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Extracellular Glycoproteins in Embryogenic Culture of Pumpkin (*Cucurbita pepo* L.)

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Summary

The extracellular proteins in three distinctly induced embryogenic lines of pumpkin (*Cucurbita pepo* L.) cultivated in four MS media modified regarding the nitrogen composition or auxin presence/absence have been analyzed. Extracellular glycoproteins containing α-D-mannose were specifically detected by the lectine concavalin A. During the cultivation of embryogenic tissue in the medium supplemented with reduced nitrogen, the embryos were mostly arrested at preglobular and globular developmental stages, which coincide with the absence of protein secretion. Secreted glycoproteins of 76, 68, 37 and 34 kDa were detected only if any of the three lines were cultivated in the medium that stimulates embryo development, irrespectively of the addition of 2,4-dichlorophenoxyacetic acid or tunicamycin. The glycoprotein of 64 kDa was detected in all lines cultivated in hormone-free MS medium with conventional nitrogen sources and it appears to be associated with embryo maturation. Tunicamycin treatment did not influence embryogenesis, although it specifically affected glycosylation of proteins in the investigated lines. Our results show that besides auxin, the source of nitrate is of great importance for proper protein glycosylation, excretion and developmental transition of pumpkin somatic embryos.

Key words: Cucurbita pepo L., extracellular glycoproteins, hormone-free medium, nitrogen sources, pumpkin, somatic embryogenesis

Introduction

Plant cells are able to form adventitious or somatic embryos by a process similar to zygotic embryogenesis. The process known as somatic embryogenesis begins with the initiation phase, when cells dedifferentiate and become competent for embryogenesis. Further, cells become determined to form embryos during the induction phase. In the last phase, the embryogenic potential is expressed by development of embryos through stages characteristic for zygotic embryogenesis. Unfortunately, understanding the mechanisms of embryogenesis, either zygotic or somatic, has progressed slowly due to several reasons. First, the egg cell or early zygotic embryos are deeply embedded into maternal tissue and the access to them is hampered. Moreover, there is no simple procedure to detect gene expression in a single cell such as egg or cell competent for somatic embryogenesis (*1*). In the case of somatic embryogenesis, which has been studied *in vitro* on numerous plant species, embryos develop asynchronously and at low frequency (*2*,*3*), so genes that can serve as reliable markers of embryogenesis are insufficiently expressed in the mass of different cell types and it has been almost impossible to detect their expression.

During somatic embryogenesis, the environment of the cells mimics the conditions that exist in the ovule.

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An embryo-promoting effect of conditioned media has been shown in different plant species (*4,5*), which indicates that besides the exogenous plant growth regulator components secreted from developing tissue, either embryogenic or non-embryogenic, have an important role in stimulation of embryo development. Up to now different classes of molecules have been identified as embryo-stimulating factors. For example, the oligosaharides released into the medium from plant cell walls are shown to have proembryo-proliferating activity in Norway spruce (*6*). Furthermore, excreted phytosulfokines during carrot somatic embryogenesis increase the number of embryogenic cells and accelerated somatic embryo formation (*7*).

The larger secreted molecules supposed to have a role in embryogenesis are arabinogalactan proteins (AGPs) and pathogenesis-related (PR) proteins. AGPs have been identified in the culture medium supporting somatic embryogenesis in carrot (*8,9*), *Cichorium* (*10*), rose (*11*), barley (*12*) and maize (*13*). AGPs have also been detected during zygotic development of carrot and maize (*13,14*), and they were secreted into culture media of endosperm cells (*15*).

The occurrence of PR enzymes such as chitinases or glucanases has been noticed in carrot suspension cultures (*16–19*), and in some other species such as barley (*20*), grapevine (*21*), *Citrus* (*22*), *Cichorium* (*23*) and maize (*13*). For some of the above mentioned substances, it has been proven that they are effective environmental stimuli for the onset of embryogenesis or embryo development (*6,7,13,14,24,25*).

