

# Comparative proteomic analysis of the H99 inbred maize (*Zea mays* L.) line in embryogenic and non-embryogenic callus during somatic embryogenesis

Lifang Sun · Ying Wu · Hongda Zou ·  
Shengzhong Su · Shipeng Li · Xiaohui Shan ·  
Jinghui Xi · Yaping Yuan

Received: 21 August 2012 / Accepted: 3 November 2012 / Published online: 6 December 2012  
© Springer Science+Business Media Dordrecht 2012

**Abstract** A proteomic approach based on two-dimensional electrophoresis (2-DE) and mass spectrometry was performed to investigate somatic embryogenesis in the H99 inbred maize line by comparing embryogenic and non-embryogenic callus. Protein spots ( $n = 42$ ) were differentially expressed between embryogenic calli and non-embryogenic calli according to our image analysis. Among them, 33 proteins were differentially expressed by at least threefold, with 15 up-regulated and 18 down-regulated in the embryogenic callus versus the non-embryogenic callus. However, only nine proteins were expressed in either of the calli. Twenty-nine protein spots were identified using mass spectrometry analysis and classified into several categories based on the matrix-science MASCOT and NCBI databases. These categories included cell proliferation (10.34 %), transcription and protein processing (17.24 %), stress response (10.34 %), signal transduction (3.45 %), metabolism and energy (48.28 %) and hypothetical function (10.34 %). Their putative roles are discussed according to their relevance in somatic embryogenesis. Real-time reverse transcription polymerase chain reaction analysis

revealed that the expression levels of five selected genes were consistent with the profiles detected in the 2-DE gels, further confirming the proteomic analysis. This study is the first comparative proteome analysis between the embryogenic callus and the non-embryogenic callus of the H99 inbred line. Our results show the differentially expressed proteins between the two callus types and reveal some key proteins that may have significant roles in molecular events during somatic embryogenesis in this species.

**Keywords** *Zea mays* L. · Immature embryos · Somatic embryogenesis · Embryogenic callus · MALDI-TOF-MS · Proteomics

## Introduction

Somatic embryogenesis (SE) is a complex developmental process that offers great potential in plant propagation. The process of SE is similar to that which occurs during formation of the zygotic embryo (Zimmerman 1993). However, zygotic embryos are tiny and deeply embedded in maternal tissues, and are therefore difficult to obtain. Thus, SE serves as a suitable model system to research the plant embryogenesis formation mechanisms with accessible experimental manipulation. For many plant species, embryogenic calli are the most suitable material for regeneration and genetic transformation. However, success in culturing embryogenic calli and regeneration through SE is affected by many factors, such as hormones, genotypes and the concentrations of various substances in the induction medium (Ptak et al. 2010; Filippov et al. 2006). Over the past 20 years, much research on SE has been conducted, however most of these works has focused on improving the culturing technologies,

**Electronic supplementary material** The online version of this article (doi:10.1007/s11240-012-0255-1) contains supplementary material, which is available to authorized users.

L. Sun · Y. Wu · H. Zou · S. Su · S. Li · X. Shan · J. Xi ·  
Y. Yuan (✉)  
College of Plant Science, Jilin University, 5333 Xi'an Road,  
Changchun 130062, China  
e-mail: yapingyuan@yahoo.com.cn

L. Sun  
e-mail: sunlifang2010@hotmail.com

L. Sun  
College of Agronomy, Heilongjiang Bayi Agricultural  
University, Development Zone, Daqing 163319, China

therefore the molecular basis of SE remains poorly understood.

Recently, efforts have been made to elucidate the patterns of gene expression that may play a critical role in the process of SE as these genes may aid in the elucidation of SE mechanisms (Zimmerman 1993; Fehér et al. 2003). Numerous genes associated with SE, such as *SERK* (Steiner et al. 2012; Zhang et al. 2010; Pérez-Núñez et al. 2009), *LEAFY COTYLEDON* (Ledwoń and Gaj 2009; Stone et al. 2008), *BABY BOOM* (Casson et al. 2005; Boutilier et al. 2002), *WUS* (Busch and Benfey 2010; Su et al. 2009) and *PICKLE* (Henderson et al. 2004; Ogas et al. 1999) have been identified. Moreover, proteomic and transcriptomic approaches have been used to research embryogenesis at the molecular level in several plant species, such as *Vitis vinifera* (Marsoni et al. 2008), *Elaeis guineensis* (Lin et al. 2009), *Phoenix dactylifera* L. (Sghaier-Hammami et al. 2009), Cotton (Wu et al. 2009), *Zea mays* L. (Che et al. 2006), *Cyclamen persicum* (Winkelmann et al. 2006), *Acca sellowiana* (Cangahuala-Inocente et al. 2009), longan (Wang et al. 2010) and Brazilian pine (Schlögl et al. 2012). 2-D electrophoresis (2-DE) combined with mass spectrometry (MS) has been used to identify differential expression of proteins and provide accurate analysis of changes in protein levels during various stages of plant development (Tan et al. 2012; Chen and Harmon 2006). This approach should be suitable for the identification of proteins associated with SE, and should provide insight into the process of SE. Although several expressed proteins have been identified in the embryos of a few species, there have been few reports focused on SE in maize.

Maize (*Zea mays* L.) is an important food crop. However, maize cultivar improvement through conventional genetic breeding is hindered by several factors, such as biotic and abiotic stresses, and is very time-consuming. As a receptor material, SE can provide a convenient way for improving transgenic efficiency in tissue culture and genetic transformation, which may to some degree overcome these obstacles, thereby providing valuable enlargement of maize germplasm resources.

Immature embryos have been frequently used as a source of explants in maize tissue cultures (Binott et al. 2008). The inbred line H99, has demonstrated that a high regeneration rate in transgenic maize plants can be achieved by somatic embryogenesis (Brettschneider et al. 1997). Thus, the H99 inbred was used in this study to research the mechanism of formation of embryogenic calli.

In this study, we used 2-DE and matrix assisted laser desorption ionization-time of flight (MALDI-TOF) MS analysis to investigate the differential expression of proteins between embryogenic calli (EC) and non-embryogenic calli (NEC) in the H99 inbred line. We identified several proteins associated with the process of SE. Additionally, we correlated

the expression levels of particular key proteins identified with the transcriptional expression levels of the corresponding genes using quantitative reverse transcription polymerase chain reaction (qRT-PCR). These results should provide new insights into understanding the molecular mechanisms of embryogenic calli formation at the proteome level in maize EC versus NEC, and should provide guidelines for further research.

## Materials and methods

### Plant materials and tissue culture conditions

Immature embryos (1.8–2.0 mm) of inbred line H99 (Illinois Synthetic 60 C) (Henderson 1976) were used in this study. The ears were harvested 12–14 days after self-pollination and sterilized three times with 75 % ethyl alcohol. According to the procedure used by Jiménez and Bangerth (2001), immature embryos were aseptically dissected from seeds to placed side down onto the N6 induction medium (Chu et al. 1975) supplemented with 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 700 mg/L L-proline, 450 mg/L casein hydrolysate, 100 mg/L D-myo-inositol, 230 mg/L L-aspartic acid, 20 g/L D-mannitol, 3 % sucrose and 7.5 g/L agar. The pH value of the medium was adjusted to 5.8 prior to autoclaving at 121 °C for 20 min. A total of 20 embryos per plate were used in about 30 plates.

