J Plant Growth Regul (2008) 27:353–369
DOI 10.1007/s00344-008-9061-8

Embryo-specific Proteins in *Cyclamen persicum* Analyzed with 2-D DIGE

Ragnhild Lyngved · Jenny Renaut · Jean-François Hausman · Tor-Henning Iversen · Anne Kathrine Hvoslef-Eide

Received: 7 April 2008 / Accepted: 21 July 2008 / Published online: 14 September 2008

Abstract Somatic embryogenesis can be used to produce artificial seeds of *Cyclamen persicum,* one of the most important ornamental plants for the European market, both as a potted plant in northern Europe and a bedding plant in the cool winters in southern Europe. The aim of this study was to obtain new insights into the molecular biology of somatic embryogenesis, which in turn can be useful for the improvement of tissue culture methodology. Total proteins were characterized from two isogenic cell lines of *Cyclamen persicum*, one that was embryogenic and one that never has shown any embryogenic capacity. The extracted proteins were separated by two-dimensional differential gel electrophoresis (2-D DIGE) and selected proteins were treated using the ETTAN Dalt Spot Handling Workstation. Protein identification was performed using MALDI-TOF-MS. More than 1200 *Cyclamen* proteins were detected; 943 proteins were common to both lines. The different protein patterns of the embryogenic and non-embryogenic cell lines were obvious: One hundred eight proteins were more abundant in the embryogenic cells, and 97 proteins in the non-embryogenic cells. Among the differentially expressed proteins, 128 were identified. MALDI-TOF-MS analysis enabled 27 spots to be proposed as candidates for embryo-specific proteins, as they were unique to the embryogenic cell line. The proteins identified are involved in a variety of cellular processes, including cell proliferation, protein processing, signal transduction, stress response, metabolism, and energy state, but the majority are involved in protein processing and metabolism. The main functions of the putative embryo-specific proteins have been discussed in proportion to their role in the somatic embryogenesis process.

Keywords *Cyclamen persicum* · Suspension culture · Somatic embryogenesis · Electrophoresis · 2-D DIGE · Proteomics

R. Lyngved and J. Renaut contributed equally to this work.

Electronic Supplementary Material The online version of this article (doi:10.1007/s00344-008-9061-8) contains supplementary material, which is available to authorized users.

R. Lyngved · T.-H. Iversen
The Plant Biocentre, Department of Biology,
Norwegian University of Science and Technology,
7491 Trondheim, Norway

J. Renaut · J.-F. Hausman
Department of Environment and Agrobiotechnologies,
Centre de Recherche Public - Gabriel Lippmann,
41, rue du Brill, 4422 Belvaux, Luxembourg

A. K. Hvoslef-Eide
The Plant Cell Laboratory, Department of Plant
and Environmental Sciences, Norwegian University
of Life Sciences, P.O. Box 5003, 1432 Aas, Norway

Present Address:
R. Lyngved (✉)
Sør-Trøndelag University College, Rotvoll Allé 1,
7004 Trondheim, Norway
e-mail: ragnhild.lyngved@hist.no

Introduction

Cyclamen (*Cyclamen persicum* Mill.) is one of the most important ornamental potted plants for the European market. Roughly 200 million *Cyclamen* plants are produced annually on a worldwide basis, of which about 150 million are grown in Europe (Schwenkel 2001). The commercial propagation of *Cyclamen* is still through F_1-hybrid seeds, but the production of hybrid seeds is connected to inbreeding depressions and heterogeneity in some

cultivars. Manual labor for seed production results in high seed prices. The high prices facilitate commercial potential for vegetative (clonal) propagation. It has been shown that in vitro somatic embryogenesis can be applied successfully (Winkelmann and others 2000) so now the general aim is to produce synthetic seeds in vitro. So far, the production system is not sufficiently successful (Winkelmann and others 2004a, b; Seyring and Hohe 2005); therefore, comparative studies on embryo development and germination of zygotic and somatic embryos of *Cyclamen* have been conducted (Schmidt and others 2006) to improve the production of synthetic clonal seeds.

Somatic embryogenesis is regarded as a model system for the study of morphologic, physiologic, molecular, and biochemical events occurring during the onset and development of embryogenesis in higher plants (Quiroz-Figueroa and others 2006). Any progress in the research of somatic embryogenesis can indirectly contribute to the improvement of crop species and the establishment of efficient plant propagation technologies, for example, the use of artificial seeds (Dudits and others 1995). By identifying candidates for embryo-specific proteins in *Cyclamen*, it is possible to develop antibodies for the positive identification of such proteins in culture at an early stage of development. This approach may be used to identify embryogenic genotypes very early in cell cultures and breeding programs, and environments that promote embryogenic development in cell cultures. This would replace the time- and labor-intensive in vitro testing.

Large amounts of proembryogenic masses (PEMs) are produced in tissue cultures after induction on media containing auxin. After the callus or suspension cultures are transferred to growth regulator-free medium, cells in the PEMs respond by developing into somatic embryos (Halperin 1966). This is true for *Cyclamen* as well (Winkelmann and others 2000). Several studies have dealt with genetic regulation or changes in protein patterns during somatic embryogenesis, with the examination of gymnosperms, angiosperms, and monocotyledonous and dicotyledonous plants (for example, Hendriks and de Vries 1995; Hvoslef-Eide and Corke 1997; von Arnold and others 2005; Rose and Nolan 2006; Singla and others 2007). However, the signalling pathways, the genes, and the proteins that are required to induce and develop somatic embryos are still not well defined.

It is generally believed that in the continued presence of exogenous auxin, the PEMs in the culture synthesize all gene products necessary to complete the globular stage of embryogenesis and that the PEMs also contain many other mRNAs and proteins whose continued presence inhibits the continuation of the embryogenesis program (Zimmerman 1993). It follows that the removal of auxin results in the inactivation of a number of genes such that the embryogenesis program is able to proceed. Studies in carrot also suggest that new gene products are needed for the transition to the heart stage and that these new products are synthesized only when exogenous auxin is removed (Zimmerman 1993). Somatic embryo receptor-like kinases (SERKs) (Hecht and others 2001), late embryogenesis abundant (LEA) proteins (Chugh and Khurana 2002), heat shock proteins (HSPs) (Györgyey and others 1991), GTP-binding proteins (Dudits and others 1995), lipid transfer proteins (LTPs) (Chugh and Khurana 2002), arabinogalactan proteins (AGPs) (von Arnold and others 2002), and chitinases and peroxidases (Hendriks and De Vries 1995) are among the gene products that have been studied in and are important to somatic embryogenesis.

Presently, there is limited information about biochemical changes during *Cyclamen* somatic embryogenesis. Recently, Rensing and others (2005) identified approximately 90 candidate genes that influenced the somatic embryogenesis in *Cyclamen* using EST sequencing, whereas Winkelmann and others (2006) have performed proteomic analyses of somatic and zygotic embryos. So far there are no reports available on the comparison between an embryogenic and a non-embryogenic cell line of *Cyclamen*, at neither the level of gene expression nor the proteome level.

The aim of the present study was to identify specific proteins produced in embryogenic and non-embryogenic isogenic cell suspensions of *Cyclamen,* to describe the differentially accumulating proteins, to obtain new insights into early events in somatic embryogenesis, and to determine if there are proteins that might be used as molecular markers.

Materials and Methods

Plant Material

Embryogenic (E, VIII) and non-embryogenic (NE, 12G) cell lines of *Cyclamen persicum* (Fig. 1) were obtained as callus cultures in vitro from the Institute for Vegetable and Ornamental Crops, Kuehnhausen, Germany, through COST822 cooperation. The cultures were initiated from unpollinated ovules of flower buds from one individual plant of *Cyclamen persicum* 'Giganteum' Mill. cv. 'Purple Flamed' (genotype 3738), as described by Schwenkel and Winkelmann (1998) and Winkelmann and others (1998a, b). The material allows direct comparison of competent and noncompetent cells from the same genotype.

Medium, Growth Conditions, and Sampling of Cell Cultures

Callus growth was maintained on a modified MS medium [half strength Murashige and Skoog (1962) macro- and micronutrients, NaFe-EDTA full strength, 250 mg L^{-1}

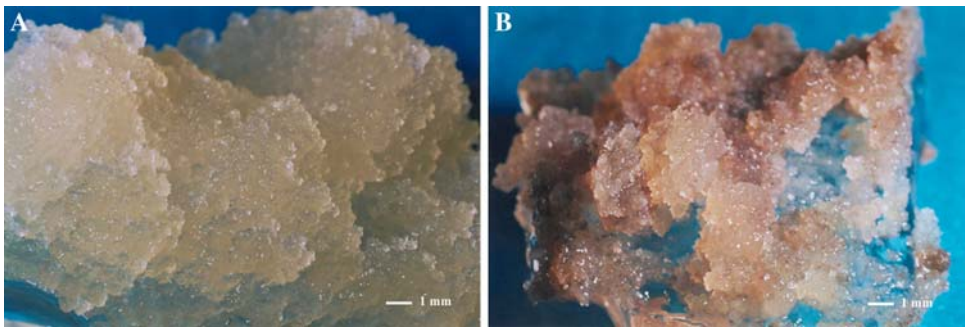

Fig. 1 Appearance (1.64×) under the light microscope of the non-embryogenic (NE, 12G) (**a**) and embryogenic (E, VIII) (**b**) callus cultures of *Cyclamen persicum* Mill., cultured in darkness for 4 weeks on modified MS medium containing plant growth regulators

casein hydrolysate, 30 g L^{-1} sucrose, 2 g L^{-1} glucose, 2.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.8 mg L^{-1} 6-(γ-γ-dimethylallylamino)-purine (2iP), and Murashige and Skoog (1962) vitamins]. pH was adjusted to 5.5 and the medium was solidified with 3.7 g L^{-1} Phytagel in accordance with Schwenkel and Winkelmann (1998). The callus cultures (E and NE) were transferred to fresh medium monthly and maintained at 24°C in the dark.

