Characterization of an Endochitinase Able to Rescue the Carrot Somatic Embryo Variant ts11

Anke J. de Jong

Characterization of an Endochitinase Able to Rescue the Carrot Somatic Embryo Variant ts11

Proefschrift

ter verkrijging van de graad van

doctor in de landbouw- en milieuwetenschappen,

op gezag van de rector magnificus,

dr. C. M. Karssen,

in het openbaar te verdedigen

op vrijdag 6 mei 1994

des namiddags te vier uur in de Aula

van de Landbouwuniversiteit te Wageningen

Stellingen

1. Plantechitinases zijn betrokken bij de ontwikkeling van de plant en niet alleen bij de afweer van pathogenen.

   Dit proefschrift.

2. In tegenstelling tot wat de titel suggereert, toont Lueng niet aan dat een plantechitinase betrokken is bij de sexuele voortplanting van hogere planten.

   Lueng (1992) Involvement of plant chitinase in sexual reproduction of higher plants. Phytochemistry 31, 1899-1900.

3. Het is onjuist het ECP31 eiwit een 'embryogenic cell protein' te noemen.

   Kioysue et al. (1992) Planta 186, 337-342.

   Kioysue et al. (1991) Plant Physiol. 95, 1077-1083.

4. 'Pathogenesis Related' (PR) eiwitten zijn niet per definitie gerelateerd aan pathogenese.

   Lotan and Fluhr (1989) Plant Cell 1, 881-887.

   Neale et al. (1990) Plant Cell 2, 673-684.

   Ori et al. (1990) EMBO J. 9, 3429-3436.

   Vögeli-Lange et al. (1994) Plant J. 273-278.

5. Perrett et al. gaan geheel voorbij aan het feit dat het al dan niet aantrekkelijk vinden van een persoon ook bepaald wordt door persoonlijkheid.

   Perrett et al. (1994) Nature 368, 239-242.

6. Het feit dat alleen de ammoniak uitstoot en niet de nitraat uitspoeling ten grondslag ligt aan de voorgeschreven bemestingsmethoden illustreert treffend het Nederlandse landbouw-, natuur- en milieubeleid: de natuur noch de boer is erbij gebaat.

7. Eigenbelang is de drijfveer van al het menselijk handelen.

8. De tanende interesse voor religie en de huidige individualisering van de westerse maatschappij versterken de vraag naar sociale hulpverlening.

Stellingen behorende bij het proefschrift: "Characterization of an Endochitinase Able to Rescue the Carrot Somatic Embryo Variant ts11",
te verdedigen door Anke de Jong op 6 mei 1994.

Foar heit en mem

Contents

Outline

Cultured carrot cells secrete proteins, many of which are glycosylated, into the culture medium. A correlation has been found between somatic embryogenesis and the presence or absence of some of these secreted proteins, and evidence has been obtained that one or more secreted glycoproteins are actually essential for somatic embryo formation. The starting point of the experiments described in this thesis was the temperature-sensitive carrot cell line ts11, originally identified on the basis of the temperature-sensitive arrest in the transition of globular to heart stage somatic embryos. The arrest in ts11 embryo development at the nonpermissive temperature could be lifted by addition of medium proteins, secreted by wild-type cells, to the culture medium. The major goal of the study presented in this thesis was to identify the secreted proteins, that were able to rescue the arrested ts11 embryos.

In chapter 1 a brief introduction in zygotic and somatic embryogenesis is presented, followed by an overview of what is currently known about the first essential steps of the development of the zygotic embryo and of the formation of embryogenic cells and somatic embryos in vitro. Based on these studies, it is discussed whether analogous cellular mechanisms control early zygotic embryogenesis and the formation of embryogenic cells in tissue culture.

In chapter 2 the experiments are described that demonstrate that ts11 embryos can be rescued by a single secreted protein of 32 kD. The amino acid sequences of two tryptic peptides of this protein shared homology with several plant endochitinases. Biochemical analysis showed that the 32-kD protein is an acidic endochitinase.

In chapter 3 the results of a search for putative products of endochitinase activity effective in ts11 rescue, are presented. A molecule produced by *Rhizobium*, the *N*-acetylglucosamine-containing lipo-oligosaccharide, NodRlv-V(Ac, C18:4), appeared to be effective in stimulating the formation of ts11 embryos with a similar efficiency as the 32-kD endochitinase.

In chapter 4 evidence is presented that a decreased amount of an otherwise fully functional endochitinase is closely correlated with the window of sensitivity of ts11 cells to addition of the 32-kD endochitinase. Morphological observations suggest that the original ts11 mutation is quite pleiotropic and does not only affect embryogenesis in this line.

In chapter 5 experiments are described to identify a 32-kD endochitinase cDNA. The deduced amino acid sequence of the isolated cDNAs was found to be nearly identical to the amino acid sequences of the 32-kD endochitinase-derived peptides. The EP3 cDNA sequences suggested that the 32-kD endochitinse is a class IV chitinase.

Finally, in chapter 6 the significance of chitinases and lipo-oligosaccharides for plant development in general is discussed.

chapter 1

Early events in higher-plant embryogenesis

INTRODUCTION

Somatic cells of many plant species can be cultured and induced to form embryos that are able to develop into mature plants. This process, termed somatic embryogenesis, was originally described in carrot (*Daucus carota* L.). Although somatic embryos usually lack a suspensor, they are structurally quite similar to zygotic embryos and, in dicots, pass through the same successive developmental stages of globular, heart, torpedo and cotyledonary stage. It is important to note that like their zygotic counterparts, somatic embryos are bipolar structures and have no connection with the parental tissue (Raghavan, 1976). In a somatic embryo both apical meristems, root and shoot meristem respectively, are present and connected by vascular tissue. There is much less clarity on the earlier preglobular stages of somatic embryos, a problem that has created confusing terminology, caused in part by the lack of suitable stage- and cell-specific markers for somatic embryos.

In this chapter, the emphasis will be on a number of selected studies that deal with events in the first and crucial steps of the development of the zygotic embryo and with events in the transition of somatic cells into embryogenic cells. In the first section, early zygotic embryo mutants of *Arabidopsis* will be highlighted. In the second section, essential steps in the formation of embryogenic cells and somatic embryos will be discussed. Based on these studies, the question will be raised which cellular mechanisms control early zygotic embryogenesis and whether analogous mechanisms are involved in the formation of embryogenic cells in tissue culture.

ZYGOTIC EMBRYOGENESIS

The detailed description of both gametogenesis and zygotic embryo development has been the subject of recent studies (Cresti et al., 1992; Jürgens and Mayer, 1992; Mansfield and Briarty, 1990a, 1990b and 1991; Mansfield et al., 1991; Webb and Gunning, 1990) and will only be recapitulated briefly.

During the complex process of plant sexual reproduction, the male gametophytes or pollen grains are formed in the anther. The female gametophyte or embryo sac, is formed in the pistil and consists of seven cells: the egg cell, two synergids, the central cell and three antipodal cells. The polarized egg cell and synergids are positioned at the micropylar pole of the embryo sac. Polarity of the egg cell is evident from the position of the nucleus and most of the cytoplasm at the chalazal side of the cell, while the micropylar part is highly vacuolated. Dual fertilization of the diploid central cell and the haploid egg cell results in the endosperm and the zygote respectively. The first zygotic division is asymmetrical and yields a small apical cell and a large basal cell. The basal cell remains positioned at the micropylar pole of the embryo sac, so the polarity of the unfertilized egg cell appears to predict the future longitudinal axis of the embryo. Development of the *Arabidopsis* embryo from fertilization, through the octant, globular, triangular, heart, torpedo and bent-cotyledon

stages, to the mature dessicated embryo has been subdivided into a sequence of 20 different stages (Jürgens and Mayer, 1992). The various classes of genes expressed during plant embryogenesis have been reviewed elsewhere (Goldberg et al., 1989; Sterk and De Vries, 1992).

Generation of the Embryo Body Pattern

In order to ultimately identify genes that direct the formation of the zygotic embryo, a large collection of embryo mutants is required. This has been established for *Arabidopsis* (Errempalli et al., 1991; Jürgens et al., 1991; Meinke, 1991) and for *Zea* (Clark and Sheridan, 1991).

The morphology and ultrastructure of a number of embryo lethal mutants has been described (Patton and Meinke, 1990). Classification, description of the morphological aberrations, establishment of complementation groups, as well as mapping and cloning of the mutated genes is currently in progress (D.W. Meinke, personal communication).

On the basis of a, most likely saturating, genetic screen for embryo mutations, it was estimated that about 3500 different genes are necessary to complete embryo development (Jürgens et al., 1991). Of these, approximately 40 genes may direct the formation of all body pattern elements in the *Arabidopsis* embryo (Mayer et al., 1991). Because this number is not very much lower than the number of genes essential for embryo pattern formation in *Drosophila* (Schiavone and Racusen, 1991), it may indicate that the apparent morphological simplicity of the plant embryo, when compared to animal embryos, is deceptive.

Jürgens *et al*. (1991) have selected for *Arabidopsis* embryo mutants in which germination and seedling development was still possible, with the aim to obtain mutants that were affected in pattern formation rather than mutants affected in more common cellular mechanisms. Based on the mutant phenotypes obtained, a division of the young embryo along the longitudinal axis into an apical, central and basal region was made (Jürgens et al., 1991; Mayer et al., 1991). A second, radial pattern, superimposed on the apical-basal pattern and consisting of the vascular, ground and epidermal tissues, was proposed. Because in the mutants disturbed in the apical-basal pattern, the three tissue types that make up the radial pattern were all formed, the apical-basal pattern and the radial pattern appear to be established independently. In a separate class of mutants, that exhibited an altered seedling shape rather than a deletion of pattern elements, all pattern elements of the wild-type seedling were still present. The existence of these mutants, in which the shape and spacing of cells in the embryo are abnormal, clearly indicates that changes in cell shape are not essential for the generation of the main pattern elements of the plant embryo.

A very detailed description of the *Arabidopsis* embryo mutant *gnom* was recently completed by Mayer *et al*. (1993). *Gnom* is a terminal pattern mutant (Mayer et al., 1991) and exhibits a highly variable phenotype, that ranges from a ball-shaped seedling with a total

lack of root and cotyledon development to a cone-shaped seedling with a clear apical-basal pattern. In all 24 *gnom* mutant alleles investigated, the entire range of phenotypes was found. By tracing the *gnom* phenotype back to the zygote, it was found that the first cell division of the *gnom* zygote is abnormal. Instead of an asymmetric division, resulting in a small apical cell and an elongated basal cell, a nearly symmetric division occurred in the *gnom* zygote. Not only the position, but also the plane of this division appeared to be abnormal, being prone to a variable degree of deviation from the plane of division in wild-type zygotes, which is always perpendicular to the longitudinal axis. Individuals of the same *gnom* mutant allele exhibited variation, both in the position and plane of the first zygotic division, and this most likely accounts for the phenotypic variation observed for each *gnom* mutant allele at seedling stage. The fact that in none of the *gnom* mutant phenotypes a normal root meristem is formed, may be the result of the observed failure in *gnom* to form the hypophysal cell, the direct progenitor of part of the root meristem initial. Whether this points to a continued requirement of the *gnom* gene in all asymmetric divisions in the early embryo, or is due to the previous failure to perform the first asymmetric division of the zygote, is not clear. In several of the *gnom* mutant alleles, it was observed that the first visible event after fertilization of the egg cell, expansion of the zygote in the direction of the future longitudinal axis of the embryo, was suppressed. Consequently partitioning of the zygote, into a cytoplasm-rich apical part with the nucleus and a vacuolated basal part, might not have taken place. The resulting aberrant first zygotic division in *gnom* could therefore also be the result of a failure of correct directional cell expansion. Thus, it appears that the two most important determinants of plant morphogenesis, the correct position of the plane of cell division and the controlled directional cell expansion (Lloyd, 1991; Lyndon, 1990) are directly affected by the *gnom* gene.

In a mutant of the class of basal pattern mutants, *monopteros* (Mayer et al., 1991), the entire seedling root and hypocotyl is deleted, but in contrast to *gnom*, the cotyledons are formed normally. Based on the *gnom* - *monopteros* double mutant phenotype, it appears that *gnom* is epistatic to *monopteros* (Mayer et al., 1993). Thus, without the prior activity of the product of the *gnom* gene, which apparently has to be active in the unicellular zygote, where it may control correct cell elongation and plane of division, the *monopteros* gene is not able to give rise to the basal part of the seedling. In an experiment similar to that performed by Schiavone and Racusen (1991), who have shown that the apical part of transected *Daucus* somatic embryos were able to regenerate the entire missing root part, cut *gnom* seedlings did not regenerate a root (Mayer et al., 1993). This result suggests an important role for the ability to perform asymmetric cell divisions in (root) regeneration, and it also indicates that the function of the *gnom* gene is not restricted to the embryo.

Cellular Mechanisms in Zygotic Embryogenesis
Maternally acting genes and zygotic genes

The role of the *gnom* gene in the establishment of apical-basal polarity in the *Arabidopsis* embryo marks it as one of the earliest-acting genes sofar described. Genetic analysis has indicated that the *gnom* gene is a zygotically acting gene (Mayer et al., 1991 and 1993). Cytological observations clearly indicate that the unfertilized egg cell is highly polarized (Cresti et al., 1992), suggesting that maternally expressed genes are involved. Although Meinke (1982) found evidence for an overlap between male gametogenesis and a lethal embryo phenotype in some mutants, to date no typical maternal effect mutations that affect pattern formation in the zygotic plant embryo have been reported. Two female-sterile ovule mutants in *Arabidopsis*, *bell* and *sin1*, have recently been described (Robinson-Beers et al., 1992). In these mutants, the formation of the integuments is aberrant. Although megasporogenesis was not affected, a normal mature embryo sac did not develop in these mutants, most likely as a result of the aberrant formation of the integuments. In the *sin1* mutant the defect appeared to be the result of a failure of the integument cells to properly expand after division. This appeared to be a more general effect, in view of reduced internode length observed in the mutant plants. Therefore, putative maternally acting genes that affect oogenesis or direct pattern formation in the early embryo have not been reported in plants. This appears in contrast with the generation of pattern during animal embryogenesis, where, except for mammals, at least one axis and, as for instance in *Drosophila*, two axes of the future embryo are established in the unfertilized egg cell (Gurdon, 1992; St Johnston and Nusslein-Volhard, 1992). Whether this reflects a fundamental difference between plant and animal embryo pattern formation or is due to the technical difficulties in isolating such mutants in plants, is at present unclear. The conventional argument that the possibility of somatic embryogenesis precludes an important role for maternal effect genes in plant embryogenesis, seems to be of limited use in view of the fact that very little is known about the molecular mechanisms that underly the transition of a somatic cell into an embryo-forming cell (see next section).

Embryonic induction and asymmetric cell division

Two mechanisms appear to be universally used in animal embryogenesis to initiate cell differentiation. These are the interaction between an inducing cell or tissue and a responding cell or tissue, and asymmetric cell division (Gurdon, 1992).

No direct evidence is available that cell inductive processes are of importance in the formation of plant gametophytes. A sequential and transient expression of an arabinogalactan-protein (AGP) epitope, recognized by the monoclonal antibody JIM8, was observed in the plasma membranes of diverse parts of both male and female reproductive tissues in *Brassica* . This included sperm cells and the egg cell, the embryo up to early

globular stage, and the suspensor and hypophysal derivatives of later embryo stages (Pennell et al., 1991). AGPs are proteoglycans with poly- and oligosaccharide units covalently attached to a central protein core (Van Holst and Klis, 1981). They are found in plasma membranes, cell walls and in the intercellular spaces of plant tissues (Fincher et al., 1983). In the absence of any clear correlation between a particular differentiation event and the expression of the JIM8 plasma membrane epitope, Pennell *et al.* (1991) speculated that this epitope might actually be a marker for a cell-inductive process in plants.

Asymmetric cell division occurs frequently in plants, and the analysis of the *Arabidopsis gnom* mutant clearly shows that this mechanism is indeed of crucial importance in plant embryogenesis. In animal cells the plane of cell division is controlled by the positioning of the mitotic spindle. This is in turn mediated by the positioning and anchoring of the centrosome by means of microtubules to a site at the anterior cortex. The 'default' plane of division in animal cells is 90° to the previous plane. This is explained by division and subsequent movement of the daughter centrosomes to opposite sides of the nucleus in the case of the 90° default orientation and alternative or additional movements in the case of deviations of this rule (Strome, 1993). The asymmetric first division of the *Caenorhabditis* zygote is essential to form daughter cells that differ in their cytoplasmic determinants, such as maternally produced mRNA (Horvitz and Herskovitz, 1992; Strome, 1993). As a consequence, these two cells follow different developmental fates. Whether the first asymmetric division of the plant zygote serves the same purpose seems quite reasonable to assume. It is not difficult to envisage that variability in the first zygotic division (Mayer et al., 1993) automatically leads to a variability in the amount of cytoplasmic determinants in each of the resulting daughter cells. However, the nature of these determinants remains to be established.

It is of interest to note that there is a certain analogy between the early phenotype of the *Arabidopsis gnom* mutant and the *Caenorhabditis par* mutants. *Par* mutants show defects in spindle orientation that result in aberrant partitioning of cytoplasmic components during the first few divisions. If this analogy is valid, the apical cell of the plant zygote would be equivalent to the AB cell, and the basal cell equivalent to the P1 cell of the two-celled nematode zygote. Phenocopies of *par* mutants could be obtained after treatment with microfilament inhibitors (Hill and Strome, 1990).

Control of cell expansion
It is clear that, during early zygotic embryogenesis, cell expansion is rigorously controlled. After the unidirectional expansion of the zygote and the first asymmetric division, the resulting apical cell does not increase in size. Instead, three cleavage-like divisions occur, and in these, but also in the following tangential divisions that form the protodermal precursor cells, no or hardly any increase in the size of the apical part of the embryo occurs.

At least one *Arabidopsis* embryo mutant, *emb 101-1*, has been described where cell expansion in the embryo is totally out of control, resulting in giant cells that fill the entire seed (D. W. Meinke, personal communication).

Cell lineage

Fate maps from egg to embryo have been constructed by direct observation of the cell lineage in *Caenorhabditis* (Sulton et al., 1983). Laser ablation and cell transplantation experiments have shown the presence of groups of cells with a similar competence (Horvitz and Herskovitz, 1992). From these studies, it appears that embryogenesis continues according to a rigidly fixed program, initially dependent on the regional localization of cytoplasmatic determinants by asymmetric cell division, but also including cell-inductive processes, in the determination of the fate of each individual cell. In plants, no evidence has been found for the existence of such a rigid cell lineage in, for instance, the functioning of the shoot apical meristem. Instead, cell position rather than previous developmental history is considered to be essential for the formation of the somatic tissues (Dawe and Freeling, 1991; Poethig, 1989). Although the fate of cells in the shoot apical meristem of *Arabidopsis* is predictable to a certain degree (Furner and Pumfrey, 1992), Irish and Sussex (1992) suggested the term "probability map" rather than "fate map" to emphasize the absence of a rigid cell lineage. Studies aimed to determine cell lineage in the generation of the embryo body plan in *Zea* and *Gossypium*, also demonstrated a general but not an absolute predictability in the final position of cells in the embryo (Christianson, 1986; Poethig, 1986).

The systematic genetic dissection of plant zygotic embryogenesis has only recently been initiated on a large scale. It is therefore not yet possible to predict whether these studies will reveal cellular mechanisms analogous to those found for animal model systems such as *Drosophila* or *Caenorhabditis*. It is clear from the description of the *Arabidopsis gnom* mutant, that asymmetric cell division is a key process in plant embryogenesis. It is also evident that this is only one of the cellular processes employed. Whether maternally acting genes, cell-inductive processes and cell lineages are also important in plant embryogenesis, remains to be determined. The role of the cell wall in the controlled directional expansion of cells and the formation of the endosperm may represent aspects of plant embryogenesis that do not have a clear counterpart in animal cells.

SOMATIC EMBRYOGENESIS

Somatic or asexual embryogenesis is the process by which somatic cells develop into plants through characteristic morphological stages. For dicots these are the globular, heart and torpedo stages. This process occurs naturally in several species such as *Malaxis*, where somatic embryos form spontanously on the leaf tips (Taylor, 1967), but it can also be

induced by experimental manipulation. Under *in vitro* conditions somatic embryos can either form directly on the surface of an organized tissue such as a leaf or stem segment, from protoplasts or from microspores, or indirectly via an intermediary step of callus or suspension culture (Williams and Maheswaran, 1986).

Here the focus will be on what is not only the most important, but also the least understood part of somatic embryogenesis, the transition of somatic cells into cells, referred to as embryogenic cells, that are capable of forming an embryo. In animals, the ability to form embryos is restricted to a specific set of stem cells, the germ cells. Germ cells are separated from somatic cells at a very early stage of embryogenesis. *Drosophila* eggs contain a class of maternally provided gene products, like *oskar* (Ephrussi and Lehmann, 1992), that function in the formation of germ cells. In plants, where the ability to form embryos is not restricted to the germ cells, somatic embryos are used extensively as convenient alternatives for zygotic embryos in many biochemical and molecular studies.

Description of Embryogenic Cells

Since its first demonstration (Reinert, 1959), somatic embryogenesis has been most widely studied in suspension cultures of *Daucus* (Backs-Hüsemann and Reinert, 1970; Halperin, 1964; Komamine et al., 1990; McWilliam et al., 1974; Van Engelen and De Vries, 1992) and *Medicago* (Dudits et al., 1991). Because a certain amount of confusion exists in the literature on terminology, it may be useful at this point to explain the terms that will subsequently be used in this review. Although suspension cultures are often described as "undifferentiated", a better term is probably "unorganized", because in many cultures subpopulations of cells exist that retain characters found to be associated with specific differentiated cell types *in planta* (Van Engelen and De Vries, 1993). Also, use of the term "embryogenic cell or cells" will be limited to describe only those cells that have completed the transition from a somatic cell or cells to a state where no further externally applied stimuli are necessary to produce the somatic embryo. Following from this, a culture or tissue with a variable number of cells in it that have responded to external stimuli will be called "embryogenic culture or tissue". Depending on the experimental conditions, the ratio of embryogenic to total cells under these conditions can vary between zero and the theoretical maximum of 1. One of the advantages of this terminology is that the difference between direct and indirect somatic embryogenesis is no longer of importance. Direct embryogenesis on explants, or indirect embryogenesis on callus or clusters of embryogenic cells in suspension cultures probably represent different sides of the same coin (Williams and Maheswaran, 1986).

In *Daucus*, the usual strategy to start an embryogenic suspension culture is to expose explants to a high concentration of auxin. After reinitiation of cell division and a period of proliferation of the released explant cells in the presence of auxin, embryogenic cells appear

in the culture (De Vries et al., 1988). These are usually in the form of clusters of small cytoplasmic cells, referred to as proembryogenic masses (Halperin, 1966). It is of importance to note that in almost all embryogenic *Daucus* cultures, the percentage of cells that actually are embryogenic is fairly low, and never amounts to more than about 1-2 % (De Vries et al., 1988). The remainder of the suspension cells are not directly capable of forming somatic embryos.

By using time-lapse photography, Backs-Hüseman and Reinert (1970) have described an elongated single vacuolated suspension cell able to develop into a somatic embryo. Using cell purification techniques, Nomura and Komamine (1985) described a much smaller, almost spherical and cytoplasmic suspension cell, designated a type 1 cell, as being able to develop into a somatic embryo. Because both require preculturing in auxin, neither of these cell types can be called embryogenic under our definition. In the case of the type 1 cell, the derived state 1 cell cluster (Komamine et al., 1990) would be the first to contain embryogenic cells in the pathway leading to somatic embryos from single cells.

Identification of Embryogenic Cells

Several molecular markers have been reported that are able to distinguish between embryogenic and non-embryogenic cell cultures (Pennell et al., 1992; Sterk and De Vries, 1992). One of these is the *Daucus* EP2 gene (Sterk et al., 1991). Employing *in situ* mRNA localization, the EP2 gene was found to be exclusively expressed in peripheral cells of proembryogenic masses and in the protoderm of somatic embryos. In *Daucus* zygotic embryos, EP2 expression was detected in a protoderm-specific fashion as early as a 60-celled globular embryo. The EP2 gene encodes a secreted lipid transfer protein, postulated to function in cutin synthesis (Sterk et al., 1991; E. Meijer and T. Hendriks, manuscript submitted).

Another marker for embryogenic cultures consists of a cell wall epitope in *Daucus* suspension cells, that is recognized by the monoclonal antibody JIM8 (Pennell et al., 1992). The JIM8 epitope has been located on three different plasma-membrane AGPs (Pennell et al., 1991), on secreted AGPs in *Daucus* suspension cultures (Knox et al., 1991) and on an unidentified cell wall molecule present in a sub-population of *Daucus* suspension cells. It is unclear whether the molecule that bears the JIM8 cell wall epitope is related to the plasma membrane AGP epitope described previously (Pennell et al., 1991) or the epitope present on secreted *Daucus* AGPs (Knox et al., 1991). The presence of the cell wall JIM8 epitope in *Daucus* suspension cell cultures is highly correlated with the presence of embryogenic cells. Surprisingly, immersion immunofluorescence showed that several morphologically different cells react with the JIM8 antibody, but not the proembryogenic masses (Pennell et al., 1992). Instead, mainly small single cells, including cells morphologically similar to the type 1 cells were recognized. The hypothesis put forward by Pennell *et al.* (1992) is therefore

that the JIM8 cell wall epitope marks a transitional state in the formation of embryogenic cells. Because the number of JIM8 reactive single cells exceeds by far the number of single cells that are able to develop into an embryo, apparently only few cells in this transitional state are actually able to reach the status of the embryogenic state 1 cell cluster. Although the JIM8 plasma membrane epitope, as observed in *Brassica* flowers (Pennell, 1991), is most likely present on a molecule different from the JIM8 cell wall epitope observed in *Daucus* suspension cultures, the observation that both visualize a transient developmental process, not restricted to a particular set of morphologically recognizable cells, represents an intriguing parallel.

Formation of Embryogenic Cells

It has often been observed that the developmental stage of the explant is of prime importance for the transition of somatic cells into embryogenic cells (Ammirato, 1983; Conger et al., 1983; Wernicke and Brettell, 1980). However, it is not clear whether these observations reflect genetic differences in the ability of somatic cells to become embryogenic, or whether they are due to the frequency of a particular responsive cell type in these tissues.

The fact that almost all cells of mature organs in plants, including *Arabidopsis* (Galbraith et al., 1991) are polyploid has led to the question whether polyploidy is negatively correlated with the ability to regenerate. However, in *Zea*, no evidence was found that this is indeed the case (Dolezelová et al., 1992). In *Daucus* suspension cultures a correlation was found between the tetraploid state and the inability to produce somatic embryos (Coutos-Thevenot et al., 1990; Smith and Street, 1974). Based on the occurrence of meiotic-like cell division configurations and the presence of a limited number of haploid nuclei in newly initiated cultures of *Daucus*, Nuti-Ronchi *et al.* (1992a, 1992b) postulated a requirement for DNA reducing mechanisms in the formation of embryogenic cells. Support for this hypothesis is the observation that, after chemical mutagenesis of embryogenic *Daucus* suspension cultures, an unexpectedly high number of recessive mutants were recovered (Giuliano et al., 1984). Definite evidence for the occurrence of reductional divisions in tissue culture awaits segregation analysis in the regenerants.

Although auxins are the best studied inducers for obtaining embryogenic cells (Abdullah et al., 1986; Halperin, 1964; Jones and Rost, 1989; Mórocz et al., 1990; Song et al., 1990; Vasil et al., 1990; Wernicke and Brettell, 1980), they are certainly not unique in the ability to mediate the transition of somatic cells into embryogenic cells. For example in *Citrus* suspension cultures, a change in carbon source is sufficient (Gavish et al., 1991) and for *Brassica* microspores a temperature shock is employed to render cells embryogenic (Pechan and Keller, 1988). In *Medicago*, the ability of cells to become embryogenic appeared to depend on their sensitivity to auxin, as illustrated by the totally different response to 2,4-D

of leaf protoplasts derived from a genotype that readily forms embryogenic cells *in vitro* and one that does not (Bögre et al., 1990).

Recent evidence suggests that particular purified AGPs, isolated from the culture medium of embryogenic *Daucus* lines and from dry *Daucus* seeds were able to promote the formation of proembryogenic masses, even in previously non-embryogenic *Daucus* cell lines, when added in nanomolar concentrations. Other AGPs, isolated from the medium of a non-embryogenic line, acted negatively on the formation of proembryogenic masses (Kreuger and Van Holst, 1993). These results show that specific members of the family of AGPs are involved in the formation of embryogenic clusters. Although the underlying mechanisms are unclear, these observations together with earlier ones employing unfractionated conditioned medium (De Vries et al., 1988), suggest that molecules totally different from conventional plant growth regulators are able to direct the transition of somatic cells into embryogenic cells. Since cell-surface AGPs turnover very rapidly (Van Holst et al., 1981), and their expression is clearly developmentally regulated (Knox et al., 1989), they are likely candidates for molecules able to mediate developmental processes in plants, perhaps by a cell-inductive mechanism (Pennell et al., 1991).

Cell polarity and asymmetrical cell division
Several observations support the hypothesis that plant growth regulators employed to form embryogenic cells, do this by alteration of cell polarity and promotion of subsequent asymmetric divisions. When immature zygotic embryos of *Trifolium* were cultured in the presence of cytokinin, somatic embryos are produced directly from the hypocotyl epidermis. The first sign of the induction of embryogenic cells was a shift from the normal anticlinal division pattern in the epidermis, to irregular periclinal and oblique divisions (Maheswaran and Williams, 1985). The effect of the cytokinin was not entry into mitosis *per se*, but rather an alteration of the division planes, because regular anticlinal divisions persisted for some time in the absence of cytokinin. As pH gradients and electrical fields can change cell polarity (Quatrano, 1978), the positive effect on embryo development of pH shifts (Smith and Krikorian, 1990) and electrical fields (Dijak et al., 1986) may be due to their effect on cell polarity. It is plausible, but unproven, that exogenously applied plant growth regulators directly modify cell polarity, by interference with pH gradients or the electrical field around cells. Following stimulation by auxin, asymmetric cell divisions were frequently observed in leaf protoplast cultures derived from an embryogenic *Medicago* cultivar, while in protoplast cultures from a non-embryogenic cultivar cells divided symmetrically (Bögre et al., 1990; Dudits et al., 1991). The different types of cell division in *Medicago* leaf protoplast cultures appeared to be correlated with differences in microtubule organization (Dijak and Simmonds, 1988). In *Daucus*, the first division of single suspension cells capable of forming embryogenic cells is also asymmetric (Backs-Hüsemann and Reinert, 1970;

Komamine et al., 1990), and only the smaller daughter cell will ultimately develop into an embryo. As the future root pole of the somatic embryo is always oriented towards the larger cell, the polarity of the entire somatic embryo is already determined prior to the first division of an embryogenic cell.

Brassica microspores are highly polarized during normal development into pollen, *in vivo* as well as under *in vitro* conditions. Depending on the developmental stage at the time of isolation, after heat shock induction to induce the formation of microspore derived embryos, the first visible change is either a 90° shift in the orientation of the mitotic spindle, or a migration of the nucleus from an acentric to a central position (Hause et al., 1993). In both cases the result is a 90° shift of the division plane and replacement of an asymmetric cell division with a symmetric cell division. Artificially increasing the number of symmetric cell divisions by colchicine resulted in a larger number of microspores proceeding towards embryogenesis (Zaki and Kickinson, 1991). These results suggest that alteration of division symmetry is required to switch from the gametophytic to the sporophytic developmental pathway. Although many variations have been observed, the replacement of the normal asymmetric cell division with a symmetric one appears to be a general phenomenon in microspore embryogenesis (Zaki and Dickinson, 1990).

With the exception of microspore embryogenesis, the ability to perform an asymmetric cell division, based on a change in cell polarity, seems to be an important and perhaps universal mechanism in the formation of embryogenic plant cells from somatic cells. This change in cell polarity can apparently be initiated by a variety of inducers, among which plant growth regulators. As in zygotic embryogenesis (see previous section), the nature of the cytoplasmic determinants that are partitioned by asymmetrical cell divisions, remains to be identified. The fact that in microspore embryogenesis a symmetric first cell division is the first one in the sporophytic pathway may be a consequence of the previous highly specialized developmental history of the microspores.

Control of cell expansion
A second mechanism that is of importance in the formation of embryogenic cells *in vitro* is the ability to restrict cell expansion under hypotonic conditions (Fry, 1990; Taiz, 1984; Van Engelen and De Vries, 1992 and1993). The ability to control cell expansion is generally accepted to reside in the cell wall, and is probably mediated by specific sets of cell wall proteins and enzymes (Fry 1986). These enzymes may act by breaking and reforming bonds in cell wall polymers by for instance the action of glucanases, cellulases and peroxidases (Van Engelen and De Vries, 1993), and other not yet identified proteins (McQueen-Mason et al., 1992). In *Daucus*, the glycosylation inhibitor tunicamycin arrests somatic embryogenesis, perhaps by the gradual disruption of proembryogenic mass due to expansion of its outer cell layer. This effect could be counteracted by addition of a single

protein, exhibiting peroxidase activity, purified from medium conditioned by a somatic embryo culture (Cordewener et al., 1991). A mechanism that limits cell expansion may also be required at later stages of somatic embryo development, as indicated by the rescue of arrested globular embryos of the temperature-sensitive *Daucus* variant ts11c with a single secreted acidic endochitinase (De Jong et al., 1992). Addition of the endochitinase appeared to prevent the formation of an aberrant, irregular protodermal layer, consisting of enlarged, vacuolated cells. A positive effect was also seen on the formation of proembryogenic masses and globular embryos from ts11c suspension cells, which implies that more than one stage in the development of embryos is affected in ts11c.

The mechanisms by which secreted proteins influence somatic embryogenesis are unknown, but it is reasonable to postulate that their function can be explained in terms of an effect on particular cell wall polymers (Van Engelen and De Vries, 1992).

CONCLUDING REMARKS

In the preceding paragraphs, several recent approaches used to understand the molecular and cellular basis of zygotic and somatic embryogenesis in plants have been dealt with. It appeared from several studies that in the *in vitro* formation of embryogenic plant cells both asymmetric cell division and control of cell expansion are important mechanisms. There is some evidence that cell polarity and a postulated subsequent partitioning of cytoplasmic determinants can be influenced by a variety of factors among which plant growth regulators. Other molecules, that profoundly influence for instance the formation of embryogenic cells and the restriction of cell expansion characteristic for these cells, have been found with biological assays based on *in vitro* systems. Analysis of *Arabidopsis* mutants, such as *emb 101-1* and *gnom*, that are affected in early stages of zygotic embryogenesis, have also pointed to control of cell expansion and asymmetric cell division as important mechanisms. The results obtained sofar suggest that, although their starting points are quite different, the same basic cellular mechanisms are used in somatic as well as in zygotic plant embryogenesis.

ACKNOWLEDGEMENTS

REFERENCES

Abdullah, R., Cocking, E.C., and Thompson, J.A. (1986). Efficient plant regeneration from rice protoplasts through somatic embryogenesis. Bio. Technol. 4, 1087-1090.

Ammirato, P.V. (1983). Embryogenesis. In Handbook of Plant Cell Culture, D.A. Evans, W.R. Sharp, P.V. Ammirato, and Y. eds, (MacMillan Publishing Co, New York) vol 1, pp. 82-123.

Backs-Hüsemann, D., Reinert, J. (1970). Embryobildung durch isolierte einzelzellen aus gewebekulturen von *Daucus carota*. Protoplasma 7, 49-60.

Bögre, L., Stefanov, I., Ábrahám, M., Somogyi, I., Dudits, D. (1990). Differences in responses to 2,4-dichlorophenoxyacetic acid (2,4D) treatment between embryogenic lines of alfalfa. In Progress in Plant Cellular and Molecular Biology, Nijkamp, H.J.J., Van Der Plas, L.H.W., Van Aartrijk, J., eds, (Kluwer Academic Publishers, Dordrecht) pp. 427-436.

