Reduced protein secretion and glycosylation induced by ammonium stress inhibits somatic embryo development in pumpkin

A. CRNKOVIĆ1, R. GARIĆ1, D. LELJAK-LEVANIĆ2, and S. MIHALJEVIĆ1*

Institute Ruđer Bošković, Bijenička cest 54, 10000 Zagreb, Croatia1
Faculty of Science, University of Zagreb, Horvatovac 102a, 10000 Zagreb, Croatia2

Abstract

Extracellular proteins and glycoproteins secreted by ammonium- or auxin-induced somatic embryogenic cultures of pumpkin were analyzed. Despite an overall similarity in developmental characteristics between these embryogenic cultures, distinct expression patterns of extracellular proteins and glycoproteins were observed. Ammonium, when supplied as the sole source of nitrogen, caused acidification of the culture medium and significantly reduced protein secretion. Buffering pH in the ammonium-containing medium restored extracellular protein secretion and glycosylation and an enhanced cell aggregation but not the development of later embryo stages. As revealed by Concavalin A (Con A) immunodetection, extracellular glycoproteins containing α-D-mannose and α-D-glucose were most abundant in proembryogenic cultures grown in a buffered ammonium-containing medium and in a medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D). We assume that extracellular proteins (Mr 28, 31, and 44 kDa) and Con A-binding glycoproteins (Mr 26, 30, 40, 53, and 100 kDa) found in both proembryogenic cultures may have a role during somatic embryogenesis induction. The glycan components of proteins were further characterized by affinity blotting with different lectins. Binding patterns of mannosese-specific lectin from Galanthus nivalis partially correlated with those detected with Con A, whereas no signal was observed with lectins from Datura stramonium and Arachis hypogea regardless of the treatment applied. Results indicate that complex N- or O-glycans are not typical for early phases of pumpkin embryo development. The accumulation of extracellular glycoproteins with high-mannose-type glycans from 30 to 34 kDa, observed after the transfer from the ammonium- or 2,4-D-containing media into a maturation medium, appeared to be associated with development of later embryo stages. This study also revealed the presence of EP-3-like endochitinases in pumpkin embryogenic cultures, particularly in cultures grown in the buffered ammonium-containing medium, however, these proteins should be examined further.

Additional key words: auxin, cell aggregation, Concavalin A immunodetection, Cucurbita pepo, endochitinase, extracellular proteins, lectins.

Introduction

Somatic embryogenesis is a process by which somatic plant cells dedifferentiate and become competent for embryogenesis. In most plant species, somatic embryogenesis is induced by exogenously supplied auxin, particularly 2,4-dichlorophenoxyacetic acid (2,4-D). However, there is growing evidence showing other abiotic stresses (temperature, osmotic pressure, heavy metals, pH, nutritional components) may...
effectively replace exogenous auxin in the acquisition of embryogenic potential in plants (Ikeda-Iwai et al. 2003, Kikuchi et al. 2006). If so, somatic embryogenesis can be considered as a particular stress-induced morphogenetic response (Zavattieri et al. 2010). Since different stress mechanisms may result in the same morphogenetic response (i.e., somatic embryogenesis), it is assumed that the cellular basis underlying this response can involve similar molecular patterns (Potters et al. 2007, Karami and Saidi 2010). The involvement of cell wall glycoproteins and proteoglycans in the signalling pathways during the plant defense response and/or adaptation processes have been well documented (Andersen and Goochee 1994, Kaku et al. 2006). Also, many extracellular proteins mediate intercellular communication during development (Lee et al. 2004). Specific extracellular proteins and glycoproteins related to somatic embryogenesis appear in suspension cultures of many plant species, such as carrot (De Jong et al. 1992), Cichorium (Helleboid et al. 2000), and Picea abies (Mo et al. 1996). The correct glycosylation and concentration of extracellular proteins were shown to be essential for the rescue of an aberrant embryogenic cell line of carrot (Lo Schiavo et al. 1990), as well as regular cultures in which embryogenesis is blocked by tunicamycin (Cordewener et al. 1991).