Suspension cultures (ES and NES) were established in accordance with Winkelmann and others (1998a) and subcultured every 2 weeks, using the same medium as for callus cultures, without the solidifier. The liquid cultures were maintained in Erlenmeyer flasks (culture volume = 1/5 of the total volume) on a rotary shaker (100 rpm) at 24°C in the dark. For protein extraction, the following cell material was collected: embryogenic and non-embryogenic suspension cultures 2 weeks after subcultivation on plant growth regulator (PGR)-containing medium. Ten flasks of each cell line were mixed before sieving and sampling. The cells, with a size fraction of 100–1000 µm, were quickly frozen in portions of 0.5 g fresh weight in liquid N_2 and stored at −80°C until extraction.

Embryogeneity Tests

The viability of the embryogenic and non-embryogenic suspension cultures was measured at the time of sampling using the TTC assay (Bennett and Loomis 1949; Towill and Mazur 1975) (10 samples per cell line). The cell fractions that were eliminated from this study (<100 µm and >1000 µm) and the cells that were analyzed (100–1000 µm) were induced for embryo development in Petri dishes. All samples, except for those less than 100 µm, were adjusted to 10% PCV in liquid, modified MS medium without PGRs and plated on top of a filter paper (Whatman no1) (1 ml jar^{-1}, 5 replicates). The cells were incubated in Petri dishes on modified, solidified MS medium without PGRs for 8 weeks at 22°C in darkness. The cells were considered embryogenic if embryos with tubers and clearly developed roots were observed.

Protein Extraction

Proteins were extracted according to Corke and Roberts (1997). Cells were homogenized with drill (1.5-ml Eppendorf tubes, 1 min) before boiling in extraction buffer [1.15% sodium dodecylsulfate (SDS), 125 mM Tris-HCl pH 6.8, 50 mM dithiothreitol (DTT)] for 5 min. Samples were centrifuged (10 min, 4°C, 21000*g*), the supernatant collected, and the proteins precipitated with ice-cold acetone (1:9, −80°C, 20 min). The precipitated proteins were pelleted (5 min, 4°C, 21000*g*), washed twice with 1 ml ice-cold acetone, dried for 5-10 min in a laminar flow hood, and stored at −80°C.

Proteins were isolated from samples taken 2 weeks after subcultivation. For both cell lines, ten independent extractions from ten frozen Eppendorf tubes with cell suspension were performed, and three random extracts were analyzed using two-dimensional differential gel electrophoresis (2-D DIGE).

Two-Dimensional Differential Gel Electrophoresis (2-D DIGE)

Before 2-D electrophoresis, the protein pellets were resuspended in labeling buffer [7 M urea, 2 M thiourea, 30 mM Tris, 4% w/v 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS)] on a thermomixer (18°C, 900 rpm, 1 h). The pH of each sample was adjusted to 8.5 with NaOH, and the protein concentration was determined using a quantification kit (2-D Quant Kit, GE Healthcare, Buckinghamshire, UK). An internal standard was made by mixing 25 µg of each sample. The samples (50 µg) and the internal standard (50 µg) were labeled with CyDye DIGE Fluor minimal dyes (400 pmol per 50 µg protein, GE Healthcare), according to the experimental design (Table 1), and incubated on ice for 30 min in the dark. Lysine (1 µl, 10 mM) was then added to stop the reaction, and the samples were left on ice for 10 min in the dark. The two samples (50 µg) for each batch (Table 1) and the internal standard (50 µg) were mixed before adding 2 × lysis buffer [7 M urea, 2 M thiourea, 4% w/v CHAPS,

Table 1 Experimental design for the 2-D DIGE analysis of the embryogenic (E) and non-embryogenic (NE) cell line at day 0 (DO)

Batch	Cy5	Cy3	Cy2
A	E DO, 1	NE DO, 1	IS
B	NE DO, 2	E DO, 2	IS
C	E DO, 3	NE DO, 3	IS

The internal standard (IS) was labeled with Cy2 and the samples were labeled with Cy3 and Cy5, according to the table. Numbers 1-3 indicate biological replicates

12 µl ml^{-1} DeStreak Reagent (GE Healthcare)] to a final volume of 150 µl, and 2% v/v ampholytes immobilized pH gradient (IPG) buffer (pH 4-7, GE Healthcare). The batch was left on ice for 10 min in the dark before loading onto Immobiline Dry Strip gels for isoelectric focusing (IEF). The current experiment thus contained three biological replicates.

For analytical gels, Immobiline Dry Strip gels (pH 4-7, 24 cm, GE Healthcare) were rehydrated in a reswelling tray (GE Healthcare) overnight with 450 µl DeStreak Rehydration Solution (GE Healthcare) containing 0.5% IPG buffer (pH 4-7, GE Healthcare). Protein batches of 150 µl were loaded on each strip via sample cups at the anodic end of the gel and covered with Dry Strip Cover Fluid (GE Healthcare). For preparative gels, rehydration loading was performed with 450 µl solution, consisting of 400 µg proteins, DeStreak Rehydration Solution (GE Healthcare) to a final volume of 450 µl, and 0.5% IPG buffer (pH 4-7, GE Healthcare).

Isoelectric focusing (IEF) was conducted at 20°C with an Ettan IPGphor system (GE Healthcare). The running conditions were successively 50 V (2 h), 500 V (2 h), 1000 V (2 h), 4000 V (3 h), 8000 V (3 h), and 8000 V (8 h), with 50 µA/strip and to a total of 85 kVh. The first and last steps were run in step mode; the remaining were in gradient mode.

The focused strips were equilibrated twice for 15 min in 15 ml equilibration solution. The first equilibration was performed in a solution containing 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% v/v glycerol, 2% w/v SDS, 0.002% w/v bromophenol blue, and 1% w/v DTT. The second equilibration was performed in a solution modified by the replacement of DTT by 2.5% w/v iodoacetamide.

Separation in the second dimension was performed on 1.0-mm gels cast by a 2DEoptimizer (NEXTGENsciences, Huntingdon, UK) using a 2DEoptimizer reagent kit (NEXTGENsciences). Glass plates used for preparative gels and picking gels were treated with Bind-Silane solution [80% ethanol, 2% acetic acid, and 0.1% Bind-Silane (GE Healthcare)] before casting. The gels were cast with a 12% resolving gel and run at 15°C overnight, with 2 W per gel, using the EttanDALT*six* system (GE Healthcare). The protein spots in the analytical gels were visualized by labeling with CyDye DIGE Fluor minimal dyes, whereas the preparative gels were stained with Coomassie blue.

Image and Data Analysis

Coomassie-stained gels were scanned using a calibrated ImageScanner II (GE Healthcare) at a resolution of 300 dpi (16 bits). Cy2-, Cy3-, and Cy5-labeled protein images were produced by excitation of gels at 488, 532, and 633 nm, respectively, and emission at 520, 590, and 680 nm, respectively, using Typhoon Variable Mode Imager 9400 (GE Healthcare) at a resolution of 100 µm. Images were analyzed using the DeCyder v6.05.11 software (GE Healthcare), first to carry out the detection and matching of the protein patterns. Next, spot selection was conducted, using the following criteria: absolute abundance variation of at least 1.5-fold, Student's t test $p < 0.001$, and the presence of the spots in all the replicates. A picking list was generated and spots of interest were excised from gels using the Ettan Spot Picker from the Ettan Spot Handling Workstation (GE Healthcare).

Spot Handling, Protein Identification, and Database Search

After washing and desalting in 50 mM ammonium bicarbonate/50% v/v methanol, followed by 75% v/v acetonitrile, spots were then digested with trypsin gold (MS grade, Promega, Madison, WI, USA; 10 mg ml^{-1} in 20 mM ammonium bicarbonate) using the Ettan Digester robot (GE Healthcare) from the Ettan Spot Handling Workstation (GE Healthcare). Automated spotting of the samples was carried out with the spotter from the same workstation. Peptides dissolved in 50% acetonitrile containing 0.5% trifluoroacetic acid (0.7 µl) were spotted on MALDI-TOF disposable target plates (4800, Applied Biosystems, Foster City, CA, USA) before the deposit of 0.7 µl of α-cyano-4-hydroxycinnamic acid (7 mg ml^{-1}, 50% v/v acetonitrile, 0.1% v/v trifluoroacetic acid; Sigma Aldrich, St. Louis, MO, USA). Peptide mass determinations were carried out using the Applied Biosystems 4800 Proteomics Analyzer. Both peptide mass fingerprinting and MS/MS in reflectron mode analysis were carried out with the samples. Calibration was done with the peptide mass calibration kit for the 4700 (Applied Biosystems). Proteins were identified by searching against the NCBI database using MASCOT (Matrix Science, London, UK, www.matrixscience.com) and against a "homemade" database containing the ESTs of "Viridiplantae" available on the NCBI website (http://www.ncbi.nlm.nih.gov/). All searches were performed using a mass window of 100 ppm for the precursor tolerance and 0.5 Da for MS/MS fragments, and with "Viridiplantae" as taxonomy. The search

parameters allowed fixed modifications for cysteine (carboxyamidomethylation) and methionine (oxidation) and variable modifications on tryptophan (double oxidation or kynurenin) as well as peptide N-terminal E or Q as pyroGlu. Two missed cleavages were allowed and the peptide charge was set at +1. All the identifications were manually verified and validated. The main functions of the proteins were found from their homologies by searching the literature and the web resources UniProtKB (http://www.pir.uniprot.org/) and KEGG (http://www.kegg.com/). Potential molecular markers (Table 2, marked g and h) were found by checking the ratio and *t*-test value, as well as the spot image on each gel.

