Induction of plant somatic embryogenesis in liquid medium

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Induction of plant somatic embryogenesis in liquid medium

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Stellingen

1; Arabinogalactaan-eiwitten met specifieke epitopen kunnen de ontwikkeling van cellen in vloeibaar medium beïnvloeden.
Dit proefschrift.

2; Een snelle opname van fytohormonen tijdens de eerste subcultures is één van de voorwaarden om in een cellijn in vloeibaar medium embryogeen vermogen te induceren.
Dit proefschrift.

3; Verschillende kenmerken van een cellijn, zoals bv. het embryogeen vermogen, die pas in de proliferatie- en embryo-ontwikkelings fase tot uiting komen, worden tijdens de initiatie fase vast gelegd.
Dit proefschrift.

4; De waarneming van Halperin dat pro-embryogene massa's zowel somatische embryo's als wortels kunnen vormen, is toe te schrijven aan de omstandigheden tijdens de initiatie fase.
Halperin, W. (1966) Alternative morphogenetic events in cell suspensions. Am. J. Bot. **53**, 443-453.

5; Het onvermogen van tetraploide cellen om somatische embryo's te vormen wordt niet veroorzaakt door het ploidie nivo, maar door de aangelegde omstandigheden tijdens de initiatie- en proliferatie-fase.
Coutos-Thevenot, P. et al. (1990) Embryogenic and non-embryogenic cell lines of *Daucus carota* cloned from meristematic cell clusters: relation with ploidy determined by flow cytometry. Plant Cell Reports **8**, 605-608.

6; De weerstand die genetisch gemodificeerde gewassen bij een deel van de bevolking oproepen, is mogelijk veroorzaakt door het gebruik van moleculair biologische technieken bij het tot stand komen van deze gewassen.

7; Het aantal door de mens gemaakte mutanten van *Arabidopsis* overstijgt langzamerhand zo zeer het aantal natuurlijk voorkomende varianten, dat gesproken kan worden van een intensief veredeld gewas.

8; De effectieve manier waarop carnivore planten zich nutrienten verschaffen, kan gebruikt worden om het mestoverschot te verlagen, mits vliegen en andere insecten bemiddeling willen verlenen.

9; Niets maakt meer onwetend, dan denken iets te weten.

10; De ware muziekliefhebber herkent men aan de dikte van de luidspreker snoeren.

Stellingen behorende bij het proefschrift 'Induction of plant somatic embryogenesis in liquid medium', te verdedigen door Marc Kreuger op 9 februari 1996.

Contents

Chapter 1
Scope of the investigation

Plants can be propagated via seeds or vegetatively, the first producing new genotypes, the second ensuring the conservation of the genotype. Breeding companies use both methods for the production of new varieties, but for many crops only seed propagation can be used. Vegetative propagation can accelerate breeding programs, since genetically identical plant populations can be obtained that may be hard to obtain using seed propagation. Genetic homogeneity of plant and seed lots is becoming increasingly important and in many crops the demands for uniform productivity are higher than breeding companies can produce.

For those crops and varieties that do not posses the genetic homogeneity that is desired, propagation using somatic embryogenesis may be a way of obtaining plant populations of the *same genetic constitution. In contrast to classical tissue culture, somatic embryogenesis can* probably result in large numbers of plants at a relative low cost and is labour extensive.

Somatic embryos were first described by Reinert in 1959. While culturing carrot cells he observed structures very similar to the zygotic embryos developing in seeds after fertilization. Both zygotic and somatic embryos pass through the globular, heart and torpedo-shaped stages. The term somatic indicates that the cells producing the somatic embryo are not derived from the generative tissues in the flowers. Somatic embryos always contain two meristems, the shoot and root meristem, and are able to form complete plantlets.

Somatic embryogenesis has not widely been applied on a large scale, so far. One of the major problems is obtaining embryos with a high efficiency from plants of any species desired. On a small scale the production of somatic embryos has been described for many species, but the used methods often involve a callus phase, which makes the process laborious and expensive. Moreover, genetic instability is observed which is very undesirable when plants are to be cloned.

In this thesis some conditions, required for the large scale production of somatic embryos, are examined. The ideal basis for somatic embryo production is the use of liquid medium, from the initiation of cell cultures, up to the embryo production phase. It was investigated whether general factors existed, essential for initiating embryogenic cell lines, directly in liquid medium. We have used three crops in our study, carrot, which has been used since long as a model plant for the study of somatic embryogenesis, cyclamen, of which some reports of the small scale production of somatic embryos exist, but with genetic instability, and cucumber, of which embryo production in liquid medium has been described, but also with extensive genetic instability. For the latter two, the large scale production of genetically identical somatic embryos, has not been achieved yet.

For a large scale production, embryogenic and genetic stability are essential. It was investigated whether some components of the extracellular medium, the arabinogalactan-proteins, were able to influence the embryogenic potential of cell lines. There was reason to believe that these molecules were able to influence the development of cells.

It was further investigated whether the production and maintenance of embryogenic cell lines in liquid medium could result in genetic stability. This should be combined with optimal

growth of cell lines in liquid medium and a high efficiency of embryo formation. Also the stability of embryogenic potential had to be ensured. Less attention was paid to the plantlet formation, since this area is already being investigated thoroughly.

In Chapter 2 a review of the literature on the arabinogalactan-proteins (AGPs) is given, describing results obtained so far on chemical properties of AGPs and occurrence in plants. A possible function and role of AGPs in somatic embryogenesis is discussed.

In chapter 3 it is demonstrated that carrot cell lines of different age and embryogenic potential, excrete different mixtures of AGPs. It is also described that purified AGPs can influence the embryogenic potential of carrot cell lines. Embryogenic potential can be induced in non-embryogenic cell lines, and in embryogenic cell lines it can be increased when carrot seed AGPs are added.

In chapter 4 a further purification of biologically active AGPs is performed with the use of two monoclonal antibodies. It is shown that the activity of AGPs depends on the presence of epitopes in the molecules, and that the activity of AGPs is not species specific. It is also shown that different AGP molecules can have different activities, and that the activity of an AGP mixture depends on the ratio of molecules with different activities. These first chapters show that AGPs are involved in the process of somatic embryogenesis, and for the first time a function of AGPs is demonstrated.

In chapter 5 a procedure for the large scale production of cyclamen somatic embryos is presented. Critical factors of the process are discussed, of which the initiation phase is probably the most important. A protocol is described, all in liquid medium, ensuring high embryogenic potential and genetic and embryogenic stability.

Chapter 6 demonstrates that large scale production of cucumber embryos can be obtained in a very similar manner as for cyclamen. The same factors as described for cyclamen, are involved in the induction and maintenance of embryogenic potential in cucumber, but also differences between the two crops were observed. It is further described how genetic stability is obtained and how this can be established.

Chapter 7 discusses the application of the process for the industrial production of somatic embryos. Essential steps and necessary controls are evaluated.

Chapter 2
Arabinogalactan-proteins and plant differentiation

Marc Kreuger and Gerrit-Jan van Holst

This chapter was, in another version, submitted to Plant Molecular Biology

Introduction.

Arabinogalactan-proteins (AGPs) are found in higher plants and in liverworts (Basile and Basile 1987, Clarke et al. 1979). The AGPs are proteoglycans occurring mainly on cell membranes, and to a lesser extent in the extracellular matrix and in gum exudates. One of the best known AGPs is Gum Arabic which is excreted by *Acacia senegal* upon wounding. Already some centuries ago, Gum Arabic was produced in east Africa and traded by Arabs. Nowadays gums, containing similar AGPs as Gum Arabic or AGP-related compounds, are still used in food industry as additives, for their aggregating and gelling capacity.

Little is known about the function of AGPs in living cells. They do not have a structural function and are often excreted in large amounts, for instance, in gums. In the latter case they might act as a physical barrier by forming a gel upon wounding, thus preventing attacks by pathogens. Since AGPs occur in many different tissues and are not exclusively produced upon wounding, it seems likely that they do have other, as yet unknown, functions.

AGPs are rich in hydroxyproline and, therefore, they are classified as hydroxyproline rich glycoproteins, HRGPs (Showalter 1993). This large family of cell wall proteins include the extensins, the repetitive proline-rich proteins (RPRPs), some nodulins and AGPs (See for review; Kieliszewski and Lamport 1994). The extensins and the RPRPs are structural cell wall proteins (Showalter 1993) the synthesis of which is developmentally regulated (Ye and Varner 1991, Ye et al. 1991, Smallwood et al. 1994). The extensins and RPRPs give strength to cell walls by cross-linkages between the chains of different molecules, mostly by the oxidation of hydroxyl (-OH) groups (Cosgrove 1993). Oxidations of monomers lead to the formation of a network of protein-protein molecules or proteins linked to other wall components (Fry 1986), and thereby stiffening of the cell wall. Cell wall growth and stiffening are important events and are strictly regulated during cell growth and differentiation. Some early nodulins are hydroxyproline rich proteins which could be cell wall proteins involved in the formation of nodules (Scheres et al. 1990). If, on the other hand, AGPs are rich in hydroxyproline they differ from the other HRGPs, in that they are not a structural component of cell walls (Van Holst et al. 1981), although they may be involved in cell wall formation.

Chemical properties and composition of AGPs.

The majority of the AGPs, characterized so far, have a protein content of less than 10% and contain more than 90% carbohydrate. The protein part of AGPs is rich in hydroxyproline, alanine, glycine and serine (reviewed by Clarke et al. 1979 and Fincher et al. 1983) but the content of the aminoacids may vary between AGPs of different species and tissues (Clarke et al. 1978, Jermyn and May Yeow 1975). The carbohydrate side chains are mainly attached to the serine and hydroxyproline residues. The protein core often contains several Ala-Hyp repeats or closely related repeats (Gleeson et al. 1989, Jermyn and Guthrie 1985) but hydroxyproline-less AGPs have also been found (Baldwin et al., 1993). Recently, the first

cDNAs coding for the protein portions of AGPs have been cloned (Chen et al. 1994, Du et al. 1994., Loopstra and Sederoff 1995).

After deglycosylation of the AGPs in a purified preparation, the remaining protein produced bands after separation by SDS-polyacrylamid gel electrophoresis, ranging in size from 30 to 70 kD (Baldwin et al. 1993, Gleeson et al. 1989, Kieliszewski et al. 1992). This indicated that the sizes of the protein cores of different AGPs in a preparation of total AGPs do not widely vary.

With a protein content of less than 10% and the apparent molecular masses found for the protein cores of AGPs, this would suggest a molecular mass of 300 kD or more for AGPs. However, AGPs are often found to be much smaller, less than 200 kD (Fincher et al. 1983). This apparent discrepancy should be attributed to the unreliable determination of the mass of the protein core. Complete deglycosylation of AGPs is difficult to achieve. Even if 95% of the carbohydrate is removed, the remaining protein will still contain 10% carbohydrate and this may influence the electrophoretic mobility of the protein. Besides, the deglycosylated AGP protein may show anomalous mobility in SDS-polyacrylamid gel electrophoresis, due to the unique aminoacid composition. The high hydroxyproline content may lead to less binding of SDS, which will lead to an overestimation of the mass of the protein (Gleeson et al. 1989). Indeed, the protein mass caculated from the aminoacid sequence deduced from the cDNAs cloned so far, indicate proteins of 13 to 15 kD (Chen et al. 1994, Du et al. 1994). Assuming AGPs have a protein portion of about 10%, this would indicate a molecular mass of 100 to 200 kD, which is in fair agreement with the results of direct determinations of the masses of AGPs.

Arabinose and galactose are the major sugar residues in AGPs and are present in oligosaccharide moieties linked by an O-glycosidation to the OH group of hydroxyproline or serine residues in the protein core (Fincher et al. 1983). Arabinose and galactose each represent 30 to 40% of the sugars, besides, uronic acids are often present in small amounts (Kieliszewski et al. 1992, Komalavilas et al. 1991, Van Holst et al. 1981). To a limited extent has the structure of the carbohydrate side-chains been determined and for certain carbohydrate units the structure has been proposed (Clarke et al. 1979, Tsumuraya et al. 1984). Basically, a ß1-3 galactan chain is linked to the protein with branches consisting of ß1-6 linked galactose and terminal ß1-3 linked arabinose or ß1-6 linked glucuronic acid residues (Fig. 1). The carbohydrate side-chains may consist of more than 50 residues (Clarke et al. 1979). In principle, the variation in the type of branching of the side chains can be unlimited. As a consequence there are few data on the precise structures of the side chains. The most intriguing and difficult aspect of AGPs, the heterogeneity of the polysaccharide side-chains is not yet understood. The complicated structure and heterogeneity of the carbohydrate side-chains raises the interesting question in which respect the protein of AGPs differ from other members of the HRGP group. Several authors make classifications within the HRGP group on basis of hydroxyproline content, aminoacid sequences and protein and sugar content

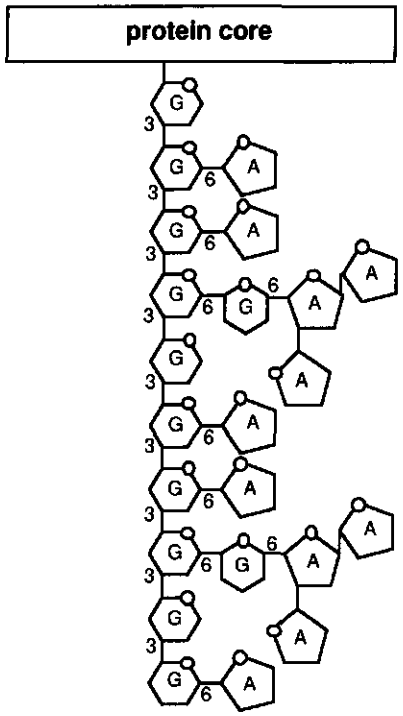

Fig. 1. Possible structure of the sugar side-chain of an AGP molecule. G = galactosyl residue, A = arabinosyl residue.

(Knox 1990, Showalter 1993). The glycosylation of the HRGPs ranges from minimal (RPRPs) to very extensively (AGPs). Each class of the HRGPs contains characteristic repetitive protein sequences that could have originated from one ancestral protein. However, proteins have been found with characteristics of both extensins and AGPs (Kieliszewski et al. 1992, Lind et al. 1994), making classifications rather difficult. There may well be proteins containing domains from more than one of the HRGP groups. The HRGP group may therefore be a continuous spectrum of hydroxyproline rich proteins, which are related but functionally different (Kieliszewski and Lamport 1994). Still, the question remains; what distinguishes AGPs from the other members of the HRGP group?

A very characteristic feature of AGPs is that they can be specifically precipitated by the ß-glycosyl Yariv reagents (Yariv et al. 1962, 1967) providing a consequent operational definition of AGPs. The ß-glucosyl Yariv reagent (1,3,5-tris [4-ß-D-glucopyranosyl-oxyphenyl azo] 2,4,6 trihydroxybenzene) is shown in Fig. 2. In practice this simple definition works quite well. The specificity of the ß-glucosyl Yariv reagent for AGPs has been confirmed by several authors (Clarke et al. 1978, Jermyn and May Yeow 1975, Van Holst and Clarke 1985). Closely related

molecules like arabinogalactans, which resemble the carbohydrate part of AGPs but do not contain a protein core, are not precipitated with the ß-glucosyl Yariv reagent. However, they can interfere with the precipitation of AGPs (M. Kreuger, unpublished results). An exception is the glucose binding lectin ConA which is precipitated by the ß-glucosyl Yariv reagent. Precipitation of ConA is however inhibited in the presence of 1% w/v glucose (Van Holst and Clarke 1985). Other compounds may also be able to interfere in the precipitation of AGPs, but are never precipitated themselves.

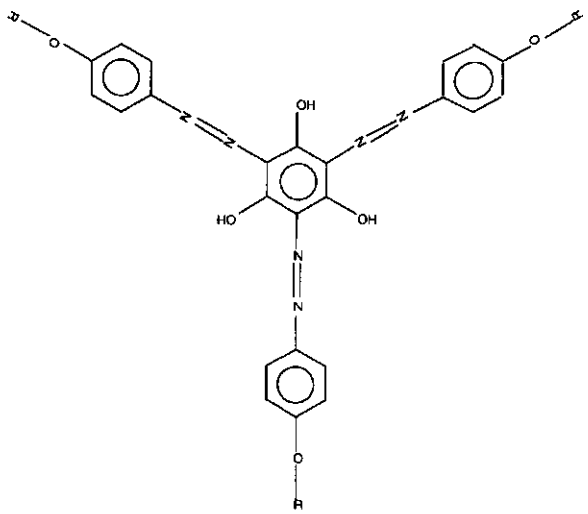

Fig. 2. Structure of the ß-glucosyl Yariv reagent. R is a glucosyl residue.

What then makes the ß-glucosyl Yariv reagent so specific for AGPs? A speculation of what might happen during precipitation is the following (Fig. 3). Yariv reagent molecules easily form aggregates in aqueous solutions (Jermyn and May Yeow 1975). The flat molecules with their hydrophobic centre will form stacks with each molecule slightly turned compared to its neighbours. The result is a helical structure containing three identical grooves. The ß1-3 galactan chains of the AGPs can also form a helical structure with each complete turn of the galactan chain containing about eleven residues (Rees 1977). This galactan helix might fit in the grooves of the Yariv stacks. Although it is unknown how many Yariv molecules are needed for one turn, it is plausible that the galactan helix fits in the grooves of the helical stacked Yariv reagent molecules. The galactan-Yariv stacks interconnected with the protein core of the AGP form a large network that precipitates. Both the protein core of the AGP and the polysaccharide chains are essential for precipitation. Interfering molecules like arabinogalactans might be able to interact with the Yariv reagent but miss the protein core to built up a network that precipitates. So, not the binding but the formation of a large network

defines the specific precipitation of AGPs with the Yariv reagent. Precipitation is optimal at a 1 : 1 ratio (w/w) of AGP to Yariv reagent (Jermyn and May Yeow 1975).

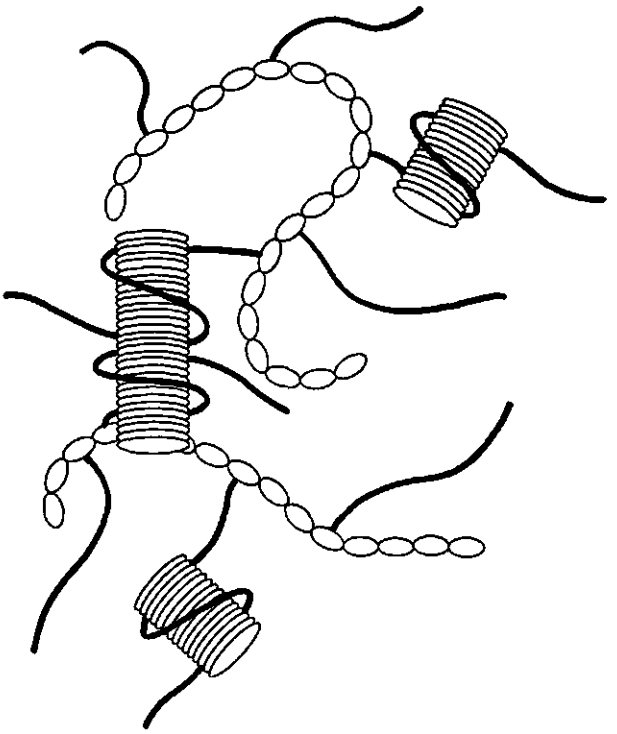

Fig. 3. Schematic representation of the precipitation of AGPs with the ß-glucosyl Yariv reagent. The Yariv molecules form stacks, as visualised by the cilinders. The AGPs consist of a protein portion, represented as the string of ovals, the amino acids, and polysaccharide side-chains, represented as black lines. The polysaccharides form helical structures around the Yariv stacks, thereby forming a large Yariv-AGP-Yariv network which will precipitate.

The ß-glucosyl Yariv reagent can not only be used for isolating AGPs but also provides a simple method for measuring AGP concentrations in a modified Mancini assay (Van Holst and Clarke 1985). If AGPs diffuse into an agarose gel containing the ß-glucosyl Yariv reagent, the optimal ratio of AGP to Yariv reagent will be reached at a certain place in the gel, dependent on the concentrations of both compounds. The result is a precipitation circle. The area covered by the circle is proportional to the amount of AGPs originally put into the well.

Analysis of AGPs.

Electrophoresis of AGPs in SDS-polyacrylamid gels resulted in the formation of a smear of AGPs with an apparent molecular weight of about 100,000 (Baldwin et al. 1993, Knox et al. 1989, 1991, Komalavilas 1991, Pennell et al. 1989). Using other techniques, like ion-

exchange chromatography or iso-electric focusing smears of AGPs were also obtained (Anderson et al. 1977, Jermyn and Guthrie 1985, Kieliszewski et al. 1992, Samson et al. 1984). If, on the other hand, AGPs were separated by gel filtration techniques, on basis of their size only, they eluted in rather distinct peaks (Anderson et al. 1977, Komalavilas et al. 1991, Van Holst et al. 1981).

These results indicate that AGPs in different fractions or isolations are more heterogeneous in charge than in size. Since the protein core of an AGP isolation shows a fairly sharp band using SDS-PAGE electrophoresis (Baldwin et al. 1993, Gleeson et al. 1989, Kieliszewski et al. 1992), and the size differences within a fraction are rather small, this indicates that the widely different electrophoretic mobilities are mainly caused by charge differences in the sugar moiety, probably due to different amounts of glucuronic acids. How these different amounts of uronic acids are achieved is unknown, nor is the precise function of the uronic acids.

A precise determination of the molecular weight using techniques other than gel filtration is not possible. The mobility of AGPs in SDS-PAGE is not proportional to the molecular weight. The SDS binds to every two aminoacids, which gives a complex of SDS with a denatured protein and a large negative charge that is proportional to the molecular weight of the protein. The negative charge acquired on binding SDS is usually much greater than the charge of the native protein; this native charge is thus rendered insignificant. Due to the very small size of the protein (only 10% of the molecular mass) and very large, highly negative side-chains, this does not apply to AGPs. The AGP molecules are therefore not only separated on basis of the length of the polypeptide chain but also by their charge.

Separation of native molecules in agarose gels, is commonly used for DNA and RNA, and has proved to be very consistent and reproducible. Native AGPs can also be separated in agarose gels. Since the AGPs are separated on basis of their size as well as their charge, the separation results in a smear. This form of gel electrophoresis can yet be useful for analyzing AGPs, by so-called crossed-electrophoresis (Van Holst and Clarke 1986). This involves electrophoresis of native AGPs in an agarose gel resulting in a smear of AGPs (Fig. 4) which may represent a continuous spectrum of many different but closely related molecules. A second electrophoresis perpendicular to the first direction, into a gel containing the ß-glucosyl Yariv reagent results in the formation of a precipitation line. The height of the precipitation line is determined by the amount of AGPs with a certain mobility in the first dimension.

By crossed-electrophoresis differences in the relative amount of AGPs with a certain mobility can be observed, and different AGP preparations will show a specific pattern. Crossed-electrophoresis appears so-far to be the only technique able to reveal differences between AGP mixtures. It could be demonstrated that different plant tissues and organs contain mixtures of AGPs with a characteristic composition and that the composition of the mixtures changed during development (Cassab 1986, Gell et al. 1986, Van Holst and Clarke 1986). During differentiation of cells and tissues, peaks in the pattern obtained by crossed-

electrophoresis, could change in height and new peaks could arise resulting in a change of the pattern. Generally the crossed-electrophoresis patterns of AGPs from a defined source, contain one or more wide peaks with specific R_f-values (the relative mobility compared with a colour dye). For example in *Lycopersicon peruvianum* (Mill) tissues three or four groups of AGPs with specific R_f-values can be found (Fig. 5). The ratio of the different groups depended strongly on the tissue, and some tissues contained only one or two groups (Van Holst and Clarke 1986). With the same technique Gell et al. (1986) showed that during flower maturation of tobacco specific AGPs were formed with a very distinct R_f-value.

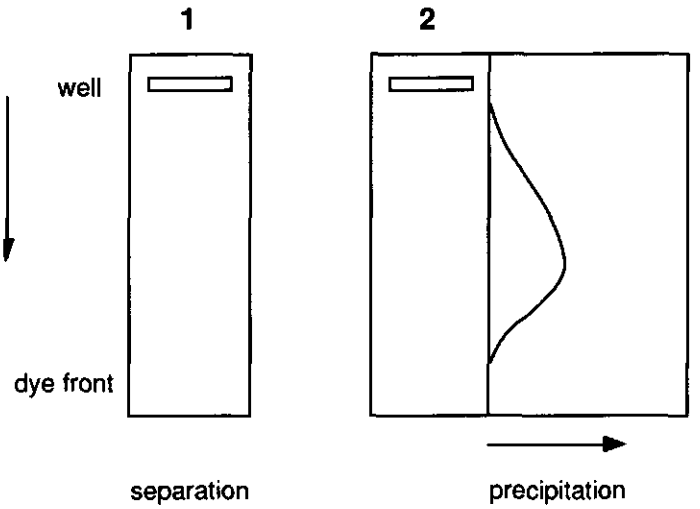

Fig. 4. Separation of AGPs by crossed-electrophoresis. AGPs are first separated in an agarose gel (1). The lane containing the AGPs is cut out and a new agarose gel containing the ß-glucosyl Yariv reagent is poured next to the AGP lane. In the second electrophoresis the AGPs move perpendicular to the first electrophoresis into the Yariv containing gel, thereby forming a precipitation line (2).

It is clear that major differences in AGPs occur during differentiation. Assuming that the AGP mixtures contain a limited number of different protein cores as is indicated by the single protein band of deglycosylated AGPs, the heterogeneity is caused by the sugar moiety. In accordance with this is the recent finding that AGP-like genes do not form a family of related genes, and are therefore present in low copy numbers (Pogson and Davies 1995). The charge of the sugar side-chains are developmentally regulated since each tissue has its own AGP mixture. This could be due to tissue specific partial degradation of the carbohydrate side-chains or changes in the production of the side-chains. During the synthesis of the AGP side-chains, monosaccharides are attached to form polysaccharides. Subsequently, the carbohydrate chains can be partially broken down by exoglycosidases, and simultaneously, branches can be initated. Obviously, many enzymes will be involved, creating the possibilty of

a tissue specific regulatory mechanism. The number of possible different AGP molecules is almost infinite, making analysis and detection or isolation of specific molecules very laborious or even impossible. This makes it very difficult, if possible at all, to isolate an AGP fraction containing molecules of defined size and structure.

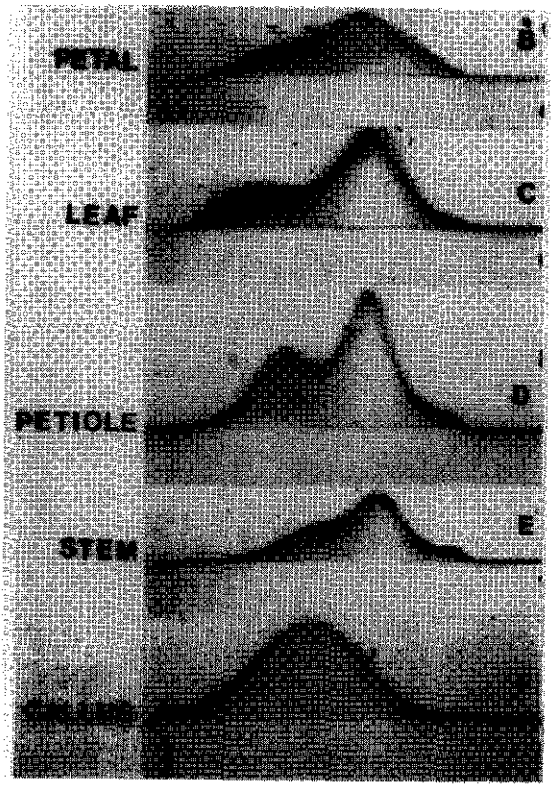

Fig. 5. Crossed-electrophoresis of AGPs from different tissues of tomato (*Lycopersicon peruvianum* Mill.). From Van Holst and Clarke (1986).

