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# Arabinogalactan proteins are essential in somatic embryogenesis of *Daucus carota* L.

**Marc Kreuger* and Gerrit-Jan van Holst**

Zaadunie BV, Biotechnology Department, P.O. Box 26, 1600 AA Enkhuizen, The Netherlands

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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, as can be determined by crossed electrophoresis with the specific AGP-binding agent, 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-month-old embryogenic cell lines usually contain less than 30% of dense, highly cytoplasmic cells, i.e. the embryogenic cells, but when carrot-seed AGPs were added this percentage increased to 80%. Addition of carrot-seed AGPs to a two-year-old, non-embryogenic cell line resulted in the re-induction of embryogenic potential. These results show that specific AGPs are essential in somatic embryogenesis and are able to direct development of cells.

**Key words:** Arabinogalactan protein – *Daucus* – Somatic embryogenesis

## 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 the intercellular spaces of tissues. In cell cultures, AGPs are excreted into the medium (for a review see, Fincher et al. 1983).

Arabinogalactan proteins are proteoglycans with poly- and oligosaccharide units covalently attached to their protein moiety (Van Holst and Klis 1981). These units mainly consist of a 1,3-β-D-galactopyranosyl backbone and side chains of (1,3-β- or 1,6-β-) 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 usually constitutes less than 10% (w/w) of the molecule. The total molecular weight is around or above 100000. Arabinogalactan proteins 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 can be determined 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, 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 which changes during the development of tissues and organs, even before this development or differentiation is visible. This indicates a role for 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 references therein). Extracellular proteins proved to be essential for somatic

* To whom correspondence should be addressed; FAX: 31 (22 80) 663 48

*Abbreviations:* AGP = arabinogalactan protein; 2,4-D = 2,4-dichlorophenoxyacetic acid

embryogenesis of carrot (De Vries et al. 1988a), showing the importance of the extracellular matrix in this system. We have 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 (*Daucus carota* L., cv. Trophy-Flakkese or Nantes from Zaadunie BV., Enkhuizen, The Netherlands) seeds were surface-sterilised and germinated on B–5 medium (Gamborg et al. 1968) supplemented with 0.6% agar, at 23° C in the dark. Cell cultures were derived from sliced 10-d-old hypocotyls. Approximately 2 g of hypocotyl tissue was added to 50 ml of Gamborg's B–5 medium supplemented with 2 μM 2,4-dichlorophenoxyacetic acid (2,4–D) in a 250-ml flask. The flask was incubated at 23° C in a daily regime of 16 h light 8 h darkness, 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 slices were removed (De Vries et al. 1988b). From this point the culture was subcultured every two weeks. About eight weeks after initiation the culture became embryogenic. The formation of somatic embryos was induced by selecting cells and aggregates that passed through 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 at a cell density of 20000 cells. $\cdot$ ml$^{-1}$ (De Vries et al. 1988a). Cell cultures were also initiated from somatic embryos. Two grams of sliced two-week-old somatic embryos were incubated in 50 ml of 2,4–D-containing B–5 medium. Further procedures were as described above.

*Isolation of AGPs from seeds and culture medium.* Seeds of *Daucus carota* L. (cv. Trophy-Flakkese) were ground in a coffee mill and extracted with water. Cell debris was removed by filtration through a 3-μm filter and then centrifuged at 10000 $\cdot$ *g* for 15 min. The AGPs were precipitated by adding the β-glucosyl Yariv reagent (1,3,5-tris-[4–β-D-glucopyranosyl-oxyphenylazo]-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 β-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 10 000 $\cdot$ *g* for 15 min. 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 β-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 β-glucosyl Yariv reagent directly to the cell-free medium. The precipitation and purification procedures were identical to those 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 β-glucosyl Yariv reagent concentration in the second electrophoresis dimension was 20 μg $\cdot$ 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 19 d).