Somatic embryogenesis in pumpkin can be induced on a medium supplemented with 1 mg dm⁻³ 2,4-D or on a hormone-free medium supplemented with 1 mM NH₄Cl as the sole source of nitrogen (Leljak-Levanić et al. 2004). Both media sustain multiplication of embryogenic cell mass without further development into later embryo stages. Embryo maturation proceeds following auxin removal in the former, and nitrate addition in the latter case. The advantage of the hormone-free ammonium system for the induction and maintenance of somatic embryogenesis in pumpkin lies in providing highly embryogenic and synchronized cultures. This system also allows us to control embryo development in pumpkin by combining different nitrogen sources without exogenous hormone applications. Despite an overall similarity in morphological and developmental characteristics of 2,4-D- and NH₄⁺-induced somatic embryogenic cultures, it was recently shown that these two embryogenic cultures differ in their physiological response to culture conditions (Mihaljević et al. 2011) as well as in the amount and expression pattern of extracellular proteins secreted into cultivation media (Leljak-Levanić et al. 2011).

The purpose of this study was to broaden our knowledge on the involvement of protein secretion and glycosylation in the process of cellular adaptation to 2,4-D or NH₄⁺ as stressors capable of inducing somatic embryogenesis in pumpkin. Furthermore, we investigated whether the secretion of specific glycoproteins from embryogenic tissues into the liquid medium may be affected by acidic pH because culture medium acidification is one of the earliest cellular responses to NH₄⁺ uptake (Britto and Kronzucker 2002). The effects of changes in nitrogen or auxin supply, and culture medium pH were examined by monitoring N- and O-glycosylation patterns of extracellular glycoproteins. We assumed that extracellular (glyco)proteins found in both stress-induced embryogenic cultures of pumpkin may have a specific role during somatic embryo development. In addition, the expression pattern of EP3-like endochitinases, the extracellular proteins known to promote embryogenic cell formation in carrot and Arabidopsis (Van Hengel et al. 1998, Passarinho et al. 2001), was analyzed and discussed with respect to the ability of pumpkin embryogenic cultures to maintain the proembryogenic state or to form somatic embryos.

Materials and methods

Two types of embryogenic tissue cultures of pumpkin (Cucurbita pepo L.), induced and established from mature zygotic embryo explants (Leljak-Levanić et al. 2004), were analyzed and compared. The first embryogenic culture was induced and maintained on a Murashige and Skoog (1962; MS) medium supplemented with 1 mg dm⁻³ 2,4-D (denoted as MS2,4D medium); this embryogenic line (DEC) was mostly comprised of proembryogenic state or to form somatic embryos.

Acid (MES) and denoted as MSHH4MES medium. Development of later embryo stages was achieved after embryogenic tissue transfer from MSHH4 or MS2,4D into the hormone-free MS medium (denoted as MS0) with a standard nitrogen content (18 mM KNO₃ and 20 mM NH₄NO₃). A hormone-independent habituated line (HEC) was used as control for later developmental stages. This line was derived from the 2,4-D-induced line, but continuously subcultured on the hormone-free MS0 medium for more than 5 years and was comprised of embryos of all developmental stages.

All tested media were supplemented with 250 mM glucose. The pH of the medium was adjusted to 5.8 by adding a few drops of 0.2 M KOH. The embryogenic cultures were maintained on solid media prepared by adding 0.8 % (m/v) washed agar (Sigma–Aldrich, St. Louis, MO, USA) to the liquid medium and subcultured every four weeks. Small-scale suspension cultures were initiated by inoculating 1 g of fresh embryogenic tissue into the hormone-free MS medium (denoted as MS0) with a standard nitrogen content (18 mM KNO₃ and 20 mM NH₄NO₃). A hormone-independent habituated line (HEC) was used as control for later developmental stages. This line was derived from the 2,4-D-induced line, but continuously subcultured on the hormone-free MS0 medium for more than 5 years and was comprised of embryos of all developmental stages.