The use of antibodies to characterize AGPs.

A powerful tool to unravel and characterize AGPs are polyclonal or monoclonal antibodies. Many groups have produced antibodies against AGPs (Anderson et al. 1984, Kikuchi et al. 1993, Knox et al. 1989, 1991, Miskiel and Pazur 1991, Norman et al. 1990, Pennell et al. 1989, 1991). All antibodies were, at least partly, directed against the sugar moiety of the AGPs and their binding could be inhibited by mono- and disaccharides (Anderson et al. 1984, Pennell et al. 1989) or by more complex oligo- and polysaccharides, like Gum Arabic and AGPs from larch and wheat (Anderson et al. 1984, Knox et al. 1989, Pennell et al. 1989).

Monoclonal antibodies have been used to localize AGPs in different tissues. The JIM4 antibody (Knox et al. 1989) was obtained after immunization with carrot protoplasts. The antibody recognized AGPs from the medium of suspension cultured carrot cells and Gum

Arabic, but also other, possibly non-AGP or AGP-like molecules. It was demonstrated that the epitope was located at a specific set of cells during the development of the root apex of carrot. The expression of the antigen occurred well before the pattern formation was visible, and was restricted to two small groups of cells in the future vascular bundle and was maintained through the root system. The epitope was also found in the vascular tissue of the cotyledons, exclusively on the outer side of the plasma membrane. The same epitope has been localized in somatic embryos of carrot (Stacey et al. 1990). Here, the epitope was restricted to the protoderm of early somatic embryos and to the provascular tissue of the root apex and the cotyledons. The JIM4 epitope therefore seemed to accompany pattern formation during the differentiation of the vascular tissue.

The JIM8 antibody was obtained after immunization with sugarbeet protoplasts (Pennell et al. 1991) and was selected for its binding with Gum Arabic. The antibody bound to AGPs from suspension cultured carrot cells, but also to rhamnogalacturonans, which are non-AGP cell wall components, and to other non-AGP gums. The epitope was localized in oilseed rape (*Brassica napus*) flowers. During flower development the epitope was located on the sexual organs and appeared progressively along several tissues, up to the egg cell and sperm cells. The epitope was present on specific cell types only during a short period, after which it disappeared. The JIM 8 epitope was also expressed during the early stages of embryogenesis in the whole embryo, and at later stages only in the suspensor cells. The same epitope was located on a specific set of single cells in embryogenic cell suspensions of carrot (Pennell et al. 1992). It was suggested that this cell type might be a transition stage between somatic and embryogenic or competent cells, although this was not actually proven. These data suggested a role for the JIM8 epitope at or near the phases of the plant cycle where embryogenic cells are formed.

The monoclonal antibodies JIM 13 and 15 were raised against two AGP fractions isolated from the conditioned medium of an embryogenic carrot cell suspension and showed to be specific for AGPs (Knox et al. 1991). JIM 13 and 15 recognized complementary patterns in the developing carrot root. JIM 13 recognized the epidermal cells and the future xylem axis, but not the cortex. The JIM 15 epitope was complementary to this pattern and bound to the cortex, but not to the epidermis, with a small overlap with JIM 13 in the stele. The same epitopes were found on cells in the developing xylem of maize, where they seemed to be markers of those cells which were committed to programmed cell death during xylem formation (Schindler et al. 1995).

Monoclonal antibodies have been used for detecting epitopes on the cell surface (Knox et al. 1989, 1991, Pennell et al. 1991, Pennell and Roberts 1990, Schindler et al. 1995, Stacey et al. 1990) or extracellular in cell walls or in the medium of suspension cultures (Knox et al. 1991, Li et al. 1992, Pennell et al. 1989, Sedgley and Clarke 1986, Van Aelst and Van Went 1992) or both simultaneously (Herman and Lamb 1992, Kikuchi et al. 1993, Knox et al. 1991, Pennell et al. 1992, Schindler et al. 1995). Occasionally epitopes have been detected

intracellular, predominantly in multivesicular bodies and/or associated with the vacuole (Herman and Lamb 1992, Pennell et al. 1992, Schindler et al. 1995, Sedgley and Clarke 1986). With these antibodies patterns of expression of membrane bound AGPs has been demonstrated, but also the presence of AGPs intra- and extracellular. The significance of the finding that AGPs are found both on the cell membrane and in the cell wall is not clear, but it indicates that AGPs are very abundant in the surroundings of all cells.

The data show that specific epitopes are present only on a limited number of cells or cell types during their differentiation and that some epitopes may show only transient expression. The outer side of cells contains sets of AGPs which can change of constitution during differentiation, meaning that cell lineages will show subsequent appearance and disappearance of specific epitopes (Knox 1993, Pennell et al. 1991). The AGP epitopes may reflect a tissue pattern which is determined by cell position. They also show extensive modulation of cell surface AGPs during cell development (Knox et al. 1991). The extensive control mechanism for the marking of cell position, may be an absolute necessity for the correct development of a multicellular organism.

This raises the question whether the different AGPs are merely a result of differentiation or whether they can cause these events, or possibly both. Is differentiation accompanied by the formation of new AGP epitopes, resulting in a change in the surface of the cells? Or can newly formed AGPs, after changing the surface, direct the development of cells, possibly by an altered interaction with the neighbouring cells? This intriguing question of what came first, the AGP or the differentiation, may be hard to answer. It must be clear however that only the detection of an epitope does not exclude other molecules than the AGP. Questions can be raised on the specificity of the antibodies used. How can we know that the localized epitopes are in fact belonging to AGPs? Screening and selection of antibodies should therefore always be performed with great care. Antibodies binding to a wide range of AGPs will have little specificity since the epitope is very common. They are therefore likely to bind other, non-AGP molecules with the same common epitope. Obviously the common epitopes exist of only a few aminoacids or sugar residues, which can be very abundant in many different types of molecules.

Stability and turn-over of AGPs.

AGPs are chemically very stable, they are resistant against high temperature and alkali treatment (G.J. van Holst and M. Kreuger, pers. comm.). In pulse-chase experiments with radioactive hydroxyproline the incorporation in AGPs and subsequent disappearance of the label was studied (Van Holst et al. 1981). Suspension cultured *Phaseolus vulgaris* L. showed labelled hydroxyproline in the AGP fraction already after 5 minutes, indicating a high production rate. The half-life of the AGPs was about 10 to 15 minutes indicating a rapid removal of the labelled molecules. In hypocotyl segments of *Phaseolus vulgaris* L. the half-life was longer than in suspension cultured cells, about 120 minutes.

The short presence of AGP molecules in tissues is probably not due to their instability, but to an active system of removal or degradation. The purpose of this short life of AGPs is not known, but it might be achieved through an active system present in all plant cells. Another group of molecules, the oligosaccharins, presently regarded as signal molecules in different processes, with a molecular weight of about 2000 (Fry et al. 1993), have shown to be internalised by means of the so-called receptor mediated endocytosis to multivesicular bodies and are deposited into the vacuole (Horn et al. 1989). Multivesicular bodies are vesicles containing several smaller vesicles and are formed after invaginations of the plasmamembrane. This form of endocytosis is mediated by receptors and is therefore called receptor mediated endocytosis. This process and multivesicular bodies are well documented in animal cells where it is generally thought that it functions as a degradation process (Stahl and Schwartz 1986). AGPs can also be found on the internal membranes of multivesicular bodies and in the vacuole (Herman and Lamb 1992, Pennell et al. 1992, Schindler et al. 1995, Sedgley and Clarke 1986). The multivesicular bodies can fuse with the tonoplast (Herman and Lamb 1992, Tanchak and Fowke 1987) or in styles they can fuse with the plasmamembrane releasing vesicles containing AGPs into the extracellular space (Sedgley and Clarke 1986).

It was demonstrated that large extracellular molecules, added to protoplasts, were incorporated in vesicles and multivesicular bodies (Tanchak et al. 1984). This indicates the presence of a mechanism for removal of large extracellular molecules. A similar system may exist for AGPs. Membrane bound and extracellular AGPs could be internalized by receptor mediated endocytosis, leading to the formation of multivesicular bodies (Tanchak and Fowke 1987). The content of the multivesicular bodies is then deposited in the vacuole or in the extracellular matrix, both eventually leading to degradation of the AGPs. The fact that AGPs are often found on multivesicular bodies may illustrate this active system of degradation and recycling, and may explain the high turn-over of AGPs.

A possible function of AGPs.

Actual proof of a function of AGPs is still absent. Hydroxyproline containing proteins, possibly AGPs, appear to have a morphoregulatory role in Bryophytes (Basile 1990) where they suppress growth at specific places on developing leaves, leading to a species-specific leaf and branching morphology. When the suppression of growth is de-suppressed by addition of hydroxyproline, a new plant morphology is formed which can resemble more to the morphology of other species. The addition of hydroxyproline to plants might interfere in the correct synthesis of hydroxyproline containing proteins, like AGPs and extensins, since proline is hydroxylated after the protein is synthesized (Pollard et al. 1981). The de-suppression of growth by hydroxyproline would suggest a role in plant morphology for the HRGPs. Several authors have ascribed certain activities to AGPs of which cell-cell

recognition and interaction, cell identity and participation in morphogenesis are the most mentioned (Clarke et al. 1979, Fincher et al. 1983, Knox 1993).

The presence of AGPs on the outer side of the plasma membranes may indicate that they play a role in the interface of the membrane and the extracellular matrix, and certain epitopes may be involved in the deposition of macromolecules. AGPs in the cell wall might be involved in crystallisation of macromolecules thereby directing planes of growth and development and cell shape (Kieliszewski and Lamport, 1994). Both events, as being dynamic and susceptible to a changing environment, will need tools for modifying the cell wall, which will change the molecular composition of the wall by removing 'old' molecules and producing new molecules. Transport of AGPs or AGP epitopes through cell walls from one cell type to another may lead to an altered wall composition. This might have an impact on the differentiation, possibly resulting in a new developmental path.

As mentioned previously AGPs were found on membranes as well as in the extracellular matrix. In one case (Komalavilas et al. 1991) differences were found between membrane bound and excreted AGPs of suspension cultured rose cells. The composition was very similar but membrane bound AGPs were larger and more negatively charged suggesting that the excreted AGPs are cleavage products or partly degraded or processed (Komalavilas et al. 1991). This puts forward the question of the function of the AGPs. Are the membrane bound AGPs active or the extracellular, or both, or do they have different activities? In animal cells a family of extracellular glycoproteins called the Cell Adhesion Molecules have shown to be involved in morphoregulatory processes (Edelman and Crossin 1991). These developmentally regulated molecules are located on the cell surface and play a role in tissue boundary formation, embryonic induction and migration, tissue stabilisation and regeneration. In all these events cell to cell communication is required for the correct differentiation. Although the precise activity is unknown it is generally accepted that the extracellular matrix plays an essential role in these processes. An analogous system in plant cells is likely to exist.

The molecular composition of AGPs provides the intrinsic possibility of many different signals. Intercellular signalling between cells is very important for a correct differentiation. Cell position as well as cell lineage determines cell fate (Irish 1991, Sussex 1989) and this is, at least partly, determined by the cell wall (Berger et al. 1994). In multicellular organisms all cells will depend on and influence each other. Cells which have entered a new cell layer by division will differentiate according to their new position or new cell layer rather than their origin. When isolated protoplasts of embryos of *Fucus spiralis* were placed in contact with the cell wall of another cell type, their fate was switched (Berger et al. 1994). This sudden change in development clearly needs cell-cell communication to trigger these events, and cell walls appear to have the ability to alter cell fate. How, and, whether AGPs can mediate this communication is unclear. It is unlikely that the membrane bound AGPs of a cell are able to

influence or signal an adjacent cell. Studies on the fate of extracellular AGPs might reveal whether they have a signalling function or not.

If AGPs are to communicate with an adjacent cell, they must be able to pass freely through the cell walls, although their size seems to prevent this. The high hydroxyproline content of the protein and the type of glycosylation can force the AGP-molecule into a linear conformation thereby forming a rod-like molecule (Kieliszewski and Lamport 1994, Van Holst and Varner 1984). This would make the passage of AGPs through the cell wall more plausible. Carrot extracellular AGPs of 70 to 100 kD had a size of 25 nm by 15 nm (Baldwin et al. 1993). Suspension cultured cells of *Glycine max* (L.) Merr. showed to take up very large molecules (20-140 kD) after treatment with saponin, a compound perturbing membranes (Meiners et al. 1991) indicating that the cell wall does not exclude these molecules. In the same system it was shown that the size exclusion limit of the cell walls is determined by pectin and is 6.6-8.6 nm (Baron-Epel et al. 1988). Treatment with pectinase could enlarge the size of the channels. These results indicate that fairly large molecules can pass through cell walls and that this flow of molecules can be increased without affecting cell viability (Baron-Epel et al. 1988, Meiners et al. 1991). The fact that cell suspension cultures excrete proteins as well as AGPs (De Vries et al. 1988a, Knox et al. 1991, Pennell et al. 1989) indicates that the size exclusion limit of cell walls might be larger than AGPs. The idea of AGPs passing the complete cell wall for exhibiting their function might therefore not be that strange at all.

Another plausible manner for AGPs to function is as substrate for the production of a oligosaccharin by an enzyme. The small oligosaccharin can then exert its function as a signal molecule, and the remainder of the AGP can be quickly recycled, which would be in accordance with the observed high turn-over. Indeed the processing of AGPs, thereby possibly releasing biologically active compounds, has been reported (Kjellbom et al. 1994). The type of message or signal would not only depend on the AGP-molecule, but also on the enzymes present in the cell wall. One AGP-molecule could, after processing by a number of different enzymes, generate several different oligosaccharins. If so, this would also imply the presence of specific receptors or target sites for AGP-derived molecules. As yet, there are, however, no indications that AGPs have a part in a signal transduction pathway.

In analogy with the oligosaccharins, the AGPs can be degraded inside the vacuole, thereby removing the putative signal function of the AGP. The result is, besides the high turn-over, a short period in which the AGP can exert its function. Any signal given to a cell has to be removed after 'delivering the message' and cells appear to have active systems of degradation. This enables the cell to react dynamically to a changed environment, just as we observe it doing it.

The highly regulated modulation of AGP epitopes as visualized by the many monoclonal antibodies, and the large charge variations of the AGP molecules, as visualized by the crossed-electrophoresis, strongly suggests the possibility of AGPs being involved in cell

identity. If cell-cell communication exists, highly regulated differentiation of AGPs may be a way to obtain this.

A possible role of AGPs in somatic embryogenesis.

Plant cells are well known for their ability to regenerate new tissues. Cells or cell aggregates are able to differentiate into shoots or roots in *in vitro* cultures, generally under control of one or several growth regulators. Under the appropriate conditions cells can develop into bipolar structures, containing a shoot as well as a root meristem, connected by a strand of vascular tissue. The development of these structures is similar to the zygotic embryo development and is therefore called somatic embryogenesis. Carrot (*Daucus carota* L.) is the plant species in which somatic embryogenesis was first observed by Reinert (1959). The paper of Halperin (1966) is an early report of the development of carrot somatic embryos from small cell clumps, through the globular, heart and torpedo stages. In carrot, somatic embryogenesis is easly induced and is, probably for that reason, most frequently studied.

If pieces of carrot tissue are cultured on solid or in liquid medium, in the presence of an auxin and a cytokinin, the cells of the tissue divide and form callus. Carrot cell cultures can easily be established directly in liquid medium (De Vries et al. 1988b) and large amounts of somatic embryos can be obtained. The cell cultures contain several cell types including large vacuolated cells, and small, dense, highly cytoplasmic cells, often present in small clumps, the pro-embryogenic masses (Halperin 1966). The pro-embryogenic masses (PEMs) can develop into somatic embryos with a very high efficiency. This allows detailed studies on embryogenic development and differentiation.

The used carrot embryogenic cell suspensions proved to be a very useful system to study differentiation events. The liquid medium in which the cells are grown can be regarded as an extended extracellular matrix. This extracellular matrix is important in somatic embryogenesis since extracellular proteins showed to be essential (De Vries et al. 1988a). When the production of these proteins was blocked by the addition of tunicamycin, a compound interfering the N-glycosylation of extracellular glycoproteins, the formation of somatic embryos was completely arrested. The addition of these proteins to arrested cell cultures restored somatic embryogenesis showing the potential of this system. By changing the extracellular matrix the development of cells can be changed indicating a morphoregulatory role.

It is well known that suspension cultured cells and protoplasts need a minimal cell density below which the cells no longer divide and grow. A way to avoid this to happen is the use of a feeder layer, a cell suspension growing in the same medium but physically separated. The excreted compounds of the cell suspension can diffuse to the protoplasts, or the cells at low density, resulting in increased growth rates. Excreted compounds, or conditioning the medium by most likely proteins and AGPs, appears therefore of vital importance for cells in suspension to grow and differentiate. The addition of conditioned medium to establishing cell

suspension cultures resulted in a faster development of embryogenic potential (De Vries et al. 1988a, b).

Control of *in vitro* morphogenesis by manipulation of the extracellular matrix using biologically active molecules like oligosaccharins has been described several times (Eberhard et al. 1989, De Jong et al. 1993, Tran Thanh Van et al. 1985). The accessibility of the extracellular matrix makes *in vitro* systems very attractive for studying these compounds, thereby including AGPs.

As mentioned previously different AGP epitopes are found on the membranes, in the cell walls and in the culture medium (reviewed by Knox 1993). The addition of AGPs, isolated from seeds, to an old carrot cell line which had lost its embryogenic potential, restored the ability to produce somatic embryos (Kreuger and Van Holst, chapter 3). In a similar experiment, the addition of AGPs to an embryogenic cell line resulted in an increase in embryogenic potential. By changing the AGPs surrounding the cells, it was possible to alter their development, demonstrating a biological activity for AGPs.

Conclusions.

AGPs might have a role in cell-cell signalling and/or cell identity. A role in developmental processes is supported by a vast amount of circumstantial evidence. The structure of AGPs has a possibility of creating many different type of molecules, which all can have a different function. The localization on the plasmamembrane and at the cell wall, suggests that they may be involved in developmental processes. The fact that each tissue, and possibly each cell type, has its own set of AGPs, indicates a relation with cell identity. The high turn-over of AGPs will enable the cells to react quickly to a changed environment, by rapid synthesis and removal of new types of AGPs. However, the true function of AGPs is still unclear, as the possible occurence of a receptor for AGPs or AGP-derived molecules.

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Chapter 3
Arabinogalactan-proteins are essential in somatic embryogenesis of *Daucus carota* L.

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Abstract.

Daucus carota L. cell lines secrete a characteristic set of arabinogalactan-proteins (AGPs) into the medium. The composition of this set of AGPs changes with the age of the culture. This is determined by crossed-electrophoresis with the specific AGP binding agent, the ß-glucosyl Yariv reagent. Addition of AGPs isolated from the medium of a non-embryogenic cell line to an explant culture initiated the development of the culture to a non-embryogenic cell line. Without addition of AGPs or with addition of carrot seed AGPs an embryogenic cell line was established. Three months old embryogenic cell lines usually contain less than 30% of dense, highly cytoplasmic cells, i.e. the embryogenic cells. When carrot seed AGPs were added this percentage increased to 80%. Addition of carrot seed AGPs to a 2 years old, non-embryogenic cell line resulted in the reinduction of embryogenic potential. These results show that specific AGPs are essential in somatic embryogenesis and are able to direct development of cells.

Introduction.

Arabinogalactan-proteins (AGPs) are known to be present in higher plants and in their exudates. They are only present on cell membranes, in cell walls and in intercellular spaces of tissues. In cell cultures, AGPs are excreted into the medium (for a review see Fincher et al. 1983).

AGPs are proteoglycans with poly- and oligo-saccharide units covalently attached to their protein moiety (Van Holst and Klis 1981). These units mainly consist of a 1,3-ß-D-galactopyranosyl backbone and side chains of (1,3-b- or 1,6-b-) D-galactopyranosyl and L-arabinofuranosyl residues (Keegstra et al. 1973; Fincher et al. 1983). Other sugars and uronic acids have also been detected, although at low levels. The protein moiety is hydroxyproline rich and is usually less than 10% (w/w). The total molecular weight is around or above 100000. AGPs isolated from tissues and exudates generally show a large heterogeneity in net charge when analysed by electrophoresis (Jermyn and Guthrie 1985; Van Holst and Clarke 1986). Although a lot of work has been done on the structure of AGPs, little is known about their function.

Recently, the presence of AGPs in the plant during its development has been studied by a number of authors. Using crossed electrophoresis it has been shown that different organs of *Lycopersicon peruvianum* L. and *Glycine max* L. contain different AGPs (Van Holst and Clarke 1986; Cassab 1986). Although AGPs are very heterogeneous, each tissue contains a specific set of AGPs. This results in a tissue specific AGP pattern which is shown by crossed electrophoresis.

By using monoclonal antibodies directed to specific epitopes of AGPs, it has been shown that a polymorphism of AGPs exists during the development of carrot (*Daucus carota* L.) roots (Knox et al. 1989 and 1991). Using one of these antibodies, Stacey and colleagues (1990) showed that in embryogenic cell cultures of carrot, specific patterns of expression of

the epitope exist during the development of somatic embryos. These results show that during differentiation cells are surrounded by a specific set of AGPs. The composition of this set of AGPs changes during the development of tissues and organs, even before this development or differentiation is visible. This suggests a role of AGPs in the establishment of pattern formation during the development of plants.

The importance of the extracellular matrix and its role in development and differentiation is becoming clearer (Roberts 1989; Knox 1990, and ref. therein). Extracellular proteins proved to be essential for somatic embryogenesis of carrot (De Vries et al. 1988a), showing the importance of the extracellular matrix in this system. We examined the role of AGPs in the establishment of embryogenic cell cultures of carrot and the influence of AGPs on the development of cells.

Materials and methods.

Plant material and cell culture. Carrot seeds (*Daucus carota* L., cv. Trophy-Flakkese or Nantes from Zaadunie BV., Enkhuizen, The Netherlands) were surface sterilised and germinated on Gamborg's B-5 medium supplemented with 0.6% agar and 58 mM sucrose, at 23°C in the dark. Cell cultures were derived from sliced ten days old hypocotyls. Approximately two grams of hypocotyl parts were incubated in 50 ml Gamborg's B-5 medium in a 250 ml flask, supplemented with 2 µM 2,4-dichloro-phenoxyacetic acid (2,4-D) and 58 mM sucrose, in a 16 h day light period at 23°C, on a rotary shaker at 100 rpm (G10 gyrotory shaker, New Brunswick Scientific, Edison, N.J., USA). Nineteen days after the initiation of the culture, the hypocotyl parts were removed (De Vries et al. 1988b). From this point the culture was subcultured every two weeks. About eight weeks after the initiation the culture became embryogenic. The formation of somatic embryos was induced by selecting cells and aggregates that passed a 150 µm nylon sieve and were retained on a 50 µm nylon sieve. This cell fraction was subcultured in auxin free B-5 medium supplemented with 58 mM sucrose, at a cell density of 20000 cells.ml^{-1} (De Vries et al. 1988a). Cell cultures were also initiated from somatic embryos. Two grams of sliced two weeks old somatic embryos were incubated in 50 ml of 2,4-D containing B-5 medium. Further procedures were as described above.

Isolation of arabinogalactan-proteins from seeds and culture medium. Seeds of *Daucus carota* L. (cv. Trophy-Flakkese) were grounded in a coffee mill and extracted with water. Cell debris was removed by filtration through a 3 µm filter and then centrifuged at 10000 x g for 15 min. The AGPs were precipitated by adding the ß-glucosyl Yariv reagent (1,3,5-tris [4-ß-D-glucopyranosyl-oxyphenyl-azo] 2,4,6-trihydroxybenzene) to a final concentration equal to the AGP concentration and NaCl to a final concentration of 0.15 M (Yariv et al. 1967). The preparation of the ß-glucosyl Yariv reagent was done essentially as described by Yariv et al. (1962). The AGP concentration was determined by the single radial diffusion method as

developed by Van Holst and Clarke (1985). The AGP-Yariv complex was precipitated at 4°C for at least 3 h, and then centrifuged at 10000 x g for 15 minutes. The complex was resuspended in 0.15 M NaCl and reprecipitated to remove impurities. After centrifugation the complex was dissolved in 0.1 M NaOH, 1.7 M NaCl to dissociate the complex. The AGPs were separated from the ß-glucosyl Yariv reagent on a Sephadex G-50 column (Pharmacia, Uppsala, Sweden), equilibrated with 0.1 M NaOH, 1.7 M NaCl, and desalted on another Sephadex G-50 column equilibrated with water.

Isolation of AGPs from culture medium could be achieved by adding NaCl and the ß-glucosyl Yariv reagent directly to the cell-free medium. The precipitation and purification was identical as described above. The AGP preparations were free of proteins as determined by the Bio-Rad protein assay (Bio-Rad Laboratories, München, FRG).

Crossed electrophoresis of AGPs. Crossed electrophoresis of AGPs was done essentially as described by Van Holst and Clarke (1986). The ß-glucosyl Yariv reagent concentration in the second electrophoresis was 20 $\mu g.ml^{-1}$. Crossed electrophoresis patterns of medium AGPs were generated from AGPs isolated from the culture medium two weeks after subculturing (except for the explant culture which was subcultured after nineteen days).

Complementation experiments with AGPs. Complementation experiments in which AGPs were added to cell cultures were performed in 10 ml cultures in 50 ml flasks or in 5 ml cultures in 60 mm petridishes. AGPs were filter sterilized (0.22 μm, Flowpore, ICN Biomedicals LTD, Irvine, UK) and added to the culture. Experiments in which the percentage of embryogenic cells was determined were done in 60 mm petridishes (in triplicate). The percentage of embryogenic cells was determined by counting single cells and small aggregates (aggregates of less than 15 cells), discriminating embryogenic cells (small, dense, highly cytoplasmic) from non-embryogenic cells (large, highly vacuolated) (Halperin 1966; Halperin and Jensen 1967; Williams and Maheswaran 1986).

Results.

Crossed-electrophoresis of AGPs isolated from cell culture media and tissue. The AGPs secreted into the medium of a cell culture were analysed from the initiation of the culture to the point at which, after more than one year in culture, it had lost its embryogenic potential. Nineteen days after the initiation of the culture, the hypocotyl parts were removed. The AGP pattern of the medium (Fig. 1C) at that stage resembled the AGP pattern of hypocotyls (the explant material, Fig.1B). During aging of the culture, the AGP pattern changed to a single peak (Fig.1D). At this point the culture was becoming embryogenic. AGPs isolated from carrot seeds showed a pattern (Fig. 1A) similar to an early embryogenic culture. In both AGP isolates the pattern had a maximum at a R_f-value of 0.6. This peak contained the majority of AGPs in both isolates.

