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

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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 mannose-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

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; Con A - concavalin A; DEC - embryogenic line induced and maintained on MS2,4D medium; DIG - digoxigenin; DSA - lectin from *Datura stramonium*; GNA - lectin from *Galanthus nivalis*; HEC - habituated embryogenic line; MES - 2-(N-morpholino)-ethane-sulfonic acid; MS - Murashige and Skoog; MS2,4D - MS medium supplemented with 2,4-D; MSNH4 - hormone-free MS medium supplemented with NH_4^+ as the sole source of nitrogen; MSNH4MES - hormone-free MS medium supplemented with NH_4^+ as the sole source of nitrogen and buffered with MES; NBT/BCIP - nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate; PEDC - embryogenic line induced and maintained on the MSNH4 medium; PNA - lectin from *Arachis hypogea*; PVDF - polyvinylidene difluoride membrane; SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis.
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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 morphogenic response (Zavattieri *et al.* 2010). Since different stress mechanisms may result in the same morphogenic 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 proteoglycanes 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 NH_4Cl 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 NH_4^+-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 NH_4^+ 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 NH_4^+ 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 embryos in preglobular and globular stages (Fig. 1*A*). The second embryogenic culture was a proembryogenic determined cell line (PEDC), induced and maintained on a hormone-free MS medium supplemented with 1 mM NH_4Cl as the sole source of nitrogen (denoted as MSNH4 medium); this line was mostly comprised of proembryogenic determined cells, preglobular embryos, and a small portion of globular embryos (Fig. 1*B*). In order to distinguish the effects of NH_4^+ ions from the effects of culture medium acidification, the MSNH4 medium was buffered with 25 mM 2-(N-morpholino)-ethane-sulfonic acid (MES) and denoted as MSNH4MES medium. Development of later embryo stages was achieved after embryogenic tissue transfer from MSNH4 or MS2,4D into the hormone-free MS medium (denoted as MS0) with a standard nitrogen content (18 mM KNO_3 and 20 mM NH_4NO_3). 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

from a 7-d-old solid culture into a 100-cm^3 Erlenmeyer flask containing 30 cm^3 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 $P \leq 0.05$.

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 *Centriprep* 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 proteins were extracted by grinding 0.3 g of fresh tissue in liquid nitrogen and then homogenized in 1.5 cm^3 of 0.05 M ice-cold Tris/HCl, pH 7.5, 150 mM NaCl, 1 % (v/v) *Nonidet P-40*, 0.5 % (m/v) sodium deoxicolate, and a complete EDTA-free protease inhibitor cocktail (*Roche Diagnostics*, Mannheim, Germany). Following homogenate centrifugation at 13 000 *g* and 4 °C for 20 min, the supernatant was collected. Total soluble cellular and extracellular proteins were quantified according to Bradford (1976).

Protein samples were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in 12 % (m/v) polyacrylamide gels using the Laemmli buffer system (1970). Prior to electrophoretic separation, the protein samples were denatured by heating at 96 °C for 5 min using a 0.125 M Tris buffer (pH 6.8) containing 5 % (v/v) β-mercaptoethanol and 2 % (m/v) sodium dodecyl sulphate (SDS). Approximately 2 μg of protein was loaded per lane. Protein bands were visualized by silver staining, and the gels were scanned as 200-ppi grayscale tiff images with an *HP Scanjet 3010* scanner (*Hewlett-Packard*, Palo Alto, CA, USA).

Proteins separated by SDS-PAGE were electroblotted to a polyvinylidene difluoride (PVDF) membrane (*Immobilon-P*, *Millipore*) as described by Balen *et al.* (2007). Glycoproteins were detected with Concavalin A (Con A) and peroxidase binding. The bound peroxidase was visualized with 4-chloronaphthol (Towbin *et al.* 1979). Con A specifically binds α-D-mannose and with low affinity α-D-glucosyl residues (Hrubá and Tupý 1999, Kaji *et al.* 2003). Carboxypeptidase Y and transferrin were applied as positive control, whereas fetuin and asialofetuin were used as negative controls (all from a *DIG* glycan differentiation kit, *Roche Diagnostics*).