Christianson, M.L. (1986). Fate map of the organizing shoot apex in *Gossypium*. Am. J. Bot. 73, 947-958.

Clark, J.K., and Sheridan, W.F. (1991). Isolation and characterization of 51 embryo-specific mutations of maize. Plant Cell 3, 935-951.

Conger, B.V., Hanning, G.E., Gray, D.J., and McDaniel, J.K. (1983). Direct embryogenesis from mesophyll cells of orchardgrass. Science 221, 850-851.

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

Coutos-Thevenot, P., Jouanneau, J.P., Brown, S., Petiard, V., and Guern, J. (1990). Embryogenic and non-embryogenic cell lines of *Daucus carota* cloned from meristematic cell clusters: relation with cell ploidy determined by flow cytometry. Pl. Cell Rep. 8, 605-608.

Cresti, M., Blackmore, S., and Van Went, J.L. (1992). Atlas of sexual reproduction in flowering plants. (Springer Verlag, Berlin).

Dawe, R.K., and Freeling, M. (1991). Cell lineage and its consequences in higher plants. Plant J. 1, 3-8.

De Jong, A.J., Cordewener, J., Lo Schiavo, F., Terzi, M., Vandekerckhove, J., Van Kammen, A., and De Vries, S.C. (1992). A carrot somatic embryos mutant is rescued by chitinase. Plant Cell 4, 425-433.

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

De Vries, S.C., Booij, H., Janssens, R., Vogels, R., Saris, L., Lo Schiavo, F., Terzi, M., and Van Kammen, A. (1988b). Carrot somatic embryogenesis depends on the phytohormone-controlled presence of correctly glycosylated extracellular proteins. Genes Dev. 2, 462-476.

Dijak, M., and Simmonds, D.H. (1988). Microtubule organization during early embryogenesis from mesophyl protoplasts of *Medicago sativa*. Pl. Sci. 58, 183-191.

Dijak, M., Smith, D.L., Wilson, T.J., and Brown, D.C.W. (1986). Stimulation of direct embryogenesis from mesophyll protoplasts of *Medicago sativa*. Pl. Cell Rep. 5, 468-470.

Dolezelová, M., Dolezel, J., and Nesticky, M. (1992). Relationship of embryogenic competence in maize (*Zea mays* L.) leaves to mitotic activity, cell cycle and nuclear DNA content. Pl. Cell Tiss. Organ Culture 31, 215-221.

Dudits, D., Bögre, L., and Györgyey, J. (1991). Molecular and cellular approaches to the analysis of plant embryo development from somatic cells *in vitro*. J. Cell Sci. 99, 475-484.

Ephrussi, A., and Lehmann, R. (1992). Induction of germ cell formation by *oskar*. Nature 358, 387-392.

Errempalli, D., Patton, D., Castle, L., Mickelson, L., Hansen, K., Schnall, J., Feldmann, K., and Meinke, D. (1991). Embryonic lethals and T-DNA insertional mutagenesis in *Arabidopsis*. Plant Cell 3, 149-157.

Fincher, G.B., Stone, B.A., Clarke, A.E. (1983). Arabinogalactan proteins; structure, biosynthesis and function. Annu. Rev. Plant Physiol. 34, 47-70.

Fry, S.C. (1986). Cross-linking of matrix polymers in the growing cell walls of angiosperms. Ann. Rev. Plant Physiol. 37, 165-186.

Fry, S.C. (1990). Roles of the primary cell wall in morphogenesis. In Progress in Plant Cellular and Molecular Biology, H.J.J. Nijkamp, L.H.W. Van Der Plas, J. Van Aartrijk, eds, (Kluwer Academic Publishers, Dordrecht) pp. 504-513.

Furner, I.J., and Pumfrey, J.E. (1992). Cell fate in the shoot apical meristem of *Arabidopsis thaliana*. Development 115, 755-764.

Galbraith, D.W., Harkins, K.R., and Knapp, S. (1991). Systemic endopolyploidy in *Arabidopsis thaliana*. Plant Physiol. 96, 985-989.

Gavish, H., Vard, A., and Fluhr, R. (1991). Extracellular proteins and early embryo development in *Citrus* nucellar cell cultures. Physiol. Plant. 82, 606-616.

Giuliano, G., Lo Schiavo, F., and Terzi, M. (1984). Isolation and developmental characterization of temperature-sensitive carrot cell variants. Theor. Appl. Genet. 67, 179-183.

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

Gurdon, J.B. (1992). The generation of diversity and pattern in animal cells. Cell 68, 185-199.

Halperin, W. (1964). Morphogenetic studies with partially synchronized cultures of carrot embryos. Science 146, 408-410.

Halperin, W. (1966). Alternative morphogenetic events in cell suspensions. Amer. J. Bot. 53, 443-453.

Hause, B., Hause, G., Pechan, P., and Van Lammeren, A.A.M. (1993). Cytoskeletal changes and induction of embryogenesis in microspore and pollen cultures of *Brassica napus* L. Cell. Biol. Intern. **17**, 153-168.

Hill, D.P., Strome, S. (1990). Brief cytochalasin-induced disruption of microfilaments during a critical interval in 1-cell *C. elegans* embryos alters the partitioning of developmental instructions to the 2-cell embryo. Development **108**, 159-172.

Horvitz, H.R., Herskovitz, I. (1992). Mechanisms of asymmetric cell division: two Bs or not two Bs, that is the question. Cell **68**, 237-255.

Irish, V.F., and Sussex, I.M. (1992). A fate map of the *Arabidopsis* embryonic shoot apical meristem. Development **115**, 745-753.

Jones, T.J., and Rost, T.L. (1989). The developmental anatomy and ultrastructure of somatic embryos from rice (*Oryza sativa* L.) scutellum epithelial cells. Bot. Gaz. **150**, 41-49.

Jürgens, G., and Mayer, U. (1992). *Arabidopsis*. In A colour atlas of developing embryos. J. Bard London, ed., (Wolfe Publ).

Jürgens, G., Mayer, U., Torres Ruiz, R.A., Berleth, T., and Miséra, S. (1991). Genetic analysis of pattern formation in the *Arabidopsis* embryo. Development Suppl. **91.1**, 27-38.

Knox, J.P., Day, S., and Roberts, K. (1989). A set of cell surface glycoproteins forms an early marker of cell position, but not cell type in the root apical meristem of *Daucus carota* L. Development **106**, 47-56.

Knox, J.P., Linstead, P.J., Peart, J., Cooper, C., and Roberts, K. (1991). Developmentally regulated epitopes of cell surface arabinogalactan proteins and their relation to root tissue pattern formation. Plant J. **1**, 317-326.

Komamine, A., Matsumoto, M., Tsukahara, M., Fujiwara, A., Kawahara, R., Ito, M., Smith, J., Nomura, K., and Fujimura, T. (1990). Mechanisms of somatic embryogenesis in cell cultures-physiology, biochemistry and molecular biology. In Progress in Plant Cellular and Molecular Biology, H.J.J. Nijkamp, L.H.W. Van Der Plas, J. Van Aartrijk, eds, (Kluwer Academic Publishers, Dordrecht) pp. 307-313.

Kreuger, M., and Van Holst, G.J. (1993). Arabinogalactan proteins are essential in somatic embryogenesis of *Daucus carota* L. Planta **189**, 243-248.

Lloyd, C.W. (1991). How does the cytoskeleton read the laws of geometry in aligning the division plane of plant cells. Development Suppl. **91.1**, 55-65.

Lyndon, R.F. (1990). Plant Development. The cellular basis. (Unwin Hyman, London).

Maheswaran, G., and Williams, E.G. (1985). Origin and development of somatic embryos formed directly on immature embryos of *Trifolium repens in vitro*. Ann. Bot. **56**, 619-630.

Mansfield, S.G., and Briarty, L.G. (1990a). Development of the free-nuclear endosperm in *Arabidopsis thaliana* L. *Arabidopsis* Inf. Scrv. **27**, 53-64.

Mansfield, S.G., and Briarty, L.G. (1990b). Endosperm cellularization in *Arabidopsis thaliana* L. *Arabidopsis* Inf. Serv. **27**, 65-72.

Mansfield, S.G., Briarty, L.G. (1991). Early embryogenesis in *Arabidopsis thaliana*. II. The developing embryo. Can. J. Bot. **69**, 461-476.

Mansfield, S.G., Briarty, L.G., and Erni, S. (1991). Early embryogenesis in *Arabidopsis thaliana*. I. The mature embryo sac. Can. J. Bot. **69**, 447-460.

Mayer, U., Torres Ruiz, R.A., Berleth, T., Miséra, S., and Jürgens, G. (1991). Mutations affecting body organization in the *Arabidopsis* embryo. Nature **353**, 402-407.

Mayer, U., Büttner, G., and Jürgens, G. (1993). Apical-basal pattern formation in the *Arabidopsis* embryo: studies on the role of the *gnom* gene. Development????.

McQueen-Mason, S., Durachko, D.M., Cosgrove, D.J. (1992). Two endogenous proteins that induce cell wall extension in plants. Plant Cell **4**, 1425-1433.

McWilliam, A.A., Smith, S.M., and Street, H.E. (1974). The origin and development of embryoids in suspension cultures of carrot (*Daucus carota*). Ann. Bot. **38**, 243-250.

Meinke, D.W. (1982). Embryo-lethal mutants of *Arabidopsis thaliana*: Evidence for gametophytic expression of the mutant genes. Theor. Appl. Genet. **63**, 381-386.

Meinke, D.W. (1991). Embryonic mutants of *Arabidopsis thaliana*. Dev. Genet. **12**, 382-392.

Mórocz, D.J., Donn, G., Németh, J., and Dudits, D. (1990). An improved system to obtain fertile regenerants via maize protoplasts isolated from a highly embryogenic suspension culture. Theor. Appl. Genet. **80**, 721-726.

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

Nuti Ronchi, V., Giorgetti, L., Tonelli, M., and Martini, G. (1992a). Ploidy reduction and genome segregation in cultured carrot cell lines. I. Prophase chromosome reduction. Pl. Cell Tiss. Organ Culture **30**, 107-114.

Nuti Ronchi, V., Giogetti, L., Tonelli, M., and Martini, G. (1992b) Ploidy reduction and genome segregation in cultured carrot cell lines. II. Somatic meiosis. Pl. Cell Tiss. Organ Culture **30**, 115-119.

Patton, D.A., and Meinke, D.W. (1990). Ultrastructure of arrested embryos from lethal mutants of *Arabidopsis thaliana*. Am. J. Bot. **55**, 807-819.

Pechan, P.M., and Keller, W.A. (1988). Identification of potentially embryogenic microspores in *Brassica napus*. Physiol. Plant. **74**, 377-384.

Pennell, R.I., Janniche, L., Kjellbom, P., Scofield, G.N., Peart, J.M., and Roberts, K. (1991). Developmental regulation of a plasma membrane arabinogalactan protein epitope in oilseed rape flowers. Plant Cell **3**, 1317-1326.

Pennell, R.I., Janniche, L., Scofield, G.N., Booij, H., De Vries, S.C., and Roberts, K. (1992). Identification of a transitional cell state in the developmental pathway to carrot somatic embryogenesis. J. Cell. Biol. **119**, 1371-1380.

Poethig, R.S. (1989). Genetic mosaic and cell lineage analysis in plants. Trends Genet. **5**, 273-277.

Poethig, R.S., Coe, E.H., and Johri, M.M. (1986). Cell lineage patterns in maize embryogenesis: a clonal analysis. Dev. Biol. **117**, 392-404.

Quatrano, R.S. (1978). Development of cell polarity. Annu. Rev. Plant Physiol. **29**, 487-510.

Raghavan, V. (1976). Adventive embryogenesis: Induction of diploid embryoids. In Experimental Embryogenesis in Vascular Plants, V. Raghavan, ed, (Academic Press, London) pp. 349-381.

Reinert, J. (1959). Über die Kontrolle der Morphogenese und die Induktion von Adventivembryonen an Gewebekulturen aus Karotten. Planta **53**, 318-333.

Robinson-Beers, K., Pruitt, R.E., and Gasser, C.S. (1992). Ovule development in wild-type *Arabidopsis* and two female-sterile mutants. Plant Cell **4**, 1237-1249.

Schiavone, F.M., and Racusen, R.H. (1991). Regeneration of the root pole in surgically transected carrot embryos occurs by position-dependent, proximodistal replacement of missing tissues. Development **113**, 1305-1313.

Smith, D.L., and Krikorian, A.D. (1990). Somatic proembryo production from excised, wounded zygotic carrot embryos on hormone-free medium: evaluation of the effects of pH, ethylene and activated charcoal. Pl. Cell Rep. **9**, 468-470.

Smith, S.M., and Street, H.E. (1974). The decline of embryogenic potential as callus and suspension cultures of carrot (*Daucus carota* L.) are serially subcultured. Ann. Bot. **38**, 223-241.

Song, J., Sorensen, E.L., and Liang, G.H. (1990). Direct embryogenesis from single mesophyll protoplasts in alfalfa (*Medicago sativa* L.). Pl. Cell Rep. **9**, 21-25.

Sterk, P., and De Vries, S.C. (1992). Molecular markers for plant embryos. In Synseeds: Applications of Synthetic Seeds to Crop Improvement, K. Redenbaugh, ed, (Boca Raton, Florida, USA: CRC Press), pp. 115-132.

Sterk, P., Booij, H., Schellekens, G.A., Van Kammen, A., and De Vries, S.C. (1991). Cell-specific expression of the carrot EP2 lipid transfer protein gene. Plant Cell **3**, 907-921.

St Johnston, D., and Nusslein-Volhard, C. (1992). The origin of pattern and polarity in the *Drosophila* embryo. Cell **68**, 201-219.

Strome, S. (1993). Determination of cleavage planes. Cell **72**, 3-6.

Sulton, J.E., Schierenberg, E., White, J.G., and Thomson, J.N. (1983). The embryonic development of *Caenorhabditis elegans*. Dev. Biol. **100**, 64-119.

Taiz, L. (1984). Plant cell expansion: regulation of cell wall mechanical properties. Ann. Rev. Plant Physiol. **35**, 585-657.

Taylor, R.L. (1967). The foliar embryos of *Malaxis paludosa*. Can. J. Bot. **45**, 1553-1556.

Van Engelen, F.A., and De Vries, S.C. (1992) Extracellular proteins in plant embryogenesis. Trends Genet. **8**, 66-70.

Van Engelen, F.A., and De Vries, S.C. (1993). Secreted proteins in plant cell cultures. In Markers of Plant Morphogenesis, K.A. Roubelakis-Angelakis, ed, NATO-ASI series, (Plenum, New York), pp. 181-200.

Van Holst, G.J., and Klis, F.M. (1981). Hydroxyproline glycosides in secretory arabinogalactan proteins of *Phaseolus vulgaris* L. Plant Physiol. **68**, 979-980.

Van Holst, G.J., Klis, F.M., De Wildt, P.J.M., Hazenberg, C.A.M., Buijs, J., and Stegwee, D. (1981). Arabinogalactan protein from a crude cell organelle fraction of *Phaseolus vulgaris* L. Plant Physiol. **68**, 910-913.

Vasil, V., Redway, F., and Vasil, I.K. (1990). Regeneration of plants from embryogenic suspension culture protoplasts of wheat (*Triticum aestivum* L.). Bio. Technol. **8**, 429-434.

Webb, M.C., and Gunning, B.E.S. (1990). Embryo sac development in *Arabidopsis thaliana*. I. Megasporogenesis, including the microtubular cytoskeleton. Sex. Plant Reprod. **3**, 244-256.

Wernicke, W., and Brettell, R. (1980). Somatic embryogenesis from *Sorghum bicolor* leaves. Nature **287**, 138-139.

Williams, E.G., and Maheswaran, G. (1986). Somatic embryogenesis: Factors influencing coordinate behavior of cells as an embryogenetic group. Ann. Bot. **57**, 443- 462.

Zaki, M.A.M., and Dickinson, H.G. (1990). Structural changes during the first divisions of embryos resulting from anther and free microspore culture in *Brassica napus*. Protoplasma **156**, 149-162.

Zaki, M.A.M., and Dickinson, H.G. (1991). Microspore-derived embryos in *Brassica*: the significance of division symmetry in pollen mitosis I to embryogenic development. Sex. Pl. Reprod. **4**, 48-55.

chapter 2

A carrot somatic embryo variant is rescued by chitinase

Anke J. de Jong, Jan Cordewener, Fiorella Lo Schiavo, Mario Terzi, Joël Vandekerckhove,
Ab van Kammen and Sacco C. de Vries

ABSTRACT

At the nonpermissive temperature, somatic embryogenesis of the temperature-sensitive (ts) carrot cell variant ts11 does not proceed beyond the globular stage. This developmental arrest can be lifted by the addition of proteins secreted by wild-type cells to the culture medium. From this mixture of secreted proteins, a 32-kD glycoprotein, designated extracellular protein 3 (EP)3, was purified that allows completion of somatic embryo development in ts11 at the nonpermissive temperature. On the basis of peptide sequences and biochemical characterization, EP3 was identified as a glycosylated acidic endochitinase. The addition of the 32-kD endochitinase to ts11 embryo cultures at the nonpermissive temperature appeared to promote the formation of a correctly formed embryo protoderm. These results imply that a glycosylated acidic endochitinase has an important function in early plant somatic embryo development.

INTRODUCTION

In carrot cell cultures, somatic embryos develop from single embryogenic cells or from small clusters of embryogenic cells designated proembryogenic masses (Halperin, 1966; Nomura and Komamine, 1985). Several reports have indicated that proteins secreted into the medium of carrot cell cultures accompany the formation of embryogenic cells and somatic embryos. In one instance, an extracellular protein (EP1) has been identified that is only secreted by nonembryogenic cells (Van Engelen et al., 1991). Sterk et al. (1991) reported that another extracellular protein (EP2), identified as a lipid transfer protein, was only synthesized by embryogenic cells and somatic embryos. From these and other extracellular proteins described (Satoh and Fujii, 1988), it emerges that the developmental state of carrot suspension cells is reflected in the type of secreted proteins synthesized by these cells.

To answer whether these and other secreted proteins are directly involved in somatic embryo development, two different assay systems have been developed. The first of these made use of the fungal antibiotic tunicamycin that prevents N-glycosylation of proteins. Tunicamycin was found to inhibit somatic embryo development at an early, preglobular stage. This inhibition could be overcome by the simultaneous addition of correctly glycosylated proteins to the culture medium (De Vries et al., 1988a). The responsible glycoprotein was purified and identified as a cationic peroxidase (Cordewener et al., 1991). Based on the observed expansion of small embryogenic cells in the presence of tunicamycin and the identification of a peroxidase activity that prevents this expansion, a model has been presented that identifies the peroxidase-mediated restriction of cell size as an important prerequisite for successful somatic embryogenesis to occur (Van Engelen and De Vries, 1992). A second assay system was based on the observation that the phenocritical period in temperature-sensitive (ts) arrest at globular stage in the carrot cell variant ts11 coincided with the period of sensitivity to replacement of the conditioned medium by fresh growth medium.
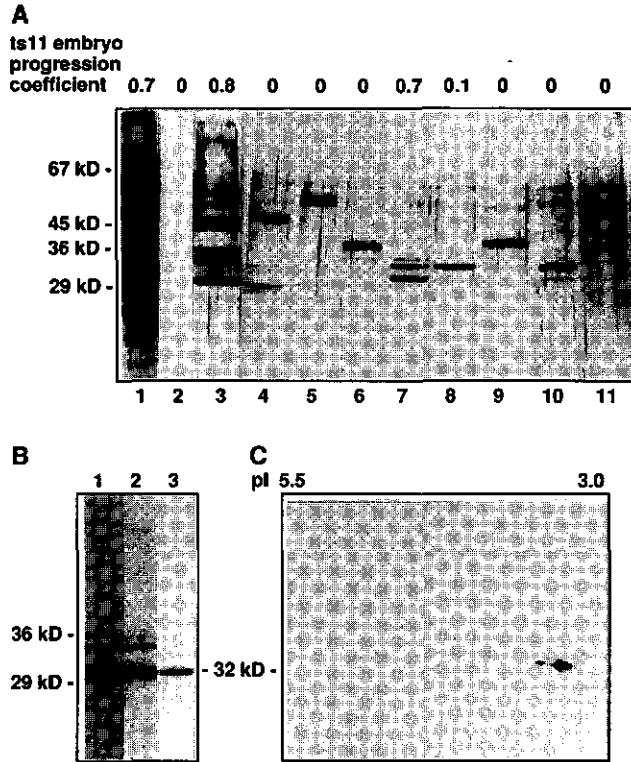
Figure 1. Purification of Secreted Medium Proteins Able to Rescue Arrested Somatic Embryos of the Variant ts11.

(A) The effect of secreted medium proteins on the development of arrested ts11 globular embryos. Embryo rescue is expressed as a progression coefficient, which is the ratio of ts11 embryos developed past the globular stage (heart, torpedo and plantlet) to the total number of embryos (Lo Schiavo et al., 1990). Embryo rescue assays have been performed as described in Methods. Proteins present in the different fractions analyzed in the course of the purification are visualized on silver-stained polyacrylamide gels. Lane 1, unfractionated conditioned medium; lane 2, basal B5 medium; lane 3, DEAE-Sepharose column fraction with ts11 embryo-rescue activity; lane 4 to lane 11, fast-protein liquid chromatography Mono Q column peak fractions derived from the DEAE-Sepharose column fraction shown in lane 3.

(B) The 32- and the 35-kD proteins analyzed for the presence of sugar side chains. Lane 1, silver-stained pattern of fraction shown in (A), lane 7; lane 2, proteins reacting with concanavalin A lectin; lane 3, proteins reacting with *Ulex europaeus* agglutinin I, a fucose-specific lectin.

(C) The purified 32-kD glycoprotein analyzed by silver staining after two-dimensional SDS-PAGE.

When medium conditioned by a wild-type cell line was added to the ts11 culture medium, arrest at the globular stage under nonpermissive temperatures was lifted and embryo development in ts11 was completed up to torpedo stage, resulting in the formation of plantlets. This effect was found to be protease-sensitive, suggesting that secreted proteins were the causative component of the conditioned medium (Lo Schiavo et al., 1990).

In this paper, we present the purification of the secreted protein that is responsible for the observed rescue of ts11 embryos arrested at the globular stage. Partial protein sequences obtained from the purified protein, as well as biochemical characterization, identified this extracellular protein, designated EP3, as a 32-kD glycosylated acidic endochitinase. These results indicate that, apart from their postulated role in the plant defense response, at least one member of the family of plant proteins with chitinase activity has a function in somatic embryo development.

RESULTS

Arrested ts11 Embryos Exhibit Aberrant Protoderm Formation and are Rescued by a Single Secreted Glycoprotein of 32 kD

Cell-free conditioned medium obtained from a 10-day-old embryo culture of the wild-type line A[+] was reported to enable completion of ts11 embryo development (Lo Schiavo et al., 1990). This effect was observed when globular ts11 embryos, developed at 24°C, were transferred to the nonpermissive temperature of 32°C in medium conditioned by an A[+] embryo culture. Prior treatment with trypsin abolished the effect of medium conditioned by an A[+] embryo culture. To confirm this observation, ts11 embryo cultures were grown at 24°C and globular embryos were manually isolated from the embryo culture. Thirty individual ts11 embryos were collected and incubated at 32°C in 2 mL of fresh basal medium alone or in the presence of concentrated medium proteins isolated from the medium of a 10-day-old embryo culture of another wild-type line, 10 (De Vries et al., 1988b). This preparation of secreted proteins allowed 21 of the 30 individual ts11 globular embryos to develop into torpedo stage embryos at 32°C, resulting in an embryo progression coefficient of 0.7 as shown in Figure 1A, lane 1. This value is approximately 75% of the value observed with medium conditioned by an A[+] embryo culture (Lo Schiavo et al., 1990). No development of ts11 embryos beyond the globular stage was observed with unsupplemented basal medium (Figure 1A, lane 2).

Concentrated medium proteins from a 10 day old embryo culture of line 10 were applied to a cation exchange column. The bound fraction contained 30% of the amount of protein applied and could be completely eluted with a linear gradient of 0 to 0.1 M KCl. None of the eluted fractions, including those that contained peroxidase activity (Cordewener et al., 1991), had a positive effect on ts11 embryo development. The embryo rescue activity remained present in the protein fraction not bound to the cation exchange column (data not
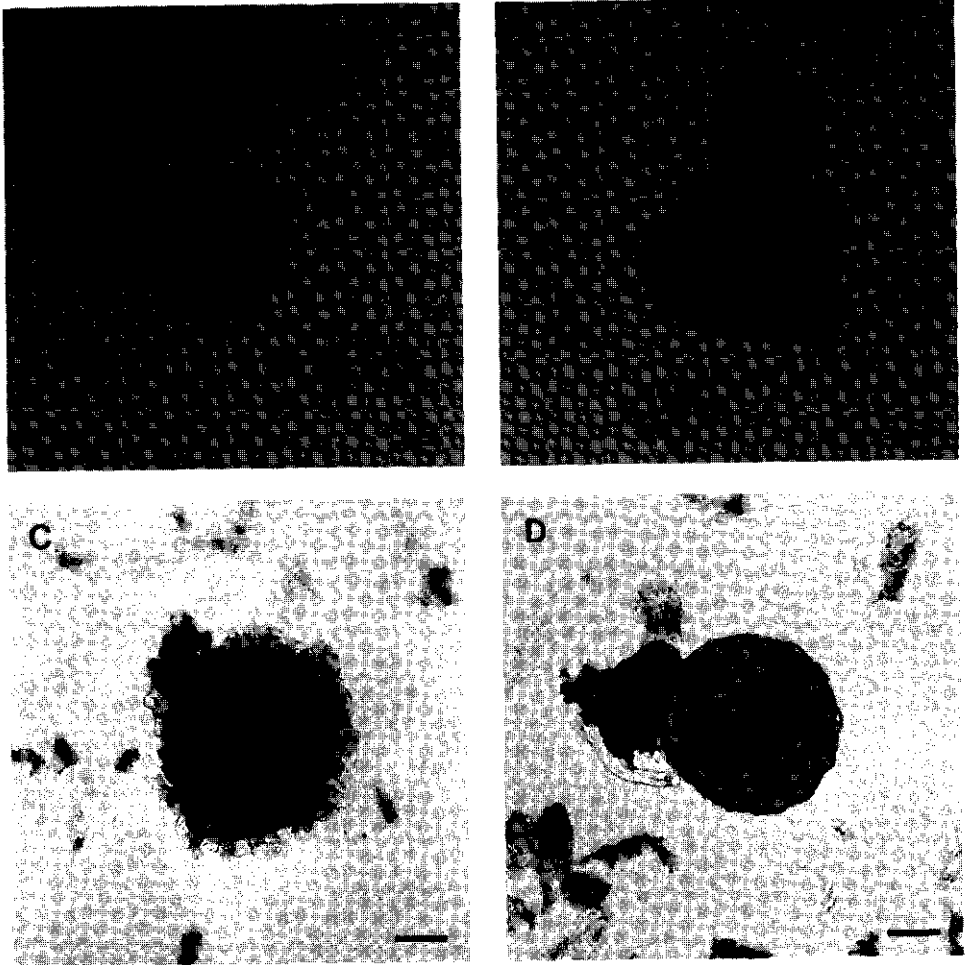
Figure 2. Effect of Secreted Proteins Purified from Wild Type Embryo Culture Medium on the Development of ts11 Embryos at the Nonpermissive Temperature of 32°C.

(A) Aberrant globular embryo in a 4-week-old ts11 embryo culture. Bar = 180 μm.

(B) Globular and heart stage embryos in a 4-week-old embryo culture to which 0.1 μg each of the 32- and 35-kD glycoproteins had been added per milliliter of culture medium. Bar = 200 μm.

(C) Globular embryo in a 14-day-old embryo culture to which 0.1 μg of pure 35-kD glycoprotein had been added per milliliter of culture medium. Bar = 180 μm.

(D) Globular embryo in a 14-day-old embryo culture to which 0.1 μg of pure 32-kD glycoprotein had been added per milliliter of culture medium. Bar = 180 μm.

shown). These proteins were then applied to a DEAE anion-exchange column. Elution of the bound proteins with a linear gradient of 0 to 0.25 M KCl yielded seven major A_{280} peaks. All of the seven anion exchange column peak fractions were tested for ts11 embryo rescue activity. Only one protein fraction eluting around 0.12 M KCl exhibited a rescue effect comparable to the effect of unfractionated medium proteins. This peak fraction contained several proteins (Figure 1A, lane 3). Separation of these proteins by Mono Q anion exchange chromatography yielded eight different fractions (Figure 1A, lanes 4-11), each of which was analyzed for ts11 embryo rescue activity. Only one fraction, eluting at 0.145 M KCl, promoted the transition of globular into torpedo shaped embryos with an efficiency comparable to the unfractionated mixture (Figure 1A, lane 7). SDS-PAGE electrophoresis of this fraction showed two protein bands with apparent molecular weights of 32 and of 35 kD. The adjacent fraction (Figure 1A, lane 8) contained predominantly the 35-kD protein and only a low amount of the 32-kD protein. The latter fraction had a marginal effect on ts11 embryo progression, which suggested that the 32-kD protein is mainly responsible for the embryo rescue effect. This was further supported by the fact that, whereas both the 32- and 35-kD proteins reacted with the glucose- and mannose-specific lectin concanavalin A, only the 32-kD protein reacted with a fucose specific lectin (Figure 1B). The involvement of the 32-kD protein in the ts11 embryo rescue would therefore be in line with the observation of Lo Schiavo et al. (1990) that, whereas core glycosylation of secreted proteins in ts11 is not affected, glycoprotein processing steps that result in the addition of fucose are affected in ts11 at 32˚C. Media supplemented only with the appropriate amounts of column buffer did not have an effect on ts11 embryo development.

So far we had only looked at the effect of the protein fractions upon the transition from globular to heart stage embryos. When the 32-kD glycoprotein-containing fractions were added directly to a newly initiated ts11 embryo culture, a quite dramatic effect on the development of globular ts11 embryos was observed as shown in Figure 2. In a ts11 embryo culture grown at 24˚C, normal globular stage embryos developed, while at 32˚C irregular shaped globular embryos developed (Figure 2A). But when the 32-kD glycoprotein-containing fractions were added, normal globular embryos that were able to develop into heart (Figure 2B) and later into torpedo stage embryos were formed. This result indicated that the rescue activity of the added proteins may also be necessary before the late globular embryo stage.

To confirm that the same proteins able to rescue ts11 embryos are also present in the embryogenic carrot cell line A+, we have fractionated and tested secreted proteins from this line. Presumably due to the presence in reduced amounts of the 35-kD glycoprotein in media of the line A+, we could use the basic purification scheme as described above to obtain a protein fraction that contained only the 32-kD glycoprotein as seen on a silver-stained SDS-polyacrylamide gel (not shown). The purity of this 32-kD glycoprotein preparation was

further analyzed by two-dimensional PAGE and showed the presence of two protein spots (Figure 1C). V8-protease digestion of the individual protein spots cut from a two-dimensional gel showed identical digestion profiles, which indicated that both glycoproteins share the same protein core and represent two closely related variants that only differ in their isoelectric points. Both glycoproteins were fucosylated as revealed by appropriate lectin staining (data not shown).

Both the 32- and the 35-kD glycoprotein were added separately to newly initiated ts11 embryo cultures at day 0. The results, as presented in Figures 2C and 2D, clearly showed that only in the presence of the 32-kD glycoprotein correctly formed ts11 globular embryos developed. These globular embryos were subsequently able to develop into the heart and torpedo stage. This result confirmed the previous assumption (cf. Figure 1A, lane 7) that only the 32-kD glycoprotein is required for embryo rescue. Optimal rescue activity of the 32-kD glycoprotein was observed as a concentration-dependent optimum in a broad range between 0.035 and 0.25 µg/mL or 1 to 9 nM. Heat treatment (10 min at 80°C) abolished the rescue activity. The addition of neither the 32-kD nor the 35-kD glycoprotein to manually isolated globular embryos from wild-type lines had any visible effect on embryo development in this lines.

The main morphological effect of the 32-kD glycoprotein on the ts11 globular embryo appeared to be a restoration of the embryo protoderm, as opposed to the lack of proper protoderm formation in ts11 cultures supplemented with only the 35-kD glycoprotein (cf. Figure 2C). Apart from the aberrant protoderm, accumulation and oxidation of probably phenolic compounds is also a feature of arrested ts11 embryos. This phenomenon, visible as a dark brown color of ts11 embryos, did not occur in ts11 embryos supplemented with the pure 32-kD glycoprotein and might be a secondary effect of the lack of a proper protoderm.

We conclude from these results that rescue of ts11 embryos at the nonpermissive temperature of 32°C can be achieved by the addition of a single 32-kD glycoprotein secreted into the culture medium. Because the rescued embryos are able to develop into the heart stage, we propose that the lack of correct protoderm formation at the preglobular stage is the cause of the inability of ts11 embryos to develop beyond globular stage.

The Secreted 32-kD Glycoprotein is an Acidic Endochitinase

To identify the 32-kD glycoprotein, we tried to determine its amino acid sequence. As it was blocked at the N-terminus, the protein was blotted onto a polyvinylidene difluoride (PVDF) membrane after two-dimensional PAGE. Both spots (cf. Figure 1C) were cut together from the membrane, pooled from several two-dimensional gels, and digested with trypsin. The resulting tryptic fragments were eluted from the membranes, separated by HPLC, and subjected to N-terminal sequencing according to Bauw et al. (1987). No evidence was found for more than one protein core, which supports the results after V8-protease digestion of the

```
              165        175        185
         PEPTIDE I      PEPTIDE II
Carrot    PLQLTFNYNYIDAGKSNQFDGLNNPDIVASDA
Bean      .I.ISW....GQC.RAIGV.L..K..L..T.S
Tobacco   .I...NQN..EK..NAIRQ.LV....L..T..
Rice      .I..SY.F..GP..QAIGA.L.GD..L.....
Sugar beet ...I.W.....P...R.IG.....A.ET..NN.
```

Figure 3. Amino Acid Sequences of Two Adjacent 32-kD Peptides.

Regions of homology between several plant chitinases and two peptides of the 32-kD glycoprotein are shown. The homology on the amino acid level is 47% with a basic bean endochitinase (Broglie et al., 1986), 56% with an acidic tobacco chitinase (Linthorst et al., 1990), 53% with a basic rice chitinase (Zhu and Lamb, 1991) and 69% with a basic sugar beet chitinase (Mikkelsen et al., 1992).

individual spots. The amino acid sequences of the two 32-kD peptides analyzed were found to share homology with several plant endochitinases as shown in Figure 3.

To determine whether the 32-kD glycoprotein exhibits chitinase activity, two different enzyme assays were performed. After electrophoresis of the purified 32-kD glycoprotein in a native polyacrylamide gel, the gel was overlayed with a glycol chitin gel. Clearing of the glycol chitin substrate coincided with the single silver-stained band of the 32-kD glycoprotein, which indicates that the 32-kD glycoprotein indeed has chitinase acitivity as shown in Figure 4A, lanes 1 and 2. The second assay, employing ^{3}H-labeled chitin, showed the release of ^{3}H-N-acetylglucosamine with a specific activity of 60 nkat/mg for the 32-kD chitinase (Figure 4B). Analysis by thin-layer chromatography of the soluble products, derived from ^{3}H-labeled high-molecular mass chitin after prolonged incubation with the 32-kD chitinase, showed the presence of dimers, trimers and tetramers but no monomers of ^{3}H-N-acetylglucosamine (Figure 4C). This indicates that the 32-kD glycoprotein only has endochitinase activity. Isoelectric focusing of the purified 32-kD endochitinase revealed an isoelectric point of 3.6; therefore, it belongs to the secreted acidic endochitinases. The 32-kD endochitinase had the ability to bind high molecular weight chitin, but a lysozyme-like activity could not be detected (A.J. De Jong, K.M. Kragh and S.C. De Vries, unpublished results). An unusual feature of the 32-kD endochitinase is the presence of complex glycans, as indicated by staining with several lectins (cf. Figure 1B). All chitinases thus far described are devoid of complex type N-linked carbohydrates.