The immature embryos were incubated in the dark at 26 °C. Buds generated from the immature embryos were removed after 7 days and the embryo-scutellum that formed primary calli were transferred to fresh induction medium after 20 days. The calli were then sub-cultured every 2 weeks on the same medium. After sub-culturing four times, the EC and NEC appeared, and were selected, separately, from 10 plates. To rule out individual differences, we mixed them together then stored them at –80 °C until required.

### Histological staining

To observe the histology of SE, the EC and NEC sub-cultured four times were fixed in FAA solution (5 % formaldehyde, 5 % acetic acid and 50 % ethyl alcohol) for 48 h. After the fixation, the samples were dehydrated in a graded ethyl alcohol series (75, 85, 95, 100 and 100 %) for 2 h per concentration. When the samples were transparent, they were incubated in xylene for 1 h, embedded in paraffin and cut into 10- $\mu$ m-thick sections. The paraffin sections were then deparaffinized with xylene for 3 h and hydrolyzed in different concentrations of ethyl alcohol (100, 100, 95, 85, 75 and 65 %) for 3 min per concentration. Finally, the paraffin sections were stained with 1 %

safranin T and 1 % fast green FCF. The sections were observed and photographed under a microscope (Nikon eclipse 50i).

#### Scanning electron microscope observations

To observe the appearance of the calli epidermal surfaces, EC and NEC sub-cultured four times were collected and fixed in FAA solution for 48 h. After the fixation, the samples were dehydrated in a graded ethyl alcohol series (75, 85, 95, 100 and 100 %) for 2 h per concentration, and dried in a vacuum dryer for 2 h. Samples were then coated with gold using a Coater EIKO ID-5 (EIKO ID-5; Tokyo, Japan). Finally, samples were scanned using a scanning electron microscope XL-30FE-ESEM FEG (XL-30FE-ESEM FEG; Pennsylvania, USA).

#### Protein extraction

The EC and NEC sub-cultured four times were used to extract the total protein content according to the modified trichloroacetic acid/acetone method (Natarajan et al. 2005). Frozen samples (a total of 1 g fresh weight) were ground in liquid nitrogen and equal amounts of powdered tissue packed in different tubes, then ten times the volume (w/v) of cold acetone with 10 % trichloroacetic acid and 0.7 %  $\beta$ -mercaptoethanol was used to re-suspend the tissue. After thoroughly mixing the samples, the tubes were stored at  $-20^{\circ}\text{C}$  for 1 h and centrifuged ( $15,000\times g$ , 15 min,  $4^{\circ}\text{C}$ ). The pellet was re-suspended in ten times the volume (w/v) of cold acetone with 0.7 %  $\beta$ -mercaptoethanol, two times every hour, and then washed with 80 % cold acetone for 1 h. Each time, the mixture was stored at  $-20^{\circ}\text{C}$  and centrifuged as above. The pellet was air dried for 30 min, and then dissolved in solubilization buffer (7 M urea, 2 % 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate (CHAPS), 2 M thiourea, 20 mM dithiothreitol (DTT), 5 mM tris (2-carboxyethyl) phosphine, 2 % *N*-decyl-*N,N*-dimethyl-3-ammonio-1-propane-sulfonate (SB3-10), 0.002 % bromophenol blue and 2 % IPG buffer pH 3–10). After the samples were centrifuged to remove insoluble particulates, the protein concentration of the samples was determined according to the Bradford (1976) method with modifications as necessary, using bovine serum albumin as a standard. The samples were directly used for isoelectrofocusing (IEF).

#### Two-dimensional IEF/SDS-PAGE

A total of 300  $\mu\text{g}$  of the soluble protein sample was used in (IEF) by employing an immobilized nonlinear 3–10 pH gradient (Immobiline DryStrip, 13 cm; Amersham Biosciences, Uppsala, Sweden). The strips were rehydrated in 340  $\mu\text{l}$  solubilization buffer containing 300  $\mu\text{g}$  soluble

proteins. IEF was performed with the IPGphor system for 1 h at 500 V, with a gradient of 1000–4000 V over 3 h. Following this procedure the samples were run at 8000 V to give a total of 65 kV/h. Each strip was equilibrated for 15 min in 6 M urea, 2 % SDS, 30 % glycerol, 50 mM Tris-HCl (pH 8.8), 0.002 % bromophenol blue and 1 % DTT, and then for another 15 min with the substitution of the DTT with 2.5 % iodoacetamide (IAM). The protein separation in the second dimension was carried out on a 12.5 % SDS-polyacrylamide gel electrophoresis (PAGE). Electrophoresis was performed in a Laemmli running buffer containing 25 mM Tris, 192 mM glycine, 0.1 % SDS for 20 mA until the dye reached the bottom of the gel.

#### Staining and image analysis

After 2-DE separation, the proteins on the gels were visualized by silver staining for analysis and were detected with Coomassie brilliant blue G250 for use in MS analysis. The gels were scanned using an ImageScanner (Amersham Bioscience, Sweden), and the images were analyzed using the ImageMaster 2D Platinum software 5.0 (Amersham Bioscience, Sweden), which allows spot detection, quantization and spot matching among multiple gels. To produce a master image, three replicates were made for each sample. Overlapping and quantitative comparisons of protein spots between two different master gel images were carried out using the ImageMaster 2D Platinum software. The proteins were considered only in EC or NEC, or for proteins showing differential expression between EC and NEC when they display a fold change of  $\pm 3$ . These spots were used for MS analysis.

#### Protein in-gel digestion

Protein spots were excised from Coomassie brilliant blue stained gels, transferred to sterilized microcentrifuge tubes (0.5 mL) and then rinsed three times using 10  $\mu\text{l}$  of ddH<sub>2</sub>O to remove the residual SDS. The gel pieces were transferred to small microcentrifuge tubes (0.2 mL) and the gels incubated with 100  $\mu\text{l}$  of 25 mM NH<sub>4</sub>HCO<sub>3</sub> in 50 % (V/V) acetonitrile (ACN) for 30 min. This step was repeated until the gels were transparent. Then the gel pieces were air dried for 30 min under vacuum on a centrifugal evaporator (Thermo Savant SpeedVac Concentrator, USA). The dried pellets were then incubated in an amount of 25 mM NH<sub>4</sub>HCO<sub>3</sub> containing 10 mM DTT for 30 min in a 56  $^{\circ}\text{C}$  water bath, and for a further 45 min with the substitution of the DTT with 55 mM IAM, and the samples placed in the dark. The supernatant was discarded and the pellets dissolved with 25 mM NH<sub>4</sub>HCO<sub>3</sub> and 50 % ACN. For protein digestion, the pellets were digested with 3–7  $\mu\text{l}$  sequencing grade trypsin (Roche Company, Switzerland) for 1 h at

4 °C, then 5 µl of 25 mM NH<sub>4</sub>HCO<sub>3</sub> was added and incubated for 15 h at 37 °C. For extraction of the peptides, the gel pieces were incubated with 5 % trifluoroacetic acid (TFA) in 50 % (v/v) ACN for 1 h at 40 °C. The supernatant was extracted and the precipitants were incubated with 2.5 % TFA in 50 % (v/v) ACN for 1 h at 30 °C for further extraction of the peptides. The peptides were concentrated to 5–10 µl under vacuum on a centrifugal concentrator and the fragments were stored at –80 °C or used in MALDI-TOF MS analysis.

