is required for the completion of *ts11* somatic embryogenesis, because in *ts11* and its parental line A^+, in contrast to the cultivar Trophy, the class I endochitinase that can rescue arrested somatic embryos is absent, while only one EP3 isozyme was found in the medium (Hendriks and de Vries, 1995). When *ts11* was cultured at non-permissive temperatures, a transient reduction in the amount of EP3 was observed, thus leaving *ts11* cultures devoid of both the class I and EP3 endochitinases (De Jong et al., 1995) and defective in somatic embryogenesis.

The occurence of only one *Arabidopsis* homologue, that, just like carrot *EP3*, is expressed in developing seeds (Passarinho et al., in preparation; chapter 2) suggests that only one class IV endochitinase carries out an essential biological function in developing seeds. The presence of AtEP3 in the medium of embryogenic suspension cultures and its virtual absence in non-embryogenic cultures makes this chitinase a good marker for somatic embryogenesis in *Arabidopsis*. Previously it was shown that in carrot, LTP could only be detected in embryogenic cultures and not in non-embryogenic ones (Sterk et al., 1991). Our results show that also in *Arabidopsis* secretion of high amounts of LTP is a good marker for somatic embryogenesis.

In carrot cultures *EP3* gene expression occurred in suspension cells which most likely did not generate somatic embryos, whereas in carrot seeds, *EP3* endochitinase gene expression was restricted to the maternal integuments and mature endosperm (chapter 2). These findings and the ability of the EP3 enzyme to rescue *ts11* somatic embryogenesis suggest an embryo "nursing" function for EP3 endochitinases (van Hengel et al., in press). The correlation between the presence of AtEP3 and *Arabidopsis* somatic embryogenesis as described in this chapter fully supports a necessity for endochitinases in somatic embryogenesis. It will now be of interest to determine the cell types expressing *AtEP3* in *Arabidopsis* embryogenic suspension cultures during the initiation and development of somatic embryos since this might reveal information on the specific role of this enzyme in the putative nursing of somatic embryogenesis.

Materials and methods

Cloning of chitinase cDNAs

The full length cDNAs coding for carrot EP3 endochitinases, and starting with the first AUG codon, were isolated from the plasmids E6, E7, H1 and H5 (Kragh et al., 1996) after digestion with EcoRI and SalI. A full length cDNA encoding the *Arabidopsis thaliana* class IV endochitinase AtEP3, and starting with the first AUG codon, was isolated from the plasmid TAI224 (Passarinho et al., in preparation) after digestion with XbaI and XhoI. Blunt ends were created using the Klenow fragment of *E. coli*. All cDNAs were cloned into the BamHI site of the baculovirus recombination vector pAcJR1, that was kindly provided by Dr. D. Zuidema.

Generation of plant chitinase-expressing baculoviruses

Every pAcJR1 plasmid, containing a single chitinase cDNA was gently mixed with lipofectin and linearized genomic baculovirus DNA devoid of the polyhedrin gene (InVitrogen, Leek, The Netherlands). The resulting mixtures were added to *Spodoptera frugiperda* (Sf) 21 insect cells. After 2-3 days infected cells could be identified under the microscope. The medium of the insect cells was harvested after 5 days of culturing. The virus titer of the medium was determined and a dilution series of the recombinant virus obtained was added to immobilized insect cells. After plaques had formed X-gal was added in order to detect lacZ activity. This serves as a positive control to detect infection by circular virus genomes that do not contain an (chitinase) insert. White plaques were carefully picked up and used for infection of new insect cells.

Chitinase expression and purification

The proteins that were present in the insect cells and in the medium after infection with recombinant viruses, were analysed by means of electrophoresis on denaturing polyacrylamide (PAA) gels and silver staining of the PAA gels (Blum et al., 1987). Immunological detection of EP3 chitinases on western blots was performed as described before (Kragh et al., 1996). For the detection of the *Arabidopsis* class IV endochitinase, antiserum raised against sugarbeet chitinase 4 was used (Mikkelsen et al., 1992). The presence of large amounts of fetal bovine serum (FBS) in the medium hampered purification. In order to circumvent this problem, the cells were cultured for 2 days in the presence of FBS, washed in medium without FBS and cultured without FBS until harvesting, that was done just before lysis of the cells. Isolation of the recombinant chitinases was performed by column purification using either EP3 or chit4 antibodies bound to Bakerbond ABx (J.T. Baker Inc. Phillipsburg, NJ, USA) as described by Kreuger and Van Holst (1995). Bound proteins were eluted

with 0.1 M Glycine-HCl pH 2.5. The eluates were analysed for the presence of plant chitinases by means of immunological detection on dot blots. The purity of the preparations was determined by means of silverstaining of denaturing PAA gels. Aliquots of 2 ml of the eluates were collected in 5 ml 0.1 M Tris-HCl pH 7.5 to neutralize the low pH, and subsequently desalted by pressure dialysis employing an YM5 filter (5-kD cut off, Amicon, Oosterhout, The Netherlands). Alternatively, the recombinant chitinases were isolated by preparative PAGE using a PrepCell (Biorad, Hercules, Ca, USA). Immunological detection on dot blots was used to determine the presence of EP3 endochitinases in the eluates. Desalting of the eluates was performed as described above. Native AtEP3 was isolated from the medium of *Arabidopsis* embryogenic cell suspensions by means of preparative PAGE.

Enzyme activity measurements

Chitinase activity was determined as described before (De Jong et al., 1992) using ^{3}H-labeled chitin as a substrate. Chitosanase activity was determined by means of a quantitative fluorometric assay as described by Osswald et al. (1992) using soluble chitosans as a substrate.

Plant material

Cell suspensions were initiated and maintained according to Mordhorst *et al.* (submitted). Medium of 7 days old *Arabidopsis thaliana* suspension cultured cells of wild type or the *primordia timing* (*pt*) mutant was used. Cultures of the *primordia timing* (*pt*) allele, of the *häupling* (*hp*), *altered meristem program1* (*amp1*) and the *constitutive photomorphogenic2* (*cop2*) complementation group have a much higher regeneration capacity then wild type.

Acknowledgments

We would like to thank Dr. Douwe Zuidema and Magda Usmani for the supply of pAcJR1 and for technical assistance in using the baculovirus expression system, Dr. Andreas Mordhorst for the supply of *Arabidopsis* cell suspension cultures, and Dr. Jörn Mikkelsen for the supply of chit4 antiserum.

Chapter 4

Plant N-acetylglucosamine-containing arabinogalactan proteins contain a cleavage site for carrot EP3 chitinases

Based on labeling with D-[1-^{14}C] glucosamine and N-acetyl-D-[1-^{14}C] glucosamine a subpopulation of arabinogalactan proteins (AGPs) secreted by suspension cultured carrot cells, were shown to contain N-acetylglucosamine (GlcNAc) and glucosamine (GlcN). [1-^{14}C] GlcN and [1-^{14}C] GlcNAc was only found in AGPs from embryogenic but not from non-embryogenic cultures. AGPs isolated from developing seeds were shown to contain oligosaccharides susceptible to EP3 class IV endochitinase activity. During seed development the native electrophoretic mobility of total seed AGPs increased, while AGP epitopes disappear, suggesting increased processing. Seed AGPs occur predominantly in the endosperm. Together with the previously reported presence of the EP3 endochitinase enzyme in the endosperm these results suggest that GlcNAc-containing AGPs are a natural substrate for plant chitinases.

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Introduction

The carrot cell line *ts11* is a temperature sensitive variant in which somatic embryogenesis is arrested at nonpermissive temperatures. The development of embryos at nonpermissive temperatures does not proceed beyond the globular stage. Remarkably, addition of medium conditioned by a wild-type carrot cell suspension culture lifts the embryo arrest and allows further development of the embryos into plantlets (Lo Schiavo et al., 1990). Among the proteins in the medium of the cell suspension culture is a class IV endochitinase, that is capable of somatic embryo rescue in the cell line *ts11* at the nonpermissive temperature (De Jong et al., 1992; Kragh et al., 1996). This endochitinase is referred to as extracellular protein 3 (EP3). These results showed that certain plant chitinases have a role in embryo development and also suggested that plants contain targets for chitinase activity. Chitinases (E.C. 3.2.1.14) are enzymes that hydrolyse ß (1-4) linkages between adjacent N-acetyl-D-glucosamine (GlcNAc) residues in, for instance, chitin polymers. Endochitinases require at least three consecutive ß (1-4) linked GlcNAc residues for activity (Molano et al., 1979; Usui et al., 1990).
Lipochitooligosaccharides (LCOs), produced by *Rhizobium* bacteria, are essential for root nodule formation in leguminous plants, and were also found to be able to rescue somatic embryogenesis in the temperature sensitive variant *ts11* (De Jong et al., 1993) where they mimick the effect of EP3. The active LCOs contain more than three GlcNAc residues and can be degraded by plant endochitinases (Lerouge et al., 1990; Staehelin et al., 1994). Based on this result it was speculated that in plants chitinases may release LCO-like molecules that can have a regulatory function. In an attempt to identify plant analogues of LCOs, Spaink et al. (1993) labeled *Lathyrus* stems with [^{14}C] acetate and analysed butanol extracts from labeled stems by TLC with and without treatment with bacterial chitinases. They observed a difference in pattern that was ascribed to the chitinase activity and supported the hypothesis that plants indeed produce low molecular weight chitinase-sensitive components. Sofar, the chitinase-sensitive plant products detected in these experiments have not been further identified.
We were then informed that certain purified membrane arabinogalactan proteins (AGPs) contain GlcNAc (Pennell, personal communication), although GlcNAc, nor glucosamine (GlcN) was known as a component of AGPs. This suggested to us that AGPs might contain cleavage sites for (endo)chitinases. An indication of a possible role of AGPs in somatic embryogenesis came from the work of Kreuger and Van Holst (1993), who showed that the addition of carrot mature seed AGPs to a non-embryogenic cell line resulted in reinitiation of somatic embryo development.
AGPs are proteoglycans that occur attached to membranes, in cell walls and are secreted into the apoplastic space. AGPs typically have a carbohydrate content of more than 90% of the molecular mass (Chasan, 1994; Kreuger and Van Holst, 1996). Analyses of the carbohydrate composition of AGPs have shown that arabinose- and galactose residues are the main constituents, but a large variety of other sugars was also found in minor and variable amounts (Baldwin et al., 1993; Komalavilas et

al., 1991; Mollard and Joseleau, 1994; Serpe and Nothnagel, 1994; Serpe and Nothnagel, 1996; Smallwood et al., 1996; Van Holst et al., 1981). In AGPs the polysaccharides that are attached to the protein core have numerous highly complex side chains with different terminal residues, and the monosaccharides in AGPs can be linked in a large number of different fashions (Chen et al., 1994; Mollard and Joseleau, 1994).

In the present study we have investigated the possibility that AGPs contain cleavage sites for EP3 endochitinases and might be a natural substrate. We show that cells in embryogenic suspension cultures, but not in non-embryogenic suspension cultures produce AGPs with GlcNAc and GlcN as components of their carbohydrate moiety. Furthermore, AGPs extracted from immature carrot seeds were shown to contain oligosaccharides that are endochitinase-sensitive, suggesting that at least three ß (1-4) linked N-acetylglucosamines are present. In addition, AGPs were found to be localised on a subpopulation of suspension cells and in the endosperm of immature carrot seeds. Furthermore the composition of AGPs in seeds was found to change during development.

Results

Glucosamine and N-acetylglucosamine labeling of suspension cells

Carrot suspension cells in basal medium, cultured in the presence or absence of 2,4-D were labeled with either D-[1-^{14}C] glucosamine (^{14}C-GlcN) or N acetyl-D-[1-^{14}C] glucosamine (^{14}C-GlcNAc). All cultures with 2,4-D proliferated as unorganized cell clusters whereas cultures without 2,4-D developed somatic embryos. In both types of cultures approximately 80% of the radioactivity had been taken up from the culture media (Fig 1A and 1B), and was found in the cells. During the subsequent 7 days the radioactivity in the medium of ^{14}C-GlcN labeled cultures slowly decreased further (Fig 1A). In contrast, ^{14}C-GlcNAc labeled cultures showed a slight increase of radioactivity in the medium during the same period (Fig 1B), suggesting that secretion of labeled compounds had occurred. When ß-glycosyl Yariv reagent was added to the cell-free medium of ^{14}C-GlcNAc labeled cultures, 60 - 70 % of the radioactivity in the medium precipitated (Fig 1C). The amount of Yariv-precipitable radioactivity increased with culture time, whereas the amount of non Yariv-precipitable radioactivity remained constant. This indicates that the radioactivity in the medium is most likely present in the form of AGPs. The amount of radioactivity incorporated in cells grown with or without 2,4-D remained practically the same during the period of the measurements (Fig 1C).

Radial gel diffusion assays were used for determining the AGP concentrations (Fig 2A). At day 7, the medium of cells cultured in the presence of 2,4-D contained about 15 µg / ml AGPs and about 10 µg / ml in the absense of 2,4-D. The Yariv precipitation ring (Fig 2A) of the medium AGPs from the

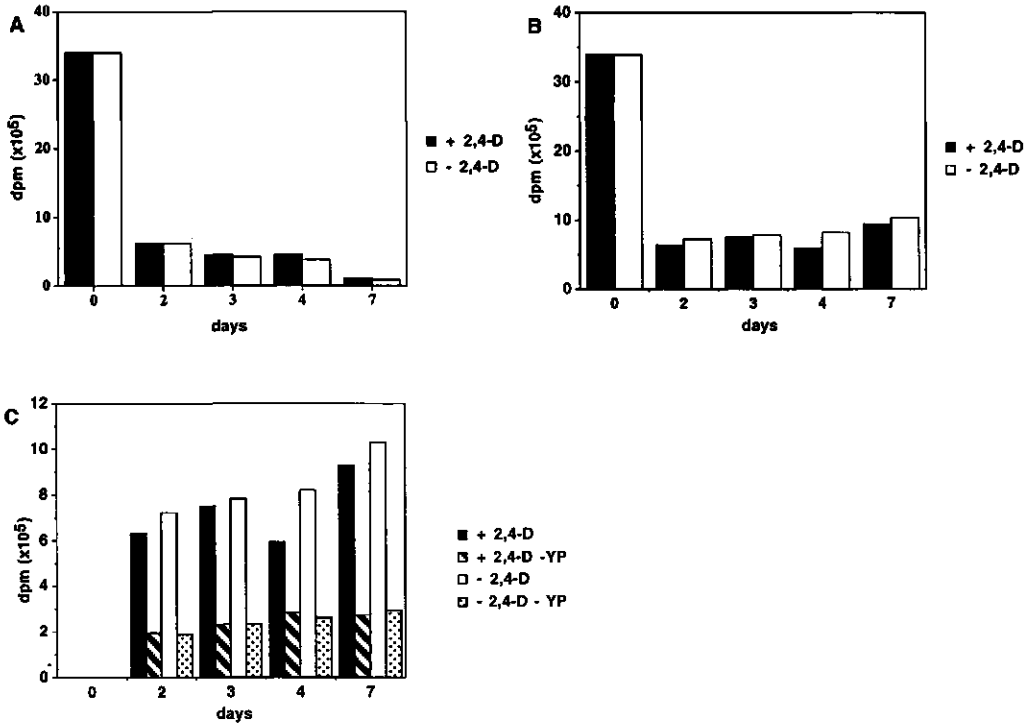


Figure 1. Total amounts of label that were present in the media of 50 ml suspension cultures at different days after initiation of labeling. **A.** Labeling with 10^{-7}M D-[1-^{14}C] Glucosamine. **B.** Labeling with 10^{-7}M N-Acetyl-D-[1-^{14}C] Glucosamine. **C.** Labeling with 10^{-7}M N-Acetyl-D-[1-^{14}C] Glucosamine before and after precipitation with the Yariv reagent. -YP is medium without Yariv precipitable material.

GlcNAc labeled culture contained radioactivity which confirmed that labeled AGPs were secreted by the suspension cells (Fig 2B).

Little difference was found between the total amount of radioactivity present in AGPs or in the specific radioactivity of AGPs as a result of culturing in the presence or absence of 2,4-D (Table 1). Therefore we conclude that culturing of carrot suspension cells in the presence of ^{14}C-GlcNAc results in the production of radioactive AGPs irrespective of the presence or absence of 2,4-D.

After 7 days of culture most of the radioactivity was taken up by the cells. Gel electrophoresis of total soluble cellular proteins of cells labeled with ^{14}C-GlcNAc showed that radioactivity was incorporated into proteins with an apparent molecular weight (Mr) of 32 to 80 kD (Fig 2C). The insoluble, crude cell wall fraction of those cells also contained radioactivity, but in contrast to the rather high incorporation of radioactivity in AGPs, little radioactivity had been incorporated into cell walls (Table 1). Neither the nature of the labeled proteins, nor that of the cell wall material were investigated further.

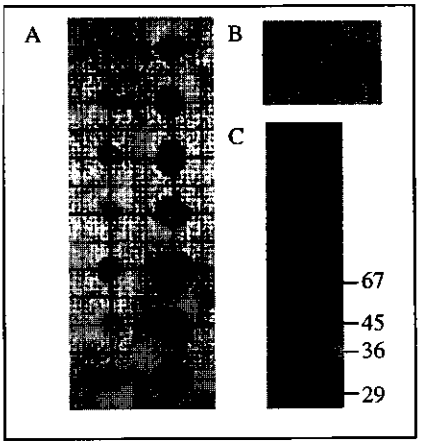


Figure 2. A. A diffusion gel containing samples of gum arabic AGPs with concentrations of 10, 20, 30, 40, 50, 60, 80, 100, 150, 200, 250, 300, 350 and 400 µg/ml. **B.** Autoradiogram of a diffusion gel containing duplo samples of AGPs from the medium of a suspension culture that was labeled for 7 days with 10^{-7}M N-Acetyl-D-[1-^{14}C] Glucosamine. This particular AGP sample had a concentration of 300 µg/ml **C.** Cellular proteins of a suspension culture that was labeled for 7 days with 10^{-7}M N-Acetyl-D-[1-^{14}C] Glucosamine, separated on a 12.5 % polyacrylamide gel.