Comparison of the unfractionated mixture of secreted glycoproteins in the medium conditioned by an A[+] wild-type embryo culture with the purified 32-kD endochitinase showed the presence of at least five other acidic chitinases (Figure 4A, lanes 3 and 4). In that the 32-kD endochitinase activity was not visible in the unfractionated protein preparation, it
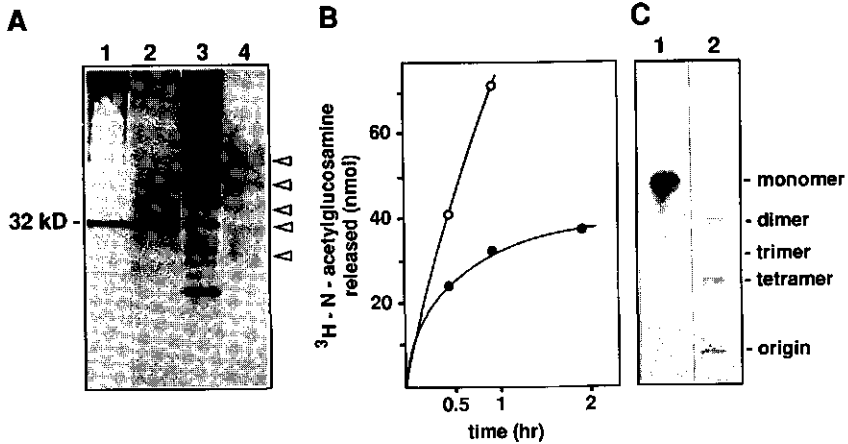
Figure 4. Chitinase Activity of the 32-kD Glycoprotein.

(A) Chitinase activity in a gel containing 0.01% (w/v) glycol chitin as substrate. Degradation of high molecular mass chitin visualized by the reduction of calco fluor white fluorescence, indicated by open arrowheads. Lanes 1 and 2 each contain 0.1 µg of the purified 32-kD glycoprotein; lanes 3 and 4 each contain 10 µg of concentrated secreted medium proteins of a wild-type embryo culture. Lanes 1 and 3 are silver-stained gel lanes, and lanes 2 and 4 show glycol chitin patterns.

(B) Formation of $[^3H]$-N-acetylglucosamine residues after incubation of ^3H-labeled chitin with 0.3 µg of carrot 32-kD glycoprotein (•–•) or with 0.2 µg of exochitinase from *Serratia marcescens* (o-o) as a function of time. $[^3H]$-N-acetylglucosamine residues released in controls with no protein added were less then 1% of the input of $[^3H]$-labeled chitin and have been subtracted.

(C) Separation by thin-layer chromatography of water soluble $[^3H]$-N-acetylglucosamine residues released after prolonged incubation of $[^3H]$-labeled chitin with the carrot 32-kD endochitinase. Lane 1, monomer $[^3H]$-N-acetylglucosamine; lane 2, released products after incubation of $[^3H]$-labeled chitin with 2 µg of the carrot 32-kD endochitinase for 4 hr at 30°C.

was evident that the 32-kD endochitinase activity is only minor compared to the total chitinase activity in the conditioned medium. None of these other carrot chitinases have been found to have rescue activity when added to arrested ts11 embryos. In addition, a bacterial exochitinase from *Streptomyces griseus*, a 36-kD basic endochitinase from barley suspension cultures (Kragh et al., 1991), a 28-kD acidic endochitinase from sugar beet (Mikkelsen et al., 1992), and a 26-kD acidic endochitinase from tomato leaves (Joosten and De Wit, 1989) were tested, but no effect was observed on the development of ts11 embryos comparable to the effect of the 32-kD carrot endochitinase (data not shown). Taken together,

-42-

we conclude that the 32-kD endochitinase has a specific function in carrot somatic embryo development that cannot be substituted by other members of the plant endochitinase family.

DISCUSSION

Previously it was shown that the temperature-sensitive arrest in the carrot cell variant ts11 can be overcome by addition to the culture medium of a mixture of proteins secreted by wild-type embryo cultures (Lo Schiavo et al., 1990). In this study, we have shown that a single secreted glycoprotein is responsible for this effect. This protein was then identified as an acidic endochitinase of 32 kD, bearing complex type carbohydrate side chains.

In many studies it has been reported that plant-produced hydrolases like chitinases and ß-1,3-glucanases are part of a defense mechanism against attack by pathogenic organisms (Schlumbaum et al., 1986; Mauch et al., 1988). Chitinase activity is increased upon wounding and by fungal elicitors (Hedrick et al., 1988; Kurosaki et al., 1990) and is part of the hypersensitive response to pathogen attack (Metraux and Boller, 1986). The biochemical basis of this function is believed to reside in the ability of some plant-produced chitinases and ß-1,3-glucanases to partially degrade fungal cell walls (Broekaert et al., 1988). However, several of the isolated plant chitinases do not possess antifungal activity in vitro (Woloshuk et al., 1991). Chitinase genes were also shown to be expressed in the absence of pathogens (Shinshi et al., 1987; Lotan et al., 1989; Kragh et al., 1990), a finding that is usually explained by assuming that they are part of a continuously present defense mechanism. Some of the chitinase genes appear to be expressed in a tissue-specific manner. A basic chitinase gene is expressed in the epidermis cells of healthy tobacco leaves (Keefe et al., 1990), whereas another basic chitinase gene is expressed in transmitting tissue of the style in tomato flowers (Harikrishna et al., 1991). In addition, chitinase genes are among the first genes whose expression is enhanced after treatment of tobacco epidermal peels with auxin and cytokinin (Meeks-Wagner et al., 1989; Neale et al., 1990). Therefore, it appears reasonable to assume that plant-produced chitinases may have other functions apart from their role in a defense mechanism.

In this paper we have not been able to demonstrate directly that the catalytic properties of the 32-kD endochitinase are required for the observed positive effect on the development of ts11 embryos. If this is indeed the case, then what is the possible substrate for this enzyme in the plant cell wall ? The presumed natural substrate for chitinases, high molecular mass chitin that consists of ß-(1,4)-linked polymers of N-acetylglucosamine, is not considered to be a constituent of higher plant cell walls. Employing cytochemical labeling, Benhamou and Asselin (1989) were able to show binding of chitinases and wheat germ agglutinin, a plant lectin that is specific for oligomers of N-acetylglucosamine, to secondary cell walls of a variety of Solanaceae in the absence of pathogens. Although no biochemical identification of this material was performed, their results imply that in plant secondary cell walls oligomers

of N-acetylglucosamine are present. If oligomers of at least three N-acetylglucosamines are present, they might be putative substrates for plant endochitinases because three residues are the minimum sugar chain length required to serve as a substrate for endochitinases (Molano et al., 1979; Usui et al., 1990). Despite the fact that the substrate specificity of a wheat germ endochitinase is restricted to ß-(1,4)-linked N-acetylglucosamines (Molano et al., 1979), it cannot be excluded that other ß-(1,4)-linked sugars or combinations of different sugars might serve as substrate for other members of the chitinase family. If for instance the two N-acetylglucosamines, flanked by asparagine and ß-1-4-mannose, as present in all the innercores of N-linked carbohydrate chains of glycoproteins could be cleaved, then this would considerably broaden the range of potential substrates for the 32-kD endochitinase described in this work.

Our morphological observations indicate that ts11 globular embryos developed after the addition of the 32-kD endochitinase have a properly formed protoderm, whereas ts11 globular embryos developed in the absence of the 32-kD endochitinase have an aberrantly formed protoderm. This implies that the putative substrate of the 32-kD endochitinase in the plant cell wall has to be accessible, which points to a location at the cell walls of the peripheral cells of the embryo and that this substrate is required for the formation of a proper protoderm. As was previously noted for the ability of a 38-kD peroxidase to restore somatic embryogenesis in tunicamycin-inhibited cultures (Cordewener et al., 1991), a concentration-dependent optimum of the rescue activity was found. Although, at present, we lack a clear explanation for these findings, it may point to a dual activity of these proteins, either in terms of their substrate specificity or in their temporal requirement during embryo development. Further evidence for the disturbed protoderm formation in ts11 globular embryos was obtained when the presence of glycoproteins reacting with concanavalin A was analyzed in sections of arrested ts11 embryos (Lo Schiavo et al., 1990). In that work it was argued that concanavalin A-stainable glycoproteins were distributed in a diffuse manner throughout the arrested ts11 globular embryo, instead of being restricted to the protoderm, as was found in wild-type globular embryos. A similar result was obtained by in situ localization of the epidermis-specific EP2 mRNA. In wild-type embryos, the EP2 mRNA was restricted to the embryo protoderm, but appeared more diffuse in several subprotodermal cell layers in arrested ts11 globular embryos (Sterk et al., 1991). Taken together, we conclude that protoderm formation is an essential step in the formation of globular somatic embryos and a prerequisite for the development to subsequent embryonic stages.

METHODS

Plant Material and Culture Conditions

The embryogenic wild-type carrot suspension cultures 10 and A$^+$ as well as the temperature-sensitive variant ts11 were maintained as described previously (Giuliano et al. 1984; De Vries et al. 1988a).

Ts11 Embryo Rescue Assays

Rescue assays were performed with ts11 globular embryos or with newly initiated ts11 embryo cultures. ts11 embryo cultures were initiated by resuspending the 70 to 170-μm fraction, enriched for proembryogenic masses, of a 6-day-old ts11 suspension culture. Globular embryos were manually isolated from ts11 embryo cultures grown at 24°C. Unfractionated (50-100 μg/mL) and fractionated (1-10 μg/mL) extracellular proteins were mixed in three different amounts (10, 20 and 40 μl) with 1 mL of basal B5 medium (Gamborg, 1970), filter sterilized (0.2 μm; type FF030/3; Schleicher & Schuell), and added to 35-mm-diameter petri dishes (Greiner, Alphen a/d Rijn, The Netherlands, Tissue Culture quality) in duplicate. Subsequently 1 mL of basal B5 medium with 30 isolated ts11 globular embryos or 1 mL of a diluted proembryogenic mass-enriched fraction at 6000 cell clusters per milliliter (Lo Schiavo et al., 1990) was added. Each series of experiments included negative controls of unsupplemented basal B5 medium and basal B5 medium to which an appropriate column buffer had been added. In assays with *Streptomyces griseus* exochitinase (Sigma) and heterologous chitinases, the proteins were added to ts11 embryo cultures in the same concentration range as the 32-kD carrot endochitinase. All assays were then incubated at the nonpermissive temperature of 32°C. Positive control cultures consisted of ts11 globular embryos in unsupplemented basal B5 medium or unsupplemented newly initiated ts11 embryo cultures incubated at 24°C. The newly initiated ts11 embryo cultures were examined after 14 days for the formation of proper globular embryos under a microscope. All cultures were followed at regular intervals for up to 2 months after initiation. Only the results obtained from at least three independent experiments with a protein concentration that gave optimal rescue activity, are presented here.

Protein Purification

Conditioned medium was obtained from a 9-day-old embryo culture by filtration through Whatmann 1 MM paper followed by a 0.2 μm Durapore (polyvinylidene difluoride) filter using a Minitan pressure filtrator (Millipore, Etten-Leur, The Netherlands). The resulting cell-free conditioned medium was concentrated approximately 200 times by pressure dialysis employing an YM5 filter (5-kD cutoff; Amicon, Oosterhout, The Netherlands) and equilibrated with the appropriate starting buffer for column fractionation. The extracellular proteins were then applied to a cation exchange column (S-Sepharose FF; Pharmacia, LKB

Biotechnology, Uppsala, Sweden) equilibrated in 50 mM Mes buffer, pH 5.8, and the absorbed proteins were eluted with a continuous linear salt gradient from 0 to 0.1 M KCl (total volume 100 mL; flow rate 40 mL/hr). Fractions of 4 mL were collected and monitored at 280 nm. Unabsorbed proteins were applied to a DEAE-Sepharose FF (Pharmacia) anion exchange column equilibrated in 25 mM Tris buffer, pH 8.5, and eluted with a linear salt gradient from 0 to 0.25 M KCl with a total volume of 100 mL. Fractions of 4 mL were collected at a flow-rate of 40 mL/h. Final purification was performed by fast-protein liquid chromatography (Pharmacia) employing anion exchange chromatography with a Mono Q (Pharmacia) column equilibrated in the same buffer as used for the DEAE-Sepharose column. For large-scale purification of the 32-kD endochitinase, batch-wise absorption to DEAE-Sepharose FF resin was used. The cell-free conditioned medium was filtrated as described above and then adjusted to pH 8.6 with solid Tris. Fifty milliliters of DEAE-Sepharose FF suspension, equilibrated in 25 mM Tris buffer, pH 8.5, was added to 3 L of cell-free conditioned medium and incubated for 1 hr at 4°C on a rotary shaker. The DEAE-Sepharose FF resin was separated from the medium by filtration through a 10-µm mesh filter and incubated for 15 min in 30 mL of 25 mM Tris buffer, pH 8.5, 0.2 M KCl. The eluate was then diluted with 3 volumes of 25 mM Tris buffer, pH 8.5 and loaded on a DEAE-Sepharose FF column. Further purification was then performed as described above.

Amino Acid Sequence Determination
The 32-kD glycoprotein containing fast-protein liquid chromatography fractions were concentrated by centrifugation using Centricon tubes (10-kD cutoff; Amicon). Concentrated proteins were subsequently separated on two-dimensional polyacrylamide gels as described by De Vries et al.(1982). The isoelectric focusing was performed within the pH range of 3-6. Two-dimensional gels were blotted overnight at 4°C on polyvinylidene difluoride (PVDF) membranes with 25 mM Tris, 150 mM glycine, and 20% ethanol as transfer buffer. After blotting, the PVDF membranes were washed twice in PBS (6.7 mM NaH_2PO_4, 150 mM NaCl, pH 7.2) to remove residual glycinate. Protein spots were visualized after amido black staining, cut out, and stored at -20°C in Eppendorf tubes. In situ membrane tryptic digestions, separation of peptides, and amino acid sequence determination were done as described by Bauw et al. (1987).

Biochemical Characterization
SDS-PAGE was carried out according to Laemmli (1970). After electrophoresis, the gels were silver stained according to Blum et al. (1987). Protein determination was done by comparing the intensity of the Coomassie Brilliant Blue R-250 stained protein band in a polyacrylamide gel with a concentration range of bovine serum albumin in the same gel.

Lectin staining of the proteins on blots was performed according to Clegg (1982). Biotinylated lectins (concanavalin A, specific for glucose and mannose, and Ulex europaeus agglutinin I, specific for fucose; both from Sigma) were used in a concentration of 5 µg/mL in PBS followed by an incubation with 1 µg/mL avidin-labeled peroxidase (Sigma) in PBS. The blots were subsequently stained for peroxidase activity using 0.2 mg/mL 3-amino-9-ethylcarbazole in 50 mM NaAc, pH 5.0, 0.01% H_2O_2.

To detect chitinase activity of the 32-kD glycoprotein in the gel, the protein was run on a native polyacrylamide gel (Laemmli, 1970, without SDS and ß-mercaptoethanol). A glycol chitin gel was overlayed on the native gel and incubated at 30°C for 2 hr. The glycol chitin was stained with calcofluor white M2R, and chitinase activity was visible as a nonfluorescent dark band, in contrast to the fluorescent intact glycol chitin (Trudel and Asselin, 1989).

The radiometric chitinase activity assay employed regenerated ^{3}H-labeled chitin as a substrate (Molano et al. 1977). The specific radioactivity of the ^{3}H-chitin used was 338 cpm/nmol N-acetylglucosamine equivalents or 1.7×10^6 cpm/mg ^{3}H-labeled chitin. The reaction mixture consisted of 50 µl of ^{3}H-labeled chitin suspension containing 200,000 counts per minutes, 50 mM potassium phosphate, pH 6.3, and protein in a final volume of 300 µL. After incubation at 30°C for 0.5 to 2 hr, 300 µL of 10% trichloroacetic acid was added. After centrifugation for 10 min at 16,000 g, the radioactivity of 300 µL of the supernatant was used for scintillation counting. The exochitinase from *Serratia marcescens* (Sigma) was used as a control. The products of the chitinase reaction were analyzed on thin-layer chromatography according to Boller et al. (1983).

Chitin binding activity was determined by using a column of regenerated chitin according to Molano et al. (1979). To detect possible lysozyme activity of the 32-kD endochitinase, a native polyacrylamide gel was overlayed with a polyacrylamide gel containing lyophilized *Micrococcus luteus* cells. Lysozyme activity was then visible as clear or opalescent bands within the opaque greyish substrate (Audy et al. 1988).

ACKNOWLEDGMENTS

REFERENCES

Audy, P., Trudel, J., and Asselin, A. (1988). Purification and characterization of a lysozyme from wheat germ. Plant Science 58, 43-50.

Bauw, G., De Loose, M., Inzé, D., Van Montagu, M., and Vandekerckhove, J. (1987). Alterations in the phenotype of plant cells studied by NH_2-terminal amino acid sequence analysis of proteins electroblotted from two-dimensional gel-separated total extracts. Proc Natl Acad Sci USA 84, 4806-4810.

Benhamou, N., and Asselin, A. (1989). Attempted localization of a substrate for chitinases in plant cells reveals abundant N-acetyl-D-glucosamine residues in secondary walls. Biology of the Cell 67, 341-350.

Blum, H., Beier, H., and Gross, H.J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8, 93-99.

Boller, T., Gehri, A., Mauch, F., and Vogeli, U. (1983). Chitinase in bean leaves: Induction by ethylene, purification, properties and possible function. Planta 157, 22-31.

Broekaert, W.F., Van Parijs, J., Allen, A.K., and Peumans, W.J. (1988). Comparison of some molecular, enzymatic and antifungal properties of chitinases from thorn-apple, tobacco and wheat. Physiol. Mol. Plant Pathol. 33, 319-331.

Broglie, K.E., Gaynor, J.J., and Broglie, R.M. (1986). Ethylene-regulated gene expression: Molecular cloning of the genes encoding an endochitinase from *Phaseolus vulgaris*. Proc. Natl. Acad. Sci. USA 83, 6820-6824.

Clegg, J.C.S. (1982). Glycoprotein detection on nitrocellulose transfers of electrophoretically separated protein mixtures using concanavalin A and peroxidase: Application to arenavirus and flavivirus proteins. Anal. Biochem. 127, 389-394.

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

De Vries, S.C., Springer, J., and Wessels, J.G.H. (1982). Diversity of abundant mRNA sequences and patterns of protein synthesis in etiolated and greened pea seedlings. Planta 156, 129-135.

De Vries, S.C., Booij, H., Janssens, R., Vogels, R., Saris, L., Lo Schiavo, F., Terzi, M., and Van Kammen, A. (1988a). Carrot somatic embryogenesis depends on the phytohormone-controlled expression of correctly glycosylated extracellular proteins. Genes Devel. 2, 462-476.

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

Gamborg, O.L. (1970). The effects of aminoacids and ammonium on the growth of plant cells in suspension culture. Plant Physiol. 45, 372-375.

Giuliano, G., Lo Schiavo, F., and Terzi, M. (1984). Isolation and developmental characterization of temperature sensitive carrot cell variants. Theor. Appl. Genet. 67, 179-183.

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

Harikrishna,K., Jampates-Beale, R., and Charles, S. (1991). A basic chitinase is expressed at high levels in the transmitting tissue of tomatoes. In: Molecular Biology of Plant Growth and Development, Program and Abstracts, R.B. Hallick, ed (Tucson, Department of Biochem., Univ. of Arizona), abstract nr. 511.

Hedrick, S.A., Bell, J.N., Boller, T., and Lamb, C.J. (1988). Chitinase cDNA cloning and mRNA induction by fungal elicitor, wounding and infection. Plant Physiol. **86**, 182-186.

Joosten, M.H.A.J., and De Wit, P.J.G.M. (1989). Identification of several pathogenesis-related proteins in tomato leaves inoculated with *Cladosporium vulvum* (syn. Fulvia fulva) as 1,3-ß-glucanases and chitinases. Plant Physiol. **89**, 945-951.

Keefe, K. Hinz, U., and Meins, F., Jr. (1990). The effect of ethylene on the cell-type-specific and intracellular localization of (1-3) ß-glucanase and chitinase in tobacco leaves. Planta **182**, 43-51.

Kragh, K.M., Jacobsen, S., Mikkelsen, J.D., and Nielsen, K.A. (1990). Tissue specific expression and differential induction of chitinase isoforms in barley (*Hordeum vulgare* L.). Ph.D. Thesis K.M. Kragh, Frederiksberg, Denmark, Royal Veterinary and Agricultural University, pp. 65-78.

Kragh, K.M., Jacobsen, S., Mikkelsen, J.D., and Nielsen, K.A. (1991). Purification and characterization of three chitinases and one ß-1,3-glucanase accumulating in the medium of cell suspension cultures of barley (*Hordeum vulgare* L.). Plant Sci **76**, 65-77.

Kurosaki, F., Tashiro, N., and Nishi, A. (1990). Chitinase induction in carrot cell cultures treated with various fungal compounds. Biochem. Internatl. **20**, 99-106.

LaemmLi, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680-685.

Linthorst, H.J.M., Van Loon, L.C., Van Rossum, C.M.A., Mayer, A., Bol, J.F., Van Roekel, J.S.C., Meulenhoff, E.J.S., and Cornelissen, B.J.C. (1990). Analysis of acidic and basic chitinases from tobacco and petunia and their constitutive expression in transgenic tobacco. Mol. Plant-Microbe Interact. **3**, 252-258.

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

Lotan, T., Ori, N., and Fluhr, R. (1989). Pathogenesis-related proteins are developmentally regulated in tobacco flowers. Plant Cell **1**, 881-887.

Mauch, F., Mauch-Mani, B., and Boller, T. (1988). Antifungal hydrolases in pea tissue. Plant Physiol. **88**, 936-942.

Metraux, J.P., and Boller, T. (1986). Local and systemic induction of chitinase in cucumber plants in response to viral, bacterial and fungal infections. Physiol. Mol. Plant Pathol. **28**, 161-169.

Mikkelsen, J.D., Berglund, L., Nielsen, K.K., Christiansen, H., and Bojsen, K. (1992). Structure of endochitinase genes from sugar beets. In: Proceedings on the 5th International Conference on Chitin and Chitosan, C. J. Brine, ed. (Amsterdam: Elsevier). pp. 344-353.

Molano, J., Duran, A., and Cabib, A. (1977). A rapid and sensitive assay for chitinase using tritiated chitin. Anal. Biochem. 83, 648-656.

Molano, J., Polacheck, I., Duran, A., and Cabib, E. (1979). An endochitinase from wheat germ. Activity on nascent and preformed chitin. J. Biol. Chem. 254, 4901-4907.

Neale, A.D., Wahleithner, J.A., Lund, M., Bonnett, H.T., Kelly, A., Meeks-Wagner, D.R., Peacock, W.J., and Dennis, E.S. (1990). Chitinase, ß-1,3-glucanase, osmotin and extensin are expressed in tobacco explants during flower formation. Plant Cell 2, 673-684.

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

Satoh, S., and Fujii, T. (1988). Purification of GP57, an auxin-regulated extracellular glycoprotein of carrots and its immunocytochemical localization in dermal tissues. Planta 175, 364-373.

Schlumbaum, A., Mauch, F., Vogeli, U., and Boller, T. (1986). Plant chitinases are potent inhibitors of fungal growth. Nature 324, 365-367.

Shinshi, H., Mohnen, D., and Meins, F., Jr. (1987). Regulation of a plant pathogenesis-related enzyme: inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissue by auxin and cytokinin. Proc. Natl. Acad. Sci. USA 84, 89-93.

Sterk, P., Booij, H., Schellekens, G.A., Van Kammen, A., and De Vries, S.C. (1991). Cell-specific expression of the carrot EP2 lipid transfer protein gene. Plant Cell 3, 907-921.

Trudel, J., and Asselin, A. (1989). Detection of chitinase activity after polyacrylamide gel elctrophoresis. Anal. Biochem. 178, 362-366.

Usui, T., Matsui, H., and Isobe, K. (1990). Enzymic synthesis of useful chito-oligosaccharides utilizing transglycosylation by chitinolytic enzymes in a buffer containing ammonium sulfate. Carbohydr. Res. 203, 65-77.

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

Van Engelen, F.A., and De Vries, S.C. (1992). Extracellular proteins in plant embryogenesis. Trends Genet. 8, 66-70.

Woloshuk, C.P., Meulenhoff, J.S., Sela-Buurlage, M., Van den Elzen, P.J.M., and Cornelissen, B.J.C. (1991). Pathogen-induced proteins with inhibitory activity toward *Phytophthora infestans*. Plant Cell 3, 619-628.

Zhu, Q., and Lamb, C.J. (1991). Isolation and characterization of a rice gene encoding a basic chitinase. Mol. Gen. Genet. 226, 289-296.

chapter 3

Rhizobium lipo-oligosaccharides rescue a carrot somatic embryo variant

Anke J. de Jong, Renze Heidstra, Herman P. Spaink, Marijke V. Hartog, Ellen A. Meijer, Theo Hendriks, Fiorella Lo Schiavo, Mario Terzi, Ton Bisseling, Ab van Kammen and Sacco C. de Vries

ABSTRACT

At a nonpermissive temperature, somatic embryos of the temperature-sensitive (ts) carrot cell variant ts11 only proceed beyond the globular embryo stage in the presence of medium conditioned by wild-type embryos. The causative component in the conditioned medium has previously been identified as a 32-kD acidic endochitinase. In search of a function for this enzyme in plant embryogenesis, several compounds that contain oligomers of *N*-acetylglucosamine were tested for their ability to promote ts11 embryo formation. Of these compounds, only the *Rhizobium* lipo-oligosaccharides or nodulation (Nod) factors were found to be effective in rescuing the formation of ts11 embryos. These results suggest that *N*-acetylglucosamine-containing lipo-oligosaccharides from bacterial origin can mimic the effect of the carrot endochitinase. This endochitinase may therefore be involved in the generation of plant analogues of the *Rhizobium* Nod factors.

INTRODUCTION

An intriguing observation made in several recent studies is that somatic embryogenesis appears to be highly dependent on proteins that either promote (Cordewener et al., 1991; De Jong et al., 1992; Kreuger and Van Holst 1993) or inhibit (Gavish et al., 1992) somatic embryogenesis. These proteins most likely represent cell wall proteins, some of which may have a function in the control of cell expansion (Van Engelen and De Vries, 1992; Van Engelen et al., 1993). However, the cell wall substrates for these secreted proteins remain to be identified.

The carrot cell line ts11, obtained after chemical mutagenesis (Giuliano et al., 1984), was originally isolated on the basis of its temperature-sensitive arrest in the transition of globular to heart stage somatic embryos. This arrest could be overcome by the addition of a mixture of proteins secreted by embryogenic wild-type cell lines (Lo Schiavo et al., 1990). The extracellular protein 3 (EP3), which was responsible for the observed ts11 embryo rescue, was identified as an acidic endochitinase of 32 kD (De Jong et al., 1992). The effect of the 32-kD endochitinase on the development of ts11 embryos appeared to be to promote the formation of proembryogenic masses and globular embryos from ts11 suspension cells at the nonpermissive temperature as well as to relieve the temperature arrest of ts11 embryos at globular stage. These results suggested that the 32-kD endochitinase affects ts11 embryo development from proembryogenic mass formation until the transition from the globular to heart stage embryo. In contrast with the aberrantly formed protoderm of ts11 globular embryos, ts11 globular embryos formed in the presence of 32-kD endochitinase exhibited a properly formed protoderm. This suggested that the 32-kD endochitinase has a positive effect on protoderm formation of ts11 globular embryos (De Jong et al., 1992).

In view of the presumed absence of the only known natural substrate for endochitinases, polymers of ß-(1,4)-linked *N*-acetylglucosamine (chitin), in the cell walls of higher plants, it

is unlikely that the endochitinase affects an abundant structural plant cell wall polymer. As put forward previously (De Jong et al., 1992), the assumption must be made that the substrate specificity of plant endochitinases is restricted to at least three ß-(1,4)-linked *N*-acetylglucosamines. However, this has only been shown so far for a wheat germ endochitinase (Molano et al., 1979).

Recent reports have described the structure of signal molecules produced by *Rhizobium* bacteria as *N*-acetylglucosamine-containing lipo-oligosaccharides or nodulation (Nod) factors. These molecules have been identified on the basis of their ability to induce nodule organogenesis in roots of compatible legume host plants (Lerouge et al., 1990; Spaink et al., 1991; Truchet et al., 1991). They consist of an oligosaccharide backbone of four or five ß-(1,4)-linked *N*-acetyl-D-glucosamines with a C16 or C18 fatty acid group attached to the nonreducing end. Modifications of the sugar moiety such as addition of an O-acetyl group or a sulfate group and the degree of unsaturation of the fatty acid moiety determine the host range specificity of the rhizobia (reviewed by Fisher and Long, 1992; Spaink, 1992).

An alternative hypothesis for the role of the carrot 32-kD endochitinase would be that the enzyme releases a signal molecule analogous to the *Rhizobium* Nod factors from a larger and so far unidentified *N*-acetylglucosamine-containing precursor present in a low amount in the plant cell wall. To test this hypothesis, several compounds that contain oligomers of *N*-acetylglucosamine were tested for their ability to promote proembryogenic mass and globular embryo formation in ts11. Of these, the *R. leguminosarum* bv *viciae* lipo-oligosaccharide NodRlv-V(Ac, C18:4) was found to be as effective as the 32-kD endochitinase.

RESULTS

Rescue of ts11 Embryo Formation by the *Rhizobium* Lipo-oligosaccharide NodRlv-V(Ac, C18:4)

In ts11 at the permissive temperature of 24°C, 1.6 somatic embryos per 10,000 cells are formed. This is approximately 1% of the wild-type level of 135 somatic embryos per 10,000 cells when incubated under the same conditions (De Vries et al., 1988). At 32°C, only very few embryos are formed in the absence of the 32-kD endochitinase. In the presence of an optimal amount of endochitinase, the number of somatic embryos formed at 32°C is slightly higher than in unsupplemented cultures at the permissive temperature, as shown in Table 1. These results confirm and extend the previously described dependence of ts11 embryogenesis on the presence of the 32-kD endochitinase. No effect was found of solubilized high molecular mass chitin, *N*-acetylglucosamine pentamers (Table 1), nor of N-acetylglucosamine tetramers (results not shown) on ts11 embryo formation. In contrast, the *Rhizobium* lipo-oligosaccharide NodRlv-V(Ac, C18:4) at concentrations between 10^{-7} and 10^{-9} M was able to promote the formation of proembryogenic masses and globular embryos

in the ts11 line as shown in Table 1 and Figures 1C and 1D. At the optimal concentration (10^{-8} M) of NodRlv-V(Ac, C18:4), which is similar to the concentration used to induce nodule organogenesis in legume roots (Spaink et al., 1991), the *Rhizobium* molecules were as effective as the 32-kD endochitinase (Table 1 and Figures 1B to 1D). NodRlv-V(Ac, C18:1), differing only in the presence of one double bond instead of four in the fatty

Table 1. Rescue of ts11 Embryos by Endochitinase and by *N*-Acetylglucosamine-containing Compounds

Compound	Concentration (M)	Mean no. of ts11 embryos per 10,000 cells (se)[a]	n[b]	P values compared to control at 32°C[c]
control (no addition) at 24°C	-	1.6 (0.5)	13	0.001
control (no addition) at 32°C	-	0.3 (0.1)	34	-
32 kD endochitinase	5×10^{-9}	2.4 (0.5)	32	0.000
glycol chitin	0.0001% w/v	0.0 (0.0)	4	0.256
N-acetylglucosamine pentamer	10^{-9}	0.0 (0.0)	4	0.410
	10^{-8}	0.0 (0.0)	4	0.410
	10^{-7}	0.3 (0.3)	4	0.867
NodRlv-V(Ac, C18:4)	10^{-9}	1.0 (0.5)	16	0.043
	10^{-8}	2.4 (0.8)	24	0.000
	10^{-7}	1.6 (0.6)	20	0.001
NodRlv-V (Ac, C18:1)	10^{-9}	0.0 (0.0)	4	0.339
	10^{-8}	1.5 (1.5)	4	0.086
	10^{-7}	0.8 (0.8)	4	0.455
2,4-D	2×10^{-7}	0.3 (0.3)	3	0.946
2,4-D + 6-BAP	$2 \times 10^{-7} / 6 \times 10^{-8}$	0.0 (0.0)	3	0.339

[a] The effect of addition of an optimal amount of the 32-kD endochitinase and of various *N*-acetylglucosamine-containing compounds is expressed as the number of proembryogenic masses and globular embryos obtained per 10,000 suspension cells. The standard error of the mean (se) is included within parentheses.

[b] The number of individual assays (n) was obtained in 13 independent experiments.

[c] P values less than 0.05 are regarded as significantly different from the untreated control.
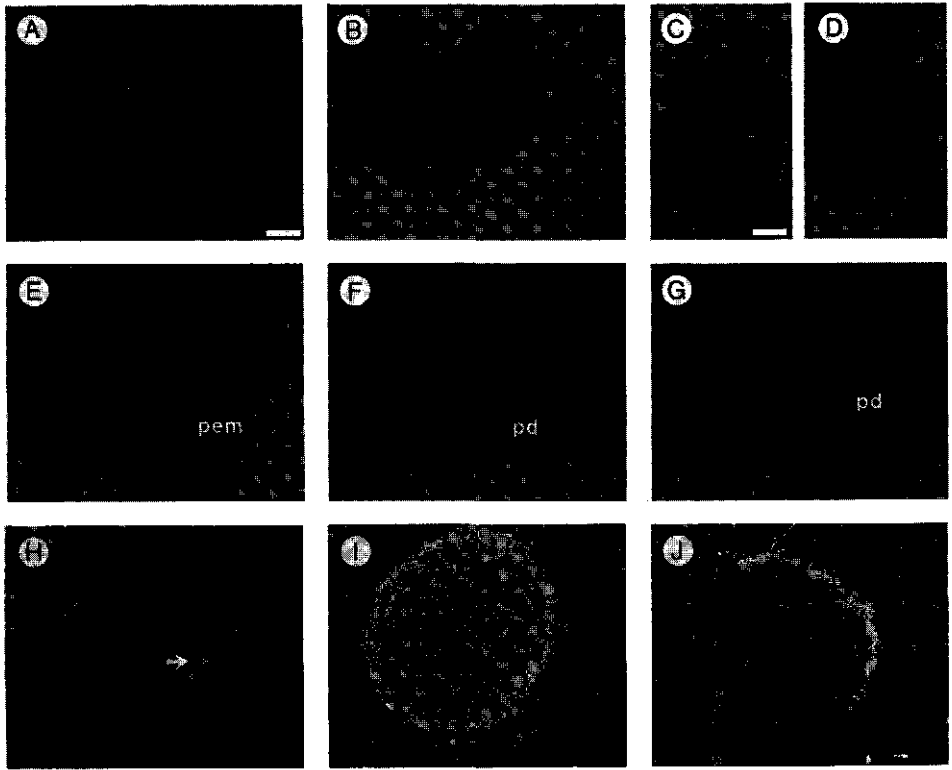
Figure 1. Rescue of ts11 Embryos.

Cells and embryos were photographed by bright-field microscopy. Sections were photographed by bright-field and dark-field microscopy. Silver grains are visible as bright white dots in dark-field images.