#### Mass spectrometry analysis and protein identification

For MALDL-TOF MS, 1 µl of digested peptides was mixed with 1 µl of matrix solution containing 5 mg of  $\alpha$ -cyano-4-hydroxycinnamic acid in 50 % acetonitrile and 0.1 % TFA. The mixture was deposited onto the instrument target plate. Tryptic peptides were analyzed using a Bruker ultraflex TOF/TOF (Bruker Daltonics Bremen, Germany) in positive-ion mode at an accelerating voltage of 25 kV equipped with a 337 nm nitrogen laser. Using trypsin autolysis products, the spectra were internally calibrated. The parameters used for MALDL-TOF MS were as follows: enzyme-trypsin; peptide mass tolerance-100 ppm; fragment mass tolerance-  $\pm$ 0.5 Da; max mass cleavages-1; and, modifications-carbamidomethyl and monoisotopic mass values. The list of peaks obtained was analyzed using Flexcontrol<sup>TM</sup> Software v3.0. The peptide mass fingerprint was submitted to MASCOT (Matrix Science, London, UK) and NCBI online databases. In addition, the selected protein spots were performed without constraining protein relative molecular weight (*Mr*) and pI.

#### RNA preparation and qRT-PCR analysis

The transcription expression levels of selected genes were measured by qRT-PCR analysis with an ABI 7500 real-time PCR system using the SYBR Green Real Master Mix with ROX kit (Takara, Japan). The gene-specific primers (Table S1) were designed and synthesized based on the mRNA sequences encoding the identified protein gene. Briefly, the total RNA of EC and NEC which were sub-cultured four times from the H99 inbred line were extracted using the RNAiso reagent kit (Takara, Japan). The quality and the integrity of the RNA were measured by absorbance and agarose electrophoresis (Fig. S1). The first-strand cDNA synthesis reaction mixture consisted of 1.5 µg of total RNA, 2 µl of oligo (dT) 18 primer (50 µM), 1 µl of M-MLV reverse transcriptase (200 U/µl), 4 µl of 5× M-MLV buffer, 4 µl of dNTPs (2.5 mM each) and 0.5 µl of RNase inhibitor (40 U/µl), supplemented with RNase-free water to a total volume of 20 µl. The above mixture was incubated at 42 °C for 1 h and then at 70 °C for 15 min; the mixture was

placed on ice immediately for 2 min. The cDNAs were then diluted three-fold with ddH<sub>2</sub>O for the qRT-PCR analysis. The endogenous reference gene (*tubulin*, GenBank accession no. X83696) was used as an internal standard for RT-PCR analysis. Each sample was run in triplicate in a final volume of 20 µl containing 2 µl of the cDNA, 5 pmol of each specific primer, 8 µl of 2.5× Real Master Mix and 1 µl of 20× SYBR solution, according to the manufacturer's instructions. Thermal cycling was performed as follows: 95 °C for 30 s, 95 °C for 5 s, 58 °C for 30 s and 72 °C for 30 s over 40 cycles. At the end of the PCR, the melt curve data were obtained to exclude the occurrence of primer dimers and non-specific PCR products (Fig. S2) based on the 7500 System software (version 2.0.5). The relative expression levels were analyzed using the 2<sup>– $\Delta\Delta C_t$</sup>  method (Livak and Schmittgen 2001).

## Results

### Calli induction

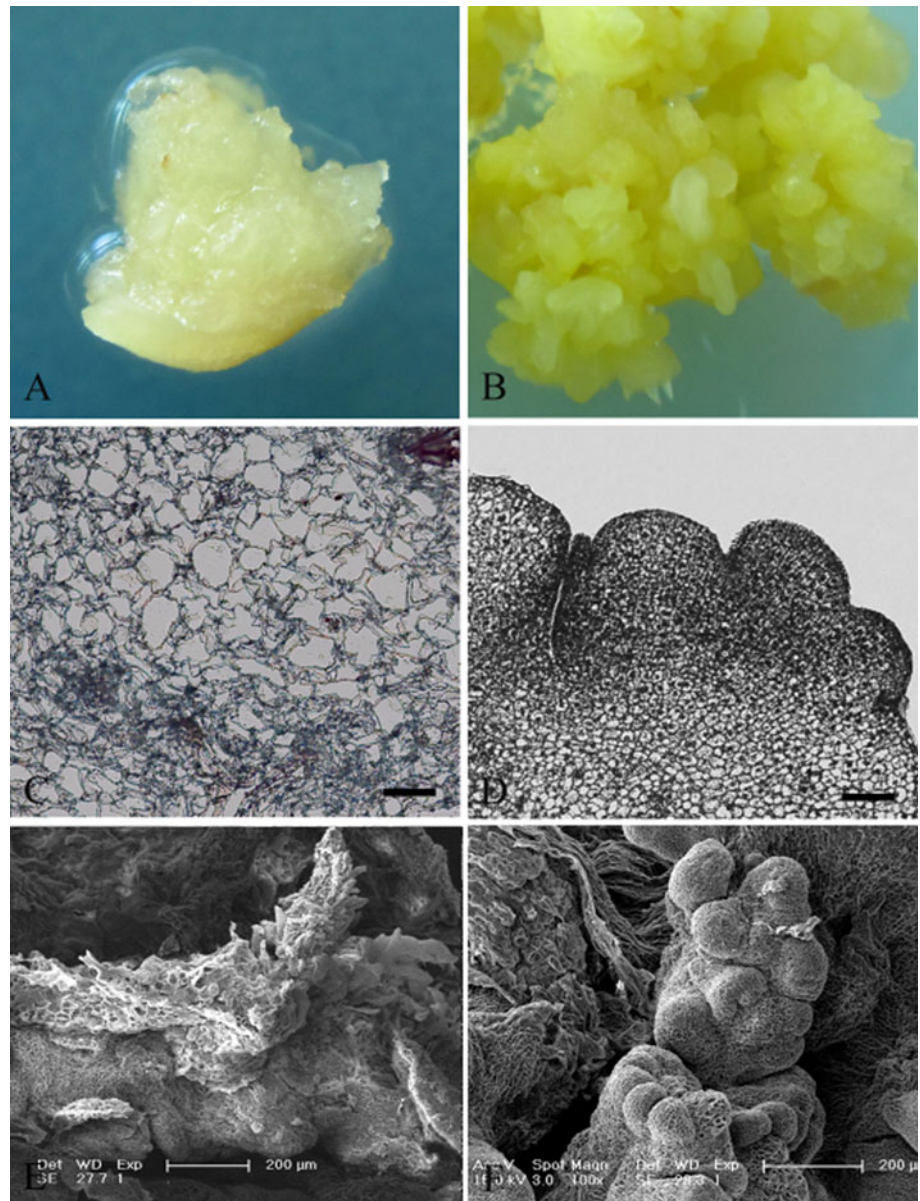
Immature embryos cultured on N6 basal medium containing 2, 4-D were sub-cultured twice. Primary calli were pale yellow, translucent, watery and sticky in appearance (Fig. 1a). The primary calli were then sub-cultured two times continuously on the same medium, and this led to the formation of EC and NEC (Fig. 1b). The EC were yellow in color and more friable, and the NEC appeared spongy, watery and brown (Fig. 1b). Histological analysis of the two calli types revealed that the embryogenic cells were small with dense cytoplasm and a high capacity for cell division (Fig. 1d). In contrast, non-embryogenic cells were large, with gaps between the cells observed (Fig. 1c). At the same time, structural differences in the epidermal cells between the EC and NEC were observed using a scanning electron microscope. The EC had a rough surface and granule structures had formed between cells, which combined tightly. Large gaps were observed between granules, which combined loosely. The cell shape was regular, and the embryogenic cells that had piled into groups were observed clearly when there was no apparent surface cover (Fig. 1f). The NEC had a loose surface structure and they snowflake. The cell shape was irregular and had a large volume (Fig. 1e). These results show that the EC and NEC are different in structure and appearance.