Induction and development of somatic embryos in pumpkin tissue (*Cucurbita pepo* L.) grown in hormone-containing media, but also in hormone-free media had been reported previously (*26–28*). The cultures established without hormones were characterized by a high embryogenic rate and an arrest in the globular stage of development, which makes it possible to analyse separately earlier and later stages of embryo development.

As very little is known on proteins involved in or responsible for the process of somatic embryogenesis of *Cucurbita pepo* L., the aim of the present study is to get an insight into developmentally specific patterns of extracellular glycoproteins in embryogenic tissue lines of pumpkin.

Materials and Methods

Nutrient media

Culture media used for the induction and maintaining of pumpkin somatic embryos were based on the basal Murashige and Skoog (MS) medium (*29*) modified as follows: (M1) $MSNH_4$ – basal MS medium containing 1 mM NH_4Cl as a sole source of nitrogen (instead of KNO_3 and NH_4NO_3), and supplemented with 3.37 μM thiamine-HCl; (M3) MSC+2,4-dichlorophenoxyacetic acid (2,4-D) – basal MS completed with MS organic supplements (MSC) and supplemented with 4.5 μM 2,4-D; (M5) MSC – basal MS completed with MS organic supplements; (Tm) MSC+tunicamycin – MSC medium supplemented with 1.0 μg/mL of tunicamycin, which is an inhibitor of protein *N*-glycosylation.

The media were liquid or were solidified by adding 0.8 % (by mass per volume) washed agar (Sigma-Aldrich, St. Louis, MO, USA). The culture media with the pH adjusted to 5.8 were autoclaved at 121 °C and 103 kPa for 15 min. All the tested media were supplemented with 250 mM of glucose.

Plant material

Excised and mechanically wounded mature embryos of pumpkin (*Cucurbita pepo* L.) detached from their cotyledons were used as initial explant material (*27*). With respect to regeneration and growth conditions described earlier (*28*), three types of embryogenic callus cultures were established: (*i*) a preembryogenic determined cell line (PEDC) consisting of preembryogenic determined cells, preglobular and globular embryos, which were induced and continuously subcultured in $MSNH_4$ medium; (*ii*) an embryogenic callus line (DEC) induced and continuously subcultured in MSC+2,4-D medium. DEC line contains mostly embryos in the early developmental stages; and (*iii*) a habituated embryogenic callus line (HEC) derived from DEC line after transferring and maintaining of embryogenic tissue in hormone-free MSC medium. This line contains embryos of all developmental stages.

All three embryogenic lines were subcultured every three weeks and cultivated in each of the above defined media (M1, M3, M5, Tm). Cultures were incubated in a culture room at (24±1) °C under an artificial light (daylight fluorescent tubes, 40 W, 400–700 nm) for 16 h per day, at light intensity of 90 $\mu E/(s \cdot m^2)$.

Protein samples

To analyse extracellular proteins, the liquid medium of four-day-old suspension cultures was decanted and passed through a mash filter to remove the cell debris. Proteins were concentrated by the addition of Sephadex G-25 to the filtrate and the reduction of its volume approx. five times. Protein content of the supernatants was determined according to Bradford (*30*). Samples were denatured in 0.125 M Tris-buffer (pH=6.8), containing 5 % (by volume) β-mercaptoethanol and 2 % (by mass per volume) sodium dodecyl sulphate (SDS).

Electrophoresis and electroblotting

Proteins were separated by SDS polyacrylamide gel electrophoresis (12 % T, 2.67 % C) (*31*). Approximately 5 μg of proteins per sample were loaded onto the gel.

Protein bands were visualized by silver staining (*32*). Extracellular proteins were transferred to a nitrocellulose membrane in a vertical tank apparatus for electroblotting. The proteins successfully transferred onto the membrane were visualised by Ponceau S (Sigma-Aldrich-Fluka, Darmstadt, Germany). Glycoproteins with α-D-mannose in their glycan component were detected by concanavalin A-peroxidase (Con A-peroxidase) and visualized by peroxidase reaction using diaminobenzidine as a substrate (*33*).