A. Control ts11 embryo culture at 32°C. Bar = 100 μm.

B. Globular embryo in a ts11 embryo culture with 5×10^{-8} M of 32-kD endochitinase. Bar = 100 μm.

C. Proembryogenic mass in a ts11 embryo culture with 10^{-8} M of NodRlv-V(Ac, C18:4). Bar = 100 μm.

D. Globular embryo in a ts11 embryo culture with 10^{-8} M of NodRlv-V(Ac, C18:4). Bar = 100 μm.

E. Bright-field photograph of sectioned ts11 control cells at 32°C. Bar = 50 μm. pem, proembryogenic mass.

F. Bright-field photograph of a sectioned ts11 globular embryo formed in the presence of the 32-kD endochitinase. Bar = 100 μm. pd, protoderm.

G. Bright-field photograph of a sectioned ts11 globular embryo formed in the presence of NodRlv-V(Ac, C18:4). Bar = 50 μm. pd, protoderm.

H. In situ localization of EP2 mRNA in the section shown in (E). The arrow points to cells which express the EP2 gene.

I. In situ localization of EP2 mRNA in the section shown in (F).

J. In situ localization of EP2 mRNA in the section shown in (G).

acid chain, is less active in the rescue of ts11 embryo formation (Table 1). This suggests that the fatty acid moiety of the lipo-oligosaccharides is important for their effect on ts11.

At reduced concentrations, the synthetic plant growth regulators 2,4-D and 6-benzylaminopurine (6-BAP) in combination promote the formation of proembryogenic masses and globular embryos in wild-type carrot cultures (Nomura and Komamine, 1985). Employed at the same concentrations, 6-BAP and/or 2,4-D did not promote ts11 proembryogenic mass and embryo formation at 32°C (Table 1). These results suggest that the effects of the 32-kD endochitinase and NodRlv-V(Ac, C18:4) cannot be mimicked by conventional plant growth regulators. Except for the cultures supplemented with 6-BAP and/or 2,4-D, in which cell proliferation was observed, all cultures were found to be similar to untreated controls with respect to the total number of cells present after 3 weeks (results not shown).

Rescued ts11 Embryos Exhibit a Wild-Type EP2 Expression Pattern

To confirm that intact ts11 somatic embryos are formed after exposure to NodRlv-V(Ac, C18:4), expression of the carrot EP2 gene, previously identified as a marker for protodermal differentiation in carrot proembryogenic masses and embryos (Sterk et al., 1991), was examined by in situ mRNA localization. The results show that, in contrast to arrested ts11 embryos that exhibit either a uniform (Figure 1H) or a diffuse subepidermal pattern of expression (Sterk et al., 1991), ts11 embryos formed in the presence of the 32-kD endochitinase as well as in the presence of NodRlv-V(Ac, C18:4) express the EP2 gene in its normal protoderm-specific fashion (Figure 1I and J, respectively).

DISCUSSION

The carrot 32-kD endochitinase EP3 has been identified based on its ability to promote proembryogenic mass and globular embryo formation and its ability to rescue arrested globular embryos of the temperature-sensitive variant ts11 (De Jong et al., 1992). In this work, several compounds were tested that contain oligomers of *N*-acetylglucosamine for their ability to promote ts11 proembryogenic mass and globular embryo formation. Of these compounds, only the metabolic products of the *R. leguminosarum nod* genes, which were identified as *N*-acetylglucosamine-containing lipo-oligosaccharides (Lerouge et al., 1990; Spaink et al., 1991; Truchet et al., 1991), were found to be active. The products of the *Rhizobium nod* genes, therefore, appear to have a biological effect on in vitro embryogenesis in a nonleguminous plant.

At first glance, root nodule development in legumes and somatic embryogenesis in carrot are two disparate systems. However, a parallel may exist in that both require reinitiation of meristematic cell division in previously nondividing plant cells. In the *Rhizobium*-legume interaction, purified *Rhizobium* Nod factors elicit root hair deformation and initiation of

cortical cell division in compatible interactions (Spaink et al., 1991). One explanation for the effect of the Nod factors on ts11 is that they may initiate meristematic cell divisions. In somatic embryogenesis meristematic cell divisions, characteristic of proembryogenic mass formation, are generally initiated by exogenous auxins. In view of the multitude of physiological effects of auxins (Palme and Schell, 1991), other signal molecules are undoubtedly also involved. In line with this is the fact that no significant effect on ts11 embryo formation at the nonpermissive temperature was observed by commonly used plant growth regulators. Morphological observations, which were confirmed by the expression pattern of the protodermal marker EP2, reveal a properly formed protoderm of ts11 globular embryos formed in the presence of the 32-kD endochitinase or NodRlv-V(Ac, C18:4), which confirms previous morphological observations (De Jong et al., 1992). Because, similar to zygotic embryogenesis (Jürgens and Mayer, 1993), the protodermal cell layer of a somatic embryo is established well before the globular stage, it is plausible that the observed positive effect on protoderm formation of the 32-kD endochitinase and NodRlv-V(Ac, C18:4) is a consequence of their promotive effect on the initiation of meristematic cell divisions giving rise to proembryogenic masses.

While both NodRlv-V(Ac, C18:4) and NodRlv-V(Ac, C18:1) elicit root hair deformation in *Vicia sativa* subsp *nigra*, only NodRlv-V(Ac, C18:4) is able to induce nodule meristems (Spaink et al., 1991). These results correlate with our finding that NodRlv-V(Ac, C18:1) is less active in the rescue of ts11 embryo formation than NodRlv-V(Ac, C18:4) and may indicate that the secondary structure of the fatty acid moiety is of importance in both nodule formation and in somatic embryogenesis. However, it is not known whether the fatty acid chain has a similar function in legume roots and carrot suspension cells.

In *Rhizobium*, the *nodABC* or common *nod* genes are essential for the synthesis of the core lipo-oligosaccharide structure (reviewed by Fisher and Long, 1992; Spaink, 1992). Introduction of the *Rhizobium nodA* and *nodB* genes in tobacco resulted in plants with altered flower and leaf morphology (Schmidt et al., 1991); this is postulated to be due to the generation of mitosis-stimulating compounds (Schmidt et al., 1991; Schmidt et al., 1988). The *Rhizobium* NodB protein has been identified as a chitooligosaccharide deacetylase (John et al., 1993). This deacetylase causes characteristic morphological alterations in transgenic tobacco plants; therefore, in tobacco substrates are apparently present that can be modified by NodB to form specific plant growth signals (Schmidt et al., 1991). There are only few indications to suggest that plant cells indeed contain oligomers of N-acetylglucosamine, other than those present in the carbohydrate moiety of glycoproteins. The results of Benhamou and Asselin (1989) indicate that chitin derivatives may occur in secondary plant cell walls of various plant species. Lipophilic molecules that can be degraded by chitinase appear to be present in uninfected Lathyrus plants (Spaink et al., 1993), but their biological relevance remains to be determined. The Xenopus gene *DG42*, transiently expressed during

early embryogenesis (Rosa et al., 1988), was found to have sequence homology with the *Rhizobium nodC* gene and with the yeast chitin synthase II (*CHS2*) and catalytic subunit of chitin synthase III (*CSD2*) genes (Sandal and Marcker, 1990; Bulawa, 1992), suggesting that *N*-acetylglucosamine-containing signal molecules may be fairly common.

It is tempting to speculate that at least one member of the large family of endochitinases has a function in the generation of plant-produced signal molecules from larger precursors. In the case of ts11, this would imply that the *Rhizobium* Nod factor mimics the activity of these putative endochitinase-produced signal molecules. Although the correlations that may exist between the carrot 32-kD endochitinase and the *Rhizobium* Nod factors remain to be elucidated, it will now be of interest to identify biologically active, plant-produced molecules, that have a similar effect as the *Rhizobium* Nod factors, with the aid of responsive in vitro systems such as described here.

METHODS

Ts11 Embryo Rescue Assays

Purification of the 32-kD endochitinase and embryo rescue assays at 32°C with the temperature sensitive mutant ts11 were performed with newly initiated ts11 embryo cultures as described by De Jong et al. (1992). *N*-Acetylglucosamine tetramers were purchased from Sigma and *N*-acetylglucosamine pentamers from Seikagaku Kogyo, Tokyo, Japan and were used without further purification. Lipo-oligosaccharides were purified to homogeneity from *Rhizobium* culture supernatants as described by Spaink et al. (1991), and dissolved to 5×10^{-5} M in 50% (v/v) acetonitrile in water. Concentrations were determined as described by Spaink et al. (1991). Dilution to 2×10^{-6} M was done in basal B5 plant culture medium. After autoclaving for 15 min, appropriate amounts were added to 2 mL of a resuspended 70- to 170-μm fraction of a 6-day-old ts11 suspension culture at 2500 clusters per mL. Control experiments, performed with appropriate serial dilutions of solvents (acetonitrile for the lipo-oligosaccharides and Tris-KCl for the 32-kD endochitinase), did not show any adverse effects on ts11 embryogenesis (results not shown).

A generalized linear model (Aitkin et al., 1991) was used to relate the number of somatic embryos both to treatment and experiment number. Because the number of somatic embryos obtained did not follow a normal distribution, the Poisson distribution was used as error distribution and the logarithm as the link function to relate the scale of measurement to the linear scale. The overall effect of treatment and experiment number was assessed by means of F tests. Significant differences between treatments ($P < 0.001$) and experiments ($P = 0.006$) could be demonstrated. Comparison of individual treatments to the unsupplemented control at 32°C resulted in the P values listed in table 1.

In Situ Hybridization

In situ hybridization with [^{35}S]-labeled antisense extracellular protein 2 (EP2) mRNA was performed on fixed, paraffin embedded, and sectioned 3-week-old ts11 embryo cultures as described previously (Cox and Goldberg, 1988; Sterk et al., 1991). Hybridization was visualized as bright white dots by dark-field illumination.

ACKNOWLEDGMENTS

REFERENCES

Aitkin, M., Anderson, D., and Francis, B. (1991). *Statistical Modelling in GLIM* (Oxford, England: Oxford University Press).

Benhamou, N., and Asselin, A. (1989). Attempted localization of a substrate for chitinases in plant cells reveals abundant *N*-acetyl-D-glucosamine residues in secondary walls. Biol. Cell 67, 341-350.

Bulawa, C.E. (1992). *CSD2*, *CSD3* and *CSD4*, genes required for chitin synthesis in *Saccharomyces cerevisae*: the *CSD2* gene product is related to chitin synthases and to developmentally regulated proteins in *Rhizobium* species and *Xenopus laevis*. Mol. Cell. Biol. 12, 1764-1776.

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

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

De Jong, A.J., Cordewener, J., Lo Schiavo, F. Terzi, M., Vandekerckhove, J., Van Kammen, A., and De Vries, S.C. (1992). A carrot somatic embryo mutant is rescued by chitinase. Plant Cell 4, 425-433.

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

Fisher R.F., and Long, S.R. (1992). *Rhizobium*-plant signal exchange. Nature 357, 655-660.

Gavish, H., Vard, A., and Fluhr, R. (1992). Suppression of somatic embryogenesis in *Citrus* cell cultures by extracellular proteins. Planta 186, 511-517.

Giuliano, G., Lo Schiavo, F., and Terzi, M. (1984). Isolation and developmental characterization of temperature sensitive carrot cell variants. Theor. Appl. Genet. **67**, 179-183.

John, M., Röhrig, H., Schmidt, J., Wieneke, U., and Schell, J. (1993) *Rhizobium* NodB protein involved in nodulation signal synthesis is a chitooligosaccharide deacetylase. Proc. Natl. Acad. Sci. USA **90**, 625-629.

Jürgens, G., and Mayer, U. (1993). *Arabidopsis.* In: A Colour Atlas of Developing Embryos, J. Bard, ed (London: Wolfe Publishing).

Kreuger, M., and Van Holst, G.J. (1993). Arabinogalactan proteins are essential in somatic embryogenesis of *Daucus carota* L. Planta **189**, 243-248.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J.C., and Dénarié, J. (1990). Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature **344**, 781-784.

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

Molano, J., Polacheck, I., Duran, A., and Cabib, A. (1979). An endochitinase from wheat germ. Activity on nascent and preformed chitin. J. Biol. Chem. **254**, 4901-4907.

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

Palme, K., and Schell, J. (1991). Auxin receptors take shape. An end to the long search for phytohormone receptors is firmly in sight. A better understanding of how plants respond to environmental changes should follow. Curr. Biol. **2**, 228-230.

Rosa, F., Sargent, T.D., Rebbert, M.L., Michaels, G.S., Jamrich, M., Grunz, H., Jonas, E., Winkles, J.A., and Dawid, I.B. (1988). Accumulation and decay of *DG42* gene products follow a gradient pattern during *Xenopus* embryogenesis. Dev. Biol. **129**, 114-123.

Sandal, N.N., and Marcker, K.A. (1990). Some nodulin and Nod proteins show similarity to specific animal proteins. In: Nitrogen Fixation: Achievements and Objectives, P.M. Gresshoff, L.E. Roth, G. Stacey, and W.E. Newton, eds (New York: Chapman and Hall), pp. 687-692.

Schmidt, J., Wingender, R., John, M., Wieneke, U., and Schell, J. (1988). *Rhizobium meliloti nod*A and *nod*B genes are involved in generating compounds that stimulate mitosis of plant cells. Proc. Natl. Acad. Sci. USA **85**, 8578-8582.

Schmidt J., John, M., Wieneke U., Stacey, G., Röhrig, H., and Schell, J. (1991). Studies on the function of *Rhizobium meliloti* nodulation genes. In: Advances in Molecular Genetics of Plant-Microbe Interactions, H. Hennecke, and D.P.S. Verma, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 504-513.

Spaink, H.P. (1992). Rhizobial lipo-oligosaccharides: Answers and questions. Plant Mol. Biol. **20**, 977-986.

Spaink, H.P., Sheeley, D.M., Van Brussel, A.A.N., Glushka, J., York, W.S., Tak, T., Geiger, O., Kennedy, E.P. Reinhold, V.N., and Lugtenberg, B.J.J. (1991). A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of *Rhizobium*. Nature 354, 125-130.

Spaink, H.P., Aarts, A., Bloemberg, G.V., Folch, J., Geiger, O., Schlaman, H.R.M., Thomas-Oates, J.E., Van De Snade, K., Van Spronsen, P., Van Brussel, A.A.N., Wijfjes, A.H.M., and Lugtenberg, B.J.J. (1993). Rhizobial lipo-oligosaccharides: Their biosynthesis and their role in the plant. In Advances in Molecular Genetics of Plant-Microbe Interactions, Vol. 2, E. Nester, ed (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 151-162.

Sterk, P., Booij, H., Schellekens, G.A., Van Kammen, A., and De Vries, S.C. (1991). Cell-specific expression of the carrot EP2 lipid transfer protein gene. Plant Cell 3, 907-921.

Truchet, G., Roche, P., Lerouge, P., Vasse, J., Camut, S., De Billy, F., Promé, J-C., and Dénarié, J. (1991). Sulphated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa. Nature 351, 670-673.

Van Engelen, F.A., and De Vries, S.C. (1992). Extracellular proteins in plant embryogenesis. Trends Genet. 8, 66-70.

Van Engelen, F.A., and De Vries, S.C. (1993). Secreted proteins in plant cell cultures. In Markers of Plant Morphogenesis, NATO-ASI series, K.A. Roubelais-Angelakis, ed (New York: Plenum Press), pp. 181-200.

chapter 4

Characterization of the temperature-sensitive somatic embryo variant ts11

Anke J. de Jong, Ellen A. Meijer, Marijke V. Hartog, Theo Hendriks,
Renze Heidstra, Maarten Penning, Fiorella Lo Schiavo, Mario Terzi,
Ab van Kammen, and Sacco C. de Vries

ABSTRACT

At the nonpermissive temperature, somatic embryos of the temperature-sensitive (ts) carrot cell variant ts11 only proceed beyond the globular embryo stage in the presence of medium conditioned by wild-type cells. The causative component in the conditioned medium has been identified as a 32-kD endochitinase.

An antiserum raised against the 32-kD endochitinase detected the 32-kD protein in culture medium from ts11 embryo cultures grown at the permissive temperature as well as at the nonpermissive temperature. No difference in biochemical characteristics, nor in effect on ts11 embryo development could be detected between the 32-kD endochitinase purified from wild-type cultures and from ts11 cultures grown at the permissive or at the nonpermissive temperature. Compared to the amount present in a ts11 embryo culture at the permissive temperature, a 2- to 5- fold transient reduction in the amount of 32-kD endochitinase was observed at the nonpermissive temperature. These results imply that the ts11 phenotype is not the result of a structural difference in its 32-kD endochitinase. Morphological observations indicate that the ts11 phenotype is pleiotropic and also affects the cell wall of non-embryogenic cells.

INTRODUCTION

In carrot (*Daucus carota*) cell cultures, somatic embryos develop from single embryogenic cells (Nomura and Komamine, 1985) or from small clusters of embryogenic cells designated proembryogenic masses (Halperin, 1966). An important finding in this system has been that cells secrete proteins into the culture medium that can influence somatic embryo development, either positively (Cordewener et al., 1991; De Jong et al., 1992; Kreuger and Van Holst, 1993) or negatively (Gavish et al., 1992; Kreuger and Van Holst, 1993).

The carrot cell line ts11, obtained after chemical mutagenesis (Giuliano et al., 1984) was isolated on the basis of the temperature sensitivity of the somatic embryo development. The temperature sensitivity of ts11, as originally determined by temperature-shift experiments, appeared to be restricted to a relatively short period in embryo development, around the transition from globular to heart stage embryos . At the permissive temperature ts11 embryos were sensitive to replacement of conditioned medium by fresh medium in the same time interval (Lo Schiavo et al., 1990). Both temperature- and fresh medium-induced arrest in ts11 embryo development could be lifted by the addition of medium proteins secreted by wild type cells to ts11 culture media (Lo Schiavo et al., 1990; De Jong et al., 1992). The causative component in the conditioned medium was purified and identified as an acidic 32-kD endochitinase (De Jong et al., 1992). The effect of the 32-kD endochitinase (EP3) appeared to be broader than only the transition of globular into heart-stage embryos. Also formation of proembryogenic masses and globular embryos from ts11 suspension cells at

the non-permissive temperature was promoted by addition of the 32-kD endochitinase (De Jong et al., 1993).

In search of a possible function of the 32-kD endochitinase in somatic embryogenesis, several *N*-acetylglucosamine-containing compounds were tested on ts11 cells. That study revealed that lipo-oligosaccharides (Nod-factors) produced by *Rhizobium leguminosarum* biovar *viciae*, an *N*-acetylglucosamine-containing compound that is able to induce nodules on legume roots, was able to promote the formation of proembryogenic masses and globular embryos in ts11. Based on this result it was suggested that the mode of action of the 32-kD endochitinase was to release a Nod-factor-like signal molecule from a larger plant-produced precursor molecule (De Jong et al., 1993).

Here we address two questions: 1. Can we determine the basis of the stimulating effect of the 32-kD endochitinase on ts11 embryo development? and 2. Is there a correlation between the mechanisms of action of the 32-kD endochitinase and the Nod-factor? The results presented in this paper show that the ts11-produced 32-kD endochitinase is fully functional. The rescue of ts11 embryo development by addition of the 32-kD endochitinase to the culture medium coincided with a transient decrease in the 32-kD endochitinase concentration. Evidence is presented that the promotion of ts11 embryo formation by addition of the Nod-factor NodRlv-V(Ac, C18:4) or 32-kD endochitinase may be induced via the same pathway.

RESULTS
Detection of the 32-kD Endochitinase in Carrot Cell Cultures
To be able to detect the presence of the 32-kD endochitinase in media conditioned by wild-type and ts11 cultures, the protein was purified by fast-protein liquid chromatography (FPLC) and preparative SDS-PAGE and subsequently used to raise a mouse polyclonal anti-serum. As shown in figure 1B, the anti-32-kD endochitinase serum reacted with the 32-kD endochitinase on a gel blot (Figure 1B, first and second lane), while preimmune serum was unreactive (Figure 1B, third lane). Of the embryo culture medium proteins, a 28-kD protein is also recognized by the anti-32-kD endochitinase serum.

The simplest explanation for the rescue effect of the 32-kD endochitinase on ts11 embryo development would be that the 32-kD endochitinase is not present in the ts11 culture medium at non-permissive temperatures. To test this hypothesis, immunoblots were made with medium proteins isolated from ts11 cultures grown for 20 days under different conditions. The 32-kD endochitinase was detected in the media of ts11 embryo cultures both at the permissive and the non-permissive temperature cultures as well as in ts11 suspension cultures (Figure 1C). This indicates that the observed rescue of ts11 embryo development by the wild-type 32-kD endochitinase cannot be due to the total absence of that protein in the ts11 culture medium at the non-permissive temperature.
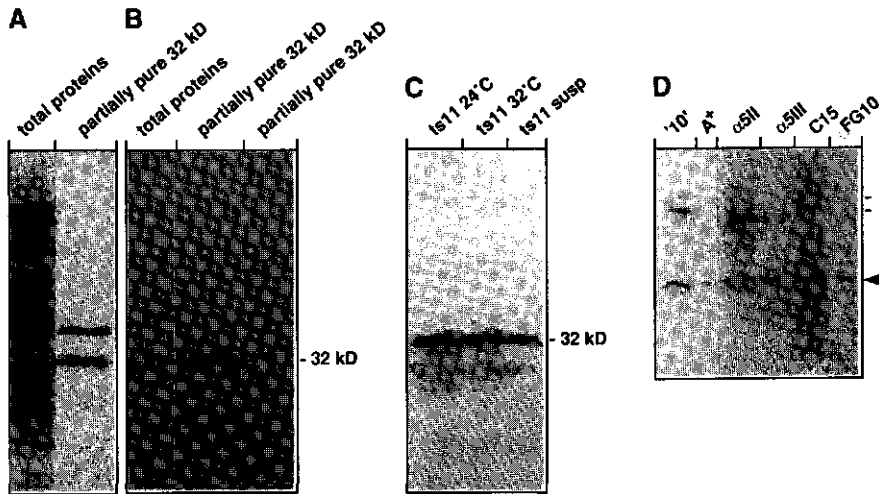
Figure 1. Detection of the 32-kD Endochitinase in Culture Media.

(A) Purification of 32-kD endochitinase via anion-exchange chromatography, visualized on a silver stained SDS-polyacrylamide gel. First lane, total medium proteins (2 μg) from A$^+$ embryo culture; second lane, 0.1 μg of partially purified 32-kD endochitinase.

(B) Specificity of the anti-32-kD endochitinase serum. First lane, immunoblot with total medium proteins (2 μg) of A$^+$ embryo culture, incubated with anti-32-kD endochitinase serum. second lane, immunoblot with 0.1 μg of partially purified 32-kD endochitinase, incubated with anti-32-kD endochitinase serum; third lane, immunoblot of 0.1 μg of partially purified 32-kD endochitinase, incubated with preimmune serum.

(C) Detection of 32-kD endochitinase in ts11 cultures. Immunoblot with total medium proteins from a ts11 embryo culture grown at 25°C or 32°C and total medium proteins from ts11 suspension culture, incubated with anti-32-kD endochitinase serum. Each lane contains ± 20 μg of protein.

(D) Detection of 32-kD endochitinase isoforms in suspension culture medium of embryogenic and nonembryogenic cell lines. The immunoblot of a nondenaturing polyacrylamide gel was incubated with anti-32-kD endochitinase serum that was raised in rabbit. The embryogenic lines are '10'-line, A$^+$ and α5^{III} and the nonembryogenic lines are α5^{II}, C15 and 'FG10' line. C15 and 'FG10' line are not able to form proembryogenic masses, while α5^{II} forms some proembryogenic masses (De Vries et al., 1988). Each lane contains ±10 μg of protein. The 32-kD endochitinase isoform that rescues ts11 embryo development is indicated by an arrow. Two other 32-kD endochitinase isoforms present in '10'-line suspension culture medium are indicated by a small bar.

Recent results have shown that at least five proteins, that can be distinguished from each other on an immunoblot of a non-denaturing polyacrylamide (PAA) gel, are present in the culture medium and react with the anti-32-kD endochitinase serum (Ellen Meijer and Theo

Hendriks, personal communication); all of these five proteins have chitinase activity. The 32-kD endochitinase that has been used to rescue ts11 embryo development and to stimulate ts11 embryo formation has a pI of 3.6 (De Jong et al., 1992) and has the highest mobility on a non-denaturing PAA gel (isoform 1). The protein with a slightly lower mobility on a non-denaturing PAA gel, isoform 2, has the same mobility on a denaturing SDS-PAA gel as isoform 1. While the 32-kD endochitinase isoform 1 elutes from a mono Q column at 125 mM KCl (De Jong et al., 1992), isoform 2 elutes at 105 mM KCl. The remaining three proteins that react with the anti-32-kD endochitinase serum have a slightly higher mobility on a denaturing SDS-PAA gel, suggesting that the molecular mass of these proteins is lower then that of isoform 1. These last three proteins are not likely to be degradation products of the 32-kD endochitinase, since the amino acid composition of one of these proteins, isoform 3, differs from the amino acid composition of the 32-kD endochitinase isoform 1 (Karsten Kragh, personal communication). To determine a possible correlation between embryogenic potential and the presence of the 32-kD endochitinases, an immunoblot of a nondenaturing PAA-gel with medium proteins from different embryogenic and non-embryogenic 10-day-old suspension cultures was made (Figure 1D). Although among the different culture lines a variation in the amount and number of isoforms present in the culture media was observed, in all cases the 32-kD endochitinase isoform 1 was found (indicated by an arrow). These results show that neither the presence of the 32-kD endochitinase isoform 1, nor one of the others is correlated with embryogenic potential.

Purification and Characterization of the 32-kD Endochitinase from ts11 Cultures

A second possible explanation for the rescue effect of the 32-kD endochitinase on ts11 embryo development would be that the protein, as present in ts11 cultures, is an altered and inactive form of the wild-type 32-kD endochitinase. To determine this, the 32-kD endochitinase isoform 1 was purified from wild-type embryo culture after two weeks of culture, from ts11 embryo culture after two weeks of culture at the permissive or nonpermissive temperature and from a wild-type and ts11 suspension culture after one week of culture. After FPLC anion-exchange chromatography, in all ts11 cultures an anti-32-kD endochitinase serum reactive 32-kD protein was found in the same peak fraction as the wild-type 32-kD endochitinase isoform 1 (results not shown) This suggests that the net charge of the 32-kD protein from ts11 is comparable to that of the wild-type 32-kD endochitinase isoform 1. On a silver stained non-denaturing polyacrylamide gel, 32-kD endochitinases isolated from wild-type and ts11 cultures were present at the same position (Figure 2 A). To determine if the ts11 32-kD protein has chitinase actitivity, a duplicate gel was overlaid with a glycol chitin gel to visualize chitinase activity (Figure 2B). The dark bands, being a result

Figure 2. Comparison of '10'-line and ts11 32-kD Endochitinases.

(A) Silver stained nondenaturing polyacrylamide gel with 32-kD endochitinase purified from '10'-line and ts11. First lane, 0.1 µg of 32-kD endochitinase partially purified from '10'-line embryo culture; second lane, 0.2 µg of 32-kD endochitinase purified from '10'-line suspension culture; third lane, 0.05 µg of 32-kD endochitinase purified from '10'-line suspension culture; fourth lane, 0.05 µg of 32-kD endochitinase purified from ts11 suspension culture.

(B) Glycol chitin overlay gel of gel shown in (A). Chitinase activity is indicated by clearing of the glycol chitin substrate.
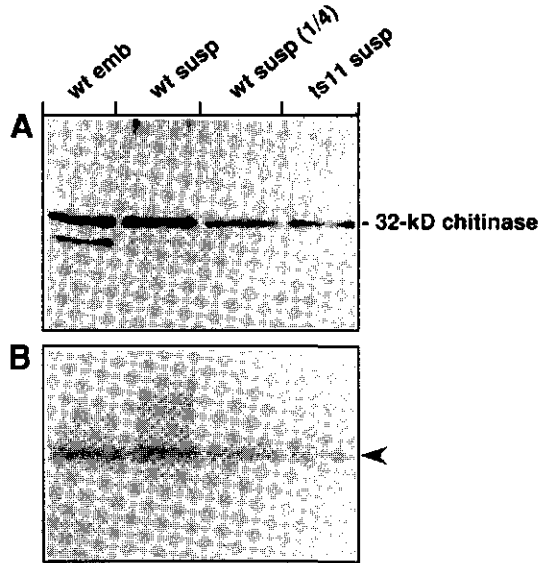
of glycol chitin breakdown, indicate that the ts11 32-kD protein indeed has chitinase acitivity. In order to compare the isoelectric point and molecular mass of ts11 and wild-type 32-kD endochitinase in more detail, two-dimensional gel analysis was performed. Two-dimensional gel electrophoresis of the 32-kD endochitinase from wild-type or partially purified from ts11 cultures at the nonpermissive temperature, followed by silver staining, revealed in both cases an identical and characteristic doublet of one small and one large spot (Figure 3A; De Jong et al., 1992). The similarity in isoelectric point and molecular mass was confirmed by two-dimensional gel electrophoresis of an equimolar mixture of partially purified wild-type and ts11 32-kD endochitinases, which showed that both wild-type and ts11 32-kD endochitinase migrate to exactly the same position on a two-dimensional gel (Figure 3B). These results indicate that the apparent molecular mass and isoelectric point of the 32-kD endochitinase isolated from wild-type or ts11 cell cultures, grown at permissive or nonpermissive temperature, are all identical.

Previously we have reported that the 32-kD endochitinase is a glycoprotein, because it reacts with concanavalin A, a glucose and mannose specific lectin and Ulex europaeus agglutinin I, a fucose specific lectin. However, binding-inhibition experiments showed that glucose, a competitor for the sugar binding site of concanavalin A, did not inhibit the binding between the 32-kD endochitinase and concanavalin A (Figure 4A and 4B), in

contrast to the concanavalin A binding to the EP1 glycoprotein, which was largely inhibited. These observations suggest that the 32-kD endochitinase does not contain glucose and mannose side chains. Also the binding of concanavalin A with the sugar beet chitinase 4 was not inhibited by glucose (Figure 4, lane 2). Montreuil et al. (1986) reported that sometimes aspecific hydrophobic interactions occur between lectins and proteins. Possibly, the observed artefactual lectin-chitinase interactions are due to non-specific hydrophobic interactions between the chitinases and the lectin. An indirect immunological assay specific for glycoproteins, based on periodate oxidation of vicinal hydroxyl groups in carbohydrates, confirmed the absence of detectable glycosylation (Figure 4C). Additional evidence comes from preliminary sequence data of a putative 32-kD endochitinase-encoding cDNA clone, that does not contain N-glycosyl-linkage consensus sequences. Thus, we have to conclude
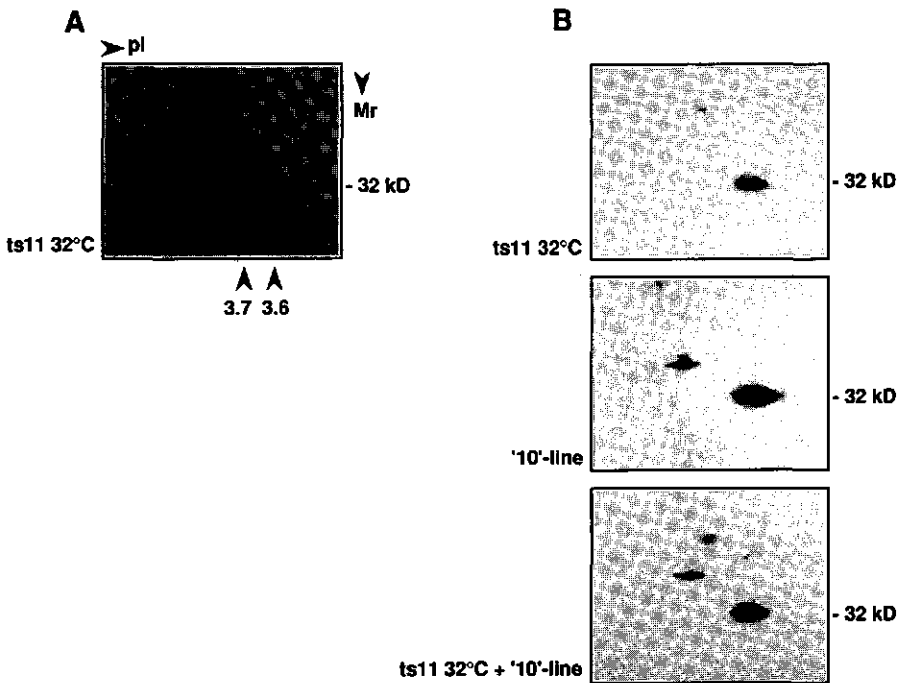
Figure 3. Two-Dimensional Gel Electrophoresis of 32-kD Endochitinase Purified from ts11 and '10'-line Cultures.

(A) Silver stained two-dimensional polyacrylamide gel of 32-kD endochitinase partially purified from a ts11 embryo culture grown at 32°C. The two 32-kD endochitinase spots are indicated by arrows.

(B) Silver stained two-dimensional polyacrylamide gels of 32-kD endochitinase partially purified from '10'-line or ts11 embryo cultures and of a mixture of partially purified 32-kD endochitinase from '10'-line and ts11 embryo cultures.
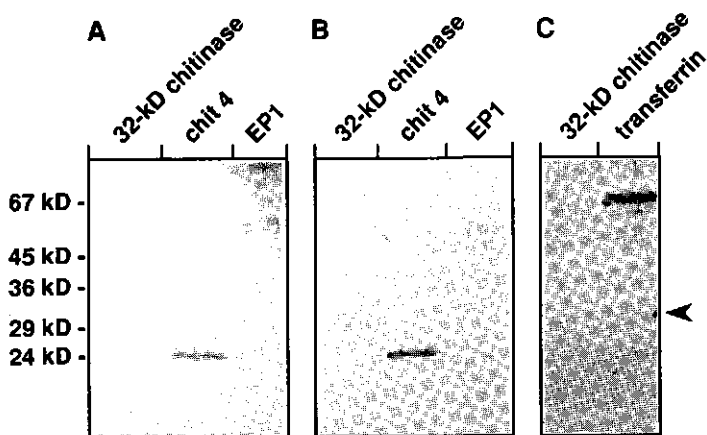
Figure 4. Verification of Glycosylation of the 32-kD Endochitinase.

(A) Proteins reacting with concanavalin A. First lane, 0.05 µg of 32-kD endochitinase; second lane, 0.3 µg of chitinase 4 from sugar beet; third lane, 0.2 µg of EP1 glycoprotein from carrot.

(B) Proteins reacting with concanavalin A in the presence of 0.5 M glucose. Lanes as in (A).

(C) Glycoprotein labeling with periodate oxidation of vicinal hydroxyl groups and conjugation to digoxigenin succinyl-ε-amido-caprioc-acid-hydrazide followed by alkaline phosphatase-conjugated secondary antibody specific for digoxigenin. First lane, 0.5 µg of 32-kD endochitinase; second lane, 0.5 µg of control glycoprotein transferrin. The position of the 32-kD endochitinase on the blot is indicated by an arrow.

that, upon closer investigation, the 32-kD endochitinase is not a glycoprotein, and therefore, that the ts11 embryo phenotype can not be due to a lack of glycosylation of the 32-kD endochitinase.

Final and conclusive evidence that the wild-type and ts11 32-kD endochitinase are identical, would be the demonstration of a similar rescue activity on ts11. Therefore, the 32-kD endochitinase purified from a ts11 embryo culture at the non-permissive temperature and from a wild-type embryo culture were compared with respect to ts11 embryo rescue activity (Table 1). The results show that both 32-kD endochitinases were active in promoting ts11 embryo formation.

Taken together, both the biochemical data and the rescue activity show that the 32-kD endochitinases from wild-type cultures and ts11 cultures are indistinguishable. We therefore conclude that the ts11 embryo phenotype cannot be the result of a structural difference in its 32-kD endochitinase.