Results and Discussion

Embryogeneity Tests

The embryogenic potential of the two cell lines was verified by culturing on medium without plant growth regulators (PGRs). In addition, a control test was performed at the time of sampling to check the viability of the two different cell lines. The TTC assay yielded no significant differences in viability between the two cell lines. The differentiation of embryos on solidified medium confirmed the embryogenic nature of cell line VIII and the non-embryogenic nature of cell line 12G. Cells from line 12G never developed into embryos (Fig. 2a). All samples from cell line VIII developed into embryos (Fig. 2b), except for the cell fraction less than 100 μm that was eliminated from the study. This fraction did not show any growth whatsoever, not in callus or embryo development, and did not seem to be important for identifying embryo-specific proteins. The fraction larger than 1000 μm developed many embryos, but because the same development was seen in the samples between 100 and 1000 μm, the largest cell lumps seemed unnecessary for inclusion in the study.

Proteomic Analysis

After 2-D DIGE separation, more than 1200 *Cyclamen* proteins were detected by digital image analysis over a pH range of 4-7 and a size range of 10-100 kDa. Roughly 1000 proteins were common to all samples, as judged by the match mode of the DeCyder 2D software. The statistical analysis showed that 205 protein spots changed significantly ($p < 0.001$) between the two cell lines E and NE (Fig. 3). Among them, 108 proteins were upregulated and 97 were downregulated in E vs. NE suspensions. Principal component analysis showed that the variability between the gels (three biological replicates) was small and that 91.8% of the variability in the gels can be explained by the differentially expressed spots between the two cell lines. Peptide-mass fingerprinting with MALDI-TOF-MS identified 128 proteins with homologies with proteins of other organisms found in databases (Table 2). The other proteins were either predicted proteins with unknown functions or their identification was not possible due to unreliable results (not enough peptides matched, protein without homology in database, or spectra of weak intensity). Many of the proteins were represented by several spots that matched the same gene sequence but had different pI and/or M_r (Table 2). These proteins were most likely the result of posttranslational modification. The ion sequences obtained are given in the Supplementary Table and the position of the spots in the 2-D gel is given in the Supplementary Figure. Furthermore, 27 proteins and 11 proteins were unique to the embryogenic cells and to the non-embryogenic cells, respectively. Unfortunately, only 12 and 4, respectively, of these were identified (Table 2, marked g and h, respectively). The unique spots could be considered molecular markers for somatic embryogenesis, although some that have been identified do not indicate rare functions. They could, however, be specific isoforms.

The suspension cultures were grown in medium containing 2,4-D prior to the sampling, and therefore the embryogenic cell line was considered competent. However, the cell samples analyzed in this article had not been initiated for differentiation. According to Zimmerman (1993), many of the genes responsible for embryo formation are already expressed in proembryogenic masses (PEMs) during unorganized growth. By comparing the PEMs with non-embryogenic cells, it was possible to investigate the very early stages of somatic embryogenesis. In this initial study, only soluble proteins within a pI range of 4-7 were analyzed. Nevertheless, valuable novel information on the molecular aspects of somatic embryogenesis has been obtained for this poorly characterized species.

The 128 identified proteins, listed in Table 2, were classified into six functional categories based on their main biological process, as suggested by Marsoni and others (2008): (1) cell proliferation (6.0% of identified proteins), (2) protein processing (14.3%), (3) signal transduction (6.0%), (4) stress response (3.8%), (5) metabolism and energy state (67.7%), and (6) hypothetical function (2.3%). The largest classes were those representing proteins implicated in protein processing and metabolism. This is in accordance with the analysis of embryogenic and non-embryogenic calluses of grapevine (Marsoni and others 2008), which gave 19 and 26%, respectively, for the same categories. Nogueira and others (2007) also found that 44% of the proteins identified from embryogenic cell suspensions of cowpea were involved in primary metabolism. Our results are discussed in five sections below according to functional categories, starting with the largest classes and

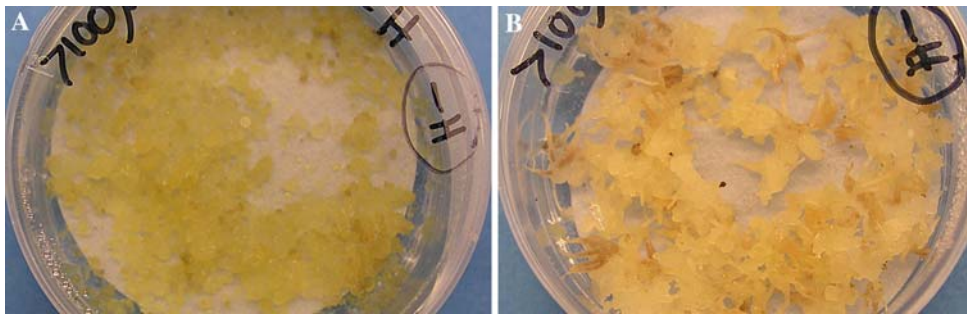

Fig. 2 Non-embryogenic (NE, 12G) (**a**) and embryogenic (E, VIII) (**b**) cells grown on modified MS medium without plant growth regulators for 9 weeks

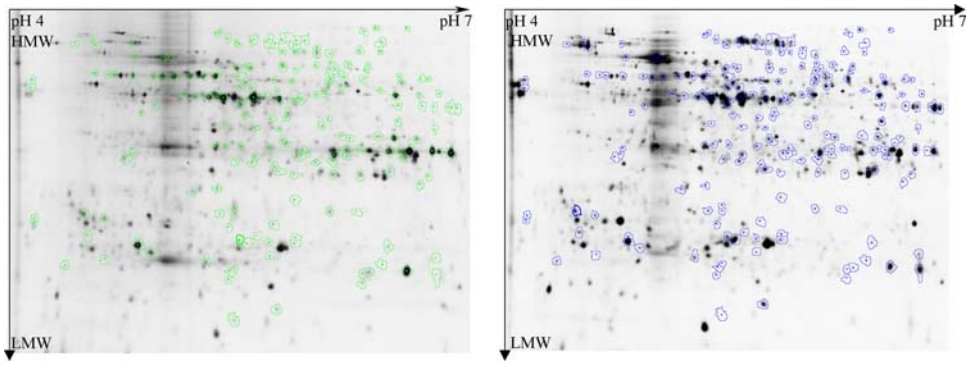

Fig. 3 Bidimensional gels on a pH 4-7. The left shows an example image from the embryogenic sample (E, VIII) (labeled with Cy3), while the right shows an example from the non-embryogenic sample (NE, 12G) (labeled with Cy5). Spots circled in green and blue were differentially expressed ($p < 0.001$) in E and NE. These spots were picked from this gel and analyzed using mass spectrometry. The spot numbers are given in the Supplementary Figure. HMW, high-molecular weight; LMW, low molecular weight

focusing on the identified proteins that were unique to the embryogenic suspensions (ES) and non-embryogenic suspensions (NES).

Metabolism and Energy State

Establishment of embryogenic competence is accompanied by active metabolic changes (Fehér and others 2003). The highest proportion (67.7%) of the proteins identified in our study were involved in metabolic processes, mainly carbohydrate metabolism, amino acid metabolism, lipid metabolism, phosphate metabolism, metabolism of cofactors and vitamins, and energy metabolism (Table 2).

A high abundance of proteins involved in carbohydrate metabolism may be evoked by the exogenous sucrose and glucose supply by tissue culture media, as mentioned in Winkelmann and others (2006). Transketolase (spot 157, unique to ES) has already been reported as more abundant in embryogenic than in non-embryogenic cultures (Marsoni and others 2008), similar to our results. GAPDH (spots 904, 907, 908, 917, 946, 961, 982) have been found in higher amounts in somatic embryos compared to zygotic embryos (Winkelmann and others 2006). In our analyses, four of these proteins were unique to ES. Nogueira and others (2007) also identified several proteins as GAPDH in proembryogenic masses of cowpea. Pyruvate dehydrogenase E1α (spot 839, unique to ES) is essential for overall control of metabolic flow, as a component of the pyruvate dehydrogenase complex (Luethy and others 2001).

Rensing and others (2005) and Winkelmann and others (2006) found that glutathione-S-transferase accumulated in embryogenic tissue and somatic embryos. In our study, putative glutamate decarboxylase (spot 530) and glutamate dehydrogenase A (spots 801, 830) were either unique to or upregulated in ES. This may indicate that the glutamate and glutathione metabolisms are involved in embryogenesis. IVD 2 (spot 900, unique to ES), involved in leucine catabolism, has also been strongly expressed in potato tubers, and Faivre-Nitschke and others (2001) suggested a possible role in sprouting.

Cell wall and membrane formation is enhanced during embryogenesis (Chugh and Khurana 2002). In agreement with this, two enzymes involved in wall and membrane synthesis (spots 890, 960) were upregulated in ES. Acyl-[acyl-carrier protein] desaturase (spot 960, unique to ES) is involved in fatty acid biosynthesis and is a key determinant of the overall level of unsaturated fatty acids in the cell (Kachroo and others 2001).

