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I am submitting herewith a dissertation written by Maureen E. McConnell entitled "Specific Proteins Potentially Associated With Somatic Embryogenesis in Orchardgrass." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Plant and Soil Science.

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Specific Proteins Potentially Associated With Somatic Embryogenesis in Orchardgrass

A Dissertation

Presented for the

Doctor of Philosophy

The University of Tennessee, Knoxville

Maureen E. McConnell

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DEDICATION

This dissertation is dedicated to the memory of my mother

Elizabeth Stella Cannova McConnell

who dedicated her life to my happiness, health and future.

My mother did not live to see me graduate

but her spirit is with me always.

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Abstract

Various biotechnological techniques studied to supplement conventional plant breeding procedures require the regeneration of whole plants from cells or tissue cultured in vitro. The objective of this study with orchardgrass (Dactylis glomerata L.) was to investigate specific protein differences associated with 'Embryogen-P', a genotype which produces somatic embryos in high numbers, and four nonembryogenic genotypes by one- and two-dimensional polyacrylamide gel electrophoresis.

Four proteins were present in Embryogen-P leaves that were not present in the leaves of the nonembryogenic genotypes. One protein appeared in the nonembryogenic genotypes that did not appear in Embryogen-P. Similar results were found in the embryogenic and nonembryogenic progeny of a cross between Embryogen-P and one of the nonembryogenic genotypes. These results support an association of specific proteins with the ability to produce somatic embryos.

Embryogenesis was supported in suspension cultures by the addition of casein hydrolysate to the medium. Five proteins were found in the protein profiles of these cultures that were not present in those not supporting

embryogenesis. Similar results were obtained in a second experiment using $(NH_4)_2SO_4$ to support embryogenesis. Although these proteins may not be directly involved in embryogenesis, they appear to be associated with the process.

Basal sections of Embryogen-P leaves were analyzed after 2, 4, 7, 10, 14, or 22 d after culture. A total of 12 proteins were found to be produced during culture, 9 at 7 d, 2 at 10 d, and 1 at 14 d after culture. Appearance of these proteins at different times throughout the culture period suggests a time course for their synthesis.

Leaves were placed on medium with abscisic acid (ABA) and removed at 1, 2, or 3 d after culture. Four proteins were found in leaves 1 d after culture and three were present after 2 d. Three proteins found in leaves 1 d after culture with ABA had the same molecular weights as three appearing at 7 d after culture on medium without ABA. These three may be promoted at an earlier time by ABA and produced in response to embryogenesis.

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1. Introduction

There is current interest in the development of various biotechnological techniques to supplement conventional plant breeding procedures (Conger and Gray, 1984; Morrish et al., 1987; Vasil, 1987). These include micropropagation, in vitro fertilization and wide hybridization, creation of haploids and somaclonal variants, and gene transfer. A basic requirement of most of these techniques is regeneration of whole plants from cells and tissues cultured in vitro.

The development of regeneration systems for species in the Poaceae has been slower and more difficult than for some other botanical families, e.g., the Solanaceae. Prior to 1980, the induction of a more or less uniform population of dedifferentiated cells and plant regeneration from those cells was considered by some workers not to be possible in cereals and grasses (King et al., 1978). The discovery of somatic embryogenesis in several poaceous species represented a major breakthrough (see reviews by Conger and Gray, 1984; Morrish et al., 1987; Vasil, 1987).

In 1982, somatic embryogenesis was obtained in orchardgrass (Dactylis glomerata L.) from both mature embryos (McDaniel et al., 1982) and leaf explants (Hanning

and Conger, 1982). The full development of somatic embryos to a germinable stage directly in a single liquid medium was reported by Gray et al. (1984) for this species. Histological evidence supported a single cell origin of embryos from both leaf explants (Trigiano et al., 1989) and suspension cultures (Conger et al., 1989).

Genotypic differences for regeneration were evident within plant species including the cereals. Among 20 rice (Oryza sativa L.) cultivars, only 16 were capable of regenerating plants (Fatokun and Yamada, 1984). Variability among genotypes of maize (Zea mays L.) to initiate differentiating cultures was found by Green and Phillips (1975). Genotypic differences in wheat (Triticum aestivum L.) were reported for callus formation and shoot production (Sears and Deckard, 1982). Significant differences in the production of regenerable callus were found among oat (Avena sativa L.) cultivars having a wide spectrum of genetic backgrounds (Rines and McCoy, 1981).

Data from two independent studies of alfalfa (Medicago sativa L.) showed that two dominant alleles likely controlled the regenerative response (Reisch and Bingham, 1980; Wan et al., 1988). This species is an autotetraploid forage legume (Bingham, 1980) and therefore similar to orchardgrass in breeding behavior and tetrasomic inheritance (Lumaret, 1988).

Results of an initial study with orchardgrass involving controlled reciprocal crosses between an embryogenic genotype, later named 'Embryogen-P' (Conger and Hanning, 1991), and two nonembryogenic genotypes showed that the somatic embryogenesis trait was transmitted sexually through both male and female gametes and expressed as a dominant nuclear gene in approximately 50% of the F_1 progeny (Gavin et al., 1989). The 1:1 ratio fits the model of the simplex (Aaaa) embryogenic genotype crossed to a nulliplex (aaaa) nonembryogenic genotype.

Genotypic differences may be detected at the DNA, RNA or protein level. A method to extract and separate proteins from orchardgrass suspension cultures on two-dimensional (2-D) gels was developed by Mayer et al. (1987). Using this technique, Hahne et al. (1988) showed protein differences in the comparison of somatic embryos, calli, leaves, and seeds of orchardgrass. These two studies suggested that differences existed which could be easily detected using 2-D gels. Protein differences between embryogenic and nonembryogenic genotypes in orchardgrass should be detectable using the Mayer et al. (1987) method.

The objective of this study was to investigate specific protein differences associated with Embryogen-P and four nonembryogenic genotypes by one-dimensional (1-D)

and 2-D gel electrophoresis. To further investigate the embryogenic response, protein profiles of Embryogen-P suspension cultures in medium supporting and not supporting embryogenesis were compared. The protein profiles of Embryogen-P leaves cultured for different lengths of time were also examined to study a possible time course of protein synthesis.

2. Literature Review

Various investigations have been performed to learn more about the process of somatic embryogenesis. In many of these studies proteins from embryogenic and nonembryogenic cultures were compared by either 1-D or 2-D polyacrylamide gel electrophoresis (PAGE) with immunological studies used to further verify the findings. These unique proteins are likely to be associated with somatic embryo development and not the products of housekeeping genes (Choi and Sung, 1984). Further characterization of these proteins may lead to an understanding of their role in embryogenesis.

Two proteins with molecular weights (MWs) of 77 and 43 kilodaltons (kD), were produced by embryogenic carrot (Daucus carota L.) suspension cultures (Sung and Okimoto, 1981) in MS (Murashige and Skoog, 1962) medium. These proteins, designated E1 and E2, were not produced in suspension cultures when embryogenesis was suppressed by 2,4-dichlorophenoxyacetic acid (2,4-D). In a later study, two proteins, C1 (MW=24 kD) and C2 (MW=20 kD), were produced by carrot callus in suspension cultures with 2,4-D in MS medium when the cell density was 2 x 10^6 cells per ml or higher (Sung and Okimoto, 1983). Cultures at a

density of 2 x 10^4 cells per ml became embryogenic without 2,4-D and produced E1 and E2.

In embryogenic cell suspensions of barley, 50 proteins were found with 30 being most prominent (Nielsen and Hansen, 1992). Of the 30 proteins, seven were constitutively produced. During embryogenesis, six proteins were either produced or increased while six others decreased. Six proteins were associated with the culture method to support embryogenesis and five were produced during prolonged growth without subculture.

In another study, four proteins appeared to be stage specific and causally involved in carrot somatic embryo development (Schnall et al., 1991). Two variants were used in which exposure to 33°C arrested embryo development at the oblong globular stage; normal development occurred at 24°C.

Using immunoblotting techniques, a protein was found to be associated with carrot cultures grown with 2,4-D in liquid MS medium (Satoh and Fujii, 1988). This protein, GP57, was produced and released into the medium by nonembryogenic carrot cell cultures in the presence or absence of 2,4-D. However, embryogenic cultures released GP57 only when supplied with 2,4-D. GP57 was also detected in the space between the embryo and the endosperm of dry and germinating carrot seeds, in the epidermis,

endodermis and periderm of mature taproots, and in the epidermis of petioles and young leaves. Since all of these tissues are known to be suberized or cutinized, GP57 was suggested to have a barrier-forming or defense activity.

A 45 kD protein found in the extracts of carrot somatic embryos was detected by monoclonal immunoassay (Smith et al., 1988). Suspension-cultured cells and explant tissues were fixed, embedded in plastic and cut into sections. The sections were then attached to glass slides and incubated with monoclonal antibodies specific for the 45 kD protein. A fluorescent dye was coupled to the antibodies. The 45 kD protein was localized in the nucleus and was strongly associated with the nucleolus. The same protein was found in rapidly growing cells, immature zygotic embryos, developing floral buds, apical shoots, and root meristems. Also, this protein, believed to be associated with cell division, was detectable in rapidly dividing cells of maize, wheat, Arabidopsis thaliana Heynh., celery (Apium graveolens var Dulce.), alfalfa, and peach (Prunus persica L. Batsch).

Using a monoclonal antibody raised against microsomal fractions from carrot embryogenic cells, a 31 kD protein was detected in embryogenic cells and in the organ segments that produced somatic embryos (Kiyosue et

al., 1990). Antigen was not detected in somatic embryos, nonembryogenic cells, crown galls, or hairy roots. In a second study, this protein was found in the peripheral cells of embryogenic cell clusters when 2,4-D was in the medium. It was not found in developing embryos without 2,4-D in the medium (Kiyosue et al., 1991). It was also located in carrot hypocotyl segments cultured for 33 days, as well as in zygotic embryos in low amounts and in provascular tissue of malformed somatic embryos.

Changes in the activity of certain enzymes were associated with somatic embryogenesis in carrots. Arginine decarboxylase activity increased in embryogenic cells and the activity of S-adenosyl methionine decarboxylase increased in nonembryogenic cells (Montague et al., 1979). Inhibition of arginine decarboxylase reduced somatic embryo formation in suspension cultures while inhibition of ornithine decarboxylase did not affect embryogenesis (Feirer et al., 1984). Baker et al. (1983) found an increase in ornithine carbamoyltransferase activity within 6 h of subculturing callus into auxin-free medium. These are key enzymes in the pathways of the production of nucleotides, amino acids and polyamines.

Cultured carrot cells undergoing differentiation and organization which were resistant to cycloheximide became sensitive at the onset of somatic embryogenesis (Sung et

al., 1981). However, somatic embryos and plantlets from this callus were resistant to the compound. Certain isoperoxidases, esterases and malate dehydrogenases, detected by isozyme assays of 1-D gels, were present in embryogenic calli but not in nonembryogenic calli of maize (Rao et al., 1990). The genes for these enzymes and cycloheximide resistance may be involved in the response to somatic embryogenesis. Studying the mechanisms that control these markers might elucidate those that control embryogenesis (Sung et al., 1984).

Using 2-D polyacrylamide gel electrophoresis (PAGE), McGee et al. (1989) discovered two proteins in Trifolium rubens L. and T. pratense L. that were associated with somatic embryogenesis. The largest of these had a MW of 45 kD and a pI of 6.2-6.3. This pI (isoelectric point or pH at which the protein has no net charge) differed from that in the embryogenic proteins of the same size found in carrots, pI 9.5-9.6 (Choi and Sung, 1984) and peas (Pisum sativum L.), pI 6.9-7.1 (Stirn and Jacobsen, 1987).

Selection of immature white spruce [Picea glauca (Moench) Voss] and Engelmann spruce (Picea engelmanni Parry ex Engelm) zygotic embryos prior to the accumulation of storage proteins increased the percentage of embryogenic callus produced via callus culture (Roberts et al., 1989). In another study with Norway spruce (Picea

abies L.), Hakman et al. (1990) showed storage protein accumulated to approximately the same quantity in zygotic and somatic embryos. Proteins with MWs of 22, 28, 33, 42 were found in both types of embryos and they had similar pIs.

Pollen grains of Nicotiana rustica L. were incubated with [^{32}P]-H_3PO_4 and sampled at 12, 24, 48, and 74 h after culture (Kyo and Harada, 1990). In comparing the autoradiographs of proteins from embryogenic pollen sampled at different times and then separated by 2-D PAGE, it was found that the major labelled proteins disappeared with increased culture time. Six spots appeared more intensely after 24 h. They remained 74 h after culture and revealed a pattern of protein phosphorylation. Protein phosphorylation was not found in normally developing pollen grains or nonembryogenic pollen grains.

In a study comparing 2-D PAGE of embryogenic and nonembryogenic Brassica napus L. microspores, certain proteins appeared to be correlated with embryogenesis (Cordewener et al., 1992). Isotypes of α- and β-tubulin and actin were detected by using antibodies specific for a set of cytoskeletal proteins and MPM-2 with the western blot technique. Using ^{32}P, differences in phosphorylation of these isotypes were found between embryogenic and nonembryogenic microspores.

Five groups of proteins were associated with meristem initiation and bud development from the callus of Petunia hybrida Hort (Renaudin et al., 1991). One group was associated with bud formation. Another group accumulated in tissue cultured on both the control and regeneration media. A third group was abundant in the meristem at the time of culture on both control and regeneration media. A fourth group was detected at low levels at day 0 but increased to day 7 and then declined. A fifth group of proteins accumulated between day 7 and day 14. This group decreased thereafter and appeared only in the controls.

In rice embryo cultures, Chen and Luthe (1987), using 1-D and 2-D PAGE, found three proteins, with MWs of 56, 54, and 36 kD, associated with somatic embryos and embryogenic callus and to a lesser extent with nonembryogenic callus. Ramagopal (1989) found 32 new proteins produced in immature embryos and root explants of two barley species, Hordeum vulgare L. and H. bulbosum L.; 15 were produced during the production of callus from the explant and another 9 during cell proliferation of callus that had been subcultured until a permanent cell line was established. The remaining were produced throughout the culture period.

Abscisic acid (ABA) promoted the synthesis and accumulation of storage proteins, GLB1 and GLB2, in

cultured maize zygotic embryos (Rivin and Grudt, 1991). These effects were believed to be part of the general water stress responses regulated by ABA. In another study, a cDNA was isolated which corresponded to an 800-nucleotide transcript in maize. It was expressed in the presence of ABA in early to mid-stage embryos (Williams and Tsang, 1991).

Whole plant and seed proteins of orchardgrass were compared with callus and embryo proteins (Hahne et al., 1988). The plants and seeds were from the cultivar 'Potomac' but not from the genotype which produced the embryogenic callus. Suspension cultures were fractionated into embryogenic aggregates and nonembryogenic callus. These fractions were used immediately or stored at -70°C until used. On silver-stained 2-D gels, 21 proteins were found in the somatic embryo fraction that were not present in the callus fraction. Six proteins were present in the callus that were not present in the somatic embryo fraction. Ten of the 21 proteins found in the somatic embryos were detected in the whole plant proteins. However, none of the six proteins found in calli were detected in any 2-D gel from whole plants. The authors compared silver-stained 2-D gels of orchardgrass seeds, calli and somatic embryos and found 3 of the 21 proteins specific for somatic embryos to be clearly present in

seeds and 4 absent in gels of zygotic embryo proteins. Storage proteins of the endosperm were numerous and obscured those from the zygotic embryo. This made locating callus and somatic embryo proteins difficult. However, one callus protein was clearly absent from the seed proteins.

Hahne et al. (1988) also used in vivo labelling with [^{35}S]-methionine. They identified ten proteins specific for embryos and eight for callus. Three were the same as previously found in the silver-stained gels and the rest were unique to the in vivo labelling experiment. Poly(A)$^+$ RNA was isolated from callus and somatic embryos and the translated products were separated by 2-D PAGE. Six proteins were identified as specific for embryos. Four were similar in MW and pI to those in the silver-stained gels and one was similar to that from the in vivo labelling experiment. Eight proteins were associated with callus in the in vitro translation products. None were the same as those in the in vivo labelling experiment or in the silver-stained gels.

3. Materials and Methods

Materials

Orchardgrass plants used in this study were from the cultivar 'Potomac.' These included Embryogen-P (Conger and Hanning, 1991), four nonembryogenic genotypes, and F_1 progeny from crosses between Embryogen-P and a nonembryogenic genotype. The ability of the progeny to produce somatic embryos was tested previously (Gavin et al., 1989). All plants were grown in a greenhouse or a growth chamber. The temperature was maintained at 21°C during the day and 13°C at night for both the chambers and the greenhouse. Ambient light was used in the greenhouse. The chambers were set for 16 h light and 8 h darkness.