Table 1. Rescue of ts11 Embryos with 32-kD Endochitinase from '10'-line or ts11 Embryo Cultures

Compound	Concentration (M)	Mean no. of ts11 embryos per 10,000 cells (se)[a]	n[b]
control at 32°C		0.25 (0.0)	4
32-kD	1.10^{-8}	1.15 (0.9)	8
32-kD ts11	1.10^{-8}	1.75 (0.7)	8

[a] The effect of addition of an optimal amount of 32-kD endochitinase is expressed as the number of proembryogenic masses and globular embryos obtained per 10,000 suspension cells. The standard error of the mean (se) is included within parentheses.

[b] The number of individual assays (n) was obtained in two independent experiments.

Evidence for a Concentration Effect

The results described above show that both wild-type and ts11 cultures accumulate the same 32-kD endochitinase after two weeks. Another explanation for the embryo rescue effect, observed after addition of the 32 kD endochitinase to a ts11 embryo culture at 32°C, could therefore be that the amount of 32-kD endochitinase present in the ts11 culture medium is too low during the temperature-sensitive time period in ts11 embryo development.

Lo Schiavo et al. (1990) estimated by shift experiments that the window of sensitivity to both temperature and fresh medium lay between 0 and 15 days after embryo-culture initiation with a maximum between days 8 and 12 . To determine first whether there was more general decrease in the secretion of proteins into the culture medium at the non-permissive temperature during this period, [^{3}H]-leucine incorporation into secreted proteins produced by ts11 and A$^+$ embryo cultures was measured. After 7 days of culture, 1 mL of ts11 or A$^+$

Table 2. Incorporation of [^{3}H]-Leucine in Extracellular Medium Proteins of Embryo Cultures at 24°C or 32°C

Daucus line	grown at 24°C[a]	grown at 32°C
Ac10	510	570
A$^+$	430	520
ts11	400	460
tsrev	350	430

[a] The amount of [^{3}H]-leucine incorporated in medium proteins secreted in a 1 ml culture with 20,000 cell clusters, is expressed in counts per minute (cpm).
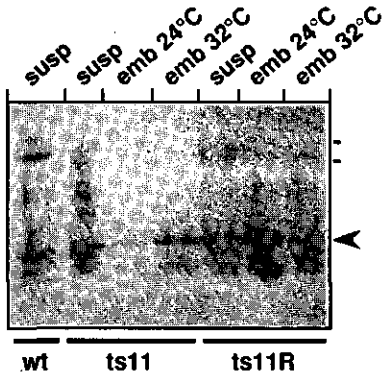
Figure 5. Detection of 32-kD Endochitinase Isoforms in ts11 and ts11R Cultures.

Immunoblot of nondenaturing polyacrylamide gel with ±10 µg of ts11 and ts11R culture medium proteins from 10-days-old embryo cultures grown at 24°C or 32°C and from a 7-days-old suspension cultures, incubated with anti-32-kD endochitinase serum raised in rabbit. The 32-kD endochitinase isoform that rescues ts11 embryo development is indicated by an arrow. Two other 32-kD endochitinase isoforms present in '10'-line suspension culture medium are indicated by a small bar.

embryo culture was grown for 16 hours in the presence of [^{3}H]-leucine, medium proteins were collected and the [^{3}H]-incorporation was measured (Table 2). The results show that the total amount of proteins secreted by ts11 into the culture medium at day 8 at the non-permissive temperature, was comparable to the amount secreted by ts11 at the permissive temperature and by the wild-type line A$^+$. This indicates that the overall rate of secreted protein synthesis is not greatly affected in ts11 at the non-permissive temperature, confirming previous observations by Lo Schiavo et al. (1990).

If the overall rate of secreted proteins is not affected, then possibly the secretion of a specific class of proteins, amongst which the 32-kD endochitinase, may be decreased. Since the presence of different 32-kD endochitinase isoforms in ts11 embryo culture medium may interfere with the detection of the 32-kD endochitinase isoform 1 in ts11 embryo culture medium on immunoblots of denaturing SDS-PAA-gels, the presence of putative different isoforms of the 32-kD endochitinase in ts11 and the ts11 revertant line (ts11R, Lo Schiavo et al., 1990) was first determined. Two weeks after culture, total medium proteins from both lines were separated on a non-denaturing PAA-gel, blotted onto membrane and incubated with anti-32-kD endochitinase serum. Figure 5 shows that, in contrast to three isoforms detected in the embryogenic cell line '10', in ts11 as well as in ts11R predominantly the 32-kD endochitinase isoform 1 is present (indicated by an arrow). These results imply that the amount of 32-kD endochitinase as detected in ts11 embryo culture medium via immunoblots of denaturing SDS-PAA gels represents mainly the 32-kD endochitinase isoform 1.

To follow the amount of 32-kD endochitinase in ts11 embryo-culture medium with time, medium proteins from 5 mL ts11 embryo cultures were precipitated with ethanol, separated on denaturing SDS-PAA gel, blotted onto membrane and incubated with anti-32-kD endochitinase serum (Figure 6A). During the first 10 days after embryo culture initiation, more 32-kD endochitinase was present in the culture medium at the permissive temperature of 24°C than at the nonpermissive temperature of 32°C (Figure 6A, first and second lanes).
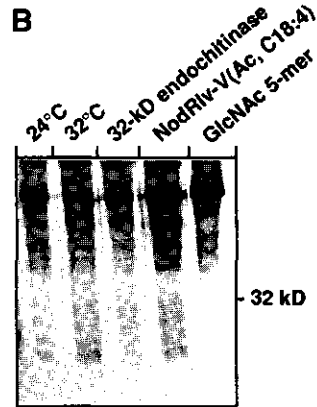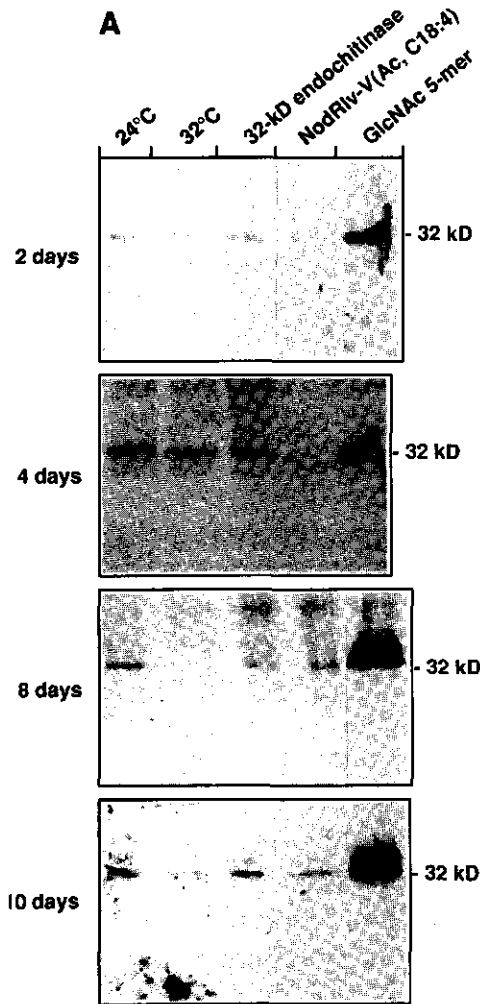
Figure 6. Effect of Temperature and the Addition of 32-kD Endochitinase and NodRlv-V(Ac, C18:4) on the Amount of 32-kD Endochitinase in ts11 Embryo Culture Medium, Followed with Time.

(A) Immunoblots with medium proteins from 1.7 mL of 2-, 4-, 8- and 10-day-old ts11 embryo cultures respectively, incubated with anti-32-kD endochitinase serum. First lane, culture grown at 25°C; second lane, culture grown 32°C; third lane, culture supplemented with 0.06 µg 32-kD endochitinase, grown at 32°C ; fourth lane, culture supplemented with 10^{-8} M NodRlv-V(Ac, C18:4), grown at 32°C; fifth lane, culture supplemented with 10^{-8} M N-acetylglucosamine pentamer and grown at 32°C.

(B) Effect of temperature and addition of 32-kD endochitinase, NodRlv-V(Ac, C18:4) or N-acetylglucosamine pentamer to ts11 embryo culture medium on the amount of proteins in the culture medium. Silver stained SDS-polyacrylamide gel with ts11 medium proteins from 8-day-old ts11 embryo cultures. Each lane contains ± 2 µg of total proteins. Lanes are as in (A).

The corresponding silver-stained gels (Figure 6B) show that the different culture conditions do not affect the overall amount or composition of proteins present in the culture medium. The amount of 32-kD endochitinase present in an unfractionated mixture such as used here,

is too low to be visualized by silverstaining. Densitometric scans of the immunoblots showed that at the nonpermissive temperature at day 2, 1.5-fold, at day 4, 1.1-fold, at day 8, 7-fold and at day 10, 5-fold less 32-kD endochitinase was present in the culture medium than at the permissive temperature (Figure 6A, first and second lanes). Addition of 32-kD endochitinase to a 5 mL ts11 embryo culture resulted in a 1.2-fold increase at 2 days and a 3-fold increase in the amount of the 32-kD endochitinase at 4, 8 or 10 days of culture (Figure 6A, third lane). At 20 days the amount of 32-kD endochitinase in the culture medium grown at the nonpermissive temperature is again similar to that of a culture grown at the permissive temperature (Figure 1B). These experiments described above show that up to 10 days after ts11 embryo culture initiation less 32-kD endochitinase is present in a culture grown at the nonpermissive temperature than in a culture grown at the permissive temperature.

The next question was whether a correlation could be found between the time period in which ts11 is temperature sensitive, the lowered amount of 32-kD endochitinase in the culture medium and the rescue activity of the 32-kD endochitinase. Since the temperature-sensitive period in ts11 was originally estimated on the basis of progression into heart and torpedo embryo stages (Lo Schiavo et al., 1990), while we have mainly studied the effect of the 32-kD endochitinase on the number of ts11 globular embryos formed (De Jong et al., 1993), the previously determined window of temperature sensitivity is difficult to compare with our more recently obtained results. Therefore it was decided to compare only the time

Table 3. Transient Susceptibility of ts11 Cells to 32-kD Endochitinase and NodRlv-V(Ac, C18:4)

No. of days[a]	No. of ts11 embryos[b] 32 kD[c]	No. of ts11 embryos[b] buffer[d]	No. of ts11 embryos[b] NodRlv-V(Ac, C18:4)[e]	No. of ts11 embryos[b] DMSO[f]
2	11	3	17	9
5	12	3	11	6
7	5	3	6	4
9	3	3	5	6
12	3	2	3	3

[a] The numbers represent the days of culture after embryo culture initiation

[b] The numbers represent the number of ts11 proembryogenic masses and ts11 globular embryos obtained per 10,000 suspension cells.

[c] The 32-kD endochitinase was diluted in 25 mM Tris-HCl, pH 7.2, 125 mM KCl.

[d] Ts11 control culture with 0.25 mM Tris-HCl, pH 7.2, 1.25 mM KCl.

[e] NodRlv-V(Ac, C18:4) was dissolved in 100% DMSO.

[f] Ts11 control culture with 0.25% DMSO.

Table 4. Dose-Dependence of the Stimulation of ts11 Embryo Formation by the 32-kD Endochitinase

Concentration (M)	No. of ts11 embryos per 10,000 cells[a]
5×10^{-10}	–
1×10^{-9}	–
2×10^{-9}	–
4×10^{-9}	2
8×10^{-9}	1
1×10^{-8}	–

[a] The effect of addition of an optimal amount of 32-kD endochitinase is expressed as the number of proembryogenic masses and globular embryos obtained per 10,000 suspension cells in one experiment.

period during which addition of the 32-kD endochitinase was able to stimulate ts11 embryo formation with the time period during which less 32-kD endochitinase was present in ts11 embryo culture medium. Addition of the 32-kD endochitinase to a ts11 embryo culture grown at the non-permissive temperature promoted embryo formation when added within 5 days after embryo culture initiation. Upon addition at or after 7 days of culture, promotion of ts11 embryo formation was no longer possible (Table 3). These results suggest that the 32-kD endochitinase is only effective in promoting ts11 somatic embryogenesis in the first week of culture. Promotion of ts11 embryo formation upon addition of 32-kD endochitinase fits with the observation that during the first 10 days, less 32-kD endochitinase was present in the culture medium than at the permissive temperature. To illustrate that the effect on ts11 embryo development of the 32-kD endochitinase in the culture medium is indeed concentration dependent, increasing amounts of the 32-kD endochitinase were added to ts11 embryo cultures. This showed that, in line with previous findings, there exists an *optimum* in the number of ts11 embryos formed (Table 4 and De Jong et al., 1992).

We have previously reported that NodRlv-V(Ac, C18:4), a lipo-oligosaccharide produced by *Rhizobium*, was able to promote ts11 embryo formation with a similar efficiency as the 32-kD endochitinase (De Jong et al., 1993). To test if NodRlv-V(Ac, C18:4) is able to promote ts11 embryo formation within the same time period as the 32-kD endochitinase does, NodRlv-V(Ac, C18:4) was also tested in the time-resolved experiment (Table 3). When NodRlv-V(Ac, C18:4) was added within 0 to 5 days after embryo culture initiation, promotion of ts11 embryo formation was observed, while upon addition at or after 7 days such promotion of ts11 embryo formation was not observed. This result showed that NodRlv-V(Ac, C18:4) and the 32-kD endochitinase are able to stimulate ts11 embryo formation within the same time period.

To recapitulate, the results obtained here suggest that the hampered ts11 embryo formation is an effect of a lower amount of the 32-kD endochitinase in ts11 culture medium. The ts11 embryo formation can be stimulated by addition of the 32-kD endochitinase itself or of NodRlv-V(Ac, C18:4). How can the effect of NodRlv-V(Ac, C18:4) on the ts11 embryo formation be explained? Either NodRlv-V(Ac, C18:4) stimulates the secretion of the 32-kD endochitinase or NodRlv-V(Ac, C18:4) compensates for the lower concentration of a putative signal molecule released by the 32-kD endochitinase from a larger plant-produced precursor, as proposed previously (De Jong et al., 1993). To examine the effect of NodRlv-V(Ac, C18:4) on the amount of 32-kD endochitinase in the culture medium, the amount of 32-kD endochitinase in the culture medium was followed in a ts11 culture with NodRlv-V(Ac, C18:4) added in the same series of experiments as described above (Figure 6A). The results show that at 2 and 4 days after embryo culture initiation there was no detectable effect of 10^{-8} M NodRlv-V(Ac, C18:4) on the level of 32-kD endochitinase in the ts11 culture medium (Figure 6A, fourth lane). In contrast, at 8 and 10 days after embryo culture initiation, an increase of the amount of 32-kD endochitinase was observed, to a level comparable to that of cultures supplemented with the 32-kD endochitinase. Densitometric scans of the immunoblots showed that addition of NodRlv-V(Ac, C18:4) results in a 5-fold increase at day 8 and a 1.6-fold increase at day 10 of the 32-kD endochitinase in the culture medium when compared to untreated controls (Figure 6A). From these results it can be concluded that initially, up to about a week after embryo-culture initiation, NodRlv-V(Ac, C18:4) does not increase the amount of 32-kD endochitinase in the culture medium, but that after 8 days there is an increase, when compared to intreated controls.

Chitin oligomers are known to be elicitors of chitinases (Roby et al., 1987; Koga et al., 1992). To examine if addition of a chitin oligomer also increases the 32-kD endochitinase concentration, 10^{-8} M N-acetylglucosamine pentamer was added to a ts11 culture in the same series of experiments as described above (Figure 6A). In contrast to NodRlv-V(Ac,C18:4), addition of 10^{-8} M N-acetylglucosamine pentamer resulted in a 10- to 40-fold increase in the amount of 32-kD endochitinase, compared to control cultures (Figure 6A, fifth lane), but does not affect ts11 development (De Jong et. al., 1993). Apparently, during the first 5 days, ts11 embryogenesis is dependent on a fairly precise optimum level of the 32-kD endochitinase in the medium, and the 10- to 40-fold increase of the 32-kD endochitinase far exceeds the concentration range of 1-9 nM in which the 32-kD endochitinase was reported to have an effect on ts11 embryo formation (De Jong et al., 1992).

Another explanation for the effect of NodRlv-V(Ac, C18:4) on ts11 embryogenesis would be that it serves as an analogue of the putative signal molecule that is released by the 32-kD endochitinase from a larger plant-produced precursor molecule. To determine whether the 32-kD endochitinase is able to hydrolyse modified N-acetylglucosamine oligomers, [^{14}C]-acetate-labeled NodRlv-V(Ac, C18:4) was incubated with 32-kD endochitinase and

the mixture was separated by TLC (Figure 7). This showed that the 32-kD endochitinase releases an N-acetylglucosamine dimer from a substituted pentamer. Thus, if a substrate for the 32-kD endochitinase is present in the plant, then this putative substrate can also be a modified N-acetylglucosamine chain.
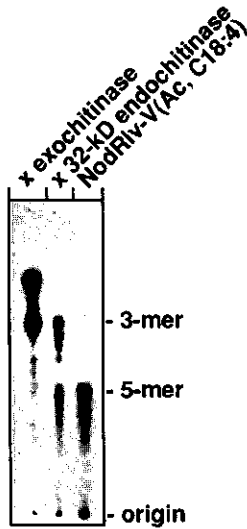
Figure 7. TLC Analysis of [^{14}C]-Acetate-Labeled NodRlv-V(Ac, C18:4) Subjected to Chitinase Digestion.

First lane, NodRlv-V(Ac, C18:4) digested with an exochitinase from *Serratia marcescens*; second lane, NodRlv-V(Ac, C18:4) digested with 32-kD endochitinase from carrot; third lane, untreated NodRlv-V(Ac, C18:4).

Non-embryogenic ts11 cells have an abberant morphology

Although the original ts11 line was selected on the basis of its temperature-arrest in embryogenesis, at both the permissive and nonpermissive temperature, a clear phenotypical difference was observed between ts11 and the '10'-line. The non-embryogenic elongated cells in a ts11 embryo culture differ morphologically from those in a '10'-line embryo culture. Even at the permissive temperature ts11 cells appear to have a less compact cell wall, with frequent protrusions, while the cells are larger than comparable wild-type cells (Figure 8). This abberant morphology is quite prominent in the vacuolated, elongated ts11 cells in an embryo culture; it is less apparent in the small cytoplasm-rich cells (Figure 8E) or in ts11 cells grown in a high-density suspension culture (not shown). Addition of the 32-kD endochitinase to ts11 culture medium does have no effect on ts11 cell walls (not shown), suggesting that the abberant morphology is not caused by the lower amount of the 32-kD endochitinase in the culture medium.
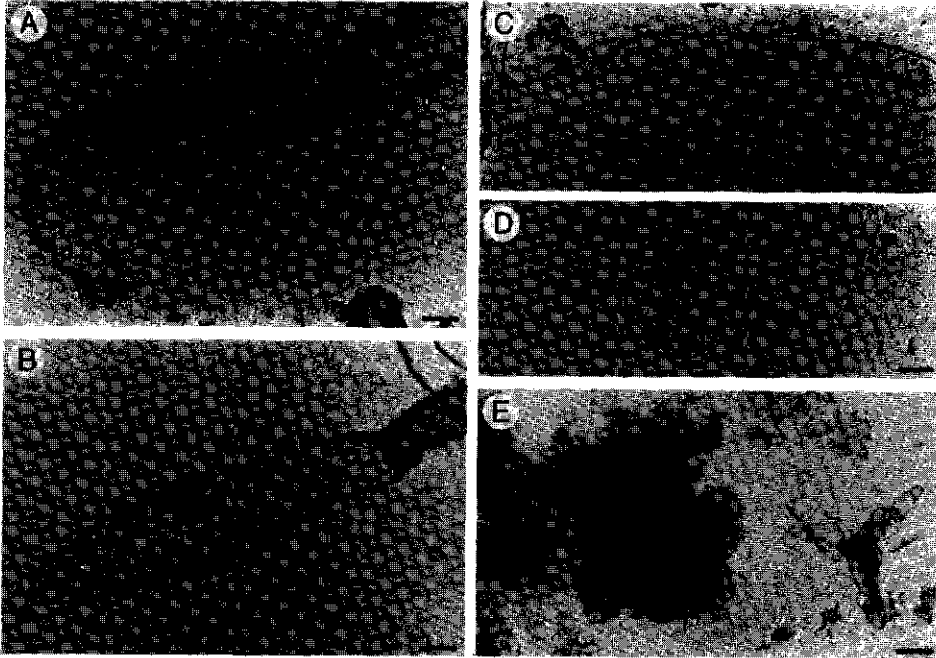
Figure 8. Comparison of Elongated Cells in ts11 and '10'-line Embryo Cultures Grown at the Permissive Temperature (25°C).

(A) and (B), elongated cells in a ts11 embryo culture. Bar = 25 μm.

(C) and (D), elongated cells in a '10'-line embryo culture. Bar = 25 μm.

(E), cluster of cytoplasma rich cells in a ts11 embryo culture (indicated by an arrow). Bar = 100 μm.

DISCUSSION

In this chapter we have addressed the question whether the basis of the previously observed stimulating effects of the 32-kD endochitinase on ts11 embryo development could be determined. Therefore, presence of the 32-kD endochitinase in ts11 cultures was examined and a 32-kD protein reactive with the anti-32-kD endochitinase serum was isolated form ts11 cultures. Biochemical properties of this protein, such as pI, enzymatic activity and mobility on denaturing or non-denaturing gels, and stimulating effect on ts11 embryo formation were compared with that of wild-type 32-kD endochitinase. In addition, protein secretion and amount of 32-kD endochitinase in the culture media of ts11 cultures was compared. The results show that a fully functional 32-kD endochitinase is present in ts11 cultures at the permissive temperature as well as at the nonpermissive temperature. At the nonpermissive

temperature, the concentration of the 32-kD endochitinase in ts11 embryo culture medium during the first 10 days of culture was shown to be lower than at the permissive temperature. The results obtained here suggest that the transient decrease of the 32-kD endochitinase concentration in ts11 embryo culture medium at the nonpermissive temperature causes the arrest in ts11 embryo development and that the rescue effect of the 32-kD endochitinase addition to the culture medium can be explained by increasing the 32-kD endochitinase concentration in the culture medium to a level seen in cultures at the permissive temperature. The importance of a precisely regulated amount of 32-kD endochitinase in ts11 embryo culture medium was further demonstrated by the concentration-dependent optimum of the rescue effect of the 32-kD endochitinase addition to ts11 embryo culture medium. Collectively, these results suggest that during the first week of ts11 embryogenesis, ts11 embryo formation is dependent on a fairly precise optimum level of the 32-kD endochitinase in the culture medium.

Morphological comparison of wild-type and ts11 embryo cultures grown at the permissive temperature of 25°C showed some striking differences, also at the permissive temperature. Besides the described abberant morphology of the cell walls of ts11 elongated cells, several characteristics of ts11 have been reported previously. The number of embryos developing per 10,000 cells in ts11 embryo cultures is approximately 1% of the number of embryos developing in wild-type embryo cultures (De Jong et al., 1993). So, apart from the arrest at globular stage, there is also a reduced capability for producing embryogenic cells. Another difference is the time needed for development of proembryogenic masses into plantlets. Four weeks after initiation of an embryo culture grown at the permissive temperature, most ts11 embryos are still in the globular stage, while in a similar wild-type embryo culture most embryos have developed into plantlets. In addition, it has been noted that at the nonpermissive temperature fucose incorporation into secreted proteins is affected in ts11 and a disturbed distribution pattern of glycosylated proteins in ts11 globular embryos was observed (Lo Schiavo et al., 1990). Hence, it is clear that the ts11 mutation is quite pleiotropic and its influence is not restricted to the globular/heart transition of ts11 somatic embryos.

The results presented here show that the total amount of proteins secreted into ts11 embryo culture medium is, both quantitatively and qualitatively not greatly affected at the nonpermissive temperature. Yet, the amount of 32-kD endochitinase in ts11 embryo culture medium is lower at the nonpermissive temperature than at the permissive temperature. The period during which the amount of the 32-kD endochitinase in ts11 embryo culture medium is low, covers the same period ts11 cells respond to addition of the 32-kD endochitinase or NodRlv-V(Ac, C18:4). In addition, the finding that addition of the 32-kD endochitinase or NodRlv-V(Ac, C18:4) only affects ts11 embryo development when added within the first 5 days of culture, seems to be in line with the previously reported temporary temperature

sensitivity of the development of ts11 embryos (Lo Schiavo et al., 1990). A possible explanation for the lower amount of the 32-kD endochitinase in ts11 embryo culture medium at the nonpermissive temperature would be that the defective protein secretion is restricted to a specific class of proteins amongst which the 32-kD endochitinase. Because it is difficult to reconcile all of the observed ts11 phenotypical changes with the transient reduction in the amount of the 32-kD endochitinase, the secretion of other proteins may well be affected too. Examples of mutants with a temperature-sensitive protein secretion are the yeast *sec* mutants (Novick et al., 1980). Most of the *sec* mutations block transport of proteins from the endoplasmic reticulum to the Golgi apparatus or from mature secretory vesicles to the plasma membrane (Novick et al., 1981). The product of the SEC14 gene is required for sustained transport of secretory glycoproteins from a late Golgi compartment and has been identified as a phosphatidyl-inositol transfer protein (Bankaitis et al., 1990), that stimulates yeast Golgi function by controlling the phospholipid content of the Golgi membranes (Cleves et al., 1991). But, upon treatment of ts11 cells with *N*-acetylglucosamine pentamers, the amount of 32-kD endochitinase is induced to a high level and 20 days after culture initiation the amount of 32-kD endochitinase in ts11 embryo cultures is similar to ts11 embryo cultures at the permissive temperature. Therefore, a defective secretion, such as found in the yeast *sec* mutants, of the 32-kD endochitinase is unlikely. Taken together, these results imply that in ts11 the regulation of the amount of the 32-kD endochitinase is affected rather than the secretion of the 32-kD endochitinase itself.

The question was raised whether the stimulation of ts11 embryo formation by addition of NodRlv-V(Ac, C18:4) to the culture medium could have been due to an increase of the 32-kD in the medium. The results have shown that in the first week after culture initiation, this is not the case. Because the 32-kD endochitinase concentration was shown to be most critical in the first week, we believe that the effect of the Nod-factor is not simply by increasing the amount of the 32-kD endochitinase. However, Western blotting is not the most sensitive technique to determine precisely the concentration of a protein, and clearly more sensitive methods are needed to provide more definite answers. The raise in amounts of the 32-kD endochitinase observed after day 7, in cultures treated with NodRlv-V(Ac, C18:4) may have been due to *N*-acetylglucosamine-containing fragments released from Nod-factor molecules by chitinase activity in present in the medium.

Chitin oligomers are known to be elicitors of chitinases (Roby et al., 1987; Koga et al., 1992). Recently, Felix et al. (1993) have shown that tomato suspension cells respond to chitin oligomers with a rapid, transient alkalinization of the culture medium and changes in protein phosphorylation. Changes in membrane permeability are characteristic for elicitor treated cells and are thought to be part of the signal chains mediating the induction of defense responses (Boller, 1989; Dixon and Lamb, 1990; Scheel et al., 1991). It is unknown if socalled defense-related proteins are induced in the ts11 embryo culture medium upon

addition of *N*-acetylglucosamine pentamers, nor if alkalinization of the culture medium occurs or protein phosphorylation changes. However, in view of the strong induction of the amount of 32-kD endochitinase in the ts11 embryo culture medium, also defense responses may be induced. Among these can be the synthesis of extensins, glycine-rich proteins and peroxidases, proteins that change the extracellular matrix, and cell wall reinforcement by deposition of callose and lignin (see Bowles, 1990). The effect of these defense responses on ts11 embryo development is unknown. However, in view of the presumed role of the primary cell wall in somatic embryogenesis (Van Engelen and De Vries, 1993), changes introduced in cell walls may affect somatic embryo development. The failure to promote ts11 embryo formation by addition of *N*-acetylglucosamine-pentamers could be due, not to the high 32-kD endochitinase concentration, but to other, possibly defense-related processes.

Taken together, ts11 embryo development can be stimulated in two ways: 1. By addition of the 32-kD endochitinase to an optimal concentration in the culture medium, and 2. By addition of NodRlv-V(Ac, C18:4), which may mimic its putative enzymatic product. The concentration-dependent optimum in ts11 embryo development seen both for the 32-kD endochitinase and the Nod-factor is reminiscent of the responses seen in other physiological responses of plant cells to growth regulators such as IAA (Ballas et al., 1993) or NAA (Barbier-Brygoo et al., 1990). NAA induces variation of the transmembrane potential difference in tobacco protoplasts and is thought to be mediated by binding to a receptor (see Napier and Venis, 1990). Also lipo-oligosaccharides like NodRlv-V(Ac, C18:4) are proposed to be perceived by the plant cell via specific receptors (see Dénarié and Cullimore, 1993). It is therefore tempting to speculate that the putative product of the 32-kD endochitinase acts as a ligand for a specific receptor, setting in motion events that are essential for the initiation of somatic embryogenesis in ts11.

METHODS
Plant Material and Cell Cultures
Carrot seedlings were grown from "Flakkese" SG 766 Trophy seeds, kindly donated by Zaadunie, Enkhuizen, The Netherlands. Embryogenic '10'-line suspension cultures were initiated from cut seedling roots and propagated in 2,4D containing B5 medium as described previously (De Vries et al., 1988). The embryogenic wild-type carrot suspension culture A[+] and the temperature-sensitive variant ts11 were maintained as described by Giuliano et al. (1984). For the initiation of '10'-line and A[+] or ts11 embryo cultures, suspension cells were sieved through nylon meshes between 50 and 125 μm or 70 and 170 μm respectively and were diluted to 20,000 cells per mL or 2500 clusters per mL respectively in hormone free B5 medium.

Ts11 Embryo Rescue Assays

Rescue assays were performed with newly initiated ts11 embryo cultures as described previously (De Jong et al., 1992; 1993). NodRlv-V(Ac, C18:4) was dissolved in 100% DMSO. Control experiments were performed with buffer (0.25mM Tris-HCl, pH 7.2, 1.25 mM KCl, final concentration) or solvent (DMSO, 0.25% final concentration) which were diluted in basal medium, autoclaved for 15 min and subsequently added to the ts11 cells.

Protein Purification, Silver Staining and Immunological Techniques

Purification of 32-kD endochitinase was performed as described previously by De Jong et al. (1992).

Electrophoresis of proteins on 12.5% denaturating polyacrylamide gels was performed according to Laemmli (1970). Silver staining of protein gels was performed essentially as described by Blum et al. (1987), except that formaldehyde was omitted from the fixing solution and ethanol was used instead of methanol. Two-dimensional gel electrophoresis was performed as described by De Vries et al.(1982). Nondenaturing gel electrophoresis was performed according to Laemmli (1970) with omission of ß-mercapto ethanol and sodium dodecyl sulfate (SDS) from the sample buffer and the SDS from the gel and the running buffer.

For anti-32-kD endochitinase serum production in balb/c mice, the 32-kD endochitinase was further purified from fast-protein liquid chromatography fractions, obtained as described by De Jong et al., 1992, by preparative SDS-PAGE, followed by brief staining with Coomassie Brilliant Blue and excision of the protein band from the gel. The gel strip was ground in PBS and the mice were immunized by three intraperitoneal injections of 2 to 5 µg of protein at 4-weeks intervals. For anti-32-kD endochitinase serum production in rabbit, the 32-kD endochitinase was isolated on a large scale according to Kragh et al. (to be published). The rabbit was immunized by four intraperitoneal injections of 35 µg of protein at 4-weeks intervals. Antisera were assayed in a dilution of 1:3000 for mouse antisera and 1:1000 for the rabbit antiserum on polyvinylidene difluoride (PVDF)-blots (IMMOBILON) from 12.5% (SDS)-polyacrylamide mini-gels, using alkaline phophatase-conjugated goat-anti-mouse IgG for detection as described by Sterk et al. (1991). Only the use of the rabbit antiserum has been metioned in the figure legends, otherwise the rabbit antiserum was used.

Two different methods were applied to detect glycostructures in proteins immobilized on PVDF-membranes. a) Lectin staining of the proteins on blots according to Clegg (1982). Biotinylated concanavalin A (Sigma), specific for glucose and mannose, was used in a concentration of 5 µg/mL in PBS followed by an incubation with 1 µg/mL avidin-labeled peroxidase (Sigma) in PBS. The blots were subsequently stained for peroxidase activity using 0.2 mg/mL 3-amino-9-ethylcarbazole in 50 mM NaAc, pH 5.0, 0.01% H_2O_2. The binding reaction was in the presence of 0.5 M glucose in case a binding-inhibition

experiment was performed. b) Periodate oxidation of vicinal hydroxyl groups in carbohydrates to aldehyde groups, followed by conjugation to digoxigenin-succinyl-ε-amido-caproic-acid-hydrazide. Digoxigenin-labeled glycoconjugates were visualized by indirect immunostaining using alkaline-phosphatase-conjugated antibody specific for digoxigenin and the colour reagents from a glycan-detection kit (Boehringer).

To follow the amount of 32-kD endochitinase in ts11 culture medium with time, 5 mL ts11 embryo cultures, with eventually added compounds, were grown in 5-cm-diameter TC dishes (Greiner). For every time point, medium proteins were precipitated out of the culture medium with 4 volumes of ethanol overnight at -20°C. Proteins were collected by centrifugation at 17.000 g for 30 minutes. The pellet was dried under vacuum and solved in 80 µl 1x sample buffer (Laemmli, 1970). Proteins in 25 µl were separated on a 12.5% SDS-PAA gel and immunoblotted.

[^{3}H]-Leucine Labeling of Medium Proteins

The number of clusters in a 7-day-old embryo culture was counted and the culture was concentrated to 20,000 clusters/mL. To 1 mL embryo culture 20 µl [^{3}H]-leucine was added (1µCi/µl) and incubated overnight either at 25°C or 32°C. The labeled medium proteins were precipitated by addition of 2.5 volumes of ethanol at 4°C and 1µg of BSA as carrier, collected by centrifugation for 30 min at 10,000g, washed with ethanol, and resuspended in 30 µl of 1x SDS-PAGE sample buffer (Laemmli, 1970). Incorporation was determined as hot TCA-insoluble material, precipitated from 3 µl out of the 30 µl.

Digestion of NodRlv-V(Ac, C18:4) with Chitinases and Chitinase Assays

Chitinase assays were performed as described by Trudel and Asselin (1989). NodRlv-V(Ac, C18:4) has been randomly labeled with [^{14}C]-acetate according to Heidstra et al. (submitted). The randomly [^{14}C]-labeled NodRlv-V(Ac, C18:4) was incubated with 300 ng of purified 32-kD endochitinase or with 2 mU *Serratia marcescens* exochitinase (Sigma) for 3 hours at 25°C in 10 mM NaPi pH 6.0 buffer in a total volume of 20 µl. After incubation, the samples were analyzed on thin layer chromatography plates (Merck, Darmstadt, Germany) using n-butanol/acetic acid/water =6:2:2 as solvent system as described by Spaink et al. (1992).

REFERENCES

Bankaitis, V.A., Aitken, J.R., Cleves, A.E., and Dowhan, W. (1990). An essential role for a phospholipid transfer protein in yeast Golgi function. Nature 347, 561-562.

Ballas, N., Wong, L.-M., and Theologis, A. (1993). Identification of the auxin-responsive element, *AuxRE*, in the primary indolacetic acid-inducible gene, *PS-IAA4/5*, of pea (*Pisum sativum*). J. Mol. Biol. 233, 580-596.

Barbier-Brygoo, H., Guern, J., Ephritikhine, G., Shen, W.H., Maurel, C., and Klämbt, D. (1990). The sensitivity of plant prototplasts to auxins: Modulation of receptors at the plasmalemma. In: Plant Gene Transfer, C. Lamb and R. Beachy, eds (New York: Alan R. Liss, Inc.), pp. 165-173.