Table 1. Incorporation of label in secreted AGPs and cell wall fractions, derived from suspension cultures that were cultured for 7 days in the presence of 10^{-7}M N-Acetyl-D-[1-^{14}C] Glucosamine, in B5 medium with, or without 0.2 µM 2,4-D. Values indicate the amount of radioactivity incorporated in AGPs as a percentage of the total amount of radioactivity supplied to the cultures, and the specific labeling of AGPs and cell wall fractions. (nd = not determined)

Non-embryogenic cell line	dpm/µg AGP
Af	$1.7\ 10^2$
Ai	$2.1\ 10^2$
Embryogenic cell line	
Am	$4.8\ 10^2$
Ar	$4.2\ 10^2$
At	$4.1\ 10^2$
Au	$4.7\ 10^2$

Since pectins are a common contaminant of Yariv-precipitated AGPs, the pectin specific monoclonal antibodies JIM5 and JIM7 were used to determine the possible occurrence of pectins in the AGP preparations used. Immunodetection of pectin epitopes was performed before and after treatment with pectinase. Dot blot analysis showed that after pectinase treatment no pectin epitopes could be detected anymore. AGP specific epitopes that are recognized by the monoclonal antibodies JIM8, MAC207 and MAC254 remained unaltered upon reprecipitation of the AGPs after the pectinase treatments (data not shown). Over 80% of the radioactivity originally present in the AGPs was retained in the reisolated pectin-free AGP fraction, indicating that indeed the radioactivity was incorporated in the AGPs secreted into the medium. Electrophoresis of AGPs both before and after pectinase treatment, followed by silver staining of PAA gels did not reveal any contaminating proteins in the AGP fraction (data not shown). We conclude that ^{14}C-GlcNAc uptake by carrot suspension cultures results in the production of AGPs with a high specific radioactivity.

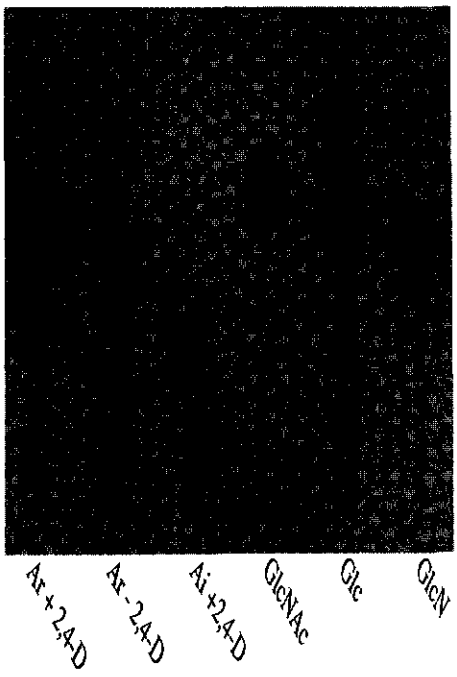
AGPs from embryogenic, but not from non-embryogenic cell lines contain GlcNAc

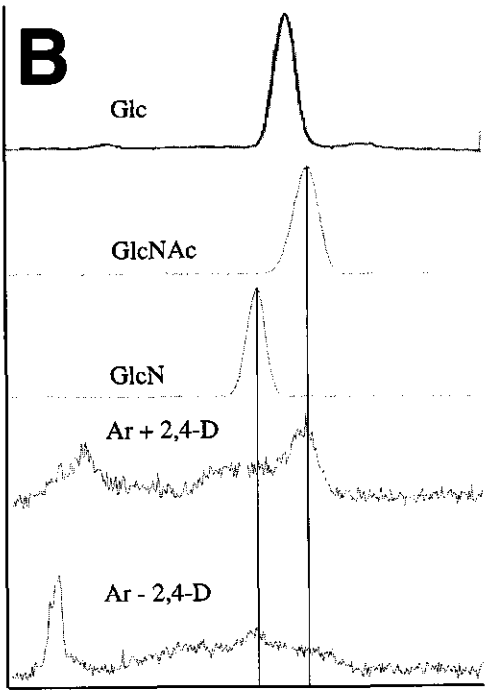
To determine whether ^{14}C-label in the newly formed AGPs occurred in GlcNAc, labeled AGPs were incubated with 2 M trifluoroacetic acid (TFA) for 45 min at 100°C or for 60 min at 120°C. Degradation was monitored by TLC followed by autoradiography and densitometric scanning of the autoradiographs.

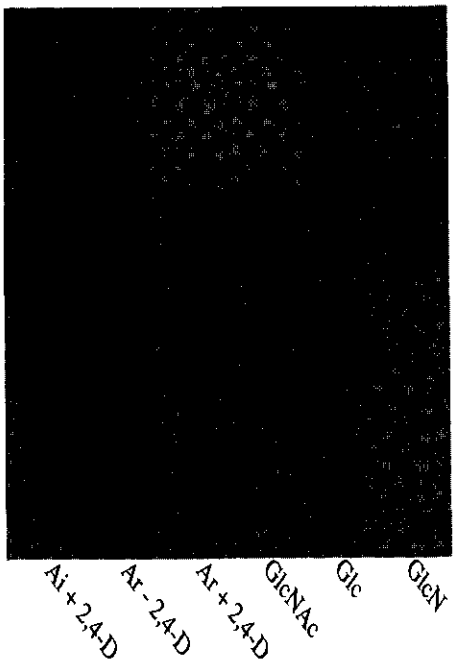
On TLC the references, labeled glucose (Glc), GlcN and GlcNAc could easily be distinguished (Fig 3A, last three lanes). After 45 min at 100°C the GlcNAc molecules remained unaltered, whereas after 60 min at 120°C GlcNAc is deacetylated and converted into GlcN. This is clearly illustrated by the behaviour of GlcNAc and GlcN used as references (Fig 3C, last three lanes). The AGP samples incubated at 100°C for 45 min were partially degraded (Fig 3A, first three lanes), but a peak of labeled compounds, coinciding with GlcNAc, was found in the hydrolysate of AGPs from the embryogenic cell line Ar (Fig 3B). No peak coinciding with GlcNAc was detected after incubation of AGPs at 120°C for 60 min, but then a larger peak was found at the position of GlcN, presumably because GlcNAc was converted into GlcN (Fig 3D). We conclude therefore that the secreted AGPs of the embryogenic cell line Ar, cultured in the presence of 2,4-D contain GlcNAc, while after culturing of the same cell line in the absence of 2,4-D secreted AGPs were found to contain GlcN. However, regarding the conversion of GlcNAc into GlcN, we assume that the latter AGPs also contain GlcNAc. Peaks coinciding with either GlcNAc or GlcN were virtually absent in hydrolysates of AGPs from the non-embryogenic cell line Ai, where labeled compounds were found at the position of Glc (Fig 3D). This indicates that deacetylation and deamination occur during the 7 day culture period, resulting in the presence of label in monosaccharides such as glucose, arabinose or galactose that all have the same mobility on TLC. The smears of labeled material as observed in Figure 3A indicate that the carbohydrate moieties of the AGPs were not completely resolved into individual monosaccharides, whereas the smears in Figure 3C may point to an incorporation of label in non-sugar components.

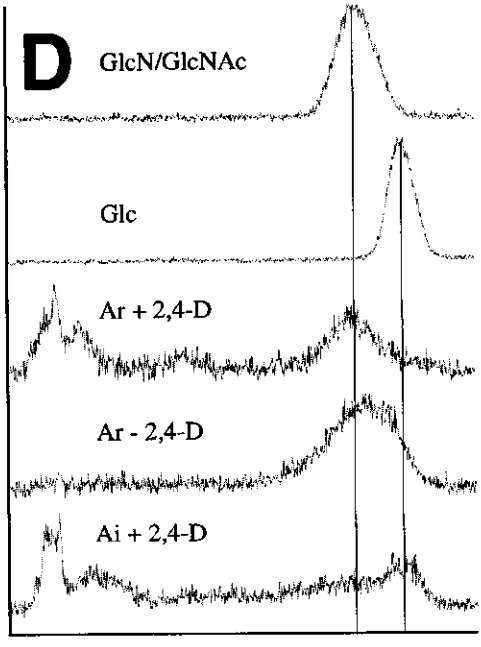
Metabolic conversion of ^{14}C GlcNAc was also apparent after degradation of cell wall fractions of an embryogenic suspension culture which showed that in the cell walls radioactivity resided in sugars with an Rf value equal to glucose (data not shown). These results suggest that the presence of GlcNAc-containing medium AGPs is restricted to embryogenic cultures. Several independently established embryogenic and non-embryogenic cell lines were labeled with ^{14}C-GlcNAc. The AGPs isolated from four embryogenic cell lines contained twice the amount of label per μg AGP when compared to AGPs isolated from two non-embryogenic cell lines (Table 2).

Figure 3. TLC analysis of the TFA degraded Yariv precipitable fractions containing the secreted AGPs of an embryogenic (Ar) and a non-embryogenic (Ai) cell line that were labeled for 7 days with 10^{-7}M N-Acetyl-D-[1-^{14}C] Glucosamine, and optical density scans of the autoradiograms. **A**. TLC of AGP degradation by means of incubation in 2M TFA at 100^0C for 45 min. **B**. Optical density scan of A. **C**. TLC of AGP degradation by means of incubation in 2M TFA at 120^0C for 60 min. **D**. Optical density scan of C.


Ar + 2,4-D
Ar - 2,4-D
Ai + 2,4-D
GlcNAc
Glc
GlcN


B
Glc
GlcNAc
GlcN
Ar + 2,4-D
Ar - 2,4-D


Ai + 2,4-D
Ar - 2,4-D
Ar + 2,4-D
GlcNAc
Glc
GlcN


D
GlcN/GlcNAc
Glc
Ar + 2,4-D
Ar - 2,4-D
Ai + 2,4-D

Degradation of AGPs by TFA, followed by TLC showed that in AGPs from all embryogenic cell lines, grown in the presence of 2,4-D, a part of the label was present as GlcNAc, while in AGPs isolated from all non-embryogenic cell lines it was only present as Glc (data not shown). There appeared to be a positive correlation between the embryogenic capacity and the amount of labeled GlcNAc incorporated in the AGPs.

Table 2. Specific labeling of AGPs isolated from the conditioned medium of several independently established embryogenic and non-embryogenic cell lines.

		% of total label incorporated	dpm/µg
+ 2,4-D	AGPs	2.0	$4.2\ 10^2$
	cell wall fraction	nd	15
- 2,4-D	AGPs	1.7	$6.0\ 10^2$
	cell wall fraction	nd	21

AGPs from developing carrot seeds contain a cleavage site for plant endochitinases

After having shown that GlcNAc occurs in AGPs, we wondered if AGPs can contain cleavage sites for EP3 endochitinases. When ^{14}C-GlcNAc labeled AGPs from embryogenic cell cultures were incubated with purified EP3 endochitinases, no reaction products could be detected upon analysis of the reaction mixture by TLC or by agarose gel electrophoresis. Because it could not be excluded that the AGPs from the conditioned medium were already cleaved by endochitinases present in the medium, which would make it impossible to observe cleavage by added EP3 endochitinases, we looked for another source of AGPs with possible endochitinase cleavage sites. As *EP3* endochitinase genes are expressed in immature carrot seeds (Van Hengel et al., in press) it appeared possible that AGPs from these tissues might contain EP3 endochitinase cleavage sites. Therefore we isolated AGPs from immature seeds, made them pectin-free and used these purified AGPs for further analysis. High performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) was used for the detection of cleavage products. Using HPAE-PAD, neutral monosaccharides are separated based on differences in pKa values, while the separation of oligosaccharides is based on the composition, sugar linkage type and the level of negatively charged residues.

Figure 4A shows the elution profile of AGPs isolated from immature seeds 7 days after pollination (DAP) upon elution from the column with a salt gradient. Only a few minor peaks with retention times between 1 and 6 minutes are observed, and no material is eluted with the salt gradient.

If the 7 DAP seed AGPs were first incubated with EP3 endochitinases for 24 hrs, the elution pattern did not show any marked changes (Fig 4B) in comparison with untreated preparations. Apparently no small oligosaccharides that could have arisen by endochitinase cleavage were produced. This result is consistent with analyses of ^{14}C labeled secreted AGPs derived from suspension cultures.

Then we considered the possibility that AGPs might contain cleavage sites that were not directly accessible to the added endochitinase. Therefore we first incubated the AGPs with a mixture of purified fungal endogalactosidase, endoarabinofuranosidase, and exoarabinofuranosidase. Incubation of AGPs with these enzymes should split off oligosaccharides from the carbohydrate content of the AGPs. Subsequently EP3 endochitinases should be able to cleave further if any endochitinase cleavage site would be present. Upon incubation of 7 DAP AGPs with the three fungal enzymes a limited number of discrete oligosaccharides were produced, as illustrated by the elution profile in Figure 4C. Upon inclusion of EP3 endochitinases in the incubation mixture, there appeared a change in the elution profile. This involved a decrease of material in a peak numbered 2, eluting at 11.8 minutes, (Fig 4C) together with an increase of material eluting at 9.1 min, in a peak numbered 1 (Fig 4D). Such a change might point at the conversion of a larger AGP fragment into a smaller one due to an additional endochitinase cleavage reaction. A similar result was obtained using AGPs from immature seeds of 21 DAP (Fig 4E and 4F). Peak 1 does not result from the added endochitinase itself, as EP3 endochitinase alone eluted in a single peak with a retention time of 1.5 min (data not shown).

By incubation of AGPs with only one fungal hydrolase with or without EP3 endochitinases we then attempted to obtain some information on the composition of EP3-sensitive oligosaccharides. The HPAE-PAD analyses shown in Figure 5A indicates that removal of terminal arabinoses from 7 DAP AGPs by exoarabinofuranosidase generated a series of oligosaccharides, whereas only one new peak at 3.8 min, corresponding to arabinose, was expected. Among the peaks that were generated by exoarabinofuranosidase activity two peaks (number 3 and 4 in Figure 5A, eluting after 7.0 and 12.2 minutes respectively) were found to disappear after incubation with a mixture of exoarabinofuranosidase and the chitinases (Figure 5B) while a new one, peak 1, appeared. This peak 1 has the same retention time (9.1 min) as peak 1 in Figure 4, that arose after incubation with the mixture of hydrolases and endochitinases. It is notable that treatment with exoarabinofuranosidase alone already enables to access the endochitinase cleavage site.

Incubation with endoarabinofuranosidase generated a large number of oligosaccharides (Fig 5C). Despite the large number of peaks, little changes were observed when incubation with endoarabinofuranosidase was combined with endochitinases. Only one peak (peak 1) was found to increase as a result of the endochitinase activity (Fig 5D).

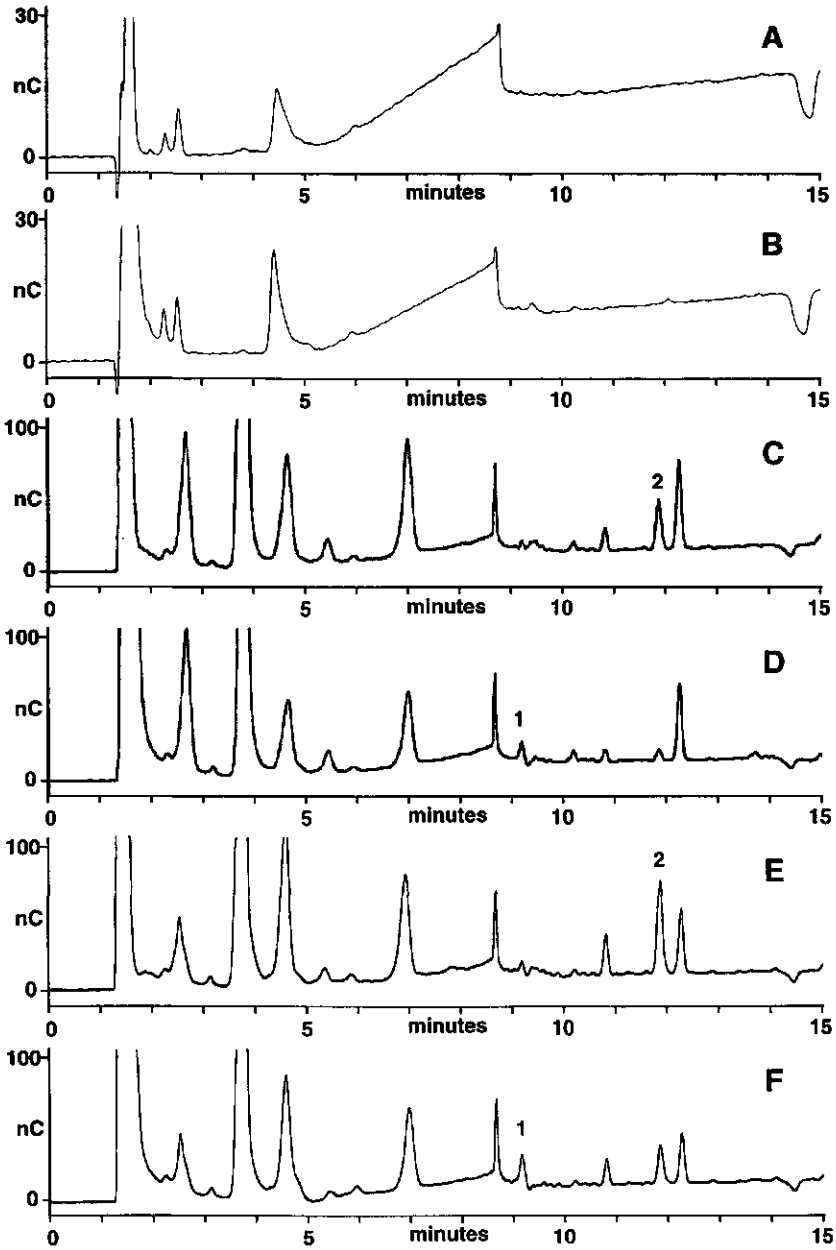


Figure 4. HPAE-PAD chromatograms of AGPs that were isolated from developing seeds and that had been incubated with several AGP degrading enzymes in the presence, or absence of the EP3 endochitinases. **A**. AGPs isolated at 7 DAP. **B**. AGPs isolated at 7 DAP and incubated with EP3 endochitinases. **C**. AGPs isolated at 7 DAP and incubated with endogalactosidase, endo- and exoarabinofuranosidase. **D**. AGPs isolated at 7 DAP and incubated with endogalactosidase, endo- and exoarabinofuranosidase in combination with EP3 endochitinases. **E**. AGPs isolated at 21 DAP and incubated with endogalactosidase, endo- and exoarabinofuranosidase. **F**. AGPs isolated at 21 DAP and incubated with endogalactosidase, endo- and exoarabinofuranosidase in combination with EP3 endochitinases. Peaks that change upon incubation with the EP3 endochitinases are numbered.