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from a 7-d-old solid culture into a 100-cm² Erlenmeyer flask containing 30 cm³ of a liquid medium, and grown on an orbital shaker (120 rpm) for 7 to 21 d in a growth chamber at a temperature of 24 ± 1 °C, an irradiance of 17 W m⁻² (400 - 700 nm; daylight fluorescent tubes, 40 W), and a 16-h photoperiod.

The size of cell aggregates was determined under a light microscope Olympus BX51 equipped with the DP70 digital camera system (Olympus Life Science Europe GmbH, Hamburg, Germany). Microscopic images were analyzed using the ImageJ v. 1.45s software (Rasband 2011). The size of cell aggregates was expressed as the average diameter of at least 70 randomly chosen aggregates per experiment. Variance of mean values was performed with the Duncan's multiple range test (Statistica 7.1, StaSoft, Tulsa, OK, USA) and significance was determined at P ≤ 0.05.

A conditioned medium was collected from 7- or 21-d-old cell suspension cultures by filtration through a nylon mesh (a 30-μm pore size), followed by centrifugation at 10 000 g for 10 min, and again filtration through a 0.22 μm filter. The resulting cell-free conditioned medium was dialyzed (6 - 8 kDa cut-off) in distilled water over-night, and then concentrated and desalted using an 8 kDa cut-off Centricon column (Millipore, Bedford, MA, USA). The samples were then lyophilized, dissolved in a 0.02 M phosphate buffer (pH 7), and desalted in a 0.02 M phosphate buffer (pH 7.5). The resulting culture medium concentrate was lyophilized and stored at -80 °C until further use. Total soluble cellular and extracellular proteins were extracted by grinding 0.3 g lyophilized and stored at -80 °C until further use. Total soluble cellular proteins were extracted by grinding 0.3 g lyophilized and stored at -80 °C until further use. Total soluble cellular proteins were extracted by grinding 0.3 g lyophilized and stored at -80 °C until further use. Total soluble cellular proteins were extracted by grinding 0.3 g lyophilized and stored at -80 °C until further use. Total soluble cellular proteins were extracted by grinding 0.3 g lyophilized and stored at -80 °C until further use. Total soluble cellular proteins were extracted by grinding 0.3 g lyophilized and stored at -80 °C until further use. Total soluble cellular proteins were extracted by grinding 0.3 g

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The glycans were further characterized according to the binding affinity of digoxigenin-labeled lectins: GNA (Galantus nivalis agglutinin) specific for mannose-α-1(3)-mannose units in high-mannose-type N-glycans; DSA (Datura stramonium agglutinin) specific for oligomers of N-acetylglucosamine or galactose-β(1,4)-N-acetylglucosamine in complex N-glycans; and PNA (peanut agglutinin) specific for galactose-β(1,3)-N-acetylgalactosamine present in O-glycans (all from the DIG kit). The concentration of lectins in the incubation solution was 1 μg cm⁻² for GNA and DSA, and 10 μg cm⁻² for PNA. Detection was performed using anti-digoxigenin alkaline-phosphatase conjugates and the nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) solution following manufacturer’s instructions. The following control glyco-proteins were applied: carboxypeptidase Y was used as positive control for GNA and as negative control for DSA and PNA; transferrin and fetuin were used as positive controls for DSA and as negative controls for PNA and GNA; asialofetuin was used as positive control for PNA and as negative control for GNA (all from the DIG kit).