Putative metallophosphatases (spots 61, 65, 84, 89, 90, 116, 128), involved in phosphate metabolism (Olczak and Olczak 2002), were unique to or strongly upregulated in

Table 2 Differentially accumulated proteins identified by MALDI-TOF-MS, categorized by their putative biological function[a]

Spot No.	Ratio[b]	Abundance[c]	*p* value (*t* test)	Exp. pI/M_r (kDa)[d]	Theor. pI/M_r (kDa)	Protein score[e]/ ion score[f]	GI accession	Protein name	Species
Cell proliferation									
41	4.36	NE	5.7E-04	5.2/89	5.1/89.4	156/121	15232776	CDC48 (Cell division cycle 48)	*Arabidopsis thaliana*
747	2.15	NE	7.2E-04	6.7/49	5.3/41.8	125(+4)/51	58013197	Actin	*Isatis tinctoria*
1265	2.68	NE	2.0E-06	5.8/25	5.3/41.8	236(+90)/259	58013197	Actin	*Isatis tinctoria*
617	2.26	NE	4.4E-04	6.5/57	4.9/49.5	467(+84)/477	464849	Tubulin alpha chain	*Prunus dulcis*
1098	−1.80	E	5.7E-04	5.3/34	4.8/49.9	207/113	135460	Tubulin beta-2 chain (Beta-2 tubulin)	*Zea mays*
1109	−2.23	E	1.1E-04	5.1/33	4.7/49.4	243/167	5668671	Beta-tubulin	*Zinnia elegans*
1129	−2.18	E	7.6E-06	5.8/31	5.0/49.7	78/84	15241168	Tubulin alpha-3 (TUA3)	*Arabidopsis thaliana*
1450[g]	−5.82	E	2.6E-05	6.2/19	4.4/19.0	163/124	6094439	Translationally controlled tumor protein homolog (TCTP)	*Fragaria x ananassa*
Protein processing									
93[h]	16.39	NE	5.0E-06	5.8/84	5.0/80.2	296/136	38154489	Molecular chaperone Hsp90-1	*Lycopersicon esculentum*
95	−2.88	E	4.3E-05	4.7/82	5.0/80.2	456/343	38154489	Molecular chaperone Hsp90-1	*Lycopersicon esculentum*
164	−2.10	E	9.0E-05	5.8/78	5.0/80.2	77(+20)/71	38154489	Molecular chaperone Hsp90-1	*Lycopersicon esculentum*
256	−1.59	E	3.7E-05	5.5/71	4.9/92.9	79(+29)/88	544242	Endoplasmin homolog precursor (GRP94 homolog)	*Hordeum vulgare*
165	−4.44	E	5.4E-06	5.9/77	5.2/71.0	129/97	21664287	Heat shock protein 70	*Oryza sativa (indica cultivar-group)*
187	1.97	NE	8.6E-04	4.7/77	5.2/71.0	254/203	21664287	Heat shock protein 70	*Oryza sativa (indica cultivar-group)*
198	1.64	NE	5.4E-04	4.9/77	5.2/71.5	364/257	762844	Hsc70	*Lycopersicon esculentum*
199	2.84	NE	8.2E-04	5.0/77	5.1/71.0	407/281	38325815	Heat shock protein 70-3	*Nicotiana tabacum*
230	2.30	NE	1.4E-04	6.0/72	6.0/72.5	522/398	399940	Heat shock 70 kDa protein, mitochondrial precursor	*Phaseolus vulgaris*
1443	−1.98	E	1.5E-04	5.3/20	5.2/71.0	73/56	21664287	Heat shock protein 70	*Oryza sativa (indica cultivar-group)*
1491	2.46	NE	4.5E-06	5.7/17	5.2/71.0	71/57	30025966	Heat shock protein 70 (fragment)	*Nicotiana tabacum*
294	1.75	NE	1.4E-06	5.3/70	6.3/61.1	124(+173)/232	461736	Chaperonin CPN60-2, mitochondrial precursor (HSP60-2)	*Cucurbita maxima*
295	−1.50	E	6.4E-04	4.4/67	6.3/61.1	266(+110)/317	461736	Chaperonin CPN60-2, mitochondrial precursor (HSP60-2)	*Cucurbita maxima*

Table 2 continued

Spot No.	Ratio[b]	Abundance[c]	*p* value (*t* test)	Exp. pI/M_r (kDa)[d]	Theor. pI/M_r (kDa)	Protein score[e]/ ion score[f]	GI accession	Protein name	Species
308	−2.18	E	4.8E-05	4.7/67	6.3/61.1	102(+79)/123	461836	Chaperonin CPN60-2, mitochondrial precursor (HSP60-2)	*Cucurbita maxima*
313	−2.32	E	7.1E-05	5.2/67	5.3/57.8	154(+66)/127	24637539	Heat shock protein 60	*Prunus dulcis*
320	−1.82	E	3.3E-05	4.8/67	5.3/57.8	189(+122)/203	24637539	Heat shock protein 60	*Prunus dulcis*
330	1.51	NE	3.4E-04	6.1/68	5.9/63.0	393(+80)/407	2506277	RuBisCO large subunit-binding protein subunit beta, chloroplast precursor	*Pisum sativum*
387	1.87	NE	6.8E-04	5.5/66	5.9/63.0	(91)/91	2506277	RuBisCO large subunit-binding protein subunit beta, chloroplast precursor	*Pisum sativum*
661	−1.69	E	8.7E-04	4.8/54	5.0/47.5	353/272	23197864	26S proteasome AAA-ATPase subunit RPT5a	*Arabidopsis thaliana*
Signal transduction									
316	−2.61	E	7.8E-05	5.1/67	4.9/65.6	183(+131)/208	683502	Protein phosphatase 2A 65-kDa regulatory subunit	*Arabidopsis thaliana*
1188	−2.49	E	4.9E-05	4.5/28	5.1/32.7	74/68	38564727	Putative alpha-soluble NSF attachment protein	*Helianthus annuus*
1308	−1.82	E	7.7E-04	6.4/24	4.8/29.3	85/81	110278805	14-3-3-like protein GF14-D (G-box factor 14-3-3 homolog D)	*Oryza sativa (japonica group)*
360	1.52	NE	2.3E-04	6.7/67	4.5/48.1	(62)/62	11131631	Calreticulin precursor	*Beta vulgaris*
438	3.74	NE	1.7E-05	5.4/64	4.4/46.6	198/142	1009712	Calreticulin	*Arabidopsis thaliana*
1269	2.15	NE	4.2E-04	5.4/25	6.4/25.1	58(+25)/51	15241245	RAN2; GTP binding	*Arabidopsis thaliana*
1278	2.12	NE	7.9E-04	5.6/24	6.4/25.1	131/71	15241245	RAN2; GTP binding	*Arabidopsis thaliana*
1291	2.51	NE	3.2E-04	5.8/24	6.4/25.1	206/126	15241245	RAN2; GTP binding	*Arabidopsis thaliana*
Stress response									
235	2.76	NE	3.7E-04	6.2/72	5.2/62.4	355(+90)/395	300265	HSP68 = 68 kDa heat-stress DnaK homolog	*Lycopersicon peruvianum*
246	−1.85	E	5.0E-05	5.7/72	5.9/64.5	274/156	15221564	Stress-inducible protein, putative	*Arabidopsis thaliana*
251	3.80	NE	2.5E-05	6.3/73	5.8/63.6	77(+130)/203	41018257	Heat shock protein STI (Stress-inducible protein) (GmSTI)	*Glycine max*
510	1.76	NE	5.0E-04	5.9/61	6.7/23.0	70/56	6979698	Dehydrin	*Vaccinium corymbosum*
1192	−2.44	E	7.1E-04	4.0/28	7.4/57.0	151(+32)/136	3202034	Root catalase	*Mesembryanthemum crystallinum*

Table 2 continued[a]

Spot No.	Ratio[b]	Abundance[c]	*p* value (*t* test)	Exp. pI/M_r (kDa)[d]	Theor. pI/M_r (kDa)	Protein score[e]/ ion score[f]	GI accession	Protein name	Species
Metabolism and energy state									
Carbohydrate metabolism									
15	1.59	NE	2.7E-04	6.3/99	5.8/98.0	124/85	30407706	Aconitase	*Lycopersicon pennellii*
1261	1.86	NE	2.6E-05	5.5/26	5.8/98.0	67(+28)/67	30407706	Aconitase	*Lycopersicon pennellii*
157[g]	−4.48	E	1.0E-06	5.9/78	5.9/17.1	55(+58)/85	4586600	Transketolase	*Cicer arietinum*
109	3.02	NE	6.0E-04	4.3/83	5.6/82.0	(56)/56	18025340	Alpha-L-arabinofuranosidase/ beta-D-xylosidase isoenzyme ARA-I	*Hordeum vulgare*
183	5.76	NE	1.9E-04	6.0/78	5.6/82.0	94/74	18025340	Alpha-L-arabinofuranosidase/ beta-D-xylosidase isoenzyme ARA-I	*Hordeum vulgare*
261	1.86	NE	7.7E-04	6.1/72	5.9/69.7	191(+14)/159	15240075	SDH1-1 (Succinate dehydrogenase 1-1)	*Arabidopsis thaliana*
263	−2.42	E	3.1E-05	5.5/70	5.4/61.2	231(+131)/295	3914394	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	*Mesembryanthemum crystallinum*
279	2.66	NE	7.3E-04	5.8/71	5.4/61.2	80(+19)/80	3914394	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	*Mesembryanthemum crystallinum*
280	1.87	NE	3.0E-05	6.0/71	5.4/61.2	120/82	3914394	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	*Mesembryanthemum crystallinum*
323	−2.76	E	4.9E-04	5.0/67	5.3/69.7	134(+41)/161	15225438	Malic enzyme/ oxidoreductase	*Arabidopsis thaliana*
326	−1.92	E	3.4E-05	4.9/67	6.7/72.4	99/88	226027	Acetolactate synthase SuRB	*Nicotiana sp.*
329	2.87	NE	1.1E-05	6.0/68	7.0/72.4	111/106	3820612	Herbicide-resistant acetolactate synthase precursor	*Bassia scoparia*
349	2.52	NE	5.0E-04	4.0/67	6.6/73.4	79(+108)/155	15235730	Phosphoenolpyruvate carboxykinase (ATP)	*Arabidopsis thaliana*
434	2.09	NE	5.8E-04	4.0/64	6.3/61.8	(77)/77	18424599	ALDH12A1 (Aldehyde dehydrogenase 12A1)	*Arabidopsis thaliana*
502	1.78	NE	9.9E-05	5.6/61	6.9/59.3	101(+72)/160	1743354	Aldehyde dehydrogenase (NAD+)	*Nicotiana tabacum*
483	−2.53	E	4.6E-06	5.3/60	5.7/53.4	(136)/136	145328282	ALDH6B2 (Aldehyde dehydrogenase 6B2)	*Arabidopsis thaliana*
515	−1.60	E	9.4E-06	6.5/59	7.1/58.6	175/267	15228319	ALDH2B4 (Adehyde dehydrogenase 2)	*Arabidopsis thaliana*
436	1.64	NE	4.3E-04	5.5/64	5.9/64.9	106(+26)/108	15229546	Dehydratase family	*Arabidopsis thaliana*