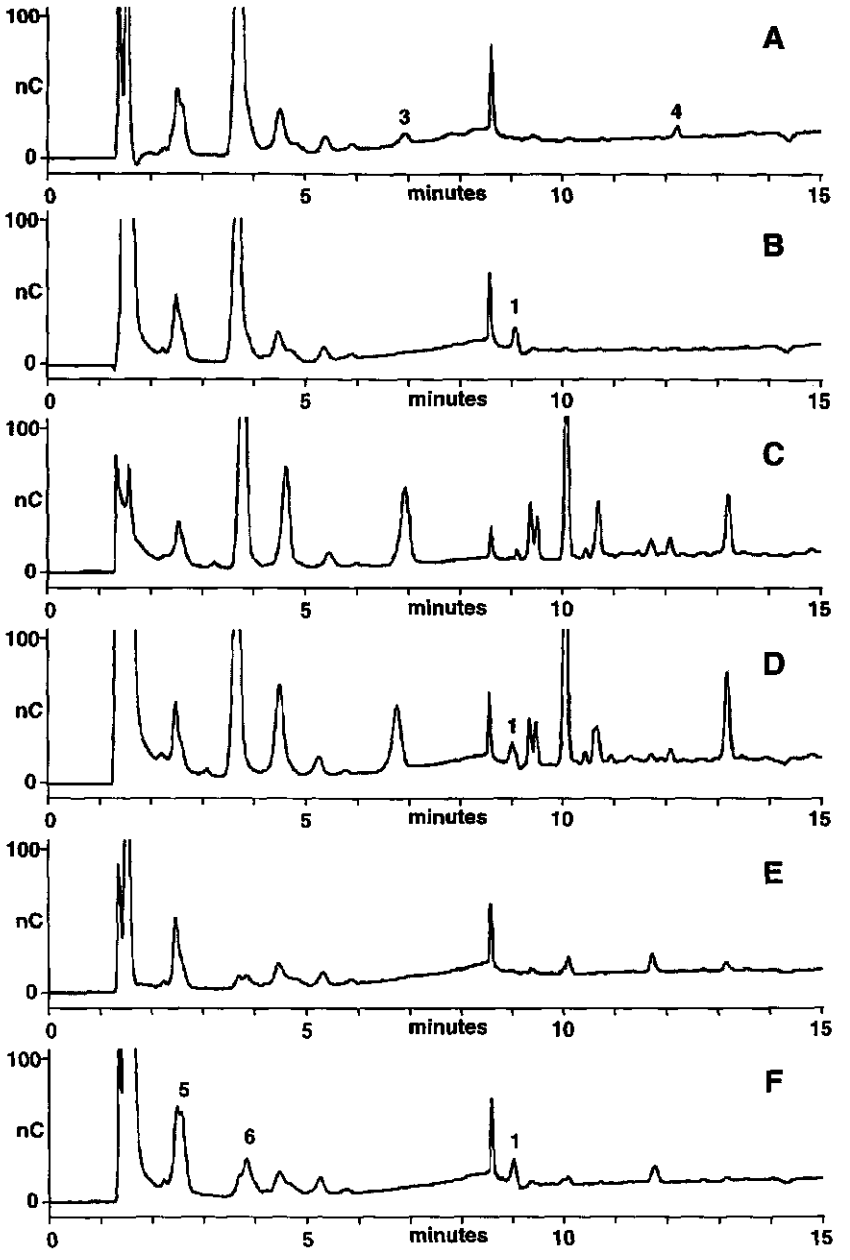


Figure 5. HPAE-PAD chromatograms of AGPs that were isolated from developing seeds 7 DAP and that had been incubated with single AGP degrading enzymes in the presence, or absence of the EP3 endochitinases.
A. AGPs incubated with exoarabinofuranosidase. **B.** AGPs incubated with exoarabinofuranosidase in combination with EP3 endochitinases. **C.** AGPs incubated with endoarabinofuranosidase. **D.** AGPs incubated with endoarabinofuranosidase in combination with EP3 endochitinases. **E.** AGPs incubated with endogalactosidase. **F.** AGPs incubated with endogalactosidase in combination with EP3 endochitinases.

The carbohydrate part of AGPs consists of chains of (1-3)-ß-D-galactose residues with side chains of variable sugar components. The (1-3)-ß-D-galactose chains are linked to the protein core (Clarke et al., 1979). Incubation with endogalactosidase should split the galactose chains into pieces and releases the side chains that were attached to the galactose backbone. A comparison of Figure 5E and 5F shows that incubation with endogalactosidase produced three oligosaccharides, peak 1, 5 and 6 arising after endochitinase activity. As a result of chitinase treatment again a peak 1 appeared with a retention time of 9.1 min. In addition two peaks, numbered 5 and 6, eluting after 2.7 and 3.9 minutes, were found to increase considerably.

These results can be summarized as follows. Exoarabinofuranosidase activity produces two AGP fragments, peaks 3 and 4 (Fig 5A), that contain an endochitinase cleavage site. After endochitinase treatment both disappear and peak 1 arises (Fig 5B). Endoarabinofuranosidase activity produces a large number of fragments (Fig 5C). Although none of these fragments disappear after endochitinase treatment, we suggest that after endoarabinofuranosidase treatment a fragment or fragments are produced that can be cleaved further by endochitinases, in turn leading to the production of peak 1 (Figure 5D). Endogalactosidase produces fragments representing sidechains of the AGP carbohydrate moiety (Fig 5E). The amount of peaks that can be observed in Figure 5E indicates either that only a few different side chains are present, or that most of the side chains are too large to be analysed by HPAE-PAD. After endochitinase treatment of the AGP side chains three peaks, 1, 5 and 6 arise.

We can conclude that the endochitinase cleavage site in intact 7 DAP AGPs is inaccessible for EP3 endochitinases and that a partial hydrolysis with one of the three different fungal hydrolases is required to make this site accessible, resulting in a peak designated 1 as the main product of the endochitinase activity. Next to peaks containing a product of endochitinase activity, in Figure 4C a peak, designated 2, containing material with an endochitinase cleavage site, was observed. This peak was not produced after incubation of AGPs with only a single fungal hydrolase, indicating that the generation of peak 2 requires the activity of at least two of the fungal hydrolases together.

In conclusion, these results indicate that AGPs can contain endochitinase cleavage sites, that become accessible after incubation with hydrolases able to degrade the carbohydrate part of AGPs.

To determine wether the occurrence of endochitinase cleavage sites in AGPs was developmentally regulated, we examined whether any changes arose during seed maturation. Figures 6A, C, E and G, show a part of the HPAE-PAD elution pattern of AGPs isolated from seeds of 4, 7, 11 and 21 DAP after incubation with all three fungal hydrolases. This part of the elution pattern presents a peak numbered 2 (eluting after 11.8 minutes) that shows a decrease between 4 and 11 DAP, followed by an increase between 11 and 21 DAP. One other peak with a retention time of 12.3 minutes shows an increase between 4 and 7 DAP and no changes between 11 and 21 DAP. Apparently the composition of the carbohydrate part of the AGPs changes during development.

If the AGPs obtained at different days after pollination were treated with EP3 endochitinase together with the three hydrolases (Fig 6B, D, F and H) the following changes were observed. There was a decrease in material of the peak numbered 2, while no changes were observed for the other peak with

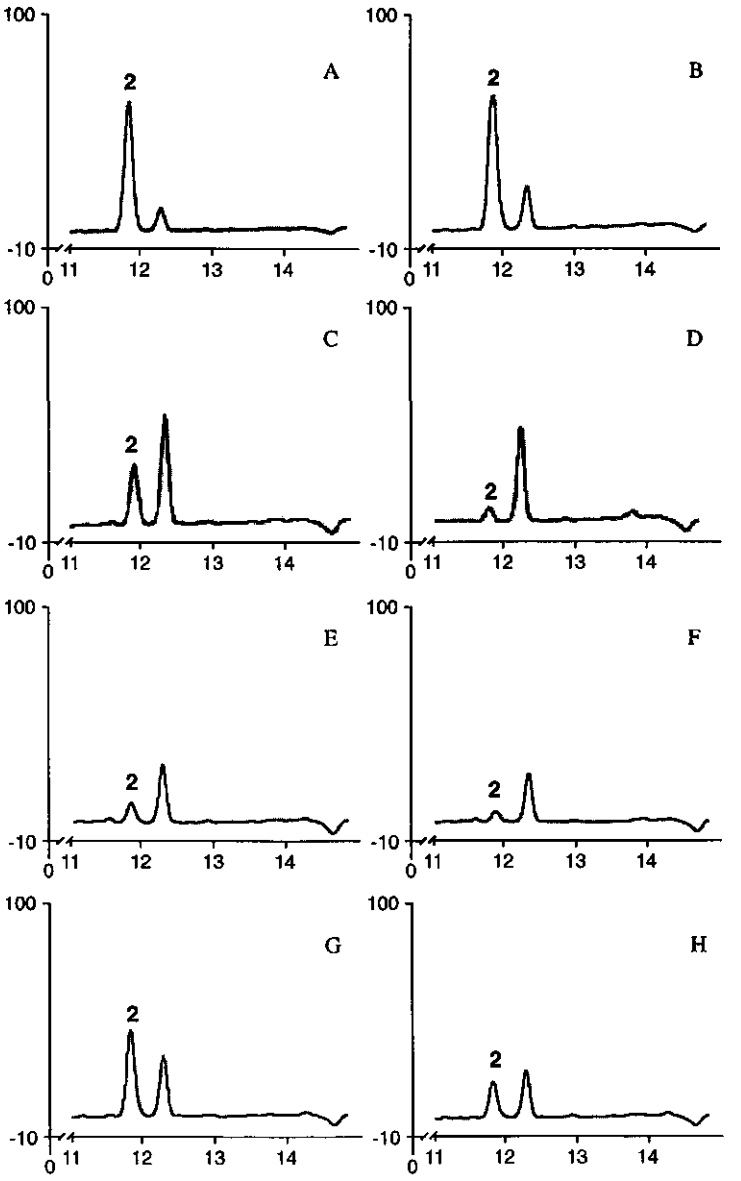


Figure 6. Details of HPAE-PAD chromatograms of AGPs that were isolated from seeds at different stages of development, and that had been incubated with several AGP degrading enzymes in the presence, or absence of the EP3 endochitinases. **A**; **C**; **E** and **G**. AGPs isolated at 4; 7; 11 and 21 DAP respectively, that had been incubated with endogalactosidase, endo- and exoarabinofuranosidase. **B**; **D**; **F** and **H**. AGPs isolated at 4; 7; 11 and 21 DAP respectively, that had been incubated with endogalactosidase, endo- and exoarabinofuranosidase in combination with the EP3 endochitinases.

a retention time of 12.3 min. However, comparing Figure 6A with Figure 6B shows that when 4 DAP AGPs were used peak 2 does not show a decrease as a result of the presence of endochitinases in the incubation mixture. This indicates that at 4 DAP the material in peak 2 is not the same as at later stages of development. We conclude that apparently the changes in the carbohydrate part of the AGPs that were observed during seed development include changes in the presence of endochitinase cleavage sites.

Characterization of AGPs in suspension cultures and at different stages of seed development

The experiments for labeling of carrot suspension cultures showed that the amount of AGPs present in the medium after 7 days of subculturing was higher if the cultures were grown with 2,4-D in the medium then if grown in the absence of 2,4-D. Here we present data on the composition of AGPs produced in suspension cultures of the embryogenic cell line Ar in medium with or without 2,4-D. The AGPs secreted in the medium after 2 weeks of culturing in the presence or absence of 2,4-D showed different patterns after crossed electrophoresis (Fig 7). AGP preparations from medium that contained 2,4-D showed a broad partition pattern in which 3 peaks can be observed having Rf values of 0.4; 0.6 and 0.8. The Rf value of the major peak of AGPs that were secreted in the medium in the absence of 2,4-D was 0.7 and that of the minor peak 0.4. This indicates that the presence of 2,4-D in the culture medium affects the production of AGPs, or results in changes in the secretion of enzymes that can degrade AGPs.

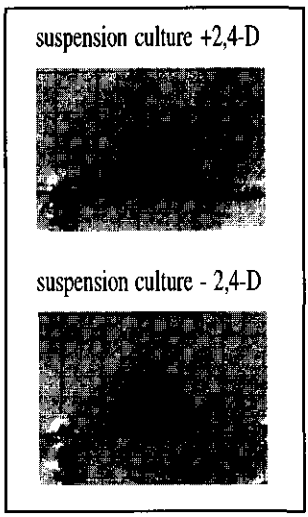


Figure 7. Crossed electrophoresis of 10 µg AGPs that were isolated from the media of an embryogenic suspension culture that was grown in the presence, or absence of 0.2 µM 2,4-D.

Suspension cells that produce AGPs can be identified with Yariv reagent, which visualizes the localisation of AGPs in plant tissues (Gane et al., 1995). Figure 8A shows Yariv staining of

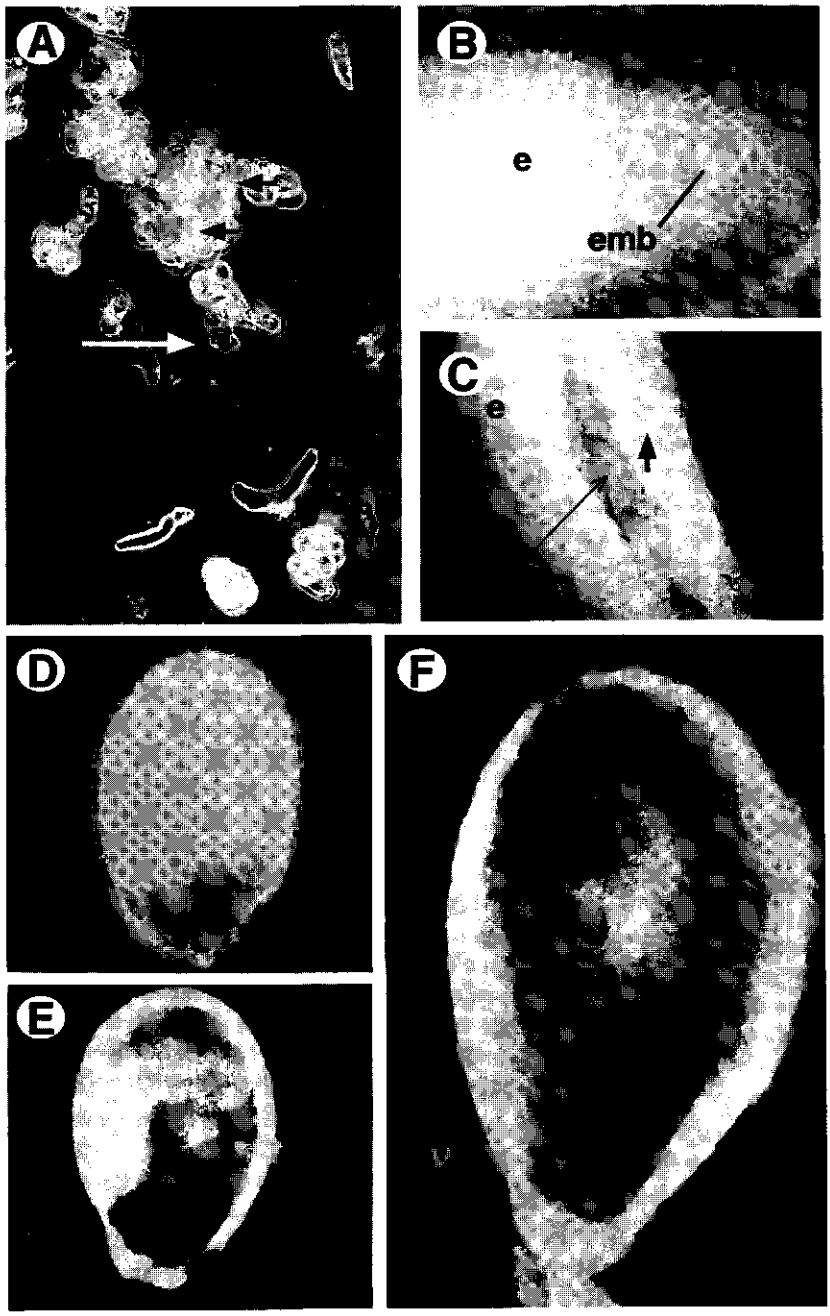


Figure 8. Plant material stained with ß-glucosyl Yariv.
A. Suspension cells of an embryogenic cell line. Arrows indicate stained cells. **B.** Detail of a longitudinal section of a immature carrot seed 21 DAP showing staining mostly associated with endosperm (e) cells, no staining in the zygotic embryo (emb) and very little staining in the seed coat (sc). **C.** Detail of a longitudinal section of a mature carrot seed, showing staining in the central space (big arrow) and around endosperm cells (small arrow). **D-F** Developing seeds of *Arabidopsis thaliana*, showing staining in the inner cell layers.

suspension cells. In the majority of the cells almost no staining was observed. Some cells showed intermediate staining (indicated by black arrows), while only few cells showed intense staining (white arrow). This indicates that there are large differences in the amount of AGPs present at the surface of individual suspension cells. If the cells that show intense staining also produce the majority of AGPs, it might be that different cells do not contribute equally to the secretion of AGPs.

In the previous section we showed that AGPs isolated at different stages of seed development differ in composition (Figure 6). This difference in composition of AGPs from seeds at different days after pollination was confirmed by using crossed electrophoresis. Figure 9 shows the differences in the native electrophoretic mobility of AGPs during development. AGPs from 11 DAP have the highest electrophoretic mobility as compared to earlier or later stages in seed development. Immunoblotting of AGPs after crossed electrophoresis allowed detection of AGP epitopes recognized by the monoclonal antibodies JIM8 and MAC207. These results are schematically represented with horizontal lines in Figure 9, and showed that a similar difference in electrophoretic mobility could be observed for the distribution of AGP epitopes that are present in the broad band of AGPs and that are recognized by JIM8 and MAC207. This indicates that during seed development the mobility of specific AGPs containing the JIM8 or MAC207 epitopes shows an increase followed by a decrease, that can be caused by changes in size or changes in charge of these AGPs.