The glycan part of proteins was 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^{-3} for GNA and DSA, and 10 μg cm^{-3} 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 glycoproteins 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 electroblotted 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^{TM} ECL^{TM} Prime Western Blotting Reagents*, *GE Healthcare*, Buckinghamshire, UK). The radiation signal emitted by the decay of the chemiluminiscent 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 MSNH4. 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).

Tissue line	Treatment	Extracellular proteins in medium [mg cm^{-3}]	Total soluble cellular proteins [mg g^{-1} (d.m.)]	Medium pH	Cell aggregate size [μm^2]
PEDC	MSNH4	b.d.l.	13.08 ± 1.99 c	3.57 ± 0.21 c	19 402 b
	MSNH4MES	7.75 ± 1.0 b	13.57 ± 3.00 c	5.13 ± 0.28 a	24 704 b
	MSNH4→MS0	1.19 ± 1.2 c	31.56 ± 9.51 b	3.70 ± 0.13 c	29 253 b
DEC	MS2,4D	9.53 ± 3.3 b	46.45 ± 6.78 a	4.82 ± 0.09 b	442 240 a
	MS2,4D→MS0	9.10 ± 3.9 b	21.08 ± 4.51 bc	5.13 ± 0.09 a	510 930 a
HEC	MS0	14.48 ± 6.7 a	47.17 ± 6.90 a	4.82 ± 0.20 b	n.d.

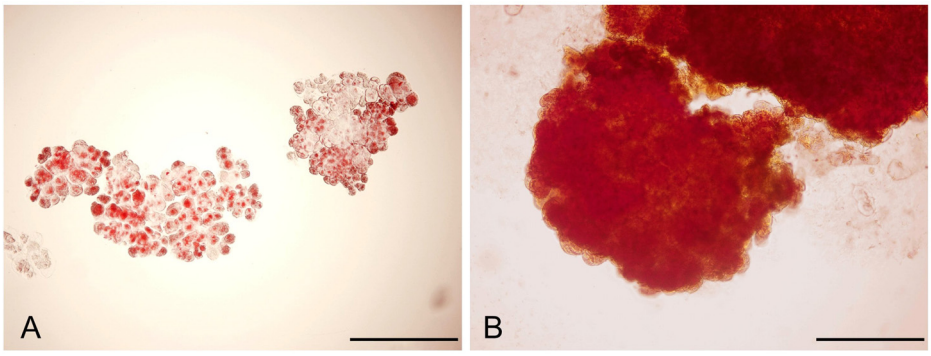

Fig. 1. Morphology of pumpkin embryogenic cultures stained with acetocarmine: *A* - small friable proembryogenic cell aggregates grown in the MSNH4 medium, *B* – a globular embryo attached to nodular embryogenic tissue grown in the MS2,4D medium; *bars* = 200 μm.

The SDS PAGE analysis of extracellular proteins revealed qualitative and quantitative differences between the proembryogenic cultures grown in the MS2,4D or MSNH4 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 MSNH4 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 MSNH4 culture medium was 10 times more concentrated then the other culture media. After buffering the MSNH4 medium with MES (MSNH4MES), 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 MSNH4MES 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

MSNH4MES medium (Fig. 2). 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.

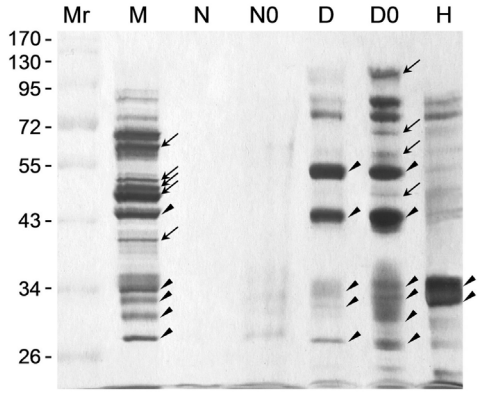

Fig. 2. The SDS-PAGE analysis of extracellular proteins from culture media of different pumpkin embryogenic suspension cultures. Proteins derived from the cell line grown for 7 d in MSNH4MES (*lane* M) or MSNH4 (*lane* N), or 7 d after the transfer from the MSNH4 into MS0 medium (*lane* N0). Proteins 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.