Methods

Extraction of Proteins

Proteins were extracted from leaves and calli using the methods described by Mayer et al. (1987). Extraction buffer, 2D-MH, contained 2% (v/v) ampholytes (Sigma Chemical, St. Louis, pH 3-10) to maintain a neutral pH, 300 mM sodium chloride, 1 mM disodium ethylenediaminetetraacetate (EDTA), 1 mM ethyleneglycol-bis-N,N,N′,N′-tetraacetic acid (EGTA), 2% (v/v) Triton

X-100, 5 mM ascorbic acid, 100 mM DL-dithiothreitol (DTT), 10 μg/ml leupeptin, and 10 μg/ml α_2-macroglobulin. Polyvinyl polypyrrolidone (PVPP) was added to the sample at a 0.5:1 (w/w) ratio prior to grinding. Samples were frozen with liquid nitrogen and ground with a mortar and pestle. After the samples were powdered, the extraction buffer was added, frozen with liquid nitrogen, and ground into a powder. For every 1 g of callus, 0.5 ml of buffer was added. For leaves, 4 ml of buffer was added to every 1 g. After the sample thawed, plant material was removed by centrifugation at 12,000 x g or by filtration through Miracloth (Calbiochem). The supernatant was shaken with 1 to 2 mg of protamine sulfate per ml for 10 min. The sample was centrifuged again and the supernatant removed. The supernatant was stored at -70°C. A Biorad (modified Bradford) assay for total protein was performed on these samples. Urea was added to the supernatant to a concentration of 9 M just prior to electrophoresis.

Polyacrylamide Gel Electrophoresis

One dimensional gel electrophoresis was used to separate proteins by size, creating a protein profile for each nonembryogenic and embryogenic plant or suspension culture. Polyacrylamide gels (PAG) were prepared according to the Laemmli (1970) procedure. Prior to

electrophoresis, 1 ml of protein extract was diluted with 2 ml of Laemmli buffer. This mixture was boiled for 3 min. Gradient PAGs of 9 to 18% (w/v) acrylamide were employed to further resolve protein bands (O'Farrell, 1975) and were stained using commassie blue (Merril et al., 1981) or silver (Wray et al., 1981). An equal volume of protein extracts containing approximately 0.23 mg of proteins from nonembryogenic and embryogenic leaves was separated by PAGE.

Protein markers with MWs of 65, 45, 36, 29, 24, 20, and 14 (Sigma Chemical, St. Louis, MO) were included in all the 1-D PAGs to determine MWs of the unknown proteins. Distances from the top of the PAG to the MW markers were measured and a linear regression was performed. The equation of the line was used to determine the MWs of the unknown proteins.

PAGs were scanned using an LKB Ultroscan densitometer. A laser beam at 632.5 nm was transmitted through the gel from the bottom to the top. The absorbance of the light by the gel was measured in optical density (O.D.) units. These measurements or absorbance values were passed to an IBM computer with the LKB Gelscan program running. This program then stored the data and produced densitograms using the absorbance values.

Two-dimensional gel electrophoresis was used to separate proteins by two criteria to further characterize the proteins. The first dimension was an isoelectrofocusing tube gel which separated proteins by pIs (O'Farrell, 1975). Samples from 2D-MH buffer extraction were loaded onto these tube gels. They were subjected to 400 V for 14 to 18 h establishing the pH gradient within the tube gel. The voltage was then increased to 800 V for 2 h. During this period of 16 to 20 h, the proteins moved through the tube gel and stopped at their pIs. The second dimension was a gradient slab gel used to separate proteins by MW within their pI ranges. The silver-staining technique used in 1-D PAGs also was used to stain 2-D PAGs. The pIs of the proteins were determined by running a tube gel with only the sample buffer. After electrophoresis, the tube gel was cut into 1 cm pieces which were placed in distilled water in an eppendorf tube. After 4 h, the pH of the water was measured. This pH was used as the average for that piece of tube gel.

Leaf Protein Experiments

In most studies, tillers were excised slightly above the soil surface and basal segments, 20 to 30 mm long, from the innermost two leaves were used for the

experiments. Proteins were extracted from these segments and separated by 1-D and 2-D PAGE. Leaves were sampled from one embryogenic and one nonembryogenic offspring from each of two crosses. In the sectioned leaf study, approximately seven samples of 20 mm length from the base to the tip were collected. Proteins were extracted and separated by 1-D PAGE. In another experiment, proteins were extracted from basal sections of all leaves within a tiller and separated by 1-D PAGE.

Suspension Culture Experiments

Suspension cultures not producing embryos were established from Embryogen-P leaf-derived callus or somatic-embryo-derived callus using the technique described by Gray et al. (1984). It has been previously reported that embryogenesis in suspension cultures occurs when casein hydrolysate (Gray et al., 1984) or ammonium sulfate (Trigiano et al., 1992) is added to the medium. The liquid medium was SH (Schenk and Hildebrandt, 1972) medium containing 30 μM of dicamba (SH-30). Cultures were maintained by adding fresh medium every 7 to 14 d. After the volume in a 125 ml flask reached 50 ml, old medium was poured off and the culture was divided and transferred into flasks of fresh medium. Embryogenesis was initiated by addition of 0.3% (w/v) casein hydrolysate (Gray et al.,

1984) or 12.5 mM ammonium sulfate, $(NH_4)_2SO_4$, (Trigiano et al., 1992) to the liquid medium at the beginning of culture establishment.

Callus pieces were removed from suspension cultures at a minimum of 22 d after subculturing in medium not supporting embryogenesis (without casein hydrolysate or ammonium). For suspension cultures in medium supporting embryogenesis, callus pieces were removed 22 d after the addition of casein hydrolysate or ammonium sulfate to the medium. Proteins were extracted and separated by 1-D PAGE.

Time Course Experiments

The basal leaf portions were split along the midvein and surface sterilized in 50% (v/v) commercial bleach (2.62% NaOCl) and 0.1% (w/v) Triton X-100 for 90 s. Each leaf half was cut transversely into six sections, approximately 3 to 4 mm long and placed on SH-30 solid medium. Leaf sections were removed at 2, 4, 7, 10, 14, and 22 d after the initiation of culture and proteins extracted. Proteins were then separated by 1-D PAGE.

Abscisic Acid Experiments

Basal leaf sections, prepared as in Time Course Experiments section, were placed on SH-30 with or without

0.1 mM ABA. These sections were removed 1, 2, and 3 d after culture. Proteins were extracted from the leaf sections and separated by 1-D PAGE.

Immunological Experiments

Antibody Production

Proteins from embryogenic and nonembryogenic plants were precipitated from extracts obtained in the Leaf Protein Experiments section with acetone at a 1:5 (v/v) ratio cooled to -20°C for 1 h. After the mixture was centrifuged at 12,000 x g for 15 min, the supernatant was removed. The pellet was either air-dried or dried with N_2 gas. Proteins were dissolved in a 0.05 M sodium phosphate buffer, pH 7.2. One ml of the protein solution was mixed with 1 ml of Freund's incomplete adjuvant and injected into individual New Zealand white rabbits to raise antibodies against these two extracts. The injections consisted of 0.3 ml of the mixture at 6 subcutaneous sites. Three rabbits were injected with proteins from Embryogen-P and three other rabbits with proteins from one nonembryogenic genotype. Blood was collected from 7 to 10 d after three injections at 2 wk intervals and after each booster shot. The serum containing antibodies was separated from the blood by centrifugation at 3000 x g followed by a second centrifugation at 5900 x g. Sodium

azide was added to sera to a 0.05% (w/v) concentration. Sera were stored at -4°C.

Enzyme Linked Immunosorbent Assay (ELISA)

The wells of a polystyrene multiwell plate (with 8 rows of wells and 12 wells per row) were coated with the proteins dissolved in sodium phosphate buffer as prepared in the Antibody Production section. The protein solution was diluted with carbonate coating buffer (0.05 M sodium carbonate, pH 9.6) so that a 1 mg/ml solution was obtained. This solution was placed in the top row of wells of the polystyrene plate. The remaining solution was serially diluted with the carbonate buffer to a 10^{-6} dilution and placed in following rows. In the bottom row, only carbonate buffer was placed in the wells as a control. The plate was covered, placed into a plastic bag with a wet towel to prevent drying and the bag was sealed. This was then incubated at 30°C for 2 h.

The plate was removed from the bag and the wells emptied and carefully washed with PBS at pH 7.3 with 0.05% (v/v) Tween 20 (PBS-Tween) three times to prevent contamination of wells. One μl of serum from the Antibody Production section above was diluted with 1000 μl of PBS-Tween. This mixture was added to all the wells. The plate was returned to the plastic bag with the paper towel

and incubated at 30°C for 2 h. Antibodies within the serum hybridized to the proteins coating the wells. The plate was emptied and washed as above.

One μl of an alkaline phosphatase-protein A conjugate in a 100 units of activity/μl solution was diluted with 1000 μl of PBS-Tween. This mixture was added to all the wells. The plate was placed into the plastic bag with the wet paper towel and incubated at 30°C for 2 h. The protein A attached to the base of the antibody with the alkaline phosphatase free to react. The plate was emptied and washed as before.

One mg of the substrate of alkaline phosphatase, p-nitrophenylphosphate, was dissolved in 1 ml of 10% (w/v) diethanolamine. This solution was added to all of the wells. The plate was covered and incubated at room temperature for 1 h. The results were analyzed by an ELISA plate reader. Serum was used in the Western Blot and Protein Purification sections below only when its corresponding ELISA plate had values between 1 and 2 optical densities (light absorbance at 450 nm) for the protein dilutions of 10^{-1} or greater.

Western Blot

Proteins from both embryogenic and nonembryogenic plants were separated using 1-D PAGE and then transferred

to Biorad nitrocellulose filters (Towbin et al., 1979). Three filter papers (Whatman No. 1) were cut to the size of the PAG and soaked in transfer buffer of 25 mM tris-HCl, 192 mM glycine and 20% (v/v) methanol at pH 8.3. The papers were placed on the bottom graphite plate of the Semidry Electroblotter (E & K Scientific Products, Inc, Saratoga, CA). A nitrocellulose membrane cut to size and soaked in buffer was placed on top of the filter papers. The PAG was then placed onto the nitrocellulose membrane. Three more filter papers cut to size and soaked in transfer buffer were placed on top of the PAG. Bubbles between the layers were removed by rolling a pipette across the stack and then the top graphite plate of the semidry electroblotter was placed on top of the stack. The stack was subjected to 1 V/cm^2 of gel for 25 min.

The Dot-ELISA technique was used to visualize the proteins on the membrane (Banttari and Goodwin, 1985). After proteins were transferred to the nitrocellulose membrane, it was placed in a pyrex dish and soaked in 0.5% (w/v) instant dry milk dissolved in TBS at pH 7.5 (TBS-IDM) for 16 h at 4°C to block the unoccupied sites. The TBS-IDM was removed from the membrane. To hybridize the antibodies to the attached proteins, 15 μl of serum from the Antibody Production section was diluted with

15 ml of TBS-IDM and added to the membrane in the dish. This was shaken on a rotary shaker for 2 h at room temperature. The membrane was rinsed with TBS-IDM five times for 6 min each on the rotary shaker.

To visualize the proteins on the membrane, 15 μl of alkaline phosphatase-protein A conjugate in a 100 units of activity/μl solution was diluted with 15 ml of PBS-IDM. This solution was added to the membrane and shaken for 2 h at room temperature. The membrane was rinsed as above except only three times. A solution of 6 mg/ml Fast Red and 0.1% (w/v) Nathol AS-MX (Sigma Chemical, St. Louis, MO) in 0.2 M tris-HCl, pH 8.2 was added to the membrane and shaken for 25 min.

Removal of Antibodies for Common Proteins

Antibodies specific for common proteins found in both the embryogenic and nonembryogenic genotypes were removed using the method of Madara et al. (1990). Proteins from Embryogen-P and one nonembryogenic genotype were separated by 1-D electrophoresis on individual PAGs. Gels were washed for 15 min with phosphate-buffered saline (PBS) at pH 7.4 and fixed for 15 h in 0.2% (v/v) glutaraldehyde-PBS. Individual whole PAGs were washed with tris-buffered saline (TBS) to remove the fixative and suspended in TBS at a ratio of 1 to 4 (w/v). PAGs were homogenized on ice

with a Sorvall Omnimixer and TBS removed by centrifugation. Sodium borohydride (20 mM) was added to the homogenized gel to quench reactive aldehyde groups and the slurry was stirred for 1 h. The homogenized gels were centrifuged and the supernatant was removed. They were then washed with TBS three times and centrifuged after each wash.

The homogenized gels, considered an immunoabsorbant (IA-PAG), were either stored at 4°C or stirred for 24 h with crude serum diluted five-fold with TBS. Crude serum containing antibodies raised against the leaf proteins of Embryogen-P was added to the IA-PAG made with leaf proteins from a nonembryogenic genotype. Crude serum with antibodies raised against the nonembryogenic genotype was added to the IA-PAG made with Embryogen-P leaf proteins. In this way, antibodies for common proteins were removed leaving those which differed between the two genotypes in the serum.

The IA-PAG was centrifuged and the diluted serum was poured into beakers so that the serum could be returned to the IA-PAG after attached antibodies were removed. The IA-PAG was washed with TBS. Antibodies for common proteins were removed from the gel by stirring with 0.2 M glycine-HCl at pH 2.0 for 30 min. The IA-PAG was centrifuged, the supernatant removed, and washed three

times with TBS. Diluted serum was returned to the IA-PAG and stirred for 24 h as before. This process of removing antibodies for common proteins was repeated three times for each diluted serum. Antibodies attached to the IA-PAG were removed as above. The IA-PAG was washed with TBS and stored at 4°C. The remaining antibodies were precipitated from the serum with 36% (w/v) sodium sulfate solution. The precipitant was washed with 18% (w/v) sodium sulfate solution and dissolved in PBS. The concentration of antibodies was determined using a Shimadzu spectrophotometer at A_{280}.

Protein Purification

The four proteins present in Embryogen-P but not in the nonembryogenic genotype were separated from the total proteins with a protocol using carboxylated-modified (surface modified, 10% solids, 0.298 μM particle diameter) latex beads (CML) from Seradyn (Indianapolis, IN). A 50 μl aliquot of CML was placed in two microcentrifuge tubes and 500 μl of PBS at pH 7.4, 100 μl of 0.4 M N-hydroxysuccinimide (NHS), and 150 μl of distilled, deionized water were added. This mixture was stirred for 5 min at room temperature. Antibodies, from the Removal of Antibodies for Common Proteins section, were dissolved in PBS at 40 mg/ml. Into these two microcentrifuge

tubes, a 100 μl aliquot of crossreacted antibodies specific for embryogenic leaf proteins was added to one tube and an aliquot of crossreacted antibodies specific for nonembryogenic leaf proteins was added to the second tube. The tubes were stirred for 15 min at room temperature. To these, 100 μl of 0.3 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC) dissolved in deionized water were added and the mixtures were stirred for at least 15 h at 4°C.

During this time, the EDAC reacts with the carboxyl group of the CML bead by nucleophilic hydrolysis to form the o-acylurea intermediate. This intermediate reacts with NHS to become an active ester that forms a stable bond between the amino group of the antibody and the carboxyl group on the CML bead.

The mixtures were then centrifuged, the supernatant removed, and the beads washed with 0.1 M phosphate buffer, at pH 7.2. To block unoccupied binding sites, CML beads were incubated for at least 2 h with 10% (w/v) instant dry milk dissolved in phosphate buffer. The beads were again washed with phosphate buffer three times.

Proteins extracted from the basal sections of Embryogen-P and nonembryogenic leaves were precipitated with acetone and dissolved in PBS at 4 mg/ml as in the Antibody Production section. One ml of the protein

solution was added to the CML beads with antibodies attached and stirred for at least 2 h at room temperature. The mixture was centrifuged and the supernatant was removed. The beads were washed with PBS three times. A 0.1 M solution of glycine at pH 2.0 was added to the beads to release the proteins from the antibodies. This mixture was stirred for 30 min at room temperature, centrifuged and the supernatant transferred to another microcentrifuge tube. The beads were again washed three times with PBS and stored at 4°C. The supernatant (released proteins) was neutralized and dialyzed against PBS.

Proteins removed from the beads were separated on a 1-D gradient gel. A vertical strip of gel was removed and stained to locate the bands containing the four proteins. The corresponding bands were cut from the unstained gel. The protein was extracted from each individual gel strip using the procedure of Mayer et al. (1987).

