Immobilization of Flax Protoplasts in Agarose and Alginate Beads

Correlation between Ionically Bound Cell-Wall Proteins and Morphogenetic Response

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Linum usitatissimum protoplast-derived colonies that are cultured in auxin-supplemented medium and immobilized in Ca\(^{2+}\)-alginate matrix form round colonies that develop into polarized, embryo-like structures. On the other hand, protoplast-derived colonies that are immobilized in agarose do not show an organized morphogenetic response, and unique, ionically bound cell-wall protein patterns match this response. Although only slight differences in neosynthesized or total constitutive polypeptides are observed, dramatic changes in ionically bound cell-wall proteins are seen. In protoplasts grown on Ca\(^{2+}\)-alginate-solidified, auxin-containing medium, several basic polypeptides were strongly induced and were found tightly bound to the cell wall. In contrast, these basic proteins were found only weakly bound to the walls of protoplasts grown on agarose-solidified, auxin-containing medium or on Ca\(^{2+}\)-alginate-solidified, auxin-free medium, in which they were released into the medium. Our results suggest that plant cells can perceive and respond to the adjacent extracellular matrix, since we show that the growth of flax cells on Ca\(^{2+}\)-alginate in the presence of auxin-containing medium may promote the binding of specific proteins to the walls. This establishes a direct correlation of an embryo-like morphogenesis with ionically bound cell-wall basic proteins in flax protoplasts grown on Ca\(^{2+}\)-alginate-solidified, auxin-containing medium.

Virtually any cell can be immobilized (Nussinovitch et al., 1994). However, the gentlest methods that allow the best cell viability must be used, and this is achieved by using agar-type polysaccharides, including agarose and alginate, a family of co-polymers, that are most frequently used for plant cell entrapment. Flax protoplasts immobilized in alginate behave differently than those immobilized in agarose. We investigated the effects of agarose and alginate matrices on the morphogenetic response of phytohormone-controlled flax protoplast-derived cells that were obtained at optimized concentrations of growth regulators (5.5 µM 2,4-D, 2.45 µM NAA, 3.45 µM BA, and 1.25 µM zeatin). We observed that cell colonies in agarose lacked defined outlines, since the clumps of actively dividing cells were surrounded by cells in an advanced state of lysis. In contrast, in alginate it was possible to observe the presence of compact and spherical colonies with actively dividing cells, as well as a periplasmic space with a very sinuous cell wall (David et al., 1994).

Because of the presence of negative charges, Ca\(^{2+}\)-alginate could affect the secretion of wall polysaccharides, thus modifying the composition and architecture of the newly formed walls, which has been reported for the increase of alkaloid synthesis by Coffea arabica cells (Haldimann and Brodelius, 1987). Indeed, it was demonstrated that alginate-induced colony differentiation is concomitant with a specific spatial and temporal distribution of acidic, methylesterified, and probably acetylated pectins (David et al., 1995a) by using the monoclonal antibody 2F4 (Liners et al., 1989), specific to a calcium-induced supramolecular conformation of homopolygalacturonic acid. We observed that the pectin content of the walls of protoplast-derived cell colonies entrapped in alginate is higher than in agarose and that its degree of esterification decreased between d 3 and d 8 of culture. Other authors have suggested that the physical and chemical characteristics of the matrices are responsible for a particular physiology and performance of immobilized cells, with the space available for cell growth being important for metabolic activity (Hahn-Hägerdal, 1989).

These results raise several questions. What is the impact of the polyanionic nature of the matrix of immobilization on the ion exchange between cells and the environment? What is the possible role of alginate as a reservoir of calcium for the influxes that are implicated in cell polarization and differentiation (Kropf, 1992)? What is the influence of the Ca-induced supramolecular conformation of alginate on the synthesis, accumulation, and secretion of proteins? To address this last question, both types of biopolymers, agarose or alginate, were used.

Alginate, an algal gel-forming cell-wall polysaccharide, is composed of alternating 1,3-linked β-D-galactopyranose

Abbreviations: CBB, Coomassie brilliant blue R-250; 2D, two-dimensional; Glc-6-PDH, Glc-6-P dehydrogenase; HV, high viscosity; NEpHGE, nonequilibrium pH gradient electrophoresis.