### Proteomic analysis

Changes in the protein profiles of EC and NEC were examined, using 2-DE analysis of the total proteins present (Fig. 2). Immobilized pH 3–10 IPG strips, molecular masses of 14.4–97.4 kDa and a silver stain were used. For



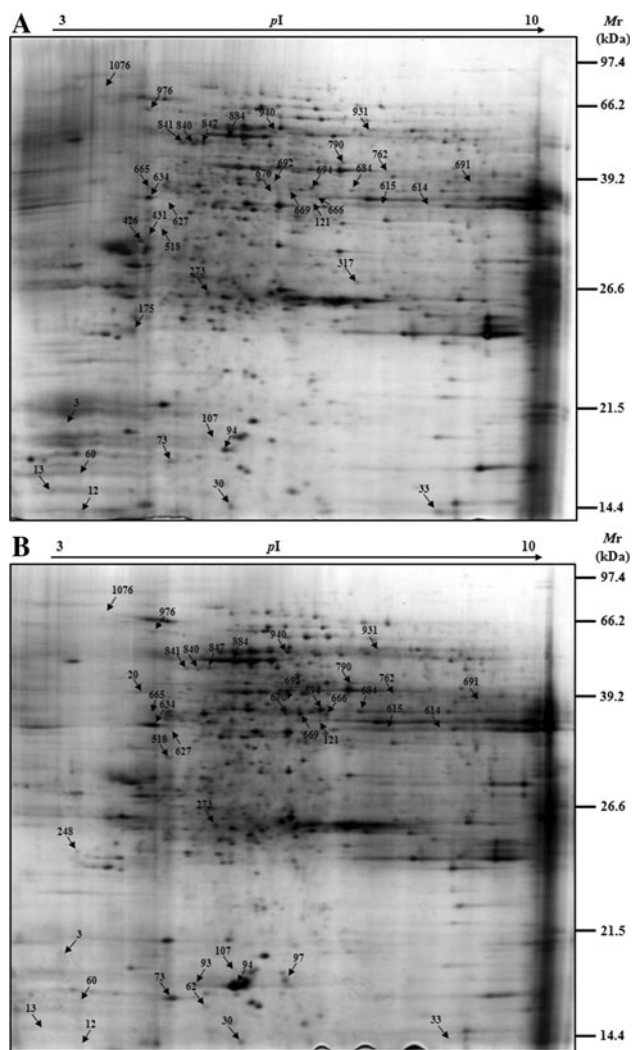
**Fig. 1** Histological and scanning electron microscope analysis of embryogenic calli and non-embryogenic calli. **(a)** Pale yellow, translucent, watery and sticky callus; **(b)** loosened, globular, yellow and fragile callus; **(c)** non-embryogenic callus (scale bar: 50  $\mu$ m) detected by histological staining; **(d)** embryogenic callus (scale bar: 80  $\mu$ m) detected by histological staining; **(e)** non-embryogenic callus on scanning electron microscope (scale bar: 200  $\mu$ m); and **(f)** globular-shape embryo on scanning electron microscope (scale bar: 200  $\mu$ m). (Color figure online)



each sample, the gel experiments were performed at least three times and showed a high level of reproducibility. Image analysis using Platinum showed that about 1,200 spots were detected on each silver-stained gel. All spots were auto-matched by gel-to-gel comparison using the Platinum software and the difference in the relative abundance of each spot was analyzed. This analysis showed that many of the protein profiles between EC and NEC were similar; however, there were a number of spots that had obvious differences in volume or abundance. Using the gels, statistical analysis showed that 42 protein spots were noticeably different ( $P < 0.05$ ). Among them, 33 proteins were differentially expressed at least threefold. Compared to the non-embryogenic calli, a total of 15 spots were

categorized as up-regulate, and 18 spots were down-regulated in the embryogenic calli. In addition, nine proteins spots were specific expression (four in EC and five in NEC).

According to the differential abundance of the spots, 38 were excised from the gels, digested with trypsin, and analyzed using mass spectrometry (MS/MS) (Table S2). The MS profiles obtained were searched against the matrix-science MASCOT and NCBI databases. Using the normal MASCOT database, 9 spots (20, 33, 60, 62, 73, 93, 97, 175 and 426) showed no good matches, whereas others were identified with an identification success rate of 78.95%. According to their homologies, the 29 identified proteins (Table 1) were classified into six functional groups, including



**Fig. 2** Two-dimensional gels of embryogenic callus (a) and non-embryogenic callus (b). Proteins extracted by cold acetone protocol; 300  $\mu$ g soluble proteins were separated in the first dimension on an immobilized nonlinear 3–10 pH gradient and in the second dimension on a 12.5 % acrylamide-SDS gel

cell proliferation (10.34 %), transcription and protein processing (17.24 %), stress response (10.34 %), signal transduction (3.45 %), metabolism and energy (48.28 %) and hypothetical function (10.34 %) (Fig. 3). Among these groups, the largest class is implicated in cellular metabolism (48.28 %), whereas in the signal transduction class only one protein was identified. Additionally, some of the identified proteins showed discrepancies with their theoretical molecular weight (*Mr*) or pI. We identified three proteins (spots 518–691–364) that matched the same sequence but had different *Mr* and pI values. These phenomena are commonly found on 2-DE gels and are invariably due to post-translation modification or protein degradation (Ahsan et al. 2008).

### Assessment of protein transcript levels using qRT-PCR analysis

To validate the proteomic results, qRT-PCR was performed to assess the transcription level of 10 genes. After sub-culturing four times, the expression levels of *pathogenesis related protein 10 (PR10)*, *ascorbate peroxidase (APX)*, *glutamine synthase (GS)*, *S-adenosylmethionine synthase (SAMS)* and *26S proteasome regulatory particle triple-A ATPase (26S-RPT)* showed clear correlation (Fig. 4) with the profiles detected in the 2-DE gels of the EC and NEC. Similar mRNA levels for *glycine-rich RNA binding protein (GRP)* and *protein disulfide isomerase (PDI)* were detected in two samples. However, the gene expression levels of  $\alpha$ -1,4-glucan-protein synthase, *ATP synthase  $\beta$  subunit* and *enolase* was in contrast to the results of the proteomic analysis, presumably due to the proteomic approach focusing on protein isoforms, whereas the gene expression levels analyzed by PCR may verify the quantification of the abundance of related transcripts. The best explanation for these discrepancies could be because of post-translational modification or protein degradation.

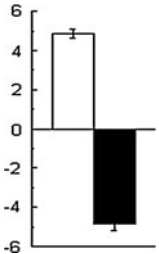
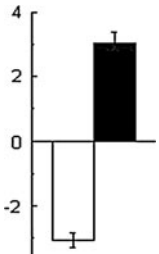
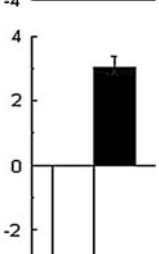
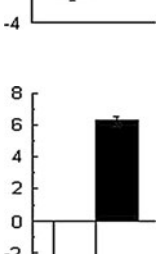
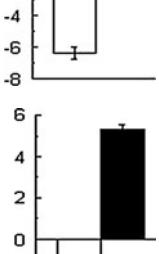
### Discussion

The process of SE represents the beginning of asexual reproduction that is usually achieved via in vitro induction of explants (Zimmerman 1993). Since the rapid development of biotechnology for asexual production, agricultural systems have been hugely influenced by this process. To obtain new insights into *Zea mays* L. SE, a proteomic approach was performed to investigate the protein patterns of EC and NEC induced by 2, 4-D. Our results showed that the percentage of differentially expressed protein is in accordance with the results obtained for *Vitis vinifera*, *Medicago truncatula* and *Picea glauca* (Marsoni et al. 2008; Imin et al. 2005; Lippert et al. 2005). We describe below some of the most important proteins identified and their putative biological functions.