*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-diameter Petri dishes. The AGPs were filter-sterilized (0.22 μm, Flowpore; ICN Biomedicals, Irvine, UK) and added to the culture. Experiments in which the percentage of embryogenic cells was determined were done in 60-mm-diameter Petri dishes (in triplicate). The percentage of embryogenic cells was determined by counting single cells and small aggregates (aggregates of less than 15 cells), and by 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 carrot 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 slices of hypocotyl 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 ageing of the culture, the AGP pattern changed to a single peak (Fig. 1D). At this point the culture was becoming

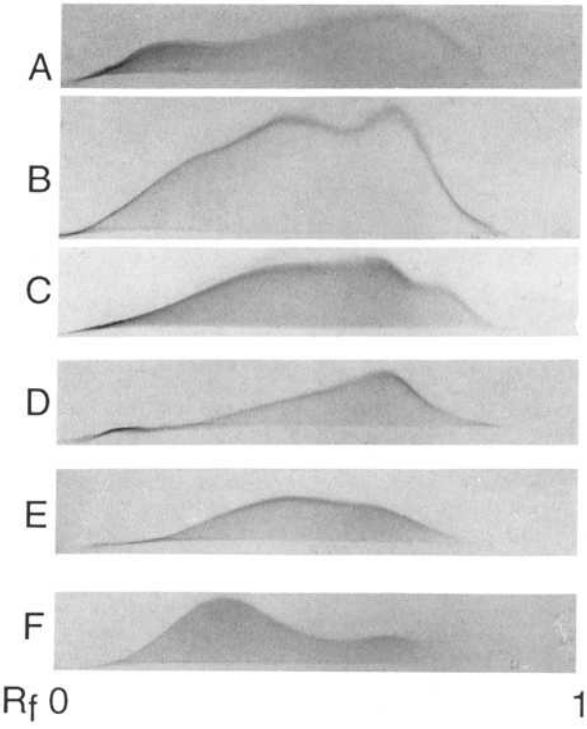


**Fig. 1A–F.** Crossed-electrophoresis patterns of AGPs from carrot cell-culture medium, hypocotyl tissue or seeds. The AGPs were isolated at different ages of a cell line and the amount of AGP supplied to the gel was between 3 and 10 μg, as indicated below. Also indicated is the concentration of AGPs in the analysed media and tissues. **A** Seeds, 6 μg, 0.28 mg AGP per g seeds. **B** Hypocotyl (explant), 10 μg, 0.16 mg AGP per g FW. **C** Nineteen-day-old cell line, 10 μg, 31 mg AGP per litre medium. **D** Seven-week-old cell line, 3 μg, 13 mg AGP per litre medium. **E** Three-month-old cell line, 6 μg, 24 mg AGP per litre medium. **F** Two-year-old cell line, 7 μg, 180 mg AGP per litre medium

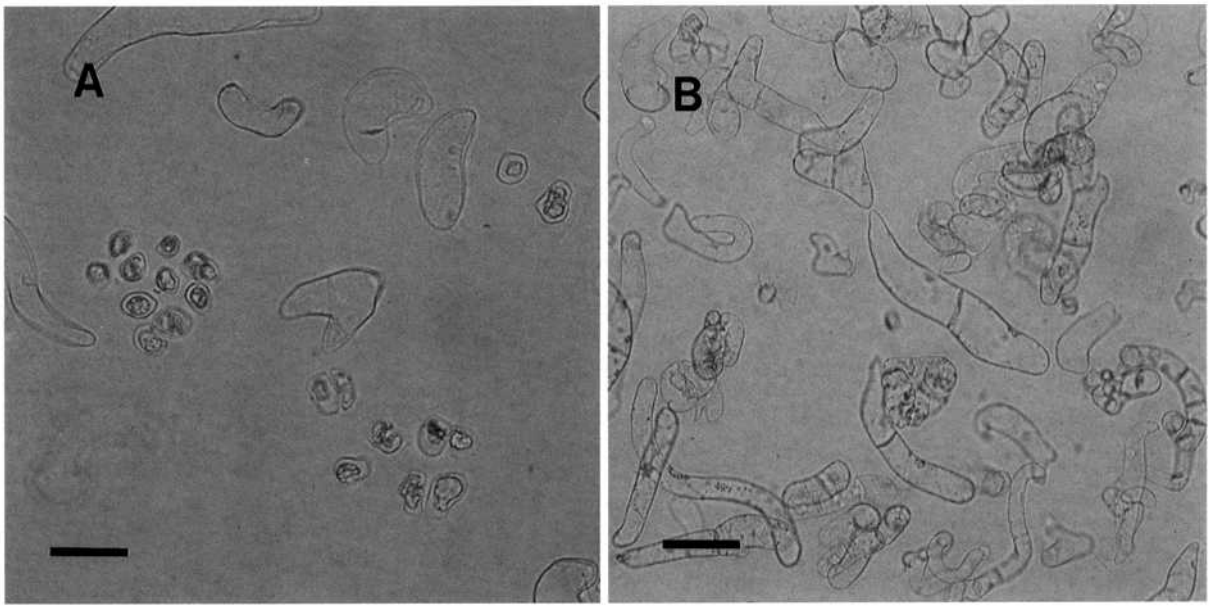


**Fig. 2A–B.** Young cell cultures established from one carrot hypocotyl in 10 ml of medium. × 200; bars = 50 μm. **A** Control culture 19 d after initiation. Small, single cells are present. **B** Culture with added AGPs from a non-embryogenic cell line. Virtually no small, single cells are present

embryogenic. Arabinogalactan proteins 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 an $R_f$ value of 0.6. This peak contained the majority of AGPs in both isolates.