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and 1,4-linked 3,6-anhydro-α-L-galactopyranose. The sulfate content is usually low (0.04/0.07%) and this biopolymer is considered neutral (Pochet et al., 1991, 1993). Its simple gelation occurs by raising and lowering the temperature. However, this process does not exclude possible heat damage to the entrapped cells (Nussinovitch et al., 1994). Among the polyuronides, natural ion exchangers, the widely used alginate is a 1,4-linked linear polymer of β-D-mannurionate and α-L-guluronate in different proportions and sequential arrangements. The strength of the gels that are formed with divalent cations, usually Ca\(^{2+}\), increases with the increasing content of polyguluronate, whereas polymannuronate sequences remain soluble in the presence of Ca\(^{2+}\) (Morris et al., 1978). Within the gel, the polyguluronate sequences locked in a 2-fold conformation are known as the egg-box model (Grant et al., 1973). Thus, gelation of alginate is inotropic, with di- and multivalent cations, and has limitations, such as breakdown, in the presence of chelating agents (Nussinovitch et al., 1994). In this study Ca\(^{2+}\)-alginate and agarose were used for the entrapment of flax protoplasts and subsequent analysis of the neosynthesized, accumulated, and secreted proteins of the protoplast-derived cell colonies.

Electrostatic trapping of proteins inside a polyanionic matrix is a known phenomenon, with the diffusion rate decreasing with the increasing size of the proteins while the diffusion rate increasing as a function of guluronic acid content in alginate, leading to larger pore sizes (Martinsen et al., 1987; quency of flax protoplast-derived cells (David et al., 1994). The unique colony formation observed in Ca\(^{2+}\)-alginate-solidified, auxin-concentration (35 rpm) overnight in dim light at 24 ± 1°C in an enzyme solution containing 1.2% (w/v) cellulase Onozuka RS (Yakult Honsha, Tokyo, Japan) and 0.2% (w/v) pectinase PY-23 (Seishin Pharmaceutical, Tokyo, Japan) in a salt solution (David et al., 1982) containing 1 mM CaCl\(_2\), 0.2 mM KH\(_2\)PO\(_4\), 1 mM KNO\(_3\), and 1 mM MgSO\(_4\) supplemented with 0.5 M Glc and 1% (w/v) BSA (Sigma). The osmolality was 650 mOsm (kg H\(_2\)O\(^{-1}\)). The protoplast preparation was filtered through a 60-μm-pore nylon sieve (Sefar, Rüschlikon, Switzerland) and centrifuged for 10 min at 900 rpm. The pelleted protoplasts were resuspended in 5 mL of 21% Suc in the salt solution mentioned above (enzyme-solubilization solution), and 5 mL of the salt solution was poured on top. After the sample was centrifuged (155g for 10 min) the liquid layer at the interface containing most of the protoplasts was siphoned off. Following two more cycles of centrifugation and washing with salt solution, pelleted protoplasts were ready for subsequent immobilization.

**Protoplast Isolation and Purification**

Two gelling agents were used: 0.75% (w/v) agarose (type VII low-gelling temperature, Sigma A-4018) and 0.4% (w/v) sodium alginate (HV, Sigma A-2033). These different concentrations allowed the corresponding beads to have similar strengths. For immobilization in agarose after the final centrifugation, protoplasts were carefully mixed at 40°C with the agarose-containing culture medium and occasionally with the agarose-containing salt solution, which was used for both enzyme dissolution and protoplast purification but was supplemented with 10 mM CaCl\(_2\), the concentration that was used for alginate gelation (see below). The protoplast suspension was dispensed as 10-μL beads into 60- × 15-mm Petri dishes. After cooling, 20 beads were submerged into 4 mL of AE- culture medium, which was derived from medium G (David et al., 1984) with the following modifications: 4.1 mM CaCl\(_2\), 2.5 mM NH\(_4\)NO\(_3\), 6.3 mM KNO\(_3\), 20 mM Glu, 40 μM spermidine, 1.25 μM zeatin, 3.5 μM N\(^6\)-BA, 2.5 μM NAA, 5.5 μM 2,4-D, and 0.45 μM Glc. The measured osmolality was 550 mOsm (kg H\(_2\)O\(^{-1}\)), and the pH was adjusted to 5.7. For embedding in HV alginate the biopolymer was dissolved in the same salt solution as above except that the CaCl\(_2\) concentration was 1 mM. Keeping 10 mm CaCl\(_2\) within the test tube resulted in the immediate gelation of alginate; therefore, occasionally, the mixture was maintained at 40°C. After gentle homogenization of the protoplasts, the suspension (final density of 3 × 10\(^5\) protoplasts mL\(^{-1}\)) was extruded dropwise (10 μL) in the salt solution that was used for embedding but containing 10 mM CaCl\(_2\). After 30 min