Results

Embryo development

It has previously been shown that pumpkin embryogenic lines PEDC, DEC and HEC respond similarly to the same composition of nitrogen salts and exogenous 2,4-D. In each line sustained subculturing of embryogenic tissue in a medium with NH_4Cl as the sole source of nitrogen enabled the establishment of highly uniform cultures in which no further development into mature embryo stages occurred. Globular embryos proceeded to maturity when a combination of reduced (NH_4) and unreduced (NO_3) forms of nitrogen was provided in the medium (*28*).

In this work the development of pumpkin somatic embryos from preglobular to cotyledonary stage was controlled exclusively by changing the ratio of reduced and unreduced nitrogen or by the presence or absence of auxin. The frequency of the single embryo stage shown in Fig. 1 is expressed as a percentage of the total embryos counted for each medium composition.

The callus cultures that were established in the presence of 2,4-D (DEC and HEC lines) during the cultivation on the medium containing 2,4-D (M3) had nodular structure with most embryos arrested in globular stage. Few globules continued to develop into later embryo stages. MSC medium without 2,4-D (M5) was favourable for embryo development to later stages. When tissue was cultured on $MSNH_4$ medium (M1), only proembryogenic cells and embryogenic globules survived, therefore the cultures consisted of preglobular and globular embryos (Fig. 2, bottom row). The effect of unreduced nitrogen became more expressive after prolonged subculturing on M1 medium (more than six successive subcultures), when the rate of globular-heart stage transition additionally decreased (data not shown). Embryonic PEDC line obtained in the $MSNH_4$ induction medium (M1) was organized in friable nodules, and embryos were arrested in globular stage. After transferring PEDC callus to MSC medium supplemented with 2,4-D (M3), some of the globular embryos developed to more mature embryos. Subcultivation of PEDC tissue in MSC medium (M5) enabled globular embryos to develop further to heart and torpedo stage embryos (Fig. 2, upper row). After a prolonged subculturing in MSC medium (supplemented with conventional nitrogen and without 2,4-D), the embryos of PEDC line developed to mature stages with higher frequency than in DEC and HEC lines.

The addition of glycosylation inhibitor tunicamycin did not block embryogenesis even after a long-term treatment (Fig. 1).

Extracellular proteins and α-mannose glycoproteins in embryogenic lines

During the cultivation of embryogenic tissue in the media with conventional nitrogen sources (M3 and M5), we have detected 5 dominant, clearly visible and reproducible bands belonging to extracellular proteins. As revealed in Figs. 3a and b, none of the lines in $MSNH_4$ medium (M1) secreted enough proteins or glycoproteins that could be detected by silver staining or Con A-peroxidase staining of protein blots.