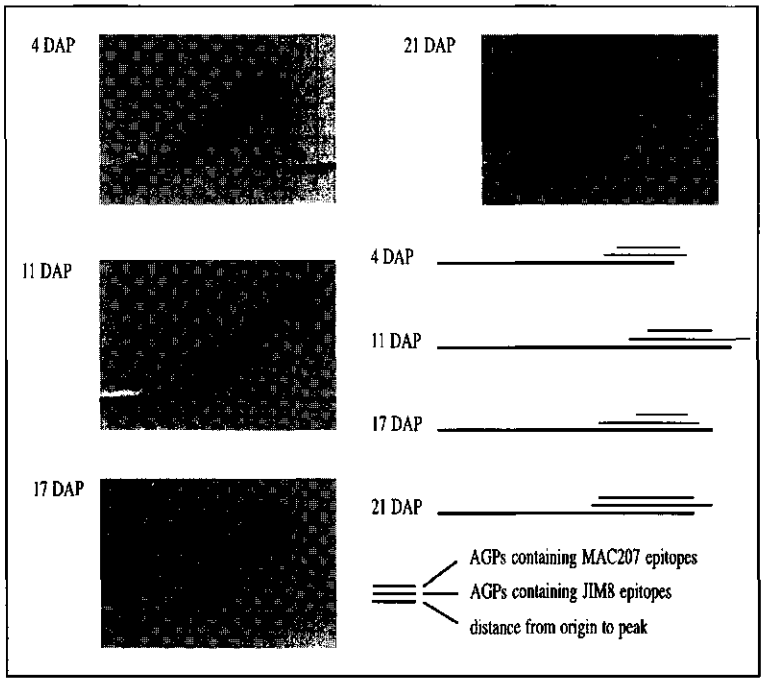


Figure 9. Crossed electrophoresis of 10 µg AGPs that were isolated from developing seeds of 4; 11; 17 or 21 DAP,. and a comparison of the native electrophoretic mobility of the AGPs that contain JIM8 or MAC207 epitopes.

The Yariv reagent was also used to localise AGPs in immature carrot seeds. Figure 8B shows staining around cells of the endosperm at 21 DAP, while the embryo in the seed did not show any staining. The presence of AGPs in the endosperm is supported by the fact that AGPs could be isolated from dissected endosperms of immature carrot seeds. In mature carrot seeds AGPs were visualised in the central space in which the embryo is located (Figure 8C; big arrow) and around the endosperm cells (Figure 8C; small arrow). This implies that endosperm cells produce large amounts of AGPs.

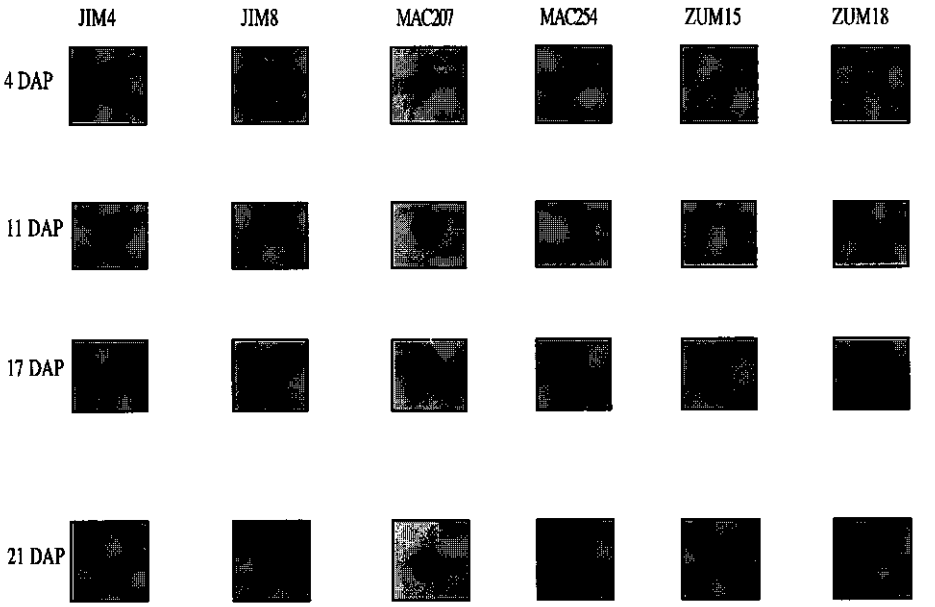


Figure 10. Dot blot analysis of 10 µg samples of AGPs that were isolated at different stages of seed development (4; 11; 17; 21 DAP) The antibodies JIM4; JIM8; MAC207; MAC254; ZUM15 and ZUM 18 were used for the detection of specific AGP epitopes

Dot blot analysis using several monoclonal antibodies that recognize AGP epitopes provides additional information on the composition of seed AGPs. Figure 10 shows that the epitopes that are recognized by JIM4, JIM8, MAC207, MAC254, ZUM15 and ZUM18 are all present in AGPs that were derived fom developing seeds at 4 DAP, when the carrot endosperm is not yet cellularised (Gray et al., 1984). Cell wall formation starts 7 days after pollination and is completed at 21 DAP (Gray et al., 1984). In between 4 and 17 DAP there is a gradual decline in the amount of epitopes that are recognized by JIM4 and JIM8, while MAC254, ZUM15 and ZUM18 epitopes are essentially gone by 11 DAP. At 21 DAP, when large amounts of AGPs are present in and most likely produced by the endosperm, there is an increase of JIM8 and MAC254 epitopes. The epitope recognised by MAC207 remains present in comparable amounts between 4 and 21 DAP, but MAC207 binds a somewhat wider variety of AGPs than other monoclonals such as for instance JIM4 (Yates and Knox, 1994).

These results can be summarized as follows. Between 4 and 11 DAP there is a decline in the epitopes that are recognised by the monoclonal antibodies JIM4, JIM8, MAC254, ZUM 15 and ZUM 18, while the native electrophoretic mobility of the total population of AGPs increases during the same period. Since AGPs are components of the cell surface and the endosperm is not yet, or hardly cellularised between 4 and 11 DAP we conclude that AGPs are maternally produced and that degradation of these AGPs during development results in smaller sizes and the loss of epitopes. At 21 DAP the population of AGPs that can be isolated from immature seeds seems to be largely derived from the endosperm that is cellularised by this time. This coincides with an increase of the sizes of AGPs between 11 and 21 DAP and in the reappearance of JIM8 and MAC254 epitopes.
Visualisation of AGPs in developing seeds of another plant species confirmed the localisation of AGPs in the endosperm. *Arabidopsis* developing seeds show staining inside the seeds (Figure 8D, 8E and 8F), while no staining was observed in the seed coat. The observed pattern of staining seems to correlate with the development and cellularization of the endosperm in this species as described by Mansfield and Briaty (1990).

Discussion

Secreted AGPs contain GlcNAc

In this chapter we demonstrated that embryogenic carrot suspension cells secrete AGPs that contain N-acetylglucosamine into the culture medium, while no or hardly any GlcNAc can be detected in AGPs secreted by non-embryogenic cell lines. The presence of GlcNAc in AGPs was demonstrated by labeling embryogenic cell cultures with [^{14}C] GlcNAc. When [^{14}C] GlcNAc was added to suspension cells, the labeled compound was rapidly taken up and metabolised by the cells. A small amount of label was subsequently secreted into the medium and found to be present mainly in AGPs. Presumably some [^{14}C] GlcNAc was used as a direct precursor of the GlcNAc incorporated in AGPs, because labeled GlcNAc and labeled GlcN were found after AGP hydrolysis.
The occurrence of GlcNAc in AGPs has not been observed before in studies on the total sugar composition of AGPs (Baldwin et al., 1993; Komalavilas et al., 1991; Mollard and Joseleau, 1994; Serpe and Nothnagel, 1994; Serpe and Nothnagel, 1996; Smallwood et al., 1996; Van Holst et al., 1981). It is possible that GlcNAc has sofar been overlooked if GlcNAc is a rather rare component of AGPs. But in view of the tissue-, and stage specificity of AGP epitopes (Knox et al., 1991; Knox et al., 1989) and our finding that only embryogenic cell lines contain AGPs with GlcNAc it is quite well possible that the presence of GlcNAc in AGPs is restricted to only a few plant tissues and in a limited developmental time-span. This is the more attractive hypothesis since it might point to a functional significance of the occurrence of GlcNAc in AGPs.

The presence of GlcNAc in the AGPs secreted by embryogenic cell lines only may also indicate a more functional role of GlcNAc containing AGPs in carrot somatic embryogenesis.

Seed AGPs contain cleavage sites for endochitinases

We have not been able to demonstrate endochitinase cleavage sites in the AGPs fom suspension cultures. However, we have been able to demonstrate endochitinase cleavage sites in AGPs purified from immature carrot seeds. The presence of such cleavage sites could only be demonstrated after treatment of AGPs with endogalactosidase, endoarabinofuranosidase and, or exoarabinofuranosidase. Apparently the complex carbohydrate structure of AGPs had to be opened before cleavage sites become accessible for endochitinases.

Endochitinases require at least three adjacent GlcNAc residues for cleavage activity (Molano et al., 1979; Usui et al., 1990). In view of this specificity we feel confident that the AGP cleavage that was demonstrated upon chitinase treatment is a reflection of the presence of at least three GlcNAc residues. However, at present we cannot exclude that the observed chitinase mediated hydrolysis of AGPs is based on the presence of arrays of at least three GlcN residues instead of GlcNAc. In the previous chapter we have shown that, like some citrus chitinases (Osswald et al., 1993), EP3 endochitinases also have some chitosanase activity. It is evident that the occurrence of endochitinase cleavage sites is a relatively rare event, because only very few differences were observed after endochitinase treatment. Enzymatic degradation of AGPs by hydrolytic enzymes such as endogalactosidase and arabinofuranosidases as we have used here to generate partially degraded AGPs shown to contain an endochitinase cleavage site, has also proven to be a useful tool for controlled degradation in structural studies of AGPs (Gleeson and Clarke, 1979; Saulnier et al., 1992; Tsumuraya et al., 1984; Tsumuraya et al., 1990). The enzymes and the combinations that we have used imply that GlcNAc is present in side chains of AGPs. However, they do not provide enough information to allow a more precise identification of the oligosaccharides that contain an endochitinase cleavage site. In addition to EP3 endochitinases, the presence of a ß-galactosidase and an α-arabinofuranosidase in the conditioned medium of carrot suspension cultures (Konno and Katoh, 1992; Konno et al., 1994) suggests that AGPs that are present in the medium of suspension cultures may also be processed by such enzymes. *In vivo*, hydrolytic enzymes of both fungal and plant origin have been shown to be capable of degrading AGPs, suggesting that a stepwise AGP degradation mechanism occurs by means of individual hydrolytic enzymes (Nothnagel, 1997). Determination of the *in planta* localisation of such enzymes in relation to AGPs might provide important evidence concerning the turnover or processing of AGPs. Furthermore AGP hydrolases might provide information on the biological function of both AGPs and their breakdown products. The hypothesis that AGPs can function as the locked-up form of signalling molecules as postulated by Bacic et al.

(1988), implies that co-localisation of both AGPs and hydrolytic enzymes is essential for the release and / or degradation of such signaling molecules.

EP3 endochitinases and AGPs are both secreted molecules and co-localise in supension cultures and in seeds. The EP3 endochitinase protein is secreted by the maternal integument cells and has been detected in the endosperm of developing carrot seeds (Van Hengel et al., in press), so in carrot seeds both the EP3 endochitinases and AGPs are localised in the same tissues. This supports the hypothesis that processing of AGPs by EP3 endochitinases occurs in the developing seed.

Our results support earlier observations that the composition of AGPs is developmentally regulated (Pennell et al., 1991; Pennell et al., 1989). In suspension cultures the removal of 2,4-D was shown to result in a change in AGPs coinciding with the formation of somatic embryos. Previously it was shown that the removal of auxin from the medium of a carrot cell suspension culture is correlated with the expression of a plasma membrane epitope (Stacey et al., 1990). The application of monoclonal antibodies that recognise specific AGP epitopes has led to the finding that in general the presence of AGP epitopes can be tissue- and stage specific and that AGPs are developmentally regulated (Knox et al., 1991; Knox et al., 1989; Pennell et al., 1991; Pennell et al., 1989). Using monoclonal antibodies we have shown that also in carrot seeds the presence of AGP epitopes changes during development. The disappearance and reappearance of AGP epitopes and the observed increase and subsequent decrease in the native electrophoretic mobility of AGPs during seed development suggest a degradation, and a new synthesis of AGPs. Before fertilization and during the initial stages of seed development AGPs are predominantly found in maternal tissues such as the extracellular mucilage of the transmitting tissue and ovaries (Gane et al., 1995; Hoggart and Clarke, 1984). This suggests that in the initial stages of carrot seed development AGPs that were isolated from these seeds were mainly derived from the transmitting tissue and ovaries. In addition we have shown that in carrot later in development the newly cellularised endosperm contains large quantities of AGPs.

In an earlier paper we have demonstrated that the expression of the EP3 genes changes during seed maturation. In seeds the integuments that surround the developing endosperm express the EP3 genes and the highest expression has been found around 10 DAP (Van Hengel et al., in press). Between 7 and 21 DAP, when the AGPs in the seeds contain an endochitinase cleavage site relatively large amounts of EP3 endochitinases are present in these seeds.

In conclusion there appears to be a striking coincidence in the temporal and spatial regulated transient presence of both EP3 endochitinases and AGPs that contain an endochitinase cleavage site. Both are secreted inside the developing carrot seed and present in the endosperm in close vicinity of the early zygotic embryo. Together with the fact that EP3 can only rescue *ts11* embryogenesis during the phenocritical period (De Jong et al., 1995), points towards a developmentally regulated processing of AGPs that is important for the process of embryogenesis. In the next chapter we shall present biological activity tests using the AGPs characterised here and the effects of endochitinases on the biological activity of AGPs.

Chitinases are involved in plant development

Earlier it was proposed that chitinases have a major role as plant defence enzymes. This was based on their inducibility after challenge with fungal pathogens (Herget et al., 1990; Kurosaki et al., 1987; Meins and Ahl, 1989). Growth retardation of fungi by dissolution of wall polymers offered a convenient explanation for the presence of many types of chitinases in apoplastic fluids and in vacuoles (Ariorio et al., 1992; Boller et al., 1983; Schlumbaum et al., 1986; Wubben et al., 1992). Based on inducibility of chitinases by exposure to non-chitin-containing pathogens (Meins and Ahl, 1989) and after abiotic stresses (Bronner et al., 1991; Van Damme et al., 1993) this idea was broadened and proposed a role for chitinases in more general stress responses in plants. However no clear model on such a function was ever put forward. In this paper we describe the occurrence of endochitinase cleavage sites in plant produced AGPs. Based on this finding we propose that endochitinases primarily function in plant development.

AGPs are generally assumed to function in plant development, but also other roles have been proposed. Amongst these are involvement in plant-pathogen or plant-symbiont interactions and in wound healing (Clarke et al., 1979; Gollotte et al., 1995; Whistler, 1993). Remarkably these processes are also known to increase chitinase gene expression. If AGPs that are produced upon infection or wounding also contain endochitinase cleavage sites, endochitinases may generate AGP fragments that might not primarily function in attacking pathogens, but rather in the plants' recovery after wounding, either by abiotic or mechanical agents or by infection. While both infection and wounding, induce defence reactions, plant recovery is equally important and depends upon renewed development, requiring AGPs as well as perhaps endochitinases as a means to generate AGP derived signalling molecules.

Materials and methods

Plant material

Daucus carota cell suspensions of cv. Autumn King /Trophy (S&G Seeds, Enkhuizen, The Netherlands) were initiated and maintained as described before (De Vries et al., 1988). One week old suspension cultures were used for labeling experiments.

AGP isolation

AGPs were isolated from suspension cultures and developing carrot seeds (S&G) by precipitation with Yariv reagent (Yariv et al., 1962) as described by Kreuger and Van Holst (1993). Pectin-free

AGP fractions were obtained by incubating 1 mg of AGPs in 50 mM NaAc pH 5.0 containing 10 units pectinase (Sigma) for 16 hrs followed by a second AGP isolation. The AGP concentration was determined by the radial gel diffusion method as described by Van Holst and Clarke (1985).

Labeling of suspension cultures and degradation of labeled AGP fractions

Carrot suspension cells (2 ml packed cell volume) were cultured for 1 week in 50 ml B5 medium containing 0.2 µM 2,4-D. The cells were washed with and transfered to B5 medium with, or without 0.2 µM 2,4-D and grown in the presence of 10^{-7}M D-[1-^{14}C] glucosamine, or N-acetyl-D-[1-^{14}C] glucosamine. Medium samples were taken after 2, 3, 4 and 7 days.
The degradation of the labeled AGP fractions was done by incubation with 2M trifluoroacetic acid for 45 at 100^{0}C or 60 min at 120^{0}C. After degradation the samples were analysed by TLC (n-butanol: acetic acid: water 6: 2: 2) next to the reference compounds D-[1-^{14}C] glucose, D-[1-^{14}C] glucosamine and N-acetyl-D-[1-^{14}C] glucosamine that had been subjected to the same degradation reactions.
Detection and quantification of label was done using a PhosphorImager (Molecular Dynamics).

Cell wall isolation, protein extraction and gel electrophoresis

Cell wall fractions were obtained using the method described by Brown and Fry (1993). Cellular proteins were obtained by grinding cells in extraction buffer containing 50 mM phoshate buffer pH 7.0, 10 mM EDTA, 0.1% triton, 0.1% sarkosyl, 10 mM ß-mercaptoethanol supplemented with polyvinylpyrrolidone and powdered glass. The resulting slurry was centrifuged for 30 min and the supernatant was used for analysis.
Standard SDS-PAGE (Laemmli, 1970) 12.5% gels were used. Gels were silver stained according to the method of Oakley et al. (1980), dried under vacuum and exposed in a PhosphorImager cassette.