**Protoplast Immobilization and Culture**

Seeds of *Linum usitatissimum* cv Ariane were surface-sterilized by a 1-min immersion in 70% ethanol followed by a 20-min immersion in 2% sodium hypochlorite containing a few drops of polyoxyethylene-sorbitan mono-oleate (Tween 80, Sigma P-4780) and three to four washes in sterile tap water. The seeds were germinated on a Phytagel (gellan gum; agar substitute gelling agent; Sigma P-8169) solidified (0.25%) Murashige-Skoog medium (Murashige and Skoog, 1962) lacking growth regulators and vitamins, under continuous dim light (2.8 μmol m\(^{-2}\) s\(^{-1}\), PAR) at 22 ± 1°C.
of polymer activity, Ca\(^{2+}\)-alginate gel beads were washed and submerged in 3 mL of auxin-containing and auxin-free AE\(^+\) culture media. Immobilized protoplasts were incubated under the following conditions: 16 h of dim light (4 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) PAR) at 28 ± 1°C and 8 h of dark at 25 ± 1°C.

**In Vivo Protein Labeling**

In vivo labeling of proteins neosynthesized by protoplast-derived cell colonies 6 d after entrapment of protoplasts was performed as follows. Sixty beads were transferred into 3 mL of fresh culture medium with 1.85 MBq of \([^{35}\text{S}]\)Met solution (Amersham SJ 204 specific activity >37 TBq mol\(^{-1}\)). The incubation conditions were 6 h at 40 rpm in dim light (3 \(\mu\)mol m\(^{-2}\) s\(^{-1}\), PAR) and 28 ± 1°C. Later, the beads were rinsed thoroughly with fresh culture medium and used for protein extraction.

**Extraction of Total Proteins**

Proteins were phenol extracted according to the method of Meyer et al. (1988). Phenol extraction allows most of the proteins to be recovered, except the highly glycosylated ones, and the polysaccharidic biopolymers that are used for immobilization can easily be discarded. Sixty beads were pooled, washed with fresh culture medium on a 120-μm-pore nylon sieve, frozen in liquid nitrogen, powdered in a precooled mortar, and homogenized in 12 mL of an emulsion of 50% (w/v) phenol in 0.1 M Tris-HCl, pH 8.0, containing 5% β-mercaptoethanol. After 30 min of stirring on a magnetic stirrer at room temperature, the phenol solution was centrifuged to separate the emulsion (5000 g for 15 min at 4°C). The phenol phase was re-extracted three times for 5 min with 1 volume of 0.1 M Tris-HCl, pH 8.0, saturated with phenol containing 5% β-mercaptoethanol, and centrifuged (5000 g for 15 min at 4°C). The proteins in the phenol phase were further precipitated overnight by 4 volumes of methanol containing 0.1 M ammonium acetate at -20°C. The precipitate was recovered by centrifugation and washed five times with 0.1 M ammonium acetate (−20°C) and once with 90% cold acetone (−20°C) acetone. The pellet was dried and considered a “phenol extract.” The extract was subsequently dissolved and homogenized in a lysis buffer containing 9.2 M urea, 2% (w/v) 3-(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propane sulfonate, 5% ampholines (Pharmacia, pH range 3.5-10.0), and 5% β-mercaptoethanol, and either used immediately for NEpHGE or stored at −20°C for further experiments.