Blum, H., Beier, H., and Gross, H.J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8, 93-99.

Boller, T. (1989). Primary signals and second messengers in the reaction of plants to pathogens. In: Second Messengers in Plant Growth and Development, D.J. Morré, and W. Boss, eds (New York, A.R. Liss, inc.), pp. 227-255.

Bowles, D.J. (1990). Defense-related proteins in higher plants. Annu. Rev. Biochem. 59, 873-907.

Clegg, J.C.S. (1982). Glycprotein detection on nitrocellulose transfers of electrophoretically separated protein mixtures using concanavalin A and peroxidase: Application to arenavirus and flavivirus proteins. Anal. Biochem. 127, 389-394.

Cleves, A.E., McGee, T.P., Whitters, E.A., Champion, K.M., Aitken, J.R., Dowhan, W., Goebl, M., and Bankaitis, V.A. (1991). Mutations in the CDP-choline pathway for phospholipid biosynthesis bypass the requirement for an essential phospholipid transfer protein. Cell 64, 789-800.

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

De Jong, A.J., Cordewener, J., Lo Schiavo, F., Terzi, M., Vandekerckhove, J., Van Kammen, A., and De Vries, S.C. (1992). A carrot somatic embryo mutant is rescued by chitinase. Plant Cell 4, 425-433.

De Jong, A.J., Heidstra, R., Spaink, H.P., Hartog, M.V., Meijer, E.A., Hendriks, T., Lo Schiavo, F., Terzi, M., Bisseling, T., Van Kammen, A., and De Vries, S.C. (1993). *Rhizobium* lipooligosaccharides rescue a carrot somatic embryo mutant. Plant Cell 5, 615-620.

Dénarié, J., and Cullimore, J. (1993). Lipo-oligosaccharide nodulation factors: A minireview. New class of signaling molecules mediating recognition and morphogenesis. Cell 74, 951-954.

De Vries, S.C., Springer, J., and Wessels, J.G.H. (1982). Diversity of abundant mRNA sequences and patterns of protein synthesis in etiolated and greened pea seedlings. Planta 156, 129-135.

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

Dixon, R.A., and Lamb, C.J. (1990). Molecular communication in interactions between plants and microbial pathogens. Annu. Rev. Plant Physiol. Plant Mol. Biol. **41**, 339-367.

Felix, G., Regenass, M., and Boller, T. (1993). Specific perception of subnanomolar concentrations of chitin fragments by tomato cells: induction of extracellular alkalinization, changes in protein phosphorylation, and establishment of a refractory state. Plant J. **4**, 307-316.

Gavish, H., Vard, A., and Fluhr, R. (1992). Suppression of somatic embryogenesis in *Citrus* cell cultures by extracellular proteins. Planta **186**, 511-517.

Giuliano, G., Lo Schiavo, F., and Terzi, M. (1984). Isolation and developmental characterization of temperature sensitive carrot cell variants. Theor. Appl. Genet. **67**, 179-183.

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

Koga, D., Hirata, T., Sueshige, N., Tanaka, S., and Ide, A. (1992). Induction patterns of chitinases in yam callus by inoculation with autoclaved *Fusarium oxysporum*, ethylene, and chitin and chitiosan oligosaccarides. Biosci. Biotech. Biochem. **56**, 280-285.

Kreuger, M., and Van Holst, G.J. (1993). Arabinogalactan proteins are essential in somatic embryogenesis of *Daucus carota* L. Planta **189**, 243-248.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680-685.

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

Montreuil, J., Bouquelet, S., Debray, H., Fournet, B., Spik, G., and Strecker, G. (1986). Glycoproteins. In Carbohydrate Analysis. A Practical Approach, M.F. Chaplin and J.F. Kennedy, eds (Oxford: IRL Press), pp 143-205.

Napier, R.M., and Venis, M.A. (1991). From auxin-binding protein to plant hormone receptor? Trends Biochem. Sci. **16**, 72-75.

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

Novick, P., Field, C., and Schekman, R. (1980). Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell **21**, 205-215.

Novick, P., Ferro, S., and Schekman, R. (1981). Order of events in the yeast secretory pathway. Cell **25**, 461-469.

Roby, D., Gadelle, A., and Toppan, A. (1987). Chitin oligosaccharides as elicitors of chitinase activity in melon plants. Biochem. Biophys. Res. Commun. **143**, 885-892.

Scheel, D., Colling, C., Hedrich, R., Kawalleck, P., Parker, J.E., Sacks, W.R., Somssich, I.E., and Hahlbrock, K. (1991). Signals in plant defense gene activation. In: Advances in Molecular Genetics of Plant-Microbe Interactions, H. Hennecke, and D.P.S. Verma, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), **1**, pp. 373-380.

Spaink, H.P., Aarts, A., Stacey, G., Bloemberg, G.V., Lugtenberg, B.J.J. and Kennedy, E.P. (1992). Detection and separation of *Rhizobium* and *Bradyrhizobium* Nod metablites using thin layer chromatography. Mol. Plant Microbe Ineract. 5, 72-80.

Sterk, P., Booij, H., Schellekens, G.A., Van Kammen, A., and De Vries, S.C. (1991). Cell-specific expression of the carrot EP2 lipid transfer protein gene. Plant Cell 3, 907-921.

Trudel, J., and Asselin, A. (1989). Detection of chitinase activity after polyacrylamide gel electrophoresis. Anal. Biochem. 178, 362-366.

Van Engelen, F.A. and De Vries, S.C. (1993). Secreted proteins in plant cell cultures. In Morphogenesis in Plants: Molecular Approaches, NATO-ASI Series, K.A. Roubelais-Angelakis, ed (New York: Plenum Publishers), pp. 181-200.

chapter 5

The carrot 32-kD endochitinase (EP3) is a class IV chitinase

Anke J. de Jong, Marcel A.J. Toonen, Nandor Bucherna, Ab van Kammen
and Sacco C. de Vries
submitted

ABSTRACT

In this chapter experiments are presented aimed at identifying a 32-kD endochitinase cDNA. Three highly homologous cDNA clones, Extracellular Protein (EP) 3A, B and C were isolated. The cDNA clone EP3B fully overlapped cDNA clone EP3A. The 32-kD endochitinase peptide sequences differed in 3 out of 32 amino acids with the corresponding deduced amino acid sequence of the cDNA clones. The 32-kD endochitinase and the EP3B encoded peptide was recognized by an antiserum raised against the 32-kD endochitinase. Comparison of the deduced amino acid sequence of EP3B and EP3C with amino acid sequences of class I, II and IV chitinases revealed that the EP3 sequences share homology with the primary structure of class IV chitinases, suggesting that the 32-kD endochitinase is a class IV chitinase. The EP3 genes are members of a multigene family and are expressed in most parts of the plant including flowers, immature seed and endosperm.

INTRODUCTION

Chitinase genes have been found in all higher plant species investigated, and their expression is under complex developmental and hormonal control (Shinshi et al., 1987; Lotan et al., 1989; Samac and Shah, 1991). Primarily based on their induction after pathogen attack and their catalytic properties, chitinases have been suggested to be involved in the defense mechanism against plant pathogens (Boller, 1987; 1988). However, chitinase activity is also markedly increased by wounding, ethylene and a range of abiotic stresses (Mauch and Staehelin, 1989; Kurosaki et al., 1989; Broglie et al., 1986), suggesting that their role is not restricted to the degradation of chitin-containing cell walls of pathogens. According to their primary structure, plant chitinases have been divided into at least four distinct classes (Shinshi et al., 1990; Collinge et al., 1993). Class I chitinases are enzymes with a highly conserved main structure and a N-terminal cysteine-rich domain that is separated from the main structure by a hypervariable, glycine and proline rich, 'hinge' region. The cysteine-rich domain is homologous to hevein, wheat germ agglutinin and the proteins encoded by the Win 1 and Win 2 genes of potato (Lucas et al., 1985; Stanford et al., 1989) and is thought to serve as a chitin-binding domain (Chrispeels and Raikhel, 1991). Furthermore, class I chitinases have a C-terminal extension of about six amino acids that is both necessary and sufficient for vacuolar localization (Neuhaus et al., 1991). Class II chitinases lack the N-terminal cysteine-rich domain, the hinge region and the C-terminal extension, while the amino acid sequence homology to the main structure of class I chitinases is greater than 40% (Perrakis et al., 1993). Class III chitinases show no sequence similarity to enzymes in class I or II and are thought to have evolved from another ancestor gene than the classes I, II, and IV (Perrakis et al., 1993). They lack a cysteine-rich domain and have structural homologies to a bifunctional lysozyme/chitinase from *Parthenocissus quinquifolia* (Bernasconi et al., 1987; Métraux et al., 1989). Class IV chitinases contain a cysteine-rich domain and a

conserved main structure which resembles those found in class I and II chitinases, but the proteins are significantly smaller due to the presence of four deletions, of which the position is highly conserved in all class IV chitinases. Only a few of the plant chitinases that have been studied belong to the rare class of plant exochitinases (Roby and Esquerre-Tugaye, 1987; Kurosaki et al., 1989; Kirsch et al., 1993). Sofar it is unknown if these exochitinases belong to one of the classes I-IV or to a not yet identified class of plant chitinases.

In several studies it has been observed that somatic embryogenesis in carrot cell cultures is dependent on proteins that are secreted into the culture medium (De Vries et al., 1988a; Cordewener et al., 1991; De Jong et al., 1992; 1993; Kreuger and Van Holst, 1993). One of the secreted proteins with a positive effect on somatic embryogenesis has been identified as a 32-kD acidic endochitinase (De Jong et al., 1992). This 32-kD endochitinase has been observed to have two effects: 1. stimulation of the formation of ts11 globular embryos of the temperature sensitive (ts) variant ts11 (De Jong et al., 1993) and 2. the ability to lift the temperature sensitive arrest in the transition of globular to heart stage ts11 embryos (De Jong et al., 1992). The 32-kD endochitinase was first identified as such by the amino acid sequence of two peptides derived from it (De Jong et al., 1992). These peptides were from a region sharing extensive homology between class I, II and IV chitinases, and did not allow classification of the 32-kD endochitinase into one of these three classes. In order to obtain the complete coding sequence of the EP3 endochitinase mRNA and to determine the expression pattern of the EP3 gene both in cell cultures and in plants, an attempt was made to isolate the corresponding full-length cDNA. Southern analysis showed that the 32-kD endochitinase EP3 gene is member of a small gene family. Northern analysis indicated that the EP3 chitinase genes are expressed throughout the carrot plant including flowers, immature seeds and the endosperm.

RESULTS

Isolation of EP3 cDNA

The amino acid sequences of two tryptic peptides of the 32-kD endochitinase were used to derive two degenerate oligonucleotide probes (Figure 1). The following rules of thumb were applied in the design of the oligos: 1) two unique bases at both ends, 2) in case of two possibilities, incorporation of either one or the other, 3) in case of three or four possibilities, incorporation of inosine, 4) no more than a six fold degeneracy per oligonucleotide and 5) a length of around 30 bases. This resulted in two different oligonucleotide mixtures; probe I with 64 different oligonucleotides of 29 bases including 3 inosines, and probe II with 48 different oligonucleotides, also of 29 bases including 4 inosines. Screening of an amplified λZAPII cDNA library was performed with both oligonucleotide mixtures simultaneously. This yielded one positive clone with an insert length of 197 base pairs, designated EP3A, out of 225,000 phage plaques. DNA and amino acid sequence analysis showed that half of

```
Peptide 1:
 P    L    Q    L    T    F    N    Y    N    Y    I    D    A    G    K
Pro  Leu  Gln  Leu  Thr  Phe  Asn  Tyr  Asn  Tyr  Ile  Asp  Ala  Gly  Lys

oligo I:    5'- ACI TTT AAT TAT AAT TAT ATI GAT GCI GG -3'
                     C   C   C   C   C       C


Peptide 2:
 S    N    Q    F    D    G    L    N    N    P    D    I    V    A    S    D    A
Ser  Asn  Gln  Phe  Asp  Gly  Leu  Asn  Asn  Pro  Asp  Ile  Val  Ala  Ser  Asp  Ala

oligoII:             5'- AAT AAT CCI GAT ATT GTI GCI TCI GAT GC -3'
                          C   C       C   C                   C
                                          A

PCR-oligo (antisense)   3'- TTA TTA GGI CTI TAI CAI CG CCTAGG CCC -5'
                            G   G                       |
                                                   BamHI site
```

Figure 1. Amino Acid Sequence of Two 32-kD Endochitinase Tryptic Peptides and Composition of the Derived Oligonucleotides. I = inosine.

the encoded peptide had homologous to plant endochitinases, while the other half had no homology at all with plant endochitinases. This abrupt change in homology suggests that EP3A is a mosaic cDNA clone (a cDNA clone containing fragments of different cDNAs ligated to each other). The part homologous to chitinases contained part of the amino acid sequence of peptide 1 and the entire amino acid sequence of peptide 2. Among the 26 overlapping amino acids, 23 amino acids were identical (Figure 2). A lysine and a glutamine in the peptide sequence were exchanged for an asparagine and an aspartic acid respectively in the cDNA derived sequence. In the next cDNA library screening, the EP3A insert was preferred as a probe instead of the mixture of oligonucleotides, because the screening could be performed at more stringent conditions. Screening of an additional 160,000 recombinant phages of the same library with the EP3A insert yielded one clone, designated EP3B, with


```
peptides .    .    .    .    .    .    .    .    K    .    .    Q    .    .    .    .    .    .    .    .
          N    Y    N    Y    I    D    A    G    N    S    N    D    F    D    G    L    N    N    P    D
EP3A      AACTATAACTACATTGATGCCGGAAACAGTAATGATTTTGATGGGCTAAACAATCCGGAC
                 220                      240                    260

peptides .    .    .    .    .    .
          I    V    A    S    D    A    V    V    S    F    K    T    A    L    W    Y    W    K
EP3A      ATCGTGGCTTCAGATGCAGTGGTGTCCTTCAAGACGGCCTTGTGGTACTGGAAG
                 280                    300                    320
```

Figure 2. Alignment of the EP3A-Deduced Amino Acid Sequence with the Peptide Amino Acid Sequences. Nucleotide sequence and the deduced amino acid sequence of the EP3A cDNA was aligned with the amino acid sequence of the 32-kD endochitinase-derived peptides.

```
                              20                  40                  60
            S   Y   S   S   F   G   A   S   G   S   A   D   D   S   K   R   E   I   A   A
EP3C        TCCTACTCCTCGTTCGGAGCTTCTGGTTCTGCTGATGATTCTAAGCGTGAGATTGCAGCC
EP3B        ............................
            .   .   .   .   .   .   .   .   .


                              80                 100                 120
            F   F   A   H   A   T   H   E   T   G   Y   F   C   H   K   E   E   T   N   G
EP3C        TTTTTCGCCCATGCTACCCATGAGACCGGATACTTCTGTCATAAAGAAGAGACAAATGGT
EP3B        ............................................................
            .   .   .   .   .   .   .   .   .   .   .   .   .   .


                             140                 160                 180
            R   D   K   N   Y   C   E   S   K   A   G   Y   P   C   N   A   N   V   K   Y
EP3C        CGCGACAAGAATTACTGCGAATCAAAGGCAGGGTACCCATGCAACGCAAATGTGAAGTAT
EP3B        .................................G..........................
            .   .   .   .   .   .   .   .   .   .   .   .   .   .


                             200                 220                 240
peptides            .   .   .   .   .   F   .   .   .   .   .   .   .   K   .
            F   G   R   G   P   L   Q   L   T   W   N   Y   N   Y   I   D   A   G   K   S
EP3C        TTTGGCCGCGGCCCTCTTCAGCTAACATGGAACTATAACTACATTGATGCCGGAAAGAGT
EP3B        .........................................................C...
            .   .   .   .   .   .   .   .   .   .   .   .   .   .   N   .


                             260                 280                 300
peptides    .   Q   .   .   .   .   .   .   .   .   .   .
            N   E   F   D   G   L   N   N   P   D   I   V   A   S   D   A   V   V   S   F
EP3C        AATGAGTTTGATGGGCTAAACAATCCCGACATCGTGGCTTCAGATGCCGTGGTGTCCTTC
EP3B        .....T...................G...................A.............
            .   D   .   .   .   .   .   .   .   .   .   .   .


                             320                 340                 360
            S   R   A   L   W   Y   W   K   V   K   V   Q   S   V   T   T   Q   G   F   G
EP3C        AGCAGGGCCTTGTGGTACTGGAAGGTCAAAGTTCAGTCTGTCACTACCCAAGGTTTCGGG
EP3B        .AG.CA...................................T..C.G.............
            K   T   .   .   .   .   .   .   .   .   .   .   S   .   .   .


                             380                 400                 420
            A   T   I   R   A   I   N   S   I   E   C   N   G   G   S   P   D   A   V   N
EP3C        GCAACCATCCGTGCCATCAACAGCATTGAGTGCAACGGTGGAAGCCCGGATGCTGTCAAC
EP3B        ............................................................
            .   .   .   .   .   .   .   .   .   .   .   .   .   .


                             440                 460                 480
            S   R   V   S   L   Y   N   P   Y   C   S   K   F   G   V   A   P   G   D   N
EP3C        TCGCGTGTTAGTCTTTACAACCCGTATTGTAGTAAATTTGGTGTAGCCCCTGGTGATAAT
EP3B        ........C...........TT.A.....C...............A.............
            .   .   .   .   .   .   .   .   .   .   .   .   .   .


                             500                 520                 540
            Q   R   C   *
EP3C        CAGCGGATGCTAGAACATCAAAGTGCTACTATATTTCTATTTCAATTATGCCATAATAAGT
EP3B        ............G...................
            .   .   .   *


                             560                 580                 600
EP3C        CCTCCCAGAGAGAAGAAATTGTACTATCAATAAACACATAACCTTCCCTAGTAAAAAAAA
```

an insert length of 740 base pairs. Sequence analysis of the deduced amino acid sequence of EP3B showed that 482 base pairs code for a protein homologous to plant chitinases and included the two 32-kD endochitinase peptide sequences completely (Figure 3). The nucleotide sequences of EP3B corresponding with EP3A differ in only one nucleotide; at position 306 a deoxyguanylate in the EP3B sequence is exchanged by a deoxyadenylate, but this alteration does not result in an amino acid change (not shown). The remainder of the EP3B sequence had no open reading frame and shared no homology with plant chitinases, indicating that EP3B was also a mosaic clone. With a 230 base pair NruI-StyI fragment of EP3B, that contained the overlapping sequence of the two peptide sequences, another 150,000 phages of the nonamplified λZAPII cDNA library were screened. This yielded 1 new clone, designated EP3C, with an insert length of 620 base pairs. The open reading frame in the EP3C cDNA clone is a 490 nucleotides fragment with a poly(A) tail, but it lacks the 5'-end of the messenger. The open reading frame is preceeded by a stopcodon, suggesting that also EP3C is a mosaic clone. The open reading frame of the EP3C cDNA clone represents a 163 amino acid peptide. The calculated molecular mass of that peptide is 15.2 kD, suggesting that, in view of the 32 kD for which the messenger should encode, at least another 500 nucleotides were missing. Based on the total number of phages screened (> 535,000) it was considered unlikely that the available libraries contain a full-size cDNA clone corresponding to the 32-kD endochitinase (EP3). A final attempt to isolate the missing 5'-part of the clones was made by direct PCR amplification of the entire λZAPII library. A defined primer (Figure 1) corresponding to the EP3 peptides and a primer corresponding to the polylinker of the Bluescript vector DNA were used to amplify the 5'-end of the EP3B clone. Amplified products that hybridized to a EP3B fragment were cloned into Bluescript vectors. The insert lengths and position of internal restriction sites were compared to those of EP3B (not shown). All PCR products showed a restriction pattern identical to the EP3B clone, confirming that no full-length EP3 cDNA clones are present in the λZAPII library.

Alignment of the nucleotide sequences of EP3B and EP3C and the deduced amino acid sequences of these cDNA clones showed that the clones are highly homologous to each other (Figure 3); on the amino acid level as well as on the nucleotide level the homology between EP3B and EP3C is 96%. Five of the 19 differences between the nucleotide sequence of EP3B and EP3C result in an amino acid exchange. The EP3B- and EP3C-deduced amino acid sequences overlap with the amino acid sequences of the 32-kD endochitinase-derived peptides. Only 3 out of these 32 amino acids are different. Both

Figure 3. Comparison of the EP3B and EP3C cDNA Clones.
Alignment of the nucleotide sequences of the chitinase-homologous fragments of EP3B and EP3C. The distinct nucleotides and amino acids of EP3B are depicted below the EP3C nucleotide sequence. The different amino acids are underlined and printed in bold.

```
          1                                                         60
beanIV:   MGNKLVLVLVAVALV*MG-PKNVSAQNCG-------CAEGLCCSQYGYCGTGEDYC
bean I:   MKKNRMMMMIWSVGVVWMLLLVGGSYG*EQ-CGRQAGGALCPGGNCCSQFGWCGSTTDYC
tob II:     MEFSGSPMALFCCVFFLFLTGSLA*Q---------------------------
barley:      MRSLAVVVAVVATVAMAIGTARG*----------------------------

          61                                                        120
EP3C:                                                              SYS
beanIV:   G-GCQQGPCTTASPPPSNNVNADILTADFLNGIIDQAGSGCAGKNFYTRDAFLSALNSYT
bean I:   GPGCQSQCGGPSPAPTDLSAL-ISRSTFDQMLKHRNDGA-CPAKGFYTYDAFIAAAKAYP
tob II:   ----------------GIGSIVTSDLFNEMLKNRNDGR-CPANGFYTYDAFIAAANSFP
barley:   ----------------SVSSIVSRAQFDRMLLHRNDGA-CQAKGFYTYDAFVAAAAFP

          121                                                       180
EP3C:     SFGTSGSADDSKREIAAFFAHATHET-G------Y--F----CHKEETNGRDK-NYC-E-
beanIV:   DFGRVDSEDDSKREIAAAFAHFTHET-G------H--F----CYIEEIDGASK-DYCDEE
bean I:   SFGNTGDTATRKREIAAFLGQTSHETTGGWATAPDGPYAWGYCFVRER--NP-STYCSA-
tob II:   GFGTSGDDTARRKEIAAFFGQTSHETTGGSLSAE--PFTGGYCFVR-Q--NDQSD-----
barley:   GFGTTGSADAQKREVAAFLAQTSHETTGGWATAPDGAFAWGYCFKQERGASS--DYC---

          181                                                       240
EP3C:     SK-AGYPCNANVKYFGRGPLQLTWNYNYIDAGKSNEFD-GLNNPDIVASDAVVSFSRALW
beanIV:   SI-AQYPCSSSKGYHGRGPIQLSWNFNYGPAGSANNFDHGLGAPETVSNDVVVSFKTALW
bean I:   TP--QFPCAPGQQYYGRGPIQISWNYNYGQCGRAIGAD-LLGDPDLVASDATVSFDTAFW
tob II:   -----------RYYGRGPIQLTNNYEKA--GNAIRQD-LVNNPDLVATDATISFKTAIW
barley:   TPSAQWPCAPGKRYYGRGPIQLSHNYNYGPAGRAIGVD-LLANPDLVATDATVGFKTAIW

          241                                                       300
EP3C:     YWKVKVQS--------V---------------TTQGFGATIRAINS-IECNGGSPDAVNS
beanIV:   YWM---QHVRP-----VI--------------NQGFGATIRAINGALECDGANPTTVQA
bean I:   FWMTP-QSPKPPSCNAVATGQWTPSADDQRAGRVPGYGVITNIINGGLECGHGEDDRIAD
tob II:   FWMTP-QDNKPSSHD-VIIGSWTPSAADQSANRAPGCGVITNIINGGIECGVGPNAAVED
barley:   FWMTA-QPPKPSSH-AVIAGQWSPSGADRAAGRVPGFGVITNIINGGIECGHGQDSRVAD

          301
EP3C:     RVSLYNSYCSKFGVAPGDNQRC*
beanIV:   RVNYYTEYCRQLGVATGDNLTC*
bean I:   RIGFYKRYCDILGVSYGANLDCYSQRPSAPPKLRLPSFHTVINNH*
tob II:   RIGYYRRYCGMLNVAPGDNLDCYNQRNFAQG*
barley:   RIGFYKRYCDILGVGYGNNLDCYSQRPFA*
```
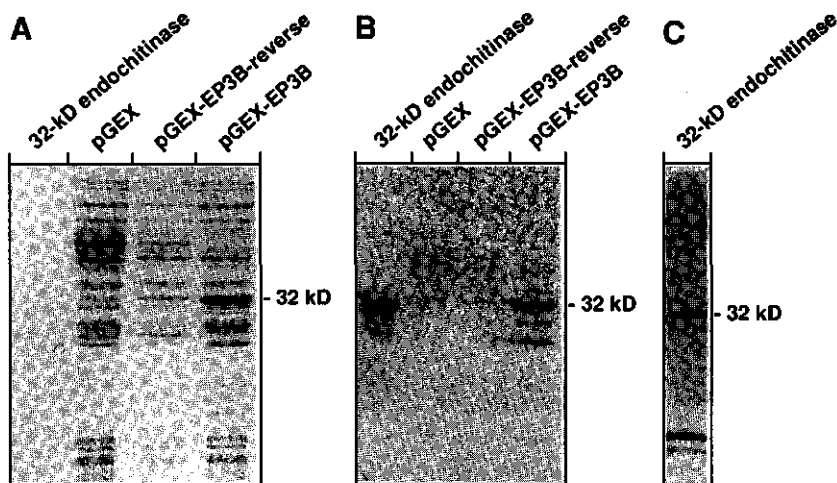
Figure 5. Correlation Between EP3B-Encoded Polypeptide and 32-kD Endochitinase.

(A) Coomassie Brilliant Blue stained SDS-PAA gel with total protein produced by *E.coli* containing pGEX, PGEX-EP3B-reverse or pGEX-EP3B plasmid.

(B) Immunoblot of gel shown in (A), incubated with anti-32-kD endochitinase serum.

(C) Recognition of the 32-kD endochitinase by antiserum against EP3B-encoded fusion protein on immunoblot.

cDNAs have at the same position a tryptophane instead of a phenylalanine. A glutamine was replaced by an aspartic acid in EP3B and by a glutamic acid in EP3C, and a lysine was replaced by an aspartic acid in EP3B but not in EP3C. The exchange from a glutamine to a glutamic acid is the result of the change of the third base, while the change from a phenylalanine to a tryptophane results from a two base change.

Comparison of the EP3B and EP3C deduced amino acid sequences with known amino acid sequences of plant chitinases from class I, II and IV chitinases, revealed that the EP3 sequences contain three out of four deletions at conserved positions that are characteristic for class IV chitinases (Figure 4). The fourth deletion is not in the sequenced part of the chitinase. To determine whether EP3B indeed encodes a class IV chitinase, an EP3B-

Figure 4. Amino Acid Sequence Homology of the EP3C-Encoded Peptide with Plant Chitinases.

Alignment of the EP3C-deduced amino acid sequence with a bean class IV chitinase (bean IV) (Margis-Pinheiro et al., 1991), with a bean class I chitinase (bean I) (Broglie et al., 1986), with a tobacco class II chitinase (tob II) (Payne et al., 1990) and with a class II chitinase from barley seeds (barley) (Leah et al., 1991). Gaps were introduced for optimal alignment.

encoded fusion protein was obtained. The 230 base pair NruI-StyI fragment from EP3B was cloned into a pGEX-2T expression vector. While the pGEX-2T encodes the 26-kD glutathione S-transferase, the pGEX-EP3B recombinant plasmid produced a fusion protein of 34 kD (Figure 5A), which was expected on the basis of the derived 32-kD endochitinase amino acid sequence. On an immunoblot, the EP3B-encoded fusion protein was recognized by an antiserum raised against the class IV chitinase 4 from sugar beet (Mikkelsen et al., 1992), but not by the antiserum raised against the class I chitinase K from barley (Kragh et al., 1991) (Figure 6B), similar to the results obtained for the 32-kD endochitinase purified from the culture medium (Figure 6A). Previous observations have shown that class I and class IV chitinases are immunologically distinguishable (Mikkelsen et al., 1992). Therefore our results confirm that the EP3B-encoded peptide corresponds to a class IV chitinase. Additional immunological evidence indicate that EP3B corresponds to a protein highly homologous to the 32-kD endochitinase. Antisera against the 32-kD endochitinase isoform 1 or 2 from the culture medium, raised in rabbit or mouse respectively, both recognized the
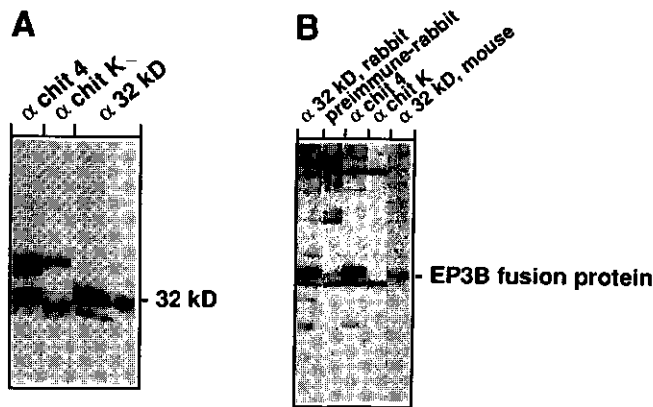

Figure 6. Correlation Between Recognition of 32-kD Endochitinase and EP3B-Encoded Fusion Protein by Different Antisera.

(A) Immunoblots of total proteins of suspension culture incubated with antisera raised against chitinase 4 from sugar beet, against chitinase K from barley, or against the 32-kD endochitinase. First three lanes, 20 μg of total protein; fourth lane, purified 32-kD endochitinase.

(B) EP3B-encoded fusion protein recognized by different antisera on immunoblot. Each lane contains total proteins produced by *E.coli* containing pGEX-EP3B plasmid. First lane, anti-32-kD endochitinase serum, raised in rabbit; second lane, pre-immune of anti-32-kD endochitinase serum of the same rabbit; third lane, anti-chitinase 4 (sugar beet) serum; fourth lane, anti-chitinase K (barley) serum; fifth lane, anti-32-kD endochitinase serum, raised in mouse.
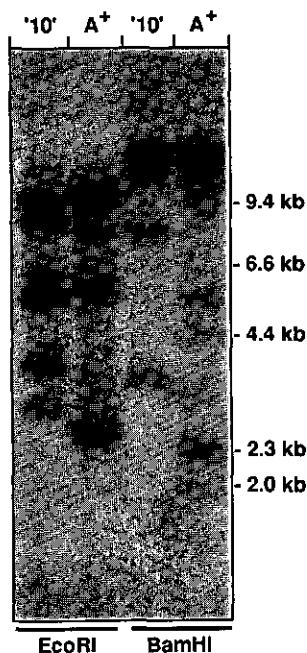
Figure 7. Determination of the Number of EP3-Related Genes per Carrot Genome.
Southern blot analysis of carrot genomic DNA, isolated from '10'-line or A$^+$ cells, digested with EcoRI or BamHI. The filter was hybridized with [^{32}P]-labeled EcoRI insert of EP3A.

glutathione S-transferase-EP3B fusion protein, but not the glutathione S-transferase alone (Figure 5B and 6B). Preimmune sera from the same animals did not recognize the fusion protein. Vice versa, a rabbit antiserum raised against the EP3B-encoded fusion protein, recognized the purified 32-kD endochitinase isoform 1 on an immunoblot of a SDS-PAA gel (Figure 5C). Taken together, these results show that the 32-kD endochitinase is a class IV chitinase and that the isolated cDNA clones correspond to highly homologous isoforms of the 32-kD endochitinase.

The EP3 Gene is a Member of a Gene Family

To determine the number of EP3 genes that are present in the Daucus genome, DNA gel blot analysis was performed. Total genomic DNA from both cultivar Flakkese ('10'-line) and S. Valery (A$^+$) was digested with EcoRI or BamHI, both of which do not cut within the partial EP3 sequences obtained, and subjected to Southern blot hybridization with the 200 base pair EcoRI insert of EP3A as probe. Four to seven hybridizing bands were observed in the lanes with '10'-line DNA (Figure 7, lanes 1 and 3), while in the lanes with A$^+$ DNA three to four hybridizing bands were observed (Figure 7 lanes 2 and 4). The part of the EP3A-EcoRI insert that is not homologous to chitinases does not influence the hybridization pattern, since the same hybridization pattern was observed with an EP3B fragment containing solely the chitinase encoding part (results not shown). The results of the genomic Southern blot show that the 32-kD endochitinase EP3 gene is member of a small gene family.

-103-

EP3 Gene Expression

The expression pattern of the EP3 genes was examined by RNA gel blot analysis of total RNA of different cell cultures with a NruI-SpeI 465 base pair fragment from EP3C as probe (Figure 8). Because of the high homology between the different EP3 clones, all the different EP3 mRNAs will be detected with the EP3C probe and may reflect the expression of all EP3
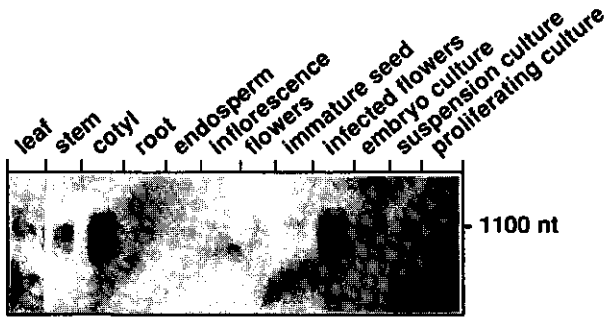

Figure 8. RNA Gel Blot Analysis of EP3-Messengers in Tissue Cultures and Plants.

For each lane 10 µg of total RNA was loaded on gel. The probe was a 465 base pairs, [^{32}P]-labeled, NruI-SpeI fragment of EP3C. Expression of EP3 genes was detected in embryo culture, and suspension- and proliferating cell cultures of the '10'-line. Plant parts that were investigated were leaf, cotyl, root, endosperm, young flower, old flower, immature seeds and mildew-infected flower. In all parts EP3 gene expression was detected, with an induced expression in cotyls and in mildew infected flowers.

genes. The expression of EP3 genes was detected in embryo cultures, and in proliferating cell cultures as well as in suspension cell cultures of the '10'-line. The EP3C probe hybridized to mRNAs of about 1100 nucleotides, a length that is expected for a protein of 32-kD. No significant difference in expression level of the EP3 genes could be observed between embryo cultures or proliferating- or suspension cell-cultures.

To determine EP3 gene expression in carrot plants, RNA gel blot analysis was perfomed with total RNA isolated from different parts of the plant (Figure 8). A very low expression of EP3 genes was detected in seedling leaves, endosperm, flower buds, flowers and immature seeds, a slightly increased expression in stem and a high expression in seedling cotyls and mildew-infected flowers. These expression studies show that the EP3 mRNAs are present in all parts of the plant including flowers and immature seeds, and that they are fairly low abundant messengers, except in seedling cotyls and mildew-infected flowers.

DISCUSSION

In this chapter our attempts to isolate a full-length cDNA corresponding to the 32-kD endochitinase are described. The deduced amino acid sequences of the isolated EP3 cDNAs differ in 3 out of 32 amino acids when compared with the 32-kD endochitinase-derived peptide sequences. The EP3B-encoded peptide is immunologically related to the 32-kD endochitinase and shares homology with the primary structure of class IV chitinases. The EP3 genes are members of a gene family which are expressed throughout the whole plant, including flowers, immature seeds and endosperm.