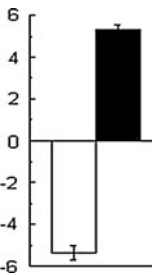
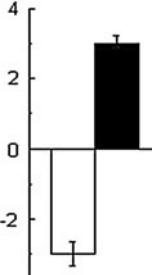
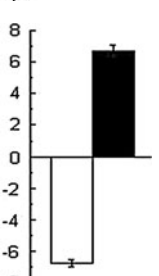
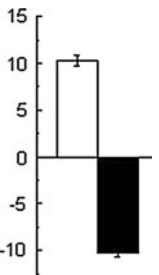
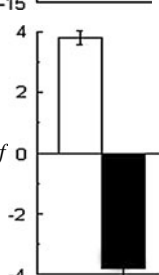
#### Stress response proteins

In vitro culture conditions that impose stress on the explants has been shown to be a potent inducer of the embryogenic response (Fehér et al. 2003), indicating that stress plays a significant role during the embryogenic switch. In this study, two stress response proteins were identified using MS: ascorbate peroxidase (APX, spot 273 which was up-regulated in NEC and spot 317 which was a specific expression spot in EC versus NEC) and pathogenesis-related protein 10 (PR10, spot 94, which was down-regulated in EC).

**Table 1** Differentially expressed proteins and their identification by mass spectroscopy

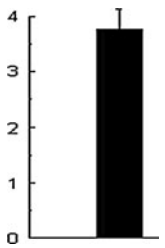
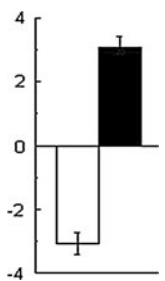
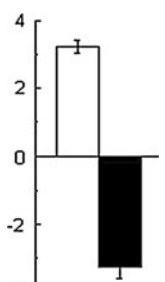
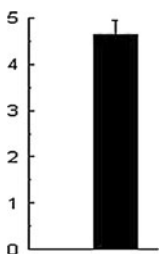
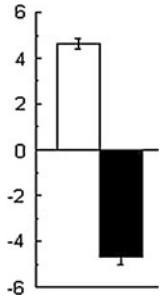
Spot no.	Protein name	GenBank accession No.	Amino acids coverage (%)	Theoretical pI/ Mr (kDa)	Experimental pI/ Mr (kDa)	Spot patterns (relative spot) □ NEC ■ EC
<i>Cell proliferation</i>						
694	Actin	XP_002530711.1 (Ricinus communis)	52.25	5.31/41.67	6.85/41.22	
841	$\alpha$ -tubulin	NP_001167663.1 ( <i>Zea mays</i> )	52.77	4.89/49.72	4.94/50.38	
976	$\beta$ -tubulin	NP-001105458.1 ( <i>Zea mays</i> )	61.57	4.77/50.0	4.73/62.4	
<i>Transcription and protein processing</i>						
3	Histone H2B.2	NP_001131654 ( <i>Zea mays</i> )	67.33	10.8/16.17	3.6/18.99	
12	Glycine-rich RNA-binding protein	ACG26657.1 ( <i>Zea mays</i> )	62.18	6.1/15.48	3.8/14.26	

**Table 1** continued

Spot no.	Protein name	GenBank accession No.	Amino acids coverage (%)	Theoretical <i>pI</i> / <i>Mr</i> (kDa)	Experimental <i>pI</i> / <i>Mr</i> (kDa)	Spot patterns (relative spot) □ NEC ■ EC
30	Glycine-rich RNA-binding protein	NP_001151562 ( <i>Zea mays</i> )	48.72	6.31/15.37	5.86/13.79	
665	40S ribosomal protein	ACG32965 ( <i>Zea mays</i> )	49.68	4.91/33.41	4.77/40.56	
1076	Protein disulfide isomerase	NP_001105754.1 ( <i>Zea mays</i> )	51.85	5.01/56.72	3.56/87.89	
<i>Stress response</i>						
94	Pathogenesis-related protein 10	AAY29574.1 ( <i>Zea mays</i> )	86.25	6.0/17.1	5.8/16.75	
273	APx1—Cytosolic Ascorbate Peroxidase	NP_001152746 ( <i>Zea mays</i> )	52.8	5.64/27.37	5.59/27.42	



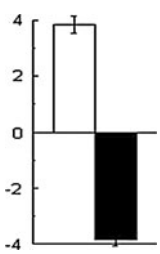
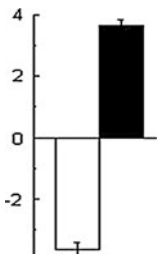
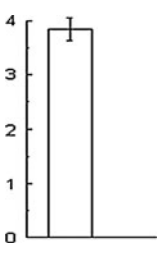
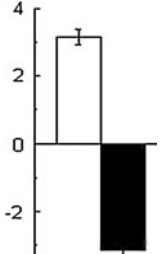
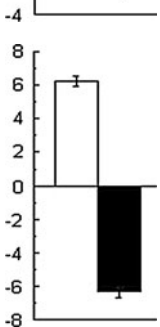
**Table 1** continued

Spot no.	Protein name	GenBank accession No.	Amino acids coverage (%)	Theoretical $pI/M_r$ (kDa)	Experimental $pI/M_r$ (kDa)	Spot patterns (relative spot) □ NEC ■ EC
317	Ascorbate peroxidase	NP_001152746 ( <i>Zea mays</i> )	57.2	5.64/27.37	7.35/27.48	
<i>Signal transduction</i>						
840	26S proteasome regulatory particle triple-A ATPase subunit5b	ABA78504.1 ( <i>Oryza sativa</i> )	74.23	5.51/21.75	5.3/50.15	
<i>Metabolism and energy</i>						
121	Cytoplasmic fructose-bisphosphate aldolase isozyme	NP_001150049 ( <i>Zea mays</i> )	71.27	6.95/38.46	6.87/38.89	
431	Enolase	ACG31732.1 ( <i>Zea mays</i> )	37.67	5.59/48.13	4.79/34.84	
518	Glyceraldehyde-3-phosphate dehydrogenase	AAA33465 ( <i>Zea mays</i> )	65.81	8.4/24.8	4.92/35.64	

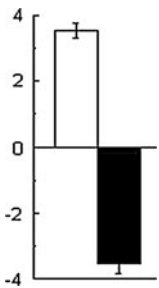
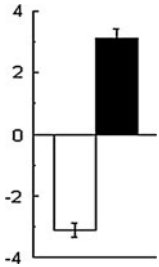
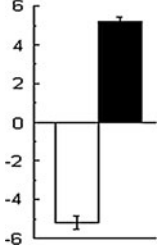
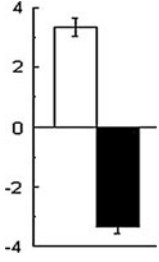
**Table 1** continued