**Preparation of Proteins Soluble in the Medium**

Proteins released into the liquid culture medium by diffusion throughout the beads were TCA (12.5%) precipitated as described above, except that the precipitation step lasted overnight instead of 4 h.

**Enzymatic Assays**

Glc-6-PDH (EC 1.1.1.49) activity was tested as a marker for cytosolic protein contamination of the extracellular samples (Boudet et al., 1981). Extractions of total and intracellular proteins were performed according to the method of Peelman et al. (1989). Glc-6-PDH activity was determined by the absorption change at 340 nm, which was caused by the Glc-6-PDH-mediated reduction of NADP\(^{+}\). Possible contamination of the extracellular fraction by intracellular proteins was deduced from direct (extracellular) as well as indirect (total and intracellular) measurements of Glc-6-PDH activity.

**Gel Electrophoresis**

Mini Protean II dual slab cells (Bio-Rad) were used for 2D PAGE. First dimension NEpHGE was performed in cylindrical gels (1.5 × 80 mm) as described by Meyer et al. (1988) using 2% (w/v) ampholine carrier ampholites, pH 3.5 to 10.0 (Pharmacia).

The second dimension SDS-PAGE was performed in 10% (w/v) acrylamide minislab gels according to the method of Domon et al. (1994). After electrophoresis, the gels were either analyzed by CBB staining or checked for radioactivity as follows: 1 h of either fixation or fixation staining (20 min) in ethanol:acetic acid (25:15, v/v) with or without 0.25% CBB, followed in some circumstances by a destaining step in 10% acetic acid (overnight) and dried for 2 h, unless otherwise stated.

Protein concentration was determined spectrophotometrically by the Bio-Rad protein assay derived from Brad-
Cell-Wall Staining

Cell walls of colonies derived from agarose or Ca\textsuperscript{2+}-alginate-immobilized protoplasts were stained with the fluorescent brightener 28 ((2.1. 40622; Calcofluor white M2R; Sigma) solution (0.001% [w/v] in AE\textsuperscript{+} culture medium) for 15 min. Staining was then stopped by introducing the dried gels to x-ray film (XAR-5, Kodak) at -80°C. When necessary, exposure times were adjusted to achieve equivalent image intensities.

RESULTS

Phenotypic Characterization of the Cell Colonies Immobilized in Agarose or Ca\textsuperscript{2+}-Alginate

The immobilization of flax protoplasts in agarose (Fig. 1B) or Ca\textsuperscript{2+}-alginate (Fig. 1C) was detrimental for 72.3 or 75.3% of the protoplasts, respectively. However, most of the protoplasts that survived the immobilization procedure started to divide within 3 d and were not affected in their subsequent development. After 6 d of culture, 76.7 or 70%, respectively, of them formed colonies. The statistical analysis indicated that plating efficiencies were not significantly different (P = 0.058) in agarose and alginate conditions. However, these data did not take into account either the cell number per colony or the phenotype of the different colony morphologies that we observed at d 6. These specific colony types observed for each condition of immobilization concerned most, if not all, of the colonies.

The morphology in agarose-grown colonies was disorganized and heterogeneous (Fig. 1, D and E), formed by a small number of actively dividing cells, highly vacuolated cells, and necrotic cell clumps. On the other hand, the general aspect of colonies grown on Ca\textsuperscript{2+}-alginate-solidified, auxin-containing medium consisted of dense and globular structures (Fig. 1, G and H). Such differences, which were enhanced by Calcofluor staining, showed that, in contrast to agarose (Fig. 1F), alginate-beaded round colonies were formed of numerous small cells (Fig. 1I). Later (10–12 d of culture) agarose-grown colonies retained the same heterogeneity as described above, whereas most of the alginate-grown colonies expressed a polarized phenotype and, occasionally, in the same culture conditions, a heart-type phenotype.