The membranes were scanned as 200-ppi grayscale tiff-images with an HP Scanjet 3010 scanner. For EP3-like endochitinase detection in a pumpkin embryogenic culture, we used a polyclonal rabbit antibody raised against the class IV EP3-1 endochitinase from carrot (Kragh et al. 1996). This antibody was generously provided by Prof. Sacco de Vries. Anti-rabbit IgG (whole molecule)-peroxidase (Sigma-Aldrich) produced in goat was used as secondary antibody. Proteins were separated by SDS-PAGE (2 μg per lane) and electrobotted onto a PVDF membrane as described for glycoprotein detection. After the transfer, the membrane was equilibrated for 1 h in 50 mM Tris/HCl (pH 7.6), 150 mM NaCl, 0.05 % (v/v) Tween 20 supplemented with 5 % (m/v) milk powder, and then incubated with the primary antibodies (1:1 000) at 4 °C overnight, and with the secondary antibodies (1:5 000) at 4 °C for 2 h. Signal detection was performed by incubating the membrane for 1 - 5 min in Luminol and peroxide solutions (Amersham™ ECL™ Prime Western Blotting Reagents, GE Healthcare, Buckinghamshire, UK). The radiation signal emitted by the decay of the chemiluminescent substrate was recorded on an X-ray film (Agfa-Gevaert N.V., Mortsel, Belgium).
Results

In this study, we found a correlation between a slower growth rate and week secretion of extracellular proteins in the culture medium (Table 1), both potentially associated with a strong acidification of the culture medium as a remarkable characteristic of embryogenic tissue grown in the medium containing NH$_4^+$ as the sole nitrogen source. Within the first week of cultivation, pH of the culture medium dropped from initial pH 5.8 to pH 3.6. When the NH$_4^+$-containing medium was stabilized at pH 5.1 by buffering with MES, the accumulation of extracellular proteins was enhanced but the embryo development remained arrested. The recovery of the extracellular protein secretion was also observed in the cultures transferred from the MSNH$_4$ medium into the hormone-free MS0 medium with a standard nitrogen composition (Table 1) which enabled cell aggregation and further embryo development (Leljak-Levanić et al. 2004). Embryogenic tissue maintained in the 2,4-D-containing medium grew faster and secreted more proteins compared to tissue cultured in MSNH$_4$. When the amount of total soluble cellular proteins was compared between the embryogenic cultures (Table 1), a significantly lower proteins content was observed in embryogenic tissue grown in the buffered or non-buffered NH$_4^+$-containing media than in tissue cultured in the MS0 or MS2,4D medium.

Table 1. Extracellular and total soluble protein content in 7-d-old embryogenic suspension cultures of *C. pepo* as affected by culture media ingredients and pH. Means ± SD. The size of cell aggregates was expressed as the average diameter of at least 70 aggregates grown for 21 d in three different cultures. Statistically significant difference at 5% was determined with the Duncan's new multiple range test (b.d.l. - below detection limit, n.d. - not determined).

<table>
<thead>
<tr>
<th>Tissue line</th>
<th>Treatment</th>
<th>Extracellular proteins in medium [mg cm$^{-3}$]</th>
<th>Total soluble cellular proteins [mg g$^{-1}$ (d.m.)]</th>
<th>Medium pH</th>
<th>Cell aggregate size [μm$^2$]</th>
</tr>
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<tbody>
<tr>
<td>PEDC</td>
<td>MSNH$_4$</td>
<td>b.d.l.</td>
<td>13.08 ± 1.99 c</td>
<td>3.57 ± 0.21 c</td>
<td>19 402 b</td>
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<tr>
<td></td>
<td>MSNH$_4$MES</td>
<td>7.75 ± 1.0 b</td>
<td>13.57 ± 3.00 c</td>
<td>5.13 ± 0.28 a</td>
<td>24 704 b</td>
</tr>
<tr>
<td></td>
<td>MSNH$_4$→MS0</td>
<td>1.19 ± 1.2 c</td>
<td>31.56 ± 9.51 b</td>
<td>3.70 ± 0.13 c</td>
<td>29 253 b</td>
</tr>
<tr>
<td>DEC</td>
<td>MS2,4D</td>
<td>9.53 ± 3.3 b</td>
<td>46.45 ± 6.78 a</td>
<td>4.82 ± 0.09 b</td>
<td>442 240 a</td>
</tr>
<tr>
<td></td>
<td>MS2,4D→MS0</td>
<td>9.10 ± 3.9 b</td>
<td>21.08 ± 4.51 bc</td>
<td>5.13 ± 0.09 a</td>
<td>510 930 a</td>
</tr>
<tr>
<td>HEC</td>
<td>MS0</td>
<td>14.48 ± 6.7 a</td>
<td>47.17 ± 6.90 a</td>
<td>4.82 ± 0.20 b</td>
<td>n.d.</td>
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Fig. 1. Morphology of pumpkin embryogenic cultures stained with aceticarmine: A - small friable proembryogenic cell aggregates grown in the MSNH$_4$ medium, B – a globular embryo attached to nodular embryogenic tissue grown in the MS2,4D medium; bars = 200 μm.