Antibody Purification

Monospecific polyclonal antibodies, specific for each of the four proteins from Embryogen-P, were purified with the use of four immunoaffinity columns. Purified proteins from Protein Purification were dissolved in Pierce (Rockford, IL) Coupling Buffer, 0.1 M phosphate pH 2.0, with the protein at a concentration of 20 mg/ml. To the

prepared immunoaffinity column with carbonyl groups, 2 ml of the protein solution and 0.2 ml of Pierce Working Reducing Solution were added. Working Reducing Solution consisted of 1 M sodium cyanoborohydride in 0.01 M sodium hydroxide. This was used to attach the proteins to carbonyl groups on the column by reductive amination. To mix the solutions thoroughly, the column was inverted several times. The mixture was shaken for 2 h at room temperature. The column was then washed several times with Coupling Buffer after the mixture was removed.

Uncoupled binding sites were blocked by adding 4 ml of Pierce AminoLink Quenching Buffer, 1.0 M tris-HCl at pH 2.4, allowing the buffer to run through the column. The bottom cap was replaced and 2 ml of Quenching Buffer were added to the column. To this mixture, 0.2 ml of Working Reducing Solution was added. The gel was resuspended in this mixture and shaken for 30 min at room temperature. The gel was washed four times with Pierce AminoLink Wash Solution, 1.0 M sodium chloride, and then washed with PBS three times. At this point, the protein was permanently attached to the column.

To purify the antibody, 1 ml of sample containing antibodies at 20 mg/ml was added to the immunoaffinity column with the bottom cap replaced. Once the sample

entered the gel, 0.2 ml of PBS was added. Another 1 ml of PBS was added and the mixture was allowed to stand for 1 h. The column was drained and washed with 16 ml of PBS. Antibodies were eluted from the column with 0.1 M glycine at pH 2.8 and collected in 1 ml fractions. These fractions were neutralized with 1M tris-HCl, pH 9.5. The protein content was determined with a Shimadzu spectrophotometer at A_{280}. Pooled fractions were dialyzed against PBS. Buffer in the column was replaced with 5 ml of degassed 0.05% (w/v) sodium azide solution. The column was sealed and stored at 4°C. The pooled fractions of antibodies were then used immediately in the Western blot technique or stored at -4°C.

4. Results

Results of these studies are presented as 1- and 2-D PAGs and Western blots of leaf proteins hybridized with specific antibodies. The 1-D PAGs were scanned with an LKB Ultroscan densitometer and the data were processed by the Gelscan program. Unfortunately, the results are not as clear as desired. Specific bands on the 1-D gels were faint but distinct immediately after the gels were stained. However, resolution was lost and the bands are mainly nondiscernible in the final photographs. Arrows on the lanes of individual gels indicates the position of bands seen after staining. The gels were scanned while the bands of interest were still visible. Possible reasons for the loss in band resolution include instability of the staining reaction and interference by substances in the gel. The 2-D gels were mostly unsatisfactory but some spots were faintly visible immediately after staining.

Leaf Protein Experiments

Proteins extracted from the leaves of Embryogen-P and the four nonembryogenic genotypes were separated in 89 1-D PAGs. Four protein bands were found in Embryogen-P that were not present in the nonembryogenic genotypes. The MWs

of these bands, EPP1 (embryogenic plant protein), EPP2, EPP3, and EPP4, ranged from approximately 27 to 52 kD (Table 1). One band, NEPP1 (nonembryogenic plant protein), was present in the nonembryogenic genotypes but not present in Embryogen-P (Fig. 1).

Densitograms obtained by scanning these gels are shown in Fig. 2. Peaks were produced by the bands present in the PAGs and the valleys were produced by spaces between the bands. Some densitograms were represented as (a) subtracted from another densitogram (b) to produce (c). Peaks in (c) densitograms represent either peaks that only appear in (b) or differing heights of common peaks. A valley represents the lack of a peak in (b).

Approximately 15 proteins were present in the 2-D PAGs of the embryogenic genotype which were not present in PAGs of the nonembryogenic genotypes. Unfortunately, the resolution of these 2-D PAGs was also poor and further deteriorated during taking and processing the photographs (not presented). Many attempts to produce 2-D PAGs with well-stained, separated protein spots as described by Mayer et al. (1987) failed. Several modifications to this procedure were tried. One modification was changing the storage of the tube gel from a microcentrifuge tube filled with Laemmli buffer to a pocket made from parafilm. After this, a few PAGs were produced that sufficiently separated

Table 1. Molecular weights of unique proteins found in Embryogen-P and the nonembryogenic genotypes.

Protein	Approximate Molecular Weight (kD)
EPP1	52.3
EPP2	43.4
EPP3	39.6
EPP4	27.0
NEPP1	30.0

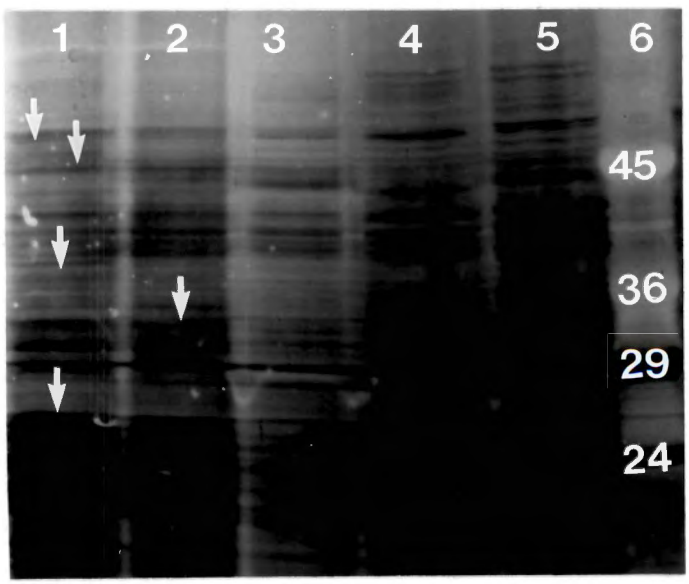

Fig. 1. One-dimensional PAG of leaf proteins. The first lane represents the profile of leaf proteins from Embryogen-P. EPP1 to EPP4 are indicated by arrows in lane 1. Lanes 2 to 5 represent the profiles of leaf proteins from the four nonembryogenic genotypes. NEPP1 is indicated in lane 2 by an arrow. Lane 6 contains known protein markers with their molecular weights indicated.

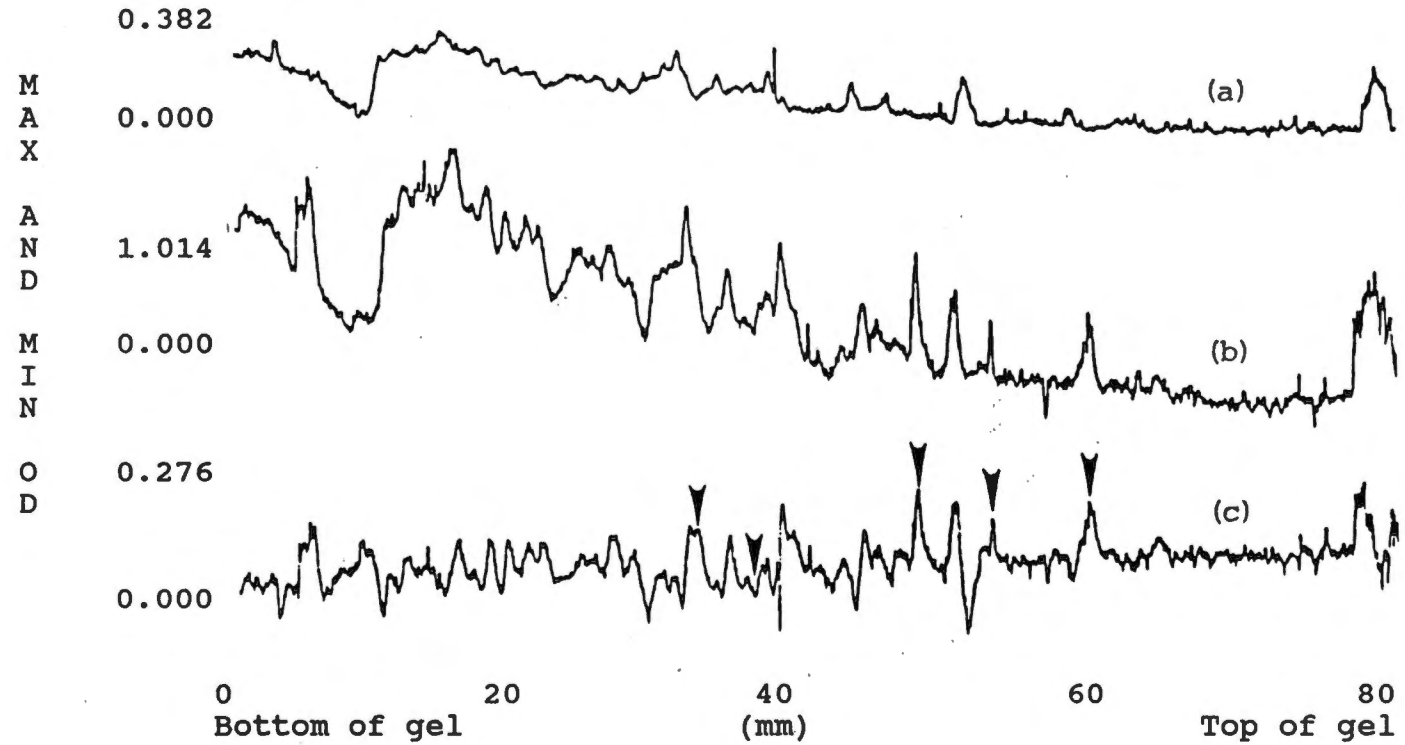

Fig. 2. Densitograms of leaf protein profiles of Embryogen-P and the four nonembryogenic genotypes. Scan (a) represents the average absorbance values (O.D.) for the four nonembryogenic genotypes and (b) the absorbance values (O.D.) for Embryogen-P. Scan (c) represents (a) subtracted from (b). Peaks at 31, 48, 51, and 58 mm of scan (c) correspond to EPP1 to EPP4 and the valley at 38 mm corresponds to NEPP1. These are indicated by arrows. Peaks at 35, 37, 41, and 49 may correspond to differences in the amounts of proteins produced by Embryogen-P and the nonembryogenic genotypes. Scan (a) corresponds to lanes 2 through 5 and scan (b) corresponds to lane 1 presented in Fig. 1.

the proteins but the protein spots were small and lightly-stained. To improve the resolution of the 2-D PAG, proteins were concentrated. The methods used were ultrafiltration, dialysis against PEG, lyophilization, dialysis against water then lyophilization, and ammonium sulfate, acetone, trichloroacetic acid, and ethanol precipitation. Increasing the amount of protein did not improve the resolution. Table 2 lists the approximate weights and isoelectric points of the 15 proteins. The range in MWs of these proteins were approximately the same as those of EPP1 through EPP4.

Polyacrylamide gels of proteins extracted from the inner two leaves of a tiller cut into 2 cm sections from the base to the tip were performed to detect protein differences between Embryogen-P and the nonembryogenic genotypes along the entire length of the leaf (Figs. 3 and 4). There was an increase in the amount of proteins from the base to the tip of the Embryogen-P leaves but there was no increase in the amount of proteins for the nonembryogenic leaf sections (Figs. 5 and 6).

Protein profiles of the basal sections of four consecutive leaves, from the inner to outer, were performed to detect protein differences within a tiller of Embryogen-P and the nonembryogenic genotypes (Figs. 7 and 8). There was a difference in the densitograms when

Table 2. Molecular weights and isoelectric points of 15 proteins found in Embryogen-P leaves separated by 2-D PAGE and not in those of the nonembryogenic genotypes.

Group No.	Approximate Molecular Weight (kD)	Approximate Isoelectric Point	No. of Proteins
1	16	5.0	1
2	18.4	5.0	2
3	28	5.0	1
4	34-36	5.0	6
5	35	5.8	1
6	35	6.1	2
7	52	6.5	2

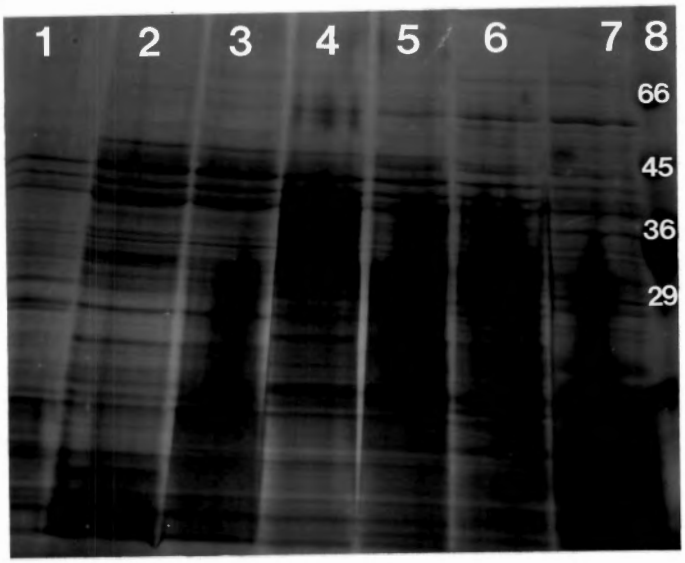

Fig. 3. One-dimensional PAG of proteins from Embryogen-P leaves sectioned from the base to the tip. Lane 1 represents the basal section and lane 7 represents the tip section. Lane 8 contains known protein markers with their molecular weights indicated.

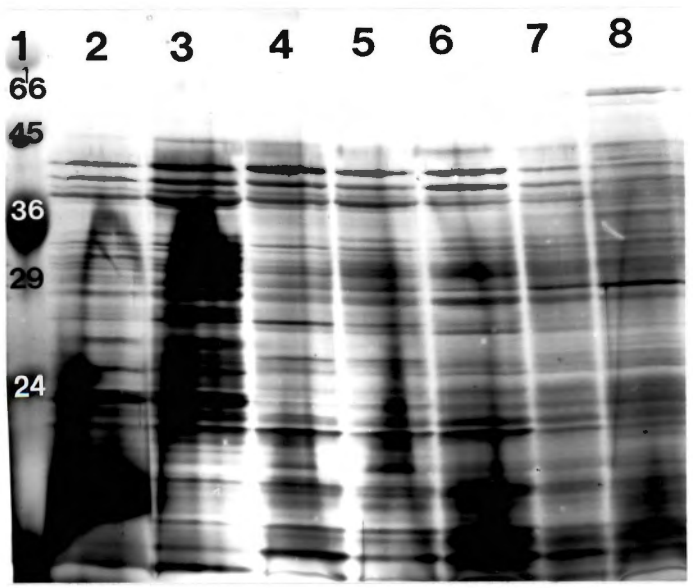

Fig. 4. One-dimensional PAG of leaf proteins from a nonembryogenic genotype. The leaves were sectioned from the base to the tip. Lane 2 represents the basal section and lane 8 represents the tip section. Lane 1 contains known protein markers with their molecular weights indicated.