Protoplasts immobilized in Ca\textsuperscript{2+}-alginate were cultured in an auxin-free medium. The derived cells were highly vacuolated, and a few cell divisions were observed at d 6. The morphology of the colonies was still highly disorganized at d 12 of culture. The frequency of colonies formed at 6 d of culture in auxin-containing AE\textsuperscript{+} medium and the specificity of the colony phenotype in a given matrix were very reproducible. We observed unique, globular structures in alginate-solidified, auxin-containing medium, high heterogeneity in agarose-solidified, auxin-containing medium, and unorganized phenotype in alginate-solidified, auxin-free medium. The protein analyses that follow were performed on structures that always showed the morphology described here.

Patterns of de Novo Synthesized Polypeptides

To identify changes in gene expression that could be associated with the specific morphogenetic responses of protoplast-derived cells in both conditions of immobilization, we analyzed neosynthesized proteins 1 and 6 d after immobilization of the protoplasts. The profiles of radioactive proteins are very different at 1 versus 6 d of culture. This allowed discrimination between the early and the late stages of development of the immobilized flax protoplasts. Whatever the biopolymer used, after 1 d of culture the protoplasts had already formed a new wall but no first divisions were seen. The overall patterns of the [\textsuperscript{35}S]Met-labeled proteins were similar (data not shown). It is likely that these total protein profiles reflected the events occurring before or at the time of the re-entry of the cells into the cell cycle. At 6 d of culture colony phenotypes in Ca\textsuperscript{2+}-alginate and agarose beads were very different (Fig. 1). In spite of that, no significant and reproducible differences were observed within the patterns of the proteins that were synthesized in vivo by cells immobilized in the alginate (Fig. 2B) compared with the agarose (Fig. 2A) matrix. These similarities may indicate that only slight changes in gene expression are associated with the morphogenetic response observed; such changes are undetectable at this level of analysis.

Patterns of CBB-Stained Polypeptides

The proteins accumulated by the 6-d-old colonies were also analyzed from total protein extracts. Almost 200 spots were detected by CBB staining. A comparison of the patterns that were obtained from the cells entrapped in the two matrices (Fig. 2, C and D) showed evident similarities. However, a closer comparison of the basic region of the gels enabled us to discriminate between alginate (HV) and agarose conditions. Thus, among the overall proteins accur-
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**Figure 1.** Immobilization of protoplasts in agarose or alginate gels and subsequent morphogenetic responses after 6 d of culture. A, Protoplasts just after isolation and purification; B and C, schematic representation of agarose and Ca-alginate gels; D and G, 6-d-old colonies in agarose and alginate (bright-field microscopy; note in D the disorganized protoplast-derived colonies in agarose as compared with the abundance of the specific, round colony type derived from alginate-immobilized protoplasts in G); E and H, same as D and G but at higher magnification; F and I, single colonies in E and H after Calcofluor white staining. Tiny cells of the round colony type in alginate (I) are compared with large cells of disorganized colonies in agarose (F). Bars: A, 55 μm; D and G, 135 μm; E and F, 45 μm; H and I, 65 μm.

Patterns of Proteins Ionically Bound to the Walls or Soluble in the Liquid Medium

Vacuum infiltration, a step in the extraction of extracellular ionically bound proteins, is known to cause little damage to the cells. To test cell integrity we measured the activity of Glc-6-PDH, an intracellular enzyme (Boudet et al., 1981). No extracellular Glc-6-PDH activity was measurable when the direct approach was attempted. We therefore measured total Glc-6-PDH activity before and after extraction of the extracellular proteins. The same values of 0.3 μkatal/bead were recorded from both extracts, providing evidence for the lack of loss of Glc-6-PDH due to potential cell lysis.

The proteins that were secreted by the 6-d-old cells developed on Ca$^{2+}$-alginate-solidified, auxin-containing or auxin-free medium, as well as those secreted on agarose-solidified, auxin-containing medium, were studied in a two-step analysis. First, the proteins ionically bound to the cell walls or the cell surfaces were considered in a global study along with those that might have been sequestered within the extracellular matrix through electro-
Figure 2. Total protein patterns from 6-d-old protoplast-derived cell colonies immobilized in agarose (A and C) or alginate (B and D) matrices. For de novo synthesized protein analysis (A and B) entrapped cell colonies were labeled with $[^{35}S]$Met for 6 h and then powdered (see "Materials and Methods" for details). The phenol extracts were subjected to 2D NEpHGE/SDS-PAGE followed by autoradiography to identify differences in the newly synthesized proteins. Equal counts were loaded onto the acidic ends for NEpHGE (50,000 cpm), and no significant differences were observed between the two matrices. At least three different experiments were performed for these analyses. Constitutive expression of total proteins (C and D) was analyzed after CBB staining following 2D NEpHGE/SDS-PAGE separation. The variation of intensity of spot 183 is the main difference between the two immobilization conditions. Sixty micrograms of protein was loaded onto the acidic ends for NEpHGE. Molecular mass markers are shown in kD. No less than four different experiments were compared for this analysis.