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. 3*A*). The patterns of extracellular glycoproteins from these embryogenic cultures had several common signals at 21, 23, 26, 30, 32, 40, 53, 65, 87, and 100 kDa (Fig. 3). 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. 3*A*). Seven days after the transfer from the MSNH4 into MS0 medium, signals at 30, 32, 40, and 53 kDa were amplified (Fig. 3*A*). 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. 3*A*). In the habituated line grown in the MS0 medium, signals of 21, 23, 32, 65, and 87 kDa (Fig. 3*A*) 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.

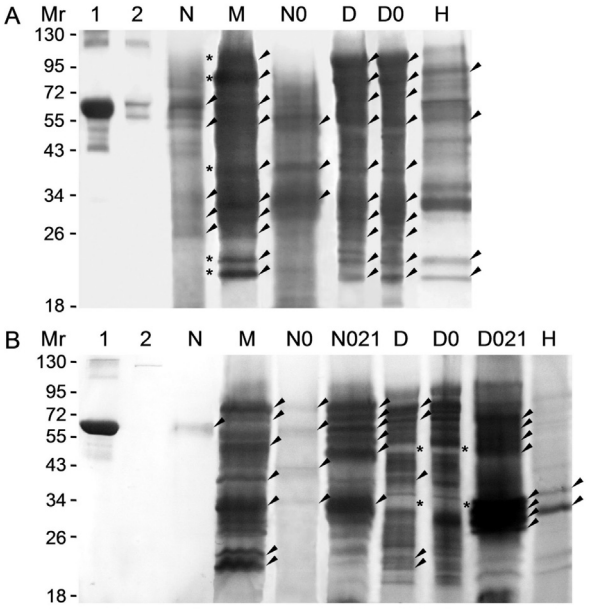

Fig. 3. The glycoprotein pattern of extracellular proteins from culture media of different pumpkin embryogenic suspension cultures after SDS-PAGE and the transfer of extracellular proteins onto a PVDF membrane determined according to binding lectins: *A* - Concavalin A, and *B* - *Galantus nivalis* agglutinin (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. 3*B*). The MSNH4MES and MS2,4D samples shared common GNA-binding proteins at 21, 23, 40, and 80 kDa (Fig. 3*B*), whereas signals at 34 and 53 kDa were missing in the MS2,4D sample (Fig. 3*B*). 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. 3*B*). 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 mannose-*N*-glycans were detected (Fig. 3*B*). 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. 3*B*). 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. 3*B*). 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. 3*B*). 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 3*B*).