Table 2 continued

Spot No.	Ratio[b]	Abundance[c]	*p* value (*t* test)	Exp. pI/M_r (kDa)[d]	Theor. pI/M_r (kDa)	Protein score[e]/ ion score[f]	GI accession	Protein name	Species
505	−1.96	E	7.3E-06	6.6/60	5.9/58.2	191(+34)/204	48093459	Putative UDP-glucose dehydrogenase 2	*Nicotiana tabacum*
559	1.96	NE	1.6E-04	5.8/59	5.6/53.1	133/116	15241704	UDP-glucose 6-dehydrogenase	*Arabidopsis thaliana*
529	−1.88	E	5.8E-05	5.5/59	7.1/41.0	244(+97)/309	17402533	UDP-glucose pyrophosphorylase	*Nicotiana tabacum*
815	1.57	NE	4.1E-05	5.7/45	6.7/52.1	(75)/75	90820120	UDP-glucose pyrophosphorylase	*Cucumis melo*
886	−2.73	E	2.4E-04	6.1/42	6.7/52.1	104(+35)/113	90820120	UDP-glucose pyrophosphorylase	*Cucumis melo*
1176	−2.13	E	2.7E-05	4.4/29	5.5/51.4	65(+107)/165	12585489	UDP-glucose pyrophosphorylase	*Musa acuminata*
560	1.75	NE	2.4E-04	5.8/59	6.2/47.7	299(+210)/476	33415263	Enolase	*Gossypium barbadense*
684	−1.80	E	1.2E-04	5.7/52	5.6/47.8	175(+223)/418	14423688	Enolase	*Hevea brasiliensis*
777	−2.71	E	1.7E-04	6.1/46	6.2/47.7	237(+102)/306	33415263	Enolase	*Gossypium barbadense*
799	2.62	NE	3.4E-04	6.2/47	5.4/47.6	221(+158)/353	3023685	Enolase	*Alnus glutinosa*
647	7.10	NE	1.5E-04	6.9/56	6.7/54.0	120(+117)/213	15320419	Monodehydroascorbate reductase	*Spinacia oleracea*
839[g]	−7.27	E	3.9E-05	5.7/44	8.0/43.5	107(+74)/129	1709449	Pyruvate dehydrogenase E1 component subunit alpha, mitochondrial precursor	*Pisum sativum*
856	−2.65	E	2.1E-05	5.4/44	5.8/42.3	113/84	121485004	Cytosolic phosphoglycerate kinase	*Helianthus annuus*
890	−3.14	E	1.4E-05	5.9/42	5.7/41.6	112/64	34582497	Alpha-1,4-glucan-protein synthase [UDP-forming] (UPTG)	*Pisum sativum*
904[g]	−3.81	E	5.7E-06	6.7/42	6.4/31.8	155(+127)/272	34099812	Glyceraldehyde-3-phosphate dehydrogenase	*Panax ginseng*
907[g]	−7.15	E	9.8E-06	6.5/42	8.3/36.7	442(+82)/484	120666	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	*Antirrhinum majus*
908[g]	−4.84	E	1.3E-06	6.4/42	8.3/36.7	(132)/132	120666	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	*Antirrhinum majus*
917	−3.30	E	2.2E-07	6.8/42	8.3/36.7	165/137	120666	Glyceraldehyde-3-phosphate d ehydrogenase, cytosolic	*Antirrhinum majus*
946	−2.55	E	9.2E-05	4.7/41	8.3/36.7	184(+164)/277	120666	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	*Antirrhinum majus*
961[g]	−6.18	E	5.8E-07	4.6/40	8.3/36.7	109/99	120666	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	*Antirrhinum majus*
982	−2.10	E	7.1E-05	5.9/39	8.3/36.7	355(+124)/463	120666	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	*Antirrhinum majus*
950	−2.49	E	2.8E-05	6.5/41	6.0/63.5	108/81	12585316	Phosphoglucomutase, cytoplasmic (PGM)	*Solanum tuberosum*

Table 2 continued

Spot No.	Ratio[b]	Abundance[c]	*p* value (*t* test)	Exp. pI/M_r (kDa)[d]	Theor. pI/M_r (kDa)	Protein score[e]/ ion score[f]	GI accession	Protein name	Species
987	1.84	NE	7.5E-04	5.5/39	6.0/38.5	243(+50)/275	113624	Fructose-bisphosphate aldolase, cytoplasmic isozyme	*Spinacia oleracea*
1036	2.46	NE	3.7E-05	6.4/37	8.9/36.2	235/193	126896	Malate dehydrogenase, mitochondrial precursor	*Citrullus lanatus*
1348	−1.51	E	3.0E-04	5.6/22	5.4/27.5	168(+157)/270	28627542	Putative ascorbate peroxidase	*Capsicum annuum*
Amino acid metabolism									
80	17.19	NE	4.1E-04	5.5/84	6.0/68.9	60(+105)/105	42568444	Hydrolase/protein serine/ threonine phosphatase	*Arabidopsis thaliana*
307	−2.46	E	1.4E-07	4.6/67	6.6/52.9	89/73	15238328	Serine carboxypeptidase-like 42	*Arabidopsis thaliana*
333	3.68	NE	1.4E-04	5.4/68	6.6/52.9	(95)/95	15238328	Serine carboxypeptidase-like 42	*Arabidopsis thaliana*
348	4.21	NE	3.8E-05	6.6/67	6.6/52.9	152(+155)/101	15238328	Serine carboxypeptidase-like 42	*Arabidopsis thaliana*
385	1.99	NE	1.0E-04	6.5/66	6.6/52.9	72(+20)/73	15238328	Serine carboxypeptidase-like 42	*Arabidopsis thaliana*
672	−3.00	E	6.6E-05	5.5/53	6.6/52.9	81/64	15238328	Serine carboxypeptidase-like 42	*Arabidopsis thaliana*
369	1.78	NE	8.2E-05	5.8/67	6.5/63.8	94(+119)/205	266346	Ketol acid reductoisomerase, chloroplast precursor	*Spinacia oleracea*
389	−1.62	E	2.1E-04	6.0/64	5.5/57.0	166(+236)/344	2554835	Chain I, acetohydroxy acid isomeroreductase complexed with Nadph, magnesium and inhibitor Ipoha	*Spinacia oleracea*
495	1.96	NE	3.1E-04	6.0/61	5.8/53.2	166(+57)/186	6094228	Adenosylhomocysteinase (S-adenosyl-L-homocysteine hydrolase) (AdoHycase)	*Mesembryanthemum crystallinum*
499	2.15	NE	8.0E-04	6.8/61	6.5/50.1	71/71	6094413	Seryl-tRNA synthetase (SerRS)	*Helianthus annuus*
626	5.17	NE	9.6E-07	6.8/57	6.8/51.7	(84)/84	15236375	Serine hydroxymethyl-transferase 4	*Arabidopsis thaliana*
530[g]	−3.70	E	1.4E-04	5.8/59	5.7/51.3	82/47	32493114	Putative glutamate decarboxylase	*Glycine max*
553	3.70	NE	5.9E-06	5.4/59	6.4/53.8	(80)/85	13430632	Putative glutathione reductase	*Arabidopsis thaliana*
613	2.32	NE	3.4E-04	4.9/57	8.1/50.7	(72)/72	4049354	Glycine hydroxymethyltransferase (EC 2.1.2.1)-like protein	*Arabidopsis thaliana*
619	2.14	NE	5.7E-06	6.7/57	8.1/50.7	(111)/111	4049354	Glycine hydroxymethyltransferase (EC 2.1.2.1)-like protein	*Arabidopsis thaliana*
669	−1.82	E	3.3E-05	5.8/54	6.2/12.7	81/65	149390699	Arginosuccinate synthase	*Oryza sativa (indica cultivar-group)*
700	1.90	NE	7.4E-04	5.6/53	6.0/45.2	133(+19)/126	9759413	Beta-ureidopropionase	*Arabidopsis thaliana*