Static linkages with Ca$^{2+}$-alginate. The high ionic strength of the solution (NaCl/CaCl$_2$) used for extraction of ionically bound proteins did not allow discrimination between their electrostatic interactions with the wall polysaccharides or the alginate polymer. Second, the soluble proteins found in the surrounding liquid medium were also analyzed.

Figure 3 represents partial electrophoretograms of the basic proteins either strongly bound to the wall or the adjacent matrix (A, D, and G) or released into the medium (B, E, and H). Sixty micrograms were loaded onto each gel. Whatever the immobilization and culture conditions, the amount of NaCl-extracted proteins resolved in A, D, and G was 0.15 mg per bead. The amounts of proteins recovered from agarose (B) or alginate (E) surrounding auxin-containing medium and alginate (H) auxin-free medium were 0.30, 0.15, and 0.50 mg per bead, respectively. Thus, the proteins resolved in A, D, E, and G correspond to cell colonies developed in 400 beads, and the proteins resolved in B and H correspond to cells grown in 200 and 120 beads, respectively.

The differences we observed in the pattern of proteins secreted in agarose and alginate auxin-containing conditions (A/B and D/E) enable us to comment on several. Although the patterns of basic proteins were qualitatively similar, most of these polypeptides differed in intensity. There was an increase of most of the ionically bound basic proteins in the alginate condition compared with the agarose condition. This was the case for the polypeptides numbered 148, 162, 166, 183, 184, and 187, ranging from 65 to 15 kD (Fig. 3, A and D). A detailed analysis shows that in agarose they were poorly maintained through electrostatic interactions (Fig. 3A); in contrast, they were abundant in the liquid medium, particularly 148, 183, and 184 (Fig. 3B). In the alginate condition these proteins were mainly ionically bound to the wall or to the matrix (Fig. 3D). On the other hand, these polypeptides were less abundant in the liquid medium (Fig. 3E). The pair of polypeptides 183 and 184 had already been detected by CBB in the total phenol extracts (Fig. 2, B and D).

Flax protoplasts were cultured in Ca$^{2+}$-alginate-solified, auxin-free AE$^+$ medium. In these conditions abnormal divisions of highly vacuolated cells were observed at d 6 (Fig. 3I, 1), and unorganized colonies developed at d 12 (Fig. 3I, 2 and 3), whereas alginate-embedded globular colonies cultured in standard auxin-containing AE$^+$ medium developed into early-stage embryos (Fig. 3F, 1–4) as mentioned earlier. It is interesting that the patterns of secreted proteins strongly bound to alginate and/or cell walls differed, depending on the presence (Fig. 3D) or the absence (Fig. 3G) of auxin in the culture medium. The overall basic proteins secreted by the round colonies (Fig. 3D) were hardly detectable among the proteins secreted by Ca$^{2+}$-alginate-beaded cells developed in auxin-free AE$^+$ medium (Fig. 3G). Particularly, polypeptides 148, 183, and 184 were found in the medium (Fig. 3H). These results make it likely that at least some of the highly charged proteins were...
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Figure 3. Comparison of partial 2D NEpHGE/SDS-PAGE electrophoretograms of basic proteins isolated from flax protoplasts grown for 6 d in agarose-solidified (A and B) or Ca$^{2+}$-alginate-solidified (D and E), auxin-containing medium and in Ca$^{2+}$-alginate-solidified (G and H), auxin-free medium. A, D, and G, ionically bound wall proteins; B, E, and H, proteins soluble in the liquid medium. The same basic region of the gels is shown in each frame (A, B, D, E, G, and H). Proteins were extracted from 400 beads (A, D, and G), corresponding to 80 ml of culture medium (E), or from one-half and 30% of this volume (B and H, respectively) to keep the same protein amount (60 /xg per cylindrical gel). Molecular mass markers are shown in kD. Four to six different experiments were compared for these analyses. Subsequent development of the corresponding colonies are shown: C in agarose at d 12, F in alginate at d 8 (F1 and F2) or d 12 (F3 and F4), and I in alginate but in AE$^+$ medium lacking auxin at d 12. Despite the same condition of immobilization (alginate, F and I), protoplasts behaved differently according to the presence (F) or the absence (I) of auxin in the culture medium. The protoplasts cultured in AE$^+$ medium lacking auxin expressed low division rates after 6 d of culture (F1) and developed into unorganized colonies of large, vacuolated cells after 12 d of culture (I2 and I3). The round-type structures derived from protoplasts cultured in auxin-containing AE$^+$ medium developed into polarized structures after 8 d of culture (F1 and F2). Heart-shaped embryos were observed after 12 d of culture (F3 and F4). The colonies developed in agarose remained poorly organized even after 12 d of culture (C1 and C2). Bars: C, 25 /xm; F1 and F2, 40 /xm; F3, 35 /xm; F4, 80 /xm; and I, 50 /xm.