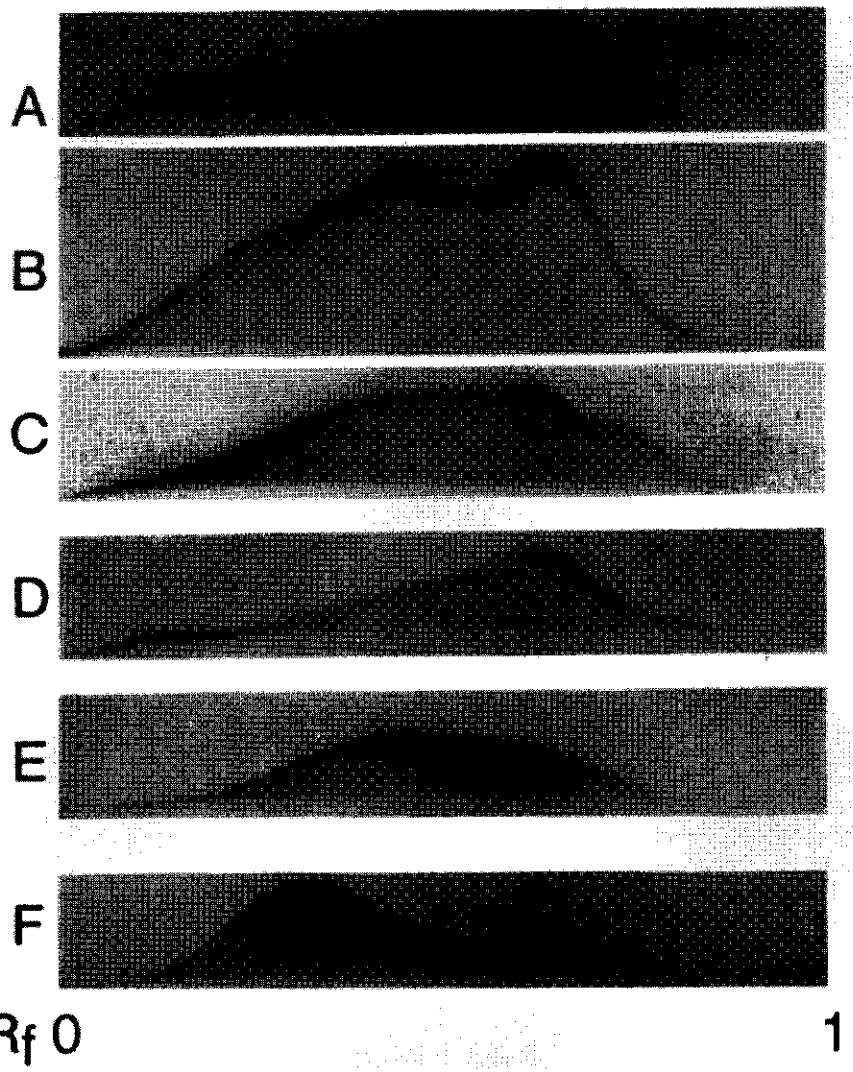

Fig 1 A-F. Crossed electrophoresis patterns of medium or tissue AGPs. The AGPs were isolated at different ages of a cell line and the amount of AGPs supplied to the gel was between 3 and 10 µg, as indicated. Also indicated is the concentration of AGPs in the analysed media and tissues. **A** Seeds, 6 µg, 0.28 mg AGPs per g seeds. **B** Hypocotyl (explant), 10 µg, 0.16 mg AGPs per g FW. **C** 19 Days old cell line, 10 µg, 31 mg AGPs per litre medium. **D** 7 Weeks old cell line, 3 µg, 13 mg AGPs per litre medium. **E** 3 Months old cell line, 6 µg, 24 mg AGPs per litre medium. **F** 2 Years old cell line, 7 µg, 180 mg AGPs per litre medium.

After about three months of culture (and subculturing every two weeks) the AGP pattern showed two wide peaks (see Fig.1E). The maximum of the largest peak had a R_f-value of 0.4, the other maximum had a R_f-value of 0.6. At this point the culture was highly embryogenic.

A cell line which had been in culture for 2 years and had lost its embryogenic potential showed a striking difference in AGP pattern (see Fig.1F). The largest peak had a maximum at a R_f-value of 0.3. This peak contained the majority of the AGPs.

The pattern of AGPs isolated from somatic embryos was very similar to the pattern of hypocotyl AGPs (data not shown). This indicates that somatic embryos, with respect to the nature of AGPs, are comparable to normal plants. The patterns of AGPs isolated from cells of embryogenic and non-embryogenic cultures were identical to each other, in contrast to the AGPs isolated from their conditioned media. Both AGP patterns of the cells showed a wide peak with a R_f-value of 0.5 (data not shown).

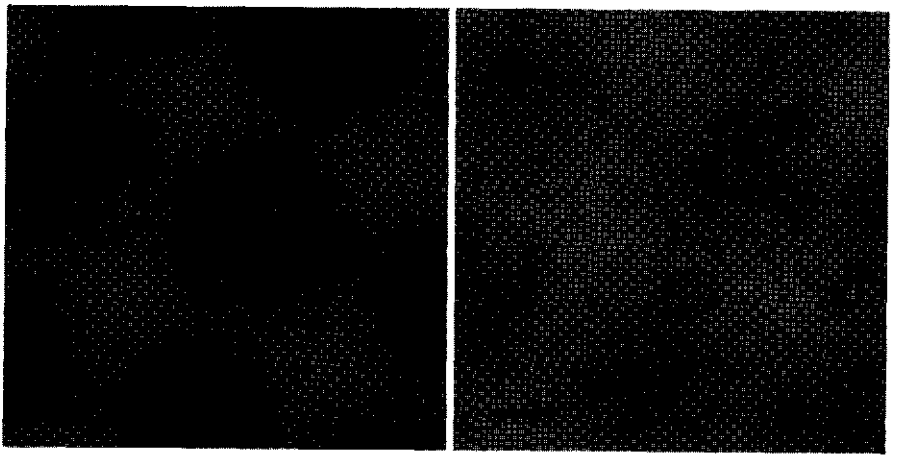

Fig. 2 A-B. *Young cell cultures established from one hypocotyl in 10 ml medium.* Bar = 50 μm; magnification 200x. **A** Control culture nineteen days after initiation. Small single cells are present. **B** culture with added AGPs from a non-embryogenic cell line. Virtually no small single cells are present.

The addition of AGPs to explant material in culture. Cell cultures were initiated by adding one sliced hypocotyl (2-3 cm) to 10 ml 2,4-D containing medium. This low explant density was used to avoid competition between added AGPs isolated from seeds or cell culture media and endogenous AGPs from the explant. In the control cultures without added AGPs, small single cells were present when the hypocotyl parts were removed after nineteen days (Fig. 2A). Subculturing of these small, single cells resulted in the appearence of pro-embryogenic masses from which somatic embryos can be generated (De Vries et al. 1988b). The addition of carrot seed AGPs to a final concentration of 10 $mg.l^{-1}$, did not show an effect. However, addition of AGPs isolated from a 2 years old, non-embryogenic cell line, to a final concentration of 10 $mg.l^{-1}$, did change the morphology. When the hypocotyl parts were removed very few small single cells were present in the culture medium. Most of the cells were large and highly vacuolated, a characteristic feature of non-embryogenic cells. After two subcultures virtually no small single cells were present (Fig. 2B). The morphology of this culture closely resembled that of the 2 years old, non-embryogenic cell line from which the added AGPs were isolated. The culture did not contain pro-embryogenic masses and was made up entirely of non-embryogenic cells. This shows that specific AGPs added to explant cultures can influence the establishment of cell cultures.

The addition of AGPs to a young establishing cell line. A cell culture was initiated by incubating 4 grams of somatic embryos in 50 ml Gamborg's B-5 medium, instead of the 2 grams used in normal cultures. After eight weeks of culture, still no embryogenic cells or pro-embryogenic masses were present, in contrast to normal cultures which are embryogenic at this age. Subsequently, the cell line was subcultured in 5 ml cultures (petridishes) in the presence of AGPs (10 $mg.l^{-1}$) and 2,4-D at a cell density of 20000 $cells.ml^{-1}$. This low cell density was used to avoid competition between the added AGPs and the AGPs produced by the cells in the culture.

The added AGPs were isolated from carrot seeds or from the conditioned medium of the non-embryogenic cell line. During the subculture the percentage of embryogenic cells was determined by counting single cells and small aggregates (aggregates of less than 15 cells), discriminating embryogenic cells (small, dense, highly cytoplasmic) from non-embryogenic cells (large, highly vacuolated) (Halperin 1966; Halperin and Jensen 1967; Williams and Maheswaran 1986). The small aggregates of embryogenic cells will eventually grow into larger clumps, the pro-embryogenic masses. One or a few cells of a pro-embryogenic mass can, at low cell density and without hormones, give rise to a somatic embryo. Since the somatic embryos predominantly originate from the pro-embryogenic masses, the amount of pro-embryogenic masses or the percentage of embryogenic cells in a culture is proportional with the embryogenic potential (De Vries et al. 1988b).

Table 1. Percentage of embryogenic cells during a subculture of a young cell line. The values given are the mean of three cultures (SD= 3%)

Days in culture	1	8	15
		% embryogenic cells	
no AGPs added	<1	8	10
non-emb. susp. AGPs added	<1	16	17
seed AGPs added	<1	21	29

During the subculture the percentage of embryogenic cells increased (Table 1). However, when AGPs isolated from seeds or cell culture media were added the increase was more pronounced compared to the control experiments, especially in the first week of the culture period. The largest increase was observed in the cultures in which carrot seed AGPs were added. Addition of 1 $mg.l^{-1}$ AGPs showed less pronounced effects (data not shown).

After prolonged subculturing (4 to 6 weeks) in the presence of 10 $mg.l^{-1}$ seed AGPs the percentage of embryogenic cells became very high (i.e. 80%) while in the control cultures, without added AGPs, the percentage of embryogenic cells did not exceed 30%. This shows that added carrot seed AGPs are able to substantially increase the percentage of embryogenic cells in a young cell line.

The addition of AGPs to a 2 years old, non-embryogenic cell line. The 2 years old, non-embryogenic cell line was subcultured in the presence of AGPs isolated from carrot seeds (10 $mg.l^{-1}$) and 2,4-D at a low cell density. This cell line normally did not contain pro-embryogenic masses. After 8 weeks (four subcultures) cell aggregates appeared, containing small, cytoplasmic cells (see Fig. 3A). The aggregates were isolated and subcultured on the same, seed AGP containing medium. This resulted in a culture which closely resembled a young embryogenic culture. After transfer of the aggregates to hormone free medium somatic embryos were generated (see Fig. 3B). The original line remained non-embryogenic. The carrot seed AGPs therefore reinduced embryogenic potential.

The addition of AGPs to an embryogenic cell line on hormone free medium. To a cell line induced to produce somatic embryos on hormone free medium, AGPs isolated from carrot seeds or from the culture medium of non-embryogenic cell lines were added, in a concentration of 1 $mg.l^{-1}$. After three weeks of culture the number of embryos formed was

counted. The added AGPs both gave an increase in the number of embryos (40% and 34% increase with seed AGPs respectively AGPs from the non-embryogenic cultures). Addition of 10 mg.l^{-1} AGPs showed a less pronounced effect (28% and 12% increase respectively).

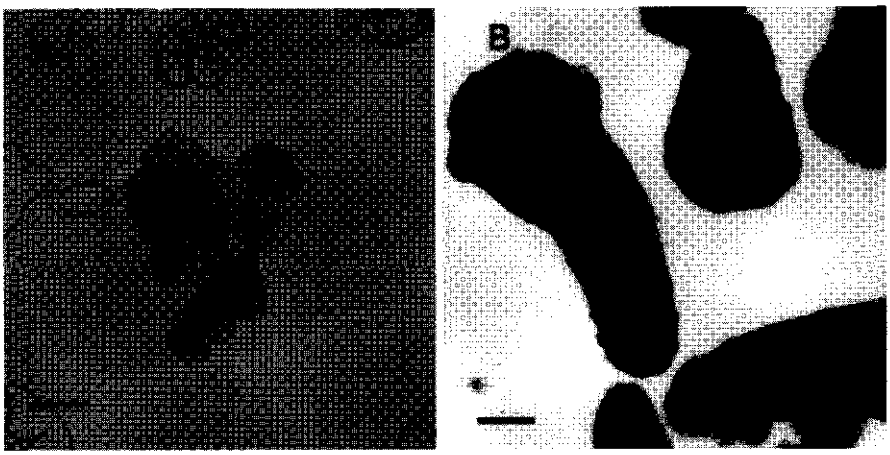

Fig 3. A Two years old cell line cultured in the presence of carrot seed AGPs. Cell aggregates containing small cytoplasmic cells are present. These cytoplasmic cells were not present when no carrot seed AGPs were added (not shown). Bar = 50 μm, magnification 200x. **B** Somatic embryos generated from the cell aggregates from **A**. Bar = 250 μm, magnification 40x.

Discussion.

The results of the crossed-electrophoresis show that the pattern of the excreted AGPs changes with the developmental stage of the cell line. At the initiation of the cell line the pattern of the medium AGPs resembles the pattern of hypocotyl AGPs (Fig. 1B,C). This is to be expected since the AGPs in the hypocotyl parts are eluted into the medium. At the same time already new types of AGPs are present (R_f value 0.7). When the culture starts growing it is becoming embryogenic. This process is accompanied by the secretion of different types of AGPs, resulting in the change of the AGP pattern. The pattern keeps changing until the cell line is non-embryogenic (Fig. 1F). In the ageing cell line not only the AGP pattern changes but also the amount of AGPs excreted in the medium increases (from 31 to 180 mg.l^{-1}, Fig. 1), probably due to higher growth rates and a changed morphology.

The AGPs isolated from seeds do represent, at least in part, an embryogenic environment and might be able to induce such an environment when added to other cells. Our experiments showed that the crossed electrophoresis patterns of AGPs isolated from seeds

and the medium of a 7 weeks old embryogenic cell line have similarities. In both AGP patterns the major peak, containing the majority of the AGPs, has a R_f-value of 0.6 (Fig. 1A and D). These types of AGPs might correlate with the presence of embryogenic cells or an embryogenic environment.

The ability of the seed AGPs to reinduce embryogenic potential in a non-embryogenic cell line shows that specific AGPs are essential for embryogenesis. These specific AGPs might have been produced by the non-embryogenic cell line, but the relative amount could have been too low to be active, as one might conclude from Fig. 1F. The pattern of the AGPs secreted by the non-embryogenic cell line contains a major peak with a R_f-value of 0.3, only a small portion has a different R_f-value. This indicates that the ratio between different types of AGPs is important for the effect of AGPs on cells. For the excreted AGPs this ratio could be a reflection of the developmental state of the cells. The added seed AGPs caused the appearance of new pro-embryogenic masses and therefore reinduced embryogenic potential. From the newly formed pro-embryogenic masses somatic embryos were generated.

When seed AGPs were added to a young cell line which was at that time developing towards an embryogenic culture, the percentage of embryogenic cells increased (Table 1). The seed AGPs added increased the number of pro-embryogenic masses and therefore increased the embryogenic potential since somatic embryos are predominantly derived from the pro-embryogenic masses (De Vries et al. 1988b). Less but still effective, are AGPs from the non-embryogenic cell line. This is in contrast with the experiment in which the AGPs from the non-embryogenic cell line were added to a culture with explant material. This culture closely resembled the non-embryogenic cell line (Fig. 2b). These cells clearly reacted differently to the AGPs, indicating that cell cultures, at different moments during their development react differently to added AGPs.

The fact that plant cells usually differentiate according to position rather than to lineage (Sussex 1989; Mayer et al. 1991) implicates that during development and differentiation cell-cell interactions do occur. We postulate that AGPs, as a soluble and diffusable component of the extracellular matrix and of the plasma membrane (Fincher et al. 1983; Samson et al. 1983 and 1984), play a role as messengers in cell-cell interactions during differentiation. This is consistent with published observations (Knox et al. 1989 and 1991; Stacey et al. 1990) as well as our observations. Herman and Lamb (1992) indicated that AGPs are internalized for vacuolar mediated disposal. For another class of signal molecules in plants, the elicitors, the same mechanism called receptor-mediated endocytosis was proposed by Horn et el. (1989). These results suggest that receptors for AGPs are present on the cell surface, making a high turnover of AGPs possible. Van Holst et al. (1981) indeed showed a high turnover of radioactivity in AGPs in what Samson and coworkers showed to be plasmamembrane bound (Samson et al. 1983) and extracellular AGPs (Samson et al. 1984).

From our experiments it is concluded that AGPs influence the development of cell cultures by the addition of very low concentrations (10 to 100 nM) of specific AGPs.

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Chapter 4
Arabinogalactan-protein epitopes in somatic embryogenesis of *Daucus carota* L.

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Planta (1995) **197**, 135-141

Abstract.

Two monoclonal antibodies called ZUM 15 and ZUM 18 directed against carrot (*Daucus carota* L.) seed arabinogalactan-proteins (AGPs) were used to isolate specific AGP fractions. For both carrot and tomato seed AGPs analysed by crossed-electrophoresis, the ZUM 15 and ZUM 18 AGP fractions showed one identical peak. However, the R_f-values for the two species were different; 0.82 for carrot seed AGPs and 0.52 for tomato seed AGPs. When the fractionated AGPs (carrot or tomato) were added to carrot cell lines they showed a dramatic effect on the culture. One AGP fraction (ZUM 15 AGPs) was able to induce vacuolation of embryogenic cells. Those cells failed to produce embryos. The other AGP fraction (ZUM 18 AGPs) increased the percentage of embryogenic cells from about 40% up to 80% within one week and this subsequently resulted in the formation of more embryos on hormone-free medium. This activity was higher than that of unfractionated carrot seed AGPs, while the optimum concentration was 50-fold lower. Since both ZUM 18 AGPs (carrot or tomato) yielded identical responses it can be concluded that the R_f-value nor the source are essential for biological activity. The dose response curve of ZUM 18 AGPs showed a sharp optimum. When the AGPs that also bound to the antibody ZUM 15 were removed the dose response curve of the remaining AGPs (containing only the ZUM 18 epitope, not the ZUM 15 epitope) resembled a saturation curve. Regardless of its concentration, the fraction in which AGP molecules contained both epitopes showed no appreciable embryogenesis-promoting activity. The biological activity of AGPs was therefore determined by the presence of embryogenesis-enhancing and -inhibiting epitopes. The inhibiting and enhancing epitopes can be localised on separate molecules and on one single AGP molecule.

Introduction.

Carrot (*Daucus carota* L.) cell cultures in which somatic embryogenesis is easily induced can be used to study the plant extracellular matrix. The medium of liquid cell cultures can be regarded as an extended extracellular matrix and it has been shown that secreted proteins are essential for correct embryogenesis to take place (De Vries et al. 1988a).

One component of the extracellular matrix are the arabinogalactan-proteins (Clarke et al. 1978). They are proteoglycans and are developmentally regulated, as was shown by their change in electrophoretic mobility during the development of tissues (Van Holst and Clarke 1986; Cassab 1986; Kreuger and Van Holst 1993). This, together with the fact that arabinogalactan-proteins (AGPs) have a high turnover (Van Holst et al. 1981) makes them candidates for being signal molecules during cell-cell interactions. Cell-cell interactions are likely to take place during development and differentiation since plant cells can differentiate according to cell position (Sussex, 1989; Mayer et al. 1991).

By using different monoclonal antibodies it has been shown that specific AGP epitopes have specific patterns of expression, both temporal and spatial (Knox et al. 1989, 1991; Stacey et al. 1990; Pennell et al. 1991, 1992; Li et al. 1992). Specific AGPs or AGP-epitopes

appear to be associated with differentiation events during the life cycle of the plant. The appearance and disappearance of these epitopes accompany changes, visible or invisible, in properties of cells. Recently, it was shown that AGPs when added to embryogenic cell cultures of carrot, were able to increase or decrease embryogenic potential (Kreuger and Van Holst 1993). In old cell lines, which had lost embryogenic potential, this was induced by adding carrot seed AGPs (Kreuger and Van Holst 1993). This shows that by changing the AGPs of the extracellular matrix of cells in culture, it is possible to alter the developmental fate of cells. The added AGPs were different mixtures of AGPs. The question was raised to what degree the different AGPs in these mixtures contribute to the biological activity.

This paper describes the use of two monoclonal antibodies raised against carrot seed AGPs in the isolation of four AGP fractions. With one antibody AGPs were isolated that increase the embryogenic potential and with the other antibody AGPs that decrease the embryogenic potential.

Materials and methods.

Plant material and cell culture. Cell cultures were initiated and maintained essentially as described by De Vries et al. (1988b). In short, cell cultures were grown in Gamborg's B-5 medium (Gamborg et al. 1968), supplemented with 2 μM 2,4-dichloro-phenoxyacetic acid (2,4-D) and 58 mM sucrose, in a 16 h day light period at 23°C, on a rotary shaker at 100 rpm (G10 gyrotory shaker, New Brunswick Scientific, Edison, N.J., USA) and were subcultured every two weeks by adding 1 ml of packed cell volume into 50 ml of medium (final volume). The packed cell volume was determined by centrifuging a sample from the culture (200 x g, 2 min). The volume of the pelleted cell mass is the packed cell volume.

The formation of somatic embryos was initiated by sieving the fraction 50 - 150 μm from a culture seven days after subculturing. To show the relationship between the percentage of embryogenic cells and the number of embryos formed two cell populations of the 50 - 150 μm fraction were obtained. This was done by rotating the cells in a large petridish. The dense embryogenic cells settled in the centre of the dish, while the non-embryogenic cells drifted towards the outer side. This resulted in two populations, one containing 68% embryogenic cells and one containing 11% embryogenic cells. The two cell populations were mixed at different ratios and inoculated at 20000 cells. ml^{-1} on hormone free medium to produce somatic embryos. The experiments were performed in duplicate in 50 ml cultures. After 10 days the number of embryos was determined.

Preparation of the ß-glucosyl Yariv reagent, 1,3,5-tris (4-ß-D-glucopyranosyloxyphenylazo) -2,4,6- trihydroxybenzene. Synthesis (personal communication F.M. Klis, University of Amsterdam, The Netherlands). Four millimoles of p-aminophenyl-ß-D-glucose were dissolved in 24 ml of 0.5 M HCl at 0°C. Four mmoles of $NaNO_2$ were added. This solution was very slowly added to a solution of 1 mmole phloroglucinol in 50 ml water (pH 9.0). The pH was

kept stable at 9.0 by adding 0.5 N NaOH (initially) and 0.1 N NaOH (later). The temperature was kept at 0°C. About 2 h after the solution was added to phloroglucinol the pH was stable.

Purification. An equal volume of methanol was added to precipitate the ß-glucosyl Yariv reagent. The precipitate was collected by centrifugation (3000 x g, 15 min) and the supernatant was discarded. The precipitate was dissolved in water and reprecipitated. The residue was dried at 50°C and stored at room temperature. Stock solutions of 1 $mg.ml^{-1}$ were also stored at room temperature.

Isolation of arabinogalactan-proteins. AGPs were isolated by precipitation with the ß-glucosyl Yariv reagent from seeds and culture medium as described by Kreuger and Van Holst (1993). After reprecipitation the AGP-Yariv complex was dissolved in water and sodiumhydrosulfite ($Na_2S_2O_4$) was added to a final concentration of 10 % (w/v) to break down the ß-glucosyl Yariv reagent. The solution was heated to 50°C until the red colour disappeared. After centrifugation (10000 x g, 10 min) the supernatant was dialysed extensively against water at 4°C. The dialysed AGPs were centrifuged (10000 x g, 10 min) to remove a small amount of undissolved material. In the supernatant the AGP concentration was determined by radial gel diffusion method as described by Van Holst and Clarke (1985). The AGP preparations were free of proteins as determined by the Bio-Rad protein assay (Bio-Rad Laboratories, München, germany).

Crossed electrophoresis of AGPs. Crossed electrophoresis of AGPs was done essentially as described by Van Holst and Clarke (1986).

Preparation of monoclonal antibodies. Monoclonal antibodies were prepared at MCA Development (Zernikepark 6e, 9747 AN, Groningen, The Netherlands). Isolated carrot seed AGPs were used as antigen. Female BALB/c mice were immunised by i.p. injection of 100 µg of AGPs in incomplete Freund's adjuvant. Three additional cycles of i.p. injections with AGPs in incomplete Freund's adjuvant were given at time intervals of one month. Reactivity of immunoserum samples towards carrot seed AGPs and inhibition experiments were determined using ELISA techniques. Hybridoma cell lines were selected that were positive in the ELISA for binding to carrot seed AGPs and that could not be inhibited by L-arabinose (100 mM), D-galactose (100 mM) and gum arabic (1.0 $mg.ml^{-1}$). Generally the cell culture supernatant contained 20 to 50 $mg.l^{-1}$ of antibody.

Immuno-affinity chromatography. For immuno-affinity chromatography monoclonal antibodies were isolated from cell culture supernatant using Bakerbond ABx (J.T. Baker Inc., Phillipsburg, NJ, USA). Purified antibodies were covalently bound to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). Isolated AGPs purified by ß-glucosyl Yariv reagent precipitation were used for fractionation. AGPs were bound to the column in PBS (10

mM sodium phosphate buffer, 0.15 M NaCl, pH 7.2). Subsequently the column was washed with several column volumes of PBS. Bound AGPs were eluted using PBS with an additional 0.35 M NaCl. After desalting on a Sephadex G-50 spin column (Pharmacia), the AGPs were tested on cell cultures.

Addition of AGPs to cell cultures . Experiments in which AGPs were added to cell cultures were performed in triplicate in 3 ml cultures in 6-well plates (Costar, Cambridge, Mass., USA). The experiments were repeated several times (in triplicate) and gave very similar results. Cell cultures were sieved (fraction <100 μm) prior to the experiments. This fraction with single cells and small cell clusters was used to facilitate reproducible counting of cells since in larger cell aggregates individual cells can not be distinguished. Cell fractions were inoculated at a cell density of 10000 cells.ml^{-1} in the same medium used for standard cell lines. This low cell density was used to avoid competition between the added AGPs and the AGPs produced by the cells. AGPs were filter sterilised (0.22 μm, Flowpore, ICN Biomedicals LTD, Irvine, UK) and added to the culture.

The embryogenicity of the cultures was expressed as the percentage of embryogenic cells rather than the number of embryogenic cells per ml. Embryogenic cells (small, dense, highly cytoplasmic) were discriminated from non-embryogenic cells (large, highly vacuolated) (Halperin 1966; Halperin and Jensen 1967; Williams and Maheswaran 1986) and the percentage of embryogenic cells was calculated by dividing the number of embryogenic cells by the total number of cells. We determined the percentage of embryogenic cells by taking at least three representative samples (10 μl) from the cultures using a pipette with a wide nozzle (diameter of about 1.5 mm) and counting all cells in the samples. This resulted in reproducible figures with low standard errors. Counting all the cells in the cultures resulted in the same percentages. Counting only parts of the culture without sampling resulted in large variations between observations due to the fact that the highly cytoplasmic cells concentrated in the centre of the culture well.

Since larger variations exist between the total number of embryogenic cells in cultures than between the percentages of embryogenic cells, we decided to determine the relative percentage instead of the number of embryogenic cells. This resulted in more accurate figures.