The SDS PAGE analysis of extracellular proteins revealed qualitative and quantitative differences between the proembryogenic cultures grown in the MS2,4D or MSNH$_4$ culture medium (Fig. 2). Despite an extensive and carefully executed procedure of the extracellular protein concentration from the suspension cultivation medium, the sample preparation from the non-buffered MSNH$_4$ cultivation medium was hindered by a very low concentration of extracellular proteins. To obtain a sufficient amount of total extracellular proteins for the analysis, the MSNH$_4$ culture medium was 10 times more concentrated then the other culture media. After buffering the MSNH$_4$ medium with MES (MSNH$_4$MES), several bands at 40, 48-51, and 62 kDa specific for this culture medium appeared (Fig. 2). An extracellular protein pattern from the MS2,4D culture medium differed from that of the MSNH$_4$MES culture medium by the appearance of a new 53 kDa protein (Fig. 2). Signals of 28, 31-34, and 44 kDa proteins were found to be common for proembryogenic tissues grown in the MS2,4D or
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Subcultivation from the MS2,4D into MS0 medium, favourable for embryo development, enhanced a signal amplification at 74, 80, and 104 kDa (Fig. 2). Most bands observed after the transfer from MS2,4D into MS0 were also observed in the MS0 culture medium of the habituated embryogenic tissue line which comprised all embryo stages, but 32 and 34 kDa proteins dominated (Fig. 2). Seven days after the transfer from the MSNH4 into MS0 medium, extracellular protein secretion was re-established (Table 1); secreted proteins were mostly smaller than 14 kDa (Fig. 2). A significant amount of low molecular mass extracellular proteins was also observed in other samples, regardless of the treatment applied.

As immunodetected with Con A, extracellular glycoproteins with α-D-mannose and α-D-glucose were most abundant in samples from the MSNH4MES and MS2,4D culture media, as well as in samples collected 7 d after the transfer from the MS2,4D into MS0 medium (Fig. 3A). The patterns of extracellular glycoproteins from these embryogenic cultures had several common signals at 21, 23, 32, 65, and 87 kDa (Fig. 3A). Despite a weak detection of extracellular proteins in the MSNH4 sample after silver staining, immunodetection with Con A revealed more than a few positive signals in this sample. Still, signals from MSNH4 were weaker than those from MSNH4MES, particularly the bands of 21, 23, 40, 80, and 100 kDa (Fig. 3A). Seven days after the transfer from the MSNH4 into MS0 medium, signals at 30, 32, 40, and 53 kDa were amplified (Fig. 3A). In the MS2,4D culture medium, an increase in the number of signals ranging from 20 to 30 kDa was observed and remained largely unchanged for 7 d after the transfer from the MS2,4D medium into MS0 (Fig. 3A). In the habituated line grown in the MS0 medium, signals of 21, 23, 32, 65, and 87 kDa (Fig. 3A) were similar to those that appeared in samples from the nonhabituated cultures, but many other signals were missing, indicating modifications in the extracellular protein glycosylation of habituated embryogenic tissue.