DISCUSSION

Previously, it was shown that a Ca$^{2+}$-alginate matrix immobilizing flax protoplasts induces a globular embryo-like phenotype from the derived cells. This response was observed at a specific growth regulator equilibrium (David et al., 1994). Hahn-Hägerdal (1989) reported that immobilization alone limited cell growth. However, in this study immobilization did not limit cell enlargement,
but it led to the formation of heterogeneous colonies that lacked a defined outline. Limitation of diffusion, the second parameter considered by Hahn-Hägerdal (1989), might control cell growth when protoplasts are immobilized in a uronate gel. For example, the alginate matrix could act as a reservoir of Ca\(^{2+}\). Extracellular Ca is involved in cell polarization of Fucus zygote following fertilization (Kropl, 1992). If a similar implication of this cation exists in the early response of the immobilized protoplasts, which behave like zygotes, the mechanisms by which Ca ions enter the cell could include their release from the polysaccharidic gel and a transient expression of plasma membrane Ca channels. This extracellular cation could be a part of the signal transduction pathway, allowing the unique morphogenetic response we observed in Ca\(^{2+}\)-alginate-solidified, auxin-containing medium.

To understand the physiological events that result from the perception, by the immobilized protoplasts, of the specific microenvironment created by the physicochemical properties of this charged matrix, i.e., the development of embryo-like structures, we compared the fate of some gene products, particularly the extracellular proteins. Specific profiles of secreted proteins have been shown to be associated with somatic embryogenesis in several groups of plants such as carrot (De Vries et al., 1988), barley (Nielsen et al., 1992), grape (Coutos-Thevenot et al., 1992), and pine (Domon et al., 1994). In some cases biological functions were demonstrated, such as their ability to reverse the development of a thermosensitive carrot mutant, which was blocked at a very early stage (Lo Schiavo et al., 1990).