Table 2 continued

Spot No.	Ratio[b]	Abundance[c]	*p* value (*t* test)	Exp. pI/M_r (kDa)[d]	Theor. pI/M_r (kDa)	Protein score[e]/ ion score[f]	GI accession	Protein name	Species
721	−3.14	E	5.0E-04	5.3/50	7.7/51.9	125/97	5731259	Alanine:glyoxylate aminotransferase 2 homolog	*Arabidopsis thaliana*
801[g]	−5.01	E	2.4E-04	6.6/45	6.6/44.8	274(+116)/371	12229785	Glutamate dehydrogenase A (GDH A)	*Nicotiana plumbaginifolia*
830	−2.40	E	2.1E-04	6.6/44	6.6/44.8	221(+40)/184	12229785	Glutamate dehydrogenase A (GDH A)	*Nicotiana plumbaginifolia*
854	−1.78	E	1.2E-04	5.3/44	7.0/46.4	70(+22)/83	1389768	Phosphoribosyl anthranilate transferase 1	*Arabidopsis thaliana*
882	1.86	NE	2.9E-04	4.7/43	5.1/39.0	184/155	18418013	GLN1;4; glutamate-ammonia ligase	*Arabidopsis thaliana*
900[g]	−5.05	E	2.3E-05	6.5/42	6.1/44.0	121(+101)/194	25453061	Isovaleryl-CoA dehydrogenase 2, mitochondrial precursor (IVD 2)	*Solanum tuberosum*
975	3.14	NE	1.8E-04	5.4/40	5.4/40.9	(105)/105	6319167	Branched-chain amino acid aminotransferase	*Solanum tuberosum*
983	−2.32	E	5.5E-04	6.1/39	5.2/40.0	(97)/82	399333	Cysteine synthase, chloroplast precursor	*Capsicum annuum*
Lipid metabolism									
960[g]	−3.47	E	2.5E-05	6.5/41	6.2/45.7	264(+44)/212	30689469	Acyl-[acyl-carrier protein] desaturase	*Arabidopsis thaliana*
1031	3.75	NE	1.2E-05	5.7/37	8.9/47.8	137(+38)/146	2204236	Enoyl-[acyl-carrier protein] reductase	*Nicotiana tabacum*
Phosphate metabolism									
61	8.67	NE	5.4E-04	5.3/86	6.1/71.0	(69)/69	52353232	Putative metallophosphatase	*Lupinus luteus*
65[h]	12.99	NE	4.1E-04	5.6/85	6.1/71.0	111(+88)/199	52353232	Putative metallophosphatase	*Lupinus luteus*
84	9.76	NE	4.0E-04	5.4/84	6.1/71.0	111(+68)/169	52353232	Putative metallophosphatase	*Lupinus luteus*
89[h]	13.18	NE	8.4E-07	5.6/84	6.1/71.0	101(+37)/123	52353232	Putative metallophosphatase	*Lupinus luteus*
90	7.63	NE	6.9E-04	5.9/84	6.1/71.0	106(+57)/163	52353232	Putative metallophosphatase	*Lupinus luteus*
116	6.61	NE	3.8E-06	5.4/83	6.1/71.0	73(+21)/81	52353232	Putative metallophosphatase	*Lupinus luteus*
128[h]	11.69	NE	2.8E-05	5.8/83	6.1/71.0	122(+50)/161	52353232	Putative metallophosphatase	*Lupinus luteus*
135	−2.16	E	5.5E-05	5.9/80	6.1/71.0	(61)/61	52353232	Putative metallophosphatase	*Lupinus luteus*
Metabolism of cofactors and vitamins									
1177	2.10	NE	3.0E-05	5.3/29	5.0/37.5	308/267	58201024	Thiamine biosynthetic enzyme	*Picrorhiza kurrooa*

Table 2 continued

Spot No.	Ratio[b]	Abundance[c]	*p* value (*t* test)	Exp. pI/M_r (kDa)[d]	Theor. pI/M_r (kDa)	Protein score[e]/ ion score[f]	GI accession	Protein name	Species
Energy metabolism									
456	3.96	NE	1.9E-05	6.4/63	6.0/55.5	81/76	114404	ATP synthase subunit alpha, mitochondrial	*Helianthus annuus*
491	−1.88	E	2.2E-04	5.0/60	5.0/54.3	619/447	15233891	Vacuolar ATP synthase subunit B2	*Arabidopsis thaliana*
1050	−1.76	E	4.6E-04	5.8/37	6.0/59.9	156/126	114421	ATP synthase subunit beta, mitochondrial precursor	*Nicotiana plumbaginifolia*
1084	−3.08	E	4.4E-06	6.6/35	6.0/59.9	501/447	114421	ATP synthase subunit beta, mitochondrial precursor	*Nicotiana plumbaginifolia*
594	1.63	NE	3.8E-05	5.4/58	5.7/54.7	(95)/95	266567	Mitochondrial-processing peptidase alpha subunit, mitochondrial precursor	*Solanum tuberosum*
1502[g]	−27.77	E	9.0E-05	5.4/15	5.8/55.1	223/200	357982	ATPase alpha, F1	*Arachis hypogaea*
Hypothetical function									
707	3.25	NE	1.2E-05	6.5/52	5.5/44.3	71(+33)/76	3775985	RNA helicase	*Arabidopsis thaliana*
1376	−1.55	E	7.6E-04	5.2/22	5.5/28.8	(104)/104	15228818	ATFER3; binding/ferric iron binding	*Arabidopsis thaliana*
1416	−4.35	E	1.6E-06	6.6/20	5.4/29.2	67(+31)/105	78128515	Ferritin	*Chorispora bungeana*

[a] Some proteins have several functions and belong to more than one of the groups in the table

[b] The ratio is the change "in fold" between the abundances. It is defined as abundance in non-embryogenic suspensions (NES) vs. embryogenic suspensions (ES). Positive ratio means increased abundance in NES vs. ES, while negative ratio means decreased abundance in NES vs. ES. Thus, for example, spot 1416, ratio −4.35 means that the abundance in NE is 4.35-fold less than in E

[c] NE: increased abundance in NE, E: increased abundance in E

[d] Values for experimental M_r are rough estimates because they were extrapolated with proteins with known M_r that were found on all the gels

[e] Protein score: (+ …) is additional ions that have been manually checked and found in the database. Just (…) means that the automatic search in the database did not provide any result, but that ions could be matched manually in the database

[f] Ion score is the sum of all the ion scores for each spot. For additional information, see the Supplementary Table

[g] Type of protein molecule unique to the embryogenic cell line (E)

[h] Type of protein molecule unique to the non-embryogenic cell line (NE)

NES. A possible function of these enzymes is unknown, but their physiologic role could be related to a regulation of the diphosphonucleotide level in plant metabolism (Olczak and Olczak 2002).

In animals, an increase in cytoplasmic pH is important for activation of later fertilization events such as movement of the pronucleus and the initiation of protein and DNA synthesis (Dudits and others 1995). Similarly, Smith and Krikorian (1990) have shown that low pH is essential for maintaining the proembryogenic stage in carrot. At low pH, the ATPase H^+ pump is stimulated (Sanders and others 1981). In our study, vacuolar ATP synthase subunit B2 (spot 491), ATP synthase subunit beta (spots 1050, 1084), and ATPase alpha F1 (spot 1502) were highly upregulated in ES compared to NES. In fact, ATPase alpha F1 (spot 1502) was the most unique embryo-specific protein found in this study with regard to the amount. This may explain the proembryogenic state of our culture. It is also probable that the embryogenic cells need more energy for their metabolic changes. Zimmerman (1993) found that ATPase was upregulated during somatic embryogenesis in carrot. Schaefer (1985), on the other hand, showed that cells of non-embryogenic alfalfa callus have a lower average intracellular pH than embryogenic cultures, and that regeneration was favored at high average intracellular pH. The 14-3-3 proteins, represented here by spot 1308, have been known as positive regulators of H^+-ATPase activity (Chen and others 2006). Our results support this theory, because the 14-3-3-like protein (spot 1308) as well as ATPase (spot 1502) were upregulated in ES.

Protein Processing

Cell reprogramming requires posttranslational modification of proteins, protein folding, and protein translocation. A range of heat shock proteins (HSPs) were found in both cell lines in our study, including members of the HSP90 family, the HSP70 family, and the HSP60 family. These proteins are chaperones and folding catalysts, and also participate in membrane transport and signal transduction (Sun and others 1996; Kiang and Tsokos 1998; Krishna and Gloor 2001). The HSP90 class participates in the masking of hormone receptors (Ellis 1990). Interestingly, a HSP90 representative (spot 93) was unique to NES and may play an important role in keeping this cell line non-embryogenic. Members of the HSP60 and HSP70 families have also been found in embryogenic tissue of *Picea glauca* (Lippert and others 2005), in the embryogenic callus of *Vitis vinifera* (Marsoni and others 2008), and in somatic embryos of *Cyclamen persicum* (Winkelmann and others 2006). The heat shock genes are also expressed during early embryogenesis in mouse (Bensaude and Morange 1983), the common fruit fly (Zimmerman and others 1983), alfalfa (Györgyey and others 1991), and white spruce (Lippert and others 2005). The HSPs are referred to as stress proteins, but recent studies show that many of the stress protein genes are activated in the absence of stress, with some HSPs appearing at specific stages of development, in specific tissues, and even during the normal cell growth cycle (Bond and Schlesinger 1987). According to Bond and Schlesinger (1987), the HSPs can play a role during cell proliferation, differentiation, and embryogenesis.