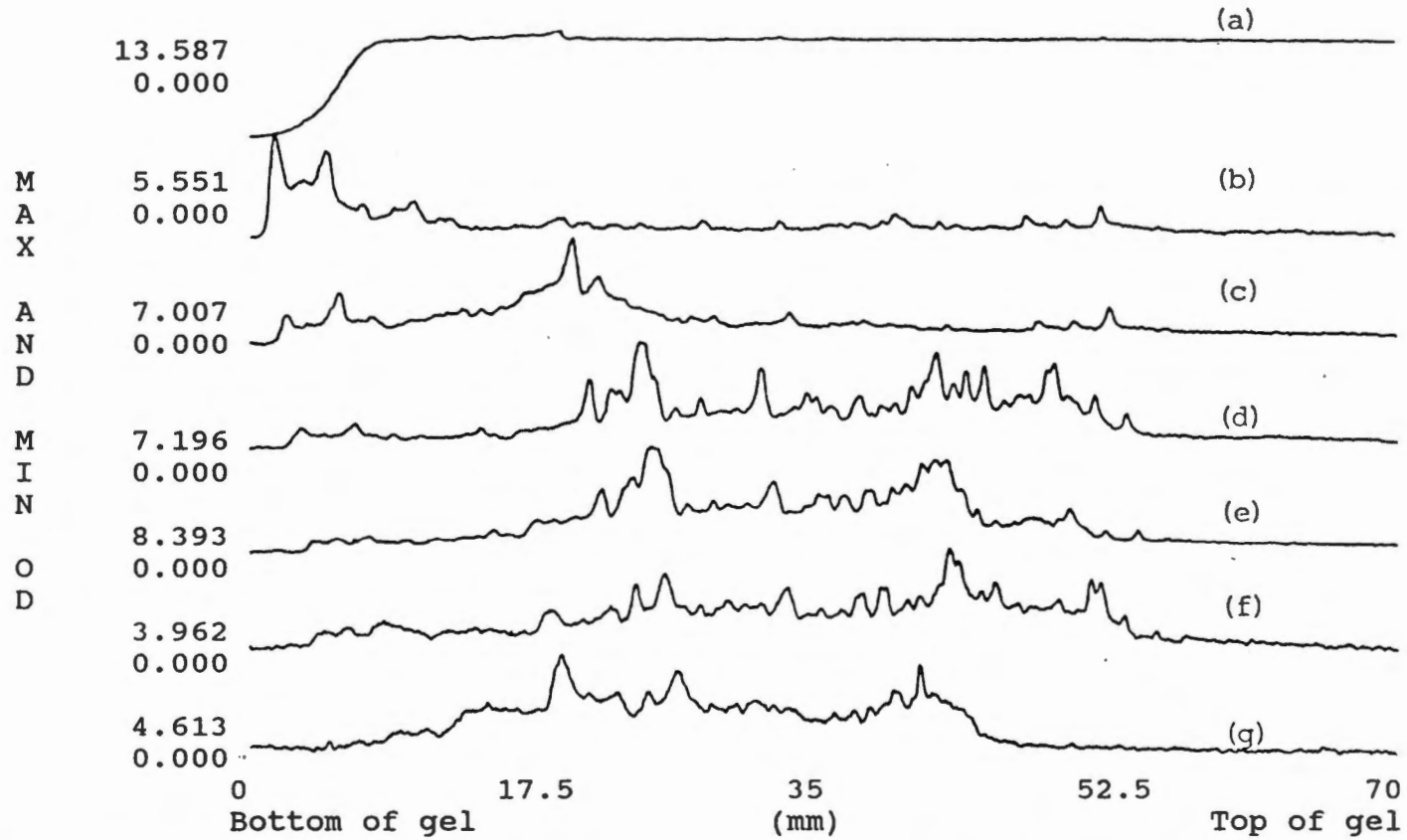

Fig. 5. Densitograms of Embryogen-P protein profiles of leaves sectioned from the base to the tip. Scan (a) is the most basal and (g) is the most distal section. The LKB Ultroscan was unable to detect bands in the profile of scan (a) due to the faint staining pattern. These scans (a through g) correspond to lanes 1 through 7, respectively, presented in Fig. 3.

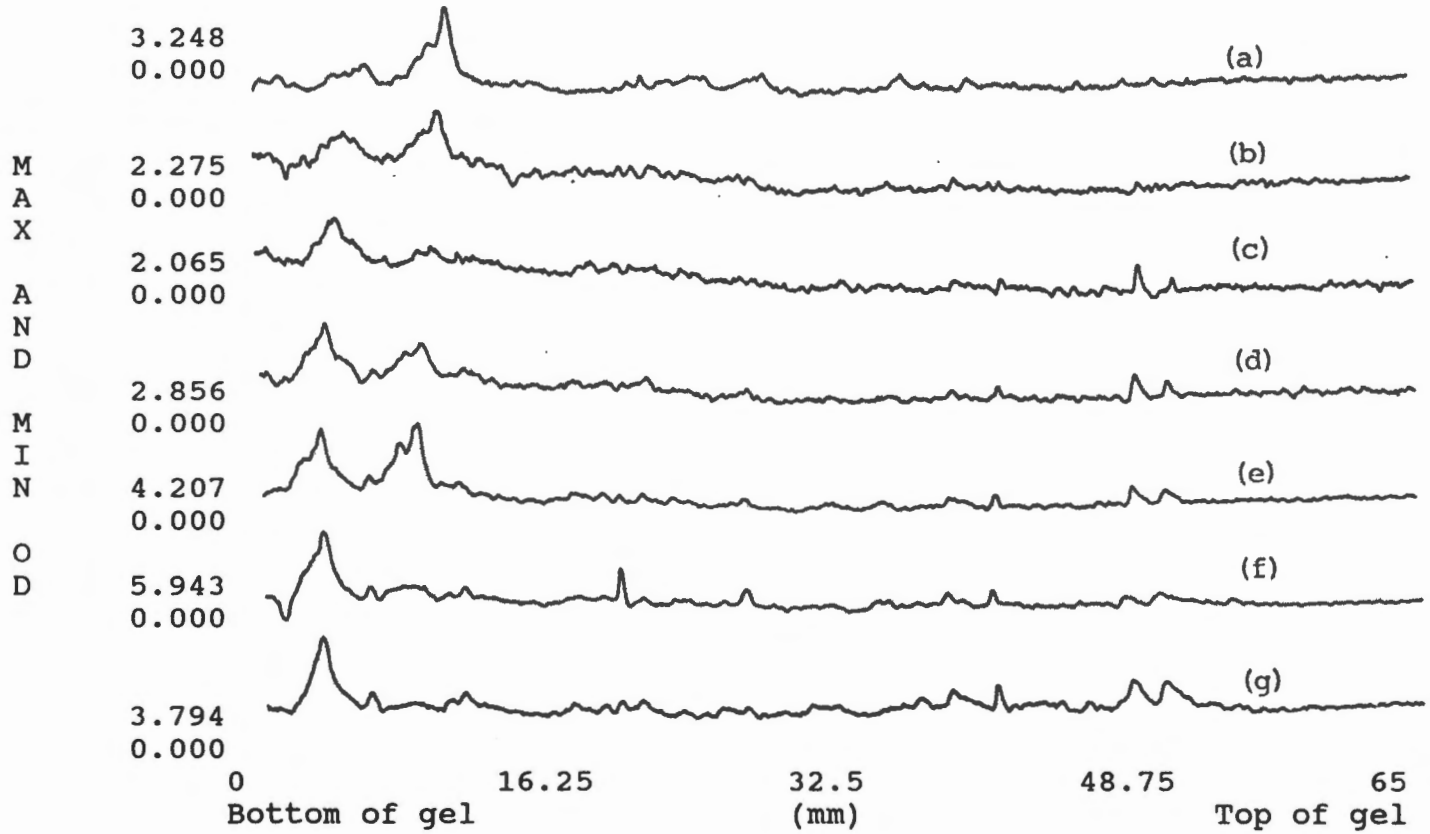

Fig. 6. Densitograms of the nonembryogenic genotype protein profiles of leaves sectioned from the base to the tip. Scan (a) is the most basal and (g) is the most distal section. These scans (a through g) correspond to lanes 2 through 8, respectively, presented in Fig. 4.

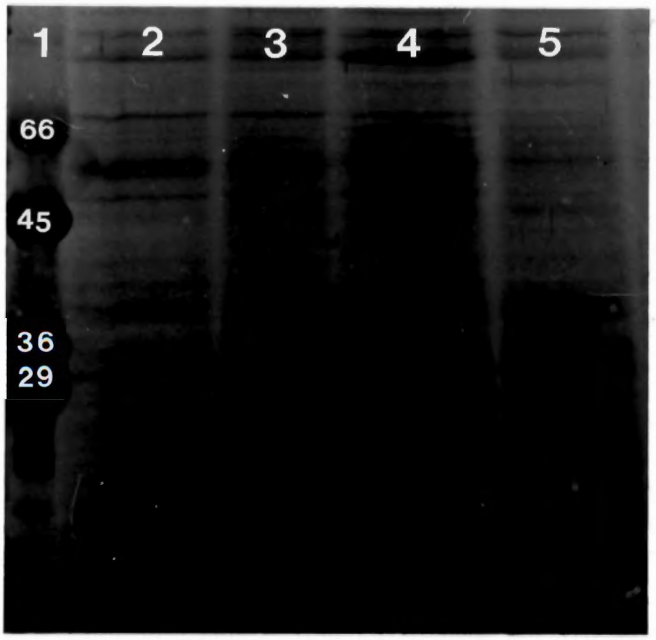

Fig. 7. One-dimensional PAG of leaf base proteins from an Embryogen-P tiller. Lanes 2 to 5 are the profiles for the inner to outer leaves, respectively. Lane 1 contains known protein markers with their molecular weights indicated.

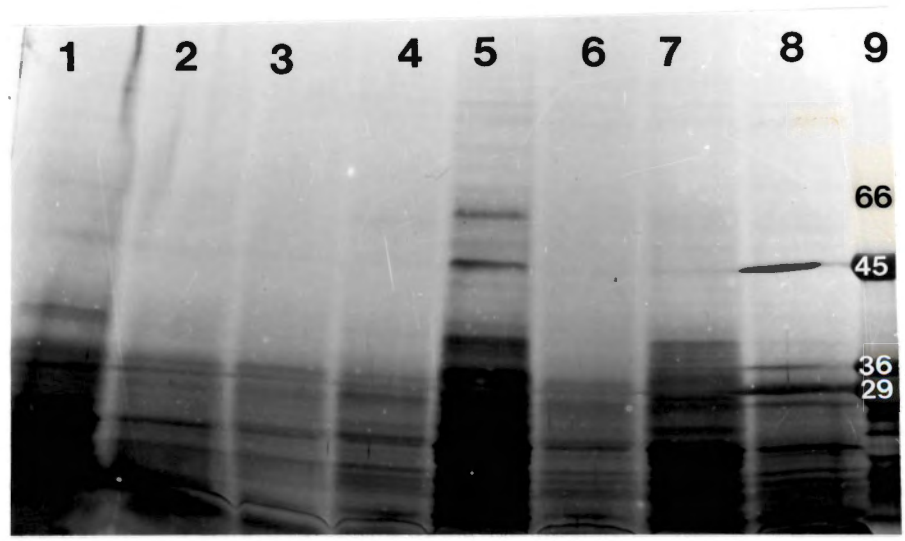

Fig. 8. One-dimensional PAG of leaf base proteins from tillers of two nonembryogenic genotypes. Lanes 1 to 4 are the profiles for the inner to outer leaf bases from a tiller of one nonembryogenic genotype and lanes 5 to 8 are the profiles of the second. Lane 9 contains known protein markers with their molecular weights indicated.

comparing the inner to the outer leaf bases of Embryogen-P (Fig. 9). There were minor differences in the densitograms of the inner to outer leaf bases of either nonembryogenic genotypes (Fig. 10).

Embryogenic and nonembryogenic F_1 offspring from crosses between Embryogen-P and a nonembryogenic genotype were compared to investigate the possibility that the five proteins found in the first study would also be found in them. Four proteins were found in the embryogenic F_1 progeny that were not present in the nonembryogenic progeny (Fig 11 and 12). The MWs of these four proteins were the same as those of the four proteins found in Embryogen-P. One protein was found in the nonembryogenic progeny that was not present in the embryogenic progeny (Table 3). Its MW was the same as that of the protein found in the nonembryogenic genotypes.

Suspension Culture Experiments

Proteins extracted from calli in suspension cultures were separated on 25 PAGs to identify those associated with embryogenesis. Five bands were found in the profiles of calli from SH-30 medium supporting embryogenesis that were not found in those from medium not supporting embryogenesis (Figs. 13 and 14, Table 4). The addition of casein hydrolysate to the medium supported embryogenesis.

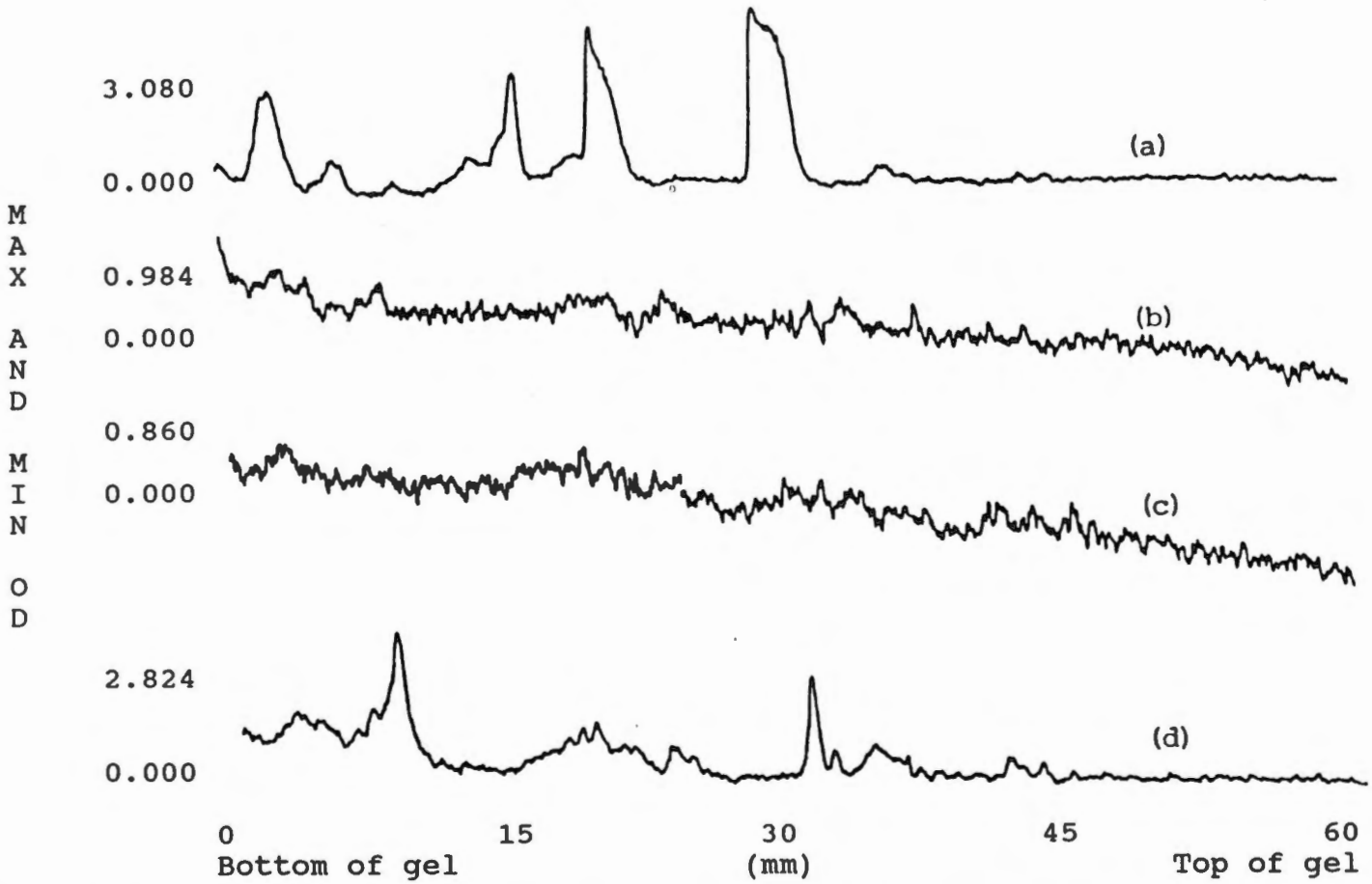

Fig. 9. Densitograms of leaf base protein profiles from Embryogen-P. Scans (a) to (d) represent the profiles of the inner to the outer leaf bases, respectively. Increases in the amount of certain proteins are indicated by peaks at 7, 17, 18, 19, 26, 31, and 33 mm in (d). Scans (a) and (b) combined correspond to scan (a) in Fig. 5.