Immunochemistry and histological techniques

JIM4, JIM8, MAC207 and MAC254 anti-AGP monoclonal antibodies and JIM5 and JIM7 anti-pectin monoclonal antibodies were kindly provided by Prof. Keith Roberts, John Innes Centre, Norwich, UK. ZUM15 and ZUM18 anti-AGP monoclonal antibodies were kindly provided by Dr. Marc Kreuger, S&G seeds, Enkhuizen, The Netherlands. AGP samples of 10 µg were blotted on polyvinylidene fluoride membranes (Millipore) and assayed for the presence of immuno-reactive

epitopes as described (Knox et al., 1991). Plant material was stained for the presence of AGPs as desribed previously (Gane et al., 1995).

Crossed electrophoresis

Two aliquots of 2 ml of packed cell volume of the embryogenic cell line Ar were washed in B5 medium with or without 2,4-D. The cells were transferred to erlenmeyer flasks with 50 ml medium with or without 2,4-D. After 1 week AGPs were isolated from the medium and analysed by crossed electrophoresis as described (Van Holst and Clarke, 1986).

Enzyme assays on AGPs and HPAE-PAD analysis

Samples of 80 µg of AGPs were incubated for 24 hrs in 10 mM MES pH 5.5 supplemented with 80-200 ng EP3 chitinases that were isolated as described before (Kragh et al., 1996) and / or with 0.050 units exo-arabinofuranosidase; 0.024 units endo-arabinofuranosidase and 0.030 units endogalactosidase, all three of which were produced by *Aspergillus niger*, a fungus capable of degrading plant material, and were kindly provided by Dr. Jaap Visser, Wageningen Agricultural University, The Netherlands.
Analysis of AGPs and enzymatically degraded AGPs was performed by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD), using the CarboPac PA-100 column (Dionex). The flow rate was 1 ml/min and the eluent consisted of 10% 0.5 M NaOH in combination with a linear salt gradient starting at t = 3 with 0% NaAc and ending at t = 18 with 80% (v/v) 0.5 M NaAc.

Acknowledgements

We would like to thank Prof. Keith Roberts for the kind supply of anti-AGP and anti-pectin antibodies, Dr Marc Kreuger for the kind supply of anti-AGP antibodies, Dr. Jaap Visser for the kind supply of enzymes that are capable of degrading AGPs, Dr. Herman Spaink and André Wijfjes for the use of the HPAE-PAD equipment and their technical assistance

Chapter 5

Chitinases and arabinogalactan proteins promote somatic embryogenesis from embryogenic wild type carrot protoplasts

EP3 endochitinases isolated from conditioned medium and arabinogalactan proteins (AGPs) extracted from immature carrot seeds can increase the number of somatic embryos formed from protoplasts obtained from wild type embryogenic suspension cultures. The immature seed AGPs were much more active then the chitinases. Pre-treatment of AGPs with EP3 endochitinases or N-acetylhexosaminidase results in optimal somatic embryo-forming activity. The carbohydrate part of the AGPs is responsible for the embryo-promoting effect. These results demonstrate that activation of immature seed AGPs by hydrolytic enzymes is an important component of embryogenesis. Apart from the increase in embryogenesis, AGPs appear to activate a subpopulation of otherwise non-dividing protoplasts. AGPs are only capable of promoting embryogenesis during a short period preceeding cell wall formation.

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Introduction

In the carrot somatic cell variant *ts11* the temperature or fresh medium-induced arrest of somatic embryogenesis at the globular stage could be lifted by addition to the culture medium of an endochitinase, designated EP3 (De Jong et al., 1992). The rescue effect appeared to be due to a transient lack in sufficient endochitinase in the conditioned medium during the period when globular *ts11* somatic embryos were most sensitive to either temperature shock or to cultivation in fresh medium (De Jong et al., 1995). A second effect of the application of EP3 endochitinases to *ts11* cells was the formation of more embryogenic cell clusters (De Jong et al., 1993). The EP3 endochitinase was found to be a member of a small multigene family of class IV chitinases. Two of the carrot class IV EP3 endochitinases were tested and found to be effective in *ts11* rescue, as was a related class I endochitinase (Kragh et al., 1996). The phenotype of *ts11* is rather pleiotropic and is thought to result from a defect in the secretory apparatus. As a result, cell wall formation is disturbed (Baldan et al., 1997).

The biological function of the EP3 endochitinases in embryogenesis is not known. Bacterial lipochitooligosaccharides (LCOs) were able to increase the number of embryogenic cells in *ts11* as efficiently as the EP3 chitinases. Based on this observation it was proposed that the function of the endochitinases was to release an LCO-like molecule from plant origin (De Jong et al., 1993).

In the previous chapter we have presented evidence that a sofar unknown group of molecules consists of N-acetylglucosamine (GlcNAc)- or glucosamine (GlcN)-containing arabinogalactan proteins (AGPs) and can be found in the medium conditioned by embryogenic wild type carrot suspension cells. In addition we have found that AGPs isolated from immature carrot seeds contain endochitinase cleavage sites (chapter 4).

In the present work we aimed to answer two questions: 1.) Whether the observed effect of endochitinases and LCOs on somatic embryogenesis is restricted to *ts11* or that also wild type somatic embryogenesis is promoted, and 2.) Whether the previously observed cleavage of GlcN and GlcNAc-containing AGPs by chitinases is of biological significance in somatic embryogenesis. In order to answer these questions a reliable and sensitive assay system based on wild type carrot cells is essential. However, no reproducible increase in the number of somatic embryos was ever found after addition of EP3 endochitinases or LCOs to such cells (De Jong, Van Hengel and De Vries, unpublished results). In addition, our attempts to establish an assay system to demonstrate the effect of AGPs on embryogenic cell formation in wild type cultures proved cumbersome (Toonen et al., 1997), not withstanding earlier successes reported by Kreuger and van Holst (1993). The demonstration of the effect of chitin fragments and LCOs on cell division in tobacco protoplasts (Röhrig et al., 1995), prompted us to establish a biological assay based on protoplasts prepared from embryogenic carrot suspension cultures. The results reported here show that EP3 endochitinases increase the number of somatic embryos that develop from protoplasts obtained from wild type

embryogenic suspension cells. LCOs were however not effective on wild type protoplasts. Immature seed AGPs were observed to increase the number of embryos formed more than one hundred-fold. These results demonstrate that chitinases, but not LCOs, have a more general effect in somatic embryogenesis. It appears that the biological effect of chitinases is to fully activate the embryogenic cell forming potential of GlcN and GlcNAc containing AGPs in immature seeds.

Results

A protoplast-based assay for testing chitinaceous molecules on somatic embryogenesis

In *ts11*, the addition of both the EP3 endochitinases as well as the LCO NodRIv-V(Ac, C18:4) resulted in an eight-fold increase in the number of somatic embryos produced (De Jong et al., 1993). Protoplasts derived from a wild type embryogenic suspension culture responded to addition of endochitinases, resulting in an aproximately ten-fold increase in the number of somatic embryos formed (Table 1). Addition of LCOs only increased the number of somatic embryos at the most about two-fold. This increase was not statistically significant according to the F-test on the average of the mean (Table 1). These results demonstrate that the effect of endochitinases on somatic embryogenesis is not restricted to *ts11* cells, but can be extended to wild type protoplasts. In contrast, the activity of LCOs on somatic embryogenesis (De Jong et al., 1993) is apparently restricted to *ts11* cells. Nevertheless we concluded that the wild type protoplast assay system was useful to further study the biological significance of chitinases and molecules that contain endochitinase cleavage sites such as AGPs (chapter 4).

Compound	Concentration (M)	Mean no. of embryos per 10.000 protoplasts	se	*n*	P values compared to no additions
no additions		0.075	0.04	4	
EP3	3 x 10-9	0.78	0.38	4	0.020
no additions		1.4	0.11	5	
LCOs	10-11	2.1	0.40	3	0.471
	10-10	2.3	0.22	2	0.062
	10-9	2.4	-	1	
	10-8	1.6	-	1	

Table 1. Effect of EP3 and LCOs on the number of somatic embryos formed from carrot protoplasts.
The effect of addition of EP3 and LCOs is expressed as the number of globular-, heart- and torpedo stage embryos obtained per 10,000 protoplasts. The standard error of the mean (se) is included. The number of individual assays (*n*) was obtained in 4 independent experiments. P values less than 0.05 are regarded as significantly different from the untreated controls.

Immature seed AGPs promote somatic embryogenesis and can be activated by chitinases

We next compared the effect of carrot immature seed derived AGPs on wild type cells and protoplasts. The results are shown in Table 2 and show that when immature carrot seed AGPs were added to an embryogenic suspension culture, no significant increase compared to unsupplemented controls could be observed (Table 2A). Addition of immature seed AGPs to protoplasts resulted in a clear increase in the number of somatic embryos formed (Table 2B).

	Compound	Concentration	Mean no. of embryos per 10.000 suspension cells	se	*n*	P values compared to no additions	
A							
	no additions		17.8	2.62	2		
	17 DAP AGPs	2 µg / ml	21.6	2.62	2	0.811	
		10 µg / ml	16.4	1.65	2	0.781	
	Compound	Concentration	Mean no. of embryos per 10.000 protoplasts	se	*n*	P values compared to no additions	
B							
	no additions		0.7	0.7	2		
	17 DAP AGPs	0.3 µg / ml	4.7	3.5	2	0.131	
		3.0 µg / ml	31.4	13	2	0.016	
		30 µg / ml	> 100	nd	2	nd	
	medium AGPs (+2,4-D)	15 µg / ml	40.2	_	1	_	
	medium AGPs (-2,4-D)	15 µg / ml	53.9	_	1	_	
							P value compared
C		Treatment					to 17 DAP AGP
	no additions		1.1	0.1	2		
	17 DAP AGPs		42	0.5	2	0.000	
	17 DAP AGPs	+ EP3	5.5		1	_	
	17 DAP AGPs	EP3 preincubated	68	2.5	8	0.000	0.030

Table 2. A. Effect of AGPs on the number of somatic embryos formed from suspension cells. **B.** Effect of AGPs on the number of somatic embryos formed from protoplasts. **C.** Effect of EP3 endochitinase pretreatment of immature seed AGPs. For 2C, the concentration of AGPs used was 15 µg / ml and the concentration of chitinases used was 200 ng / ml. The effect of addition of AGPs is expressed as the number of globular-, heart- and torpedo stage embryos obtained per 10,000 suspension cells or per 10,000 protoplasts. The standard error of the mean (se) is included. The number of individual assays (*n*) was obtained in 2 independent experiments. P values less than 0.05 are regarded as significantly different from the untreated controls (nd = not determined).

Figure 1 shows a representative example of the effect of addition of AGPs to protoplasts. Whereas AGP concentrations between 0.3 and 30 µg / ml were all capable of increasing the number of somatic embryos, this effect is clearly dose dependent. In comparing the results obtained with intact suspension cells and protoplasts derived from them, it is important to note that in untreated controls the number of somatic embryos obtained with protoplasts is 10 to 20-fold lower than with intact

suspension cells. This suggests that part of the effect of AGPs on protoplasts is the restoration of embryogenic potential, that was lost by removal of the cell walls (Table 2, compare A and B). At higher AGP concentrations protoplasts could produce significantly more embryos than the cells from which they were derived. Another source for AGPs is the conditioned medium of suspension cultures. Medium AGPs of an embryogenic culture grown in the presence or absence of 2,4-D were isolated and pectinase treated as described before (chapter 4). Addition of these medium AGPs to carrot protoplasts of the same cell line resulted in an increase in the number of somatic embryos as compared to controls (Table 2B). The results show that protoplast-derived somatic embryogenesis can be promoted by AGPs from the conditioned medium of suspension cells as well as by AGPs from immature carrot seeds.

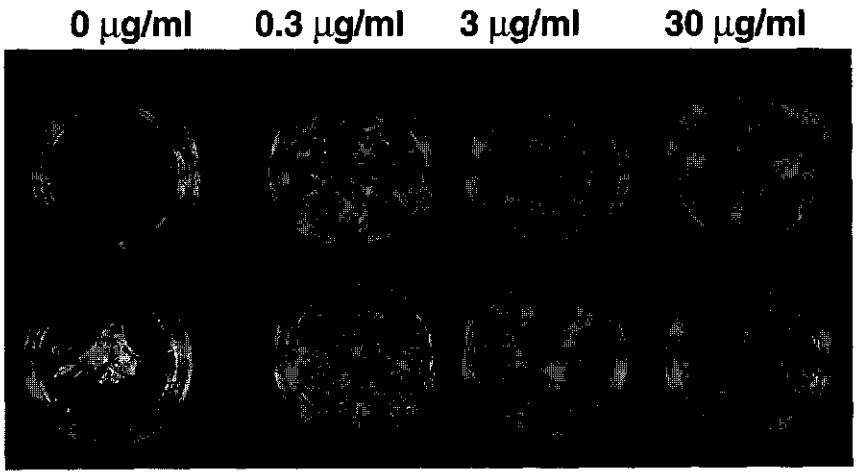

Figure 1. Petri dishes containing somatic embryos obtained after addition of immature carrot seed AGPs to freshly isolated carrot protoplasts.
AGPs were added in three different concentrations (0.3, 3.0 and 30 µg / ml), while no AGPs were added to controls. After the formation of heart- and torpedo stage embryos, the plant material was tranferred to fresh B5 medium without mannitol for further embryo development into plantlets. (Duplicates of one single experiment are shown)

In the previous chapter it was shown that AGPs isolated from immature carrot seeds contain a cleavage site for EP3 endochitinases. It was therefore of interest to determine the possible biological significance of such AGP cleavage on somatic embryo formation. A mixture of immature seed AGPs and EP3 endochitinases was added to protoplasts. Surprisingly, the number of embryos that developed from these protoplasts was greatly reduced in comparison to the number of embryos that developed after addition of AGPs alone (Table 2C). It appeared that the presence of the chitinase completely counteracted the promoting effect of the immature seed AGP preparation. This result suggested that cleavage of GlcN or GlcNAc-containing oligosaccharide side chains of AGPs, by EP3 endochitinases, inactivates the AGPs.

To determine that this inactivation was due to a structural alteration in the AGP molecule, AGPs were preincubated with EP3 endochitinase for 16 hrs and re-isolated from this mixture by Yariv

precipitation. This procedure removes chitinases (as determined by immuno dot blot analysis) and allows to recover the AGPs. Upon addition of chitinase-treated and then reisolated AGPs to protoplasts it appeared that the promotive effect of the AGPs on somatic embryogenesis was not only completely restored, but about 50% more somatic embryos were produced in comparison to non chitinase treated but Yariv-reisolated AGPs (Table 2C). Based on these results, we conclude that endochitinase treatment renders AGPs more effective in promoting somatic embryogenesis, but simultaneously a small inhibiting compound, presumably containing GlcN or GlcNAc, that must be removed before the increased promoting effect of the AGPs becomes apparent.

GlcNAc residues in AGPs are terminally located on the carbohydrate side chains

Endochitinases require at least three consecutive ß1-4 N-acetylglucosamines for hydrolytic activity. It was of interest to compare the effect of EP3 endochitinases on immature seed AGPs with that of an exochitinase from jack bean that has been described as a ß-N-acetylhexosaminidase. That enzyme removes at pH 5.5 terminal GlcNAc residues that are β1-2,3,4 or 6-R linked, whereas at pH 3.5-4.0 terminal GalNAc is preferentially removed (Li and Li, 1970). Immature seed AGPs treated at pH 5.5 with the ß-N-acetylhexosaminidase and then reisolated were more effective than endochitinase treated AGPs (Table 3A). This suggests that a strech of at least three ß (1-4) linked GlcNAc residues is located terminally in 17 DAP AGPs and is not internally embedded in an oligosaccharide side chain. Pre-incubation of AGPs with a combination of EP3 endochitinases and N-acetylhexosaminidase did not further increase the number of somatic embryos (Table 3A), suggesting that the enzymatic activation of immature seed AGPs is complete and involves removal of at least two consecutive, terminally located GlcNAc residues. The results obtained with ß-N-acetylhexosaminidase suggest that GlcNAc rather than GlcN residues are involved.

To determine whether removal of terminal GlcNAc residues has consequences for the structure of AGPs, native crossed electrophoresis was applied. The mobility of AGPs in agarose gels is determined by both the molecular charge and size of the AGP molecules. Crossed electrophoresis results in a pattern of Yariv-precipitated AGPs in which peaks with specific Rf-values can be observed. It is not clear whether the peaks contain single molecules or aggregates of AGPs that consist of different or similar molecules. Figure 2A shows that immature 21 DAP seed AGPs run as a single broad peak. Incubation of these AGPs with N-acetylhexosaminidase resulted in the appearance of several extra peaks besides the observed broad peak (Fig 2B). Incubation of AGPs with EP3 endochitinases also resulted in some changes (Fig 2D) in comparison to untreated AGPs (Fig 2C), but they were minor changes in comparison to the changes after N-acetylhexosaminidase treatment. The difference between the endochitinase and the N-acetylhexosaminidase treated AGPs might be explained by the fact that endochitinase activity requires at least three consecutive GlcNAc residues, and will always yield AGPs that still contain terminal single or double GlcNAc residues. Such

A	Compound	Preincubated with	Promoting efficiency
	no additions		1
	17 DAP AGPs	_	38
	17 DAP AGPs	EP3	61
	17 DAP AGPs	HexNAc-ase	83
	17 DAP AGPs	EP3 + HexNAc-ase	81

B	Compound	Promoting efficiency
	17 DAP AGPs BaOH treated	36
	17 DAP AGPs +exoA, endoA, endoG	2
	23 DAP AGPs added after 1 day	0.83
	23 DAP AGPs added after 2 days	2
	23 DAP AGPs added after 3 days	1
	Gum Arabic AGPs	3.1
	4 DAP AGPs	2.5
	11 DAP AGPs	4.2
	23 DAP AGPs	18
	17 DAP endosperm AGPs	153

Table 3. A. Effect of EP3 endochitinase treatment and ß-N-acetylhexosaminidase (HexNAc-ase) treatment of AGPs on promoting the efficiency of somatic embryogenesis from protoplasts. **B.** Effects of BaOH treatment of AGPs, endogalactosidase, endo and exoarabinofuranosidase (endoG, endoA and exoA respectively) treatment of AGPs, AGPs added at different time points after protoplast preparation, and of AGPs derived from seeds at different stages of development on promoting the efficiency of somatic embryogenesis from protoplasts. Due to limited availability of AGPs the results in this table were obtained in single experiments, which did not allow statistical analysis. The promoting efficiency is expressed as the number of somatic embryos formed in dishes containing 100,000 protoplasts with AGPs, divided by the number of somatic embryos formed in dishes without AGPs. In all experiments the concentration of AGPs used was 15 µg / ml. Enzyme treated AGPs were re-isolated after incubation with 3×10^{-9} M EP3 endochitinase or 0.1 M HexNAc-ase.

terminal GlcNAc residues can still be cleaved off by N-acetylhexosaminidase. It is not clear what the observed additional peaks with altered mobility represent. A possible explanation might be that spontaneous self-assembly of AGPs into larger complexes requires GlcNAc mediated interactions. Such self-assembly would then only be partionally affected by endochitinase activity.