After determination of the percentage of embryogenic cells at day 7, cells were washed twice by centrifugation in hormone-free B5 medium and cultured in this medium in the original container. After three weeks the number of embryos formed was counted. It was essential to culture the cells in the original containers after the transfer to hormone-free medium to minimise the loss of cells due to sticking of cells and cell aggregates to the containers.

Results.

Selection of monoclonal antibodies. Previous reports showed that hapten inhibition of antibodies with mono- and oligosaccharides as well as Gum Arabic and other polysaccharides was common in many cases, resulting in antibodies recognising a broad spectrum of molecules (Anderson et al. 1984; Pennell et al. 1989, 1991; Miskiel and Pazur 1991; Kikuchi et al. 1993). Nine hybridoma cell lines were selected for the production of antibodies with no affinity for L-arabinose, D-galactose and Gum Arabic and a high affinity for carrot seed AGPs. The selection was aimed at obtaining antibodies binding to only a defined fraction of AGPs. The antibodies were designated ZUM antibodies.

The nine antibodies were purified using ABx columns and subsequently coupled to CNBr-Sepharose and used to isolate carrot seed AGP fractions using affinity chromatography. AGPs purified by precipitation with Yariv reagent were used as a source for isolation of AGP fractions. It is unknown whether other compounds are able to bind to the antibodies. Since the aim of this study was to obtain highly purified fractions of AGP mixtures only AGPs purified by the Yariv reagent were applied to the antibody columns. Several papers described the sugar and amino acid composition of molecules precipitated by the Yariv reagent as being AGPs (Jermyn and May Yeow 1975; Clarke et al. 1978). The high specificity of the Yariv reagent for AGPs has also been shown (Van Holst and Clarke 1985).

The AGP fractions bound by the nine antibodies were added in two concentrations (0.1 and 1.0 $mg.l^{-1}$) to cell cultures. After one week the percentage of embryogenic cells in the cultures was determined. In the control experiments no AGPs were added. This first screening of the nine ZUM bound AGP fractions showed differences in activities. Two AGP fractions showed no activity in the concentrations used, two fractions decreased the percentage of embryogenic cells and five fractions increased the percentage of embryogenic cells (data not shown) compared to the control cultures.

One of the repressing AGP fractions (called ZUM 15 AGPs) and three of the promoting fractions (ZUM 17, 18 and 21 AGPs) were further characterised. To see whether cross-reactivity existed between the antibodies, the four AGP fractions were applied to columns of the other antibodies. Table 1 shows that all antibodies bound subfractions of the AGPs also recognised by at least one other antibody. For instance, of the ZUM 15 AGPs 27% were retained by the ZUM 18 column. This cross-reactivity was in most cases higher between antibodies that bind embryogenesis promoting AGPs, ZUM 17, 18 and 21 than between ZUM 18 and the embryogenesis inhibiting ZUM 15 AGPs.

All four antibodies were of the IgM class. The antibodies ZUM 15 and 18 were used for further studies.

Characterisation of monoclonal antibody binding AGPs. Carrot seed AGPs were applied to affinity columns with ZUM 15 or 18 antibodies. About 2.7% of the total seed AGPs bound to ZUM 15 and about 2.5% bound to ZUM 18.

Table 1. Size of AGP subfractions using two subsequent affinity columns. The columns represent the AGP fractions that were isolated with the antibodies ZUM 15, 17, 18 or 21. The rows represent the type of antibody in the second affinity column. Values given are the percentages of AGP fractions bound by the second affinity chromatography

	AGPs			
Antibody	15	17	18	21
ZUM 15	100		36	
ZUM 17		100	36	52
ZUM 18	27	48	100	38
ZUM 21		80	43	100

When the unfractionated carrot seed AGPs were analysed by crossed-electrophoresis they showed two wide peaks (Fig. 1A). When the carrot seed AGPs that were bound by ZUM 15 and 18 were analysed they surprisingly showed similar AGP patterns (Fig. 1,B and C). ZUM 15 and 18 bound AGPs isolated from media of embryogenic and non-embryogenic carrot cell lines also showed similar AGP patterns (data not shown). All patterns have a distinct peak with a R_f-value of 0.82 and a minor peak with a lower R_f-value of 0.59.

The columns with ZUM 15 and 18 antibodies could also be used to fractionate AGPs purified from seeds of tomato (*Lycopersicon esculentum* Mill), 2.3% and 0.6% of the AGPs bound respectively. ZUM 15 and 18 tomato seed AGPs show a different pattern (Fig. 1E and F), a wider peak with a different R_f-value (0.52). Also in this case, the pattern is different from the unfractionated tomato seed AGPs (Fig. 1D). The fractionated AGPs all show an AGP pattern with one peak indicating that their charge and size distribution is rather homogeneous and crop specific. The antibody binding AGPs from carrot and tomato clearly differ from each other since they have different R_f-values. It can also be concluded that the selection procedure resulted in highly specific antibodies recognising only small fractions of purified AGPs.

Addition of monoclonal antibody bound AGPs to cell cultures. ZUM 15 and 18 bound carrot seed AGPs and ZUM 18 tomato seed AGPs were added to a three to six months old embryogenic carrot cell line. The unfractionated carrot and tomato seed AGPs were added to cultures in parallel experiments as a control. After one week of culture the percentage of embryogenic cells was determined. As is shown in Figure 2A where the AGP concentration is plotted against the percentage of embryogenic cells, the unfractionated carrot seed AGPs were able to increase the percentage of embryogenic cells compared with the control cultures as was described previously (Kreuger and Van Holst 1993). The effect is concentration dependent and the highest percentage of embryogenic cells (68%) was obtained at an AGP

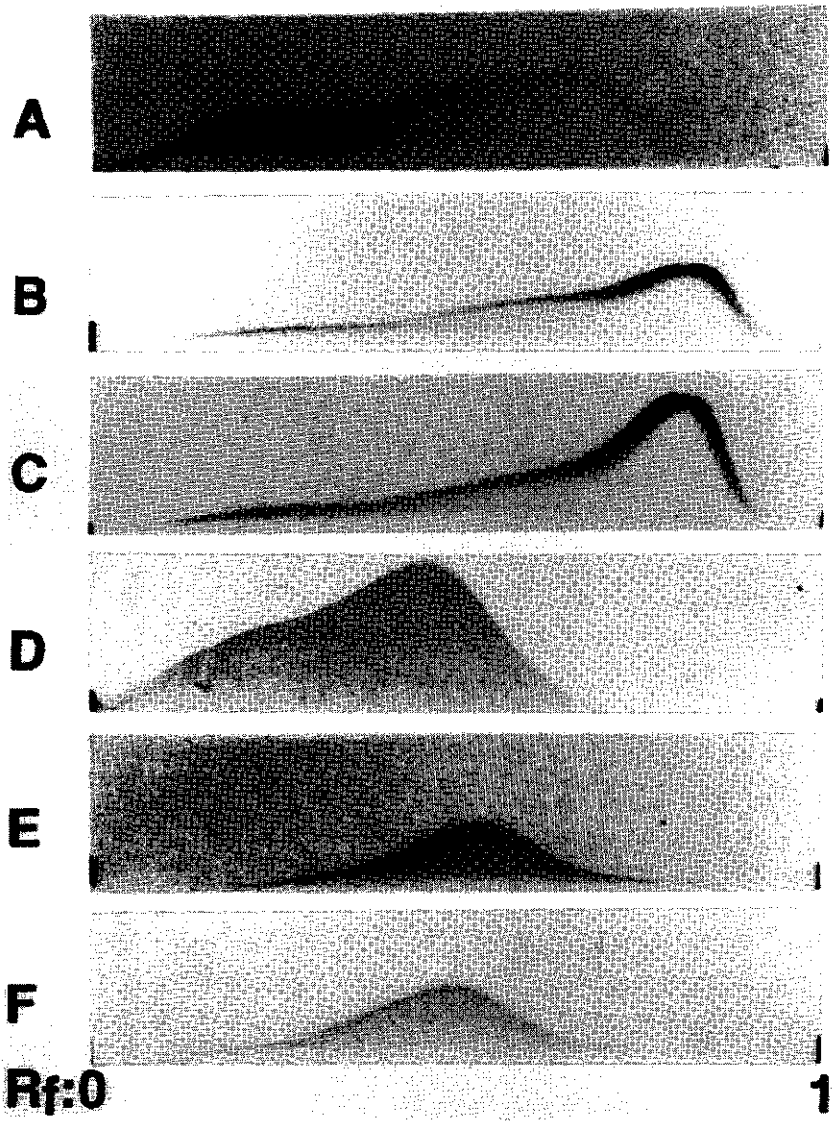

Fig. 1 A-F. crossed-electrophoresis of unfractionated and fractionated AGPs. AGPs were first electrophorated from left to right and than into a gel contain the ß-glucosyl Yariv reagent resulting in the precipitation line. R_f-values (0 to 1) indicate the mobility of the AGPs. **(A)** 6.0 µg carrot seed AGPs, **(B)** 3.6 µg carrot ZUM 15 AGPs, **(C)** 4.6 µg carrot ZUM 18 AGPs, **(D)** 4.6 µg tomato seed AGPs, **(E)** 1.5 µg tomato ZUM 15 AGPs, **(F)** 1.8 µg tomato ZUM 18 AGPs.

concentration of 10 mg.l^{-1}. The unfractionated tomato seed AGPs do not show a strong promoting effect, in some concentrations the percentage of embryogenic cells is even decreased.

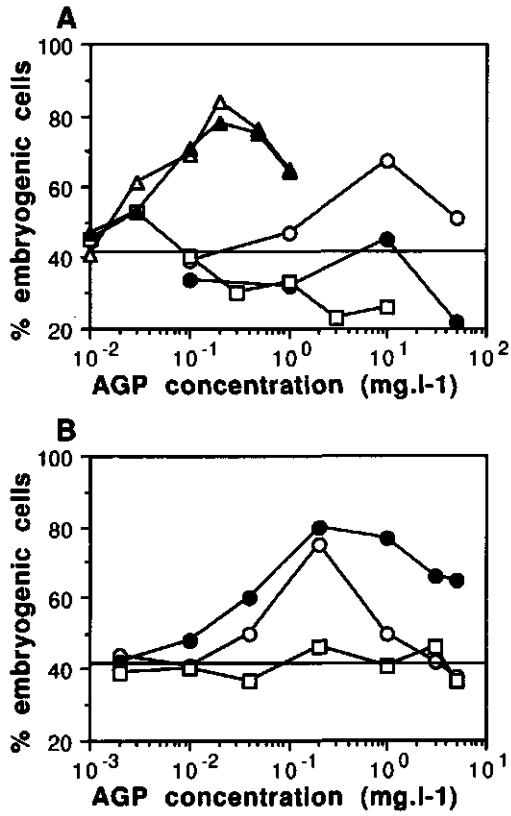

Fig. 2A, B. percentage of embryogenic cells after one week in the presence of AGPs. **(A)** AGPs added were from; carrot seeds (open circles), tomato seeds (closed circles), carrot ZUM 15 (open squares), carrot ZUM 18 (open triangles) and tomato ZUM 18 (closed triangles). The horizontal line represents the percentage of embryogenic cells in the control experiments in which no AGPs were added. SD was 4%, experiments were performed in triplicate. **(B)** AGPs added were; ZUM 18 (open circles), fraction 18-15 (closed circles) and fraction 18+15 (open squares). The horizontal line represents the percentage of embryogenic cells in the control experiments in which no AGPs were added. SD was 3%, experiments were performed in triplicate.

The ZUM 15 carrot seed AGPs have a negative effect on the culture. At the higher concentrations used (2 mg.l^{-1}) the percentage of embryogenic cells is decreased from 40% to almost 20%. The effect of the ZUM 18 carrot AGPs is opposite to that of the ZUM 15 AGPs

(Fig. 2A). The percentage of embryogenic cells is increased to about 80 % in one week at the optimum concentration of 0.2 $mg.l^{-1}$. This optimum concentration is about fifty fold lower than the optimum concentration of unfractionated carrot seed AGPs. Their specific activity is therefore much higher. ZUM 18 AGPs could also be isolated from culture media AGPs of carrot cell lines and have the same effect as the ZUM 18 seed AGPs (data not shown). Surprisingly the tomato seed ZUM 18 AGPs have the same dose response curve as the carrot seed ZUM 18 AGPs (Fig. 2A). This is in sharp contrast to the unfractionated tomato seed AGPs. Apparently the source of the ZUM 18 AGPs is unimportant.

The effect of ZUM 15 AGPs on embryogenic cells or pro-embryogenic masses is illustrated in Figure 3A. After one week of culture embryogenic cells are completely vacuolated in contrast to the control cultures (Fig. 3B) where the cells remain dense and highly cytoplasmic. In the cultures to which the ZUM 18 AGPs were added several cell clusters were observed (Fig. 3C) which contain small embryogenic cells attached to the end of an elongated non-embryogenic cell. Structures like these were hardly ever observed in the control cultures suggesting that they were induced by the added AGPs. An unequal division of the non-embryogenic cell could have formed the embryogenic cells.

A further fractionation of the ZUM 18 AGPs was done by using the cross-reactivity between ZUM 15 and 18 antibodies (Table 1). The ZUM 18 AGPs were applied to the ZUM 15 antibody column. The AGPs not retained bound not to ZUM 15 and only to ZUM 18. This was the fraction (18-15). The fraction eluted from the column using the high salt buffer bound to ZUM 15 and 18. This was the fraction (18+15). These two fractions were added in several concentrations to a cell line in a similar experiment as described previously. Not further fractionated ZUM 18 AGPs were added at several concentrations as a control. After one week of culture the percentage of embryogenic cells was determined. As is shown in figure 2B the AGPs binding to both antibodies (18+15) had no or little activity in all concentrations tested. The ZUM 18 AGPs not bound by ZUM 15 (fraction 18-15) showed to have a dose response curve much more like a saturation curve than the not further fractionated ZUM 18 AGPs. Even though the specific activity was not increased, the dose response curve changed after a further purification of the epitope.

The fact that ZUM 18 AGPs have an optimum concentration (Fig. 2A) may indicate that this is the result of two opposing activities, one promoting and one suppressing embryogenic potential. Part of the ZUM 18 AGPs can bind to the ZUM 15 antibody (Table 1) indicating that the ZUM 18 AGPs are in fact a mixture of molecules containing both ZUM 15 and ZUM 18 epitopes.

Relationship between the percentage of embryogenic cells and embryogenic potential. Two cell populations were obtained, one containing a high percentage of embryogenic cells (68%) the other containing a low percentage of embryogenic cells (11%). The populations were mixed at several ratios and cultured on hormone free medium and at low cell density to form

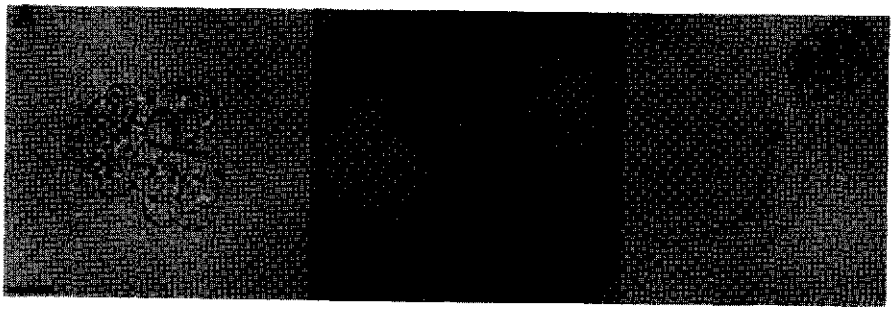

Fig. 3A-C. Carrot suspension cells in the absence and presence of added AGPs. Bar = 5 μm. Magnification 2000 x. **A** Pro-embryogenic mass after one week in the presence of ZUM 15 AGPs. **B** Embryogenic cells in control cultures without added AGPs. **C** Cell clump after one week in the presence of ZUM 18 AGPs. Small embryogenic cells attached to a vacuolated non-embryogenic cell.

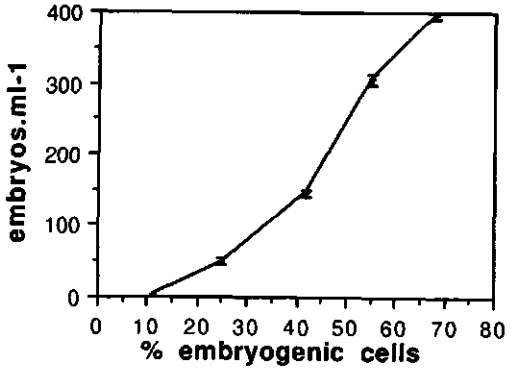

Fig.4. Relationship between the initial percentage of embryogenic cells and the number of embryos formed on hormone free medium after 10 days. Cell populations with different percentages of embryogenic cells were inoculated at low cell density on hormone free medium to form somatic embryos. Experiments were performed in duplicate, SD is indicated.

somatic embryos. Above about 10% embryogenic cells the number of embryos formed after 10 days was proportional to the initial percentage of embryogenic cells (Fig. 4). Apparently there is a threshold in the percentage of embryogenic cells below which no embryos are formed.

To see whether the newly formed embryogenic cells in response to the added AGPs also resulted in increased embryogenic potential cells were grown in the presence of 2,4-D and ZUM 15 or 18 AGPs (1.0 and 0.2 mg.l^{-1} respectively). After one week the percentage of embryogenic cells was determined. The cells were subsequently washed with hormone-free and AGP-free medium to remove the 2,4-D and AGPs and cultured in hormone-free and AGP-free medium in the original containers. After three weeks the number of embryos (heart and torpedo stage) formed was determined. Table 2 shows that in the presence of 2,4-D and ZUM 18 AGPs the percentage of embryogenic cells is increased when compared to the control cultures in which no AGPs were added. When ZUM 15 AGPs were added a slight decrease was observed. The results are in accordance with the results of Figure 2A. After development of the embryos the control cultures contained 19 embryos.ml^{-1}. The cultures which had grown in the presence of ZUM 15 and 18 AGPs contained 3 and 59 embryos.ml^{-1} respectively. The added AGPs therefore clearly influenced the embryo forming capacity of the cell line. The total number of embryos formed per ml was however different from Figure 4. This was probably due to the different cell fraction and cell density used in the experiments.

Table 2. The effect of different AGP fractions on the percentage of embryogenic cells and the number of embryos produced. Cells were grown on 2,4-D and AGPs containing medium for one week. The percentage of embryogenic cells was determined and the cells were subsequently transfered to hormone-free medium. After three weeks the number of embryos formed was deteremined. Experiments were performed in triplicate, standard error is included.

	no AGPs added	ZUM 18 AGPs (0.2 mg.l^{-1})	ZUM 15 AGPs (1.0 mg.l^{-1})
% embryogenic cells after 7 d	37 ± 3	60 ± 1	31 ± 3
number of embryos per ml after 21 d on hormone-free and AGP-free medium	19 ± 4	59 ± 17	3 ± 3

Discussion.

Specific fractions of AGPs bound by monoclonal antibodies have different effects on cells in culture. The activity of the AGPs depends on the presence of embryogenesis enhancing and inhibiting epitopes. These epitopes can be located on separate molecules or on one single molecule.

Production of highly specific monoclonal antibodies. In previous reports monoclonal antibodies have mainly been used to characterise cell types and AGPs (Anderson et al. 1984; Pennell et al. 1989, 1991; Miskiel and Pazur 1991; Kikuchi et al. 1993). In these reports the antibodies showed affinity for many types of AGPs. In this report the function of individual fractions of AGPs is described.

In order to have high specificity for embryogenesis related epitopes, a selection against "common" epitopes; D-galactose, L-arabinose and gum arabic was made. This proved to be a powerful approach for our antibodies bound only a small fraction of the total carrot and tomato seed AGPs (ZUM 15; 2.7% and 2.3% respectively and ZUM 18; 2.5% and 0.6% respectively) and were therefore highly specific. It must be noted that only purified AGPs were applied to the antibody columns, therefore it is unknown whether other compounds are recognised by the antibodies. Analysis by crossed-electrophoresis showed that the isolated fractions contain AGPs with a high R_f-value (Fig. 1). The carrot AGPs, isolated with ZUM 15 or ZUM 18 from seeds and culture media, show similar AGP patterns. What caused this apparent coincidence is not clear. The selection of the hybridoma cell lines secreting antibodies with no affinity for D-galactose, L-arabinose and gum arabic might have resulted in the selection of antibodies binding carrot AGPs with a high R_f-value. The antigenicity of AGPs with a relative high charge (= high R_f-value) may be larger than of AGPs with a lower charge. Antibody-bound AGPs from carrot and tomato, however have different R_f-values. These differences in R_f-values are caused by differences in charge and/or size and are probably crop specific.

The structure of the epitopes is unknown. Due to the nature of AGPs it is very likely that the sugar moiety is involved in binding the antibody but a contribution of the protein moiety, even though it is relatively small cannot be ruled out. The sugar side chains are probably the most variable part of the molecule generating many epitopes and differences in size and charge.

Specific AGPs influence the development of cells. The fractionated AGPs showed to have a large impact on the development of cells in culture. ZUM 15 carrot seed AGPs decreased the percentage of embryogenic cells (Fig. 2A). The embryogenic cells vacuolated (Fig. 3A) resulting in structures that hardly formed somatic embryos (Table 2). In contrast, ZUM 18 AGPs increased the percentage of embryogenic cells dramatically in one week, from about 40% up to 80% (Fig. 2A,B). The ZUM 18 AGPs appear to induce a non-embryogenic cell to form a structure (Fig. 3C) very similar to the ones described by Backs-Hüsemann and Reinert (1970) capable of forming an embryo, or in the presence of an auxin, a pro-embryogenic mass. The source (either carrot seeds, carrot suspension culture medium or tomato seeds) of the AGPs proved to be unimportant. AGPs having the same epitope give the same biological response.

The percentage of embryogenic cells established after treatment with AGP fractions determined the number of embryos formed after transfer to hormone free medium (Table 2). It is clear that the added AGPs have an effect on the embryogenic potential of the cells. The decrease and increase in the number of embryos formed after treatment with ZUM 15 and 18 AGPs respectively, was more than proportional than the change in the percentage of embryogenic cells. Table 2 and Figure 4 show that not all embryogenic cells participate in the formation of the embryos and that the efficiency of the embryogenic cells to produce somatic embryos is greatly influenced by the percentage of embryogenic cells.

More functional epitopes are present on a single AGP molecule. The activity of a number of non-identical AGP fractions on cell differentiation is demonstrated in figure 2B. We have concluded from this figure that AGP molecules can carry a number of embryogenesis promoting or inhibiting epitopes. A model described by Knox (1993) shows that cells during their development contain changing sets of AGPs with specific epitopes. The epitopes are located on different AGPs. In a similar model (Fig. 5) the non-identical AGP fractions contain different sets of epitopes and one AGP molecule can contain more than one epitope. The total fraction of carrot seed AGPs stimulated the formation of embryogenic cells (Fig 2A) in accordance with the model: more embryogenesis promoting epitopes than embryogenesis inhibiting ones. Isolation of a specific fraction with ZUM 18 not only increases the ratio of promoting to inhibiting epitopes but also the total number of promoting epitopes as a function of the number of AGP molecules. If we use the ZUM 18 AGPs the embryogenesis promoting epitopes will yield their response: increasing the number of embryogenic cells. However, an optimum response is obtained due to the fact that specific AGP epitopes show saturation kinetics and increasing number of embryogenesis inhibiting epitopes are titrated into the system. The ZUM (18-15) AGP fraction only contains embryogenesis promoting epitopes and therefore shows saturation kinetics. The ZUM (18+15) AGP fraction contains almost equal amounts of both epitopes and yields no response.

Although this model explains the observations within the frame of this article, the mechanism of AGP response remains obscure. It might be that AGP serve as classical signal molecules binding onto receptor molecules on the cell membrane or play a role in the deposition of macromolecules in the cell wall (Kieliszewski and Lamport 1994). Another possibility is that enzymes are present within the cell wall that can release sugar epitopes from the AGP that yield their response (Kjellbom et al. 1994).

The heterogeneity of AGPs, however, makes it extremely difficult or even impossible to isolate a fraction consisting of identical molecules necessary in order to study their biochemical route in cellular response. They have an activity at extremely low concentrations of about 2 nM (assuming that the molecular weight is about 100,000 and the optimum concentration is 0.2 $mg.l^{-1}$ for ZUM 18 AGPs). It must however be stressed that any response of AGPs is always the result of using a mixture of AGPs.

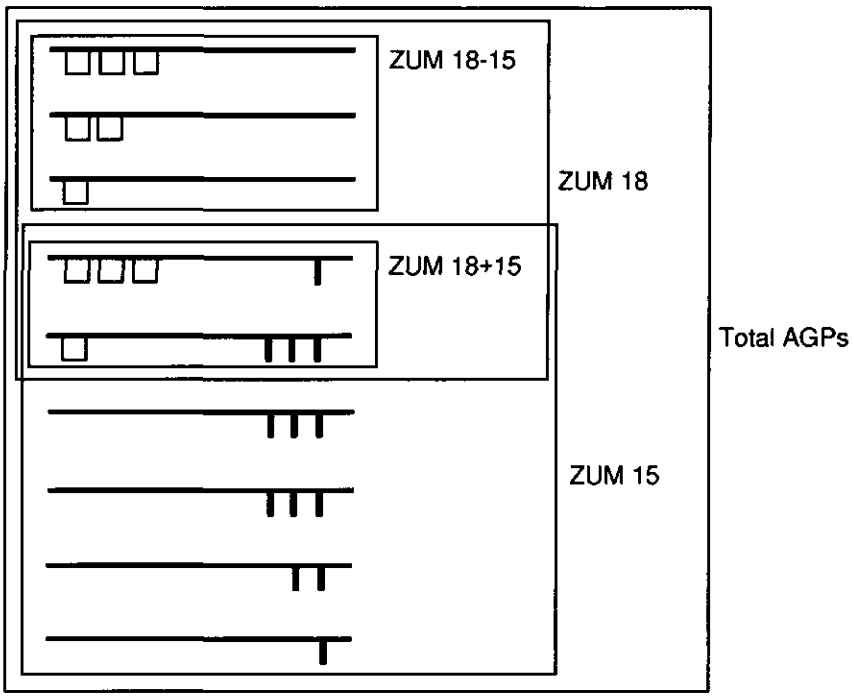

Fig. 5. Proposed model for the distribution of epitopes on AGP molecules. The AGP molecules are represented as horizontal lines, the ZUM 15 epitope as short vertical lines and the ZUM 18 epitope as squares. Each mixture or fraction contains a set of different molecules and can be defined by the affinity for one or two antibodies. The total AGPs contain, amongst many other AGPs (not included in the model), a set of ZUM 18 AGPs and a set of ZUM 15 AGPs. The subset 18+15 is the intersection of ZUM 15 and ZUM 18 AGPs.

It is clear that complex mixtures of AGPs surround cells. The presence of different AGP epitopes may be a reflection of the developmental state of cells. Cells in tissues and in vitro are in contact with AGPs produced by themselves and produced by other cells. Since the development of cells is influenced by the presence of the AGP epitopes cells can differentiate according to position as well as cell lineage (Steeves and Sussex 1989; Sussex 1989; Mayer et al. 1991).

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Chapter 5
Somatic embryogenesis of *Cyclamen persicum* in liquid medium

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Physiologia Plantarum (1995) **94**, 605-612

Abstract.