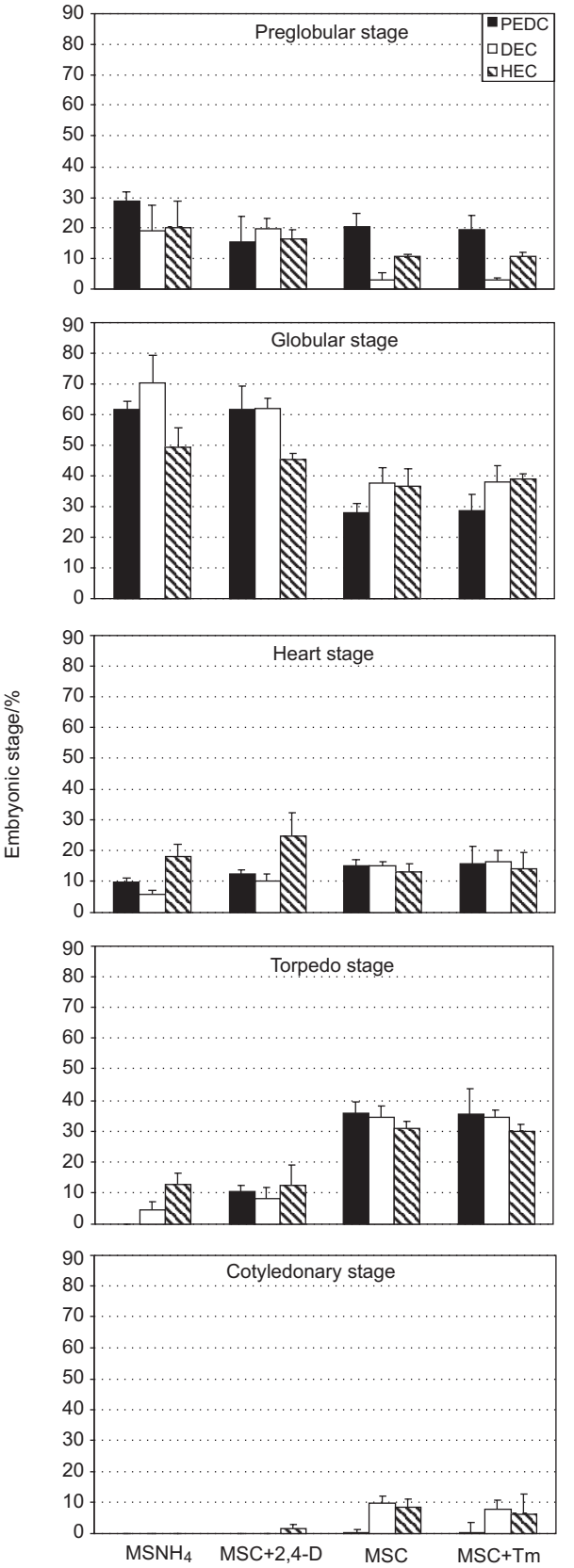

Fig. 1. Frequency of developmental embryo stages in embryogenic lines PEDC, DEC and HEC cultivated on $MSNH_4$, MSC+2,4-D, MSC and MSC+Tm media. The frequency of a single embryo stage is expressed as a percentage of total number of embryos counted for one experimental condition. The data are the average values±S.D. of 12 tubes×3 replicates

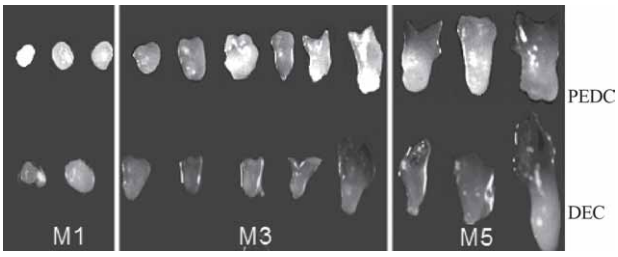

Fig. 2. Somatic embryos in various developmental stages derived from line PEDC (upper row) and line DEC (bottom row) after 9 weeks of cultivation on the media $MSNH_4$ (M1), MSC+2,4-D (M3) and MSC (M5)

Extracellular proteins of 37 and 34 kDa, detected by silver staining after SDS-PAGE electrophoresis and by Con A-peroxidase reaction, were secreted by all three lines cultivated in MSC with 2,4-D, MSC and Tm-containing MSC media (Fig. 3a, two lower arrows in Fig. 3b). Furthermore, all lines cultured in MSC medium, irrespective of 2,4-D or Tm, secreted Con A-peroxidase-reacting glycoproteins of 76 and 68 kDa (Fig. 3b, two upper arrows).

