TWO-DIMENSIONAL PROTEIN PATTERN ANALYSIS OF EXTRACELLULAR PROTEINS SECRETED BY EMBRYOGENIC AND NON-EMBRYOGENIC SUSPENSION CULTURES OF *DACTYLIS GLOMERATA* L.

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ABSTRACT

Proteins from the medium of orchardgrass (Dactylis glomerata L.) embryogenic suspension culture during defined stages of somatic embryogenesis were compared with those of non-embryogenic suspension culture during unorganized cell proliferation. After separation on two-dimensional polyacrylamide gel electrophoresis, we were able to classify 40 proteins in each of 6 groups: a group common to both embryogenic and non-embryogenic suspension cultures, a group specific only for embryogenic, respectively non-embryogenic suspension cultures and 3 groups of microcluster-, proembryogenic masses- and somatic embryo-specific proteins. One of the groups is particularly interesting as it corresponds to polypeptides that are related to the earliest stages of somatic embryogenesis-the transition of microclusters into proembryogenic masses when no morphological changes are visible yet. Using this experimental approach, we identified the major extracellular proteins involved in somatic embryo development and we discuss on the possibility for their use as early markers for somatic embryogenesis.

Introduction

The capacity for somatic embryogenesis is a remarkable property of plant cells. Somatic embryogenesis is the process by which somatic cells develop into plants through characteristic morphological stages thus rendering it a good model system for investigating early plant development. The molecular basis of this unique developmental pathway, particularly the transition of somatic cells into embryogenic ones is poorly understood (6, 20). Somatic embryogenesis in cell suspension cultures offers an alternative way to address this problem. Suspension cultures secrete into the medium glycoproteins that play an important role in somatic embryogenesis by their ability to stimulate (8, 14, 30) or inhibit (13, 18) embryo development. The characterization of extracellular protein

Abbreviations: Dicamba - 3, 6-dichloro-o-anisic acid; IEF-isoelectric focusing: PEMs-proembryogenic masses; pI -isoelectric point; SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis; SH-0 - Schenk and Hildebrandt (23) medium; SH-30 - SH medium, supplemented with 30 ìM dicamba; 2-D PAGE - two-dimensional polyacrylamide gel electrophoresis.

markers for somatic embryogenesis offers a possibility to determine the embryogenic potential of plant cells in culture long before any morphological changes have taken place. The role of these proteins has not been elucidated fully, but for some of them like lipid transfer protein EP2, acidic endochitinase EP3 and cationic peroxidase it has been shown that they play a key role in carrot somatic embryogenesis (4, 7, 25). Most information concerning molecular aspects of somatic embryogenesis has been obtained with carrot cultures (8, 24, 30). Such studies are relatively scarce in monocots and gymnospems (19, 21, 28). The relative ease to obtain cultures of defined morphological stages in *D. glomerata* L. allowed us to characterize the relative extracellular proteins. Since plant embryogenesis does not involve changes in the level of abundant genes, it is important to use a highly resolutive method to find reliable markers of different developmental stages of somatic embryogenesis.

In this paper, we show that analysis of extracellular proteins with the aid of 2-D protein gels can be used to distinguish between different stages of somatic embryogenesis and to identify putative candidates of proteins as molecular markers for somatic embryogenesis.

Materials and Methods

Plant material and suspension cultures

Callus-derived suspension cultures from an embryogenic (E_1) and a non-embryogenic (NE_1) cell line of orchardgrass (*Dactylis glomerata* L.) were initiated according to Conger et al. (3). The embryogenic E_1 line was established from callus of a highly embryogenic *D. glomerata* L. genotype, cv.'Potomac'(kindly provided by B. V. Conger). The non-embryogenic suspension culture (line NE_1) was initiated from segregated non-embryogenic sectors of the callus used for initiation of the E_1 line. All cultures were maintained in a liquid SH-30 medium in the dark at 25° C on a rotary shaker (105 rpm) and subcultured every 2 weeks.