Spot no.	Protein name	GenBank accession No.	Amino acids coverage (%)	Theoretical <i>pI</i> / <i>Mr</i> (kDa)	Experimental <i>pI</i> / <i>Mr</i> (kDa)	Spot patterns (relative spot) □ NEC ■ EC
615	Malate dehydrogenase, cytoplasmic	NP_001105603 ( <i>Zea mays</i> )	65.96	5.75/35.59	7.7/38.76	
627	Adenosine kinase	CAB40376 ( <i>Zea mays</i> )	75.83	5.23/36.03	5.04/37.68	
634	Glyceraldehyde-3-phosphate dehydrogenase	NP_001105413 ( <i>Zea mays</i> )	48.07	6.46/36.51	4.76/39.3	
684	$\alpha$ -1,4-glucan-protein synthase	NP_001105598 ( <i>Zea mays</i> )	60.71	5.75/41.2	7.35/40.95	
691	Glyceraldehyde-3-phosphate dehydrogenase	NP_001105700.1 ( <i>Zea mays</i> )	52.82	6.4/36.54	8.8/41.09	

**Table 1** continued

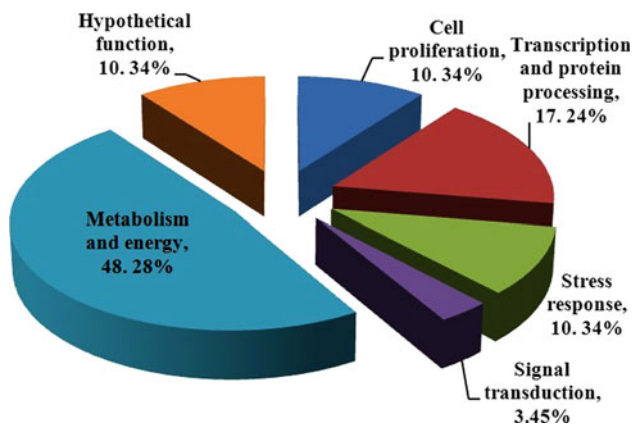
Spot no.	Protein name	GenBank accession No.	Amino acids coverage (%)	Theoretical <i>pI/Mr</i> (kDa)	Experimental <i>pI/Mr</i> (kDa)	Spot patterns (relative spot) □ NEC ■ EC
692	Glutamine synthase	ACA50923.1 ( <i>Zea mays</i> )	48.03	5.25/39.31	6.4/41.32	
762	S-adenosylmethionine synthase	ACG42196.1 ( <i>Zea mays</i> )	35.1	5.57/43.04	7.78/44.31	
790	Transaminase	NP_001159818.1 ( <i>Zea mays</i> )	54.55	6.55/49.26	7.15/45.31	
931	Enolase	ACG31732.1 ( <i>Zea mays</i> )	68.16	5.59/48.13	7.58/56.13	
884	ATP synthase beta	NP_001105340.1 ( <i>Zea mays</i> )	38.88	6.01/59.1	5.78/52.77	

**Table 1** continued

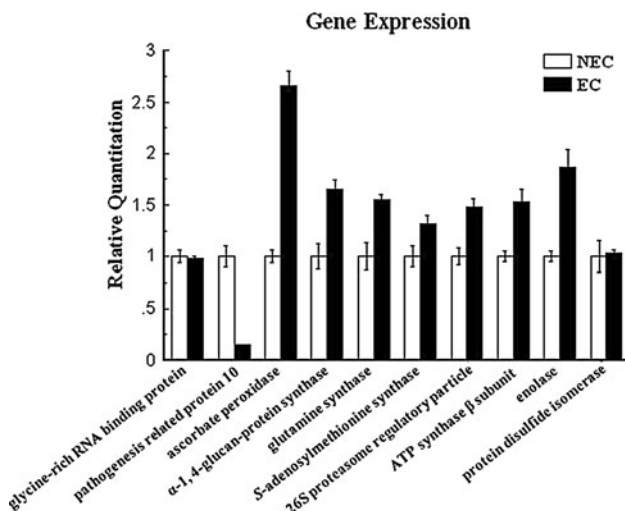
Spot no.	Protein name	GenBank accession No.	Amino acids coverage (%)	Theoretical $pI/M_r$ (kDa)	Experimental $pI/M_r$ (kDa)	Spot patterns (relative spot) □ NEC ■ EC
940	ATP synthase subunit beta	NP_001105340.1 ( <i>Zea mays</i> )	43.94	5.59/48.13	6.33/57.0	
<i>Hypothetical function</i>						
13	Predicted protein	XP_002874393 ( <i>Arabidopsis lyrata</i> )	16.04	5.45/13.69	3.3/14.34	
107	Hypothetical protein LOC100193804	NP_001132360 ( <i>Zea mays</i> )	80.71	5.33/14.98	5.67/17.92	
614	Hypothetical protein SELMODRAFT_451299	XP_002973959 ( <i>Selaginella moellendorffii</i> )	17.98	8.35/71.35	8.32/38.79	

Ascorbic acid has been reported to be a defense reaction factor against reactive oxygen species (ROS), in which ascorbate can reduce  $H_2O_2$  to water, thereby detoxifying ROS (Asada 1992). Although many papers suggest that ROS act as second messengers when stress is imposed due to the induction of embryogenesis (Maraschin et al. 2005; Ganesan and Jayabalan 2004), this signaling requires ROS-scavenging mechanisms to remove excessive reactive oxygen species (Mittler 2002). Pan et al. (2009) indicated that oxidative stress may stimulate cell differentiation to

promote somatic embryo formation, whereas other anti-oxidative proteins may serve to protect cells from toxicity caused by long term in vitro culturing. Shohael et al. (2007) reported that the expression level of APX is high in globular embryos, but low during later development, and also found that high concentrations of ROS can inhibit the development of the cotyledon embryo. In agreement with those results, our qRT-PCR results confirmed that APX is highly expressed in EC (Figs. 2 and 4), which could imply that the EC cells have the ability to adapt to cellular



**Fig. 3** Categories of proteins identified by the MS/MS analysis according to their functions



**Fig. 4** Analysis of the expression levels of multiple proteins using qRT-PCR. Quantitative reverse transcription polymerase chain reaction was performed to determine differential expression patterns of 10 selected proteins between embryogenic callus and non-embryogenic callus which were sub-cultured four times. The selected protein names are presented on the *abscissa*. Error bars were calculated from three independent determinations of mRNA abundance in each sample

stress responses through the regulation of the ROS-scavenging system.

PR10 (spot 94) was observed to be abundant in both EC and NEC. The expression level of PR10 in NEC was observed to be astonishingly high at both the protein and gene level. This result is supported by many findings in which PR10 proteins are up-regulated in NEC (Marsoni et al. 2008; Cangahuala-Inocente et al. 2009; Zhang et al. 2009). PR10 proteins belong to an intracellular defense related protein family and may have RNase activities that could function in an antiviral fashion (Moiseyev et al. 1997). PR10 proteins are widespread in various periods of

plant development, such as germination (Casacuberta et al. 1992), senescence (Hanfrey et al. 1996) and flowering (van Eldik et al. 1996). In addition, PR10 genes are highly expressed in response to biotic and abiotic stresses (Moiseyev et al. 1997). Mur et al. (2004) found that the AoPR10-GUS transgene was responsive to oxidative signals/stresses. However, the role of this protein in maize NEC cells and the high levels in NEC are not clear and requires further investigation.

### Cell proliferation

Somatic embryogenesis is accompanied by a series of stages, characteristic of morphogenesis processes, and the formation of SE occurs via two pathways: directly, from the explant without an intervening callus; or, indirectly from the callus phase (Williams and Maheswaran 1986). In the latter pathway, the formation of the EC is the first step and is based on coordinated cell division. Thus, the three proteins associated with cell division that were identified are not surprising. Among them, two forms of tubulins were up-regulated in EC (spots 841 and 976) and an actin protein was detected (spot 694) in NEC.