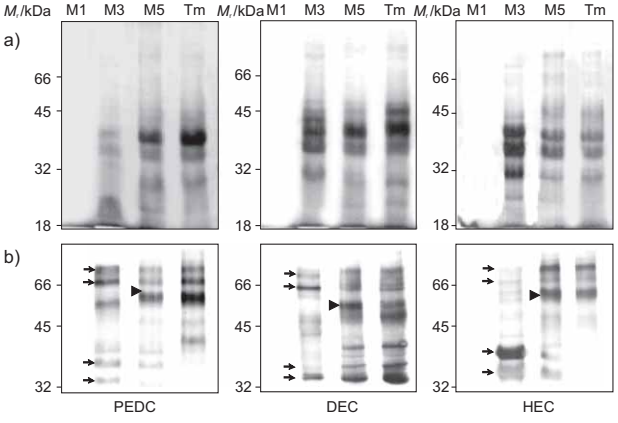

Fig. 3. Extracellular proteins (a) and glycoproteins (b) of pumpkin embryogenic lines PEDC, DEC and HEC cultivated on the media $MSNH_4$ (M1), MSC+2,4-D (M3), MSC (M5) and MSC+Tm (Tm). Proteins were separated by SDS polyacrylamide gel electrophoresis and the bands were visualized by silver staining (a). After transferring to nitrocellulose membrane, extracellular glycoproteins with α-D-mannose in their glycan component were detected and visualized by Con A-peroxidase reaction (b). Glycoproteins specific for further development of globular embryos are labelled with arrows and for embryo maturation with arrow head. M_r=molecular mass markers (kDa)

Line PEDC, which was continuously grown on $MSNH_4$ medium, secreted less proteins than the other lines immediately after its transfer to the medium with conventional nitrogen sources (MSC) and to the MSC medium supplemented with 2,4-D (MSC+2,4-D). The first secreted glycoproteins after such transfer were the ones of 37 and 34 kDa (Fig. 4, arrows), detected after at least five days of cultivation on MSC or MSC+2,4-D medium. The secretion of these proteins coincided with further maturation of preglobular and globular embryos.

Glycoprotein of 64 kDa (Fig. 3b, arrow head) was detected in all the lines on hormone-free MSC medium (irrespective of Tm addition), which was favourable for embryo maturation.

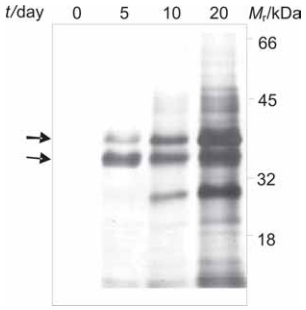

Fig. 4. Extracellular proteins of pumpkin embryogenic line PEDC, cultivated on $MSNH_4$ medium immediately (0 day), 5, 10 and 20 days after transferring the tissue from $MSNH_4$ to MSC medium. Proteins were separated by SDS polyacrylamide gel electrophoresis and the bands were visualized by silver staining. The secretion of 37- and 34-kDa glycoproteins (arrows) started shortly after transferring the tissue onto the medium that enables development of preglobular and globular embryos. M_r=molecular mass markers (kDa)

Tunicamycin treatment did not prevent protein secretion, but it affected protein glycosylation (Figs. 3a and b). Extracellular proteins with molecular mass below 48 kDa in HEC line or those with molecular mass below 39 kDa in PEDC line did not react to Con A, although they were detected in the medium by silver staining and were transferred to the membrane (visible after Ponceau S staining). These extracellular proteins secreted by DEC line were detected by Con A-peroxidase reaction, even though they were treated by tunicamycin. The tunicamycin (1.0 μg/mL) treatment that lasted for two months did not block embryo maturation. The concentration of Tm, in case when it was lower than 1.0 μg/mL (0.4; 0.75), did not affect glycosylation of Con A reacting proteins.

Proteins of molecular mass below 34 kDa, although successfully transferred onto the membrane (detected with Ponceau S), did not react to Con A, thus indicating that either they were not glycosylated or they did not contain α-D-mannose in their glycan components.

Discussion

Extracellular (glyco) proteins in three embryogenic lines of pumpkin (*Cucurbita pepo* L.) were analysed. Our results showed that changes in embryo development corresponded to differences in secretion and glycosylation of proteins. Minor quantitative differences, obtained in cellular protein patterns (date not shown), could be explained by the expression of the same set of genes during unorganised growth, in proembryogenic masses and developing embryos (*34*,*35*). These differences could also be explained by minute changes in the expression of embryogenesis-related genes that are not detectable in a mass of cellular proteins by SDS-PAGE electrophoresis (*36*). De Vries *et al.* (*17*), Nielsen and Hansen (*20*) and Domon *et al.* (*37*) reported that SDS-PAGE analysis of extracellular proteins released into the culture media showed different patterns between embryogenic and non-embryogenic tissues. Differences were also noticed among different stages of embryogenesis and among different types of cell lines. The results indicated that somatic embryogenesis is accompanied by the release of a specific set of proteins.