**Fig. 2.** The SDS-PAGE analysis of extracellular proteins from culture media of different pumpkin embryogenic suspension cultures. Proteins derived from the cell line grown for 7 d in MSNH4MES (lane M) or MSNH4 (lane N), or 7 d after the transfer from the MSNH4 into MS0 medium (lane N0). Proteins derived from the cell line grown in MS2,4D for 7 d (lane D) or 7 d after the transfer from MS2,4D into the MS0 medium (lane D0). Lane H - proteins derived from the habituated line grown in MS0 for 7 d. Mr - protein molecular mass markers (a prestained protein ladder, Thermo Scientific) in kDa. The arrows mark bands specific for a treatment. The arrowheads mark proteins common for treatments.

**Fig. 3.** The glycoprotein pattern of extracellular proteins from culture media of different pumpkin embryogenic suspension cultures after SDS-PAGE and the transfer of extracellular proteins onto a PVDF membrane determined according to binding lectins: A - Concanavalin A, and B - Galanthus nivalis agglutinin (GNA). Lane 1 - carboxypeptidase Y, lane 2 - fetuin. Proteins derived from the cell line grown for 7 d in MSNH4 (lane N) or MSNH4MES (lane M), or 7 d after the transfer from the MSNH4 into MS0 medium (lane N0). Proteins derived from the cell line grown for 7 d in the MS2,4D medium (lane D) or 7 d after the transfer from MS2,4D into MS0 (lane D0). Lane H - proteins derived from the habituated line grown in MS0 for 7 d. For the GNA analysis, two additional samples were collected: 21 d after the transfer from the MSNH4 into MS0 medium (lane N021) or from MS2,4D into MS0 (lane D021). The arrowheads mark signals common for treatments. The asterisk mark missing signals.

Glycosylation patterns detected with GNA showed some similarities with Con A patterns, although detection with GNA was more specific. After 7 d of culture, the strongest signals were detected in samples from the MSNH4MES and MS2,4D media, and after the transfer from MS2,4D into MS0 (Fig. 3B). The MSNH4MES and MS2,4D samples shared common GNA-binding proteins at 21, 23, 40, and 80 kDa (Fig. 3B), whereas signals at 34 and 53 kDa were missing in the MS2,4D sample (Fig. 3B). However, both in the MS2,4D culture medium and 7 d after the transfer into the MS0 medium, additional differential signals were observed between
20 and 26 kDa (Fig. 3B). In samples from the non-buffered MSNH4 medium as well as in samples collected 7 d after the transfer from MSNH4 into MS0, very few glycoproteins with high mannos-Ne-N-glycans were detected (Fig. 3B). GNA-binding extracellular glycoproteins from the 21-d-old cultures were analyzed in order to see whether changes remained during a prolonged period of cultivation on the maturation MS0 medium (Fig. 3B). Twenty-one days after the transfer from the MSNH4 into MS0 medium, signals at 34, 47, 55, 58, 69 and 80 kDa had the highest intensity (Fig. 3B).

A prolonged period of cultivation also enhanced the accumulation of GNA-binding extracellular proteins in the cultures transferred from MS2,4D into MS0: signals from 30 to 34 and from 47 to 69 kDa became dominant, whereas signals below 30 kDa faded (Fig. 3B). Unfortunately, the analysis of lectin-binding extracellular glycoproteins from the 21-d-cultures was limited only to the GNA-assay due to the slow growth of the MSNH4 cultures. The habituated line was characterized by only two dominant GNA signals at 32 and 34 kDa (Fig. 3B).

After immunodetection with DSA or PNA (Fig. 4A, B), A. CRNKOVIĆ et al. signals were small and faint, and therefore could not be identified with certainty as glycoproteins with galactose-β(1,4)-N-acetylglucosamine or galactose-β(1,3)-N-acetyl-galactosamine units. A potentially positive signal at 20 kDa appeared in samples from the MS2,4D culture medium, and MS0 culture medium from the habituated line (Fig. 4A, B), whereas a signal at 40 kDa appeared only in the habituated line (Fig. 4A, B).