Tubulin microtubules and actin microtubules are known to constitute the cytoskeleton. Tubulin plays an important role in the separation of the daughter chromosomes. Although tubulin is a housekeeping protein that has been widely used as a constitutive standard in quantitating gene expression, the differential expression of  $\beta$ -tubulin was demonstrated during grape development (Terrier et al. 2005) and some tubulins were shown to be up-regulated in embryogenic calli (Marsoni et al. 2008; Pan et al. 2009; Zhang et al. 2009). In this study, 2-DE results confirmed that the level of this protein is higher in EC than in NEC (Fig. 4). In addition to *tubulin*, *actin* is also used as a reference gene in gene quantitative expression. More recently, *actin* was found to be associated with cell defense mechanisms against biotic and abiotic stress. Malerba et al. (2008) found that depolymerization of the actin cytoskeleton acts as a downstream regulatory factor to adapt to stress and trigger the execution of programmed cell death. In our study, actin (spot 694) up-regulation in NEC may be associated with programmed cell death.

### Metabolism and energy

During the process of calli induction, hormones are inducers imposed on the explant, which may cause plant cells to undergo reprogramming of their metabolism and energy consumption, especially carbon and nitrogen metabolism (Fehér et al. 2003). About 48.28 % of the identified proteins differentially expressed in EC with respect to NEC were associated with metabolism and



energy. Of the 14 differentially expressed proteins identified in this group, eight were identified to be involved in carbon metabolism, three proteins are associated with nitrogen metabolism and the remaining protein is associated with energy.

Protein spots 121, 431, 518, 634, 691 and 931 are involved in metabolic pathways of glycolysis. Spot 121 is a cytoplasmic fructose-bisphosphate aldolase and it catalyzes the conversion of fructose-1, 6-diphosphate to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate, whose expression levels are lower in EC than NEC, as observed in the 2-DE gels. Fructose-bisphosphate aldolase is expressed in high abundance in the endosperm during *Lepidium sativum* L. germination (Müller et al. 2010). Massonneau et al. (2005) showed that NEC express endosperm genes and have a transitory life that aids the EC to express embryo genes and differentiate into embryos. According to these findings, the up-regulation of this protein in NEC may be essential in the formation of EC. Additionally, glyceraldehyde-3-phosphate dehydrogenase (spots 518, 634 and 691) was detected and the expression of the proteins associated with these spots was down-regulated in EC. Glyceraldehyde-3-phosphate dehydrogenase is an important enzyme in the glycolysis pathway that catalyzes key steps and participates in providing energy in the form of NADPH to the cytosol. In grape, the expression level of glyceraldehyde-3-phosphate dehydrogenase increases significantly in EC (Zhang et al. 2009), and Bustos et al. (2008) reported that the transcription level of glyceraldehyde-3-phosphate dehydrogenase was up-regulated by H<sub>2</sub>O<sub>2</sub> treatment. However, our results found the expression of glyceraldehyde-3-phosphate dehydrogenase is in contrast with previous studies and requires further investigation. We also identified a cytosolic malate dehydrogenase (spot 615) that is involved in gluconeogenesis. This enzyme catalyzes the conversion of oxaloacetate to malate, and then accomplishes the malate shuttle between the mitochondria and the cytosol. In our study, this protein was down-regulated in EC with respect to NEC.

Glutamine synthase (spot 692) involved in ammonia assimilation was identified and was up-regulated in EC. GS catalyzes the conversion of glutamic acid and ammonia to glutamine. Glutamate plays a central role in amino acid metabolism and is positioned at a 'cross-road' between carbon and nitrogen metabolism in higher plants (Forde and Lea 2007). Glutamine is not only used to assimilate ammonia, but can also be used to eliminate high concentrations of ammonia poisoning. Glutamine can also be used as a donor for glutamate ammonia synthesis. Moreover, transaminase (spot 790) involved in metabolic ammonia detoxification with glutamate and amino acids synthesis was detected in NEC. Renault et al. (2010) reported that GABA ( $\gamma$ -aminobutyric acid) transaminase was up-

regulated overall in response to NaCl in *Arabidopsis*, and found that it plays a pivotal functional role that acts in response to salt and is linked to N and C metabolism in roots. Surabhi et al. (2008) found aspartate transaminase activity increased in leaves of a susceptible mulberry under salt stress conditions. Thus, GS and transaminase may be associated with nitrogen metabolism in NEC.

The enzyme 1, 4-alpha-glucan glucanhydrolase (spot 684) was also detected. This enzyme plays a role in degrading starch into maltose and glucose for glycolysis. Alpha-amylase is one of the most important enzymes involved in rice callus differentiation, which has high levels of expression of the alpha-amylase gene in rice calli, and is under-expressed after transfer to a regeneration medium (Yin et al. 2007). However, in the current study, it was down-regulated in EC. This reason may be attributed to a genotype difference, because different varieties have different induction capacities (Filippov et al. 2006; Visarada et al. 2002).

Moreover, we identified an *S*-adenosylmethionine synthase (SAMS) (spot 762) and it was up-regulated in EC. SAMS catalyzes the formation of *S*-adenosylmethionine from Met and ATP (Horikawa et al. 1990). Raveland et al. (1998) found that adenosylmethionine is associated with several reactions that are essential for plant growth and development. Overexpression of the *Suadea SalsaS-adenosylmethionine synthase* gene in transgenic tobacco is more tolerant to salt stress than wild-type plants (Qi et al. 2010). In this study, 2,4-D imposed on immature embryos may induce the expression of SAMS to defend against stress. ATP synthase is a ubiquitous enzyme in oxidative stress, and in this condition, expression of the  $\alpha$  and/or  $\beta$  subunits of the ATP synthase has been found to be suppressed (Sweetlove et al. 2002). In our proteomic analysis, down-regulation of the  $\beta$  subunit of ATP synthase (spots 884 and 940) was observed in EC. This observation may indicate considerable damage to this subunit. A similar result was found for the  $\alpha$  subunit of ATP synthase, which was absent in grape EC gels (Zhang et al. 2009).

#### Transcription and protein processing

With observed changes in physiology and metabolism during cell reprogramming, transcriptional activity of associated genes and newly synthesized proteins is required. Glycine-rich RNA-binding protein (spots 12, 30) was detected in the EC gels, and this protein participates in post-transcriptional gene regulation. Under cold stress conditions, the mRNA levels of this protein increase (Kim et al. 2005), and a high *glycine-rich RNA-binding protein* gene expression level in EC was also found under conditions of higher oxidative stress (Zhang et al. 2009). We identified three protein synthesis-related proteins (histone

H2B.2, spot 3; 40S ribosomal protein, spot 665; protein disulfide isomerase (PDI), spot 1076) in the EC gels. These results are in agreement with the large number of proteins synthesized during the formation of calli. Histones are the archetypical class of chromatin regulatory proteins that play a role in determining a complex variety of reversible post-translational modifications (Eliuk et al. 2010). The eukaryotic 40S ribosomal protein plays a central role in protein synthesis, which binds initiation factors that facilitate the scanning of messenger RNAs and initiation of protein synthesis (Rabl et al. 2011). Protein disulfide isomerase (PDI) can catalyze disulfide bond formation and the isomerization, and acts as a chaperone that inhibits aggregation (Wilkinson and Gilbert 2004). In the process of protein maturation, disulfide bonds that chemically cross-link specific cysteines are often added to stabilize a protein or to join it covalently to other proteins. Thus, these proteins may play a significant role in the process of EC.