Fractionation of suspension cultures

Fractions of globular embryos, PEMs and microclusters from the embryogenic suspension culture were collected by passing the culture consecutively through a series of 230-, 104- and 60- µm sieves, respectively. Each of the fractions was rinsed with SH-0 medium and subcultured in a fresh SH-30 medium at a density of 1µl packed cell volume per ml medium. After 7 days in culture, the culture media were separated from the cells and were used as a source of extracellular proteins. The microclusters from the non-embryogenic culture, retained on the 60- µm sieves, were maintained in the same manner.

Protein preparation

Suspension-cultured cells at day 7 after transfer were centrifuged at 500xg for 5 min. The culture medium was recovered by passing the supernatant through Millipore 0.22 µm filter. Extracellular proteins in the medium were precipitated by the addition of 2.5 volumes of ethanol overnight at 4ºC. After centrifugation (12000xg at 4ºC for 30 min), the precipitates were vacuum-dried and stored at -70ºC or dissolved in water for immediate use (8). Intracellular soluble proteins were obtained by grinding the cells in extraction buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$ and 1 mM PMSF. The homogenate was filtered through nylon gauze and centrifuged at 15000xg at 4ºC for 30 min. The protein content was determined according to Bradford (2).

Electrophoretic procedures

Electrophoresis of the proteins was performed on SDS-PAGE (13%) according to Laemmli (17). Two-dimensional gel electrophoresis (2-D PAGE) was carried out as described by Tchorbadjieva and Odjakova (28). The samples were separated in the first dimension by IEF and in the second dimension by SDS-PAGE using 13 % separating gel. Isoelectric points (pI) were determined by using a set of broad range (pI 3.5-9.5) IEF standards (Pharmacia Biotech). Proteins were silver-stained according to Blum et al. (1). All experiments were repeated at least three times.

Results and Discussion

Somatic embryogenesis of Dactylis glomerata L.

Orchardgrass suspension cultures in contrast to the model carrot system are not inducible. Somatic embryos fully develop in a hormone-containing medium before being transferred to a hormone-free medium for regeneration. In order to establish a good reference suspension culture from the same genotype but deficient in embryogenic potential (NE_1), we used certain sectors of embryogenic callus from the embryogenic cell line E_1, which had reverted irreversibly to a soft, friable non-embryogenic callus. After several subcultures its ability to regenerate plants was definitely lost.

For convenience, we chose to divide the process of *D. glomerata* L. somatic embryogenesis into 3 phases by analogy to the carrot system (12). Two-three days after inoculation competent single cells start to divide intensively and form microclusters (phase 1). One-week later PEMs containing centers of embryonic growth form (phase 2) and after 2 weeks globular embryos begin to differentiate from the cell masses (phase 3). Single cells from the non-embryogenic culture NE_1 divide to form microclusters whose further development is blocked. Only the fractions containing defined tissue types, i. e. microclusters, PEMs and embryos were used for the identification of developmental stage –specific proteins.

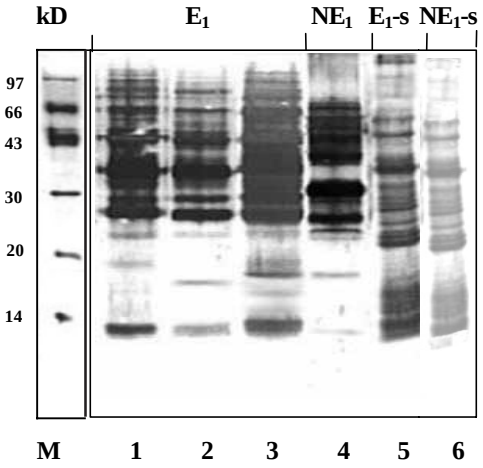

Fig. 1. 13 % SDS-PAGE of extracellular proteins secreted by: 1, microclusters; 2, PEMs; 3, somatic embryos and 5, soluble intracellular proteins of embryogenic suspension culture E_1; 4, microclusters and 6, soluble intracellular proteins of non-embryogenic suspension culture NE_1. The stage-specific proteins are shown with an asterisk.