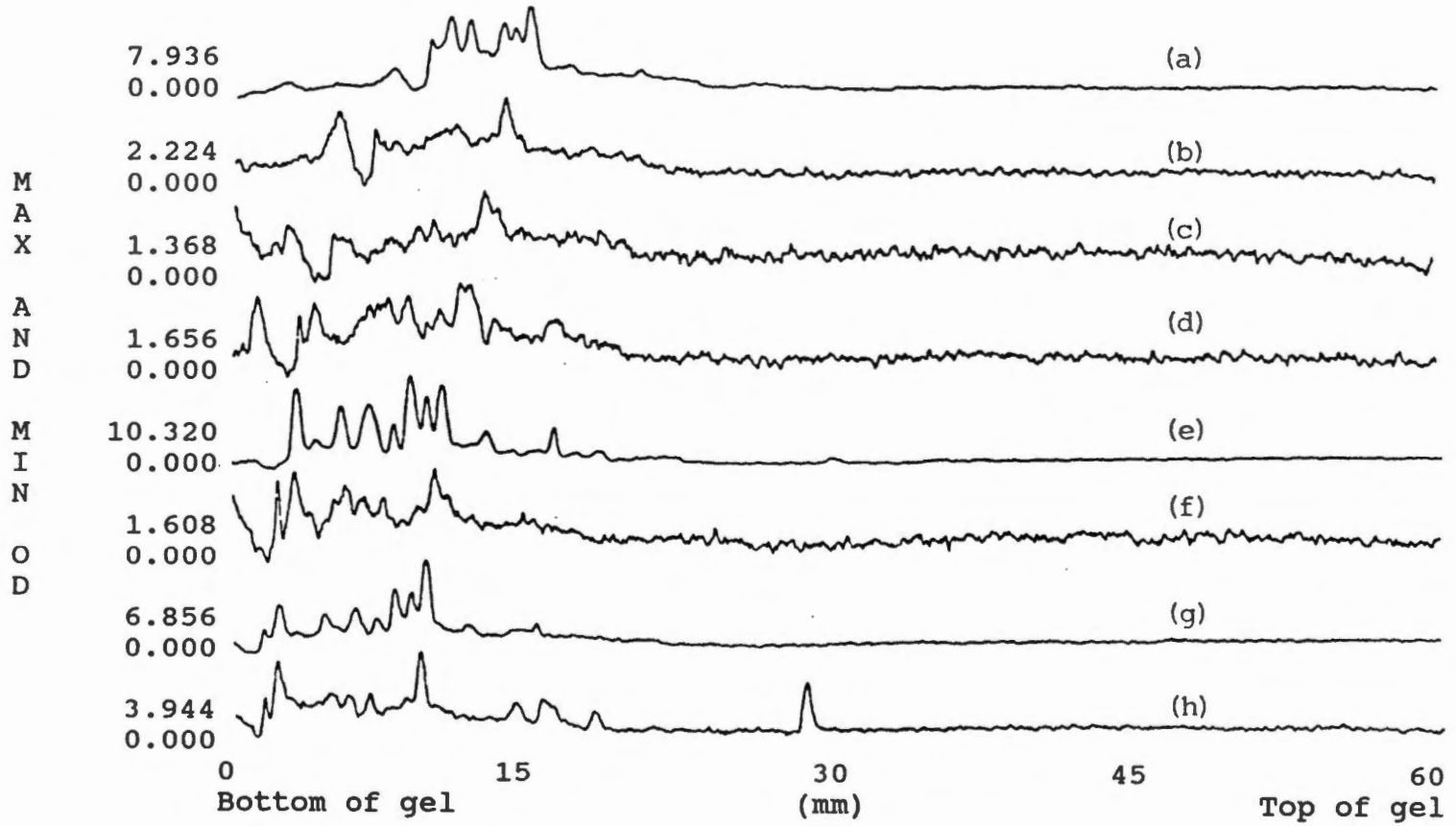

Fig. 10. Densitograms of leaf base protein profiles for two nonembryogenic genotypes. Scans (a) to (d) represent the profiles of the inner to the outer leaf bases from the tiller of one nonembryogenic genotype. Scans (e) to (h) represent the profiles of those from a second nonembryogenic genotype. Scans (a) and (b) combined and scans (e) and (f) combined correspond to scan (a) in Fig. 6.

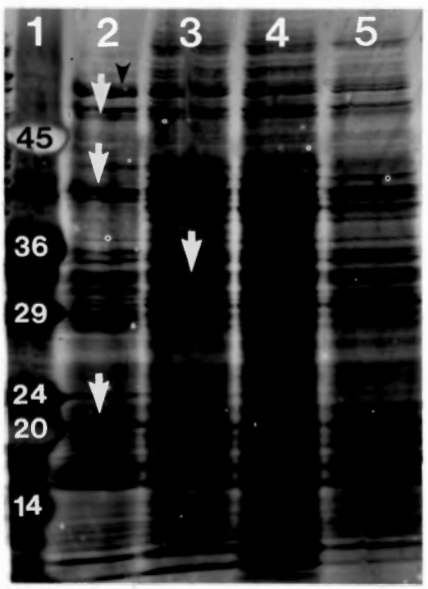

Fig. 11. One-dimensional PAG of leaf proteins from embryogenic and nonembryogenic progeny. Lanes 2 and 3 are profiles of leaf proteins from the embryogenic and nonembryogenic progeny of one cross and lanes 4 and 5 are those from the reciprocal cross. Arrows indicate EPP1 through EPP4 in lane 2 and NEPP1 in lane 3. Lane 1 contains known protein markers with their molecular weights indicated.

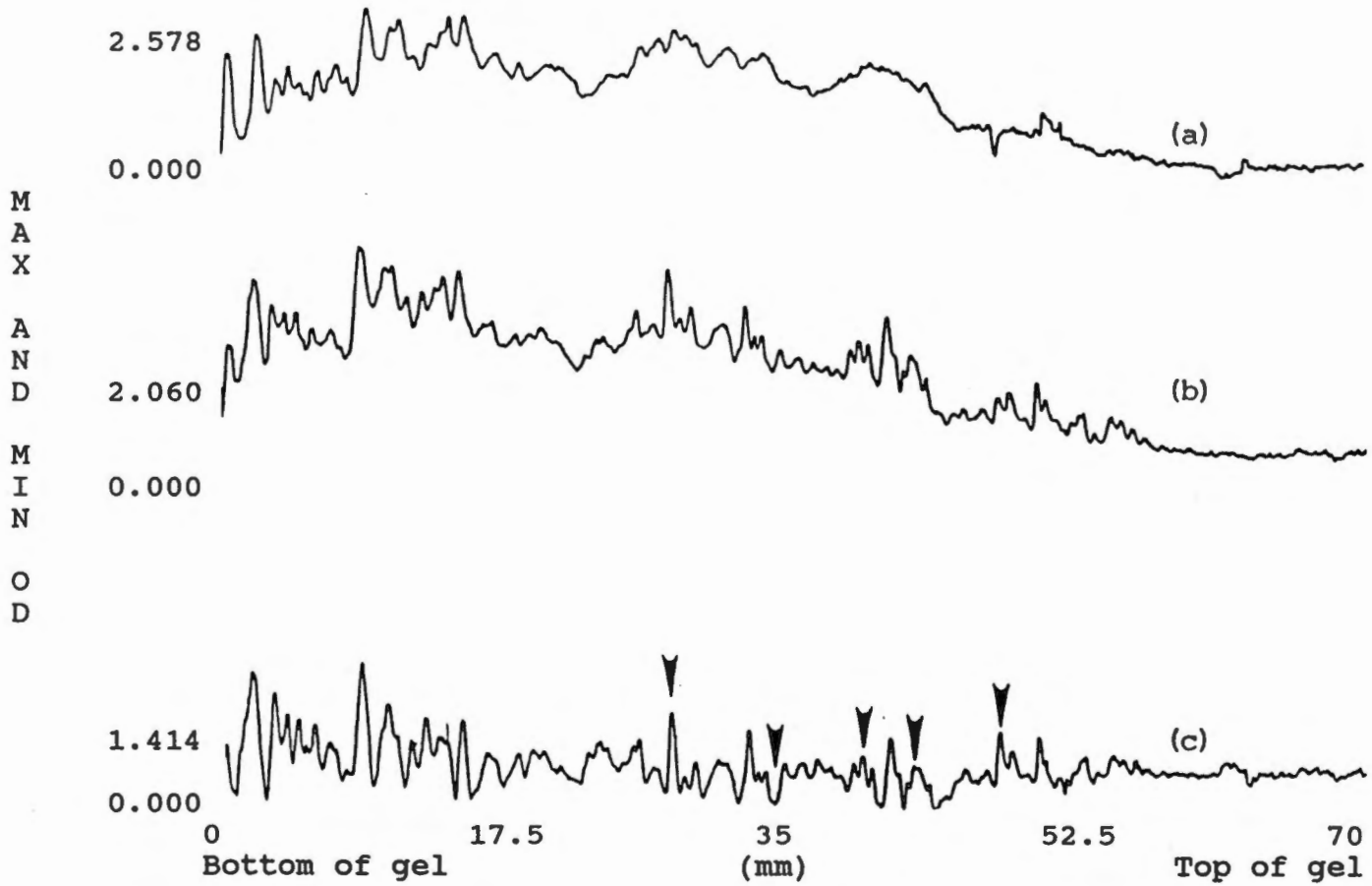

Fig. 12. Densitograms of leaf protein profiles of nonembryogenic (scan a) and embryogenic (scan b) progeny. Scan (c) is (a) subtracted from (b). Peaks at 28, 39, 42, and 47 mm in (c) represent the 4 proteins, EPP1 to EPP4 are indicated by arrows. The valley at 35 mm represents NEPP1 and is indicated by an arrow.

Table 3. Molecular weights of unique proteins found in the embryogenic (EPP) and the nonembryogenic (NEPP) F_1 plants from a cross between Embryogen-P and a nonembryogenic genotype.

Proteins	Molecular Weight (kD)
EPP1	52.3
EPP2	43.4
EPP3	39.6
EPP4	27.0
NEPP1	30.0

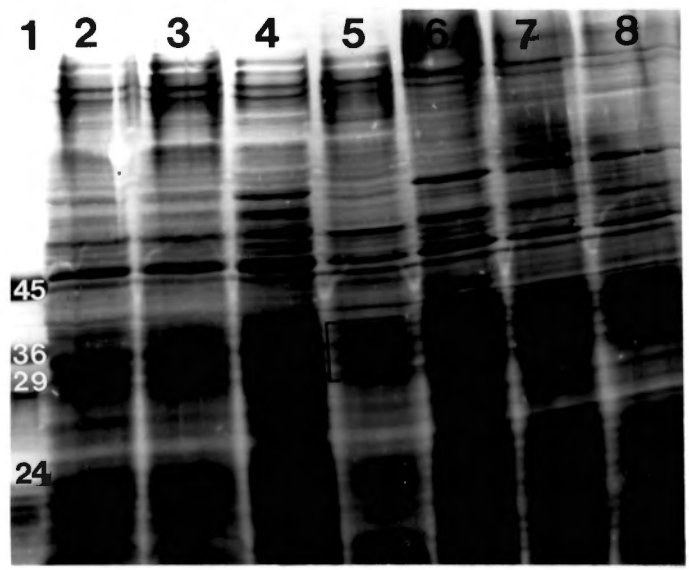

Fig. 13. One-dimensional PAG of Embryogen-P callus proteins. Lanes 2 to 4 represent the protein profiles of callus in medium not supporting embryogenesis. Lanes 5 to 8 represent profiles for callus in medium supporting embryogenesis. The area where the five proteins are present in the embryogenic callus profile is indicated by a bracket in lane 5. Lane 1 contains known protein markers with their molecular weights indicated.

Table 4. Molecular weights of embryogenic callus proteins (ESCP) found in Embryogen-P suspension cultures in medium supporting embryogenesis and not found in those in medium not supporting embryogenesis.

Proteins	Molecular Weight (kD)
ESCP1	36.0
ESCP2	35.9
ESCP3	35.8
ESCP4	35.7
ESCP5	35.5

Medium without casein hydrolysate did not support embryogenesis.

Liquid SH-30 medium containing $(NH_4)_2SO_4$ to support embryogenesis was used to determine if the callus would produce the same five proteins. Medium for the nonembryogenic cultures did not contain $(NH_4)_2SO_4$. The five proteins found in the embryogenic callus had the same MWs as ESCP1 (embryogenic suspension culture protein) through ESCP5 (Figs. 15 and 16, Table 4).

Time Course Experiments

These experiments were performed to investigate if unique proteins were produced during tissue culture which could be associated with somatic embryogenesis. When protein profiles of leaves cultured for 2, 4, 7, 10, 14, and 21 d were compared, nine bands, CLP7-1 (cultured leaf protein, number of days in culture-protein number) to CLP7-9, were found in leaves at 7 d after culture that were not present at 2 or 4 d after culture (Figs. 17 and 18, Table 5). Two proteins, CLP10-1 and CLP10-2, that did not appear after 2, 4, or 7 d, were present 10 d after culture (Figs. 17 and 19). One protein, CLP14-1, which was not found after 2, 4, 7, or 10 d, was present after 14 d (Figs. 17 and 19).

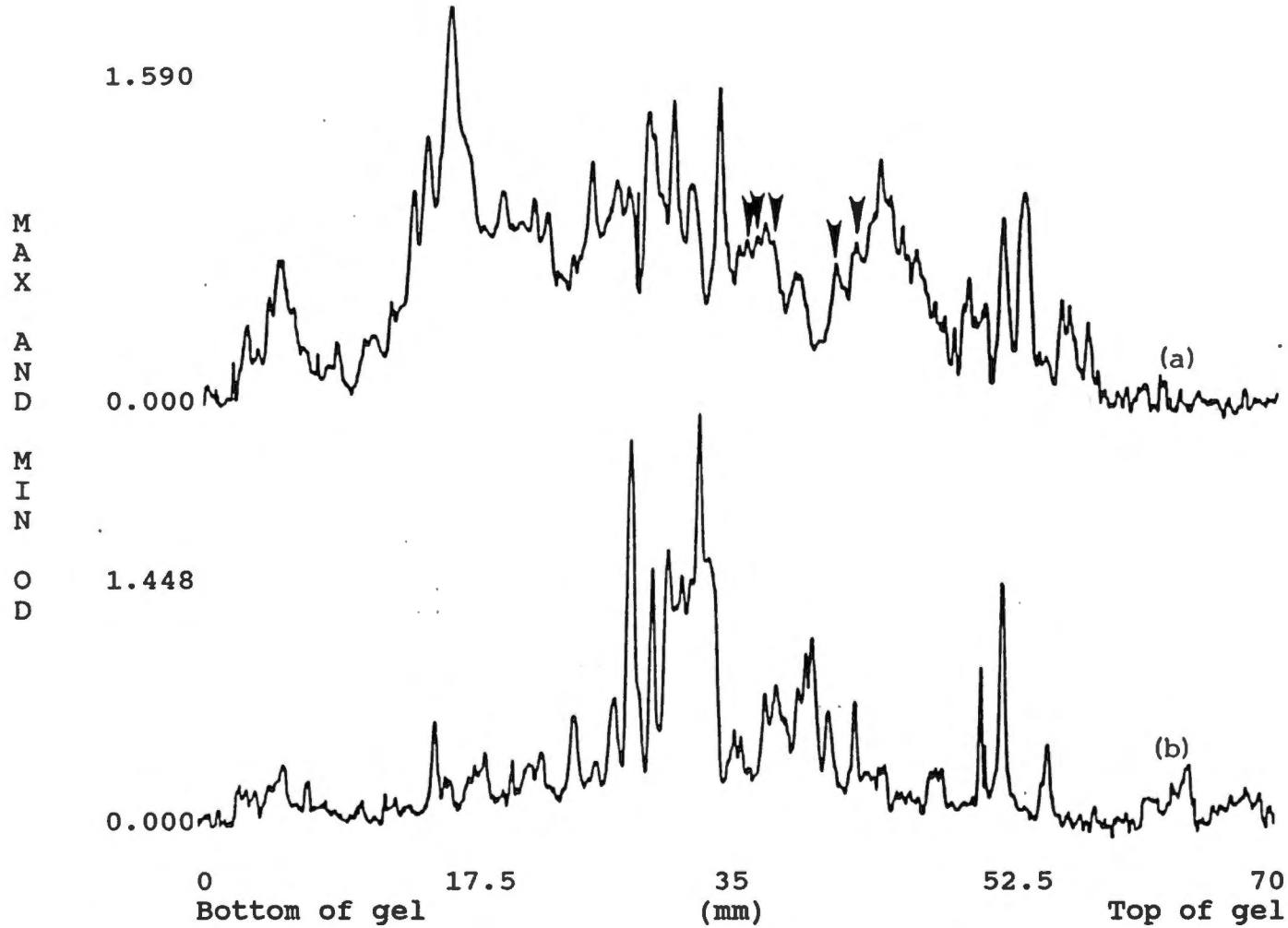

Fig. 16. Densitograms of callus protein profiles from suspension cultures with or without ammonium sulfate. Scan (a) represents the embryogenic callus protein profile and (b) represents the nonembryogenic callus. Peaks between 35 and 41 mm in (a) correspond to ESCP1 to ESCP5 and are indicated by arrows.