Immunodetection with polyclonal antibodies raised against the class IV endochitinase EP3-1 from Daucus carota (Kragh et al. 1996) revealed two extracellular pumpkin proteins of 64 and 68 kDa in samples from the MSNH4 and MS2,4D cultures (Fig. 5). These signals were particularly strong in the MSNH4MES culture medium. In a sample from the habituated cell line grown in MS0, a signal at 68 kDa was also present along with much stronger signals at 32 and 34 kDa. In samples from the MSNH4 and MS2,4D cultures, signals at 32 and 34 kDa were detectable only after a prolonged exposition of the X-ray film (data not shown). After immunodetection with EP3-1 antibodies, no positive signal was detected in the total soluble cellular protein solution from any of the examined embryogenic cultures (data not shown).

Discussion

Ammonium (NH₄⁺) provided as the sole source of nitrogen sustains growth in many plant species (Britto and Kronzucker 2002, Ali et al. 2013). However, when supplied at a stressful, but not harmful concentration,
ammonium may alter cell differentiation and trigger somatic embryogenesis induction (Smith and Krikorian 1990, Leljak-Levanić et al. 2004). The effects of ammonium assimilation on embryogenic carrot cell multiplication without development into later embryo stages have been attributed mainly to culture medium acidification (Smith and Krikorian 1992). However, the underlying mechanisms of the process are not completely understood. Physiological effects of NH$_4^+$ on an embryogenic suspension culture of pumpkin, such as callose accumulation and the arrest of embryo development at the preglobular stage point to the possible involvement of changes in cell wall properties and the associated extracellular matrix of glycoproteins in the process (Mihaljević et al. 2011).

The quantity of extracellular proteins detected in the embryogenic pumpkin cell cultures grown in the non-buffered, NH$_4^+$-containing medium was almost negligible compared to the 2,4-D-cultured cells which is in accordance with our previous observations (Leljak-Levanić et al. 2011). The low total cellular protein content in NH$_4^+$-treated embryogenic tissues was most probably due to limited nitrogen supply. Nevertheless, acidification of the culture medium caused by the uptake of NH$_4^+$ as the exclusive nitrogen source was shown to be an important factor involved in the regulation of protein secretion and glycosylation in the embryogenic pumpkin cultures. When the NH$_4^+$-medium was stabilized at pH 5.1 (MSNH4MES), the number and amounts of extracellular proteins and Con A and GNA signals increased. Extracellular glycoproteins with N-linked high-mannose glycans were also present in high concentrations in the proembryogenic cultures induced and maintained in the presence of 2,4-D, and shortly after its removal from the culture medium. The positive effect of 2,4-D on protein glycosylation and the early phase of somatic embryogenesis was reported previously (Sato et al. 1986). No signals were detected after immunodetection with lectins PNA and DSA, regardless of treatment applied. This suggests that O-glycans and complex N-glycans were not abundant in the extracellular glycoprotein fraction of the pumpkin embryogenic cultures, at least not during the early phase of embryo development. It is also known from literature that ammonium assimilation may inhibit the completion of the final sialylation reaction in the O-linked glycosylation pathway (Andersen and Goochee 1994) as well as O-linked glycosylation (Bøys et al. 1994). According to Andersen and Goochee (1994), this inhibition is pH dependent and related to the concentration of neutral ammonium in the medium. In addition, Qin et al. (2008) provided evidences that NH$_4^+$ uptake and associated acidification of the growth medium inhibit the activity of GDP-manose pyrophosphorylase in Arabidopsis and cause defective protein glycosylation which is likely to be one of the downstream molecular mechanisms important for the inhibition of Arabidopsis growth by NH$_4^+$.