After immunodetection with DSA or PNA (Fig. 4*A*,*B*),

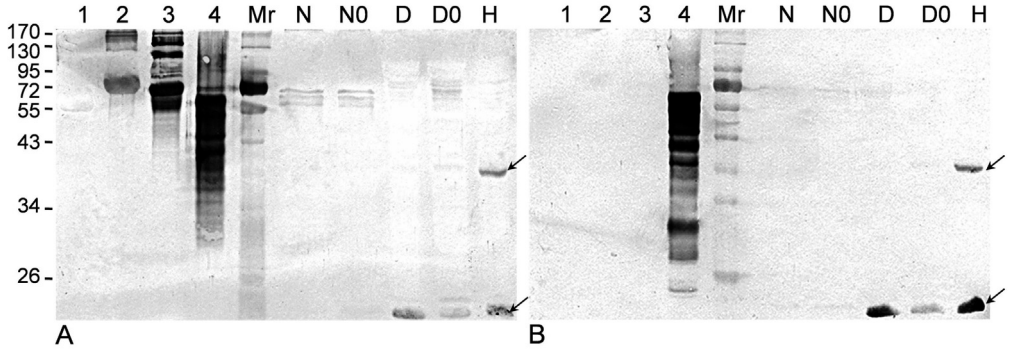

Fig. 4. The glycoprotein pattern of extracellular proteins from culture media of different pumpkin embryogenic suspension cultures after SDS-PAGE and the transfer of extracellular proteins onto a PVDF membrane determined according to lectins binding: *A* - *Datura stramonium* agglutinin (DSA), and *B* - peanut agglutinin (PNA). *Lane* 1 - carboxypeptidase Y, *lane* 2 - transfferin, *lane* 3 - fetuin, *lane* 4 - asialofetuin. Proteins derived from the cell line grown in MSNH4 for 7 d (*lane* N) or 21 d (*lane* N21), or 7 d after the transfer into the MS0 medium (*lane* N0). Proteins derived from the cell line grown in MS2,4D for 7 d (*lane* D) or 21 d (*lane* D21), or 7 d after the transfer into the MS0 medium (*lane* D0). *Lane* H - proteins derived from the habituated line grown in the MS0 medium for 7 d. The *arrows* mark putative positive signals.

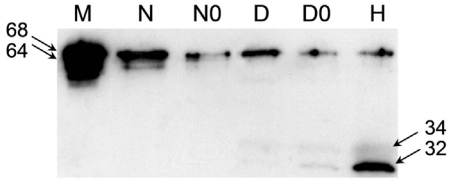

Fig. 5. The immunodetection of extracellular protein patterns of pumpkin embryogenic cell suspension cultures developed with polyclonal antibodies against carrot endochitinase EP3. Extracellular proteins from the cell line grown for 7 d in MSNH4MES (*lane* M) or in MSNH4 (*lane* N), and 7 d after the transfer from the MSNH4 into MS0 medium (*lane* N0). Extracellular proteins from the cell line grown in MS2,4D for 7 d (*lane* D), and 7 d after the transfer from the MS2,4D to MS0 medium (*lane* D0). Extracellular proteins from the habituated cell line cultured for 7 d in the hormone-free MS medium (*lane H*).

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. 4*A*,*B*), whereas a signal at 40 kDa appeared only in the habituated line (Fig. 4*A*,*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 immune-detection 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 (NH_4^+) 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 (Satoh *at al*. 1986). No signals were detected after immuno-detection with lectins PNA and DSA, regardless of the treatment applied. This suggests that *O*-glycans and complex *N*-glycanes weare 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 -linked glycosylation (Borys *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 Dougall (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 (Kragh *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 NH_4^+-induced proembryogenic cultures.

In conclusion, adaptive differences of the pumpkin somatic embryogenic cells in response to 2,4-D or NH_4^+ were reflected in the changes of extracellular protein secretion and *N*-glycosylation. The results indicate that, although both 2,4-D and NH_4^+ 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 NH_4^+ regulation of somatic embryogenesis but also in plant sensitivity to NH_4^+ in general.

References

Ali, S., Farooq, M.A., Jahangir, M.M., Abbas, F., Bharwana, S.A., Zhang, G.P.: Effect of chromium and nitrogen form on photosynthesis and anti-oxidative system in barley. - Biol. Plant. **57**: 758-763, 2013.

Andersen, D.C., Goochee, C.F.: The effect of cell-culture conditions on the oligosaccharide structures of secreted glycoproteins. - Curr. Opin. Biotechnol. **5**: 546-549, 1994.

Ary, M.B., Richardson, M., Shewry, P.R.: Purification and characterization of an insect α-amylase inhibitor/ endochitinase from seeds of Job's tears (*Cioix lachryma-jobi*). - Biochim. biophys. Acta **993**: 260-266, 1989.

Balen, B., Krsnik-Rasol, M., Zamfir, A.D., Zadro, I., Vakhrushev, S.Y., Peter-Katalinić, J.: Assessment of N-glycan heterogeneity of cactus glycoproteins by one-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. - J. Biomol. Tech. **18**: 162-172, 2007.