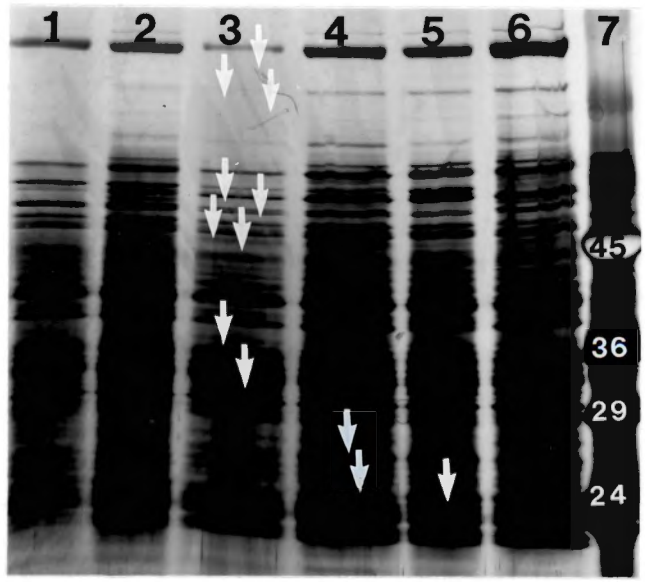

Fig. 17. One-dimensional PAG of protein profiles from cultured Embryogen-P leaves on SH-30 medium. Lanes 1 to 6 represent profiles for leaves harvested at 2, 4, 7, 10, 14, and 22 d after the initiation of culture, respectively. CLP7-1 (67.4 kD) to CLP7-9 (32.3 kD) are indicated in lane 3, CLP10-1 and CLP10-2 in lane 4, and CLP14-1 in lane 5. Lane 7 contains known protein markers with their molecular weights indicated.

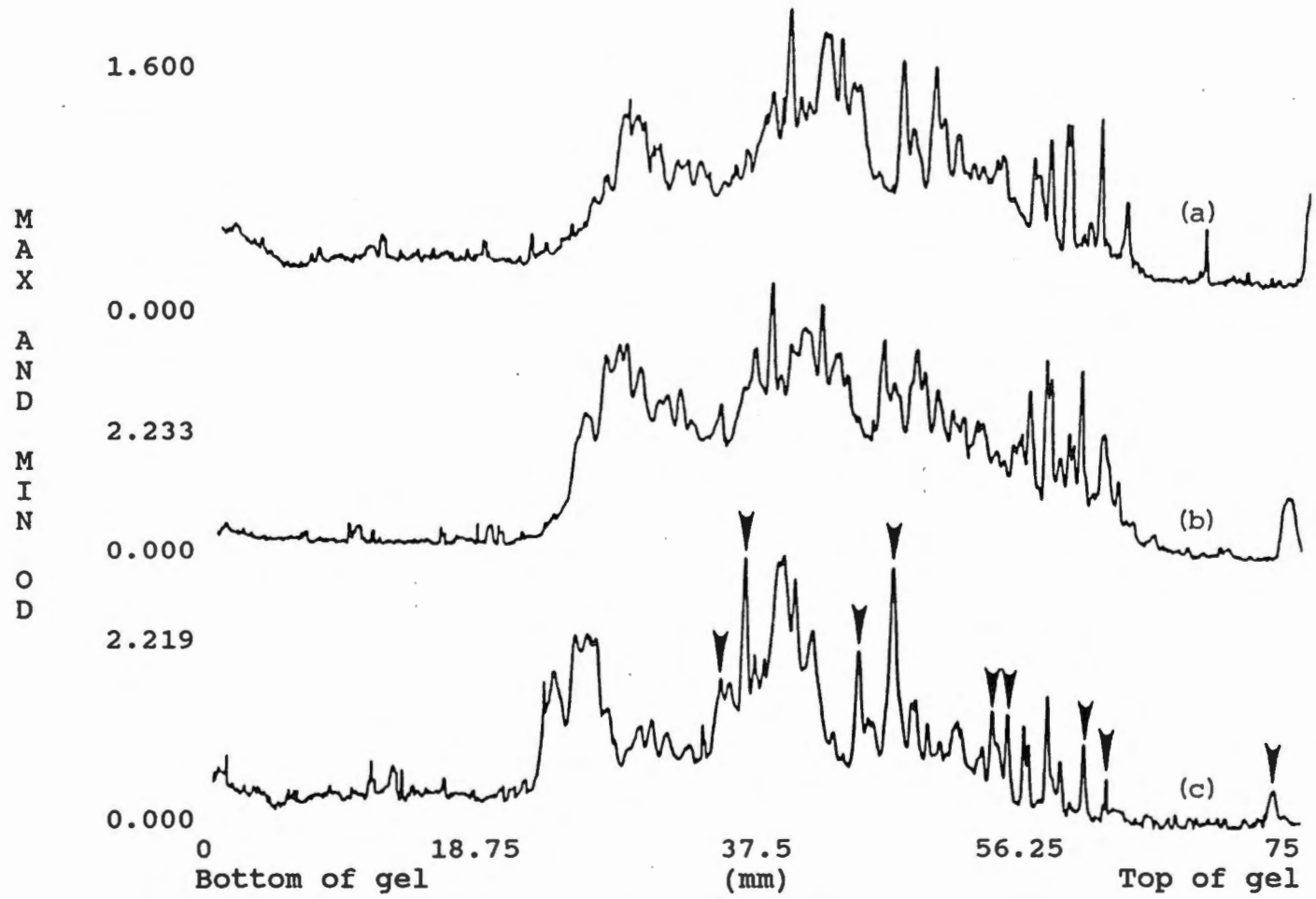

Fig. 18. Densitograms of protein profiles from Embryogen-P leaves cultured on SH-30 medium for 2, 4, or 7 d (scans a to c, respectively). Peaks at 36, 37, 45, 48, 54, 55, 62, 63, and 73 mm in (c) correspond to CLP7-1 to CLP7-9.

Table 5. Unique proteins from leaves cultured for different intervals of time.

Proteins	Days in Culture	Molecular Weight (kD)
CLP7-1	7	67.4
CLP7-2	7	65.7
CLP7-3	7	64.0
CLP7-4	7	49.0
CLP7-5	7	48.2
CLP7-6	7	46.5
CLP7-7	7	39.0
CLP7-8	7	33.1
CLP7-9	7	32.3
CLP10-1	10	27.3
CLP10-2	10	26.5
CLP14-1	14	25.6

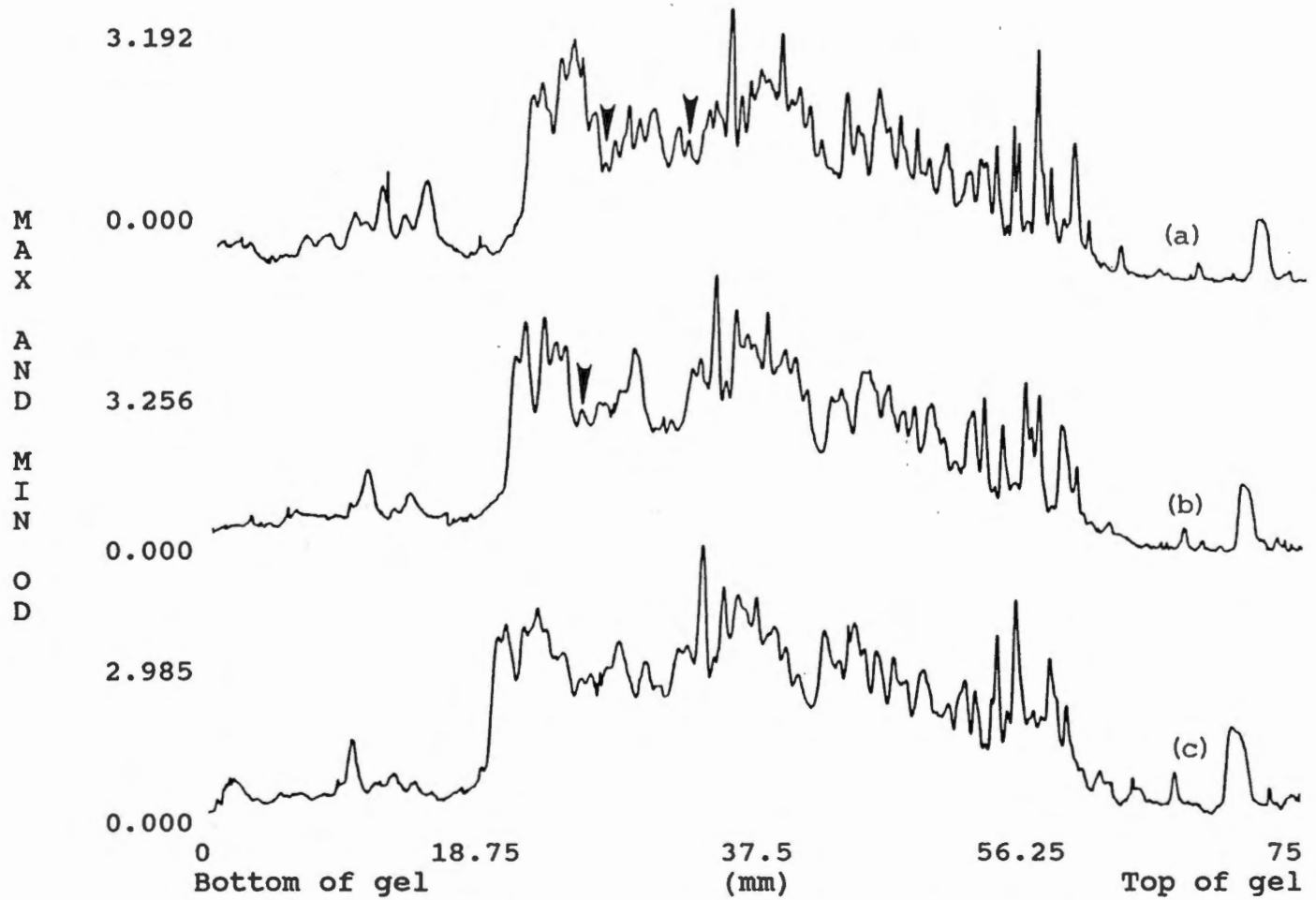

Fig. 19. Densitograms of protein profiles from Embryogen-P leaves cultured on SH-30 medium for 10, 14, or 21 d (scans a to c, respectively). Peaks at 25 and 30 mm of (a) correspond to CLP10-1 and CLP10-2. A peak at 23 mm of (b) corresponds to CLP14-1.

Abscisic Acid Experiments

Abscisic acid was added to the medium to investigate its effect on protein synthesis during culture. A total of seven proteins were found to be produced during these culture periods (Table 6). Four, ALP1-1 (abscisic acid leaf protein, number of days in culture - protein number) through ALP1-4, were found in leaves 1 d after culture (Figs 20, 21, and 22). These were not found in leaves that were not cultured. Three proteins, ALP2-1 to ALP2-3, were identified after 2 d culture that were not present at 1 d after culture or in leaves that were not cultured. Three proteins found in the ABA experiment (ALP1-1, ALP1-3, and ALP1-4) had the same MWs as three proteins from the time course experiment (CLP7-1, CLP7-5, and CLP7-6) (Fig. 23).

Immunological Experiments

These experiments were performed to confirm the results in the Leaf Protein Experiments. The titer of the rabbit serum was acceptable for enzyme-linked immunosorbent assay (ELISA) and Western blot techniques after the third or fourth booster shot. Western blots with both the embryogenic and nonembryogenic sera hybridized well with their respective protein profiles and

Table 6. Unique proteins from leaves cultured for 1 and 2 d on medium containing abscisic acid that were not present in leaves cultured on medium without abscisic acid for 1 or 2 d.

Proteins	Days in Culture	Molecular Weight (kD)
ALP1-1	1	68.6
ALP1-2	1	53.6
ALP1-3	1	48.5
ALP1-4	1	46.5
ALP2-1	2	58.6
ALP2-2	2	54.6
ALP2-3	2	43.5

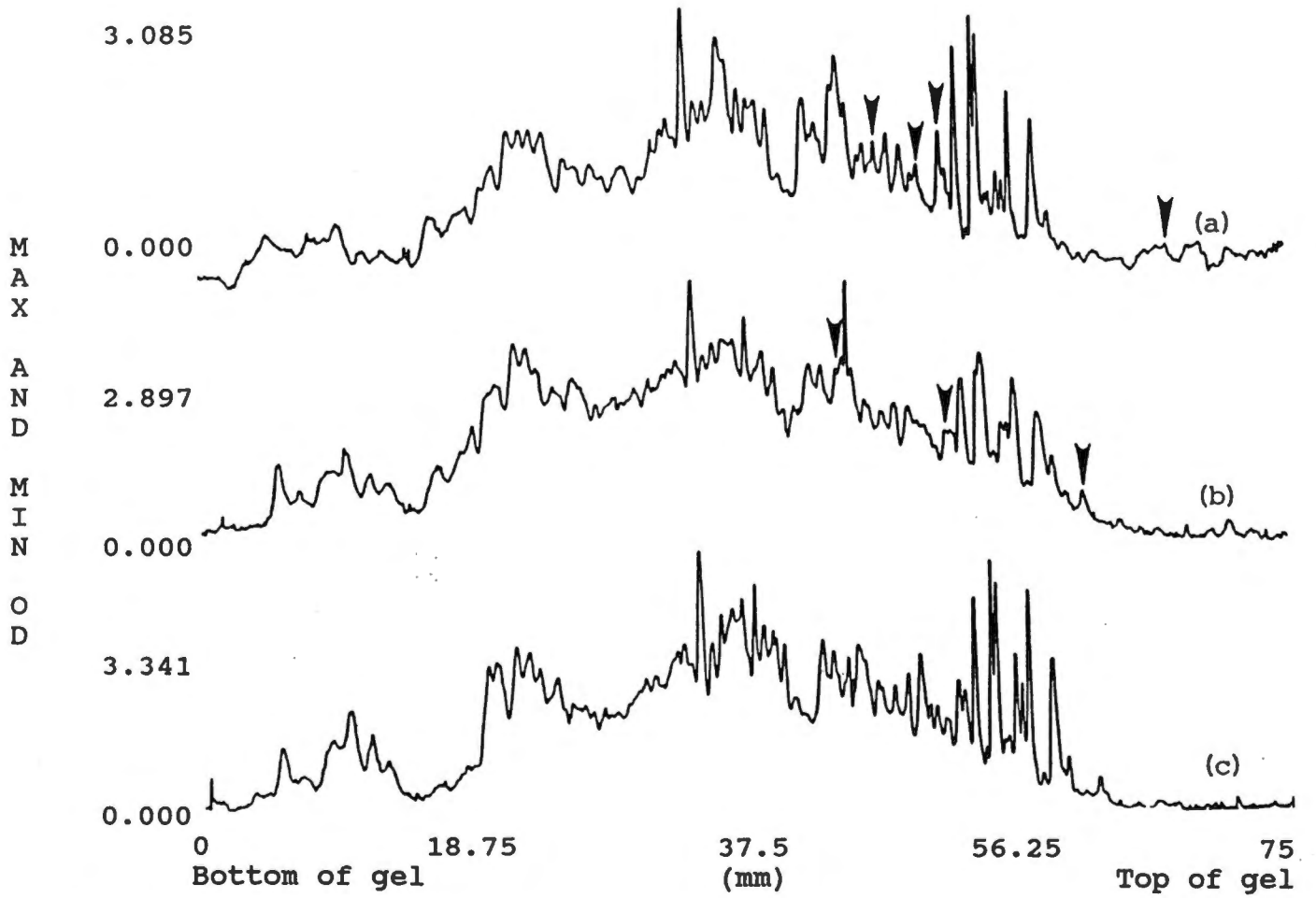

Fig. 21. Densitograms of protein profiles from Embryogen-P leaves cultured on SH-30 medium with ABA for 1, 2, or 3 d (scans a to c, respectively). Peaks at 47, 50, 52, and 60 mm of (a) correspond to ALP1-1 to ALP1-4. Peaks at 44.5, 53, and 58 mm of (b) correspond ALP2-1 to ALP2-3.