The differential accumulation of proteins may provide important clues to the function and regulation of genes associated with embryogenesis (Mishra et al. 2012). Extracellular proteins (28, 31, and 44 kDa) and Con A-binding glycoproteins (26, 30, 40, 53, and 100 kDa) that were common exclusively for the proembryogenic cultures regardless of the induction medium composition, but not for the habituated line which contained later developmental stages of embryos, might represent proembryogenic-stimulating signals. On the contrary, the accumulation of specific extracellular proteins and GNA-binding glycoproteins (32 - 34 and 47 - 69 kDa) detectable after the transfer from MS2,4-D or MSNH4 into the hormone-free MS0 medium favourable for embryo development may be considered important for the transition of early globular to later embryo stages.

In this study, enhanced extracellular protein secretion and glycosylation in the buffered NH$_4^+$-medium coincided with the appearance of larger embryogenic cell aggregates although no further differentiation into later stage embryos was observed. Steiner and Dougal (1995) explained the tendency of plant cells to remain aggregated at higher pH by a stronger cell wall and a greater cohesive force between the cells. The involvement of extracellular proteins and glycoproteins in cell-to-cell communication and somatic embryo development is well known (Lo Schiavo et al. 1990, Jamet et al. 2008) but examples of the influence of culture medium pH on protein secretion and glycosylation are limited (Andersen and Goochee 1994). Our results suggest that the acidification of the culture medium caused by assimilation of NH$_4^+$ might influence the embryogenic cell aggregation and further somatic embryo development through the modification of extracellular protein secretion and glycosylation.

Plant EP3 chitinases are extracellular proteins involved in reinitiating cell division in embryogenic cells and embryos as part of a nursing cell system that is required for embryogenesis (Van Hengel et al. 1998). They are present in a high amount in the proembryogenic cell mass of carrot, in single cells, as well as in the small clusters of ‘nursing’ cells that do not develop into embryos themselves but secrete EP3 chitinase into the culture medium promoting the transition of globular to heart stage embryo (Van Hengel et al. 1998). The EP3 chitinases also promote progression in somatic embryo development when added to the temperature-sensitive carrot cell line ts11 (De Jong et al. 1992). Interestingly, after immunoblotting pumpkin total extracellular proteins with antibodies raised against carrot EP3-1 endochitinase, the positive signals of expected Mr 32 to 34 kDa (Krugh et al. 1996) were dominant in the samples from the habituated embryogenic culture, but only faint in the embryogenic cultures grown in the MS2,4D or MSNH4 medium. However, in the presence of 2,4-D or NH$_4^+$, the EP3-like signals at 64 and 68 kDa prevailed. Plant chitinases are known to exist typically as monomers with Mr ranging from 16 to 40 kDa, although it has been suggested that some endochitinases are dimers (Ary et al. 1989) or make heterodimers with exochitinases (Shoresh
and Harman 2010). So far, no pumpkin EP3-like endochitinase has been identified, and the proteins identified in this study should be examined further. An increase in the EP3-like signals observed in the pumpkin embryogenic cultures grown in buffered and non-buffered MSNH4 compared to the MS2,4D medium might be related to the dominance of small cell aggregates in the NH4+-induced embryogenic cultures.

In conclusion, adaptive differences of the pumpkin somatic embryogenic cells in response to 2,4-D or NH4+ were reflected in the changes of extracellular protein secretion and N-glycosylation. The results indicate that, although both 2,4-D and NH4+ are capable of inducing and maintaining proembryogenic tissue in pumpkin, ammonium as the sole source of nitrogen hampered extracellular protein secretion. The negative effect of ammonium on protein secretion and glycosylation and related inhibition of cell aggregation and somatic embryo development is partially mediated by culture medium acidification. Further characterization of the extracellular glycoproteins identified in this work will provide a better understanding of the role of glycosylation in the NH4+ regulation of somatic embryogenesis but also in plant sensitivity to NH4+ in general.

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