Analysis of embryogenic and non-embryogenic cell line protein patterns by SDS-PAGE

Established suspension cultures were fractionated and the distinct morphological structures were cultured for 7 days in a fresh SH30 medium. The proteins secreted into the culture medium during subsequent developmental stages were subjected to SDS-PAGE and silver stained (**Fig. 1**). Altogether different morphological structures secrete common proteins.

However, there are stage- specific proteins like the E_1 microclusters-specific19 kDa protein, the PEMs - specific17 kDa protein or 18 kDa protein, specific for embryos (**Fig. 1, lanes 1, 2, 3**). The proteins secreted by the embryogenic cell line E_1 differ from those of the non-embryogenic one NE_1 (Fig. 1, compare lanes 1 and 4). Comparison of the extracellular protein patterns (**Fig. 1, lanes 1, 2, 3, 4**) with the intracellular ones (**Fig. 1, lanes 5, 6**) shows no corresponding bands present in the intracellular extracts. Moreover, in contrast with the extracellular profiles, strong similarities exist between the two types of cultures regarding the intracellular proteins (**Fig. 1, lanes 5, 6**). No significant differences were observed when the intracellular protein patterns of the various developmental stages were compared (data not shown). Our data are consistent with those reported by De Vries et al. (8), Domon et al. (9), Kreuger and Van Holst (16), Mo et al. (19) and Nielsen and Hansen (21) who observed main differences among the extracellular proteins between embryogenic and non-embryogenic suspension cultures. This fact served as the main argument of the authors to use the extracellular proteins as a source for embryogenic markers.

Analysis of extracellular proteins from embryogenic and non-embryogenic suspension cultures by the use of 2-D PAGE

A better resolution of the extracellular proteins has been achieved with the aid of 2-D PAGE analysis (**Fig. 2**). At least two reproducible gels were obtained for each

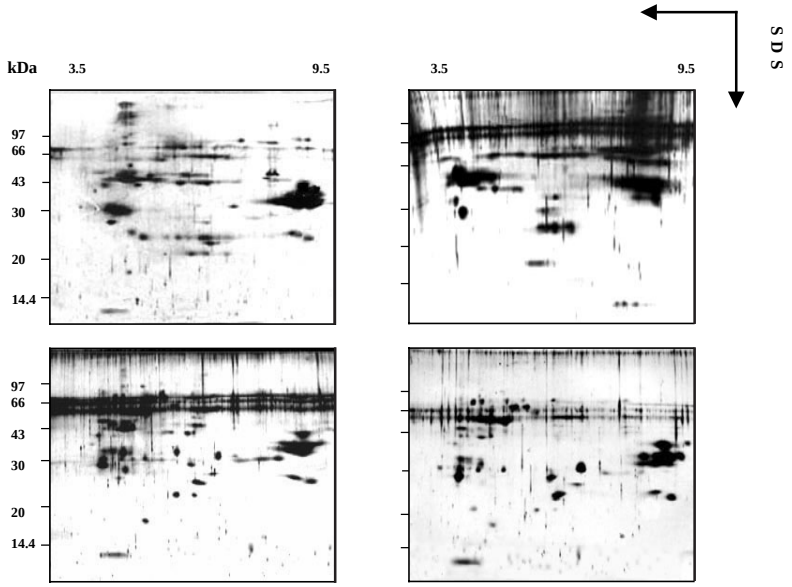

Fig. 2. 2-D PAGE patterns of extracellular proteins secreted into the medium of microclusters (A), PEMs (C), globular embryos (D) of embryogenic suspension culture E_1; microclusters of non-embryogenic suspension culture NE_1 (B). After IEF, the secreted proteins (10 µg) were separated on a 13 % SDS-PAGE and silver stained. Molecular mass markers are indicated on the left in kilodaltons.