A method is described for the production of somatic embryos of Cyclamen *persicum* Mill. in liquid medium. Five steps are involved; initiation of embryogenic cell lines, proliferation of pro-embryogenic masses (PEMs) on auxin-containing medium, development of somatic embryos on hormone-free medium with high osmolarity, germination and subsequent plantlet formation. Cell lines were initiated by culturing the explant, the seedling tuber, directly in liquid medium. Three parameters were important for obtaining embryogenic cell lines; explant density, hormone concentrations and subculture regime. The rate of uptake of the hormones 2,4-D and kinetin influenced the formation of PEMs. Highly embryogenic cell lines were obtained only when PEMs had formed within 5-7 weeks. PEMs were proliferated for at least 24 months and could be isolated from each subculture for the production of somatic embryos. A high sucrose content (175 mM) in the development medium without hormones ensured efficient embryo development from PEMs. A subsequent subculture in low sucrose concentration (58 mM) induced the formation of a tuber, thus promoting germination. Arabinogalactan-proteins (AGPs) from carrot seeds and AGPs bound by the monoclonal antibody ZUM 18 increased the number of PEMs in a culture showing that the activity of AGPs is not species specific.

Introduction.

The process of somatic embryogenesis offers a great opportunity to study events occurring during plant development. Since the first description of somatic embryos (Reinert 1958, Steward et al. 1958) many papers have been published dealing with these phenomena (Zimmerman 1993). Another aspect of somatic embryogenesis is its application in breeding and plant production. Large scale production of somatic embryos and plants from these can be very advantageous when dealing with desired genotypes or traits difficult to multiply otherwise. However, for most species the existing protocols yield only few somatic embryos.

In tissue culture callus cultures are widely used for regeneration. Callus cultures, whether organogenic or embryogenic, are grown on media solidified with agar. Several cell types can be mixed within callus clumps and often no sharp boundaries exist between embryogenic and non-embryogenic regions on the callus. Due to the size of callus clumps significant gradients of nutrients, hormones and carbon source may exist. Callus which is generally thought to be undifferentiated tissue, is multiplied on media containing mineral salts, a carbon source and one or several growth regulators. Callus clumps can be placed on other media, solidified or liquid, usually without growth regulators, to induce the formation of somatic embryos. There are some disadvantages to this system. The growth rate of callus is generally too low to produce large amounts of embryos within a certain time span. Furthermore, the production of callus is laborious and therefore expensive and can result in somaclonal variation (Evans and Sharp 1986) a most undesirable event when cloning plant tissue. In cyclamen, regeneration via a callus phase resulted in somaclonal variation (Schwenkel and Grunewald 1991).

Avoiding a callus phase in the production of somatic embryos therefore seems to be advantageous, provided there is a method available.

The model system most frequently used for somatic embryogenesis is carrot (*Daucus carota* L.) which is easily induced to form somatic embryos. Embryogenic cell lines can be established using callus but also by putting the explant directly into a liquid medium containing a low concentration of an auxin, preferably 2,4-dichlorophenoxyacetic acid (2,4-D) (De Vries et al. 1988). From the explant many cells are released into the medium and already after 1-2 weeks the so-called pro-embryogenic masses (PEMs) appear. PEMs can be defined as cell clumps able to produce somatic embryos and consisting of small, dense, highly cytoplasmic cells, the embryogenic cells (Halperin 1966, Halperin and Jensen 1967, Williams and Maheswaran 1986) and have a meristem-like morphology. The PEMs in suspension cultures will not develop into embryos due to the presence of an auxin and the high cell density (De Vries et al. 1988). They can proliferate to form large numbers of PEMs. After transfer to hormone free medium and diluting the culture the PEMs will develop into somatic embryos.

For other species that are not easily propagated via somatic embryogenesis a PEM culture was never produced or a callus phase was used to initiate cell suspensions. For *Cyclamen persicum* Mill. there are some reports of somatic embryogenesis (Kiviharju et al. 1992, Wicart et al. 1984) but both methods include a callus phase and do not easily produce large amount of somatic embryos. This paper describes the induction of embryogenic potential in *Cyclamen persicum* Mill., the production of large quantities of PEMs and the formation of somatic embryos, all in liquid medium.

Materials and methods.

Initiation of embryogenic suspension cultures. Seeds of the genotype Concerto scarlet Othello (S&G Seeds, Enkhuizen, The Netherlands) were surface sterilised with 70% ethanol (2 min) and 1% sodium hypochlorite (45 min) and washed thoroughly with sterile water. The seeds were germinated on moist paper for 2 to 5 weeks in the dark at 18°C. The emerging tuber from the seedling was used as explant material and cut in 2 to 8 pieces. The material from 3 tubers was cultured in 10 ml of standard B5 medium (Gamborg et al. 1968) supplemented with 58 mM sucrose, 25 µM 2,4-D and 5 µM kinetin in a 50 ml flask on a rotary shaker (100 rpm) in the dark at 23°C. All flasks were closed tightly with aluminium foil. After one week the cultures were diluted five fold. From this point the cultures were subcultured every two weeks by diluting 2-fold. After another one or two subcultures the cultures contained pro-embryogenic masses (PEMs) which were cultured separately from the explant by selecting them with a pipette with a wide nozzle.

From this point the cultures containing PEMs were subcultured by inoculating 1 ml of packed cell volume (PCV) in a 250 ml flask with a final volume of 50 ml medium using a pipette with a wide nozzle or in a smaller flask and culture volume, dependent on the initial

amount of PEMs present. The PCV was determined by centrifuging a sample from the culture (2 min. 700 g). The volume of the pelleted cell mass was measured and expressed in mls of packed cell volume. The growth rate of the cell line (t_d, doubling time) was determined by plotting ln(PCV) vs. time and recalculated using the formula for exponential growth (Schlegel 1976) only over the time span in which a straight line ($r^2 > 0.98$) was observed.

Development of somatic embryos. PEMs were selected by sieving the culture 8 days after subculturing. Cultures were diluted in water (2- to 5-fold) and then sieved through nylon meshes. The 150-300 μm fraction produced the highest number of single embryos.

After sieving the PEMs were centrifuged and the diluted suspension medium was replaced by the development medium. The PEMs were inoculated in the development medium, MS (Murashige and Skoog 1962) or B5 medium supplemented with 175 mM sucrose. PEMs were inoculated at a final density of 25 PEMs ml^{-1}. After 3 weeks torpedo-shaped embryos were formed which were transferred to medium with 58 mM sucrose. After an additional week the somatic embryos were used for germination experiments.

Cotyledon and plantlet formation. Germination and subsequent plantlet formation of cyclamen embryos consist of tuber formation followed by adventitious root formation and was induced by culturing the embryos in a medium with a lower sugar content. Tuber formation is defined as the development from the hypocotyl of a tuber or tuber-like structure. Liquid MS or B5 medium supplemented with 58 mM sucrose was used. The development of the cotyledon forming the first leaf was done on paper bridges in Magenta boxes (Magenta Corp., Chicago, Illinois, USA) filled with 12 ml of B5 medium supplemented with 58 mM sucrose. The cotyledon formed after 2 to 4 weeks in the dark at 18°C. A high relative humidity is preferred. Once the cotyledon is growing the plantlets can be put in the light and hardened before transfer to the greenhouse where the first true leaf is formed.

Analysis of arabinogalactan proteins and medium components. Isolation of arabinogalactan proteins (AGPs) and fractions of carrot seed AGPs using the monoclonal antibody ZUM 18 was done as described by Kreuger and Van Holst (1995). Crossed electrophoresis of AGPs was done as described by Van Holst and Clarke (1986).

Experiments in which AGPs were added to cell cultures were done essentially as described by Kreuger and Van Holst (1993). Embryogenic cyclamen cell cultures were sieved through a 250 μm nylon mesh and washed in culture medium before being inoculated in the presence of AGPs. The cell density used was about the lowest possible to ensure normal growth rates, in this case 50000 cells ml^{-1}. Cultures (3 ml, in triplicate) were grown in 6-well plates. After 2 weeks the number of PEMs per culture was determined.

Analysis of nutrients (hormones, sugars and mineral salts) in cell culture media was performed on samples from cultures taken at regular intervals during subculture. Samples

were centrifuged for 4 min. at 11000 g. The supernatant was removed and stored at -20°C before use.

Concentrations of hormones were determined using a Waters 510 HPLC (Waters, Milford, MA, USA). A reversed phase C18 column (Nova-Pak 60 A 4 μm, 3.9 x 75 mm, Waters) was used at room temperature. The column was run isocratically at 1.6 ml min^{-1} and eluted with 30% methanol and 21 mM of acetic acid in water. Kinetin was detected at 265 nm and eluted after about 3 min and 2,4-D was detected at 210 nm and eluted after about 10 minutes. The peak areas were proportional to the concentrations. The detection limits (with a 50 μl injection volume) for 2,4-D and kinetin were approximately 0.5 μM.

Sugar and salt concentrations were determined using Food Analysis kits of Boehringer (Mannheim, Germany).

Results.

Initiation of embryogenic cell cultures. Germinating seeds of Cyclamen, after emergence of the radicle, first produce a small tuber from the hypocotyl tissue. This tuber was used as explant material in all experiments. During the first three subcultures the explant fell apart releasing many cells into the medium and pro-embryogenic masses (PEMs) were formed. The PEMs can be white or light brown (Fig. 1), can vary in size, have a smooth surface and are usually spherical or globular or consist of several clumps.

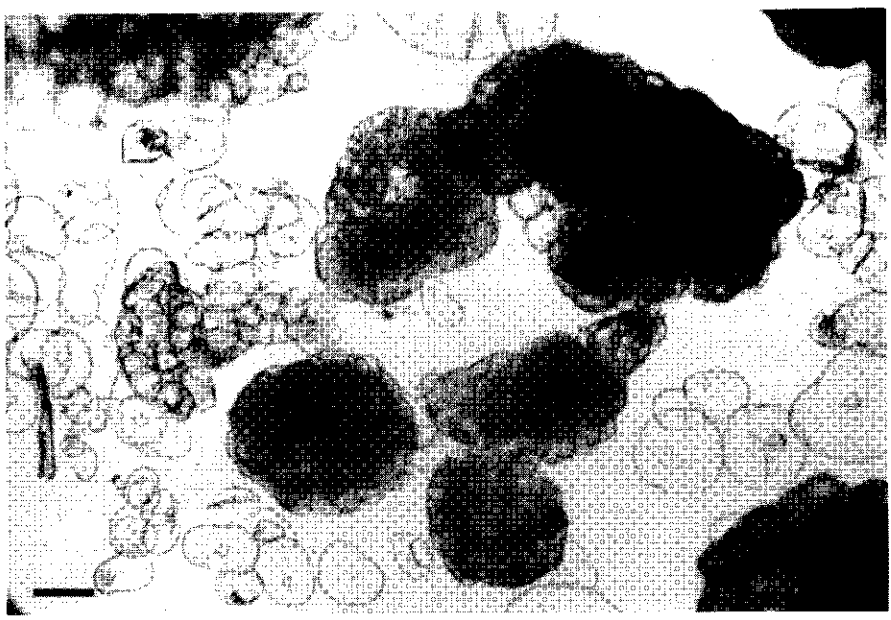

Fig. 1. Suspension culture of Cyclamen persicum. The suspension contains single cells and aggregates of large vacuolated cells. The PEMs are light brown clumps consisting of small cytoplasmic cells and have a smooth surface. Bar is 50 μm.

Chopping the explant accelerated the formation of PEMs. Cultures that did not contain PEMs 5 to 7 weeks after initiation seldom became embryogenic at a later stage. The chopped explants form large aggregates of cells originating from the cortex and epidermis of the tuber. Culturing these aggregates separately did not result in the formation of PEMs. Culturing the central tissue of the tuber containing the central pith, vascular bundles and the pericycle resulted in PEM formation. This indicates that this part of the explant contains the cells that can be induced to form (cells that form) PEMs. In general it was not necessary to separate the tissues. Purifying the PEMs and culturing them separately from the explant and large cell aggregates was advantageous for the culture morphology. This would ensure a prolonged proliferation of the PEMs.

Gamborgs B5 medium proved to be better in the induction of embryogenic potential than MS based medium containing the same amounts of hormones and sugar. One of the major differences is the different ammonium to nitrate ratio, which showed to have an effect in the embryo formation of cyclamen (Kiviharju et al. 1992).

Measurements of hormone levels in the medium during the first two subcultures showed that the explants were able to rapidly consume the available hormones. Figure 2A shows the hormone uptake of three chopped tubers in 10 ml of B5 medium with 25 μM 2,4-D and 5 μM kinetin. The cultures were diluted 5-fold after 1 or 2 weeks as indicated by the rise in hormone concentrations. The 2,4-D concentration fell to about 5 μM, but no depletion occurred. Kinetin however was depleted in both cultures. The culture diluted after one week was depleted of kinetin first at the end of the second subculture. By this time the culture contained many PEMs. The culture diluted after two weeks was depleted of kinetin at the end of both subcultures and did not become embryogenic, i.e. did not contain PEMs. This might indicate that kinetin was involved in the induction of embryogenic potential. However when cultures were initiated on B5 medium with 5 μM 2,4-D, 100 μM naphthalene acetic acid (NAA) and 5 μM kinetin and diluted 5-fold after one week, no PEMs were formed within the two subcultures or at a later stage. The hormone uptake shown in Fig. 2B,C shows that NAA was depleted in both subcultures and that both 2,4-D and kinetin were not depleted. NAA, a weak auxin, might not be able to induce embryogenic potential in these cultures. The low concentration of 2,4-D might cause the non-embryogenicity of the culture. Apparently the hormones have to be maintained at a minimal level to ensure PEM formation within the first 2 subcultures.

Chopping the explants resulted in rapid hormone uptake and subsequent PEM formation. The ratio of 25 μM 2,4-D and 5 μM kinetin was optimal for PEM induction. Lowering the two hormone concentrations lead to depletion or too low concentrations which could not induce PEM formation. Increasing the hormone concentrations, especially 2,4-D, caused extensive browning and resulted in non-embryogenic cultures.

Increasing or lowering the explant density had the same effects as respectively lowering or increasing the hormone concentrations (not shown). A prolonged first and second subculture

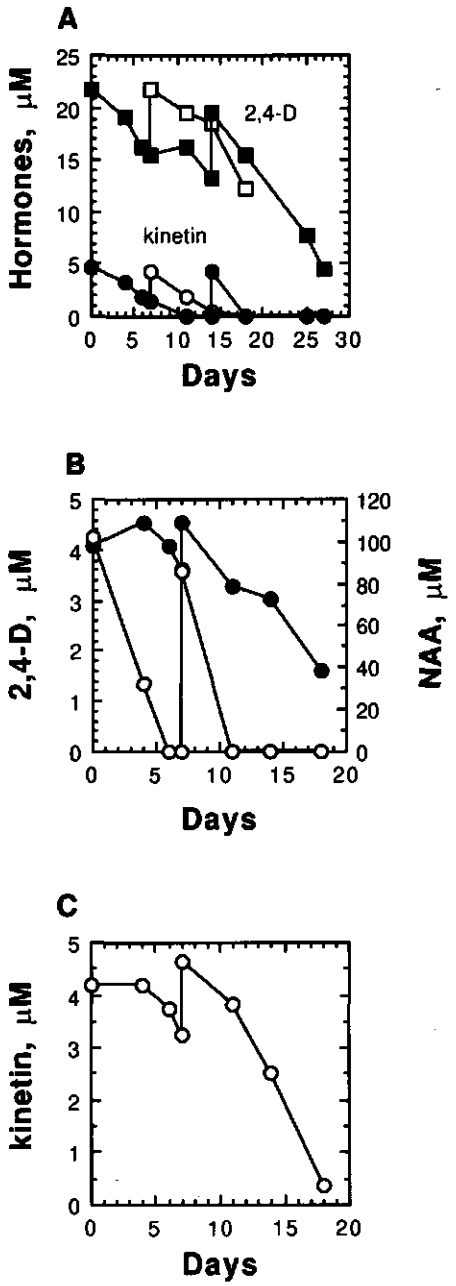

Fig. 2A-C. A. Hormone uptake during initiation of cell cultures. Three chopped tubers were cultured in 10 ml of B5 medium with 25 µM 2,4-D and 5 µM kinetin. Cultures were diluted 5-fold after 1 week (open symbols) or 2 weeks (closed symbols). **B.** Auxin uptake during initiation of cell cultures. Three chopped

tubers were cultured in 10 ml of B5 medium with 100 μM NAA (open circles), 5 μM 2,4-D (closed circles) and 5 μM kinetin. Cultures were diluted 5-fold after 1 week. **C.** Kinetin uptake during initiation of cell cultures. Three chopped tubers were cultured in 10 ml of B5 medium with 100 μM NAA, 5 μM 2,4-D and 5 μM kinetin. Cultures were diluted 5-fold after 1 week.

also lead to non-embryogenic cultures, probably caused by too low hormone concentrations at the end of the subcultures. The formation of roots or very large callus-like cell clumps by the explant was frequently observed when non-embryogenic cultures were formed, especially with prolonged subculture periods or non-optimal hormone levels.

These results indicate that explant density, hormone concentrations and subculture time should be balanced so as to lead to PEM formation within a short time.

Maintenance of embryogenic cell lines. Embryogenic cell lines were maintained by inoculating 1 ml of packed cell volume (PCV) in 50 ml of medium every 2 weeks. The PEMs tended to have a brown colour which was also excreted in the medium. The exponential growth curve of an embryogenic cell line during a subculture is shown in Fig. 3A. During the first 7 to 8 days the cell line grew with a shorter doubling time (t_d = 4.1 days) than in the last half of the subculture (t_d = 13.6 days).

Measurements of macro nutrient uptake during a subculture showed no major depletions except for ammonium which was consumed in 6 days. The depletion of ammonium coincided with the lower growth rate in the second half of the subculture (Fig. 3A). The pH of the medium showed after an initial decrease to 4.8 in the first 5 days a subsequent increase to 5.5 after ammonium was depleted. Changing the medium from B5 to MS resulted in higher growth rates and loss of the purple-brown colour of the cell line, probably because of the higher ammonium concentration. However, after a few months the embryogenic potential of the culture was decreased, making the MS medium unsuitable for proliferation of PEMs for a longer period.

Hormone measurements during a subculture showed that kinetin was depleted from the medium after 7 days and 2,4-D after 9 days (Fig. 3B). Growing the cell line on 50 μM 2,4-D and 5 μM kinetin did not change growth rates or cell morphology. The depletion of 2,4-D therefore seemed to be acceptable for PEM proliferation. However, lowering the 2,4-D concentration to 10 μM resulted in lower growth rates and loss of PEMs. Apparently both 2,4-D and kinetin had to be present in a minimal concentration at the start of the subculture to ensure PEM proliferation. Higher kinetin concentrations (in the presence of 25 μM 2,4-D) would lead to embryo development, which is not desired at this stage. Kinetin concentrations below 5 μM would lead to lower growth rates and loss of PEMs. The 2,4-D and kinetin concentrations used were therefore sufficient to ensure PEM proliferation in a reproducible and constant manner even though depletion of hormones occurred.

Sucrose was completely hydrolysed to glucose and fructose in about 9 days (data not shown). Glucose was preferentially used, as was also shown for carrot (Dijkema et al. 1988), and was not depleted during the subculture period. The nutrient measurements showed no major depletions of macro nutrients and sugars during a 14-day subculture indicating that the medium was sufficed for the demands of the proliferating cells.

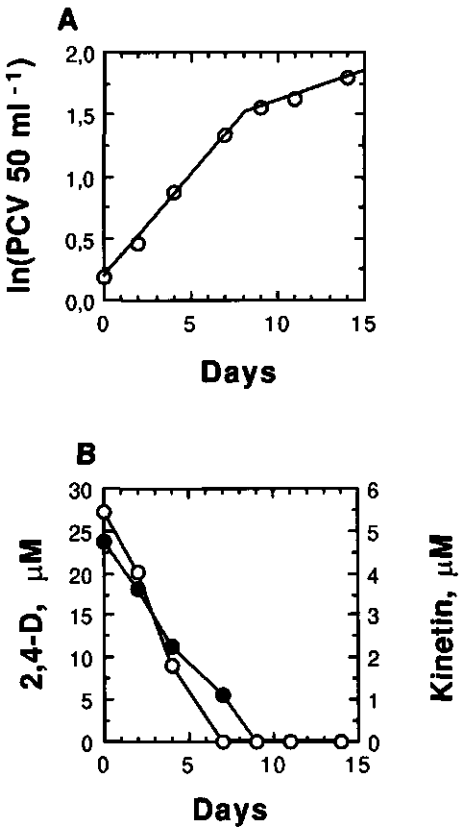

Fig. 3 A,B. A. Growth curve of embryogenic cell line during one subculture. Means of 2 PCV determinations. **B.** Hormone uptake during one subculture. 2,4-D closed circles, kinetin, open circles. Means of 2 hormone measurements.

Analysis of medium AGPs and addition of AGPs to cell cultures. AGPs excreted in the medium of cell lines were analysed by crossed electrophoresis. With this technique a precipitation line of AGPs with an AGP binding compound, the ß-glucosyl Yariv reagent, was established after two electrophoresis steps. In the first electrophoresis the AGPs were

separated on basis of their size and charge. In the second electrophoresis, perpendicular to the first direction, a precipitation line was formed. AGPs of young and old embryogenic and

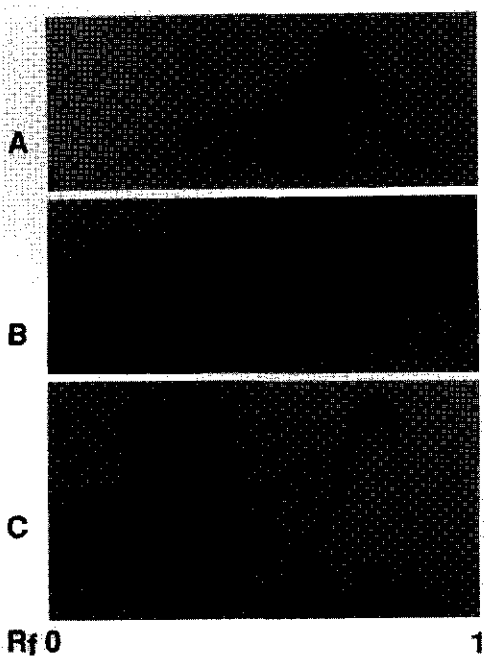

Fig. 4. Crossed electrophoresis of cyclamen arabinogalactan-proteins isolated from the culture medium at the end of the subculture. The precipitation line was formed with ß-glucosyl Yariv reagent. The R_f-value indicates the mobility of the AGPs. A, 5-weeks old embryogenic cell line, 6 µg AGPs. B, 11-month old embryogenic cell line, 7 µg AGPs. C, Non-embryogenic cell line, 12 µg AGPs.

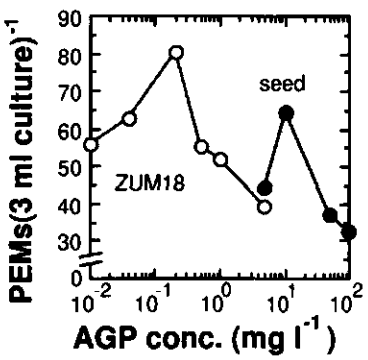

Fig. 5. Number of PEMs per culture after 2 weeks of growth in the presence of AGPs. Added AGPs were carrot seed AGPs and ZUM 18 purified carrot seed AGPs. Average SE: 8 PEMs per culture (n=3). Control cultures without added AGPs contained 36 PEMs per culture.

non-embryogenic cell lines were analysed. Both young (5 weeks) and old (11 months) embryogenic cell lines had similar AGP patterns (Fig. 4A,B). Both AGP patterns show a wide peak with a R_f-value of 0.68. This peak contains the majority of the AGPs. A small amount of the AGPs had a R_f-value of 0.27. The relative amount of these two AGP classes is different in the two cell lines, but both classes are present.

A cell line was initiated by using the cortex and epidermis of the seedling tuber as explant. This explant lead to the formation of a non-embryogenic cell line. This cell line showed a different AGP pattern (Fig. 4C). The pattern contains two peaks with R_f-values of 0.33 and 0.72 and represent different classes of AGPs. Similar differences between cell lines were shown for carrot (Kreuger and Van Holst 1993). Patterns of AGPs isolated from somatic embryos and seedling tubers were very similar (data not shown) as also shown for carrot somatic embryos and seedlings (Kreuger and Van Holst 1993).

AGPs isolated from carrot seeds and AGPs fractionated using the monoclonal antibody ZUM 18 (Kreuger and Van Holst 1995) were added to an embryogenic cyclamen cell line to test for biological activity. The experiments were performed essentially as described by Kreuger and Van Holst (1993). The percentage of embryogenic cells could not be determined due to the brown colour of the PEMs. Instead the number of PEMs in a culture was assessed after two weeks of growth (Fig. 5). Both AGP fractions doubled the number of PEMs in a culture. The optimum concentrations (10 and 0.2 mg l^{-1} for seed and fractionated AGPs respectively) were the same as for carrot cell lines (Kreuger and Van Holst 1995). This observation supports the previous finding that the activity of AGPs is not species specific (Kreuger and Van Holst 1995) and shows that AGPs binding to ZUM 18 have the same biological activity in carrot and cyclamen cell lines.

Development of somatic embryos. PEMs were isolated from cell lines to develop into somatic embryos (see Materials and methods). The size of the PEMs proved to be important for the efficiency, defined as the percentage of PEMs forming at least one somatic embryo. The larger the PEMs the higher the efficiency, however large PEMs tended to produce more than one somatic embryo. The latter is obviously not desired because double or triple embryos can produce plants with more than one shoot. Very small PEMs (smaller than 150 μm) had a very low efficiency. The fraction giving the most reproducible results and the highest number of single somatic embryos was 150-300 μm (data not shown). The point in time during a subculture of 14 days at which the number PEMs in the fraction 150-300 μm reach their maximum, expressed per ml of PCV or ml of medium was determined (Fig. 6). The first being a measure of the embryo-producing capacity of the cell line. Eight days after subculturing the highest number of PEMs (150-300 μm) per PCV were obtained as well as the highest number of PEMs per culture volume.

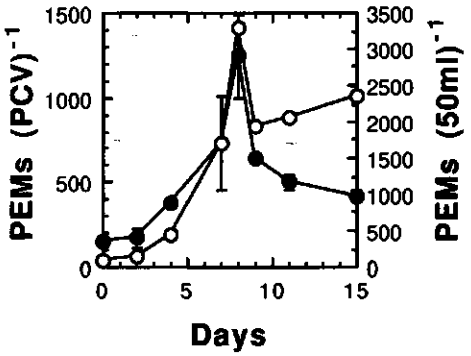

Fig. 6. PEMs per PCV during a subculture. Means ± SE (n=2). Only PEMs in the fraction 150-300 µm were counted. The number of PEMs per culture volume was calculated using the number of PEMs per PCV and the PCV per culture volume. PEMs per PCV, closed circles, PEMs per 50 ml, open circles.

The PEMs were inoculated in hormone-free medium to develop into somatic embryos. The PEM density was important for a correct development. Twenty five PEMs ml^{-1} gave good development of single, torpedo-shaped somatic embryos that resembled zygotic embryo development and was used in all subsequent experiments. A PEM density higher than 200 PEMs ml^{-1} resulted in a high density of somatic embryos which were arrested in their development and showed morphological aberrations. A PEM density lower than 10 PEMs ml^{-1} resulted in precocious germination of somatic embryos, with the formation of adventitious roots.