Based on their primary structure, Shinshi et al. (1990) and Collinge et al. (1993) proposed a division of plant chitinases into four classes. Class IV chitinases contain a cysteine-rich domain and a conserved main structure which resembles those of class I chitinases but are significantly smaller due to four deletions. The molecular mass of class IV chitinases varies from 27 kD to 30 kD, their pI is variable, their expression is not restricted to specific parts of the plant and they are expressed constitutively or only upon pathogen attack (Margis-Pinheiro et al., 1991; Araki et al., 1992; Huyhn et al., 1992; Mikkelsen et al., 1992; Rasmussen et al., 1992). In other words, class IV chitinases do not seem to have a common characteristic, except for their conserved main structure. The homology of the EP3 cDNA-encoded protein with class IV chitinases and the recognition of the EP3B cDNA-encoded fusion protein as well as the 32-kD endochitinase by an anti-class IV chitinase serum suggests that the 32-kD endochitinase is a class IV chitinase. Does the 32-kD endochitinase meet the requirements for a class IV chitinase? Recently, full-length cDNA clones of the 32-kD endochitinase isoform 1 and 2 have been obtained by Karsten Kragh by using 3' and 5' Rapid Amplification of cDNA Ends (RACE) and PCR (to be published). The mRNA corresponding to isoform 1 encodes a 26-kD protein with a chitin-binding domain at the amino terminus. This combination of a low molecular mass (26 kD) and a chitin-binding domain is characteristic for class IV chitinases. The discrepancy found between the molecular mass as calculated from the cDNA-deduced amino acid sequence (26 kD) and as determined by SDS-PAGE (32 kD) has also been found for the bean class IV chitinase PR4. Its calculated molecular mass is 27.5 kD, while its mobility on a SDS-PAA gel corresponds with a 32-kD protein (Margis-Pinheiro et al., 1991).These various results clearly demonstrate that the 32-kD endochitinase is a class IV chitinase.

Surprisingly, the EP3A-, B- and C-deduced amino acid sequences differed at exactly the same positions, suggesting that they were isoforms of the 32-kD endochitinase. Recently, it was found that there are 3 to 5 isoforms of the 32-kD endochitinase present in embryo- and in cell-suspension culture medium. These could be distinguished from each other on immunoblots of nondenaturing PAA gels (see chapter 4). The 32-kD endochitinase isoform 1 has been used to rescue ts11 embryo development, while the peptide sequences were obtained from the original 32-kD endochitinase isoform 2 (De Jong et al., 1992 and Figure

1). The amino acid sequence of the 32-kD endochitinase, including peptides covering the regions shown in Figure 1 for isoform 2, were recently obtained by Karsten Kragh (to be published). These amino acid sequences deviate from isoform 2, but also from the deduced amino acid sequence of the isolated EP3 cDNA clones. Thus, we have to conclude that the isolated EP3 cDNA clones described here do not correspond to the 32-kD endochitinase isoforms 1 or 2, but to one of the other isoforms . The fact that in our work no cDNA clones were found that fully matched the peptide sequences of either isoform 1 or 2, could be due to different factors. First, the excised plasmid from positive phage plaques was often instable and difficult to multiply in *E. coli*. It was observed that bacteria transformed with some EP3 positive plasmids grew very slowly, plasmid clones derived from the same positive phage plaque differed in some cases from each other and all of the isolated EP3 cDNA clones were mosaic clones. Possibly the EP3 gene protein product is toxic to *E.coli*, resulting in a positive selection for mosaic EP3 cDNA clones. This may also be an explanation for the fact that no positive clones were detected by immunoscreening of an expression library. Secondly, the 32-kD endochitinase is member of a multigene family. Possibly the stability of the messengers differs for each isoform.

The finding of several closely related 32-kD endochitinases is not exceptional, since most plant chitinases, except for an acidic and a basic chitinase from Arabidopsis (Samac et al., 1990) and an acidic class IV chitinase PR4 from bean (Margis-Pinheiro et al., 1991), are encoded by multigene families. Also the presence of a number of highly homologous mRNAs, as found for the carrot EP3 mRNAs, has often been observed for chitinases (Broglie et al., 1986; Payne et al., 1990; Neale et al., 1990). Other examples of proteins that are encoded by several highly homologous RNAs are the chalcone synthases (Koes et al., 1989) and 4-coumarate:CoA ligases (Lozoya et al., 1988; Uhlmann and Ebel, 1993). Two isoenzymes of 4-coumarate:CoA ligase from parsley (Lozoya et al., 1988), involved in phenylpropanoid metabolism, reveal a similar protein pattern on 2D-PAA gel as was observed for the 32-kD endochitinase isoforms 1 and 2 (chapter 2 and 4), and are biochemically indistinguishable from each other. They have a pI of 4.9 and 5.0 respectively and their corresponding mRNAs are 99% homologous. The biological significance of the existence of nearly identical mRNAs is unknown. They are most likely the result of the presence of different alleles, or of a very recent gene duplication.

Expression of the carrot EP3 genes is detectable at a low level in cell cultures and plant, including flowers, immature seeds and endosperm and, at a high level in cotyls. The low expression level of EP3 genes in the plant is in line with the inability to detect the 32-kD endochitinase in total protein extracts of seedlings on immunoblots (not shown). The similar level of EP3 gene expression in embryo cultures, and proliferating- and suspension cell-cultures indicates that neither cell density nor the presence of 2,4-D in the culture medium greatly affects the expression of EP3 genes. Immunological data indicate that the 32-kD

endochitinase is present in embryogenic as well as in non-embryogenic cell cultures (Chapter 5), suggesting that EP3 gene expression is not restricted to embryogenic cells or somatic embryos. Whether the different 32-kD endochitinase isoforms are produced by the same cells is presently unknown. An answer to this question will be quite difficult to obtain in view of the high homology of the different isoforms.

METHODS

Plant Material and Cell Cultures

Embryo cultures and suspension cell-cultures were initiated and maintained as described in chapter 4. Proliferating cell-cultures were initiated in a similar way as embryo cultures, except that the 50 to 125 μm sieved fraction, enriched for proembryogenic masses, was diluted in B5 medium with 2 μM 2,4-D instead of hormone free B5 medium. Carrot seedlings were grown from 'Flakkese' SG766 Trophy seeds and dissected when the first leaves had fully expanded. Endosperms were collected from seeds after imbibing for two hours.

Library Screening

The λZAPII cDNA library (Stratagene) was prepared against poly(A)$^+$-RNA from suspension culture cells (sieved <170 μm) and embryo culture cells (sieved <300 μm) using oligo(dT) and random primers.

The melting temperature of hybrids between an oligonucleotide and EP3 cDNA was calculated to be approximately 64°C for mixture I and 70°C for mixture II. For the cDNA library screening with a mixture of both oligonucleotide mixtures the hybridization temperature was calculated at 50°C, as it should be more than 10°C below the calculated melting temperature. After 3-4 hr prehybridisation at 50°C in 6x SSC, 5x Denhardt's (Maniatis et al., 1982), 0.5% SDS and 200 μg/mL denatured salmon sperm DNA the filters hybridized for 24 hr at 50°C in the same solution containing [^{32}P]-labeled oligonucleotides. Then the filters were washed twice for 15 min in 2x SSC at 50°C, once for 30 min in 2x SSC, 0.1% SDS at 50°C and autoradiographed. Screening with [^{32}P]-labeled DNA probes was performed according to standard conditions (Maniatis et al., 1982). Positive plaques were purified by a second screening. The Bluescript plasmid containing the insert was excised in vivo from the λZAPII phage exactly as described in the manufacturer's protocol. Inserts were further subcloned in Bluescript plasmids using standard procedures (Maniatis et al., 1982). Plasmid DNA was denatured by alkali and sequenced wiht the dideoxy chain termination method using T7 polymerase or Taq polymerase according to manufacturer's protocol (Boehringer). In addition, polymerase chain reaction (PCR) was used with a 29-mer oligonucleotide primer specifically for peptide 2 derived from the 32-kD endochitinase and the KS sequence primer (5' CGAGGTCGACGGTATCG 3') to amplify EP3 sequences

from the λZAPII library upstream the EP3 primer sequence. For this purpose, 10^8 plaque forming units of the library were extracted once with an equal volume of phenol/chloroform (1:1). Phage DNA was precipitated from the aqueous phase by adding an equal volume of 2-propanol, pelleted, and washed with 0.5 mL of 70% ethanol. The DNA was dried and redissolved in 100 μL of 1xPCR buffer (20mM Tris-HCl, pH 8.3, 25 mM KCl, 1.5 mM $MgCl_2$, 0.05 μg/mL of Tween 20, 100 μg/mL of gelatin) containing 300 ng of both primers. After addition of 2 units of *Taq* polymerase (Perkin-Elmer Cetus Instruments), 1 cycle of (2 min at 92°C, 1 min at 60°C, 3 min at 72°C), 48 cycles at (1 min at 92°C, 1 min at 60°C, 3 min at 72°C) and 1 cycle at (1 min at 92°C, 1 min at 60°C, 10 min at 72°C) were performed. The reaction products were analyzed on 1.5% agarose gels and subcloned in pBluescript SK- (Stratagene) for sequence analysis.

Oligonucleotide Synthesis and Labeling

Oligonucleotides were synthesized using ß-cyanoethyl phosphramidites with a Cyclone DNA synthesizer (Biosearch Inc.). Oligonucleotides were labeled with T4-polynucleotide kinase and [γ-^{32}P]dATP in 50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 5 mM DDT, 0.1 mM spermidine and 0.1 mM EDTA. Unincorporated [γ-^{32}P]dATP was removed on a Sephadex G25-column.

Fusion-Protein Expression and Immunological Techniques

A 227 base pairs NruI-StyI fragment from EP3B cDNA was cloned in both orientations into plasmid pGEX-2T (Amrad) (Smith end Johnson, 1988), which allows high level expression of an *E. coli* fusion protein with the C-terminus of *Schistosoma japonicum* glutathione S-transferase protein (Smith and Johnson, 1988). For fusion protein production cells were grown in LB-medium (Maniatis et al., 1982), induced with 0.1 mM IPTG and harvested after three to four hours. Proteins were analyzed on SDS-PAA gels after Coomassie Brilliant Blue staining and on immunoblots incubated with mouse EP3 antiserum as described in chapter 4. The fusion protein remains undissolved in a 1% SDS solution. A crude purification was done by precipitating the fusion protein in 1% SDS. For antiserum production, 5x50 μg of partially purified fusion protein was injected subcutaneously into a rabbit at intervals of 6 weeks.

Genomic DNA Isolation and DNA Gel Blot Analysis

Genomic DNA was isolated from cultured '10'-line suspension cells initially following the protocol for RNA isolation described by De Vries et al. (1988b). After removal of RNA by LiCl precipitation, DNA was precipitated by adding ethanol to 70% to the supernatant. After centrifugation the DNA pellet was washed twice with 70% ethanol, dried in a vacuum desiccator and dissolved in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. DNA was digested

under standard conditions and electrophoresed on a 0.8% agarose gel. After 2 min 302 nm UV irradiation of the gel, DNA was transferred to GeneScreen-*Plus* membrane with 10x SSC. The blot was hybridized with the [^{32}P]-labeled EP3A-EcoRI insert in 6x SSC (1x SSC is 0.15 M NaCl, 0.015 M sodium citrate), 5x Denhardt's (1x Denhardt's solution is 0.02% Ficoll, 0.02% PVP, 0.02% BSA), 0.5% SDS and 50 µg/mL salmon sperm DNA at 65°C. The blots were washed twice in 2x SSC, 0.1% SDS at 65°C and exposed to Kodak X-Omat AR film.

RNA Gel Blot Analysis
Total RNA was isolated from cultured cells and plant tissues as described by de Vries et al. (1988b). RNA samples were electrophoresed on 1.75% agarose gel containing formamide according to Maniatis et al. (1982) and transferred to GeneScreen membrane (Du Pont-New England Nuclear) according to the manufacturer's instructions. Hybridization with the [^{32}P]-labeled NruI-SpeI fragment of the EP3C cDNA was carried out in 5x SSC, 2x Denhardts, 1% SDS, 50% formamide and 100 µg salmon sperm DNA for 16 hr at 42°C.

REFERENCES
Araki, T., Funatsu, J., Kuramoto, M., Konno, H., and Torikata, T. (1992). The complete amino acid sequence of Yam (*Dioscorea japonica*) chitinase. A newly identified acidic class I chitinase. J. Biol. Chem. **267**, 19944-19947.

Bernasconi, P., Locher, R., Pilet, P.E., Jollès, J., and Jollès, P. (1987). Purification and N-terminal amino-acid sequence of a basic lysozyme from *Parethenocissus quinquifolia* cultured *in vitro*. Biochim. Biophys. Acta **915**, 254-260.

Boller, T. (1987). Hydrolytic enzymes in plant disease resistance. In Plant-Microbe Interactions, Molecular and Genetic Perspectives, Volume 3, T. Koshuge and E.W. Nester, eds (New York: Macmillan), pp. 385-411.

Boller, T. (1988). Ethylene and the regulation of antifungal hydrolases in plants. In Surveys of Plant Molecular and Cell Biology, Volume 5, B.J. Miflin, ed (Oxford: Oxford University Press), pp. 145-174.

Broglie, K.E., Gaynor, J.J., Broglie, R.M. (1986). Ethylene-regulated gene expression: Molecular cloning of the genes encoding an endochitinase from *Phaseolus vulgaris*. Proc. Natl. Acad. Sci. USA **83**, 6820-6824.

Collinge, D.B., Kragh, K.M., Mikkelsen, J.D., Nielsen, K.K., Rasmussen, U., and Vad K. (1993). Plant chitinases. Plant J. **3**, 31-40.

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

Chrispeels, M.J., and Raikhel, N.V. (1991). Lectins, lectin genes, and their role in plant defense. Plant Cell **3**, 1-9.

De Jong, A.J., Cordewener, J., Lo Schiavo, F., Terzi, M., Vandekerckhove, J., Van Kammen, A., and De Vries, S.C. (1992). A carrot somatic embryo mutant is rescued by chitinase. Plant Cell **4**, 425-433.

De Jong, A.J., Heidstra, R., Spaink, H.P., Hartog, M.V., Meijer, E.A., Hendriks, T., Lo Schiavo, F., Terzi, M., Bisseling, T., Van Kammen, A., and De Vries, S.C. (1993). *Rhizobium* lipooligosaccharides rescue a carrot somatic embryo mutant. Plant Cell **5**, 615-620.

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

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

Huynh, Q.K., Hironaka, C.M., Levine, E.B., Smith, C.E., Borgmeyer, J.R., and Shah, D.M. (1992). Antifungal proteins from plants. Purification, molecular cloning, and antifungal properties of chitinases from maize seed. J. Biol. Chem. **267**, 6635-6640.

Kirsch, C., Hahlbrock, K., and Kombrink, E. (1993). Purification and characterization of extracellular, acidic chitinase isoenzymes from elicitor-stimulated parsley cells. Eur. J. Biochem. **213**, 419-425.

Koes, R.E., Spelt, C.E., Van Den Elzen, P.J.M., and Mol, J.N.M. (1989). Cloning and molecular characterization of the chalcone synthase multigene family of *Petunia hybrida*. Gene **81**, 245-257.

Kragh, K.M., Jacobsen, S., Mikkelsen, J.D., and Nielsen, K.A. (1991). Purification and characterization of three chitinases and one ß-1,3-glucanase accumulating in the medium of cell suspension cultures of barley (*Hordeum vulgare* L.). Plant Sci **76**, 65-77.

Kreuger, M., and Van Holst, G.J. (1993). Arabinogalactan proteins are essential in somatic embryogenesis of *Daucus carota* L. Planta **189**, 243-248.

Kurosaki, F., Tashiro, N., Gamou, R., and Nishi, A. (1989). Chitinase isoenzymes induced in carrot cell culture by treatment with ethylene. Phytochem. **28**, 2989-2992.

Leah, R., Tommerup, H., Svendsen, I., and Mundy, J. (1991). Biochemical and molecular characterization of three barley seed proteins with antifungal properties. J. Biol. Chem. 266, 1564-1573.

Lotan, T., Ori, N., and Fluhr, R. (1989). Pathogenesis-Related proteins are developmentally regulated in tobacco flowers. Plant Cell 1, 881-887.

Lozoya, E., Hoffmann, H., Douglas, C., Schulz, W., Scheel, D., and Hahlbrock, K. (1988). Primary structures and catalytic properties of isoenzymes encoded by the two 4-coumarate: CoAligase genes in parsley. Eur. J. Biochem. 176, 661-667.

Lucas, J., Henschen, A., Lottspeich, F., Voegeli, U., and Boller T. (1985). Amino-terminal sequence of ethylene-induced bean leaf chitinase reveals similarities to sugar-binding domains of wheat germ agglutinin. FEBS Lett. 193, 208-210.

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

Margis-Pinheiro, M., Metz-Boutigue, M.H., Awade, A., De Tapia, M., Le Ret, M., and Burkard, G. (1991). Isolation of a complementary DNA encoding the bean PR4 chitinase: and acidic enzyme with an amino-terminus cysteine-rich domain. Plant Mol. Biol. 17, 243-253.

Mauch, F., and Staehelin, L.A. (1989). Functional implications of the subcellular localization of ethylene-induced chitinase and ß-1,3-glucanase in bean leaves. Plant Cell 1, 447-457.

Métraux, J.P., Burkhart, W., Moyer, M., Dincher, S., Middlesteadt, W., Williams, S., Payne, G., Carnes, M., and Ryals, J. (1989). Isolation of a complementary DNA encoding a chitinase with structural homology to a bifunctional lysozyme/chitinase. Proc. Natl. Acad. Sci. USA 86, 896-900.

Mikkelsen, J.D., Berglund, L., Nielsen, K.K., Christiansen, H., and Bojsen, K. (1992). Structure of endochitinase genes from sugar beets. In Advances in Chitin and Chitosan, C.J. Brine, P.A. Sandford and J.P. Zikakis, eds (Amsterdam: Elsevier), pp. 344-353.

Neale, A.D., Wahleithner, J.A., Lund, M., Bonnett, H.T., Kelly, A., Meeks-Wagner, D.R., Peacock, W.J., and Dennis, E.S. (1990). Chitinase, ß-1,3-glucanase, osmotin, and extensin are expressed in tobacco explants during flower formation. Plant Cell 2, 673-684.

Neuhaus, J.-M., Sticher, L., Meins, F., Jr., and Boller, T. (1991). A short C-terminal sequence is necessary and sufficient for the targeting of chitinases to the plant vacuole. Proc. Natl. Acad. Sci. USA 88, 10362-10366.

Payne, G., Ahl, P., Moyer, M., Harper, A., Beck, J., Meins, F., Jr., and Ryals, J. (1990). Isolation of complementary DNA clones encoding pathogenesis-related proteins P and Q, two acidic chitinases from tobacco. Proc. Natl. Acad. Sci. USA 87, 98-102.

Perrakis, A., Wilson, K.S., Chet, I., Oppenheim, A.B., and Vorgias, C.E. (1993). Phylogenetic relationships of chitinases. In Chitin Enzymology, Proceedings of the Indernational Symposium on Chitin Enzymology, May 10-12, 1993, Senigallia, Italy, R.A.A. Muzzarelli, ed (Italy: European Chitin Society), 217-232.

Rasmussen, U., Bojsen, K., and Collinge, D.B. (1992). Cloning and characterization of a pathogen-induced chitinase in *Brassica napus*. Plant Mol. Biol. **20**, 277-287.

Roby, D., and Esquerre-Tugaye, M.-T. (1987). Purification and some properties of chitinases from melon plants infected by *Colletotrichum lagenarium*. Carbohydr. Res. **165**, 93-104.

Samac, D.A., and Shah, D.M. (1991). Developmental and pathogen-induced activation of the Arabidopsis acidic chitinase promoter. Plant Cell **3**, 1063-1072.

Samac, D.A., Hironaka, C.M., Yallaly, P.E., and Shah, D.M. (1990). Isolation and characterization of the genes encoding basic and acidic chitinase in *Arabidopsis thaliana*. Plant Physiol. **93**, 907-914.

Shinshi, H., Mohnen, D., and Meins Jr., F. (1987). Regulation of a plant pathogenesis-related enzyme: Inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissues by auxin and cytokinin. Proc. Natl. Acad. Sci. **84**, 89-93.

Shinshi, H., Neuhaus, J.-M., Ryals, J., and Meins Jr., F. (1990). Structure of a tobacco endochitinase gene: evidence that different chitinase genes can arise by transposition of sequences encoding a cysteine-rich domain. Plant Mol. Biol. **14**, 357-368.

Smith, D.B., and Johnson, K.S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene **67**, 31-40.

Stanford, A., Bevan, M., and Northcote, D. (1989). Differential expression within a family of novel wound-induced genes in potato. Mol. Gen. Genet. **215**, 200-208.

Uhlmann, A., and Ebel, J. (1993). Molecular cloning and expression of 4-coumarate:coenzyme A ligase, an enzyme involved in the resistance response of soybean (*Glycine max* L.) against pathogen attack. Plant Physiol. **102**, 1147-1156.

chapter 6

General discussion

GENERAL DISCUSSION

One of the goals of the study presented in this thesis was to identify proteins secreted in the embryo culture medium by wild-type cells, that are able to lift the arrest in embryo development of the temperature sensitive carrot variant ts11. This was shown to be possible with a single protein, identified as a 32-kD endochitinase. In this chapter the evidence for a role of chitinases and chitin-derived signal molecules in plant development will be discussed.

Are there Possible Substrates for Chitinases in Plants?

Chitin is a linear polymer that consists of ß-1,4-linked N-acetyl-D-glucosamine residues. Enzymes that catalyze hydrolysis of N-acetyl-ß-D-glucosaminide ß-1,4-linkages in chitin and in chito-oligomers are classified as either exo- or endochitinases. Exochitinases (E.C. 3.2.1.30) liberate N-acetylglucosamine or the disaccharide diacetylchitobiose from the non-reducing end of chitin in a stepwise fashion (Molano et al., 1977). Plant chitinases generally are endochitinases (E.C. 3.2.1.14) that randomly hydrolyze internal ß-1,4-linkages of chitin and thereby release oligosaccharides of N-acetylglucosamine (Boller, 1988). In plants, chitinase activity was detected initially in sweet almond emulsions (Grassman et al., 1934). No other source was reported until 1965 when Powning and Irzykiewicz described chitinase activity in seeds of dicotyledons and Gramineae, and suggested a role for chitinases in a defence mechanism against chitin-containing parasites from the soil. As has been demonstrated many times, chitinase activity in the plant is increased by fungal elicitors (Hedrick et al., 1988; Ishige et al., 1993; Kurosaki et al., 1990; Kirsch et al., 1993) and is part of the hypersensitive response to pathogen attack (Metraux and Boller, 1986; Margis-Pinheiro et al., 1991). For some plant-produced chitinases it has been shown that they are able to inhibit fungal growth in vitro (Schlumbaum et al., 1986; Broekaert et al., 1988; Jacobsen et al., 1990; Huynh et al., 1992). Transgenic tobacco seedlings, expressing a bean chitinase gene, showed an increased ability to survive in soil infested with the fungal pathogen *Rhizoctonia solani* (Broglie et al., 1991). Some of the isolated plant chitinases only possess antifungal activity in vitro in combination with ß-1,3-glucanase activity (Sela-Buurlage et al., 1993), while several others do not have antifungal activity in vitro at all (Woloshuk et al., 1991).

Chitinase genes were also shown to be expressed in the absence of pathogens (Shinshi et al., 1987; Lotan et al., 1989; Kragh et al., 1990; Herget et al., 1990; Kaufman et al., 1992), a finding that is usually explained by assuming that they are part of a continuously present defense mechanism. The results presented in this thesis are the first direct evidence for a role of certain plant chitinases in development. At the same time these results raise the question what the natural substrate of these enzymes might be, assuming that it is the catalytic property of the 32-kD endochitinase that is required for the rescue of ts11 embryo development. However, no compounds are available that specifically inhibit the catalytic

activity of endochitinases, so it was not possible to test this directly. Therefore, an indirect approach was employed by testing putative products of chitinase activity for their effect on ts11 embryo development (see Chapter 3). One of these compounds, the *Rhizobium* nodulation factor NodRlv-V(Ac,C18:4) was able to stimulate ts11 embryo formation with a similar efficiency as the 32-kD endochitinase. Based on this finding, it was speculated that the 32-kD endochitinase acts by releasing Nod-factor-like signal molecules from plant-produced precursors. However, it must be kept in mind that presently it is not known whether the 32-kD endochitinase and NodRlv-V(Ac, C18:4) act via the same or via different mechanisms. Therefore, it remains possible that there is no direct relationship between both observations. If the 32-kD endochitinase indeed releases signal molecules from plant-produced precursor molecules, then what might be the possible substrate for the 32-kD endochitinase in the plant cell wall? When boiled homogenates of bean plants were treated with a bean endochitinase, no N-acetylglucosamine-containing fragments were detected, indicating that chitin-like substrates are not present in the plant (Boller et al., 1983). Employing cytochemical labeling, Benhamou and Asselin (1989) were able to show binding of chitinases and wheat germ agglutinin, a plant lectin that is specific for oligomers of N-acetylglucosamine, to secondary cell walls of a variety of Solanaceae. This binding was shown to be abolished by prior treatment with lipase. Although lipid-linked N-acetylglucosamine residues have not been identified as structural components of plant cell walls (Bolwell, 1988), the results of Benhamou and Asselin (1989) suggest that the N-acetylglucosamine residues detected may be present in the form of glycolipids. Incubation of membrane fractions from bean or pea stems with N-acetyl[^{14}C]glucosamine and analysis of the lipophilic fraction showed that N-acetyl[^{14}C]glucosamine was incorporated only in the highly charged dolichol-pyrophosphate-oligosaccharides, the intermediates in the biosynthesis of glycoproteins (Lehle et al., 1976; Durr et al., 1979). These intermediates contain two N-acetylglucosamines per lipid molecule at most. The minimal length of an N-acetylglucosamine chain required to serve as substrate for endochitinases is three (Molano et al., 1979; Usui et al., 1990). So the dolichol-pyrophosphate-oligosaccharides are not thought to be able to serve as substrate for chitinases. Another class of glycolipids in plants that contain N-acetylglucosamine, referred to as phytoglycolipids, are of the sphingolipid type. They are present in seeds and commonly contain complex oliosaccharides rather than a single saccharide and a variety of sugar components including N-acetylglucosamine, glucuronic acid, inositol, galactose, arabinose and fucose (Carter et al., 1964). However, a stretch of three N-acetylglucosamines has not been reported to occur in such glycolipids. A class of molecules that are also candidates to be chitinase substrates are certain arabinogalactan-proteins, proteoglycans with poly- and oligosaccharide units covalently attached to a central protein core (Van Holst and Klis, 1981). Recently, sugar composition analysis of different plasma membrane arabinogalactan-proteins from sugar beet leaf

revealed that 1-9% of the sugar molecules are *N*-acetylglucosamines. It is not known yet whether the *N*-acetylglucosamines are present as oligomers (Roger Pennell, personal communication).

To test the hypothesis that lipo-oligosaccharides analogous to the *Rhizobium* Nod factors occur in plants, Spaink et al. (1993) labeled *Lathyrus* flowers with *N*-acetyl[^{14}C]glucosamine. Some of the extracted lipophilic compounds migrated similarly to the rhizobial lipo-oligosaccharides on TLC plates. To determine the nature of the oligosaccharide chain, the lipophilic compounds were treated with commercial chitinase and analyzed on TLC plates. Although no spots disappeared, there appeared at least three new spots appearing after chitinase treatments, suggesting that lipophilic compounds had been hydrolysed by chitinases. Indirect evidence for the presence of chitin-like molecules in plants was presented by Schmidt et al. (1993), who introduced *Rhizobium* nodulation genes *nod*A and *nod*B, involved in the biosynthesis of the Nod-factors (Spaink et al., 1991), into tobacco plants. Schmidt et al. (1988) have shown that the deacetylase NodB together with the cytosolic protein NodA are sufficient to produce small, heat-stable compounds that stimulate mitosis in various protoplasts derived from legumes and non-legumes. The NodB gene product is an oligosaccharide-modifying enzyme which deacetylates the non-reducing *N*-acetylglucosamine residues of chito-oligosaccharides (John et al., 1993), while the biochemical function of the NodA protein is still unknown. Expression of the *nod*A and *nod*B genes in tobacco resulted in plants with morphological abnormalities such as wrinkled leaves, reduced growth and compact inflorescences. These experiments suggest that in tobacco substrate molecules are present that can be used by the *nod*A and *nod*B encoded proteins to synthesize growth-controlling factors.

Taken together, although no substrate molecules for chitinases have been identified yet in plants, several lines of circumstantial evidence indicate that substrates for chitinases may indeed be present, and that they may be involved in the generation of Nod-factor-like molecules.

Does the 32-kD Endochitinase Have a Distinct Substrate Affinity?
In chapter 3 it has been shown that of the different chitinases tested, none had an effect on ts11 embryo development similar to that of the 32-kD endochitinase. This suggests that the 32-kD endochitinase may be different from other plant chitinases. In order to find an indication for a possibly different substrate affinity of the 32-kD endochitinase, the deduced amino acid sequence of EP3C was aligned with amino acid sequences of several plant endochitinases. Comparison of the amino acid sequences shows that a methionine, conserved in all class I, II and IV chitinases is replaced by a lysine in the 32-kD endochitinase (Figure 1, position 243). To predict the possible consequence of this amino

```
            1                                                          60
beanIV:     MGNKLVLVLVAVALV*MG-PKNVSAQNCG-------CAEGLCCSQYGYCGTGEDYC
bean I:     MKKNRMMMMIWSVGVVWMLLLVGGSYG*EQ-CGRQAGGALCPGGNCCSQFGWCGSTTDYC
tob II:     MEFSGSPMALFCCVFFLFLTGSLA*Q-----------------------------
barley:     MRSLAVVVAVVATVAMAIGTARG*-------------------------------

            61                                                        120
EP3C:                                                               SYS
beanIV:     G-GCQQGPCTTASPPPSNNVNADILTADFLNGIIDQAGSGCAGKNFYTRDAFLSALNSYT
bean I:     GPGCQSQCGGPSPAPTDLSAL-ISRSTFDQMLKHRNDGA-CPAKGFYTYDAFIAAAKAYP
tob II:     ----------------GIGSIVTSDLFNEMLKNRNDGR-CPANGFYTYDAFIAAANSFP
barley:     ----------------SVSSIVSRAQFDRMLLHRNDGA-CQAKGFYTYDAFVAAAAFP

            121                                                       180
EP3C:       SFGTSGSADDSKREIAAFFAHATHET-G------Y--F----CHKEETNGRDK-NYC-E-
beanIV:     DFGRVDSEDDSKREIAAAFAHFTHET-G------H--F----CYIEEIDGASK-DYCDEE
bean I:     SFGNTGDTATRKREIAAFLGQTSHETTGGWATAPDGPYAWGYCFVRER--NP-STYCSA-
tob II:     GFGTSGDDTARRKEIAAFFGQTSHETTGGSLSAE--PFTGGYCFVR-Q--NDQSD-----
barley:     GFGTTGSADAQKREVAAFLAQTSHETTGGWATAPDGAFAWGYCFKQERGASS--DYC---

            181                                                       240
EP3C:       SK-AGYPCNANVKYFGRGPLQLTWNYNYIDAGKSNEFD-GLNNPDIVASDAVVSFSRALW
beanIV:     SI-AQYPCSSSKGYHGRGPIQLSWNFNYGPAGSANNFDHGLGAPETVSNDVVVSFKTALW
bean I:     TP--QFPCAPGQQYYGRGPIQISWNYNYGQCGRAIGAD-LLGDPDLVASDATVSFDTAFW
tob II:     -----------RYYGRGPIQLTNNYEKA--GNAIRQD-LVNNPDLVATDATISFKTAIW
barley:     TPSAQWPCAPGKRYYGRGPIQLSHNYNYGPAGRAIGVD-LLANPDLVATDATVGFKTAIW

            241                                                       300
EP3C:       YWKVKVQS--------V---------------TTQGFGATIRAINS-IECNGGSPDAVNS
beanIV:     YWM---QHVRP-----VI--------------NQGFGATIRAINGALECDGANPTTVQA
bean I:     FWMTP-QSPKPPSCNAVATGQWTPSADDQRAGRVPGYGVITNIINGGLECGHGEDDRIAD
tob II:     FWMTP-QDNKPSSHD-VIIGSWTPSAADQSANRAPGCGVITNIINGGIECGVGPNAAVED
barley:     FWMTA-QPPKPSSH-AVIAGQWSPSGADRAAGRVPGFGVITNIINGGIECGHGQDSRVAD

            301
EP3C:       RVSLYNSYCSKFGVAPGDNQRC*
beanIV:     RVNYYTEYCRQLGVATGDNLTC*
bean I:     RIGFYKRYCDILGVSYGANLDCYSQRPSAPPKLRLPSFHTVINNH*
tob II:     RIGYYRRYCGMLNVAPGDNLDCYNQRNFAQG*
barley:     RIGFYKRYCDILGVGYGNNLDCYSQRPFA*
```
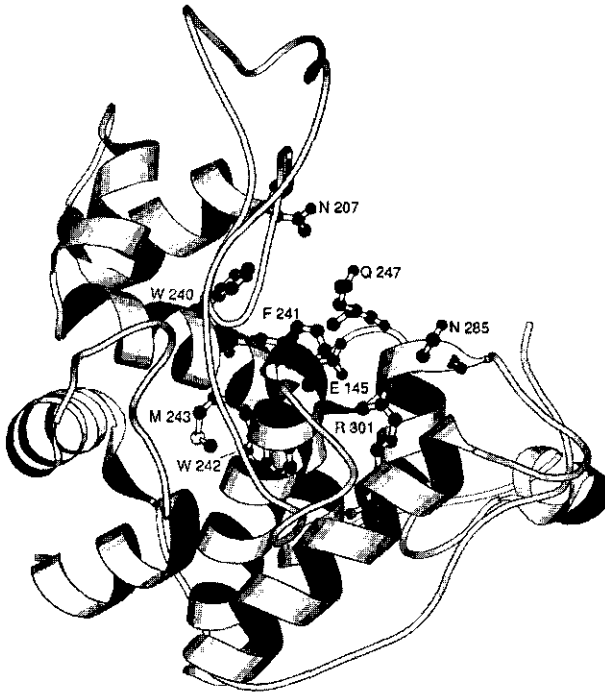
-120-

Figure 2. Ribbon Drawing of the Barley Seed Endochitinase Backbone (Per Kraulis, 1991).

The residues thought to be involved in binding and hydrolysis of the substrate (except for the methionine 243 residue) are represented in ball-and-stick. The residue position numbers are as in figure 1.

acid change, the amino acid sequence of EP3C was compared with the amino acid sequence of a 26-kD endochitinase from barley seeds (Leah et al., 1991), from which recently the crystal structure has been elucidated (Hart et al., 1993). The amino acid sequence of the EP3C encoded peptide and the 26-kD endochitinase from barley reveal 42% identity (60% similarity) at the amino acid level, suggesting that it is likely they have a similar protein structure. The protein structure of the barley endochitinase shows the presence of three

Figure 1. Alignment of the Deduced Amino Acid Sequence of EP3C with other Plant Chitinase Amino Acid Sequences.