Cell Proliferation

Accepting the fact that the initiation of somatic embryogenesis is closely linked to hormone-induced cell divisions, its molecular characterization can also be based on genes with cell cycle-dependent expression (Dudits and others 1995). One protein that is involved in cell growth and cell division through microtubule stabilization, the translationally controlled tumor protein homolog (TCTP) (Gachet and others 1999), was unique to ES (spot 1450). This protein has previously been identified in embryogenic cell suspensions of cowpea (Nogueira and others 2007).

Signal Transduction

Levels of some proteins involved in signalling were identified and were found to be higher in ES than in NES (spots 316, 1188, 1308). The 14-3-3-like protein GF14-D (spot 1308), similar to a protein found in embryogenic cell suspensions by Nogueira and others (2007), belongs to the 14-3-3 proteins, which are major regulators of primary metabolism and cellular signal transduction in plants (Comparot and others 2003; Chen and others 2006). Protein phosphatase 2A (PP2A) 65-kDa regulatory subunit (spot 316) has previously been associated with the embryogenesis process by Rensing and others (2005) and by Marsoni and others (2008). PP2A is involved during developmental processes such as seedling and floral developments, and is mostly expressed in cell-dividing tissues such as apical meristems (Zhou and others 2004). PP2A also plays a prominent role in the regulation of specific signal transduction cascades (Janssens and Goris 2001). It is important in auxin transport (Garbers and others 1996), functions as a general positive transducer of early ABA signalling (Kwak and others 2002), and is involved in differential cell elongation responses (Deruère and others 1999). Zhou and others (2004) demonstrated its role in embryogenesis and reproduction through *Arabidopsis* mutants. Dudits and others (1995) reported different protein phosphorylation patterns from cells of embryogenic and non-embryogenic genotypes. In the presence of the same concentrations of 2,4-D, the embryogenic cell population exhibited a marked increase in phosphorylation of

defined proteins compared to the non-embryogenic cells. Our results may be in accordance with this because the embryogenic suspensions expressed higher levels of PP2A and a 14-3-3-like protein, both of which play important roles in protein phosphorylation.

G proteins (spots 1269, 1278, 1291) and calreticulin (spots 360, 438) have previously been classified as promising candidates for involvement in signal tranduction in *Cyclamen* somatic embryogenesis (Rensing and others 2005).

Stress Response

Somatic embryogenesis can be considered an extreme case of adaptation, a survival strategy, and a cellular stress response to in vitro culture conditions (Dudits and others 1995; Pasternak and others 2002). Auxins, such as 2,4-D, play a critical role in the induction of embryogenic somatic cells, and the application of high exogenous concentrations of 2,4-D in the culture medium itself is a stress signal (Dudits and others 1995). The auxin treatment is thought to induce an oxidative burst which results in stress response and induction of somatic embryogenesis (Thibaud-Nissen and others 2003). In our study, two stress-related proteins were upregulated in ES (spot 246, 1192). Catalase (spot 1192) had previously been named as a candidate protein in the influence of *Cyclamen* somatic embryogenesis and is expressed as a response to oxidative stress, according to Rensing and others (2005). Catalase takes part in energy metabolism and amino acid metabolism.

The consideration of somatic embryogenesis as a specific form of stress response is supported by experimental findings that show the involvement of the heat shock systems in this developmental reprogramming (Zimmerman and others 1989; Györgyey and others 1991). Czarnecka and others (1984) found the activation of heat shock genes by 2,4-D treatment of soybean seedlings. In agreement with this, members of the HSP90, HSP70, and HSP60 families were found in our study. One of these heat shock proteins, HSP68 (spot 235), was supposed to have a stress-activated defense function (Bond and Schlesinger 1987; Neumann and others 1993).

Dehydrin (spot 510), another stress-inducible protein found in this study, is part of a large group of highly hydrophilic proteins known as LEA (late embryogenesis abundant) proteins. LEA proteins were originally shown to accumulate in plant embryos during the later stages of embryogenesis and are now widely accepted as somatic embryogenesis-related proteins (Chugh and Khurana 2002). Dehydrins accumulate late in embryogenesis and in nearly all the vegetative tissues during normal growth conditions and in response to stress leading to cellular dehydration (Rorat 2006). Several functional roles have been proposed for the different subgroups of LEA proteins, including molecular chaperones, DNA binding and repair, Ca^{2+} binding, and being a structural component of the cytoskeleton (Wise and Tunnacliffe 2004). However, despite being discovered more than 20 years ago, the precise function of the LEA proteins is still unclear (Tunnacliffe and Wise 2007).

Conclusion

There is probably no single model applicable to all plant species for differentiating into an embryo, but a number of the proteins identified in our study were similar to candidate proteins found in other studies. Differentially expressed proteins in embryogenic and non-embryogenic cell suspensions of isogenic lines were involved in cell proliferation, protein processing, signal transduction, stress response, and metabolism. The highest proportion was implicated in metabolism, especially carbohydrate metabolism. Among the proteins detected, 27 were unique to the embryogenic suspension and 12 of these were identified. The others were either predicted proteins with unknown functions or their identification was not possible due to unreliable results (not enough peptides matched, protein without homology in the database, or spectra of weak intensity). The 12 embryo-specific proteins represented different biological functions: cell proliferation, and carbohydrate, amino acid, lipid, and energy metabolism. ATPase (spot 1502), the most abundant protein, was unique to the embryogenic cell line. This may explain the pro-embryogenic state of our culture, but may also mean that the embryogenic cells need more energy for their metabolic changes. Three putative metallophosphatases (spots 65, 89, and 128) as well as a molecular chaperone Hsp90 (spot 93) were unique to the non-embryogenic cell line. These may prevent differentiation.

Further studies should include suspension cultures that are induced for differentiation and their protein expression over time. Candidate proteins could be analyzed in more detail with regard to their localization in the cell, because proteins bound to or part of the cell wall could be a good basis for developing antibodies. The possibility of antibody-covered beads as a means of cell separation will enable enrichment of embryogenic cells in bioreactors and more synchronous development of embryos. Antibodies against embryo-specific proteins in general can be useful for the identification of appropriate treatments or environments during in vitro testing.

Acknowledgments The authors acknowledge cooperation with the European Union through COST822, COST843, and the gift of the *Cyclamen persicum* Mill. line 3738 cell cultures from Dr. Hans-Georg Schwenkel, Erfurt, Germany. We also thank Tone I. Melby, engineer

in the Plant Cell Laboratory, for the growth of *Cyclamen* cultures over the years; Tor Beisvåg for taking the embryo pictures; and Pol Kieffer at the Proteomics Laboratory in CREBS for assisting with 2-D DIGE. The Research Council of Norway (project No. 132078/410), Centre de Recherche Public - Gabriel Lippmann, and Sør-Trøndelag University College funded the project.

References

Bennett N, Loomis WE (1949) Tetrazolium chloride as a test reagent for freezing injury of seed corn. Plant Physiol 24:162–174

Bensaude O, Morange M (1983) Spontaneous high expression of heat-shock proteins in mouse embryonal carcinoma cells and ectoderm from day 8 mouse embryo. EMBO J 2:173–177

Bond S, Schlesinger MJ (1987) Heat-shock proteins and development. Adv Genet 24:1–29

Chen F, Li Q, Sun L, He Z (2006) The rice 14–3-3 gene family and its involvement in responses to biotic and abiotic stress. DNA Res 13:53–63

Chugh A, Khurana P (2002) Gene expression during somatic embryogenesis–recent advances. Curr Sci 83:715–730

Comparot S, Lingiah G, Martin T (2003) Function and specificity of 14–3-3 proteins in the regulation of carbohydrate and nitrogen metabolism. J Exp Bot 54:595–604

Corke FMK, Roberts K (1997) Large changes in the population of cell wall proteins accompany the shift to cell elongation. J Exp Bot 48:971–977

Czarnecka E, Edelman L, Schöffl F, Key JL (1984) Comparative analysis of physical stress responses in soybean seedlings using cloned heat shock cDNAs. Plant Mol Biol 3:45–58

Deruère J, Jackson K, Garbers C, Söll D, DeLong A (1999) The *RCN1*-encoded A subunit of protein phosphatase 2A increases phosphatase activity in vivo. Plant J 20:389–399

Dudits D, Györgyey J, Börgre L, Bakó L (1995) Molecular biology of somatic embryogenesis. In: Thorpe TA (ed) In vitro Embryogenesis in Plants. Kluwer Academic Publishers, Dordrecht, pp 267–308

Ellis RJ (1990) Molecular chaperones: the plant connection. Science 250:954–959

Faivre-Nitschke SE, Couée I, Vermel M, Grienenberger JM, Gualberto JM (2001) Purification, characterization and cloning of isovaleryl-CoA dehydrogenase from higher plant mitochondria. Eur J Biochem 268:1332–1339

Fehér A, Pasternak TP, Dudits D (2003) Transition of somatic plant cells to an embryogenic state. Plant Cell Tiss Org Cult 74:201–228

Gachet Y, Tournier S, Lee M, Lazaris-Karatzas A, Poulton T, Bommer U-A (1999) The growth-related, translationally controlled protein P23 has properties of a tubulin binding protein and associates transiently with microtubules during the cell cycle. J Cell Sci 112:1257–1271

Garbers C, DeLong A, Deruère J, Bernasconi P, Söll D (1996) A mutation in protein phosphatase 2A regulatory subunit A affects auxin transport in *Arabidopsis*. EMBO J 15:2115–2124

Györgyey J, Gartner A, Németh K, Magyar Z, Hirt H, Heberle-Bors E, Dudits D (1991) Alfalfa heat shock genes are differentially expressed during somatic embryogenesis. Plant Mol Biol 16:999–1007