PEMs placed in B5 or MS medium with 58 mM sucrose can produce either somatic embryos or roots. The embryos (Fig. 7A) resemble zygotic embryos and the somatic embryos reported by Kiviharju et al. (1992) and Wicart et al. (1984). The roots can be attached to a tuber-like organ (Fig. 7B) or can be single (not shown). The formation of roots can be inhibited by development of somatic embryos on a medium with a higher sucrose content (Fig. 8A). The ratio of embryos to roots is determined by the sucrose concentration in the medium. The same media will increase the efficiency (Fig. 8B) with an optimum of 175 mM sucrose. The figures show that MS medium resulted in a higher efficiency than B5 medium, in contrast to the initiation and PEM proliferation phases where MS medium was not successful. The somatic embryos produced after 4 weeks on MS medium with 175 mM sucrose are white/yellow (Fig 7C), in contrast to the somatic embryos developed on B5 medium which were brown. Browning of the medium can also be severe during embryo development on B5 medium. The MS medium was therefore preferred for embryo development and sufficient to produce large numbers of somatic embryos in liquid medium.

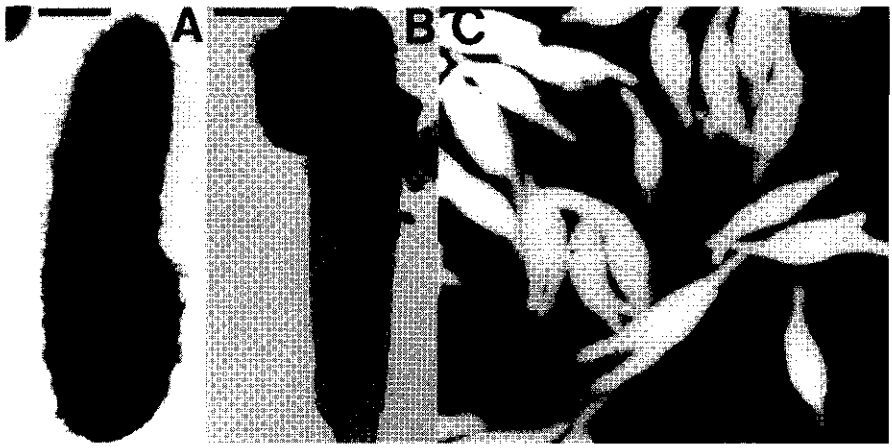

Fig. 7A.-C. A. Three-week old somatic embryos formed on MS medium with 58 mM sucrose. The upper half of the embryo is the cotyledon. Bar is 125 μm. **B.** Root formation of PEM on MS medium with 58 mM sucrose. The PEM has developed into a tuber-like organ. Bar is 125 μm. **C.** Four-week old somatic embryos developed on MS medium with 175 mM sucrose. Embryo size: 2 to 3 mm.

Germination of somatic embryos. Germination, like their zygotic counterparts involves the formation of a tuber-like organ followed by adventitious root formation and the formation of the cotyledon (Fig. 9A). Tuber formation is initiated in liquid medium by placing the embryos in MS medium with 58 mM sucrose. The embryos were germinated and converted into plantlets by placing them on paper bridges in Magenta boxes. The formation of the cotyledon was followed by the formation of true leafs of different shape and coloration (Fig. 9B), similar to normal seedlings.

The duration of the liquid medium phase of the somatic embryos was important for the germinating capacity. Germination of 4 weeks old somatic embryos on MS medium was always much lower than on B5 medium (data not shown), therefore all germination experiments were performed on B5 medium. This is again in contrast to the previous phase, the embryo development phase, where MS medium gave the best results. Up to 80% of the somatic embryos formed a cotyledon within 4 weeks (data not shown). Embryos grown for longer periods on either 175 or 58 mM sucrose germinated more poorly.

The culture conditions during germination proved to be important. The paper bridges in the Magenta boxes ensured high humidity and good aeration of the embryos without affecting the availability of water and nutrients. Using other solid media like perlite, sorbarod trays or soil resulted in lower germination frequencies. Plantlets were hardened and transferred to the greenhouse where they grew into mature plants.

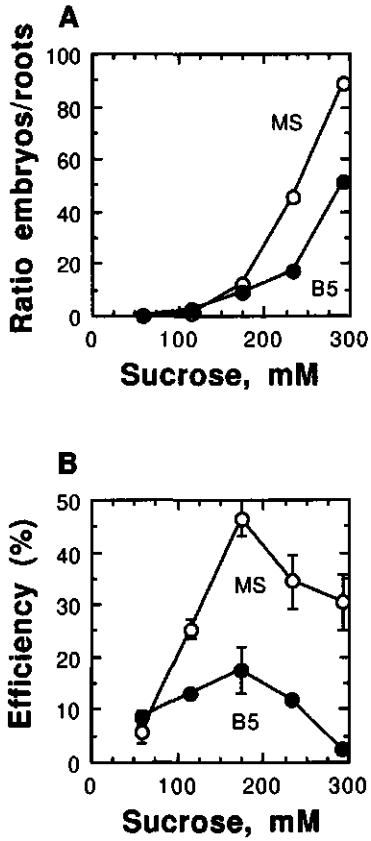

Fig. 8A,B. A. Effect of sucrose concentration on the ratio of the embryos and roots formed on B5 medium or MS medium. **B.** Effect of sucrose concentration on the efficiency of embryo formation. *Embryos developed on B5 medium or MS medium. Means ± SE (n=3).*

Five hundred embryos and 20 plants were measured for their ploidy levels using flowcytometry on at least 2000 nuclei per sample (De Laat et al. 1987). All embryos and plants as well as the embryogenic cell lines were diploid, as was the explant. The ploidy level of the cell lines remained constant for at least 24 months.

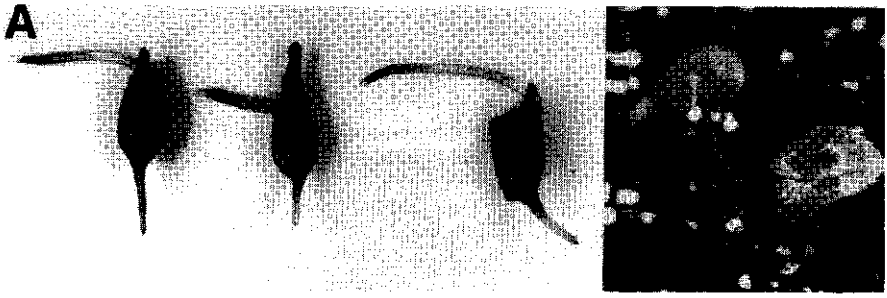

Fig. 9A,B. A. Cotyledon formation of somatic embryos in the dark. The etiolated cotyledon is red/purple and turns green after transfer to light. **B.** Plantlet formation from germinated somatic embryos. The first leaf has different shape and coloration than the cotyledon.

Discussion.

The system presented in this paper makes possible the large scale production in liquid medium of cyclamen somatic embryos. The process consists of several steps; initiation of embryogenic cultures, proliferation of pro-embryogenic masses (PEMs), development of somatic embryos, germination of embryos and the formation of plantlets. The first three are performed in liquid medium of which the initiation is of vital importance for the further development of the culture. A correct initiation of embryogenic cultures depends on explant density, hormone concentration and subculture time. Balancing these three will result in a rapid hormone uptake in the first subcultures (Fig. 2) which will lead to the formation of PEMs within a fairly short period of 5 to 7 weeks. The PEMs have a meristem-like morphology (Fig. 1). In the first subcultures the morphology of the culture is established and can hardly be changed at a later stage. Disrupting the balance between the three parameters will lead to non-embryogenic cultures. Once the PEMs are formed and enriched they can be proliferated for at least 2 years without loss of embryogenic potential or polyploidisation.

A very similar protocol is used for the carrot model system (De Vries et al. 1988). In both systems PEMs are produced at the initiation phase. The PEMs can be proliferated at high density for a prolonged time making a large scale production of somatic embryos possible. After transfer to hormone-free medium and dilution, the PEMs will develop into somatic embryos. The use of liquid medium from the beginning of the culture ensures a good availability of nutrients for all cells, something most uncertain using media solidified with agar. For cells growing on agar based media the depletion of nutrients might eventually lead to somaclonal variation.

The most important feature of this protocol is the avoidance of the callus phase. In the system presented here the regeneration events take place at the initiation phase whereas in callus cultures regeneration is often located at the plant production phase. Since PEMs do not need additional hormones to develop into somatic embryos, the only function of the auxin appears to be arresting the development of the embryo. PEMs might be regarded as being

arrested somatic embryos (Wilde et al. 1988) and therefore as differentiated structures. For cyclamen this two-step system of PEM proliferation and subsequent embryo formation is superior to conventional in vitro propagation methods (Geier et al. 1990). Regeneration in cyclamen via a callus phase showed about 5% polyploid plants (Schwenkel and grunewald 1991), while our method did not result in polyploidization in over 500 embryos.

The root formation of cyclamen PEMs (Fig. 7B) on hormone-free medium with 58 mM sucrose raises the question whether PEMs that form roots can still be regarded as PEMs. The root formation can be changed to embryo formation by high sucrose concentrations and the PEMs are therefore real PEMs. The root formation of PEMs on medium with low sucrose concentrations can be regarded as a form of precocious germination, the formation of the tuber and roots. The same phenomena are observed when somatic embryos are placed on medium with low sucrose concentrations. The tubers are able to germinate although at low levels without the addition of hormones (data not shown), indicating that they are bipolar like somatic embryos. Early tuberisation of somatic embryos has been reported before (Wicart et al. 1984) and resulted in the formation of bipolar tubers.

Analysis of medium AGPs of embryogenic and non-embryogenic cell lines showed similar results as obtained with carrot cell lines (Kreuger and Van Holst 1993). Again it is shown that differences exist between AGPs of embryogenic and non-embryogenic cell lines (Fig. 4). The finding that AGP fractions isolated from carrot seeds can influence the cyclamen cell lines shows again that the activity of AGPs is not species specific (Fig. 5). Recently it was shown that tomato AGP fractions can influence carrot cell lines (Kreuger and Van Holst 1995). The activity of the AGPs may therefore be universal.

The in vitro system for the large scale production of somatic embryos of *Cyclamen persicum* as presented, may well be applicable to other species. The many similarities between carrot and cyclamen cell lines indicate that this system is a general method for producing large quantities of somatic embryos in liquid medium.

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Chapter 6
Genetically stable cell lines of cucumber for the large scale production of diploid somatic embryos

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Abstract.

Initiation of embryogenic cucumber (*Cucumis sativus* L.) cell lines, from excised radicles, directly in liquid medium, required similar culture conditions as previously described for the initiation of cyclamen cell lines (Kreuger et al. 1995). The culture regime, explant density and hormone uptake were balanced so that pro-embryogenic masses (PEMs) were formed within about 8 weeks. The established cucumber cell lines could then be maintained for several years. The ploidy level of somatic embryos from different cucumber cell lines was either tetraploid or diploid. Embryogenic cell lines of carrot (*Daucus carota* L.), initiated from hypocotyl explants, directly in liquid medium, produced 6% tetraploid embryos. The ploidy level of the embryos depended on the ploidy level of the cell line. Cucumber cell lines that produced only diploid embryos were obtained by choice of completely diploid explant material and darkness during the initiation phase. Chimaeric explants could lead to tetraploid or mixed ploidy cell lines. Isolation and further subculturing of single PEMs resulted in either completely diploid or tetraploid cell lines, indicating that PEMs are either fully diploid or tetraploid. The embryogenic cucumber cell lines different in ploidy level, were indistinguishable in growth rate and embryogenic potential, and were genetically stable.

Introduction.

The initiation of embryogenic suspension cultures from explants, directly into liquid medium has been described for carrot (*Daucus carota* L.) (De Vries et al. 1988) and cyclamen (*Cyclamen persicum* Mill.) (Kreuger et al. 1995). Embryogenic cell suspensions of carrot and cyclamen may consist of several types of cells including aggregates of dark granular, highly cytoplasmic cells. Under the appropriate conditions, these aggregates are able to form somatic embryos and are therefore called pro-embryogenic-masses (PEMs) (Halperin 1966). The PEMs can be proliferated in a medium containing enough auxin to keep the development of embryos from PEMs arrested. After transfer to hormone-free medium and dilution, the PEMs can develop into somatic embryos.

Embryogenic cell lines of carrot tend to loose their embryogenic potential within a year (Halperin 1966, Pennell et al. 1992) and can also loose their genetic stability (Bayliss 1975). On the other hand, embryogenic cell lines of several other species, like cyclamen, retain their embryogenic potential for several years (Kreuger et al. 1995, Mo et al. 1989). It is unclear what causes these differences between cell lines of different species.

For the large scale production of somatic embryos, the use of liquid medium is generally preferred above agar based media. In callus cultures, grown on solidified media, somaclonal variation, especially polyploidization, is often observed (Custers et al. 1990, Evans and Sharp 1986, Geier et al. 1992). For cyclamen polyploidization was the most frequent observed aberration in callus derived plants (Schwenkel and Grunewald 1991). For *in vitro* plant propagation somaclonal variation is highly undesirable and should therefore be prevented.

Recently we have demonstrated for cyclamen that avoidance of a callus phase resulted in embryogenic and genetic stable cell lines producing only diploid embryos (Kreuger et al. 1995). Here we report the establishment of embryogenic cell lines of *Cucumis sativus* L., that is known to show considerable polyploidization when a callus phase is used (Custers et al. 1990). We also demonstrate that genetically stable cell lines can be obtained that produce only diploid embryos and are therefore suitable for the large scale production of somatic embryos during a longer period.

Materials and Methods.

Initiation of embryogenic cucumber cultures. Cucumber seeds (Pandorex, S&G seeds, Enkhuizen, The Netherlands) were surface sterilised with 70 % ethanol for 2 minutes and 1.4 % sodium hypochloride for 45 minutes and then thoroughly washed with sterile water (3 times). The seeds were germinated on moist filterpaper for 2 days at 23 °C in the dark. The emerged radicle from the seed was used as explant material. Fifteen radicles were cultured in 10 ml of liquid MS medium plus vitamins (Murashige and Skoog, 1962) supplemented with sucrose (58 mM), 2,4-D (9 μM) and kinetin (4.6 μM) in a 50 ml erlenmeyer. All flasks were closed tightly with two layers of aluminium foil and placed on a rotary shaker (100 rpm) at 23 °C with a 16 h light period (100-500 lux) or in the dark. When the auxin concentration fell to 0.5 μM, after about 5 days, the culture was diluted 5-fold with supplemented MS medium described above. Cultures were subcultured fortnightly by diluting the culture 2-fold with medium. After 8 to 10 weeks pro-embryogenic masses (PEMs) appeared and subsequently the explant was removed by sieving, and letting pass the fraction smaller than 250 μm. Subsequently, subcultures were performed by inoculating, at two weeks intervals, 0.4 ml of packed cell volume (PCV) in 50 ml of KK medium (described below) in a 250 ml erlenmeyer flask. The PCV was determined by centrifuging a sample (10 or 50 ml) from the culture (2 min at 700 *g*). The volume of the packed cell mass in the tube was marked and replaced by water. The weight of the water was determined and expressed as mls. of PCV. The PCV was measured at the end of each subculture to check the growth rate of the cell line.

The cucumber cell lines were maintained on cucumber specific KK medium supplemented with 45 μM of 2,4-D and 2.3 μM kinetin. The KK medium consists of: sucrose, 58 mM; NH_4NO_3, 15 mM; $(NH_4)_2SO_4$, 5 mM; KH_2PO_4, 4 mM; KNO_3, 10 mM; $MgCl_2.6H_2O$, 1.5 mM; $CaCl_2$, 2 mM, citric acid, 2.4 mM; $FeSO_4.7H_2O$, 50 μM; $CuSO_4.5H_2O$, 5 μM; $CoCl_2.6\ H_2O$, 1 μM; $MnCl_2.4H_2O$, 30 μM; $NaMoO_4.2H_2O$, 2 μM; KI, 0.5 μM; HBO_3, 50 μM; $ZnSO_4.7H_2O$, 30 μM; $NiSO_4.6H_2O$, 3 μM; myo-Inostitol 100 mg l^{-1}; Nicotinic acid, 1 mg l^{-1}; Pyridoxine HCl, 1 mg l^{-1}; Thiamin-HCl 1 mg l^{-1}. pH 5.8.

Initiation of embryogenic carrot cultures. Embryogenic carrot cell lines were initiated and maintained as described by De Vries et al. (1988).

Somatic embryo formation. For the formation of somatic embryos, cell lines, 7 - 9 days after subculturing, were centrifuged (2 min at 700 *g*) and the PCV was determined. The medium was replaced by sterile demineralized water. The cell suspensions were sieved over nylon mesh sieves of 150 µm followed by sieves of 50 µm for carrot or 100 µm for cucumber. Embryo development was performed at the same ambient conditions as the cell lines. PEMs were inoculated in hormone-free medium supplemented with 58 mM sucrose, at a density of 100 PEMs ml^{-1} for carrot or 200 PEMs ml^{-1} for cucumber. Carrot embryos were developed in B5 medium while cucumber embryos were developed in MS medium supplemented with 5 µM abscisic acid. After 11 days the embryo efficiency, defined as the percentage of PEMs forming a single torpedo-shaped embryo, was determined.

Embryo conversion and plant growth. Somatic embryos were hand-picked with a pincer that was sterilized in sodium hypochloride (1 %) followed by rinsing in sterile demineralized water and embryos were transferred to Sorbarod containers (Baumgartner Papier, Switzerland) filled with 240 ml of B5 medium (for carrot) or MS medium (for cucumber) supplemented with 58 mM sucrose and incubated at 22 °C in a 16 h day light period at 7000 lux. After 3 weeks the conversion, defined as the percentage of plantlets that has formed at least one leaf was determined. Plantlets were washed in tap water to remove the medium and sucrose present in the cellulose plug and transferred (with plug) to soil. After 3 days of nursery (high humidity) in the greenhouse the plants were treated as normal seedlings.

Measurement of ploidy levels using flowcytometry. Ploidy levels were measured on a flowcytometer (Partec CAII, Munster, Germany) as described by De Laat et al. (1987). At least 2000 nuclei were measured per sample.

Approximately 0.1 cm^3 of plant material (leaf, stem, somatic embryos) was chopped (50-100 strokes) with a razor blade in 0.2 ml of DAPI (4', 6-diamine-2-phenylindol) buffer. DAPI is a fluorescent dye intercalating in the DNA. The amount of fluorescence is proportional to the amount of DNA present in a nucleus. The composition of the DAPI buffer was; Tris, 10 mM; NaCl, 100 mM; $MgCl_2.6H_2O$, 5 mM; Na_2EDTA (ethylene-diaminetetra-acetic acid), 1 mM; Polyvinylpyrolidone (PVP), 1 g l^{-1}; Triton X-100, 2 g l^{-1}; Ascorbic acid, 10 mM; DAPI, 1 g l^{-1}. Subsequently, DAPI buffer was added to a final volume of 1 ml. This suspension was sieved (mesh size of 33 µm) and the filtrate was used for determination of the ploidy level. If necessary, the filtrate was diluted.

To determine the ploidy level of cell lines, a sample of 3-5 ml was centrifuged at 700 g for 1 min. The medium was replaced by cell wall lysing solution (Mannitol, 0.5 M; MES (2-[N-morpholino] ethane-sulfonic acid), 0.5 % (w/v); $CaCl_2.2H_2O$, 10 mM; cellulase, 0.25 % (Yakult Honsha Co-Ltd, Tokyo Japan) and Pectolyase Y23 0.1 % (Seishin Corp. Tokyo Japan). After 90 minutes of incubation the nuclei were isolated by quickly pipetting the protoplasts with a small pipette. The nuclei were separated from the cells by sieving (mesh

33μm) and to the filtrate DAPI buffer was added to a final volume of 1 ml. This suspension was used for determination of the ploidy level.

Calculation of the percentage of tetraploid cells. In order to check the genetic stability of embryogenic cucumber cell lines, the presence of tetraploid cells had to be monitored and measured. From the histograms obtained by flowcytometry, the percentage of tetraploid cells could be calculated (Fig 6). For cucumber cell lines it was found that the number of cells in G1 phase and the number of cells in G2 phase is almost equal (Fig. 6 B,C), the area covered by the G1 peak is almost equal to the area covered by the G2 peak. The ratio G1/G2 of diploid cell lines is 0.88 $\pm$ 0.24 (n= 11), the ratio G1/G2 of tetraploid cell lines is 1.04 $\pm$ 0.20 (n= 5). For the calculation of the percentage of tetraploid cells of cell lines of mixed ploidy level the following equation was derived:

If 2C, 4C and 8C are the number of cells in the diploid, tetraploid and octaploid peaks, respectively, and assuming that there are only diploid and tetraploid cells in the cell line, one can calculate that the percentage of tetraploid cells is defined as: $100\ 8C\ (2C + 8C)^{-1}$.

It is noteworthy that the 4C peak contains the diploid cells in the G2 phase and the tetraploid cells in the G1 phase. The 8C peak contains the tetraploid cells in the G2 phase. The equation has been used throughout all experiments concerning cucumber cell lines.

Determination of hormone concentrations. The concentrations of 2,4-D and kinetin in the media, were determined by HPLC on a reversed phase C 18 column (Nova-Pak 60 Å 4 μm, 3.9 x 75 mm, Waters, Milford, MA, USA) at room temperature. The column was run isocratically at 1.6 ml min^{-1} and eluted with 30 % methanol and 21 mM of acetic acid in water. Kinetin was detected at 265 nm and eluted after app. 3 minutes, 2,4-D was detected at 210 nm and eluted after app. 10 minutes. The peak areas were proportional to the concentrations. The detection limit (with a 50 μl injection volume) was approximately 0.5 μM for both 2,4-D and kinetin.

Results.

Initiation of embryogenic cucumber cell lines. The initiation of embryogenic cucumber cell lines was similar to the initiation of cyclamen cell lines (Kreuger et al. 1995). The radicles of germinated cucumber seeds were used as explants, and cultured in supplemented MS medium. Within a few days the epidermis and cortex cells separated from the rest of the tissue, containing the pericycle and the vascular tissue. Many single cells and cell aggregates were present in the medium but most of them did not seem to divide. For initiation of embryogenic cucumber cell lines the explant density, the hormone concentrations and the subculture period were balanced. PEMs were formed in a rather short time after starting the culture, i.e., about eight weeks. During the initiation phase, rapid uptake of 2,4-D in the first subcultures was observed (Fig. 1). In the following subcultures the uptake rate of 2,4-D was

much lower due to lower explant concentrations (not shown). Too low concentrations of 2,4-D or too long subculture periods resulted in root formation and non-embryogenic cultures.

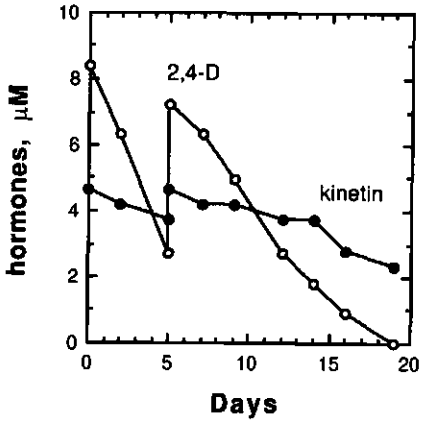

Fig. 1. Hormone uptake during the initiation of cucumber cell lines. Fifteen radicles were cultured in MS medium with 58 mM sucrose, 9.0 µM 2,4-D and 4.5 µM kinetin. After five days the culture was diluted 5-fold with fresh medium which restored the initial hormone concentrations, after which rapid consumption proceeded.

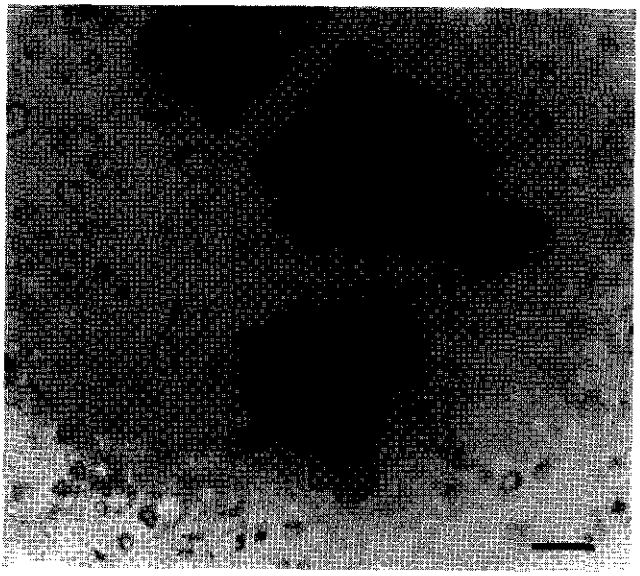

Fig. 2. PEMs of an embryogenic cucumber cell line. The PEMs are round cell clumps with a smooth surface and consist of small round cells. The bar represents 20 µm.

The PEMs in the cucumber cell lines (Fig. 2) seemed to originate from the explant, although very small PEM-like cell aggregates, which might have originated from dividing single cells, were also observed. MS medium was the most suitable for initiating embryogenic cucumber cell lines, B5 (Gamborg et al. 1968) and KK medium were less effective for that purpose.

Maintenance of embryogenic cucumber cell lines. At the end of the initiation phase, when the PEMs were formed and the explant was removed, the cell lines were grown in KK medium. For the maintenance of the cell lines, KK medium was preferred above MS medium, because of the higher growth rates and the embryogenic and genetic stability. Embryogenic cucumber cell lines maintained in KK medium showed a doubling time of approximately 2.9 days, which remained constant during the whole subculture period of 14 days. The cell lines growing in KK medium could be maintained for over 3 years, without losing embryogenic potential. The number of PEMs, in the 100-150 μm size fraction, per ml of PCV, was taken as a measure of embryogenic potential of the cell line. The PEMs in the fraction 100-150 μm form predominantly single somatic embryos. The number of PEMs in this fraction, obtained from a flask, is an indication of the cell line's ability to produce single somatic embryos, although it must be noted that larger cell clumps can also form embryos.

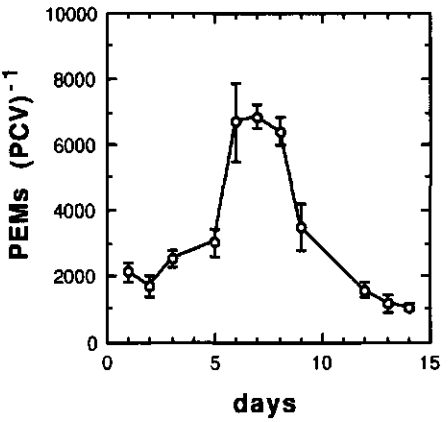

Fig. 3. Number of PEMs in the fraction 100-150 μm per ml of PCV during a 14 days subculture period of an embryogenic cucumber cell line, grown in KK medium with 45 μM 2,4-D and 5 μM kinetin. At regular intervals during the subculture period, the number of PEMs per PCV was determined, in duplicate. The data shown are the means of the two determinations, SD is indicated.

During the subculture period of two weeks, the number of PEMs, in the 100-150 μm size fraction, per PCV increased, to reach a maximum in the middle of the subculture period, and

then decreased again (Fig. 3). Similar results were obtained with cyclamen cell lines (Kreuger et al. 1995). What causes this variation is not clear, but it might be related to the changing osmotic conditions during a subculture period. The average size of the PEMs in a cell line, may vary extensively during a subculture period.