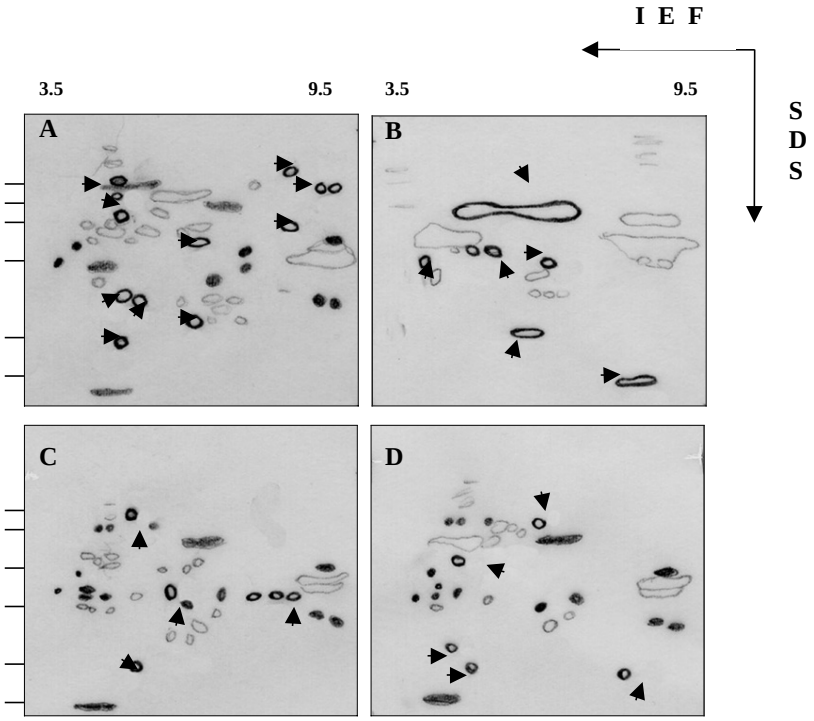

Fig. 3. Map of the reproducible spots of 2-D protein patterns of embryogenic suspension culture E_1 of microclusters (A), PEMs (C), somatic embryos (D) and microclusters of non-embryogenic suspension culture NE_1 (B). White spots are for proteins common to both embryogenic E_1 and non-embryogenic NE_1 suspension cultures, black spots are for proteins common to all stages of somatic embryo development in embryogenic culture E_1 only and spots marked with arrows indicate stage-specific proteins in the various phases of development. The molecular marker values are the same as in the previous figure.

sample. Two-dimensional gels were visually compared in order to score the presence/absence of spots. Approximately 40 polypeptides with a wide range of molecular masses (10-120 KDa) and pIs (3.5-9.5) characterized each developmental stage as well as each suspension culture (**Fig. 2**). The upper part of the gels was not considered because of poor resolution due to highly glycosylated proteins. The polypeptides detected in the various stages of development in both suspension cultures could be divided into 6 sets (**Fig. 3; Table 1**). The first set comprised a few polypeptides that were common to both embryogenic and non-embryogenic cell lines and all developmental stages suggesting that they probably refer to common metabolism and a similar response to the new culture conditions (**Table 1, group 1**). There are also proteins common to the microclusters of E_1 and NE_1 lines, which disappear at the next stages of development like 24 kDa (pI 5.85-6.8) and a group of 38, 40 and 42 kDa (pI 4.35-5.4). Probably some of them are related to cell proliferation and the non-differentiated state of these structures.

TABLE 1

Extracellular proteins of embryogenic E_1 and non-embryogenic NE_1 suspension cultures after 2-D PAGE

Group 1		Group 2		Group 3		Group 4		Group 5		Group 6	
kD	**pI**	**kD**	**pI**	**kD**	**pI**	**kD**	**pI**	**kD**	**pI**	**kD**	**pI**
27	4.35	**12**	**4.1-4.8**	19	5.3	17	5.4	15	8.3	12	8-8.8
32	8.6-9.5	24	9.3	21	6.55	30	8.2-8.5	18	5.2	18	5.5-6.2
34	8.6-9.5	25	8.7	25	5.2	33	6.0	21	4.5	31	6.2
38	8.6-9.5	27	6.3	26	5.4	80	5.3	40	5.2	32	4.3
40	4.3-5.2	**32**	**3.6**	42	6.3-6.5			85	6.0	36	5.-5.5
		32	7.2	43	8.5					50	5.2-6.8
		32/34	4.2-5	**48**	**5.2**						
		36	**3.8**	78	5.2						
		40	9.0	81	8.7-9.0						
		57	6-7.3	105	8.45						
		70	4.3-5.8								