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 an $R_f$ value of 0.4, the other maximum had an $R_f$ value of 0.6. At this point the culture was highly embryogenic.

A cell line which had been in culture for two years and had lost its embryogenic potential showed a strikingly different AGP pattern (see Fig. 1F). The largest peak had a maximum at an $R_f$ 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. The AGP patterns of both types of cell showed a wide peak with an $R_f$ value of 0.5 (data not shown).

*Addition of AGPs to explant material in culture.* Cell cultures were initiated by adding one sliced hypocotyl (2–3 cm total length) to 10 ml of 2,4-D-containing medium. This low explant density was used to avoid competition between endogenous AGPs from the explant and added AGPs isolated from the media of cell cultures or isolated from seeds. In the control cultures without added AGPs, small, single cells were present when the hypocotyl parts were removed after 19 d (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 $10\ mg \cdot l^{-1}$, did not have an effect. However, addition of AGPs isolated from a 2-year-old, non-embryogenic cell line, to a final concentration of $10\ mg \cdot l^{-1}$, 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 two-year-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 g of somatic embryos in 50 ml Gamborg's B–5 medium, instead of the 2 g 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 were embryogenic at this age. Subsequently, the cell line was subcultured in 5-ml cultures (Petri dishes) in the presence of AGPs ($10\ mg \cdot l^{-1}$) and 2,4-D at a cell density of $20000\ cells \cdot ml^{-1}$. 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 medium of the non-embryogenic cell line, two weeks after subculturing. During subculture the percent-

**Table 1.** Percentage of embryogenic cells during subculture of a young cell line of carrot. Cells were subcultured in the absence of AGPs, or in the presence of AGPs isolated from carrot seeds or from the culture medium of a non-embryogenic cell line. The values given are the mean of three cultures (SD = 3%)

| AGPs added | Days in culture | | |
|---|---|---|---|
| | 1 | 8 | 15 |
| None | <1 | 8 | 10 |
| Non-embryogenic line | <1 | 16 | 17 |
| Seed | <1 | 21 | 29 |

age of embryogenic cells was determined by counting single cells and small aggregates (aggregates of less than 15 cells), and by 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 number of pro-embryogenic masses or the percentage of embryogenic cells in a culture is proportional to the embryogenic potential (De Vries et al. 1988b).

During subculture in the absence of added AGPs the percentage of embryogenic cells increased with time (Table 1). However, when AGPs isolated from seeds or cell culture medium were added the increase was more pronounced compared with the control treatment, 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 $\cdot$ l$^{-1}$ AGPs showed less pronounced effects (data not shown).

After prolonged subculturing (four to six weeks) in the presence of 10 mg $\cdot$ 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 increase substantially the percentage of embryogenic cells in a young cell line.

*The addition of AGPs to a two-year-old, non-embryogenic cell line.* The two-year-old, non-embryogenic cell line was subcultured in the presence of AGPs isolated from carrot seeds (10 mg $\cdot$ l$^{-1}$) and 2,4–D at a low cell density. This cell line normally did not contain pro-embryogenic masses. After eight weeks (four subcultures), cell aggregates appeared that contained 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 re-induced embryogenic potential.

*The addition of AGPs to an embryogenic cell line on hormone-free medium.* Arabinogalactan proteins isolated from carrot seeds or from the culture medium of non-embryogenic cell lines were added, at a concentration of 1 mg $\cdot$ l$^{-1}$ to a cell line induced to produce somatic embryos on hormone-free medium. After three weeks of culture the number of embryos formed was counted. The added AGPs from both sources produced an increase in