If cell lines were grown in KK medium, with the same hormone combination as used during the initiation phase (9.0 μM 2,4-D and 2.3 μM kinetin), the hormones were depleted before the end of the subculture period. Depletion of 2,4-D led to differentiation of somatic embryos in the suspension culture, which was undesirable at this stage. Therefore, the concentration of 2,4-D in KK medium was increased to 45 μM, which prevented depletion during a subculture period, with the further result that the number of PEMs in the fraction 100-150 μm, per ml of PCV, was increased 6-fold (data not shown). Kinetin was allowed to be consumed to depletion since increasing the concentration of kinetin led to embryo development. It appeared that even omission of kinetin from the medium did not affect the growth rate or embryogenic potential. Even though, it was decided to maintain kinetin in the medium.

Cucumber embryo formation and plant morphology. Somatic embryos from cucumber were formed in auxin-free MS medium. This medium was preferred above KK medium, for it results in better embryos formation than KK medium, possibly due to a lower salt concentration in MS medium. The morphological appearance of these embryos improved significantly by the addition of abscisic acid (ABA) in the medium. In all cucumber cell lines, independent of hormone concentrations and ploidy level, the percentage of PEMs forming an embryo, varied between 10 and 20%. The morphological criteria by which cell clumps are designated as being PEMs, therefore seemed inadequate. On the other hand, the inability of the PEMs to produce embryos at high efficiency might be due to the culture conditions.

Torpedo shaped embryos were formed within 10 days (Fig. 4). In initial experiments, up to 10% of the embryos were tetraploid, as determined by flowcytometry, in contrast to cyclamen where all embryos were diploid (Kreuger et al. 1995).

When the cucumber embryos were placed in Sorbarod containers, up to 60%, of both diploid and tetraploid embryos, formed a plantlet. After transfer to the greenhouse they grew into mature, fertile plants. The ploidy levels were confirmed by morphological observations and flowcytometry.

Ploidy level during Initiations of embryogenic cucumber cell lines. Once the embryogenic cucumber cell line was established, the percentage of tetraploid embryos generated by the cell line, remained constant over time. It appeared that the percentage of tetraploid cells in these cell lines remained the same and was similar to the percentage of tetraploid embryos formed on hormone-free medium. This suggested that the established cell lines were indeed

Fig. 4. Ten days old somatic embryos formed from a diploid cucumber cell line. The embryos were formed in MS medium supplemented with 5 μM ABA. Embryo size is 2 to 4 mm.

genetically stable, and that the polyploidization might have been introduced at the initiation phase.

It was therefore investigated whether the initiation phase could cause differences in ploidy level of the embryogenic cell lines. A paper by Gilissen et al. (1993) demonstrated that in many organs of cucumber plants the presence of tetraploid cells is very common. The explant used for the initiation of embryogenic cell lines, a 5 mm protruded radicle, was also chimaeric, and contained diploid as well as tetraploid cells. From the histogram, obtained by flowcytometry, the number of diploid and tetraploid cells was estimated. If it is assumed that the number of cells in the G1 phase equals the number of cells in the G2 phase, then it can be calculated that the explant contained 60% tetraploid cells.

At the beginning of the initiation of a cell line, the explant pieces form the main part of the biomass in the cucumber culture, but after 8 weeks, when the remainder of the explant is removed, there are mainly newly formed cell clusters. During the first 7-14 days the percentage of tetraploid cells in the starting cell line decreased. In this phase the cells of the cortex from the explant were released into the medium and dispersed as single cells and small aggregates, leaving only the pericycle and vascular tissue intact. By the method used for preparing the samples for flowcytometry, single cells and small aggregates were not included. We therefore assume that the cortex predominantly existed of tetraploid cells that escaped flowcytometry on release from the tissue, which resulted in a decrease of the percentage of tetraploid cells.

The ploidy level of an embryogenic cell line appeared remarkably to be influenced by light during the initiation phase. If cell lines were either initiated in the light, or in the dark, the cell lines initiated in the light often became completely tetraploid, whereas most of the dark initiations became of mixed ploidy level. However, the presence or the absence of light did not always lead to the same results. In both cases, fully diploid cell lines were occasionally obtained, although this was less frequently observed in cultures in the light. The presence of light during the initiation phase therefore seems to favour growth of tetraploid cells. Octaploid embryos were never observed in any of the cell lines, suggesting that polyploidization of tetraploid cells did not occur.

Influence of explant on ploidy level. Since tetraploid cells were already present in the radicle, used as explant, they might have contributed in the formation of PEMs, and subsequently resulted in tetraploid embryos. If no polyploidization occurred during the initiation phase then a fully diploid explant should lead to a fully diploid cell line, provided the initiation is performed in the dark.

Cucumber explants without tetraploid cells can be obtained from leaves, shoot meristems and ovaries, as determined by flowcytometry. If such explants were used to initiate cell lines in the dark, following the standard initiation protocol, embryogenic cell lines could be initiated from ovaries and shoot meristems. The resulting embryogenic cell lines were completely diploid and showed the same growth rates and number of PEMs, in the fraction 100-150 μm, per ml of PCV, as the cell lines that were obtained from radicles. Five hundred somatic embryos from a fully diploid cell line were found to be all diploid.

Fractionation of embryogenic cucumber cell lines. Since cucumber cell lines of mixed ploidy level produced either diploid or tetraploid embryos and not chimaeric embryos, this suggested that the individual embryos originated from a single cell, or, if the whole PEM formed the embryo, that PEMs were either fully diploid or tetraploid. This was checked with a cucumber cell line initiated from radicles, in the dark, that contained approximately 10 % tetraploid cells, 10 weeks after initiation. In an attempt to obtain a fully diploid cell line, individual PEMs of about 1 mm were isolated. The ploidy level of 11 PEMs was analyzed and 7 PEMs were 100% diploid and 4 were 100% tetraploid. In each cell clump the majority of cells therefore contained the same ploidy-level. Twenty individual PEMs were then selected and subcultured individually in 10 ml of standard proliferation medium. After 2 weeks 18 cell lines were completely diploid and 2 were completely tetraploid (Fig 5).

From this point the diploid and tetraploid cell lines were subcultured following the standard protocol and were similar in growth rate and the PEM quantity produced. All cell lines had a doubling time of 3.0 $\pm$ 0.3 days (n=110), and the efficiency of embryo formation from PEMs was 10-20%.

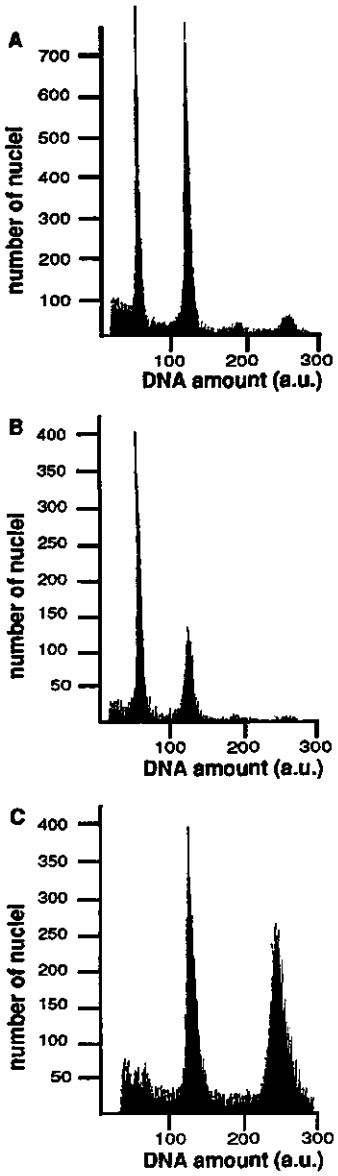

Fig. 5A-C. Production of fully diploid and tetraploid cell lines from individual PEMs. **A.** Histogram showing the cell line of mixed ploidy level from which single PEMs were cloned. The histogram shows a 2C, 4C and 8C peak, where the 4C peak contains the diploid cells in the G2 phase, and the tetraploid cells in the G1 phase. The percentage of tetraploid cells is 10%, as calculated with the equation from Materials and Methods. **B.** Histogram showing a fully diploid cell line, because it only contains a 2C and 4C peak. **C.** Histogram showing a fully tetraploid cell line, because it only contains a 4C and 8C peak.

Carrot embryo formation and plant morphology. From an embryogenic carrot cell line that had been initiated and maintained for 6 months according to De Vries et al. (1988) embryos were produced. Six % of the embryos were tetraploid. Up to 80% of both diploid and tetraploid embryos converted into mature, fertile plants. The ploidy level of the plants was confirmed by morphological observation and flowcytometry.

In carrot the number of cells in the G1 phase is much larger than the number of cells in the G2 phase (data not shown), making the detection of octaploid cells difficult. As shown in Fig. 6 the 4C peak of carrot cell suspensions of mixed ploidy level is only 5 -10 % of the 2C peak, thereby explaining the embryos formed of which 6% is tetraploid. If the percentage of tetraploid embryos and plants reflects the percentage of tetraploid cells found in the cell line, then the percentage of 8C cells would be less than 8 % of the 4C peak observed: 8 % from 6 % equals 0.5 %, which is too low to be detected.

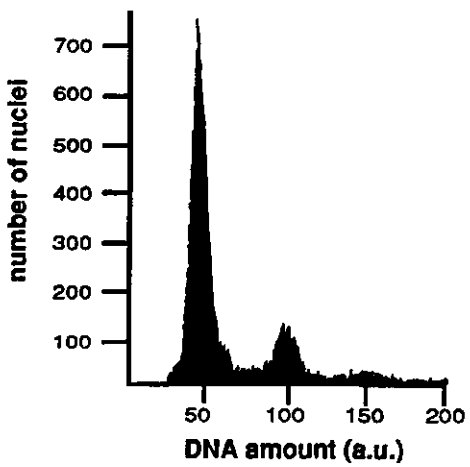

Fig. 6. Histogram of an embryogenic carrot cell line showing the large 2C, and the small 4C peak. Six % of the embryos produced by this cell line was tetraploid.

Discussion.

We have demonstrated that it is possible to generate cucumber cell lines, directly in liquid medium, that maintain their embryogenic potential. We have moreover shown that it is possible to obtain genetically stable diploid cell lines if diploid explants are used for initiating the cell line or if diploid pro-embryogenic masses (PEMs) are selected for further multiplication, from a cell line of mixed ploidy level.

The requirements for initiating cucumber cell lines were largely similar to those for cyclamen, and confirmed our previous observations. For the induction of embryogenic potential, in both cucumber and cyclamen, a rapid hormone uptake and a correct nutrient composition are desireable (Fig. 1, Kreuger et al. 1995). The latter is illustrated by the lower effectivity of B5 (Gamborg et al. 1968) and KK medium to initiate embryogenic cucumber cell lines, while on the other hand the KK medium is superior for proliferation of the embryogenic cell lines.

During the phase where the PEMs are proliferated, the medium has to meet other requirements, showing the difference between the initiation and proliferation phase. The function of 2,4-D in the proliferation phase is to arrest the development of embryos from PEMs, and this is totally different from the inductive function during initiation. In rapid growing cucumber cell lines in KK medium, high 2,4-D concentrations were necessary. These concentrations would be toxic during the initiation phase. It can be reasoned that the 2,4-D concentration at the start of a subculture period is potentially toxic and that rapid uptake, which is coupled to rapid growth, is an attempt at de-toxification. The use of high 2,4-D concentrations also resulted in increased embryogenic potential in cucumber. This is in contrast to cyclamen, where it was not necessary to increase the 2,4-D concentration (M. Kreuger, unpublished results). Even though cucumber and cyclamen have remarkable similarities during the initiation phase, also differences were observed for all phases. The contrast between the initiation and proliferation phase is best demonstrated in cucumber, where each phase has specific nutrition and hormone requirements. In cyclamen however, the initiation and proliferation can be performed with the same medium (Kreuger et al. 1995). For optimal embryo development both crops need another medium composition, again demonstrating the difference between the subsequent phases.

In both cyclamen and cucumber a remarkable optimum in the number of PEMs, in a specific fraction, was observed in the middle of a subculture period (Fig. 3, Kreuger et al. 1995). This might be the result of a continuous change in the size of the PEMs. At the start of the subculture period the hormone and nutrient concentrations are high, possibly resulting in a disintegration of large PEMs, and concomitantly in a rise in the number of small PEMs. As hormone and nutrient concentrations lower, as the subculture period proceeds, larger PEMs are formed, resulting in a fall in the number of small PEMs.

The efficiency of embryo formation remains constant over time and might be related to the ratio of cells in the G1 and G2 phase. In both carrot (Fig. 6) and cyclamen (not shown) few cells are in the G2 phase, and in both species a high percentage of the PEMs is able to form an embryo (Kreuger and Van Holst 1995, Kreuger et al. 1995). Alternatively, in cucumber the number of cells in G1 and G2 is about equal and approximately 20% of the PEMs form an embryo. A high G2 peak may result in, or be the consequence of, a low embryo-forming capacity, although the cause of this observation is unknown.

The production of embryogenic cell lines of carrot (De Vries et al. 1988) and cucumber, directly in liquid medium, can eventually result in diploid and tetraploid embryos. The production of embryogenic cell lines of cyclamen resulted, in contrast, in only diploid embryos (Kreuger et al. 1995). The formation of tetraploid embryos is highly undesirable when plants are propagated via somatic embryogenesis. The presence of tetraploid cells in explants is almost unavoidable, since they are found in many organs of mature cucumber plants (Gilissen et al. 1993). Chimaeric explants and the presence of light during the initiation phase can result in tetraploid cell lines. In this case light may have a unwanted hormone-like effect and should therefore be avoided. Still, even if cultures are initiated in the dark, isolation of individual PEMs may be necessary for obtaining embryos which all have the same ploidy level. Also in carrot, the presence of light during embryo development in liquid medium seemed to be inhibitory, although ploidy measurements were not performed (Michler and Lineburger 1987).

The production of somatic embryos via a callus phase on solid medium, often results in somaclonal variation. In the *Cucurbitaceae* and in many other species polyploidization and structural changes of chromosomes were observed (Ashmore and Shapcott 1989, Bayliss 1980, Debeaujon and Branchard 1993, Larkin and Scowcroft 1981, Pijnacker et al. 1989, Schwenkel and Grunewald 1991). In cucumber one out of eight cell lines appeared to be tetraploid (Custers et al. 1990). Tetraploid carrot cells were unable to form somatic embryos (Coutos-Thevenot et al. 1990) and similar results were obtained from callus and suspension cultures of Poinsettia (Geier et al. 1992), indicating a reduced embryogenic potential of polyploid cells. This paper however shows that embryogenic potential is independent of ploidy level. Ashmore and Shapcott (1989) demonstrated that polyploidization happens more frequently during the proliferation of callus cultures on solid medium than in suspension cultured cell lines. Since the polyploidization is observed to happen during the process, one might conclude that cell growth on solid medium eventually leads to genetic alterations and impaired embryogenic potential, not necessarily in that order. The large scale production, for a longer period, of somatic embryos which all have the same ploidy level, will therefore be hard to establish.

If, however, cell lines are obtained by selection of individual PEMs, cell lines with mixed ploidy levels are avoided and polyplidization is not observed during the proliferation phase. Even when crops are used, which are known to show polyploidization in a callus culture, like cucumber (Custers et al. 1990), genetic stability can be obtained. The much better availability of nutrients in the liquid medium may result in higher growth rates and absence of polyploidization. Provided the medium meets the nutritional demands of the cells, the use of liquid medium throughout the whole process makes long term maintenance, including genetic and embryogenic stability, possible.

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Chapter 7
General discussion

The large scale propagation of plants via somatic embryogenesis, has so far been difficult to achieve. In this thesis research is described leading to embryogenic cell lines that can be maintained for a long period, without loss of genetic stability. It is also described how embryogenic potential of cell lines can be influenced by the addition of specific arabinogalactan-proteins.

We consider the large scale production of somatic embryos to consist of five steps; initiation of embryogenic cell lines, proliferation of pro-embryogenic masses (PEMs), formation of embryos, germination of embryos and transfer of germinated embryos to the greenhouse. We have found for three crops, carrot, cyclamen and cucumber, that when the first three steps are performed in liquid medium, embryogenic cell suspensions can be obtained in a very comparable manner. The cell lines produce PEMs, that proliferate at a similar growth rate, for all the three crops, and produce somatic embryos at a high efficiency. The somatic embryos germinate and produce plantlets which grow into mature, fertile plants, again with a high efficiency. Since the first three steps are performed in liquid medium, the process is labour extensive and inexpensive. The major costs in large scale productions will then be associated with the germination of the embryos and the transfer to the greenhouse. The achievement of the research presented in this thesis is that the feasibility of somatic embryogenesis for plant propagation is demonstrated for three different crops, under conditions that preserve genetic stability and embryogenic potential of the cell lines.

Essentials for the initiation phase.

For the initiation of an embryogenic cell line, directly in liquid medium, an explant has to be chosen, which is able to produce PEMs if incubated in the proper medium. In our experience, an explant derived from a piece of the plant close to the zygotic embryo, both in space and in time, has the best chance of success (Carman 1990, Debeaujon and Branchard 1993, Williams and Maheswaran 1986). Explants from a germinating seed, like root tips from cucumber (Chapter 6) or hypocotyls from cyclamen (Chapter 5), have proven to be very useful, both in our experience and in literature (Carman 1990, Debeaujon and Branchard 1993, Williams and Maheswaran 1986).

In initiation, the nutrient composition and the hormone concentrations of the liquid medium in which the explant was placed, are very important. For each crop we observed a different optimal composition. The ammonium concentration in different media varies greatly, from 2 mM in Gamborgs B5 medium to 25 mM in KK medium. The higher concentrations might be toxic to carrot and cyclamen, but not to cucumber. Carrot and cyclamen could be initiated in B5 medium, but cucumber needed MS medium, which contains 21 mM ammonium. In all cases the media, contained an auxin, 2,4-dichlorophenoxyacetic acid (2,4-D), and usually also kinetin. These hormones were rapidly taken up by the explant (Chapter 5, Fig. 2, Chapter 6, Fig. 1) and we showed that rapid uptake of hormones is likely a prerequisite for induction of embryogenic potential.

If root tips or hypocotyls were used, the incubation of the explant resulted in the dispersion of the epidermis and cortex, leaving the vascular bundle and the surrounding cells intact. Probably, the cells surrounding the vascular tissue provide cells able to produce the PEMs. A similar conclusion was drawn by Nadolska-Orczyk and Malepszy (1987), in their study on the initiation of embryogenesis on leaf explants of cucumber. Although these experiments were performed on solid medium, embryo formation was initiated in cells surrounding the vascular tissue.

In our experience, PEM formation was achieved within a period of maximal 8 weeks, although differences between crops were observed, carrot always being the fastest and cucumber the slowest. What causes the differences between the three crops in the length of the initiation phase is unknown. PEMs were either formed on the explant or from single cells or cell aggregates in the medium, and were formed in massive amounts at an almost predictable point during the initiation phase.

The use of different explants, or media with different nutrients or hormone concentrations could lead to delay, or absence, of PEM formation, resulting in poorly, or non-embryogenic cell lines. In these cases, root formation was often observed. Already in 1966, Halperin reported similar observations in carrot cell lines. In his paper (Halperin 1966) on alternative morphogenetic events in carrot cell suspensions he stated that "Strong circumstantial evidence indicates that the same cells in culture are capable of giving rise to either a root-bearing clump or to an embryo, depending upon the particular chemical environment in which they are grown. The present data suggest that the formation of proembryos depends upon a rapid rate of cell division under conditions which prevent cell enlargement." We have found that this also holds for cyclamen and cucumber cell lines. Indeed, rapid cell division may repress cell enlargement, and thus promote the formation of small cells, which are able to become embryogenic cells.

Furthermore we have found that the nutrient composition is equally important as the concentration of the hormones. In a recent paper Preece (1995) concluded "There is an interesting relationship between nutrient salts in the medium and the plant growth regulators (PGR). However, nutrient salts will not wholly substitute for PGR, and neither will PGR completely substitute for nutrient salts. With the improper nutrient medium, chances will be very low that explants will respond as desired, regardless of the PGR and their concentrations tested. Conversely, with the improper PGR type, combination, or concentration, explants will respond poorly if the nutrient salts are less than ideal for that plant genotype or the physiological state of the plant material." Indeed, also in our experience, the type of explant determines which is the ideal combination of nutrients and hormones for obtaining maximal embryogenic potential in the initiation phase. This is further illustrated by the preference of cucumber for MS medium (Chapter 6), while cyclamen (Chapter 5) and carrot prefer B5 medium (de Vries et al. 1988).

The induction of embryogenic potential in explants, placed in liquid medium, has been described for only a limited number of plant species. Embryos of cucumber (Ziv and Gadasi 1986), cassava (Raemakers et al. 1993) and melon (Oridate et al. 1992) were obtained, on explants placed in medium containing an auxin. After an induction period in auxin containing medium, the embryos developed on the explants, which were then transferred to hormone free medium. No PEMs were observed, and no embryogenic cell suspensions were formed. This method therefore differed form the one described in this thesis. The importance of the initiation phase was also emphasized for *Hevea brasiliensis* (Micheaux-Ferriere and Carron 1989). In this case the length of time of the first two subculture periods on solidified, auxin containing medium, determined whether embryos were formed in the third subculture period, after transfer to auxin-free medium. Although PEMs were not observed, the general conclusion of Micheaux-Ferriere and Carron (1989) is in accordance with the results we have obtained for cyclamen and cucumber. The majority of cases of somatic embryogenesis described in literature use a callus phase on solid medium (Ammirato 1983, Zimmerman 1993). This seems to be the generally accepted method for obtaining somatic embryos, even when subsequently suspension cultures are made by dispersing the callus in liquid medium.

It is our conviction that a callus phase should be avoided, since it is unnecessary laborious, and may lead to genetic instability and loss of embryogenic potential, as will be discussed below. Furthermore, with callus controlled growth is hard to establish, and in contrast to starting embryogenic cell lines in liquid medium, which is now possible, the large scale production of somatic embryos from callus cultures may not be feasible.

Optimal proliferation of PEMs.

Once embryogenic cell lines are obtained the PEMs have to be proliferated in order to produce the number of embryos wanted. For proliferation it is essential that PEMs are arrested in their development to embryos. Moreover, the number of PEMs must be multiplied by growth and subsequent disintegration. A single PEM, with a doubling time of 3.5 days, will produce 1 million PEMs in 10 weeks. The doubling times of plant cells, cultured *in vitro*, reported for cells of different species, range from 1.0 to 6.3 days, but it is not known whether embryogenic cell lines were used in all cases (Taticek et al. 1991). It will be obvious that the amounts of nutrients required for this proliferation are substantial. In our system, the proliferation was performed in flasks, and the cell lines were subcultured every 14 days. This included the complete replacement of the medium and dilution of the cells to a standard cell density per flask. Apart form the hormones, also the optimal nutrient composition of the maintenance medium differs for various crops. For cyclamen and carrot B5 medium was suitable, but for cucumber KK medium was used. For cucumber the medium composition had to be changed after the initiation phase. Initiation of cucumber cell lines was done in MS medium, but MS medium proved to be inadequate for the long term maintenance of the embryogenic cell line (Chapter 6).

During a 14 day subculture period, nutrients are consumed by the growing cells. If the initial concentration of a nutrient in the medium is too low it may get depleted before the end of the subculture period, resulting in a decrease in growth rate. Indeed for cyclamen cell lines, growing in B5 medium, we observed depletion of ammonium after six days and this caused a decrease in growth rate (Chapter 5, Fig. 3). A similar observation was made for alfalfa growing in Schenk and Hildebrandt medium, containing 3 mM ammonium (McDonald and Jackman 1989). Uptake of nutrients were also measured in carrot cell lines growing in B5 medium, and it was found that phosphate could be depleted in 6 days (Ashihara and Nygaard 1989). Carrot cell lines even ceased growing, also known as the stationary phase, 10 days after subculturing due to sugar depletion (starting with 58 mM sucrose, Dijkema et al. 1988). Sugar depletion and subsequent cessation of growth were also observed in cucumber cell lines growing in MS medium with 86 mM of sucrose (Callebout and Motte 1988). Carrot cell lines showed an increased percentage of polyploid cells and loss of embryogenic potential, if subculture periods were extended and a stationary phase was reached (Bayliss 1975, Halperin 1966). Ashihara and Nygaard (1989) observed a decrease in RNA content of carrot cells during phosphate depletion. It was suggested that cells needed breakdown products of RNA, caused by reduced capacity for de novo synthesis. Depletions during the stationary phase may lead to impaired protein synthesis and affect control mechanisms, and may eventually lead to genetic instability (Bayliss 1975). Apparently it seems advantageous to avoid a stationary phase during a subculture period (Bayliss 1975).

In the proliferation phase, it is essential to prevent the formation of embryos from PEMs. When this is not adequately dealt with, premature embryo formation will occur and all PEMs may be lost. The balance between the auxin and the cytokinin, and the concentration of the auxin, determine whether the PEMs will proliferate or develop into an embryo. In contrast to the initiation phase, where the hormones function as inducing compounds of the embryogenic potential, their role in the proliferation phase is totally different. The initial hormone concentration during a subculture period is important, and in general, a high auxin to cytokinin ratio is required. Low auxin to cytokinin ratios or low auxin concentrations may lead to premature embryo formation. We found that for cyclamen this ratio should be about 5 and for cucumber about 20. In cucumber, the increase in the 2,4-D concentration from 5 μM in the initiation phase to 45 μM in the proliferation phase, had an additional effect of a 6-fold increase in the number of PEMs per PCV (Chapter 6). In cyclamen such effect was however not observed (Chapter 5).

In other studies on embryogenic cucumber cell lines (reviewed by Debeaujon and Branchard 1993), usually 2 to 5 μM of auxin is used. By comparison, the 45 μM 2,4-D used in our cucumber cell lines is very high. This difference is most probably explained by the rapid growth of our cell lines in KK medium. The actual growth rates of cucumber cell lines in other studies were never reported, but since in most cases callus cultures were used, the growth rates were probably much lower.

The proliferation of embryogenic carrot cell lines in hormone-free medium containing ammonium as the sole nitrogen source, which results in low medium pH, was reported by Smith and Krikorian (1990). In that case PEMs were maintained and proliferated without the premature formation of embryos. This remarkable medium may be an example of the relationship between nutrients and hormones, noticed by Preece (1995), and illustrates how nutrients and hormones substitute for each other in specialized circumstances in carrot cell lines.

An intriguing phenomenon was observed, in both cyclamen and cucumber cell lines. A large variation in the number of PEMs during a subculture period, in the size fractions 150-300 μm and 100-150 μm, respectively, was observed (Chapter 5, Fig. 6, Chapter 6, Fig. 3). Due to growth of the cell lines, the number of PEMs increased, but the number of PEMs in a specific fraction varied during a subculture period. The specific fractions were chosen for their ability to produce single embryos, and represent only a small part of the whole biomass. The variation in the number of PEMs in these fractions may represent a small, but significant change in average PEM size during a subculture period. This might be related to the discontinuity of the process and a possible explanation is the following. At the start of the subculture period the nutrient and hormone concentrations in the medium are high, possibly resulting in a disintegration of large PEMs, and as a result, in an increase in the number of small PEMs (Halperin and Jensen 1967). Due to growth of the cells, the concentrations of nutrients and hormones in the medium decrease, possibly preventing PEMs from disintegrating, and consequently, decreasing the number of small PEMs in the specific fractions mentioned above. McDonald and Jackman (1989) measured that during a subculture period nutrients, pH of the medium, hormones and osmotic pressure varied dramatically, possibly explaining the change in the average size of the PEMs. Similar variations in aggregate size were observed for other species, but it was not mentioned whether embryogenic cell lines were used (Taticek et al. 1991).