A second set of polypeptides is characteristic for all stages of development only in the embryogenic cell line E_1 (**Fig. 3, A, C, D; Table 1, group 2**). Most of them are low molecular mass proteins, which show quantitative changes during development. The low molecular mass of these proteins is similar in molecular mass to the previously reported embryo-specific proteins in somatic embryos of carrot (8), *Cichorium* (15) and *Pinus abies* (9). There are also polypeptides, which are specific for each of the three phases of development (**Fig. 3; Table 1, groups 3, 4, 5**). It is noteworthy that the development of PEMs from microclusters is characterized with the highest number of stage-specific proteins-11 (**Table 1, group 3**). This is the earliest stage of development when after a rapid cell division some microclusters grow and give rise to PEMs when no morphological changes are visible yet. The proteins of group 4 are specific for the stage when the globular embryos form from PEMs and those of group 5-for embryo maturation (**Table 1**). These proteins could be regarded as morphogenic markers since it is the time of formation of the primary tissues. Proteins from group 6 represent the stage-specific proteins of microclusters from the non-embryogenic suspension culture. They could act as inhibitors of somatic embryogenesis in the latter. The protein profiles observed in the medium of *D. glomerata* L. cell suspensions were similar in complexity with the profiles observed in barley suspensions where 50 different proteins were found (21). The number of low molecular mass proteins is lower in the non-embryogenic cell line NE_1 when compared with the embryogenic cell line E_1. This is in agreement with earlier reports, which indicate low molecular mass polypeptides as a feature of embryo-specific proteins (5, 6, 10, 22). The embryogenic suspension culture of *D. glomerata* L. consists of different cell types with different morphology and develop-

mental potential. 2-D analysis shows that specific proteins mark these differences. This is in accordance with the basic assumption that the cell wall and its proteins play an important role in plant development. (23). As the induced suspension cultures of E_1 and NE_1 lines had the same explant origin and were submitted to the same culture procedures, the 40 spots detected only in the embryogenic culture were considered as embryogenesis-associated ones. The absence of these polypeptides from the medium of the non-embryogenic culture could be related to their blocked synthesis in the process of loosing its embryogenic potential.

D. glomerata L. suspension cultures differentiating into somatic embryos secrete proteins into the culture medium in a stage-specific manner. Besides, there is a significant difference between the extracellular proteins isolated from the medium of common morphological structures with a different fate of development, e.g. microclusters from E_1 and NE_1 lines. This indicates that these proteins can also be regarded as marker proteins for the individual developmental stages.The limited number of extracellular proteins when compared to the whole protein pattern makes them appropriate candidates as protein markers for somatic embryogenesis. In a previous paper, we showed that a 36 kDa acidic esterase (pI 3.8) could be used as an early marker for embryogenic potential (28). It belongs to the group of embryogenesis-related proteins (**Table 1, group 2**). We have characterized some other extracellular proteins, which belong to group 2, namely a group of five 12 kDa non-specific lipid transfer proteins and a 32 kDa acidic endochitinase (pI 3.6). A 48 kDa glycoprotein (pI 5.2) was observed only in the earliest stage of formation of PEMs from microclusters (**Table 1, group 3**) (29). Currently purification of these proteins is underway in order to use them for sequence analysis and immunological studies using specific antibodies.

Future studies are required to clarify the specific functions of the embryogenic extracellular proteins. Identification and cellular localization may provide a better understanding of their involvement in somatic embryogenesis. These extracellular protein markers can be used to characterize embryogenic cultures as well as to elucidate the molecular mechanisms of cell differentiation.

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