Borys, M.C., Linzer, D.I.H., Papoutsakis, E.T.: Ammonia affects the glycosylation patterns of recombinant mouse placental lactogen-I by chinese hamster ovary cells in a pH-dependent manner. - Biotechnol. Bioeng. **43**: 505-514, 1994.

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Britto, D.T., Kronzucker, H.J.: NH_4^+ toxicity in higher plants: a critical review. - J. Plant Physiol. **159**: 567-584, 2002.

Cordewener, J., Booij, H., Van Der Zandt, H., Van Engelen, F., Van Kammen, A., De Vries, S.C.: Tunicamycin-inhibited carrot somatic embryogenesis can be restored by secreted cationic peroxidase isoenzymes. - Planta **184**: 478-486, 1991.

De Jong, A.J., Cordewener, J., Lo Schiavo, F., Terzi, M., Vandekerckhove, J., Van Kammen, A., De Vries, S.C.: A carrot somatic embryo mutant is rescued by chitinase. - Plant Cell **4**: 325-433, 1992.

Helleboid, S., Hendriks, T., Bauw, G., Inzé, D., Vasseur, J., Hilbert, J.L.: Three major somatic embryogenesis related proteins in *Cichorium* identified as PR proteins. - J. exp. Bot. **51**: 1189-1200, 2000.

Hrubá, P., Tupý, J.: *N*-glycoproteins specific for different stages of microspore and pollen development in tobacco. - Plant Sci **141**: 29-40, 1999.

Ikeda-Iwai, M., Umehara, M., Satoh, S., Kamada, H.: Stress-induced somatic embryogenesis in vegetative tissues of *Arabidopsis thaliana*. - Plant J. **34**: 107-114, 2003.

Jamet, E., Albenne, C., Boudart, G., Irshad, M., Canut, H., Pont-Lezica, R.: Recent advances in plant cell wall proteomics. - Proteomics **8**: 893-908, 2008.

Kaji, H., Saito, H., Yamauchi, Y., Shinkawa, T., Taoka, M., Hirabayashi, J., Kasai, K., Takahashi, N., Isobe, T.: Lectin affinity capture, isotope-coded tagging and mass spectrometry to identify *N*-linked glycoproteins. - Natur. Biotechnol. **21**: 667-672, 2003.

Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., Minami, E., Shibuya, N.: Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. - Proc. nat. Acad. Sci. USA **103**: 11086-11091, 2006.

Karami, O., Saidi, A.: The molecular basis for stress-induced acquisition of somatic embryogenesis. - Mol. Biol. Rep. **37**: 2493-2507, 2010.

Kikuchi, A., Sanuki, N., Higashi, K., Koshiba, T., Kamada, H.: Abscisic acid and stress treatment are essential for the acquisition of embryogenic competence by carrot somatic cells. - Planta **223**: 637-645, 2006.

Kragh, K.M., Hendriks, T., De Jong, A.J., Lo Schiavo, F., Bucherna, N., Højrup, P., Mikkelsen, J.D., De Vries, S.C.: Characterization of chitinases able to rescue somatic embryos of the temperature-sensitive carrot variant *ts11*. - Plant mol. Biol. **31**: 631-645, 1996.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. - Nature **227**: 680-685, 1970.

Lee, S.J., Saravanan, R.S., Damasceno, C.M., Yamane, H., Kim, B.D., Rose, J.K.: Digging deeper into the plant cell wall proteome. - Plant Physiol. Biochem. **42**: 979-988, 2004.

Leljak-Levanić, D., Bauer, N., Mihaljević, S., Jelaska, S.: Somatic embryogenesis in pumpkin (*Cucurbita pepo* L.): control of somatic embryo development by nitrogen compounds. - J. Plant Physiol. **161**: 229-236, 2004.

Leljak-Levanić, D., Čipčić, H., Uzelac, L., Mihaljević, S., Bauer, N., Krsnik-Rasol, M., Jelaska, S.: Extracellular glycoproteins in embryogenic culture of pumpkin (*Cucurbita pepo* L.). - Food Technol. Biotechnol. **49**: 156-161, 2011.