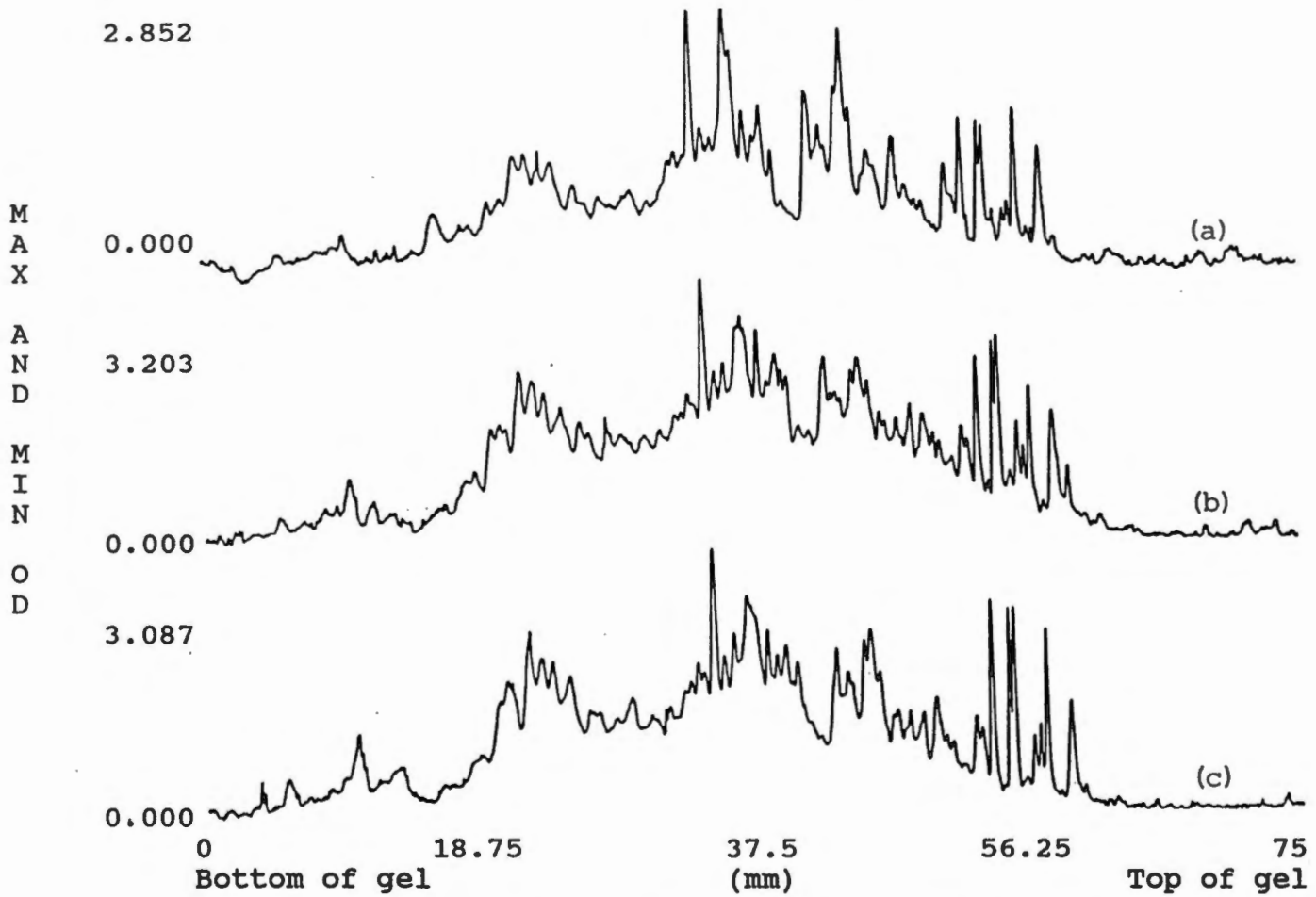

Fig. 22. Densitograms of protein profiles from Embryogen-P leaves cultured on SH-30 medium without ABA for 1, 2, or 3 d (scans a, b, and c respectively).

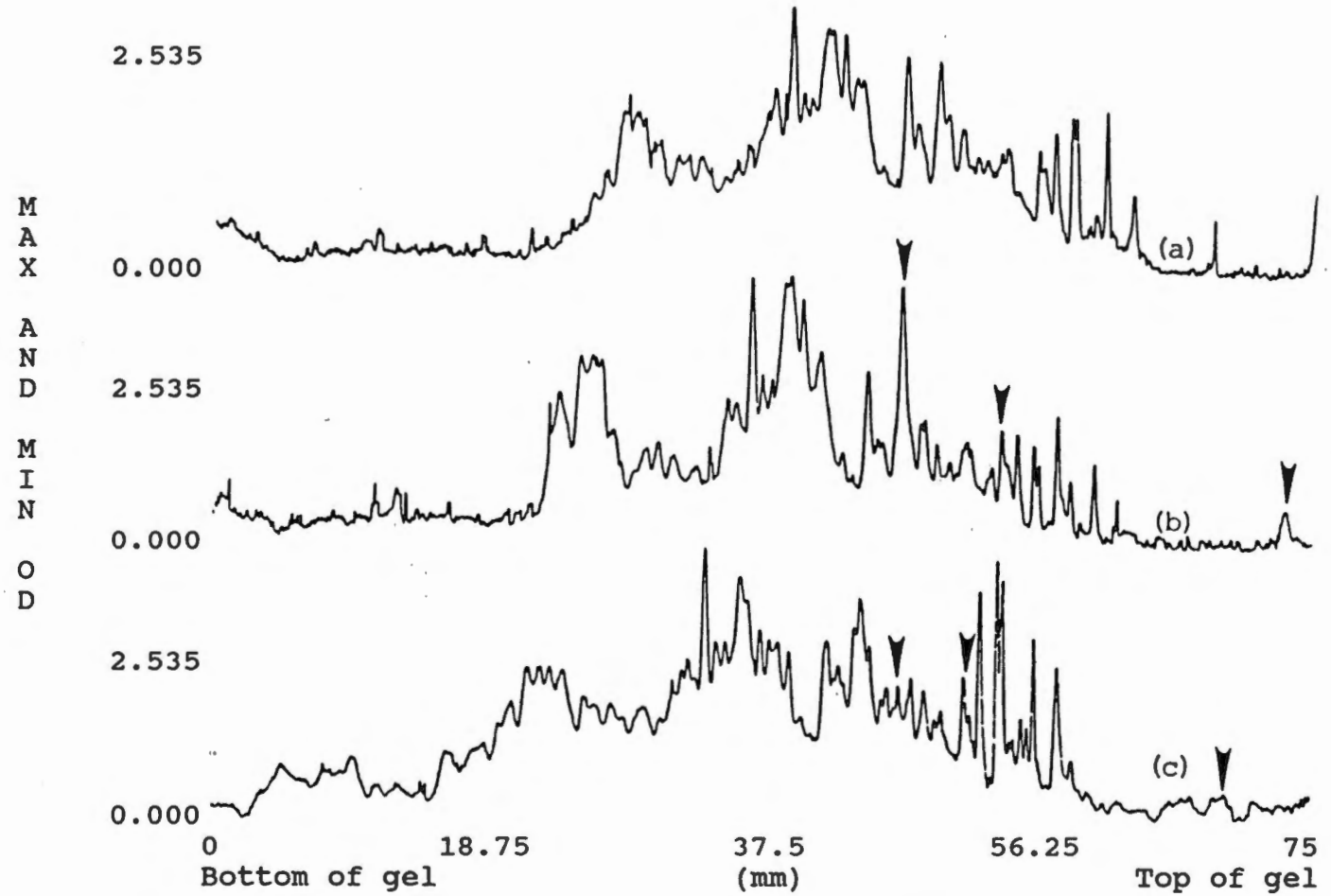

Fig. 23. Densitograms of protein profiles from Embryogen-P leaves cultured on SH-30 medium without ABA for 2 and 7 d (scans a and b) and with ABA for 1 d (scan c). Peaks at approximately 48, 54, and 73 mm on (b) and (c) correspond to similar proteins between the two studies. The right side of scan (c) is shifted slightly to the left due to two different PAGE conditions.

also with the opposite protein profiles except that some bands were missing (Figs. 24 and 25).

Sera from Embryogen-P and the nonembryogenic genotypes were crossreacted using IA-PAG. Leaf proteins of Embryogen-P and the nonembryogenic genotype were separated in 1-D PAGs and attached to nitrocellulose membranes. In the crossreacted serum specific for Embryogen-P leaf proteins hybridized to the embryogenic and nonembryogenic protein profiles, EPP1 through EPP4 were not detected (Fig. 26). Bands that were present in all the profiles may represent proteins that are produced in greater abundance in the embryogenic than in the nonembryogenic genotype.

The crossreacted serum specific for a nonembryogenic genotype leaf protein hybridized to the five protein profiles as shown (Fig. 27) by the presence of all the red bands in both the embryogenic and nonembryogenic leaf protein profiles.

The antibodies in the crossreacted sera were attached to latex beads and used to purify the EPP1, EPP2, EPP3 and EPP4 from the total embryogenic and NEPP1 from the total nonembryogenic leaf extracts. These purified proteins were separated by PAGE (Fig. 28). Bands for EPP1 through EPP4 and NEPP1 were indistinguishable in this PAG. EPP1 through EPP4 and NEPP1 proteins were used to purify

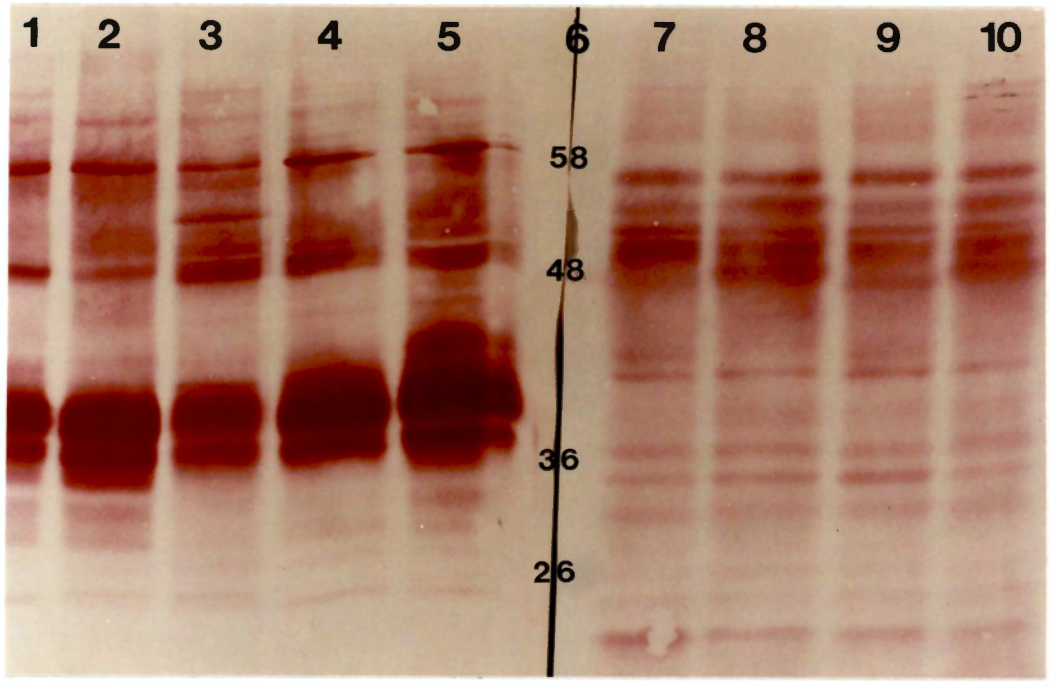

Fig. 24. Western blots of embryogenic and nonembryogenic leaf proteins hybridized with total antibodies specific for Embryogen-P leaf proteins. Lanes 1 and 7 represent the protein profiles from the basal sections of Embryogen-P leaves. Lanes 2 to 5 and 8 to 10 represent the protein profiles of basal leaf sections from the nonembryogenic genotypes. The western blot on the left represents the antibodies from one rabbit and the right blot represents those from a second rabbit. Lane 6 contains known protein markers with their molecular weights indicated.

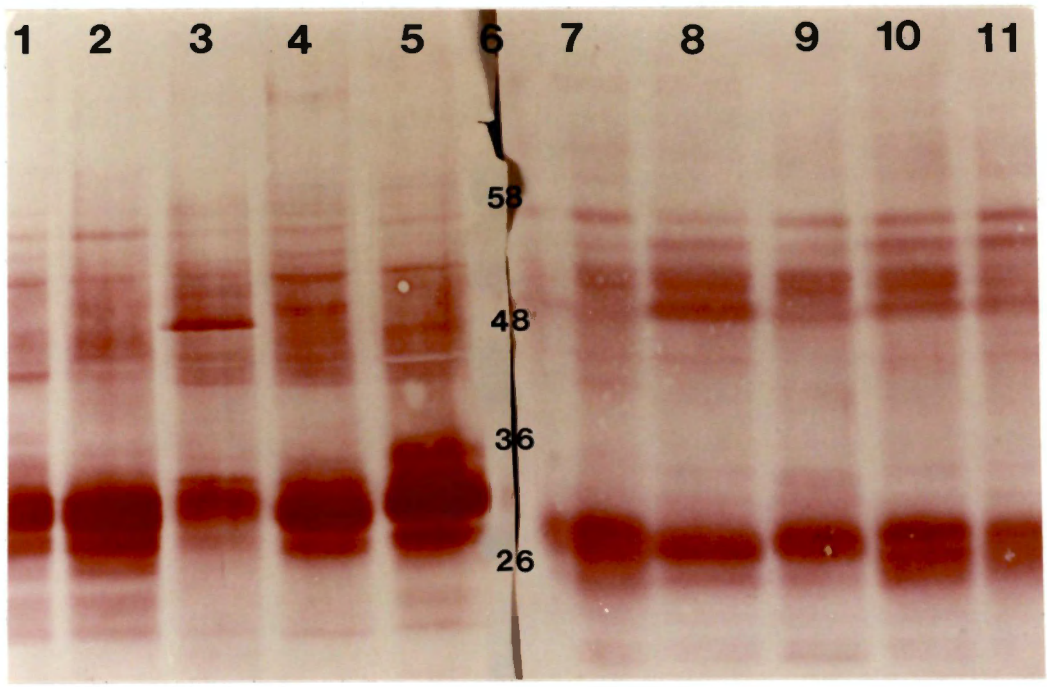

Fig. 25. Western blot of embryogenic and nonembryogenic leaf proteins hybridized with antibodies specific for the nonembryogenic leaf proteins. Lanes 1 and 7 represent the protein profiles from the basal section of Embryogen-P leaves. Lanes 2 to 5 and 8 to 11 represent the protein profiles of basal leaf sections from the nonembryogenic genotypes. The western blot on the left represents the antibodies from one rabbit and the right blot represents those from a second rabbit. Lane 6 contains known protein markers with their molecular weights indicated.

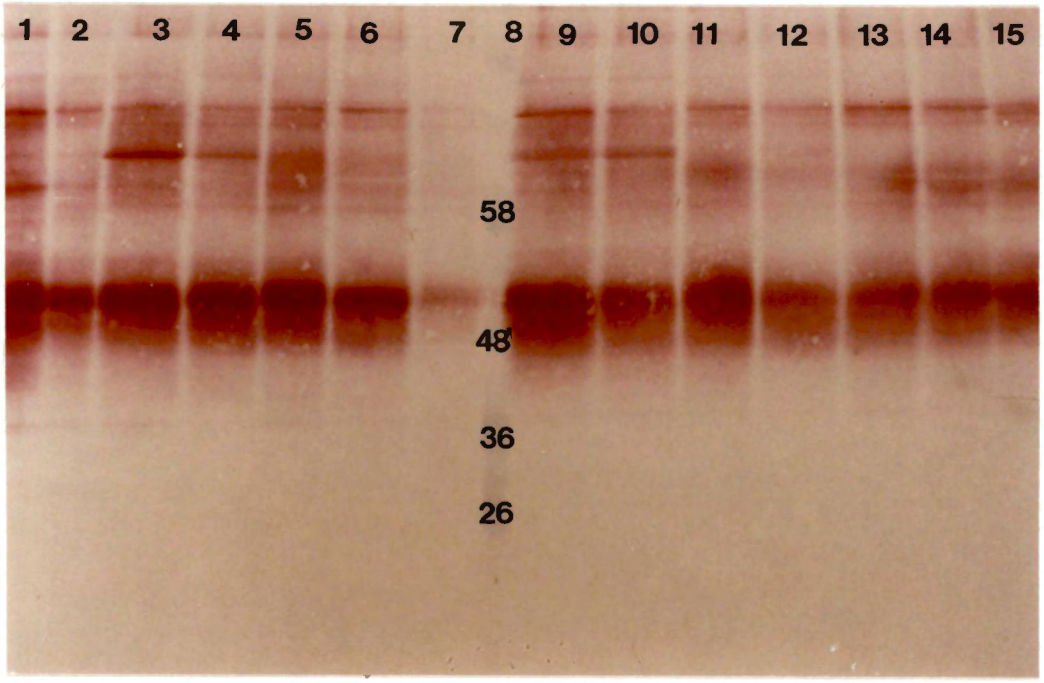

Fig. 26. Western blot of embryogenic and nonembryogenic leaves sectioned from the base to the tip hybridized with crossreacted antibodies specific for Embryogen-P leaf proteins. Lanes 1 to 7 represent the protein profiles of Embryogen-P leaves sectioned from the base to the tip, respectively. Lanes 9 to 15 represent the profiles of leaves sectioned from the base to the tip, respectively, from a nonembryogenic genotype. Proteins, EPP1 through EPP4, were not detected. Lane 8 contains known protein markers with their molecular weights indicated.