Somatic embryo promoting activity of AGPs is dependent on the carbohydrate part of the molecules

To determine whether both the protein and the carbohydrate part of AGPs are important for full biological activity, barium hydroxide hydrolysis was employed. This cleaves carbohydrate-polypeptide linkages (Lamport and Miller, 1971) and releases O-glycans. Barium hydroxide

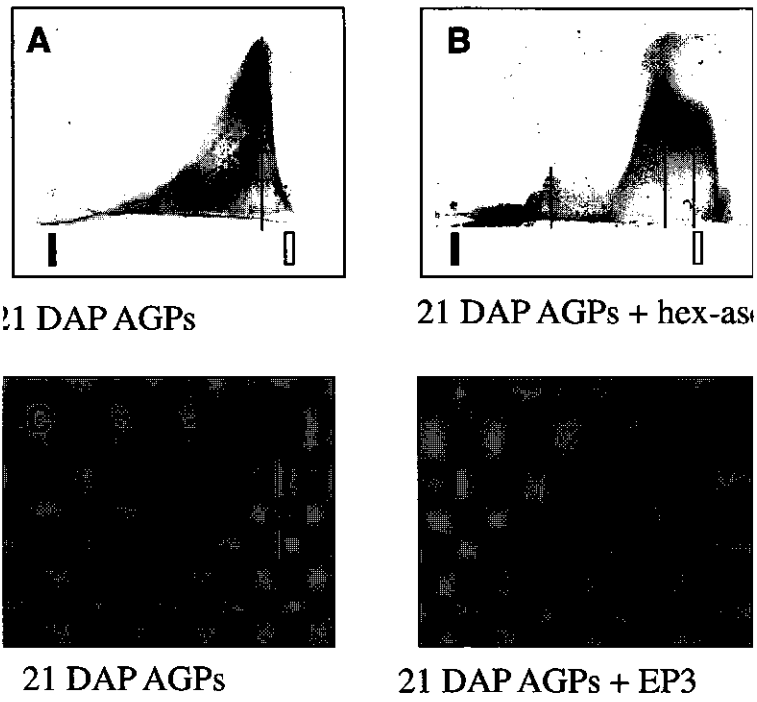

Figure 2. Crossed electrophoresis pattern of enzymatically treated AGPs isolated from carrot seeds 21 DAP.
Samples of 15 µg (EP3 endochitinase treated) or 10 µg (ß-N-acetylhexosaminidase treated) AGPs were subjected to electrophoresis directly (**A**, **C**), or after incubation with ß-N-acetylhexosaminidase (**B**) or EP3 endochitinases (**D**).
(| indicates where the AGPs have been loaded on the gel; [] indicates the position of bromophenol blue after the first electrophoresis, vertical lines indicate the position of peaks in the crossed electrophoresis pattern).

hydrolysis of developing seed AGPs prior to addition to carrot protoplasts did not affect the relative number of somatic embryos that are formed when compared to untreated AGPs (Table 3B). This result suggests that the embryo promoting effect of AGPs is present in its carbohydrate constituents. It also shows that the embryo promoting effect is unlikely to be due to proteins possibly coprecipitated with Yariv, given the conditions of pH > 10 for 6 hrs at 100°C in the hydrolysis. Treatment of AGPs with fungal endogalactosidase and exo- and endoarabinofuranosidase resulted in degradation of AGPs into several discrete oligosaccharides (chapter 4). The resulting mixture was not able to increase the number of embryos from protoplasts (Table 3B), suggesting that intact arabinogalactan carbohydrate moieties are essential for the somatic embryo promoting activity of AGPs.

In all assays described sofar, immature seed AGPs were added shortly after protoplast preparation and before cell wall regeneration was complete. To determine when AGPs were most effective, AGPs were added 1, 2 or 3 days after protoplast isolation. As a result, the relative numbers of somatic embryos formed were the same as in untreated controls (Table 3B). Within 24 hrs protoplasts have synthesised a new cell wall, as visualized by calcofluor white staining (data not shown). This

suggests that the AGPs are most effective before cell wall regeneration is complete. These results are also in line with the reported lack of embryo promoting activity on intact suspension cells (Table 1).
To get some insight into the specificity of the effects of AGPs on protoplast-derived somatic embryos, AGPs were isolated from gum arabic and from immature carrot seeds at 4, 11, 17 and 23 days after pollination (DAP). Table 3 shows that gum arabic AGPs were not active, demonstrating that there is a certain specificity in AGP-mediated somatic embryo formation. The numbers of embryos that are formed clearly show that immature seed AGPs become effective in somatic embryogenesis after about 11 DAP. In carrot seeds of this age the zygotic embryos are in the globular stage and are completely surrounded by cellularised endosperm (Gray et al., 1984). Yariv staining of hand-sectioned carrot seeds 21 DAP has shown that the cellularised endosperm contains AGPs. Most AGPs were found in the extracellular matrix of endosperm cells and in the cavity in which the embryo is located (chapter 4). AGPs isolated from manually dissected endosperms of seeds at 17 DAP were found to be highly active (Table 3B). This suggests that immature seed AGPs that are active in somatic embryogenesis are specifically located in the developing endosperm.

Early effects of immature seed AGPs on protoplasts

Sofar we have only addressed the effects of AGPs on embryogenesis in quantitative terms, which offers little insight into the possible mechanisms underlying these effects. Therefore, we employed cell tracking of protoplast-derived cells to determine whether there was a morphologically recognizable effect of the added AGPs. Cell tracking involves analysis of daily repeated video recordings made of the same area of a dish containing immobilised carrot protoplasts with and without added AGPs. Protoplasts were cultured both in the presence and in the absence of 2,4-D to compare the effect of this synthetic plant growth regulator with the effect of AGPs.
When following the development of a population of protoplasts by cell tracking, four different possible developmental patterns can be distinguished (Guzzo et al., in prep.). From an initially fairly uniform population of protoplast-derived cells, cells can 1) divide without expanding to much more then their original size, resulting in small compact clusters, 2) divide and simultaneously enlarge, resulting in loosely attached clusters of vacuolated cells, 3) enlarge, but not divide, resulting in large vacuolated cells, or 4) neither divide, nor enlarge and remain unchanged in morphology during the period of analysis. In Figure 3 examples of these four patterns are shown. Somatic embryos only derive from cells that follow pattern 1 (Guzzo et al., in prep.). In Table 4 the results of the cell tracking experiments on protoplasts, obtained from an embryogenic suspension culture, are summarized. Samples of protoplasts were immobilised and cultured with and without 2,4-D and / or 23 DAP AGPs. The results are presented as a percentage of the total number of protoplasts that follow either of the four possible developmental patterns as shown in Figure 3. For each treatment more than 600 individual protoplasts were recorded. The particular pattern followed was determined

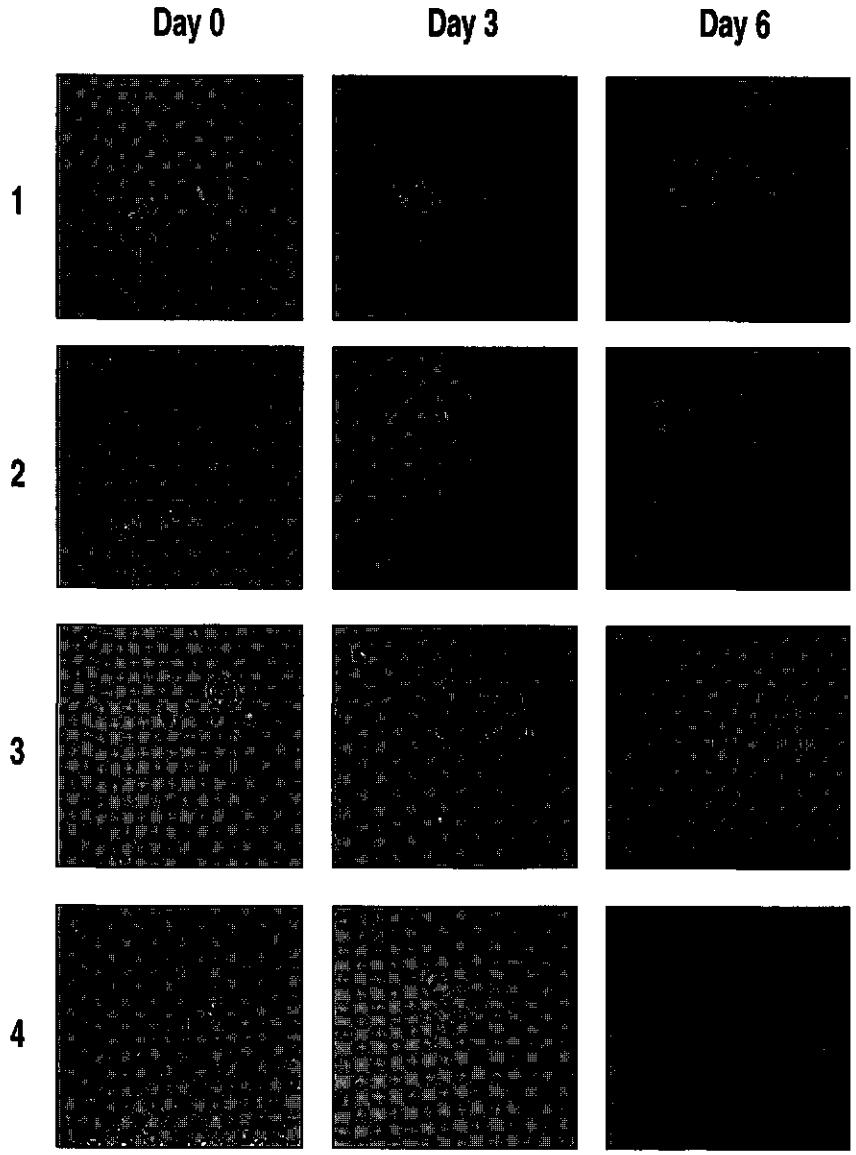

Figure 3. Development of immobilised carrot protoplasts.
Development of individual carrot protoplasts was analysed by means of video cell tracking. After comparision of images as obtained after 0, 3 and 6 days, four patterns of development were identified **1**) cells that only divide and do not enlarge **2**) cells that only enlarge **3**) cells that both enlarge and divide, and **4**) cells that do not divide, nor enlarge. Cells representing either of the developmental patterns are indicated by arrows.

from the video tapes at day 6 to ensure that all cells that could have responded, had indeed done so. Without any addition about three quarters of the protoplasts remained unchanged (pattern 4). About five percent of the protoplasts followed pattern 1 (division without elongation) and the same percentage of protoplasts was found to follow pattern 3 (elongation). The remainder, about 13%, followed pattern 2 (division and elongation). The addition of 2,4-D resulted in a significant decrease in the number of cells following pattern 4. These now responding cells entered in either of the other patterns of development with a slight preferential increase for cells that elongate. Addition of AGPs had an effect comparable to that of 2,4-D, since the same decrease in cells following pattern 4 was seen, resulting in a redistribution of cells over the other three patterns of development. This decrease of cells following pattern 4 as a result of addition of AGPs was statistically relevant (P = 0.04). A subtle difference was noted in that after addition of AGPs more cells entered into pattern 1, suggesting that AGPs are more effective in triggering cells into a rapid cell division mode, than they are in promoting cell elongation. The increase in the number of cells following pattern 1 was not statistically significant (P = 0.06). Addition of 2,4-D and AGPs gave a synergistic effect on the reduction of cells following pattern 4. In following the fate of the cells that had shifted to the other three patterns of development,synergism between 2,4-D and AGPs could only be observed for cells entering into the rapid division mode without elongation (pattern 1). No synergism was seen for cells following the developmental patterns 2 or 3. The increase of cells in pattern 1 as a result of addition of AGPs and 2,4-D, in comparison to addition of 2,4-D only was statistically relevant (P = 0.04).

Compound	developmental patterns							
	1	(P)	**2**	(P)	**3**	(P)	**4**	(P)
no additions	5.0		13.1		5.0		76.9	
2,4-D	7.8	0.12	17.1	0.04	10.4	0.04	64.7	0.04
23 DAP AGPs	10.6	0.06	15.8	0.14	8.8	0.11	64.8	0.04
2,4-D + 23 DAP AGPs	14.9	0.04 a	17.5	0.91 a	10.3	0.99 a	57.4	0.16 a

Table 4. The effect of AGPs on the development of individual carrot protoplasts as analysed by video cell tracking. The development of 1600 protoplasts of the embryogenic carrot cell line Bj and 800 protoplasts from the embryogenic carrot cell line Bg was analysed. The protoplasts were categorised into 4 different developmental pathways: 1. dividing protoplasts; **2**. dividing and enlarging protoplasts; **3**. enlarging protoplasts; **4**. protoplasts that do not divide nor enlarge. Protoplasts were cultured in the presence or absence of 2,4-D with or without AGPs that were isolated from immature carrot seeds, harvested 23 DAP. Protoplasts that follow either of the 4 developmental pathways are represented as a percentage of the total number of analysed protoplasts of one treatment. The overall effect of addition of 2,4-D or 23 DAP AGPs was assessed by means of F tests using the absolute numbers of embryos formed after addition of 2,4-D or AGPs, and the absolute number of embryos formed in controls without any additions.

[a] The overall effect of addition of AGPs in cultures containing 2,4-D was compared with control cultures containing solely 2,4-D. (P values less than 0.05 were regarded as indicative for significant differences).

We conclude that the biological effect of AGPs on protoplasts is to activate cells to enter into division and to a lesser extent to promote elongation. When AGPs are added together with 2,4-D they act synergistically on promoting the number of cells that enter into a pattern of division without enlargement (pattern 1). However, the increase in cells in pattern 1 is only about 2-3 fold, while 23 DAP AGPs alone can increase embryogenesis 25 fold. This implies that the observed shifts in developmental patterns in the entire population may not be directly causal to the AGP effect on embryogenesis.

Discussion

In this work we have demonstrated that addition of endochitinases secreted into the conditioned medium of embryogenic carrot suspension cells increases the number of somatic embryos formed from wild type carrot protoplasts. This finding implies that endochitinases have a general role in plant embryogenesis and one that is not restricted to the *ts11* line in which the chitinase was first identified. Secondly we have presented evidence that GlcNAc containing AGPs are likely target molecules for cleavage by endochitinases and that such a cleavage is biologically meaningful and results in "activated" AGPs. Thus, endochitinase-activated AGPs appear important molecules for conferring embryogenic capacity to protoplasts. After cell wall regeneration, or in the presence of intact cell walls the effect of both the chitinases and AGPs was not apparent, suggesting a requirement for chitinases and AGPs very early in embryogenesis. Thirdly we have shown that an intact AGP protein core does not seem to be essential for the biological effect of AGPs on embryogenesis, while perturbation of the structure of the arabinogalactan moieties results in loss of embryo-promoting activity of the AGPs. Finally, we have shown that addition of immature seed AGPs to carrot protoplasts triggers cells that are derived from these protoplasts to divide, while retaining a small cell size.

Biological role of AGPs in plant embryogenesis

AGPs are ubiquitous plant proteins. They are present in plasma membranes and cell walls, and are found in vast amounts as secreted molecules in conditioned media of suspension cultures (Nothnagel, 1997). Several approaches have been used to shed light on the biological role of AGPs. One of these was by the use of specific monoclonal antibodies. One of the antibodies that recognizes AGPs, JIM4, was found to label two segments of the vascular cylinder in carrot seedling roots (Knox et al., 1989). In carrot suspension cultures JIM4 was found to label a few cells at the surface of proembryogenic masses, and during development of somatic embryos JIM4 epitopes remained present at the surface of embryos. In the torpedo stage the epitope appeared in certain cells of the provascular tissue (Stacey

et al., 1990). AGP epitopes that are recognized by monoclonal antibodies such as JIM4 show a temporal and spatial localisation pattern that correlates with for instance epidermal differentiation and vascular differentiation. Based upon this finding, a function for AGPs in determining cell identity was postulated (Knox et al., 1991; Knox et al., 1989). However, formal experimental evidence that AGPs indeed have a function in cell identity is still lacking. A second approach that is used to study the role of AGPs is based on the binding of Yariv phenylglycosides to AGPs. Applying Yariv to suspension cultured rose cells resulted in an inhibition of culture growth that was based on supression of cell division. Transfer to medium without Yariv resulted in resumed cell division and culture growth (Serpe and Nothnagel, 1994). Also cell expansion can be perturbed by the addition of Yariv reagent because *Arabidopsis* roots grown in the presence of this compound were found to have only one third of the length of roots grown without Yariv. The reduction of length resulted from cells in the elongation zone that were found to be bulbous rather than elongated (Willats and Knox, 1996). A third approach to study the effect of AGPs is the direct addition of these molecules to suspension cultures. The addition of mature carrot seed AGPs to a non-embryogenic cell line has been shown to be capable of reinitiating embryogenic cell formation (Kreuger and Van Holst, 1993). This resulted in the presence of clusters of small cytoplasmic rapidly dividing cells, in line with the opposite effects reported for the addition of Yariv reagent to rose cells (Serpe and Nothnagel, 1994). A positive effect on embryogenic cell formation could also result from the removal of a class of AGPs inhibitory to embryogenic cell formation (Kreuger and Van Holst, 1995). In carrot suspension cultures removal of a subset of non-embryogenic cells resulted in a reduction of embryogenic potential. This reduction could be overcome by the addition of carrot mature seed AGPs, suggesting that in non-fractionated cultures, embryogenesis promoting AGPs are produced by this subset of non-embryogenic cells (Toonen et al., 1997). Part of the promoting effect of AGPs on embryogenic cell formation may therefore reflect a cell-cell communication system in cultured cells. Recently, compounds were isolated from carrot cell conditioned medium, based on the ability to bind to JIM8, a monoclonal antibody that recognizes AGP epitopes. Addition of these JIM8 epitope containing compounds, to a JIM8(-) cell population, that was found to be unable to form somatic embryos, allowed the formation of somatic embryos (McCabe et al., 1997). Therefore, a role for the JIM8 epitope containing AGPs in cell-cell communication during somatic embryogenesis was proposed.