Lo Schiavo, F., Giuliano, G., De Vries, S.C., Genga, A., Bollini, R., Pitto, L., Cozzani, F., Nuti-Ronchi, V., Terzi, M.: A carrot cell variant temperature sensitive for somatic embryogenesis reveals a defect in the glycosylation of extracellular proteins. - Mol. gen. Genet. **223**: 385-393, 1990.

Mihaljević, S., Radić, S., Bauer, N., Garić, R., Mihaljević, B., Horvat, G., Leljak-Levanić, D., Jelaska, S.: Ammonium-

related metabolic changes affect somatic embryogenesis in pumpkin (*Cucurbita pepo* L.). - J. Plant Physiol. **168**: 1943-1951, 2011.

Mishra, S., Sanyal, I., Amla, D.V.: Changes in protein pattern during different developmental stages of somatic embryos in chickpea. - Biol. Plant. **56**: 613-619, 2012.

Mo, L.H., Egertsdotter, U., Von Arnold, S.: Secretion of specific extracellular proteins by somatic embryos of *Picea abies* is dependent on embryo morphology. - Ann. Bot. **77**: 143-152, 1996.

Murashige, T., Skoog, F.: A revised medium for the rapid growth and bioassays with tobacco tissue culture. - Physiol. Plant **15**: 473-497, 1962.

Passarinho, P.A., Van Hengel, A.J., Fransz, P.F., De Vries, S.C.: Expression of the *Arabidopsis thaliana AtEP3/ AtchitIV* endochitinase gene. - Planta **212**: 556-567, 2001.

Potters, G., Pasternak, T.P., Guisez, Y., Palme, K.J., Jansen, M.A.K.: Stress induced morphogenic responses: growing out of trouble? - Trends Plant Sci., **12**: 98-105, 2007.

Qin, C., Qian, W., Wang, W., Wu, Y., Yu, C., Jiang, X., Wang, D., Wu, P.: GDP-mannose pyrophosphorylase is a genetic determinant of ammonium sensitivity in *Arabidopsis thaliana*. - Proc. nat. Acad. Sci. USA **105**: 18308-18313, 2008.

Rasband, W.S.: *ImageJ* (version 1.45s) software. - U.S. National Institutes of Health, Bethesda 2013 (http://imagej.nih.gov/ij)

Satoh, S., Kamada, H., Harada, H., Fujii, T.: Auxin-controlled glycoprotein release into the medium of embryogenic carrot cells. - Plant Physiol. **81**: 931-933, 1986.

Shoresh, M., Harman, G.E.: Differential expression of maize chitinases in the presence or absence of *Trichoderma harzianum* strain T22 and indication of a novel exo-endo-heterodimeric chitinase activity. - BMC Plant Biol. **10**: 136, 2013.

Smith D.L, Krikorian, A.D.: Somatic embryogenesis of carrot in hormone-free medium: external pH control over morphogenesis. - Amer. J. Bot. **77**: 1634-1647, 1990.

Smith, D.L., Krikorian, A.D.: Low external pH prevents cell elongation but not multiplication of embryogenic carrot cells. - Physiol. Plant. **84**: 495-501, 1992.

Steiner, H.-Y., Dougall, D.K.: Ammonium uptake in carrot cell structures is influenced by pH-dependent cell aggregation. - Physiol. Plant. **95**: 415-422, 1995.

Towbin, H., Staehelin, T., Gordon, J.: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. - Proc. nat. Acad. Sci. USA **76**: 4350-4354, 1979.

Van Hengel, A.J., Guzzo, F., Van Kammen, A., De Vries, S.C.: Expression pattern of the carrot EP3 endochitinase genes in suspension cultures and in developing seeds. - Plant Physiol. **117**: 43-53, 1998.

Zavattieri, M.A., Frederico, A.M., Lima, M., Sabino, R., Arnholdt-Schmitt, B.: Induction of somatic embryogenesis as an example of stress-related plant reactions. - Electron J. Biotechnol. **13**: 1-9, 2010.