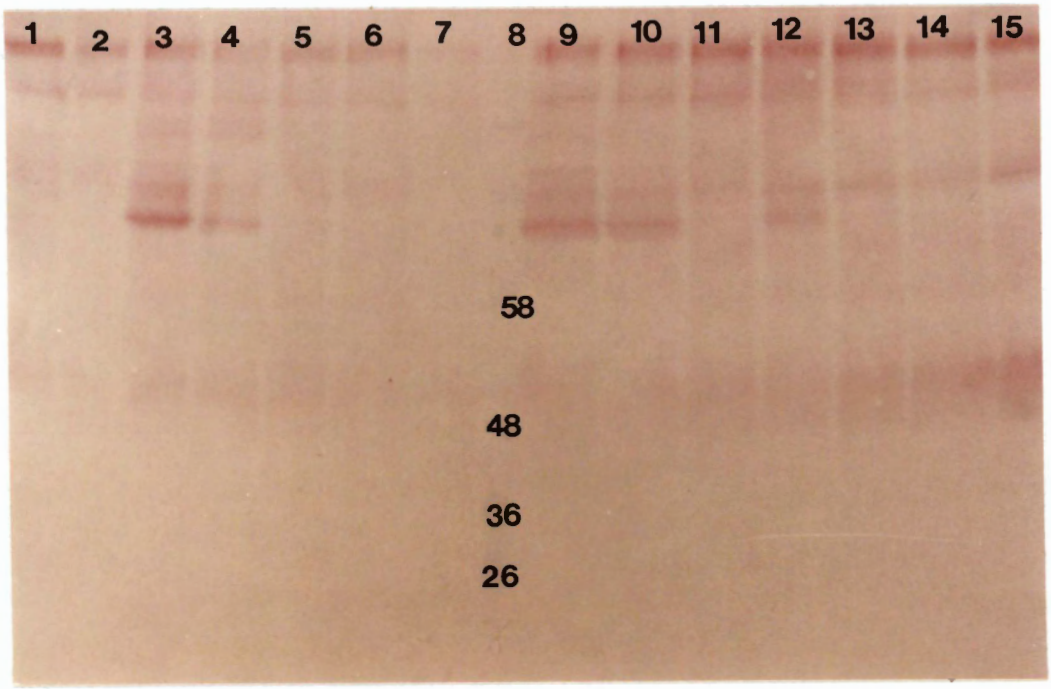

Fig. 27. Western blot of embryogenic and nonembryogenic protein profiles of leaves sectioned from the base to the tip hybridized with crossreacted antibodies specific for nonembryogenic leaf proteins. Lanes 1 to 7 represent the protein profiles of Embryogen-P leaves sectioned from the base to the tip, respectively. Lanes 9 to 15 represent the profiles of leaves sectioned from the base to the tip, respectively, from a nonembryogenic genotype. NEPP1 was not detected. Lane 8 contains known protein markers with their molecular weights indicated.

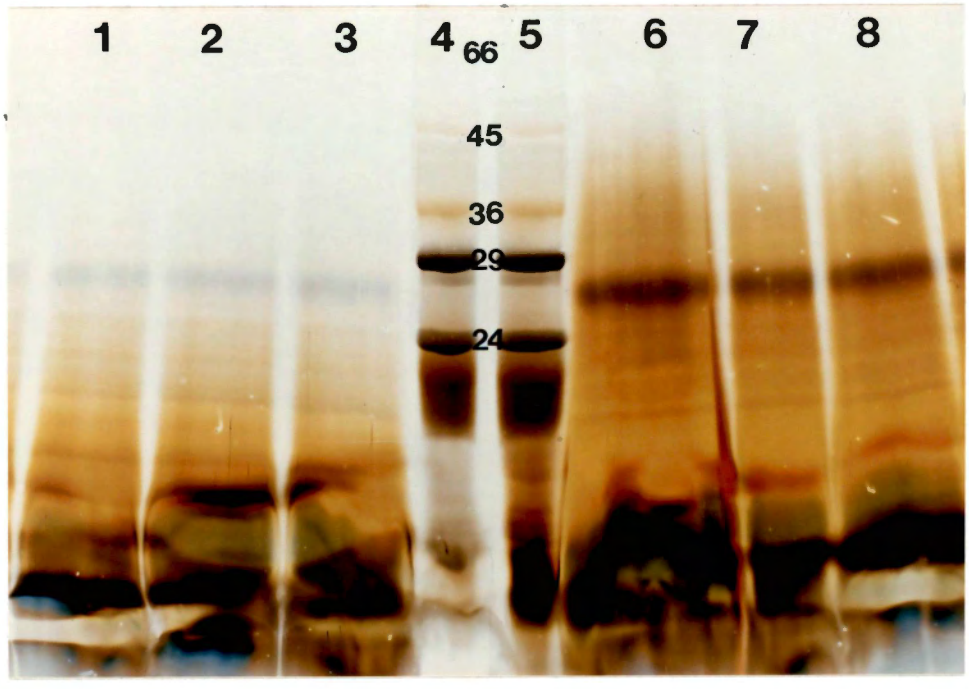

Fig. 28. One-dimensional PAG of purified proteins. Lanes 1 to 3 represent the proteins purified from total Embryogen-P leaf proteins using the crossreacted antibodies specific for Embryogen-P leaf proteins. Lanes 6 to 8 represent the proteins purified from total nonembryogenic leaf proteins using crossreacted antibodies specific for leaf proteins from a nonembryogenic genotype. EPP1 through EPP4 and NEPP1 were not detected. Lanes 4 and 5 contains known protein markers with their molecular weights indicated.

antibodies. The purified antibodies were hybridized to membranes containing both embryogenic and nonembryogenic protein profiles. The results were inconclusive.

5. Discussion

The study of genotypic differences in the ability of plant tissues and cells cultured in vitro to produce somatic embryos may help to gain an understanding of the process of regeneration. Previous studies have shown that genotypic differences in various crop species such as wheat (Sears and Deckard, 1982), maize (Green and Phillips, 1975), rice (Fatokun and Yamada, 1984), oats (Rines and McCoy, 1981), and alfalfa (Reisch and Bingham, 1980) influence the ability to regenerate plants via somatic embryogenesis. Genotypic differences for regeneration capacity existed in individual plants of 'Potomac' orchardgrass grown from seed (Hanning and Conger, 1982). Leaf sections from tillers were tissue cultured with only 9 of 50 plants producing plantlets. Although orchardgrass is an autotetraploid, it is a good species for studying genotypic differences because of its highly efficient and repeatable regeneration systems (Conger and Hanning, 1991). Investigations of differences of specific protein profiles between a genotype with a high capacity for somatic embryogenesis and nonembryogenic genotypes should provide more information on the initiation and regulation of the regeneration process.

Leaf Protein Experiments

The finding of identical specific proteins in the embryogenic parent and embryogenic F_1 progeny that were different from those in the nonembryogenic parents and F_1 progeny and visa versa, supports the association of specific proteins with somatic embryogenesis in orchardgrass. The precursors of proteins, mRNA and their genes, have been associated with the ability to produce somatic embryos in carrot. Three genes, Dc3, Dc5, and Dc13, were associated with somatic embryogenesis in carrot (Wilde et al., 1988). Another embryogenic gene, Dc8, was also identified (Franz et al., 1989). The mRNA of Dc3 was expressed in proembryogenic cell masses and somatic embryos (de Vries et al., 1988). This mRNA coded for a protein of 163 amino acids with two repeats, each with 50 amino acids (Seffens et al., 1990).

Suspension Culture Experiments

Five bands were seen in the protein profiles of callus grown in suspension culture medium supporting embryogenesis that were not present in callus cultured in medium not supporting embryogenesis. Although these proteins may not be directly involved in embryogenesis, they have been associated with embryogenesis. The MWs of the five proteins ranging from 35.5 to 36.0 were similar

to proteins identified with embryogenesis in several other studies. A 31 kD protein (Kiyosue et al., 1990, 1991) was associated with embryogenic carrot callus cells. In rice, a 54 kD protein was associated with embryogenic callus and zygotic embryos (Chen and Luthe, 1987). In another experiment with orchardgrass, six callus-specific proteins were found with MWs ranging from 22 to 51 kD (Hahne et al., 1988).

Stronger evidence for the similarity between the five proteins from my suspension culture experiments and those in carrot, rice, and barley calli might be provided by using the Western blotting technique. Antibodies for the orchardgrass proteins should hybridize with calli proteins from other species if they are similar.

The same five proteins found in the suspension cultures in which embryogenesis had been stimulated by casein hydrolysate were located in cultures stimulated by ammonium sulfate. They were absent in suspension if the medium did not contain casein hydrolysate or ammonium sulfate. This provided additional evidence for these five proteins being produced in conjunction with somatic embryogenesis. If they were synthesized in response to additives in the medium, they would not necessarily be expected to be the same because of the differences in the two additives.

Time Course Experiments

The appearance of nine new proteins in leaf explants between 4 and 7 d after culturing corresponded to the period of the initial cell divisions for somatic embryogenesis (Trigiano et al., 1989). Two other proteins appeared between 7 and 10 d, and one appeared between 10 and 14 d after the initiation of culture. Appearance of these proteins at different times throughout the culture period suggests that unique proteins are being synthesized during culture.

Several of these 12 proteins may be the same as those with similar MWs found in the suspension cultures supporting embryogenesis. Not all of the proteins in plated leaves were present in suspension calli. This may be because the leaf proteins were extracted from 2 to 22 d after culture. The suspension cultures were older than 22 d. Therefore, those not present in suspensions may have disappeared before 23 d leaving only those that were detected.

Unique proteins have been found during the culturing of several species. A total of 50 proteins were present in embryogenic cell suspensions of barley (Nielsen and Hansen, 1992). They varied from 10 to 90 kD and consisted of 5 groups. These proteins were constitutively expressed, increased or decreased in concentration during

embryogenesis, were associated with the culture method used during embryogenesis, or increased during undifferentiated growth without subculture.

Four proteins were found to be stage-specific in carrot embryo development (Schnall et al., 1991). Others were also identified as having stage-specific synthesis. However, their appearance was in only one genotype, or the explant was of a specific age, or they were unsynchronized and not associated with somatic embryo development. Of 32 barley proteins produced during culture, 24 were stage-specific (Ramagopal, 1989). Four new proteins were produced between 4 and 8 h after microspores of B. napus were cultured under embryo induction conditions and four others were produced between 8 and 16 h (Pechan et al., 1991). Hahne et al. (1988) found 50 stage-specific proteins in orchardgrass. A total of 32 unique proteins were found in somatic embryos and not in callus when using 2-D PAGE, in vivo labelling and from analyzing translated products of RNA. There were 18 unique proteins found in calli that were not present in somatic embryos.

A similar time course of protein synthesis was found in Trifolium rubens and T. pratense and the results of my study. Four groups of proteins were produced during petiole culture (McGee et al., 1989). One group was produced during the entire culture period of 20 d. A

second group decreased during culture. The third group appeared between 8 h and 5 d after culture and then disappeared after 5 d. The last group appeared in the initial part of the culture period (8 h) and remained throughout.

Abscisic Acid Experiments

Somatic embryo production in orchardgrass increased when ABA was added to the medium (Bell, 1991). When orchardgrass leaves were plated on SH-30 medium containing ABA, four new proteins appeared after 1 d in culture and three others were found after 2 d. These seven proteins may be synthesized by cells that are initiating embryogenesis or in response to the presence of the ABA in the medium. Three of the proteins had the same MWs as three appearing between 4 and 7 d after culture on medium without ABA. These three may be promoted at an earlier time by ABA and produced in response to embryogenesis.

The possibility that the genes for the proteins in this study were promoted by ABA is supported by previous studies of gene expression in the presence of ABA. A cDNA corresponding to an 800-nucleotide transcript in maize was isolated with enhanced expression in the presence of ABA in early to mid-stage embryos (Williams and Tsang, 1991).

The addition of ABA to the medium induced storage

protein gene expression in B. napus microspore embryos (Wilen et al., 1990). This induction caused the mRNA levels for the storage proteins to be comparable to the levels in zygotic embryos at the same age. Dc8, a carrot gene encoding for a 66 kD protein located in cytoplasm and cell walls of the somatic and zygotic embryos and endosperm, was found to be regulated by endogenous ABA (Hatzopoulos et al., 1990).

The types of protein differences found in suspension cultures and plated leaves support a conclusion that the expression of unique genes occurs during somatic embryogenesis. These genes may be expressed in response to environmental signals, such as wound, auxin, and reduced nitrogen. Another signal may be ABA which has been shown to promote the expression of certain proteins in zygotic embryos of peas (Barratt et al., 1989), zygotic and somatic embryos of maize (Rivin and Grudt, 1991; Williams and Tsang, 1991) and carrot (Hatzopoulos et al., 1990), and Brassica napus microspores (Wilen et al., 1990).

Immunological Experiments

The inability to produce purified antibodies may have been due to several factors. The amount of protein attached to the column may have been insufficient. An assay for the amount of protein was performed before the

column was made to calculate the proper dilution needed. Reagents such as sodium dodecyl sulfate, acrylamide, dithiothreitol, EGTA, EDTA, and mercaptoethanol within the sample may have been incompatible with the protein assay. The coupling of the proteins to the column may have been less than optimum due to an insufficient amount of protein binding or possibly to improper binding conditions.

Immunological studies such as the following in carrot have been performed to locate the proteins associated with embryogenesis and possibly determine their role. To study the physiological functions of glycoproteins and the action of auxin on their synthesis or release into the medium, antibodies for a 57 kD glycoprotein protein found in cultures were hybridized to proteins in dry and germinating seeds, mature tap roots, petioles, and young leaves (Satoh and Fujii, 1988).

A 45 kD protein associated with cell division was detected in rapidly growing carrot cells, immature zygotic embryos, developing floral buds, apical shoots, and root meristems as well as in zygotic embryos of maize and wheat, and somatic embryos of celery, alfalfa, and peach (Smith et al., 1988). An embryogenic carrot cell protein of 31 kD was located in organ segments that were producing embryos, in cultured hypocotyl segments, and in zygotic and somatic embryos (Kiyosue et al., 1990; 1991).

6. Summary and Conclusions

Problems were encountered with the 1-D gels in loss of resolution of already faint bands between the time of staining and photographing the gels. The 2-D gels were mostly unsatisfactory but some spots were faintly visible immediately after staining. Based on the best observations and gel scans, four proteins were found to be present in the protein profile of Embryogen-P from 1-D PAGE that were not present in the profiles of the nonembryogenic genotypes. One protein was present in the profiles of the nonembryogenic genotypes that was not present in Embryogen-P. Fifteen proteins were detected in the 2-D PAGE of the Embryogen-P leaf proteins that were not present in those of the nonembryogenic genotypes. The same four proteins found in Embryogen-P were also present in the embryogenic F_1 progeny and the one protein found in the nonembryogenic genotype was found in the nonembryogenic F_1 progeny from crosses between Embryogen-P and a nonembryogenic genotype.

There was an increase in the amounts of proteins from the inner to the outer leaves within a tiller of Embryogen-P. There was no increase in proteins from the leaves of a nonembryogenic genotype. There was an increase in the proteins from the base to the tip of Embryogen-P leaves but not in those of the nonembryogenic genotypes.

Five proteins were found in Embryogen-P suspension cultures in medium supporting embryogenesis by the addition of casein hydrolysate that were not present in those not supporting embryogenesis. In a second study using ammonium sulfate to support embryogenesis, five proteins with the same molecular weights were detected in the suspension cultures supporting embryogenesis that were not present in those not supporting embryogenesis.

Nine proteins were located in the protein profile of leaves cultured for 7 d that were not present in leaves cultured for 2 or 4 d. Two other proteins appeared first in the profiles of leaves cultured for 10 d and one protein first appeared in leaves cultured for 14 d. Leaves cultured on medium containing abscisic acid (ABA) for 1 d produced four proteins that were not present in leaves cultured for 1 d on medium not containing ABA. Three proteins were detected in the profile of leaves cultured for 2 d on medium with ABA that were not present in leaves cultured for 2 d without ABA or leaves plated for 1 d with or without ABA.

The identification of these unique proteins is a step toward understanding the genetic mechanisms for the development of somatic embryos in orchardgrass and perhaps also in the Poaceae. With these findings, efforts may now be focused on identifying genes specifically involved in embryogenesis. Further characterization of these proteins will help in producing specific oligonucleotides. These may

be used to screen libraries of orchardgrass DNA to identify genes associated with embryogenesis. With the use of in vivo labelling, the effects of compounds such as auxins, cytokinins, and ABA on the expression of these proteins may be identified. These effects could be investigated further by comparing RNA populations of treated and untreated orchardgrass. If such genes can be identified and the promotion of their expression understood, this technology could be used to introduce somatic embryogenesis into plants that now do not produce somatic embryos.

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Vita

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