We have shown here that addition of unfractionated AGPs, derived from immature carrot seeds, to carrot protoplasts results in several effects. There is a marked decrease in the percentage of cells that do not divide nor elongate in basal medium, suggesting a role in reactivating "resting" cells. There is a clear and concomittant increase in cell division, which is fully in line with the decrease of cell division as a result of addition of Yariv (Serpe and Nothnagel, 1994). Addition of seed AGPs did not result in a marked increase in the number of cells that elongate. However, the effects of Yariv reagent on preventing elongation were observed using intact seedlings (Willats and Knox, 1996), a system completely different from that used here.

The most important effect of AGPs was that in the absence of the cell wall, addition of AGPs promoted the formation of somatic embryos. Because removal of the cell wall resulted in a much lowered embryogenic potential, this might indicate that particular sets of AGPs are required for cells in order to remain embryogenic. AGP epitopes rapidly reappear on the surface of protoplasts after enzymic digestion of cell surface polysaccharides (Pennell et al., 1989). The accumulation of extracellular polymers, including AGPs, was found to parallel the accumulation of cell wall material on protoplasts (Mock et al., 1990). Addition of high concentrations of immature seed AGPs to carrot protoplasts could not only restore the loss in embryogenic potential, but increased it as compared to intact suspension cells. This effect was dependent upon the source of AGPs used and resided in the carbohydrate moieties of the AGPs. Our results are therefore in line with the restoration of embryogenic potential that was observed after addition of AGPs or JIM8 reactive compounds to fractionated suspension cultures (McCabe et al., 1997; Toonen et al., 1997). However, an important difference is that we employed protoplasts, while all other studies employed intact cells.
Thus, the evidence obtained sofar suggests a role for AGPs in the control of cell division and in changing the embryogenic potential of suspension cells. Possibly these two different effects can be attributed to different AGPs that are present in immature carrot seeds.

Possible modes of action of AGPs

Two main possibilities exist concerning the mode of action of AGPs in cell division or in changing embryogenic potential. The first is that the entire molecule performs a structural role, while the second is that the entire AGP or an oligosaccharide derived from it performs a signalling function. The AGPs that can promote somatic embryogenesis almost certainly contain a terminal array of GlcNAc residues, since enzymatic removal of GlcNAc residues by endochitinases or an exohexosaminidase results in a higher biological activity of the AGPs. We also showed that N-acetylhexosaminidase treatment results in the appearance of several new peaks as observed after crossed electrophoresis, suggesting that removal of terminal GlcNAc causes a dissociation of AGP complexes. AGPs tend to self-associate into aggregates as has been observed by electron microscopical imaging of carrot and tobacco AGPs (Baldwin et al., 1993; Cheung et al., 1995). The use of three different specimen preparations and a wide variety of controls supports that on the images as obtained by Baldwin et al. (1993) AGPs are observed in their native state. Aggregation of AGPs might be caused by oxidative cross linking, which was demonstrated for sugar beet plasma membrane AGPs (Kjellbom et al., 1997). AGPs are proteoglycans and an indication that proteoglycan-to-proteoglycan binding can be based on self-recognition comes from a study on cell surface proteoglycans of marine sponges (Popescu and Misevic, 1997), showing that proteoglycans that were isolated from three different species only showed homophilic recognition. The binding of these proteoglycans required a physiological concentration of Ca^{2+}. Baldwin et al. (1993) have presented indications that the

interaction between AGPs and pectins might be Ca^{2+} dependent. This might indicate that also the binding of AGPs to pectins is based on specific recognition. The observed aggregation of AGPs in large complexes might also be based on recognition of homologous or heterologous AGPs. Whether the observed increase in somatic embryo promoting activity is due to the observed dissociation of AGP complexes is not known.

Apart from the structural role for AGPs as described above, they also have been proposed to function as locked up signalling molecules (Bacic et al., 1988). We have shown that AGPs can be activated after chitinase treatment indicating that AGPs themselves are signalling molecules rather than small molecules derived from AGPs. EP3 endochitinases can partially restore the embryogenic potential in wild type suspension cell protoplasts. It is likely, but unproven, that this effect is mediated through endogeneous AGPs present, in a similar way as outlined above. Alternatively it could be that the interaction between endochitinase and GlcNAc containing AGPs as putative substrates results in the release of a chitin-like signalling molecule, representing an oligosaccharide-based way of signalling between cells. The effects of AGP addition could be observed at concentrations that did not exceed nanomolar ranges, which seems to be in line with a signalling function for AGPs. Such an oligosaccharide-based way of signalling has been shown to be important for the elicitation of plant defence responces, since structural components of the cell walls of pathogenic fungi, like chitin oligosaccharides and ß-glucan are released by plant synthesised chitinases and glucanases and enhance the plant's defence response. In addition to this the pathogens secrete enzymes that promote the release of plant cell wall polysaccharide fragments like oligogalacturonides that trigger plant defence responces (Coté and Hahn, 1994; Sharp et al., 1984).

Our experiments show that the chitinase-mediated "activation" of AGPs may also produce a small dialyzable compound that could represent such a small oligosaccharide signal molecule. Fractionation of chitinase treated and untreated immature seed AGPs should shed more light on the exact nature of the active component and on the process of chitinase-mediated activation. This is also essential to be able to attribute the observed effects of AGPs on cell division and formation to the same ot to different molecules.

A role for AGPs in zygotic embryogenesis?

The AGP-mediated promotion of somatic embryogenesis might also provide more insight in the processes that occur during zygotic embryogenesis. Especially since the AGPs that were shown to be very effective in the protoplast bioassay were derived from carrot endosperm, while the EP3 endochitinases that can activate the immature seed AGPs are also present in the endosperm, though they are produced by the maternal integuments (chapter 2). This coincidence in localisation suggests that EP3 endochitinases may provide a way of signalling between maternal tissues, endosperm and embryo. The result of this signalling most likely resides in the characteristics and the function of

AGPs. Therefore, changing AGPs by means of hydrolytic enzymes like EP3 might affect plant development by changing the identity of plant cells or by generating signal molecules.
We suggest that the EP3 endochitinase-mediated release of oligosaccharides from endosperm AGPs, or alternatively, the dissociation of AGP complexes in the endosperm might have an effect on the cells of the developing embryo, that are in the immediate vicinity of the endosperm. An intriguing question that remains to be solved is the difference in timing, since "active" AGPs and EP3 endochitinases are present around globular stage zygotic embryos, while in the protoplast assay they promote somatic embryogenesis mainly by affecting single protoplasts. Regarding the effect of AGPs on the formation of somatic embryos we propose that the biological effect of (activated) GlcNAc containing AGPs is to maintain the "embryo identity" of both the somatic and zygotic embryo.

Materials and methods

Plant material plant and cell culture

Carrot (*Daucus carota* L. cv. Trophy) suspension cultures were initiated and maintained as described before (De Vries et al., 1988). Protoplasts were obtained using a 3 days old suspension culture. The cells of the suspension culture were pelleted and overnight incubated in 1% macerozyme (Yakult Biochemicals Co. Ltd., Tokyo, Japan), 2% cellulase (Yakult Biochemicals Co. Ltd., Tokyo, Japan), 50 mM citrate-HAc pH 4.8. Protoplasts were sieved through a 50 µm nylon mesh and washed three times in 100 mM $CaCl_2$; 0.3 M mannitol. After 2 hrs the protoplasts were washed once more and transferred to B5 medium to initiate somatic embryogenesis.
Arabidopsis thaliana L. (cv. Landsberg erecta) immature siliques were used for the isolation of AGPs.

Barium hydroxide hydrolysis

Aliquots of 500 µg AGPs were incubated in 1 ml 0.1 M Barium hydroxide for 6 hrs at 100^0C. The hydrolysate was neutralised by adding 1 N H_2SO_4 until the pH was stable at 7.0. After 15 min centrifugation at maximum speed the precipitated $BaSO_4$ was omitted and the supernatant containing the hydrolysed AGPs was recovered. Hydrolysed AGPs were used in the bioassay in concentrations that were based on the amout of AGPs from which the hydrolysate was derived. Controls in which AGPs were omitted were treated in the same way.

Bioassay

AGPs were isolated as decribed by Kreuger and van Holst (1993) from carrot cell suspension cultures 7 days after subculturing of 2 ml packed cell volume in 50 ml B5 medium with or without 2 μM 2,4-D, or from immature carrot seeds. AGPs were pectinase treated as described before (chapter 4). EP3 endochitinases and the *Arabidopsis* homologue AtEP3 (Passarinho et al., to be published) were isolated as described before (Kragh et al., 1996), or produced in the Baculovirus expression system (chapter 3).

Aliquots of 30 μg of AGPs and or 400 ng EP3 were added to 100,000 freshly isolated carrot protoplasts in 2 ml B5 medium containing 0.3 M mannitol. LCOs were added (De Jong et al., 1993) to the same amount of protoplasts. Alternatively 0.1 units N-acetylhexosaminidase (Oxford Glycosystems, Oxford, UK) was used. Enzyme treatment of AGPs was performed by incubation of 100 μg AGP and 200 ng EP3 and or 0.1 units N-acetylhexosaminidase in 1 ml 20 mM citrate buffer pH 5.5 for 16 hrs, followed by a reisolation of AGPs. In the controls the enzymes were replaced by water. Aliquots of 30 μg of reisolated AGPs were added to 100,000 protoplast in 2 ml medium. Statistical analysis was done by means of F-tests on the average of the mean. The overall effect of a treatment was regarded as significantly different when calculated P values ≤ 0.05.

Crossed electrophoresis

Aliquots of 15 μg AGPs, isolated from carrot seeds 21 DAP, were incubated for 16 hrs with 0.05 units N-acetylhexosaminidase (Oxford Glycosystems, Oxford, UK) or with 500 ng EP3 in 20 mM citrate buffer pH 5.5.Crossed electrophoresis was performed according to Van Holst and Clarke (1986).

Cell tracking

Immobilisation of protoplasts of the cell lines Bj and Bg, and subsequent video cell tracking was performed as described before for single cells (Toonen and De Vries, 1997), with the difference that the medium used contained 0.3 M mannitol. 2,4-D was added to the phytagel top layer to give a final concentration of 2 μM. AGPs in 1 ml B5 medium were poured on top of the phytagel layers to give a final concentration of 13 μg / ml. Statistical analysis was done by using the SAS System based upon a generalised linear model (Aitkin et al., 1991). The overall effect of treatment was assessed by means of F tests and significant differences expressed with P values less than 0.05.

Chapter 6

Concluding remarks

The research described in this thesis addressed the question what the role of endochitinases is during somatic embryogenesis. This required the identification of plant-produced compounds that can be cleaved by endochitinases. We have shown that certain arabinogalactan proteins (AGPs) contain endochitinase cleavage sites. In addition, we found that both EP3 endochitinases and AGPs that are present in immature carrot seeds, or are secreted in the medium of suspension cultured cells can promote the formation of protoplast-derived somatic embryos. In this chapter the role of N-acetylglucosamine (GlcNAc) containing molecules in plant and animal development will be discussed. In addition, their possible relation with GlcNAc containing AGP species in plant embryogenesis will be summarized.

Chitin and plant development

Chitin consists of ß-(1-4) linked GlcNAc residues that form long, straight, insoluble polymers. Chitin is found in the exoskeletons of insects and crustacea and is one of the most abundant biopolymers on earth. In *Drosophila melanogaster* it was found that chitin not only occurs as polysaccharides, but can also occur as a polysaccharide chain attached to proteins, most probably via O-glycosidic bonds (Kramerov et al., 1986). These glycoproteins were shown to be completely composed of, or largely enriched with, GlcNAc residues that are partially O-sulfated. Whether these "chitin-proteins" play a structural role, or are involved in morphogenesis was not determined. Indications that molecules containing chitin fragments not only function in protective exoskeletons come from several reports that were published during the last decade. These reports describe molecules that consist of, or contain short arrays of GlcNAc residues and appear to function primarily in developmental processes. One family of such molecules consists of Nod factors that were identified as molecules containing a chitin fragment with an N-linked fatty acid moiety (Lerouge et al., 1990). These Nod factors are secreted by *Rhizobia* bacteria as signalling molecules that can induce nodule organogenesis in the cortex of the roots of leguminous plants. Regarding their chemical composition, Nod factors are referred to as lipochitooligosaccharides (LCOs). The external addition of LCOs promotes cell division in tobacco protoplasts. Interestingly this effect was also observed when instead of LCOs chitin tetraoses were used (Röhrig et al., 1995). This might point towards a role for chitin fragments in cell division, which is supported by the recent observation that chitin fragments can trigger cell division when they are introduced in root cells by means of ballistic microtargetting (Schlaman et al., 1997).
The intracellular mode of action of such chitin fragments remains unclear. Identification of possible endogenous intracellular compounds that contain GlcNAc oligosaccharides might provide clues on the mode of regulation of cell division as influenced by chitin fragments. The observation that wheat germ agglutinin (WGA), a lectin that binds to GlcNAc and sialic acids, blocks nucleo-cytoplasmic transport (Finlay et al., 1987) has led to the identification of proteins of the nuclear pore complex that contain terminal oligosaccharides of more than five GlcNAc residues (Heese-Peck et al., 1995).

Therefore we can speculate that chitin fragments may play a role in the assembly of nuclear pore complexes, or in transport of proteins through these nuclear pores. Therefore, chitin fragments may influence cell division indirectly by interfering with transport, over the nuclear matrix, of other compounds that interact directly with cell cycle regulators.

Apart from promoting cell division, chitin fragments may also influence plant development in another way. An indication for a role of chitin-containing molecules in plant embryo development comes from the work of De Jong et al. (1993), who have shown that in the carrot cell variant *ts11*, that is blocked in the formation of somatic embryos at restrictive temperatures, the addition of bacterial LCOs can rescue somatic embryogenesis. Indications that chitin oligosaccharides, or compounds that contain arrays of GlcNAc are also important during animal embryogenesis can be deduced from the identification of the DG42 protein. The DG42 gene is transiently expressed between midblastula and neurulation stage in *Xenopus*, zebrafish and mouse embryogenesis, and found to be homologous to NodC, the *Rhizobium* chitin oligosaccharide synthase (Bulawa and Wasco, 1991; Semino et al., 1996). Microinjection of antiserum raised against the *Xenopus* DG42 protein into fertilized eggs of zebrafish, leads to severe defects in trunk and tail development. The same effect was obtained after injection of the *Rhizobium* NodZ enzyme, that specifically fucosylates oligosaccharides that contain at least two GlcNAc residues at the reducing terminus (Bakkers et al., 1997).

In conclusion, while high molecular weight polymers of GlcNAc serve as structural components in the exoskeleton of insects and crustacea, compounds containing short arrays of GlcNAc are able to promote cell division and / or execute as yet unknown functions during early embryogenesis in animals and plants.

Arabinogalactan proteins and development

Arabinogalactan proteins are plant glycoproteins that contain a protein core, accounting for at most 10% of the molecular weight. Attached to the protein core are large oligosaccharides that consist of chains of ß-(1-3)-linked D-galactose residues with side chains of variable sugar components (Clarke et al., 1979). The carbohydrate content consists mainly of arabinose and galactose residues. Although the composition of AGPs is highly complex and no single AGP has been characterized to the extent that its complete chemical structure is known, studies on the structure of AGPs have revealed a large body of information on the core polypeptides or the carbohydrate moieties (for review see Nothnagel, 1997). The introduction of monoclonal antibodies that recognize specific AGP epitopes has led to the observation that the presence of AGP epitopes is temporally regulated in a tissue-specific manner (Knox et al., 1991; Knox et al., 1989). The structural complexity of AGPs and the developmentally regulated expression of AGP epitopes are the main arguments that have led to the general hypothesis that AGPs function in plant development. A role for AGPs was suggested in plant reproductive development (Pennell et al., 1991), pattern formation in roots (Knox et al., 1991; Knox et al., 1989)

or maize coleoptiles (Schindler et al., 1995), and somatic embryogenesis (Pennell et al., 1992). Also several other functions have been suggested. Based on a variety of experimental systems AGPs have been implicated in cell division (Serpe and Nothnagel, 1994), cell expansion (Willats and Knox, 1996) and cell death (Schindler et al., 1995). It is possible, but not proven that the effects of AGPs on these basic cellular functions underly their proposed role in plant developmental processes. In Chapter 5 we have presented another example of such a dual effect of AGPs through the addition of immature carrot seed AGPs to carrot protoplasts. This resulted in a reduction of protoplasts that neither divide nor elongate, and an increase in the number of protoplasts that divided without elongation. No clear effect on elongation was observed, so the main effect of AGPs on the entire population of protoplasts in our system appeared to be on the release of the cell cycle arrest. A second effect of AGPs, but now on a minority of the protoplasts, was seen in the form of a substantial increase in the number of protoplast-derived somatic embryos. Because the increase in the number of embryos was about 10-20 fold higher compared to the increase in the number of dividing cells, a direct relation between both effects is unlikely. Thus, as in the other systems described, the true cellular function of AGPs remains to be determined. Based on the numerical discrepancy observed between the effects of AGPs on promoting cell division and somatic embryogenesis, one possibility is that certain AGPs directly influence cell identity.

Arabinogalactan proteins and cell identity

In animal cells, carbohydrates are known to participate in cell-cell recognition. Evidence that cell surface carbohydrates are involved in cell-cell recognition has been obtained in several systems. A compelling example is the development of the nervous system, where the diversity and the selective spatial expression of cell surface carbohydrates seem to guide the formation of neural cicuits (reviewed by Jessell et al., 1990).