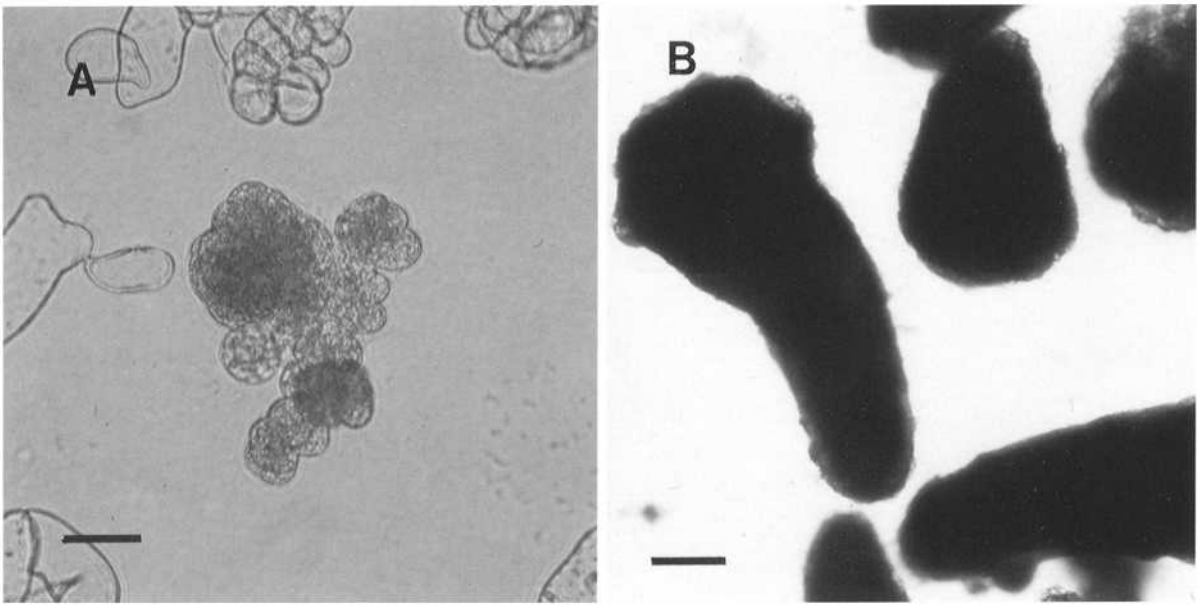


**Fig. 3. A** Two-year-old cell line of carrot cultured in the presence of carrot-seed AGPs. Cell aggregates containing small cytoplasmic cells are present. These cytoplasmic cells were not present in the absence of carrot-seed AGPs (not shown). × 200; bar = 50 μm. **B** Somatic embryos generated from the cell aggregates from **A**. × 40; bar = 250 μm

the number of embryos (40% and 34% increase, respectively, with seed AGPs, and AGPs from the non-embryogenic cultures). Addition of 10 mg · $l^{-1}$ AGPs resulted in a less pronounced effect (28% and 12% increase, respectively).

## Discussion

The results of the crossed-electrophoresis analyses 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 AGPs in the medium resembles the pattern of hypocotyl AGPs (Fig. 1B, C). This is to be expected since the AGPs in the sliced hypocotyl explants are eluted into the medium. At the same time, new types of AGPs are already present ($R_f$ value 0.7). As the culture starts growing it becomes embryogenic. This process is accompanied by the secretion of different types of AGPs, resulting in a 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 AGP excreted into the medium increases (from 31 to 180 mg · $l^{-1}$, Fig. 1), probably as a consequence of higher growth rates and a changed morphology.

The AGPs isolated from seeds are derived, at least in part, from 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 seven-week-old embryogenic cell line have similarities. In both AGP patterns the major peak, containing the majority of the AGPs, has an $R_f$ value of 0.6 (Fig. 1A, D). These types of AGPs might correlate with the presence of embryogenic cells or an embryogenic environment.

The ability of the seed AGPs to re-induce 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 an $R_f$ value of 0.3; only a small proportion has a different $R_f$ value. This indicates that the relative proportions of the different types of AGPs are important for the effect of AGPs on cells. For the excreted AGPs these proportions 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 re-induced embryogenic potential. Somatic embryos were generated from the newly formed pro-embryogenic masses.

When seed AGPs were added to a young cell line which was at that time developing into 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). The AGPs from the culture medium of the non-embryogenic cell line were still effective, but less so. This is in contrast to the experiment in which AGPs from the culture medium of the non-embryogenic cell line were added to a culture which contained explant material and which 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) implies that cell-cell interactions occur during development and differentiation. 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 other published observations (Knox et al. 1989, 1991; Stacey et al. 1990) as well as our own. 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 al. (1989). These results indicate 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 the development of cell cultures can be influenced by the addition of very low concentrations (10–100 nM) of specific AGPs.

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