Recently, three ionically bound wall polypeptides identified as germins were shown to be early markers of somatic embryogenesis (Domon et al., 1995), as well as early markers of zygotic embryogenesis, in pine (David et al., 1995b). We show here that a cluster of basic polypeptides that were solubilized from the walls by extraction with 1 M NaCl/0.4 M CaCl\(_2\) were present when the immobilization matrix had charged groups (Ca\(^{2+}\)-alginate) but were absent with uncharged groups (unsulfated agarose). Since conditions under which protoplasts are embedded in alginate or agarose are different, we included the agarose heat treatment for alginate-embedded cells and a Ca concentration in agarose-embedded cells similar to that used for alginate gelation. The original morphologies in agarose or alginate and the contents and patterns of extracellular proteins were not affected by these treatments (data not shown). This subsequently allowed the direct comparison of the protein patterns. Jauneau et al. (1994) reported the importance of Ca in the biochemistry of the plant cell wall, particularly in the regulation of enzyme activities within this external compartment. Since the egg-box conformation of Ca\(^{2+}\)-alginate (chains of G units associated cooperatively) is analogous to the Ca\(^{2+}\)-induced conformation of pectates within the primary cell wall, it was tempting to speculate about an interaction of extracellular, ionically bound, basic proteins with the polyanionic biopolymer of immobilization. Velings and Medstagh (1994) reported the adsorption of two, basic, low-molecular-weight proteins in alginate gel beads, and the behavior of these proteins in Ca\(^{2+}\)-alginate as a function of their molecular weight and the polyelectrolyte character of the gel and pH was demonstrated. The authors showed that the interaction was electrostatic, the affinity between both kinds of polyelectrolytes (proteins and polysaccharide) controlled by the balance of charges and the total deprotonation of alginate reached at a pH of about 6.5 to 7.0 (physiological pH values). In our case specific ionic bindings of basic proteins were observed when Ca\(^{2+}\)-alginate-entrapped protoplasts were cultured in an auxin-containing medium compared with an agarose-containing medium.

Basic polypeptides may interact with pectic substances abundantly secreted in the primary walls of the embryo phenotype, where a polygalacturonic network is created (David et al., 1995a). Kurosaki et al. (1992), studying the interactions between an extracellular β-1,3-glucanase and wall fractions of cultured carrot cells, showed that this protein is maintained within cell-wall structures by a noncovalent acid-base interaction with polygalacturonic acid. To establish a direct correlation between the reported morphology with the polypeptide changes (i.e., strong binding to the wall and not to Ca\(^{2+}\)-alginate), the same matrix (alginate) was used for the embedding of protoplasts that were cultured in an auxin-free medium. Recently, we showed that auxin and 2,4-D are prerequisites for induction of somatic embryos from cotyledonary explants of flax (J.L. George, E. Lainé, H. David, and A. David, unpublished data). On the other hand, the 2,4-D plus NAA-containing AE\(^+\) medium is optimal for flax protoplast-derived cell division.

By analyzing the proteins that were solubilized by NaCl/CaCl\(_2\) extraction from cells that were developed in Ca\(^{2+}\)-alginate-solidified, auxin-free AE\(^+\) medium (Fig. 3, G and H), we found that, despite the presence of the Ca\(^{2+}\)-alginate matrix, the basic protein profiles were very different from those of cells cultured in standard auxin-containing AE\(^+\) medium (Fig. 3, D and E). The secretion of these basic polypeptides dramatically decreased: some of them were almost undetectable, one was mainly retained within the bead (166), and three (148, 183, and 184) were mainly diffused into the liquid medium, in spite of the electrical properties of the alginate matrix. Indeed, the basic proteins shown in Figure 3 (ionically bound in A, D, and G and medium soluble in B, E, and H) are resolved from equal amounts (60 μg). If we take into account that the protein contents on a per bead basis are 0.15 μg in A, D, E, and G, 0.30 μg in B, and 0.50 μg in H, it is clear that the three basic polypeptides 148, 183, and 184 escape from the walls of poorly organized cell colonies (Fig. 3, C and I), whereas they are strongly bound to the walls of the embryo-like phenotype (Fig. 3F). This suggests that the growth of plant cells on an alginate matrix in the presence of auxin-containing medium may promote the binding of some basic proteins to specific wall polymers such as acidic pectins (David et al., 1995a) rather than to Ca\(^{2+}\)-alginate. These results allow the direct correlation of the high level of some basic, ionically bound cell-wall proteins with the round-type phenotype of the colonies developed in the alginate matrix.
In an attempt to understand the mechanisms that regulate the development of different colony morphologies, we found that a specific pattern of ionically bound wall proteins could be related to the unique, globular, embryo-like phenotype. Such a phenotype is only observed for highly dividing cells derived from alginate-beaded protoplasts cultured in an auxin-supplemented medium. To characterize these basic proteins the next step will be to use microsequencing to identify these polypeptides that are correlated with this morphology. This work contributes to the comprehension of the physiology of plant cells faced with a specific microenvironment and aids in our understanding of how plant form is determined.

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