The GlcNAc-specific lectin wheat germ agglutinin (WGA) can mask compounds on the muscle cell surface and thereby perturb the behaviour of growing neurites (Iglesias et al., 1996). This suggests that GlcNAc-bearing cell surface molecules have functional roles in the guidance of neurites. Thus, cell surface carbohydrates are involved in recognition of animal cells and can help to determine the identity of a cell.

The temporal and spatial expression of AGP epitopes that are present on the cell surface, and the functions in plant development that have been proposed for AGPs, suggest that in plants the identity of cells or tissues might be reflected by the AGPs present in the cellular matrix (Nothnagel, 1997). If so, the production of AGPs that reflect cellular identity must be correlated to cell differentiation. This hypothesis is supported by the observation that MAC207 epitopes, that are present on a large number of AGPs, are lost from cells involved in sexual reproduction and are absent in early zygotic embryos, where the MAC207 epitope reappears after the embryos reach the heart stage (Pennell et al., 1989).

An almost inverse pattern was found using the JIM8 monoclonal antibody. In oil seed rape, AGPs containing JIM8 epitopes were localised in gametes, some cells in anthers and ovules, and in the early embryo (Pennell et al., 1991). Taken together, the presence of MAC207 and JIM8 epitopes demonstrates that the expression of certain AGP epitopes is tightly connected to flower development and suggests that AGPs might be involved in the regulation of differentiation.

Assuming that AGPs are involved in determining cell identity and differentiation implies that the experimental removal of AGPs from the cell wall influences cellular identity and therefore might be considered as a part of a dedifferentiation process.

Evidence that the presence of plant cell walls can influence cellular differentiation by altering the way in which a plant cell develops, comes from a study on the development of Fucus. In Fucus, after laser ablation of the rhizoid cell of the early embryo, contact between the wall of the rhizoid cell and thallus cells of the early Fucus embryo is required for rhizoid development. In the absence of the rhizoid cell wall, only thallus development occurs (Berger et al., 1994). Thus, cell wall material can change the developmental fate of cells in Fucus embryos. The causative component for this change in developmental fate has not been identified yet. The polar distribution of sulfated polysaccharides in the cell wall of Fucus zygotes suggests that carbohydrates may be involved in the observed change of developmental fate (Shaw and Quatrano, 1996). In chapter 5 we have shown that the removal of the cell wall of suspension cultured carrot cells prior to addition of AGPs is required for the AGP-mediated promotion of somatic embryogenesis. Previously it was shown that one of the effects of cell wall degrading enzymes is to induce the presence of lectin binding sites on the surface of maize protoplasts (Sun et al., 1992). This may indicate that the lectins present on the surface of protoplasts can bind carbohydrates such as those present in AGPs. If AGPs indeed determine cell identity, the addition of immature carrot seed AGPs as presented in chapter 5 might change cell identity via binding of AGPs to lectins present on protoplasts. Based on their binding by Yariv phenylglucosides, that contain glucosyl, galactosyl, xylosyl, cellobiosyl, lactosyl, or maltosyl residues in a ß-anomeric linkage, AGPs were also given the name ß-lectins (Anderson et al., 1977). Thus, lectin binding of AGPs is very well feasible.

The presence of JIM8 epitopes was shown to have a polar localisation in the cell wall of individual carrot suspension cells (McCabe et al., 1997; Pennell et al., 1992). The function of this JIM8 reactive material is unknown. Previously it was suggested that cells containing the JIM8 epitope are an intermediairy cell type in somatic embryogenesis (Pennell et al., 1992). However, following the development of living cells decorated with the JIM8 antibody by cell tracking, revealed that the JIM8 cell wall epitope does not coincide with the ability of single suspension cells to form somatic embryos (Toonen et al., 1996). The release of compounds containing the JIM8 epitope from JIM8-labeled cells was suggested to function as a soluble signal that may activate non-JIM8 decorated cells to enter into the embryogenic pathway. The removal of the cell population carrying JIM8 epitopes resulted in a decrease in the embryogenic potential in the remaining cell culture (McCabe et al., 1997). Addition of the JIM8 epitope containing soluble signals might compensate for the lack of this cell population. In

chapter 5 we have shown that the removal of the cell wall of suspension cultured cells also results in a decreased embryogenic potential, which can be restored by the addition of AGPs isolated from medium conditioned by embryogenic suspension cells.

Function of chitinases

While conventional models assume that plant chitinases are involved in protection against pathogens, also several other functions have been proposed for these enzymes. We will not discuss these conventional models in detail (because they have been extensively discussed in a review by Collinge et al. 1993) and instead will discuss some of the more recently proposed functions.

LCOs are shown to be substrates for plant endochitinases in vitro, and an interesting model was proposed in which plant chitinases act to remove excess LCOs in order to prevent continued activation of cell division in target root tissues (Staehelin et al., 1994; Vasse et al., 1993). Such a model is attractive because in principle it explains why specific chitinases in susceptible roots are induced by LCOs.

Recently it was observed that chitin fragments can trigger cell division when they are introduced in root cells by means of ballistic microtargetting (Schlaman et al., 1997). This suggests that the chitin moiety, rather than the fatty acid, is the active component in initiation of cell division. The external addition of chitin tetraose increases cell division in tobacco protoplasts (Röhrig et al., 1995), representing evidence that the response to chitin fragments is a general response of plant cells. Clearly, control of appropriate levels of chitin fragments is then important to prevent unscheduled cell division. Chitinases are likely candidates for reducing the level of active chitin fragments.

In chapter 2 we have shown that EP3 endochitinases are transiently expressed in the maternal integuments surrounding zygotic embryos, and in the middle of the endosperm in mature seeds. It is not known whether chitin fragments occur in the endosperm and in the integuments of immature seeds. As a consequence, we do not know whether seed chitinases such as EP3 function in the control of cell division through modulation of the level of active chitin fragments. The occurrence of endochitinase cleavage sites in immature seed AGPs, as we have shown in chapter 4, suggested that GlcNAc-containing AGPs could be the source of the sofar elusive chitin fragments or LCO-like molecules in plants. As shown in chapter 5, the AGP molecules themselves are also capable of initiating cell division in carrot protoplasts. While not conclusive, these results do not provide evidence that small GlcNAc-containing oligosaccharides are a principal element in inactivating or sustaining cell division in developing embryos. It is clear from the results presented in chapter 5, that the seed AGPs, especially after activation by chitinases, are most active in promoting somatic embryogenesis. Whether this second effect operates solely through initiation of division is unlikely. Thus, we suggest that seed EP3 chitinases control the presence of certain active AGP species with a sofar unknown function in plant embryogenesis. In line with this hypothesis is the occurrence of

AGPs with GlcNAc residues in the medium of embryogenic carrot suspension cultures and not in the medium conditioned by non-embryogenic, yet rapidly dividing suspension cells (chapter 4).

Processing of AGPs by hydrolytic enzymes as a means to control their activity

Several recent studies suggest that AGPs or AGP fragments can function as signal molecules (McCabe et al., 1997; Toonen et al., 1997). Therefore, carbohydrate hydrolysing enzymes may in principle regulate the activity of AGPs. However, there is no evidence, except the experiments reported in this thesis, that AGP modification by hydrolytic activity is biologically meaningful. The "activation" of carrot immature seed AGPs by means of EP3 endochitinases or N-acetylhexosaminidases, described in chapter 5, is the first indication of the importance of hydrolytic processing of AGPs.

A better understanding of the biosynthesis of AGPs might elucidate the relationship between their core polypeptides and the carbohydrate moieties attached to it. It is for instance not clear whether AGP core polypeptides are always glycosylated in the same manner. Ectopic expression of AGP core polypeptide encoding genes resulted in an underglycosylation of these polypeptides in most tissues (Cheung et al., 1996), indicating that the correct glycosylation of AGPs is a tissue-specific process. Unfortunately, sofar only a single plant polysaccharide synthase, cellulose synthase, has been unequivocally identified (Arioli et al., 1998). This shows the lack in knowledge on the biosynthesis of complex plant polysaccharides such as attached to AGPs.

Apart from the two examples with endochitinase and hexosaminidase reported here, other hydrolases may be involved in activating AGPs as well. This may also be important in generating the soluble signals containing JIM8 epitopes (McCabe et al., 1997). Information on the *in planta* degradation of AGPs is largely lacking. A better understanding of the processing of AGPs and the components of the AGP molecules that are required to carry out their functions is clearly essential to understand their potential biological role as signalling molecules.

The carrot protoplast assay

Using carrot protoplasts for the initiation of somatic embryogenesis, as described in chapter 5, provides a powerful method to study the biological effect of AGPs and has provided evidence on the role of AGPs in somatic embryogenesis. Apart from that, the carrot protoplast assay also allows to study the relationship between AGPs and enzymes that modify this specific class of proteoglycans. Using enzymes that are capable of degrading AGPs in a controlled manner by having a narrow substrate specificity might be very helpful to obtain more information on the glycosylation of AGPs and might eventually lead to the identification of active sites on AGPs, or on oligosaccharides that are

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Samenvatting

In plantenzaden bevinden zich embryo's. Zo'n embryo ontstaat nadat een eicel door pollen bevrucht is. Een andere manier om plantenembryo's te krijgen maakt gebruik van losse plantencellen of klustertjes van zulke cellen die, wanneer ze in een voedingsoplossing gekweekt worden, door toevoegen en vervolgens verwijderen van synthetische plantenhormonen aangezet kunnen worden tot het ontwikkelen van embryo's. Deze embryo's, die niet via de geslachtelijke weg ontstaan, worden somatische embryo's genoemd. Het proces van somatische embryogenese biedt een modelsysteem om het ontstaan en de ontwikkeling van embryo's te bestuderen en kan mogelijk meer inzicht geven over de processen die in planten leiden tot de vorming van embryo's.
Peen (*Daucus carota* L.) is een plantensoort die veel gebruikt wordt voor somatische embryogenese. Door veranderingen aan te brengen in de erfelijke informatie van peencellen is een cellijn verkregen waarin bij hogere temperaturen slechts weinig embryo's ontstaan. De weinige embryo's die gevormd worden ontwikkelen zich niet tot zaailingen, maar hun ontwikkeling stopt al in een vroeg stadium. Het toevoegen van een endochitinase, een enzym dat chitine af kan breken, kan er voor zorgen dat er meer embryo's ontstaan, bovendien kunnen deze zich dan ook ontwikkelen tot zaailingen. Dit endochitinase, dat EP3 genoemd wordt, wordt gemaakt door de peencellen en kan gezuiverd worden uit de voedingsoplossing waarin deze cellen gekweekt worden. Aangezien er in planten nooit chitine of moleculen die chitinefragmenten bevatten zijn aangetoond, was het onduidelijk wat de exacte rol van dit enzym kan zijn. Het toevoegen van lipochitooligosacchariden (LCOs) heeft hetzelfde effect als EP3 endochitinases. Deze LCOs worden gemaakt door bacteriën en fungeren als signaalstoffen tijdens de communicatie tussen deze bacteriën en de planten waarmee ze in symbiose leven. Dit leidde tot de veronderstelling dat in planten chitinases nodig zijn voor het vrijmaken van signaalstoffen die een funktie zouden kunnen hebben tijdens de embryogenese.

In hoofdstuk 1 wordt beschreven van welke signaalstoffen er bekend is dat ze de embryogenese van planten kunnen beïnvloeden. Naast de plantenhormonen waarvan reeds lange tijd bekend is dat ze dit proces kunnen beïnvloeden wordt ook de rol van chitinases en arabinogalactaneiwitten (AGPs) besproken. AGPs zijn moleculen die maar voor een klein deel uit eiwitten bestaan. De rest van deze moleculen bestaat uit carbohydraten.

In hoofdstuk 2 staan experimenten beschreven waaruit afgeleidt kan worden welke cellen het vermogen hebben om EP3 endochitinases te produceren. Hiervoor zijn zowel cellen die gekweekt waren in een voedingsoplossing, als intakte planten gebruikt. In intakte planten bevinden de cellen waarin we transcriptie van EP3 genen konden ontdekken zich in het moederlijk weefsel rondom het embryo. Ook in suspensiecultures bevinden zulke cellen zich in de nabijheid van embryo's en soms zitten ze vast aan celklustertjes waaraan ook somatische embryos vastzitten. Aangezien de embryo's zelf geen EP3 maken suggereert deze localizatie dat in wild-type peen EP3 endochitinases belangrijk zijn tijdens de embryogenese en een embryo-verzorgende funktie hebben.

In hoofdstuk 3 staat de productie van EP3 in insectencellen beschreven. Hiervoor werden de genen die coderen voor verscheidene EP3 endochitinases van peen en een van de zandraket (*Arabidopsis thaliana* L.) ingebracht in het baculovirusgenoom. Vervolgens werden insektencellen geïnfecteerd met deze recombinante virussen. Dit resulteerde in de produktie van enzymatisch aktieve EP3 endochitinases. Na zuivering van de verschillende EP3 endochitinases die op deze manier geproduceerd waren werd de enzymatische aktiviteit van deze chitinases vergeleken. Hierbij ontdekten we dat al deze EP3 endochitinases naast chitine ook chitosan af kunnen breken. Verder bleek dat in suspensiecultures van cellen van de zandraket EP3 alleen aan te tonen is in een voedingsoplossing waarin zich embryogene cellijnen bevinden en niet in voedingsoplossingen met niet-embryogene cellijnen. Dat impliceert dat de aanwezigheid van EP3 in de voedingsoplossing indicatief is voor het vermogen van zandraket suspensiekulturen om somatische embryo's te kunnen maken.

In hoofdstuk 4 wordt weergegeven dat er N-acetylglucosamine voorkomt in AGPs die uitgescheiden worden door peencellen die gekweekt worden in een suspensiekultuur. N-acetylglucosamine is een monosaccharide en fungeert als de bouwsteen van chitine. Verder wordt aangetoond dat endochitinases carbohydraten af kunnen splitsen van uit onrijpe peenzaden geïsoleerde AGPs. De aanwezigheid van AGPs die gesplitst kunnen worden door endochitinases, in combinatie met de geobserveerde veranderingen in de totale populatie AGPs in onrijpe zaden als gevolg van de ontwikkeling van deze zaden, doen vermoeden dat de AGPs in onrijpe peenzaden onderhevig zijn aan continue processen van aanmaak en afbraak.

Hoofdstuk 5 handelt over het effect dat het toevoegen van EP3 endochitinases of AGPs heeft op de vorming van somatische embryos. Wanneer deze stoffen toegevoegd worden aan protoplasten afkomstig van peencelcultures, ontstaan er in die cultures meer somatische embryo's. Indien AGPs uit onrijpe zaden voorbehandeld worden met chitinases en vervolgens toegevoegd worden aan de protoplasten neemt het aantal embryo's dat ontstaat nog sterker toe. Deze proeven wijzen er op dat AGPs het proces van somatische embryogenese beïnvloeden en misschien reguleren, terwijl chitinases op hun beurt de aktiviteit van deze AGPs kunnen beïnvloeden.

In hoofdstuk 6 wordt de rol die N-acetylglucosamine bevattende moleculen spelen tijdens de ontwikkeling van planten en dieren bediscussiëerd. Hierbij wordt nader ingegaan op AGPs die N-acetylglucosamine bevatten en de functie die deze molekulen toegedacht wordt tijdens de embryogenese van planten.

Curriculum vitae

Arjon van Hengel werd geboren op 5 december 1966 te Barendrecht. In 1986 slaagde hij voor het eindexamen VWO aan het Ulenhof College te Doetinchem. In hetzelfde jaar begon hij met de studie Biologie aan de Rijksuniversiteit te Utrecht. Het doctoraal examen werd afgelegd in januari 1991 en omvatte de afstudeervakken Moleculaire Genetica (Prof. Dr. P.J. Weisbeek) en Botanische Oecologie. Voor dit laatste vak werd een stage uitgevoerd bij de vakgroep Biotechnologie van TNO te Zeist (Dr. H.J. Wichers en Dr. P.G.M. Hesselink). In 1991 begon hij aan zijn militaire dienstplicht die in 1992 uitmondde in een vrijwillige uitzending naar het voormalig Joegoslavië. Daar heeft hij, voornamelijk te Sarajevo, gefunctioneerd als gewondenverzorger / radiotelexist binnen de VN-vredesmacht UNPROFOR. Vanaf september 1993 was hij als onderzoeker in opleiding verbonden aan het laboratorium voor Moleculaire Biologie van de Landbouwuniversiteit Wageningen, alwaar het onderzoek dat in dit proefschrift beschreven staat werd uitgevoerd onder leiding van Dr. S.C. de Vries en Prof. Dr. A van Kammen. Dit onderzoek werd gefinancieerd door de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO). Sinds 1 maart 1998 is hij werkzaam als onderzoeksmedewerker (post-doc) bij de vakgroep Cell Biology van het John Innes Institute te Norwich, U.K.

Nawoord

Op deze plaats wil ik iedereen die bij heeft gedragen aan het tot stand komen van dit proefschrift danken. Een aantal van hen wil ik hier met name noemen.

Zewdie Tadesse, an Msc student who did a lot of work and in that way prevented me of doing more experiments. Mijn co-promotor Sacco de Vries, die mij de mogelijkheid heeft geboden om in 'zijn' embryogenesegroep te kunnen werken en die mij wist te stimuleren door altijd een kritisch oog op mijn werk te houden. Mijn promotor Ab van Kammen voor de vurige wijze waarop hij de goede lijn er bij mij in pobeerde te pompen.

Verder wil ik iedereen bedanken die mij een ontspannende en vriendschappelijke atmosfeer heeft geboden en die, hoewel ze vaak niets met de tot standkoming van dit proefschrift te maken hebben, voor mij toch erg belangrijk zijn geweest.

Ten slotte wil ik opmerken dat na het leven in de oorlogsrealiteit van Sarajevo het beginnen aan een promotieonderzoek een welkome verandering was. Alhoewel deze twee periodes van mijn leven te ver van elkaar afstaan om paralellen te kunnen trekken ben ik er van overtuigd dat ik na mijn ervaringen in Sarajevo weet dat de waarde van een proefschrift en het werk dat er aan verbonden is zeer relatief zijn.