The maintenance of cell lines should be one of the best controlled phases, for the cell line is the source of all the embryos and has to be maintained for a prolonged period. PEMs have to be proliferated without loss of embryogenic potential and without premature embryo formation. We have shown that control of this phase can be achieved by medium adaptations and controlled subculture regimes.

Production of somatic embryos.

For producing somatic embryos, PEMs are sieved from a cell line, and then inoculated in hormone-free medium. In our studies on carrot, as well as cyclamen and cucumber, we observed that large PEMs tend to produce more than one embryo which are usually interconnected. On the other hand, very small PEMs tend to be less efficient in embryo formation, and a low percentage of these small PEMs actually forms an embryo. Chee and Cantliffe (1992) obtained similar results in sweetpotato, but their cell lines were produced

from dispersed callus which may be the reason for the observed low efficiency of embryo formation.

In order to produce as many single embryos as possible, and at the same time a high efficiency of embryo formation, for cyclamen and cucumber the best results with PEMs between 50 and 300 μm. The formation of embryos from PEMs, does not require exogenous hormones or other specific compounds, indicating that PEMs are fully capable of producing an embryo-like structure with as well a root and a shoot meristem. After transfer to hormone-free medium and dilution, the morphogenetic potential is 'released' and the embryo is formed, almost by itself. Successively, the PEMs develop into a globular, heart and torpedo shaped embryo. In cyclamen this is difficult to recognize due to the monocot-like nature of this dicot. It is our view that PEMs can be regarded as embryos arrested in their development, and the induction of embryo formation is therefore not induced in the hormone-free medium, but already during the initiation phase.

The efficiency of embryo formation, expressed as the percentage of PEMs forming an embryo, can be high. In cucumber this usually was 10 to 20%, but in carrot and cyclamen it was more than 60%. Dijkema et al. (1988) showed that the efficiency of embryo formation of carrot cell lines, varied during a subculture period, with an optimum at 7 days. We have never observed such variation during a subculture period with cyclamen and cucumber. This might be related to the observed cessation of growth of the carrot cells at the end of the subculture period, under the conditions used by Dijkema et al. (1988). Suboptimal growth conditions during the proliferation phase apparently decreased the ability of PEMs to form embryos. It suggests that the quality of the PEMs is related to the growth circumstances in the proliferation phase.

In our experience the nutrient composition required for optimal embryo development, may differ from that of the maintenance medium. With both cyclamen and cucumber the maintenance medium, B5 medium for cyclamen and KK medium for cucumber, had to be changed into MS medium for optimal embryo development. The low initial cell density during embryo development will undoubtedly determine the suitability of a medium. Apparently the developing embryo has nutrient demands different from the proliferating PEMs. For cucumber ABA has been reported to improve embryo morphology (Ladyman and Girard 1992), but ABA is not generally applied (Debeaujon and Branchard 1993). For cyclamen, a high sucrose content favoured the formation of embryos, and prevented the formation of only roots (Chapter 5, Fig. 8). High sucrose concentrations were also used by Wicart et al. (1984) in the formation of cyclamen embryos on callus. Specific additions, like hormones (ABA) or high sucrose concentrations, may be required to improve the efficiency or germination, but we found that, in principle, hormone-free medium and low cell densities are sufficient for embryo development.

Genetic stability is essential.

In Chapter 5 and 6 we showed that, when the generation of embryogenic cell lines was performed in liquid medium, genetic stable embryogenic cell lines were obtained, which could be maintained for years without loss of embryogenic potential.

For the large scale propagation of plants, using in vitro techniques, genetic stability is an essential prerequisite, since plants that differ from the mother plant, have little value. In tissue culture, somaclonal variation is widespread (Bayliss 1980, Lee and Phillips 1988), and often found when 'undifferentiated' tissue, designated callus, is propagated on solidified medium. Whether callus is undifferentiated can be argued, but it is clear that growth of cell clumps on a solidified medium differs from growth of PEMs in liquid medium. Callus on agar plates often shows polyploidization (Ashmore and Shapcott 1989, Custers et al. 1990, Ezura and Oosawa 1994, Pijnacker et al. 1989, Schwenkel and Grunewald 1991). In *Haplopappus gracilis* it was demonstrated that callus cultures resulted in more polyploidization than suspension cultures (Ashmore and Shapcott 1989). In a callus clump, only a minor part of the cells is in contact with the medium and directly receives the nutrients required for growth. The majority of the cells will receive nutrients via the intercellular spaces or via cell-cell contact. The supply of nutrients will therefore be limited by diffusion and concentration gradients, and there may be shortages of essential nutrients. This may result in a lower growth rate, or even absence of exponential growth. The stress imposed on the cells in this way, may possibly result in polyploidization or other deviations in genetic constitution.

Our observation that during the initiation of cucumber cell lines, growth of tetraploid cells is favoured above diploid cells, is remarkable. Starting from chimaeric explants, consisting of diploid and tetraploid cells, fully tetraploid cell lines could be obtained. No octaploid PEMs were detected, showing that polyploidization did not occur, and that the cell lines were genetically stable. The ploidy level of cell lines of a mixed ploidy level did not change after removing the explant during proliferation of the PEMs in the maintenance medium. As was shown in Chapter 6, the growth rate of diploid and tetraploid cells was therefore equal. PEMs are either diploid or tetraploid and fully diploid cucumber cell lines could be obtained by selection and further proliferation of individual PEMs (Chapter 6).

In light, more fully tetraploid cell lines were formed than in the dark, and light therefore seemed to favour growth of tetraploid cells. However, cultures initiated in the dark could also show tetraploidization, indicating that light is not decisive. Since polyploidization was not observed in established embryogenic cucumber cell lines growing in the light, it seems that light only exerts its action on cells which are relatively undifferentiated, like cells of callus, or cells in the explant material dedifferentiating in liquid medium from which embryogenic cell lines are established. Indeed, initiation of carrot cell lines directly in liquid medium, in the light, if done according to De Vries et al. (1988), resulted in partial polyploid cell lines (Chapter 6). In accordance with this is the polyploidization observed in cucumber callus cultures during

growth in the light, even when the cultures were initiated in the dark (Custers et al. 1990). Light during callus growth may also have caused the polyploidization reported for carrot (Coutos-Thevenot et al. 1990), melon (Ezura and Oosawa 1994) and cyclamen (Schwenkel and Grunewald 1988, 1991). It must be noted, that in these cases polyploidization in the cell cultures does not necessarily mean the tetraploidization of diploid cells, but can also have arisen from the faster growth of tetraploid cells, compared with diploid cells.

We have found that, once fully diploid cell lines were obtained, genetic and embryogenic stability were ensured, provided the cells were maintained in a medium producing optimal growth. This proves that the use of liquid medium is superior to solidified media for the whole process of somatic embryogenesis, and makes clonal propagation without genetic instability possible.

Factors influencing embryogenic potential.

We further showed that, in addition to culture conditions and subculture regime, other compounds are able to influence the embryogenic potential of cell lines. In carrot cell lines, which had lost their potential to produce embryos, somatic embryogenesis was re-induced, if the cells were grown in the presence of arabinogalactan-proteins (AGPs) isolated from carrot seeds (Chapter 3). The same AGP preparation could increase the embryogenic potential during the proliferation of carrot PEMs. Similar results were recently obtained with *Picea abies*, in which the addition of seed AGPs to cell lines, previously unable to produce mature somatic embryos, resulted in a further maturation of somatic embryos (Egertsdotter and Von Arnold 1995).

Different fractions of carrot seed AGPs, characterized by their binding to the monoclonal antibodies ZUM 15 and 18, had different activities (Chapter 4). The fractionated ZUM 18 AGPs had a hormone-like dose response curve, and increased the embryogenic potential of carrot cell lines at the very low concentration of about 2 nM. On the other hand, ZUM 15 AGPs decreased embryogenic potential. The activity of the ZUM 18 AGPs did not depend on the species from which they were isolated. Tomato ZUM 18 AGPs could also effect the embryogenic potential of carrot cell lines, and carrot ZUM 18 AGPs could influence the embryogenic potential of cyclamen cell lines. In contrast, unfractionated tomato seed AGPs had a low ability to increase embryogenic potential in carrot cell lines. The presence and ratio of different AGP epitopes therefore determined the overall effect of AGPs on the cells, and different epitopes appeared to have different activities. The different epitopes could be located on different, but also on the same molecule.

The composition of the AGP mixture, excreted by carrot cell lines into the medium, changed as the cell lines got older (Chapter 3, Fig. 1), as demonstrated by the change in the crossed-electrophoresis patterns. Cell lines of different ages contain different AGPs and might therefore not respond in the same manner to subsequent, identical experimental conditions. When the AGPs of a cell line change this might result in a different response. It is

our view that any experiment should be performed with cell lines of the same age, rather than with the same cell line over and over again.

The conditions in the experiments, in which the activity of AGPs was demonstrated, were very different from the culture conditions used for embryogenic cell lines, as described in Chapter 5 and 6. In order to detect activity of AGPs, cells were grown at the lowest cell density allowing growth. Under these conditions competition between the added AGPs and the AGPs produced by the cells is avoided.

The biological activity of AGPs remains intriguing. We showed that the development of cells, and even the expression of totipotency, can be altered by the addition of specific AGPs or AGP-epitopes. AGPs, derived from tissues containing embryogenic cells, i.e. the seeds, caused other cells to form somatic embryos. This indicates that AGPs may be involved in cell-cell communication and/or cell identity. How AGPs exert their activity is still unknown, but specific epitopes seem to be involved. The assay system described in Chapter 3 and 4 and the monoclonal antibodies ZUM 15 and 18 might be used for further exploration of the role of AGPs and the different activities of different AGP-epitopes.

Induction of embryogenic potential.

Addition of specific AGPs to carrot cell lines can induce embryogenic potential, but some other AGPs can delay the onset of the induction (Chapter 3). Extracellular proteins excreted by embryogenic carrot cell lines are able to shorten the path towards an embryogenic carrot cell line as was demonstrated by De Vries et al. (1988). Various types of stress, like osmotic, salt and heavy metal treatment, are just as well able to induce embryogenic potential in carrot cell lines (Harada et al. 1990). Among the plant hormones, auxins are considered to be very effective in the induction of embryogenesis (Ammirato 1984, Zimmerman 1993). Also in our experience, the powerful auxin 2,4-D is able to induce cell division in certain parts of the explants, while in other parts, cells only elongate and the tissues disintegrate (Chapter 5 and 6). It seems that the onset of cell division in a specific set of cells, surrounding the vascular tissue, may lead to the formation of somatic embryos or PEMs. What causes these cells to divide and form embryos is not known, but their position in the explant and ability to react to a changed environment are likely important factors. It is our view that AGPs and extracellular proteins may have a function in this process.

During the initiation phase of an embryogenic carrot cell line, meiotic-like cell divisions have been observed, leading to somatic embryos derived from segregants (Giorgetti et al. 1995). During the initiation phase, haploidization of the cell line was observed, resulting in carrot cells containing 1 pg of DNA, being half of the 2 pg for diploid carrot cells reported by Bennet and Smith (1976). In contrast to this is the more recent finding of our and other laboratories, that diploid carrot cells contain 1 pg DNA (C. Kreuger, I Dijkstra, unpublished results and Bennet and Leitch 1995). Giorgetti et al. (1995) used Feulgen staining to quantify the DNA content, while we and Bennet and Leitch (1995) used flowcytometry. The

discrepancy may be caused by the difficulty of staining carrot DNA with fluorescent dyes, although the cause of this is unknown. In flowcytometry more nuclei are measured and other dyes are used to stain the DNA, possibly resulting in more accurate determinations. As a consequence, the observed haploid cells by Giorgetti et al. would be diploid, originating from a tetraploid explant. Still, the segregation, as confirmed using RFLP and RAPD techniques, is not explained. Also in embryogenic callus of melon haploid cells were detected, starting from a diploid explant, but no relationship with the induction of embryogenic potential was made (Ezura and Oosawa 1994).

Plants derived from segregants are not clones, and are therefore not desired. The cyclamen plants derived from somatic embryos, which were produced in our lab, do not show segregation at all. All plants are copies of the initial genotype, and any segregant would be easy to recognise. Meiotic cell divisions, if occurred at all, did therefore not contribute to the formation of the PEMs and embryos in cyclamen.

Comparison of somatic embryogenesis with zygotic embryogenesis reveals many morphological similarities. However, the induction appears to be totally different. In the egg cell, fertilization starts the process of embryogenesis, no stress is involved at first glance. On the other hand, the change in the plant life cycle from sporophyte to gametophyte is accompanied by an extensive separation of the two generations (Bell 1992). In some cases plasmodesmata are no longer present for connection and cell to cell contact. In fact, the two generations might be regarded as opposing tissues. A similar isolation was observed during the formation of somatic embryos (Bell 1992). This isolation, either in response to a change of generations, or to an imposed stress, may cause cells to follow a specific developmental path, resulting in embryogenic potential and finally the formation of an embryo. In our view the formation of somatic embryos from the explant, is an answer to the stress, to escape the imposed conditions. Somatic embryogenesis may ensure the continuity of the individual, and may be a way to circumvent conditions lethal to the explant.

The occurrence of meiotic cell divisions in explants or in establishing cell lines, may similarly be a response to the environmental conditions. However, embryos or plants derived from segregants have not been found in our or any other laboratory. This may be due to several reasons. Each laboratory has its own protocol for maintaining cell lines and a wide variation of media and culture regimes are applied, especially during the initiation phase. Different initiation protocols may also involve different ways of inducing embryogenic potential. The amount of stress used, or the types of stress may vary, possibly resulting in different responses of the explants. The formation of embryos by somatic cells might be achieved with or without meiotic cell divisions, depending on the inductive events during the initiation phase.

Towards a large scale production of somatic embryos.

The formation of somatic embryos by plants is, from a scientific point of view, a very attractive way to study the development of embryos, without the constraints of surrounding tissues. The other important aspect of somatic embryogenesis is its application in plant breeding. The cloning of superior plants may enhance breeding strategies and increase plant production. The research described in this thesis may contribute to both aspects of somatic embryogenesis. In the first part of the thesis it is shown that AGPs have a role in the embryogenic potential of cells. In the second part is described how correct initiation, directly in liquid medium, and controlled proliferation of PEMs lead to genetic and embryogenic stable cell lines. These conditions are essential for the large scale production of plants via somatic embryogenesis.

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Samenvatting.

Planten bezitten een groot regeneratievermogen en daar wordt in de weefselkweek veelvuldig gebruik van gemaakt. Somatische embryogenese is een vorm van regeneratie, waarbij plantecellen uitgroeien tot structuren die erg lijken op een zygotisch embryo, zoals zich dat ontwikkelt na bevruchting. Embryos zijn somatisch als zij niet het gevolg zijn van bevruchting, maar zijn ontstaan uit 'gewone' cellen. Zygotische en somatische embryo's doorlopen overeenkomstige stadia en vormen achtereenvolgens het globulaire, hart en torpedo vormige embryo.

De grootschalige opkweek van planten via somatische embryogenese kan veel voordelen opleveren boven uit zaad verkregen planten. De planten uit somatische embryos zijn in principe genetisch identiek, iets wat bij planten uit zaad niet altijd mogelijk is. Wanneer er bij somatische embryogenese uitgegaan wordt van een plant met een superieur genotype, kan op deze manier dit genotype vermeerderd worden, wat de methode extra betekenis geeft. De interesse voor industriele toepassing van somatische embryogenese lijkt dus voor de hand te liggen.

Er is reeds veel gepubliceerd over de vorming van somatische embryo's, maar grootschalige produktie van planten via somatische embryogenese is nog maar voor een paar gewassen beschreven. Voor grootschalige produktie zijn een aantal dingen noodzakelijk: stabiliteit van het embryogene vermogen, genetische stabiliteit, efficiente embryovorming en efficiente kieming van de embryo's. Voorts is het wenselijk dat van veel gewassen en veel varieteiten embryo's kunnen worden gemaakt. Factoren die hierop een invloed kunnen hebben zijn in dit proefschrift onderzocht.

In hoofdstuk 2 worden de eigenschappen beschreven van arabinogalactaan-eiwitten (arabinogalactan-proteins; AGPs). AGPs worden door de meeste plantecellen uitgescheiden en vormen een zeer heterogene 'familie' van moleculen. Er zijn verschillende aanwijzingen dat AGPs een rol spelen bij de interactie tussen cellen en het lijkt zeer wel mogelijk dat zij ook een rol spelen tijdens de somatische embryogenese.

In hoofdstuk 3 wordt beschreven hoe wij de invloed van specifieke AGPs op cellijnen van wortel (*Daucus carota* L.) hebben onderzocht. Het is bekend dat wortel cellijnen het embryogene vermogen verliezen nadat ze ongeveer een jaar in cultuur zijn. Het embryogene vermogen kon weer geinduceerd worden in voorheen niet-embryogene cellijnen, door de cellen te laten groeien in aanwezigheid van wortel zaad AGPs. Ook kon het embryogene vermogen van reeds embryogene cellijnen vergroot worden door toevoeging van dezelfde zaad AGPs aan het cultuur medium.

AGPs geisoleerd uit het medium van niet embryogene lijnen konden ook het embryogene vermogen vergroten, maar het effect was minder groot. Dit laat zien dat verschillende AGPs

verschillende effecten kunnen hebben. Wortel cellijnen scheiden een specifiek mengsel van AGPs uit; de samenstelling van dit mengsel veranderd als de cellijnen ouder worden.

Hoofdstuk 4 beschrijft de fractionering van wortel zaad AGPs om hieruit de actieve componenten te isoleren. Hiervoor werden monoclonale antilichamen gemaakt, gericht tegen wortel zaad AGPs. Twee antilichamen bonden elk een kleine fractie van de AGPs, die elk een ander effect hadden op wortel cellijnen. De ZUM15 AGPs, die gebonden werden door het ZUM15 antilichaam, waren in staat het embryogene vermogen van de wortel cellijnen te verkleinen, terwijl de ZUM 18 AGPs, die gebonden werden door het ZUM18 antilichaam, het embryogene vermogen sterk vergrootten. De dosis-effect curve van de ZUM18 AGPs vertoonde een scherp optimum, met een optimum concentratie van 0.2 mg/l. Omdat de twee antilichamen cross-reactiviteit vertoonden d.w.z. een deel van de moleculen die gebonden werden met het ene antilichaam, bond ook met het andere antilichaam, was een verdere zuivering van AGPs mogelijk. De AGP fractie, waarvan alle moleculen bonden aan beide antilichamen, vertoonde geen, of nauwelijks activiteit in de concentraties die getest werden. Een andere fractie, waarvan alle moleculen bonden aan ZUM 18 maar niet aan ZUM 15, liet een dosis-effect curve zien die erg leek op een verzadigingskromme, waarbij maximaal effect werd bereikt bij 0.2 mg/l. Deze experimenten tonen aan dat verschillende AGP fracties verschillende effecten kunnen hebben. Doordat AGPs zo heterogeen zijn zal het uiteindelijke effect bepaald worden door de balans van de verschillende activiteiten. De verschillende activiteiten kunnen gelocaliseerd zijn op een of verschillende moleculen.

De productie van somatische embryo's kan verdeeld worden in vijf fases, initiatie, proliferatie, embryo vorming, kieming en plant vorming. Een van de voorwaarden voor de grootschalige productie is, dat zo veel mogelijk stappen van het proces in vloeibaar medium gebeuren. Het modelgewas voor somatische embryogenese is al geruime tijd wortel (*Daucus carota* L.), waarvan de initiatie tot en met de embryo vorming in vloeibaar medium effectief kan plaats vinden. Er is onderzocht of dit ook voor andere gewassen mogelijk is.

In hoofdstuk 5 wordt de productie van somatische embryo's van cyclamen beschreven. Ook hier vindt vanaf de initatie tot en met de embryo vorming, alles plaats in vloeibaar medium. De initiatie fase blijkt van groot belang te zijn. Explantaat dichtheid, hormoon concentratie en subcultuur regime moeten zo gebalanceerd zijn dat binnen een korte tijd embryogene klompjes (pro-embryogenic masses: PEMs) worden gevormd. Deze PEMs worden vervolgens vermenigvuldigd in de proliferatie fase. Door PEMs van een bepaalde grootte te isoleren, en te enten in hormoon vrij medium, kunnen zij tot embryovorming worden aangezet. Factoren, zoals de samenstelling van het medium, de opname van hormonen tijdens de proliferatie fase en de cel dichtheid tijdens de embryo vorming, zijn van belang voor een efficiente embryovorming. De beschreven methode heeft geresulteerd in langdurig embryogene en genetisch stabiele cellijnen in cyclamen, waaruit embryos en planten zijn verkregen met hetzelfde ploidie nivo als het oorspronkelijke uitgangsmateriaal.

In hoofdstuk 6 is onderzocht of de methode zoals die gebruikt is voor cyclamen, ook gebruikt kan worden voor komkommer. Dit bleek inderdaad in grote lijnen het geval te zijn. Dezelfde parameters bleken van belang tijdens de initiatie van komkommer cellijnen in vloeibaar medium, als bij cyclamen celllijnen. Ook van komkommer kunnen grote hoeveelheden embryo's gemaakt worden in vloeibaar medium, echter een deel daarvan kan tetraploid zijn ten gevolge van in het explantaat aanwezige tetraploide cellen en de aanwezigheid van licht tijdens de initiatie. Door uit te gaan van een compleet diploid explantaat, dat dus geen tetraploide cellen bevat, en de initiatie in het donker uit te voeren, kan een volledig diploide cellijn verkregen worden. Ook kunnen individuele PEMs vermeerderd worden, waaruit vervolgens volledig diploide of volledig tetraploide cellijnen verkregen kunnen worden. Cellijnen met een zeker percentage tetraploide cellen, blijven dit behouden tijdens de proliferatie fase, wat er op wijst dat de cellijnen genetisch stabiel zijn. Door de genetische stabiliteit en het embryogeen vermogen te controleren, is de grootschalige productie van komkommer somatische embryo's mogelijk geworden.

De grote overeenkomsten in de procedures van wortel, cyclamen en komkommer om embryogene cellijnen te verkrijgen, duiden erop dat veel aspecten van het proces voor verschillende gewassen gelden. De vijf fases van het proces stellen elk hun eigen eisen aan media en subcultuur regime.

Het feit dat AGPs een biologische werking vertonen een bevestiging van eerdere hypotheses dat AGPs een rol spelen in cel-cel interacties, en mogelijk een aanzet voor verder onderzoek naar de functie van AGPs.

De in dit proefschrift beschreven resultaten van onderzoek naar de productie van somatische embryo's, voor gewassen waarvan dit op deze schaal nog niet eerder bekend was, laat zien dat door de productie van embryogene cellijnen direct in vloeibaar medium te beginnen, deze methode superieur is boven andere methodes die vaak gebruik maken van callus en vast medium. De toepassing van somatische embryogenese voor de vermeerdering van planten lijkt daarmee dan ook tot de reele mogelijkheden te horen.

Curriculum vitae.

Marc Kreuger werd geboren op 4 september 1963 te Berkel en Rodenrijs, onder de rook van Rotterdam. De middelbare school doorliep hij in Zoetermeer, op het Erasmus College, alwaar het VWO diploma in 1981 behaald werd. Daarna volgde een jaar Scheikundige Technologie aan de TH Delft, maar dit was het toch niet helemaal. Daarom werd in 1982 begonnen met de studie Biologie aan de Landbouwhogeschool in Wageningen. Al vrij snel werd besloten de orientatie 'cel' te nemen met als hoofdvakken moleculaire biologie en plantencytologie en -morfologie. Als rode draad door beide vakken liep de somatische embryogenese, iets waar dus al vroeg kennis mee gemaakt werd.

In januari 1988 werd het diploma behaald, waarna, tijdens het zoeken naar een leuke baan, enige tijd op de vakgroep Virologie van de LUW werd gewerkt. De hier aanwezige kruiwagen bracht hem in kontakt met Zaadunie BV, en dit leidde tot de aanstelling als onderzoeker bij de sectie ontwikkelingsbiologie.

Sinds 1 mei 1989 werkt hij hier aan somatische embryogenese voor de vermeerdering van planten. Een deel van dat onderzoek heeft tot twee patent aanvragen geleid, welke op hun beurt weer tot een aantal publicaties leidden die in dit proefschrift staan.

Nawoord.

Zoals alle proefschriften is ook deze alleen gemaakt voor de voldoening van de promovendus zelf. Het was natuurlijk wel moeilijk geworden als niet een aantal mensen eraan mee geholpen hadden. Enige bedankjes zijn dus wel op hun plaats. In tegenstelling tot andere proefschriften worden de belangrijksten nu eerst genoemd, omdat zij altijd op de eerste plaats horen te staan. Coby en Tomas hebben er voor gezorgd dat ook de laatste loodjes niet al te zwaar waren, vooral als ik geen zin had om te schrijven (zoals nu bij het schrijven van dit nawoord). Dit boekje is dus ook een beetje voor hen, al zal de laatste er nog weinig van snappen.

Als goeie tweede komt Gerrit-Jan die mij de wereld van de AGPs heeft laten zien. Mede door zijn enthousiasme zijn we daar nog steeds niet uitgekeken. Samen met de sectie ontwikkelingsbiologie, tegenwoordig Somatic Plants (met hoofdLetters): Rob Abbestee, Yvon Brouwer, Ingrid Dijkstra, Wiert van der Meer, Erik Postma, Natasja Raaijmakers en Manja Rodenburg, en ook de sectie biochemie (Hans van Doorn en Gert van der Kruk) hebben we een aardige basis gelegd voor de toekomst, al moet er nog wel een en ander gebeuren.

Verder zijn er natuurlijk een hoop mensen bij S&G Seeds (voorheen Zaadunie) die dit project geinitieerd en gesteund hebben, zoals Ad Kool (toen nog manager Biotechnologie), Ed Veltkamp (toen nog directeur Research) en de gehele afdeling (Bio)Technologie. Zij allen hebben een onmisbare bijdrage geleverd.

Ook wil ik Sacco de Vries bedanken die mij in 1986 op het embryogenese pad heeft gezet. Ik heb heel veel van hem geleerd (en afgeleerd) en hoop dat nog lang te doen. Zijn kijk op het geheel heeft een grote invloed op dit proefschrift gehad.

Mijn promotor, Ab van Kammen wil ik erg bedanken voor al z'n tijd en geduld. Hij heeft mij geleerd (dat hoop ik dan maar) hoe je iets duidelijk kunt opschrijven, terwijl het toch nog een wetenschappelijke tekst blijft. Bovendien weet ik nu dat het altijd bij je hebben van startkabels erg nuttig kan zijn, is het niet meteen, dan wel in de toekomst.

Als een na laatste, maar daarom niet de minst belangrijke, familie en vrienden, die, al begrepen ze niet wat ik nou eigenlijk deed al die tijd, altijd achter mij stonden en mij steunden in de strijd.

Als laatste Guust en JoostvanSmitaandeoverkant, die geen enkel benul hebben van wat dan ook, maar die al gauw tevreden zijn met iets op een schaaltje, de een binnen, de ander buiten.