The role of 2,4-D in establishing embryogenic cultures of carrot and other species is well known (*2*,*19*,*38*). After cell dedifferentiation in 2,4-D-containing medium, continuous cell divisions resulted in the occurrence of masses of small isodiametric cells or cell clusters that contribute to the development of embryogenic cultures (*39*,*40*), but the disadvantage is that only 1–2 % of cells in embryogenic cultures are embryogenic (*2*) and only they can form mature somatic embryos without application of growth regulators (*18*). Non-embryogenic carrot cultures cultivated in 2,4-D-free medium differ in their extracellular proteins in comparison with embryogenic ones (*17*). Coutos-Thevenot *et al*. (*21*) report that even two cell lines with different embryogenic capacity exhibited differences in extracellular protein patterns as well.

Our system of establishing and maintaining pumpkin embryogenic cultures enabled the development of highly embryogenic cultures on hormone-free MS medium with modified nitrogen sources (NH_4Cl instead of NH_4NO_3 and KNO_3) and enabled the control of embryo maturation exclusively after transferring the tissue into MSC medium with conventional nitrogen sources (*28*,*29*). Three pumpkin embryogenic lines examined in this paper responded similarly to the same medium composition. Embryogenesis was blocked in the globular stage during cultivation in NH_4Cl-containing medium. In this medium none of the lines secreted enough proteins to be detected by the combination of silver staining and lectin reaction, methods that are known as a useful tool for detection of specific glycoproteins expressed at a low level (*41*). A lack of extracellular proteins and glycoproteins may be the cause of developmental blockage in the early stages, considering the fact that different glycoproteins are involved in embryogenesis.

After transferring the tissue with embryos blocked in the globular stage to MSC medium with conventional nitrogen composition, the secretion of proteins and development of embryos started. The first detectable proteins were those of 37 and 34 kDa. The results suggested that these proteins play a role in further development of preglobular and globular stages. The protein of apparent size of 34 kDa might belong to the family of endochitinases. In carrot embryogenesis it is shown that purified class IV EP3 chitinases can stimulate somatic embryo development of the temperature-sensitive cell line *t11* (*18*,*24*,*42*,*43*). The responsible mechanism is largely unknown but the expression of *EP3* gene in the endosperm or in a single cell in suspension and not in zygotic or somatic embryos suggests its 'nursing' function (*44*). This 'nursing' activity involves endochitinase cleavage of glucosamine and *N*-acetyl-D-glucosamine AGPs and the release of GlcNAc-containing molecules from plant cell wall, which then might contribute to the nutritition of developing embryos (*45*), or may function as signals to developing embryos (*46*).

Therefore, the lack of protein secretion in NH_4^+-containing medium might cause starvation and developmental arrest, while appropriate protein secretion in MSC medium coincides with appropriate growth and development.

In general, the secretion of properly glycosylated glycoproteins seems to be the first step associated with further development of globular embryos, although it was not crucial for the onset of embryogenesis.

Conclusion

In pumpkin tissue culture, somatic embryogenesis has been induced in two different ways: (*i*) by exogenous auxin 2,4-D and (*ii*) by changing the composition of inorganic nitrogen in nutrient media. In both cases somatic embryo development was closely associated with the presence of specific glycoproteins secreted into the culture medium. It could be stated that the non-hormonal nitrogen affects the same metabolic pathways as does the auxin 2,4-D. Therefore, nitrogen in the medium is not only a nutrient, since the balanced simultaneous application of reduced and unreduced nitrogenous compounds seems to be important for the subsequent changes in gene expression, extracellular protein pattern and capability of plant embryo development.

Moreover, the regulatory molecules that appear in nutrition media after the exchange of exogenous nitrogen source might represent embryo maturation-stimulating signals that, in zygotic embryogenesis, emanate from surrounding endosperm. Determination of their nature and function will provide us with better understanding of embryo development *in vitro* as well as *in planta*.

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