Halperin W (1966) Alternative morphogenetic events in cell suspensions. Am J Bot 53:443–453

Hecht V, Vielle-Calzada J-P, Hartog MV, EdDL Schmidt, Boutilier K, Grossniklaus U, de Vries SC (2001) The Arabidopsis *SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1* gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. Plant Physiol 127:803–816

Hendriks T, de Vries SC (1995) The role of secreted proteins in carrot somatic embryogenesis. In: Terzi M, Cella R, Falavigna A (eds) Current Issues in Plant Molecular and Cellular Biology. Kluwer Academic Publishers, Dordrecht, pp 359–368

Hvoslef-Eide AK, Corke FMK (1997) Embryogenesis specific protein changes in birch suspension cultures. Plant Cell Tiss Org Cult 51:35–41

Janssens V, Goris J (2001) Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. Biochem J 353:417–439

Kachroo P, Shanklin J, Shah J, Whittle EJ, Klessig DF (2001) A fatty acid desaturase modulates the activation of defense signaling pathways in plants. Proc Natl Acad Sci U S A 98:9448–9453

Kiang JG, Tsokos GC (1998) Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. Pharmacol Ther 80:183–201

Krishna P, Gloor G (2001) The Hsp90 family of proteins in *Arabidopsis thaliana*. Cell Stress Chaperones 6:238–246

Kwak JM, Moon J-H, Murata Y, Kuchitsu K, Leonhardt N, DeLong A, Schroeder JI (2002) Disruption of a guard cell-expressed protein phosphatase 2A regulatory subunit, *RCN1*, confers abscisic acid insensitivity in Arabidopsis. Plant Cell 14:2849–2861

Lippert D, Zhuang J, Ralph S, Ellis DE, Gilbert M, Olafson R, Ritland K, Ellis B, Douglas CJ, Bohlmann J (2005) Proteome analysis of early somatic embryogenesis in *Picea glauca*. Proteomics 5:461–473

Luethy MH, Gemel J, Johnston ML, Mooney BP, Miernyk JA, Randall DD (2001) Developmental expression of the mitochondrial pyruvate dehydrogenase complex in pea (*Pisum sativum*) seedlings. Physiol Plant 112:559–566

Marsoni M, Bracale M, Espen L, Prinsi B, Negri AS, Vannini C (2008) Proteomic analysis of somatic embryogenesis in *Vitis vinifera*. Plant Cell Rep 27:347–356

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Neumann D, Emmermann M, Thierfelder J-M, zur Nieden U, Clericus M, Braun H-P, Nover L, Schmitz UK (1993) HSP68—a DnaK-like heat-stress protein of plant mitochondria. Planta 190:32–43

Nogueira FCS, Gonçalves EF, Jereissati ES, Santos M, Costa JH, Oliveira-Neto OB, Soares AA, Domont GB, Campos FAP (2007) Proteome analysis of embryogenic cell suspensions of cowpea (*Vigna unguiculata*). Plant Cell Rep 26:1333–1343

Olczak M, Olczak T (2002) Diphosphonucleotide phosphatase/phosphodiesterase from yellow lupin (*Lupinus luteus* L.) belongs to a novel group of specific metallophosphatases. FEBS Lett 519:159–163

Pasternak T, Prinsen E, Ayaydin F, Miskolczi P, Potters G, Asard H, van Onckelen H, Dudits D, Fehér A (2002) The role of auxin, pH and stress in the activation of embryogenic cell division in leaf protoplast-derived cells of alfalfa (*Medicago sativa* L.). Plant Physiol 129:1807–1819

Quiroz-Figueroa FR, Rojas-Herrera R, Galaz-Avalos RM, Loyola-Vargas VM (2006) Embryo production through somatic embryogenesis can be used to study cell differentiation in plants. Plant Cell Tiss Org Cult 86:285–301

Rensing SA, Lang D, Schumann E, Reski R, Hohe A (2005) EST Sequencing from embryogenic *Cyclamen persicum* cell cultures identifies a high proportion of transcripts homologous to plant genes involved in somatic embryogenesis. J Plant Growth Regul 24:102–115

Rorat T (2006) Plant dehydrins–tissue location, structure and function. Cell Mol Biol Lett 11:536–556

Rose RJ, Nolan KE (2006) Invited review: genetic regulation of somatic embryogenesis with particular reference to *Arabidopsis thaliana* and *Medicago truncatula*. In vitro Cell Dev Biol Plant 42:473–481

Sanders D, Hansen UP, Slayman CL (1981) Role of the plasma membrane proton pump in pH regulation in non-animal cells. Proc Natl Acad Sci U S A 78:5903–5907

Schaefer J (1985) Regeneration in alfalfa tissue culture. Characterization of intracellular pH during somatic embryo production by solid-state P-31 NMR. Plant Physiol 79:584–589

Schmidt Th, Ewald A, Seyring M, Hohe A (2006) Comparative analysis of cell cycle events in zygotic and somatic embryos of *Cyclamen persicum* indicates strong resemblance of somatic embryos to recalcitrant seeds. Plant Cell Rep 25:643–650

Schwenkel H-G (2001) Introduction: botany-economic importance-cultivars-micropropagation of *C. persicum*. In: Schwenkel H-G (ed) Reproduction of *Cyclamen persicum* Mill. through somatic embryogenesis using suspension culture systems: COST Action 822, Report of Working Group 2. EUR 19697. European Commission, Luxembourg, pp 3–7

Schwenkel H-G, Winkelmann T (1998) Plant regeneration via somatic embryogenesis from ovules of *Cyclamen persicum* Mill. Plant Tiss Cult Biotechnol 4:28–34

Seyring M, Hohe A (2005) Induction of desiccation-tolerance in somatic embryos of *Cyclamen persicum* Mill. J Hortic Sci Biotechnol 80:65–69

Singla B, Tyagi AK, Khurana JP, Khurana P (2007) Analysis of expression profile of selected genes expressed during auxin-induced somatic embryogenesis in leaf base system of wheat (*Triticum aestivum*) and their possible interactions. Plant Mol Biol 65:677–692

Smith DL, Krikorian AD (1990) Somatic proembryo production from excised, wounded zygotic carrot embryos on hormone-free medium: evaluation of the effects of pH, ethylene and activated charcoal. Plant Cell Rep 9:34–37

Sun S-W, Chung M-C, Lin T-Y (1996) The structure and expression of an *hsc70* gene from *Lycopersicon esculentum*. Gene 170:237–241

Thibaud-Nissen F, Shealy RT, Khanna A, Vodkin LO (2003) Clustering of microarray data reveals transcript patterns associated with somatic embryogenesis in soybean. Plant Physiol 132:118–136

Towill LE, Mazur P (1975) Studies on the reduction of 2,3,5-triphenyltetrazolium chloride as a viability assay for plant tissue cultures. Can J Bot 53:1097–1102

Tunnacliffe A, Wise MJ (2007) The continuing conundrum of the LEA proteins. Naturwissenschaften 94:791–812

von Arnold S, Sabala I, Bozhkov P, Dyachok J, Filonova L (2002) Developmental pathways of somatic embryogenesis. Plant Cell Tiss Org Cult 69:233–249

von Arnold S, Bozhkov P, Clapham D, Dyachok J, Filonova L, Högberg K-A, Ingouff M, Wiweger M (2005) Propagation of Norway spruce via somatic embryogenesis. Plant Cell Tiss Org Cult 81:323–329

Winkelmann T, Hohe A, Scwenkel H-G (1998a) Establishing embryogenic suspension cultures in *Cyclamen persicum* 'Purple Flamed'. Adv Hort Sci 12:25–30

Winkelmann T, Sangwan RS, Scwenkel H-G (1998b) Flow cytometric analyses in embryogenic and non-embryogenic callus lines of *Cyclamen persicum* Mill.: relation between ploidy level and competence for somatic embryogenesis. Plant Cell Rep 17:400–404

Winkelmann T, Hohe A, Pueschel A-K, Schwenkel H-G (2000) Somatic embryogenesis in *Cyclamen persicum* Mill. Curr Top Plant Biol 2:51–62

Winkelmann T, Meyer L, Serek M (2004a) Desiccation of somatic embryos of *Cyclamen persicum* Mill. J Hortic Sci Biotechnol 79:479–483

Winkelmann T, Meyer L, Serek M (2004b) Germination of encapsulated somatic embryos of *Cyclamen persicum*. HortScience 39:1093–1097

Winkelmann T, Heintz D, van Dorsselaer A, Serek M, Braun H-P (2006) Proteomic analysis of somatic and zygotic embryos of *Cyclamen persicum* Mill. reveal new insights into seed and germination physiology. Planta 224:508–519

Wise MJ, Tunnacliffe A (2004) POPP the question: what *do* LEA proteins do? Trends Plant Sci 9:13–17

Zimmerman JL (1993) Somatic embryogenesis: a model for early development in higher plants. Plant Cell 5:1411–1423

Zimmerman JL, Petri W, Meselson M (1983) Accumulation of a specific subset of *D. melanogaster* heat shock mRNAs in normal development without heat shock. Cell 32:1161–1170

Zimmerman JL, Apuya N, Darwish K, O'Carrol C (1989) Novel regulation of heat shock genes during carrot somatic embryo development. Plant Cell 1:1137–1146

Zhou H-W, Nussbaumer C, Chao Y, DeLong A (2004) Disparate roles for the regulatory A subunit isoforms in Arabidopsis protein phosphatase 2A. Plant Cell 16:709–722