These chitinases are a bean (class) IV chitinase (Margis-Pinheiro et al., 1991), a bean (class) I chitinase (Broglie et al., 1986), a tobacco (class) II chitinase (Payne et al., 1990) and a class II chitinase from barley seeds (Leah et al., 1991). Gaps were introduced for optimal alignment. Cysteine residues forming disulfide bonds are boxed. Residues postulated to be involved in substrate binding and catalysis are underlined and printed in bold.

disulfide bonds, resulting in a compact globular structure of the protein (Hart et al., 1993). These six cysteine residues forming the disulfide bonds are conserved in class I and IV chitinases and are boxed in Figure 1. In the globular structure of the protein an elongated cleft is present, that is presumed to be the region responsible for substrate binding and catalysis (Hart et al., 1993). Polar residues present in this cleft determine the specificity of the enzyme and may be essential for binding and hydrolysis of the substrate. Based on the location of the methionine-lysine residue exchange, no change of the protein structure nor of the catalytic site seems to be expected (Figure 2). A few amino acids with polar residues are, based solely on their location near the putative substrate binding site, good candidates to be involved in the binding and hydrolysis of the substrate. The glutamate 145 and the arginine 301 residues (Figure 2, position numbers according to Figure 1) can participate in an electrostatic interaction which could be involved in hydrolysis of the substrate. The asparagine 207 and 285 and glutamine 247 residues may form hydrogen bonds with the substrate, while the tryptophane 240 and 242 and the tyrosine or phenylalanine 241 residues may undergo hydrophobic interactions with the substrate. These interactions may be important for the optimal position of the substrate in the elongated cleft. The histidine 144 residue (not marked in Figure 2) may be responsible for the pH-dependent catalytic activity of plant endochitinases (Boller et al., 1983). All these amino acids appear to be highly conserved in the class I and IV chitinases, including the carrot 32-kD endochitinase (see Figure 1). This may indicate that these amino acid residues are involved in the substrate binding and hydrolysis. Thus, based on the primairy structure, there is no reason to assume that the 32-kD endochitinase has an affinity for an entirely different substrate than the known class I and IV plant chitinases. Definite answers should come from comparative substrate analysis with different amine-containing sugars.

Are Lipo-Oligosaccharides a Novel Class of Plant Growth Regulators?

Besides the *Rhizobium* lipo-oligosaccharides, in several studies it has been shown that oligosaccharides, at concentrations several orders of magnitude below those of more conventional plant growth regulators, can influence plant growth and development. These oligosaccharides are termed oligosaccharins (Darvill et al., 1992; Aldington and Fry, 1993). One of these, XXFG (formerly XG9), inhibits 2,4-D-stimulated elongation of pea stem segments at a concentration of 10^{-9} M (York et al., 1984), while at 10^{-7} M the inhibitory effect was lost (York et al., 1984; McDougall and Fry, 1989). Hence, oligosaccharides can act either as intermediates in the growth hormone regulating mechanism or interact with a consecutive site somewhere in the cascade of events normally triggered by auxin. Purified Nod-factors, when applied to legume seedlings at concentrations as low as 10^{-12} M, stimulate differentiation of epidermal cells into roothairs, deformation of roothairs, induction of early nodulation genes related to the infection process in root epidermal cells, and

induction of cell divisions in the inner cortex of the roots (see for reviews, Fisher and Long, 1992; Spaink, 1992; Vijn et al., 1993). These events normally occur during the early nodulation process. Induction of early nodulin gene expression, cortical cell division, and subsequent meristem formation can also be triggered by auxin transport inhibitors (Hirsch et al., 1989; Van De Wiel et al., 1990), suggesting that a change in the endogenous balance of growth regulators plays a role in the initiation of nodule formation. In carrot somatic embryogenesis 2,4-D is needed for the transition from non-embryogenic to embryogenic cells (Nomura and Komamine, 1985), but is inhibitory to later stages of embryogenesis. In carrot somatic embryogenesis, auxin transport inhibitors produced morphological abberations in the embryos (Schiavone and Cooke, 1987). This was recently also shown for zygotic embryos (Liu et al., 1993). As in the case of nodule development, it is as yet unclear whether the stimulating effect of Nod-factors on ts11 cells can contribute to an alteration in the endogenous growth regulator balance. Following this line of thought, it will now be of great interest to identify plant substrates for chitinases, because they may act as inactive precursors of lipo-oligosaccharides, with chitinases in the role of enzymes releasing these molecules. If plant chitinases indeed are able to release signal molecules from plant-produced precursor molecules, they may not only be able to release, but also to inactivate lipo-oligosaccharide signal molecules (Vasse et al., 1993). More insight into these clearly fundamental questions will perhaps also help to explain in part the large diversity in chitinases found in all plants investigated.

The Morphological Changes Induced by the 32-kD Endochitinase
In chapter 3 and 4 it has been shown that addition of the 32-kD endochitinase to ts11 embryo culture medium has two different effects on ts11 embryo development. 1. The arrest in the transition from globular to heart stage embryos is lifted. 2. The formation of proembryogenic masses and globular embryos is stimulated. The question now arises which developmental step is influenced by the 32-kD endochitinase. In chapter 3 it was proposed that the 32-kD endochitinase is involved in the formation of a correctly formed protoderm. This was based on the finding that arrested globular ts11 embryos do not have a smooth outer cell layer (De Jong et al., 1992), and also exhibited an abberant, non-protodermal expression pattern of the epidermal marker EP2, while upon addition of the 32-kD endochitinase both the protoderm morphology and the EP2 expression became normal (Sterk et al., 1991; De Jong et al., 1993). Because the formation of the embryo protoderm is a very early event, both in zygotic as well as in somatic embryogenesis, it cannot be ruled out that the positive effect of the 32-kD endochitinase may have been more at the level of a correct initiation of protoderm development in the preglobular stages, rather then a correction of its abberant development in the late globular stage. Without cell-tracking experiments, to be able to follow the precise response of individual ts11 cells and cell clusters to addition of the 32-

kD endochitinase, this is difficult to demonstrate. However, it could well be that formation of properly-formed proembryogenic masses and globular embryos is the common basis of the two observed effects on ts11. Except for ts11, no effect has sofar been observed upon addition of either the 32-kD endochitinase or NodRlv-V(Ac, C18:4) to any other *Daucus* cell line (Theo Hendriks, unpublished results). Whether this is related to a perhaps enhanced susceptibility of ts11 cells in particular to enzymes present in the culture medium, is not known. The observed loosely formed cell walls in non-embryogenic ts11 cells may point in this direction. Without identification of the genetic basis of the ts11 phenotype, this will be difficult to unravel. Similarly, it is unknown whether there is any zygotic embryo mutant phenotype associated with inactivation of a chitinase gene.

Is ts11 a Good Tool to Study Somatic Embryogenesis?

In order to ultimately identify genes that direct the formation of somatic embryos, temperature-sensitive variants arrested at various stages during somatic embryogenesis have been isolated from somatic cell cultures of carrot (Breton and Sung, 1982; Terzi et al., 1982; Giuliano et al., 1984; Schnall et al., 1988). Ts11 is a temperature-sensitive variant (LoSchiavo et al., 1990) and was the starting-point of the experiments described in this thesis. As discussed by Zimmerman (1993), the limitations of the carrot somatic embryo system have to be recognized when it is used in an approach involving mutagenesis and identification of defective phenotypes. Of any given cell line, the proportion of cells that actually enters somatic embryogenesis is highly variable and decreases with increasing time in culture. Furthermore, prolonged time in culture can lead to the accumulation of mutations (somaclonal variation; Widholm, 1984). These properties of the somatic embryo system have made it very difficult to keep the ts11 phenotype originally described. This has severely limited the time span over which experiments could be conducted. Regular cycling of the cell line through embryogenesis and selection of normal plantlets for the reinitiation of the line was necessary to retain the ts11 phenotype. In spite of these precautions, difficulties were encountered in maintaining ts11 sufficiently embryogenic. To illustrate this, the number of embryos formed per mL of ts11 cell culture at the permissive temperature dropped about 120-fold over a period of three years (Lo Schiavo et al., 1990; De Jong et al., 1993). In addition to this, as shown in chapter 4, the original mutation in ts11 does not only affect the ability to develop embryos, but also the morphology of non-embryogenic cells. Although the precise reason is unknown, the result of this instability of the ts11 phenotype encountered was that the response of ts11 cells to the 32-kD endochitinase as well as to the Nod-factors was highly variable. Therefore, embryo rescue assays had to be repeated many times. The effect on ts11 embryo development of the 32-kD endochitinase and NodRlv-V(Ac, C18:4), both of which are statistically significant despite these ts11 stability problems, is therefore quite certain. However, in future experiments the instability of ts11 may present a serious

problem, and may require reisolation of ts11-like cell lines, that have both a more pronounced and more stable phenotype.

ACKNOWLEDGEMENT

We thank John Hart (Department of Chemistry and Biochemistry, University of Texas) for providing the X-ray coordinates of the 2.8 Å structure of the barley seed endochitinase and Jacques Vervoort (Department of Biochemistry, Wageningen Agricultural University) for his help with the interpretation of the 3D-structure of the barley 26-kD endochitinase.

REFERENCES

Aldington, S., and Fry, S.C. (1993). Oligosaccharins. Adv. Bot Res. **19**, 1-101.

Benhamou, N., and Asselin, A. (1989). Attempted localization of a substrate for chitinases in plant cells reveals abundant N-acetyl-D-glucosamine residues in secondary walls. Biology of the Cell **67**, 341-350.

Boller, T., Gehri, A., Mauch, F., and Vogeli, U. (1983). Chitinase in bean leaves: Induction by ethylene, purfication, properties and possible function. Planta **157**, 22-31.

Boller, T. (1988). Ethylene and the regulation of antifungal hydrolases in plants. Oxford Surv. Plant Mol. Cell Biol. **5**, 145-174.

Bolwell, G.P. (1988). Synthesis of cell wall components: aspects of control. Phytochemistry **27**, 1235-1253.

Breton, A.M., and Sung, Z.R. (1982). Temperature-sensitive carrot variants impaired in somatic embryogenesis. Dev. Biol. **90**, 58-66.

Broekaert, W.F., Van Parijs, J., Allen, A.K., and Peumans, W.J. (1988). Comparison of some molecular, enzymatic and antifungal properties of chitinases from thorn-apple, tobacco and wheat. Physiol. Mol. Plant Pathol. **33**, 319-331.

Broglie, K.E., Gaynor, J.J., Broglie, R.M. (1986). Ethylene-regulated gene expression: Molecular cloning of the genes encoding an endochitinase from *Phaseolus vulgaris*. Proc. Natl. Acad. Sci. USA **83**, 6820-6824.

Broglie, K., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S., Mauvais, C.J., and Broglie, R. (1991). Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science **254**, 1194-1197.

Carter, H.E., Betts, B.E., and Strobach, D.R. (1964). Biochemistry of the sphingolipids. XVII. The nature of the oligosaccharide component of phytoglycolipid. Biochemistry **3**, 1103-1107.

Darvill, A.G., Augur, C., Bergmann, C., Carlson, R.W., Cheong, J-J., Eberhard, S., Hahn, M.G., Ló, V-M., Marfà, V., Meyer, B., Mohnen, D., O'Neill, M.A., Spiro, M.D., Van Halbeek, H., York, W.S., and Albersheim, P. (1992). Oligosaccharins-oligosaccharides that regulate growth, development and defence responses in plants. Glycobiology **2**, 181-198.

De Jong, A.J., Cordewener, J., Lo Schiavo, F. Terzi, M., Vandekerckhove, J., Van Kammen, A., and De Vries, S.C. (1992). A carrot somatic embryo mutant is rescued by chitinase. Plant Cell 4, 425-433.

De Jong, A.J., Heidstra, R., Spaink, H.P., Hartog, M.V., Meijer, E.A., Hendriks, T., Lo Schiavo, F., Terzi, M., Bisseling, T., Van Kammen, A., and De Vries, S.C. (1993). Rhizobium lipooligosaccharides rescue a carrot somatic embryo mutant. Plant Cell 5, 615-620.

Durr, M., Bailey, D.S., and Maclachlan, G. (1979). Subcellular distribution of membrane-bound glycosyltransferases from pea stems. Eur. J. Biochem. 97, 445-453.

Fisher, R.F., and Long, S.R. (1992). *Rhizobium*-plant signal exchange. Nature 357, 655-660.

Giuliano, G., Lo Schiavo, F., and Terzi, M. (1984). Isolation and developmental characterization of temperature-sensitive carrot cell variants. Theor. Appl. Genet. 67, 179-183.

Grassman, W., Zechmeister, L., Bender, R., and Toth, G. (1934). Über die chitin-spaltung durch emulsin-präparate (III. Mitteil über enzymatische spaltung von polysacchariden). Ber. Chem. Ges. 67, 1-5.

Hart, P.J., Monzingo, A.F., Ready, M.P., Ernst, S.R., and Robertus, J.D. (1993). Crystal structure of an endochitinase from *Hordeum vulgare* L. seeds. J. Mol. Biol. 229, 189-193.

Hedrick, S.A., Bell, J.N., Boller, T., and Lamb, C.J. (1988). Chitinase cDNA cloning and mRNA induction by fungal elicitor, wounding and infection. Plant Physiol. 86, 182-186.

Herget, T., Schell, J., and Schreier, P.H. (1990). Elicitor-specific induction of one member of the chitinase gene family in *Arachis hypogaea*. Mol. Gen. Genet. 224, 469-476.

Hirsch, A.M., Bhuvaneswari, T.V., Torrey, J.G., and Bisseling, T. (1989). Early nodulin genes are induced in alfalfa root outgrowths elicited by auxin transport inhibitors. Proc, Natl., Acad. Sci. USA 86, 1244-1248.

Huynh, Q.K., Hironaka, C.M., Levine, E.B., Smith, C.E., Borgmeyer, J.R., and Shah, D.M. (1992). Antifungal proteins from plants. Purification, molecular cloning, and antifungal properties of chitinases from maize seed. J. Biol. Chem. 267, 6635-6640.

Ishige, F., Mori, H., Yamazaki, K., Imaseki, H. (1993). Cloning of a complementary DNA that encodes an acidic chitinase which is induced by ethylene and expression of the corresponding gene. Plant Cell Physiol. 34, 103-111.

Jacobsen, S., Mikkelsen, J.D. and Hejgaard, J. (1990). Characterization of two antifungal endochitinases from barley grain. Physiol. Plant. 79, 554-562.

John, M., Röhrig, H., Schmidt, J., Wieneke, U., and Schell, J. (1993). *Rhizobium* NodB protein involved in nodulation signal synthesis is a chitooligosaccharide deacetylase. Proc. Natl. Acad. Sci. USA 90, 625-629.

Kaufmann, H., Kirch, H., Wemmer, T., Peil., A., Lottspeich, F., Uhrig, H., Salamini, F., and Thompson, R. (1992) Sporophytic and gametophytic self-incompatibility. In: Sexual Plant Reproduction, M. Cresti and A. Tiezzi, eds (Springer-Verlag), pp. 115-125.

Kirsch, C., Hahlbrock, K., and Kombrink, E. (1993). Purification and characterization of extracellular, acidic chitinase isoenzymes from elicitor-stimulated parsley cells. Eur. J. Biochem. 213, 419-425.

Kragh, K.M., Jacobsen, S., Mikkelsen, J.D., and Nielsen, K.A. (1990). Tissue specific expression and differential induction of chitinase isoforms in barley (*Hordeum vulgare* L.). Ph.D. Thesis K.M. Kragh, Frederiksberg, Denmark, Royal Veterinary and Agricultural University, pp. 65-78.

Kurosaki, F., Tashiro, N., and Nishi, A. (1990). Chitinase induction in carrot cell cultures treated with various fungal compounds. Biochem. Internatl. 20, 99-106.

Leah, R., Tommerup, H., Svendsen, I., and Mundy, J. (1991). Biochemical and molecular characterization of three barley seed proteins with antifungal properties. J. Biol. Chem. 266, 1564-1573.

Lehle, L., Fartaczek, F., Tanner, W., and Kauss, H. (1976). Formation of polyprenol-linked mono- and oligosaccharides in *Phaseolus aureus*. Archiv. Biochem. Biophys. 175, 419-426.

Liu, C.-M., Xu, Z.-H., and Chua, N.-A. (1993). Auxin polar transport is essental for the establishment of bilateral symmetry during early plant embryogenesis. Plant Cell 5, 621-630.

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

Lotan, T., Ori, N., and Fluhr, R. (1989). Pathogenesis-related proteins are developmentally regulated in tobacco flowers. Plant Cell 1, 881-887.

Margis-Pinheiro, M., Metz-Boutigue, M.H., Awade, A., De Tapia, M., Le Ret, M., and Burkard, G. (1991). Isolation of a complementary DNA encoding the bean PR4 chitinase: an acidic enzyme with an amino-terminus cysteine-rich domain. Plant Mol. Biol. 17, 243-253.

McDougall, G.J., and Fry, S.C. (1989). Structure-activity relationships for xyloglucan oligosaccharides with antiauxin activity. Plant Physiol. 89, 883-887.

Metraux, J.P., and Boller, T. (1986). Local and systemic induction of chitinase in cucumber plants in response to viral, bacterial and fungal infections. Physiol. Mol. Plant Pathol. 28, 161-169.

Molano, J., Duran, A., and Cabib, A. (1977). A rapid and sensitive assay for chitinase using tritiated chitin. Anal. Biochem. 83, 648-656.

Molano, J., Polacheck, I., Duran, A., and Cabib, E. (1979). An endochitinase from wheat germ. Activity on nascent and preformed chitin. J. Biol. Chem. 254, 4901-4907.

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

Payne, G., Ahl, P., Moyer, M., Harper, A., Beck, J., Meins, F., Jr., and Ryals, J. (1990). Isolation of complementary DNA clones encoding pathogenesis-related proteins P and Q, two acidic chitinases from tobacco. Proc. Natl. Acad. Sci. USA 87, 98-102.

Per Kraulis, J. (1991). MOLSCRIPT: A program to produce both detailed and schematic plots of protein structures. J. Appl. Cryst. 24, 946-950.

Powning, R.F., and Irzykiewicz, H. (1965). Studies on the chitinase systems in bean and other seeds. Comp. Biochem. Physiol. **14**, 127-133.

Schiavone, F.M., and Cooke, T.J. (1987). Unusual patterns of somatic embryogenesis in the domesticated carrot: Developmental effects of exogenous auxins and auxin transport inhibitors. Cell Differ. **21**, 53-62.

Schlumbaum, A., Mauch, F., Vogeli, U., and Boller, T. (1986). Plant chitinases are potent inhibitors of fungal growth. Nature **324**, 365-367.

Schmidt, J., Wingender, R., John, M., Wieneke, U., and Schell, J. (1988). *Rhizobium meliloti nodA* and *nodB* genes are involved in generating compounds that stiumlate mitosis of plant cells. Proc. Natl. Acad. Sci. USA **85**, 8578-8582.

Schmidt, J., Röhrig, H., John, M., Wieneke, U., Stacey, G., Koncz, C., and Schell, J. (1993). Alteration of plant growth and development by *Rhizobium nod*A and *nod*B genes involved in the synthesis of oligosaccharide signal molecules. Plant J. **4**, 651-658.

Schnall, J.A., Cooke, T.J., and Cress, D.E. (1988). Genetic analysis of somatic embryogenesis in carrot cell culture: Initial characterization of six classes of temperature-sensitive variants. Dev. Genet. **9**, 49-67.

Sela-Buurlage, M.B., Ponstein, A.S., Bres-Vloemans, S.A., Melchers, L.S., Van Den Elzen, P.J.M., and Cornelissen, B.J.C. (1993). Only specific tobacco (*Nicotiana tabacum*) chitinases and ß-1,3-glucanases exhibit antifungal activity. Plant Physiol. **101**, 857-863.

Shinshi, H., Mohnen, D., and Meins, F., Jr. (1987). Regulation of a plant pathogenesis-related enzyme: inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissue by auxin and cytokinin. Proc. Natl. Acad. Sci. USA **84**, 89-93.

Spaink, H.P. (1992). Rhizobial lipo-oligosaccharides: Answers and questions. Plant Mol. Biol. **20**, 977-986.

Spaink, H.P., Sheeley, D.M., Van Brussel, A.A.N., Glushka, J., York, W.S., Tak, T., Geiger, O., Kennedy, E.P., Reinhold, V.N., and Lugtenberg, B.J.J. (1991). A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of *Rhizobium*. Nature **354**, 125-130.

Spaink, H.P., Aarts, A., Bloemberg, G.V., Folch, J., Geiger, O., Schlaman, H.R.M., Thomas-Oates, J.E., Van De Snade, K., Van Spronsen, P., Van Brussel, A.A.N., Wijfjes, A.H.M., and Lugtenberg, B.J.J. (1993). Rhizobial lipo-oligosaccharides: Their biosynthesis and their role in the plant. In Advances in Molecular Genetics of Plant-Microbe Interactions, Vol. 2, E. Nester, ed (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 151-162.

Sterk, P., Booij, H., Schellekens, G.A., Van Kammen A., and de Vries, S.C. (1991). Cell-specific expression of the carrot EP2 lipid transfer protein gene. Plant Cell **3**, 907-921.

Terzi, M., Giuliano, G., Lo Schiavo, F., Nuti Ronchi, V. (1982). Studies on plant cell lines showing temperature-sensitive embryogenesis. In Embryogenic Development, part B, M. Burger, ed (New York: Alan Liss), pp. 521-534.

Usui, T., Matsui, H., and Isobe, K. (1990). Enzymic synthesis of useful chito-oligosaccharides utilizing transglycosylation by chitinolytic enzymes in a buffer containing ammonium sulfate. Carbohydr. Res. **203**, 65-77.

Van Holst, G.J., and Klis, F.M. (1981). Hydroxyproline glycosides in secretory arabinogalactan proteins of *Phaseolus vulgaris* L. Plant Physiol. **68**, 979-980.

Van de Wiel, C., Norris, J.H., Bochenek, B., Dickstein, R., Bisseling, T., and Hirsch, A.M. (1990). Plant Cell **2**, 1009-1017.

Vasse, J., De Billy, F., and Truchet, G. (1993). Abortion of infection during the *Rhizobium meliloti*-alfalfa symbiotic interaction is accompanied by a hypersensitive reaction. Plant J. **4**, 555-566.

Vijn, I., Das Neves, L., Van Kammen, A., Franssen, H., and Bisseling, T. (1993). Nod factors and nodulation in plants. Science **260**, 1764-1765.

Widholm, J.M. (1984). Induction, selection and characterization of mutants in carrot cell cultures. In Cell Culture and Somatic Cell Genetics of Plants, vol. 1, I.K. Vasil, ed (New York: Academic Press), pp. 563-570.

Woloshuk, C.P., Meulenhoff, J.S., Sela-Buurlage, M., Van den Elzen, P.J.M., and Cornelissen, B.J.C. (1991). Pathogen-induced proteins with inhibitory activity toward *Phytophthora infestans*. Plant Cell **3**, 619-628.

York, W.S., Darvill, A.G., and Albersheim, P. (1984). Inhibition of 2,4-dichlorophenoxyacetic acid-stimulated elongation of pea stem segments by a xyloglucan oligosaccharide. Plant Physiol. **75**, 295-297.

Zimmerman, J.L. (1993). Somatic embryogenesis: A model for early development in higher plants. Plant Cell **5**, 1411-1423.

Samenvatting

De ontwikkeling van een plant begint in de bloem met de bevruchting van een eicel. De bevruchte eicel of zygote deelt en ontwikkelt zich via globulair- en hart-vormig stadium naar een torpedo-vormig embryo. Na het kiemen van het zaad groeit het embryo uit tot een plant. Morfologisch is deze ontwikkeling goed beschreven, maar van de biochemische processen die ten grondslag liggen aan de ontwikkeling van een plant uit een enkele cel is nog weinig bekend. Biochemisch en moleculair biologisch onderzoek om eiwitten en genen te achterhalen, welke een rol spelen bij de embryo ontwikkeling van een plant, wordt belemmerd door het moeilijk verkrijgbaar zijn van voldoende plantemateriaal. Planten kunnen echter ook via een andere, niet-geslachtelijke weg embryo's vormen. Hiertoe wordt een stukje weefsel van de plant gesneden en in een kweekmedium gebracht met een hoge concentratie van de groeiregulator auxine. Cellen aan het wondoppervlak gaan vervolgens delen en de zo ontstane cellen vormen een celsuspensie. Wanneer deze cellen onder hoge dichtheid groeien in de aanwezigheid van het synthetisch auxine 2,4-D, dan ontwikkelen zich in deze suspensiecultuur clusters van cytoplasmarijke cellen. Deze clusters worden ook wel aangeduid als pro-embryogene massa's. Na verdunning in auxine-vrij medium kunnen zich embryo's ontwikkelen uit deze clusters. Deze niet geslachtelijke wijze van embryo-ontwikkeling wordt somatische embryogenese genoemd. Via somatische embryogenese zijn van *Daucus carota* (peen) grote hoeveelheden embryo's in de verschillende stadia te verkrijgen. Daarom wordt de somatische embryo-ontwikkeling van *Daucus* gebruikt als modelsysteem voor de bestudering van de moleculaire basis van de embryo-ontwikkeling van de plant.

Eerder onderzoek heeft aangetoond dat de cellen in een embryo-cultuur eiwitten uitscheiden in het kweekmedium (het medium 'conditioneren'), en dat deze eiwitten de embryo-ontwikkeling beïnvloeden. Door een onderzoeksgroep onder leiding van professor Terzi (Italië) zijn, door kunstmatig veranderingen aan te brengen in het DNA met behulp van ethylmethaan sulfonaat (EMS), een aantal *Daucus* lijnen gemaakt van welke de somatische embryo-ontwikkeling temperatuurgevoelig is. Eén van deze varianten is ts11. Ts11 embryo's ontwikkelen zich normaal bij 24°C, maar bij 32°C blijft de embryo-ontwikkeling steken in het globulaire stadium. De ontwikkeling van ts11 embryo's kan hersteld worden door aan de ts11 cultuur geconditioneerd kweekmedium van een embryogene lijn toe te voegen. Het doel van dit onderzoek was om uit geconditioneerd kweekmedium de actieve component, welke in staat is om de embryo-ontwikkeling van ts11 te herstellen, te isoleren en deze biochemisch en moleculair biologisch te karakteriseren.

Na het zuiveren van eiwitten uit geconditioneerd kweekmedium van een embryogene (wild-type) lijn en het afzonderlijk testen van deze eiwitten op globulaire ts11 embryo's bleek dat een enkel 32 kDa eiwit in staat is de embryo ontwikkeling van ts11 embryo's te

herstellen (hoofdstuk 2). Verder bleek dat na toevoegen van het gezuiverde 32 kDa eiwit aan een pas ingezette ts11 embryo cultuur bij 32°C meer globulaire embryo's worden gevormd. Het protoderm van deze globulaire embryo's is beter ontwikkeld dan die van ts11 globulaire embryo's welke bij 32°C zijn gevormd zonder het 32 kDa eiwit toe te voegen. Deze resultaten duiden erop dat het 32 kDa eiwit niet alleen een invloed heeft op de ontwikkeling van het globulaire naar het hart stadium, maar dat het 32 kDa eiwit al betrokken is bij de embryo-ontwikkeling voor het globulaire stadium.

Van twee 32 kDa eiwit peptiden is de aminozuur volgorde bepaald, welke overeen bleek te komen met reeds bekende chitinase sequenties. Biochemische karakterisering van het 32 kDa eiwit maakte duidelijk dat het 32 kDa eiwit endochitinase-activiteit heeft en een zure endochitinase is. De observatie dat een chitinase betrokken is bij de embryo-ontwikkeling van planten is een opmerkelijke waarneming, omdat chitinases tot nu toe alleen in verband zijn gebracht met de afweer van planten tegen pathogenen. Mede daardoor is een substraat voor chitinases in planten tot nu toe niet aangetoond. Onze resultaten echter, suggereren dat er wel degelijk een substraat voor chitinases in planten aanwezig moet zijn.

Op zoek naar een mogelijke functie van het enzym, zijn een aantal N-acetylglucosamine bevattende verbindingen toegevoegd aan een ts11 embryo cultuur en is de ts11 embryo-ontwikkeling gevolgd. De resultaten van deze experimenten staan beschreven in hoofdstuk 3. Getest zijn onder andere glycolchitine, oligomeren van N-acetylglucosamine en een nodulatie(Nod)factor van *Rhizobium*. De laatste component is een oligomeer van N-acetylglucosamines met een vetzuurketen aan de eerste suiker. De Nodfactoren worden uitgescheiden door *Rhizobium* bacteriën en induceren de vorming van knollen op wortels van vlinderbloemige planten. Van de geteste N-acetylglucosamine bevattende componenten heeft alleen de Nodfactor NodRlv-V(Ac, C18:4) een stimulerend effect op de formatie van ts11 embryo's bij 32°C, welke overeen komt met het effect van 32 kDa endochitinase op de ts11 embryo-ontwikkeling.

In hoofdstuk 4 worden experimenten beschreven die laten zien dat een normaal functionerend 32 kDa endochitinase in ts11 embryocultures aanwezig is. Toegevoegd aan een ts11 embryo cultuur bleek het 32 kDa endochitinase geïsoleerd uit ts11 kweekmedium een zelfde effect op de ts11 embryo-ontwikkeling te hebben als het wild-type 32 kDa endochitinase. Het effect van het 32 kDa endochitinase op de ontwikkeling van ts11 embryo's is dus niet te verklaren door de afwezigheid van dat eiwit of door andere eigenschappen van dat eiwit. Mogelijk dat het effect op de ts11 embryo-ontwikkeling te verklaren is door een te lage concentratie van het 32 kDa endochitinase in het ts11 kweekmedium. Om deze mogelijkheid te toetsen is de hoeveelheid 32 kDa endochitinase aanwezig in ts11 kweekmedium in de tijd gevolgd. Met behulp van immunoblots is aangetoond dat bij 32°C in de eerste tien dagen minder 32 kDa endochitinase in het kweekmedium aanwezig is dan bij 24°C. Door toevoegen van 32 kDa endochitinase

verschuift de hoeveelheid 32 kDa eiwit in het kweekmedium naar het niveau van een ts11 embryocultuur bij 24°C. Deze resultaten suggereren dat de ts11 cultuur bij 32°C het medium niet voldoende kan conditioneren met het 32 kDa endochitinase, waardoor de embryo-ontwikkeling verstoord wordt. Inmiddels is gebleken dat meerdere isovormen van het 32 kDa endochitinase in het medium worden uitgescheiden. Hiervan is echter nog niet bekend of deze ook een stimulerend effect op de ontwikkeling van ts11 embryo's hebben.

Hoofdstuk 5 omvat experimenten die zijn uitgevoerd om 32 kDa endochitinase cDNA te isoleren en te karakteriseren. Screenen van een cDNA bank met oligo's, afgeleid van de aminozuur volgorde van de twee 32 kDa peptiden, leverde twee cDNA kloons op welke zowel op nucleotiden als aminozuur niveau voor 96% homoloog zijn met elkaar. In het 32 kDa peptiden overlappende gedeelte van de beide kloons verschillen respectievelijk twee en drie van de 32 aminozuren. Deze kleine verschillen wijzen erop dat de geisoleerde kloons coderen voor nauw aan het 32 kDa endochitinase verwante isovormen. Chitinases zijn gebaseerd op hun aminozuurvolgorde ingedeeld in vier klassen. Vergelijking van de afgeleidde aminozuur volgorde van de cDNAs met de aminozuur volgorde van klasse I, II III en IV chitinases laat een sterke homologie zien met klasse IV chitinases. Het reageren van zowel het anti-32 kDa serum als een anti-klasse IV chitinase serum met het 32 kDa endochitinase op immunoblots wijst erop dat het 32 kDa endochitinase inderdaad een klasse IV chitinase is. Klasse IV chitinases bevatten, net als de klasse I chitinases, een chitine-bindend gebied, maar onderscheiden zich van de klasse I chitinases door enkele deleties in de eiwitsequentie. Genomische DNA blots tonen aan dat het 32 kDa endochitinase gen lid is van een genfamilie, en resultaten van RNA blots laten zien dat in alle delen van de plant het 32 kDa endochitinase gen tot expressie komt. Echter, door de hoge homologie van beide kloons is het niet mogelijk op RNA blots onderscheid te maken tussen de expressie van de nauw verwante 32 kDa endochitinase genen.

In hoofstuk 6 wordt tenslotte de rol van chitinases en van chitine-afgeleidde signaal moleculen in de ontwikkeling van de plant bediscussieerd. Bovendien is getracht aanwijzingen te vinden voor een mogelijk afwijkende substraatspecificiteit van het 32 kDa endochitinase. Daartoe is de driedimensionale eiwit structuur van een klasse I chitinase uit gerst bekeken en zijn aminozuren aangewezen die mogelijk betrokken zijn bij de binding en de splitsing van het substraat. Deze aminozuren blijken allen zowel in het 32 kDa endochitinase als in klasse I en IV chitinases geconserveerd te zijn. Op basis van deze waarnemingen is er dan ook geen reden om aan te nemen dat het 32 kDa endochitinase affiniteit heeft voor een geheel ander substraat dan de bekende klasse I en IV chitinases.

Curriculum vitae

Anke de Jong werd geboren op 8 maart 1964 te Ylst. In 1982 slaagde zij voor het eindexamen VWO aan het Bogerman College te Sneek en in hetzelfde jaar werd begonnen met de studie planteziektenkunde aan de Landbouwuniversiteit te Wageningen. De ingenieursstudie, die werd afgesloten in november 1988, omvatte afstudeervakken bij de vakgroepen Nematologie, Moleculaire Biologie en Plantenfysiologie en een stage periode werd doorgebracht op het Institut National de la Recherche Agronomique (INRA), Antibes, Frankrijk. Van december 1988 tot december 1992 werkte zij bij de vakgroep Moleculaire Biologie van de Landbouwuniversiteit te Wageningen aan het onderzoek dat in dit proefschrift beschreven staat. Sinds 1 februari 1994 is zij als post-doc werkzaam bij de onderzoeksschool Experimentele Plantenwetenschappen bij de vakgroepen Fytopathologie en Plantencytologie en -morfologie van de Landbouwuniversiteit te Wageningen.

Nawoord

Dit proefschrift is tot stand gekomen dankzij de inzet en medewerking van velen. Al deze mensen wil ik graag bedanken en enkelen met naam noemen.

Allereerst, Sacco de Vries, mijn copromotor, je inzicht, scherpzinnigheid en enthousiasme heb ik als zeer positief ervaren en zijn een grote hulp geweest bij het in goede banen leiden van dit onderzoek. Bovendien zijn de resultaten en de daaruit voortvloeiende veronderstellingen en conclusies dankzij jouw oneindige inspanningen verwerkt tot een leesbaar geheel. Ik heb er veel van geleerd en zal je daar altijd dankbaar voor zijn.

Ten tweede, Ab van Kammen, mijn promotor, je objectiviteit en kritische kijk op de experimenten en teksten zijn van belang geweest voor het stroomlijnen van het onderzoek en het helder formuleren van de resultaten.

De mensen van de penengroep, Hilbert Booij, Peter Sterk, Fred van Engelen, Jan Cordewener, Roger Pennell, Marijke Hartog, Ellen Meijer, Theo Hendriks, Ed Schmidt en Arina Kippers en 'mijn' studenten Marcel Toonen, Maarten Penning en Annet Haselhoff, die in het kader van een afstudeervak een belangrijke bijdrage aan dit onderzoek geleverd hebben; bedankt voor de grappen, buffers, en de vele handige tips.

Jacques Vervoort van de vakgroep Biochemie en Dominique van der Straeten en Joël Vandekerckhove uit Gent voor hun hulp bij specifieke onderdelen van het hier beschreven onderzoek. Also I would like to thank Mario Terzi and Fiorella LoSchiavo from Padua and Jørn Mikkelsen and Karsten Kragh from Copenhagen for their help with specific parts of this research project and for the warm welcome at their labs.

Peter van Druten voor verricht fotowerk en natuurlijk alle Molbiollers. De goede sfeer, onontbeerlijk voor plezier in het werk, en de gezellige koffie- en thee-uurtjes hebben de afgelopen vijf jaar tot een plezierige tijd gemaakt.

Verder mijn familie en vrienden, jullie zijn mijn ruggesteun en uitlaatklep en zijn een grote hulp geweest, vooral tijdens de minder gemakkelijke tijden. Lanie, de vele uren samen lachen, huilen, feesten, borrelen, kletsen, en uitblazen op Terschelling hebben de afgelopen vijf jaar een waardevolle, extra dimensie gegeven. Bart Jan, in de afgelopen twee jaar heb jij er nog een schepje bovenop gedaan. Van je warmte en rust hoop ik nog heel lang te kunnen genieten.