### Signal transduction

One spot (840) corresponding to the signal transduction subunit, 26S proteasome regulatory particle triple-A ATPase subunit5b, was up-regulated in EC at both the transcriptional and translational levels. In plants, the ubiquitin–proteasome system can regulate nearly every aspect of growth and development, such as the cell-cycle, embryogenesis, defense, environmental responses and hormone signaling (Vierstra 2009). However, in this system, ubiquitin is covalently attached to target proteins and regulates their function by the multisubunit 26S proteasome, which is an ATP dependent protease that can degrade polyubiquitinated proteins (Voges et al. 1999). Brukhin et al. (2005) reported that the regulatory particle of the 26S proteasome is encoded by two paralogous genes, *RPN1a* and *RPN1b*, and disruption of *RPN1a* may cause embryo lethality, while *RPN1b* mutants showed no obvious abnormal phenotypes. However, complementation of the *rpn1a* mutation with the coding region of *RPN1b* expressed under the control of the *RPN1a* promoter indicates that the two RPN1 isoforms are functionally equivalent, which indicates that *RPN1* is essential during embryogenesis. Therefore, based on the above researching, further studying the functions of the spot 840 may provide new insights to shed light on the molecular mechanism of maize embryogenic calli formation.

### Conclusions

A high-resolution 2-DE proteome map was generated and this provided valuable information on the different protein expression levels in EC and NEC of maize. Analysis using MS showed that somatic embryogenesis undergoes a

complex process, and a variety of proteins were identified. This included proteins involved in cell proliferation, transcription and protein processing, metabolism and stress. Further functional analysis of these differentially expressed proteins showed that stress related proteins may play an important role in SE. 2, 4-D probably causes an oxidative burst and may represent a key factor for obtaining embryogenic competence of callus cells in plant cultures, whereas the ROS-scavenging system will remove excessive reactive oxygen species to protect the culturing cells from toxicity by means of APX. According to this, we hypothesize that auxin and stress signaling may restart somatic cell reprogramming and division, and this leads to the differential expression of proteins associated with transcription and metabolism. Currently, our results provide a better understanding of the formation of the SE in maize and the results provide insight into the possibility of improving SE of this species in an in vitro culture.

**Acknowledgments** This research was supported by the National S&T Major Projects—Breeding of New Varieties for Transgenic Biology of China (2009ZX08003-024B).

### References

- Ahsan N, Lee DG, Lee KW, Alam I, Lee SH, Bahk JD, Lee BH (2008) Glyphosate-induced oxidative stress in rice leaves revealed by proteomic approach. *Plant Physiol Biochem* 46:1062–1070
- Asada K (1992) Ascorbate peroxidase—a hydrogen peroxide-scavenging enzyme in plants. *Physiol Plant* 85:235–241
- Binott JJ, Songa JM, Ininda J, Njagi EM, Machuka J (2008) Plant regeneration from immature embryos of Kenyan maize inbred lines and their respective single cross hybrids through somatic embryogenesis. *Afr J Biotech* 7:981–987
- Boutillier K, Offringa R, Sharma VK, Kieft H, Ouellet T, Zhang L, Hattori J, Liu CM, van Lammeren AAM, Miki BLA, Custers JBM, van Lookeren Campagne MM (2002) Ectopic expression of *BABY BOOM* triggers a conversion from vegetative to embryogenic growth. *Plant Cell* 14:1737–1749
- Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein dye binding. *Anal Biochem* 72:248–254
- Brettschneider R, Becker D, Lörz H (1997) Efficient transformation of scutellar tissue of immature maize embryos. *Theor Appl Genet* 94:737–748
- Brukhin V, Gheyselinck J, Gagliardini V, Genschik P, Grossniklaus U (2005) The RPN1 subunit of the 26S proteasome in *Arabidopsis* is essential for embryogenesis. *Plant Cell* 17:2723–2737
- Busch W, Benfey PN (2010) Information processing without brains—the power of intercellular regulators in plants. *Development* 137:1215–1226
- Bustos DM, Bustamante CA, Iglesias AA (2008) Involvement of non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase in response to oxidative stress. *J Plant Physiol* 165:456–461
- Cangahuala-Inocente GC, Villarino A, Seixas D, Dumas-Gaudot E, Terenzi H, Guerra MP (2009) Differential proteomic analysis of developmental stages of *Acca sellowiana* somatic embryos. *Acta Physiol Plant* 31:501–514

- Casacuberta JM, Raventós D, Puigdoménech P, San Segundo B (1992) Expression of the gene encoding the PR-like protein *PRms* in germinating maize embryos. *Mol Gen Genet* MGG 234:97–104
- Casson S, Spencer M, Walker K, Lindsey K (2005) Laser capture microdissection for the analysis of gene expression during embryogenesis of *Arabidopsis*. *Plant J* 42:111–123
- Che P, Love TM, Frame BR, Wang K, Carriquiry AL, Howell SH (2006) Gene expression patterns during somatic embryo development and germination in maize Hi II callus cultures. *Plant Mol Biol* 62:1–14
- Chen SX, Harmon AC (2006) Advances in plant proteomics. *Proteomics* 6:5504–5516
- Chu CC, Wang CC, Sun CS, Hus C, Yin KC, Chu CY, Bi FY (1975) Establishment of an efficient medium for another culture of rice through comparative experiments on nitrogen source. *Sci Sin* 18:659–668
- Eliuk SM, Maltby D, Panning B, Burlingame AL (2010) High resolution electron transfer dissociation studies of unfractionated intact histones from *Murine* embryonic stem cells using on-line capillary LC separation. *Mol Cell Proteomics* 9:824–837
- Fehér A, Pasternak TP, Dudits D (2003) Transition of somatic plant cells to an embryogenic state. *Plant Cell Tissue Organ Cult* 74:201–228
- Filippov M, Miroshnichenko D, Vernikovskaya D, Dolgov S (2006) The effect of auxins, time exposure to auxin and genotypes on somatic embryogenesis from mature embryos of wheat. *Plant Cell Tissue Organ Cult* 84:213–222
- Forde BG, Lea PJ (2007) Glutamate in plants: metabolism, regulation and signalling. *J Exp Bot* 58:2339–2358
- Ganesan M, Jayabalan N (2004) Evaluation of haemoglobin (erythrogen): for improved somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum* L. cv. SVPR 2). *Plant Cell Rep* 23:181–187
- Hanfrey C, Fife M, Buchanan-Wollaston V (1996) Leaf senescence in *Brassica napus*: expression of genes encoding pathogenesis related proteins. *Plant Mol Biol* 30:597–609
- Henderson CB (1976) Maize research and breeders manual, no. 8. Illinois Foundation Seeds Inc, Champaign
- Henderson JT, Li HC, Rider SD, Mordhorst AP, Romero-Severson J, Cheng JC, Robey J, Sung ZR, De Vries SC, Ogas J (2004) *PICKLE* Acts throughout the plant to repress expression of embryogenic traits and may play a role in gibberellin-dependent responses. *Plant Physiol* 134:995–1005
- Horikawa S, Sasuga J, Shimizu K, Ozasa H, Tsukada K (1990) Molecular cloning and nucleotide sequence of cDNA encoding the rat kidney S-adenosylmethionine synthetase. *J Biol Chem* 265:13683–13686
- Imin N, Nizamidin M, Daniher D, Nolan KE, Rose RJ, Rolfe BG (2005) Proteomic analysis of somatic embryogenesis in *Medicago truncatula*. Explant cultures grown under 6-benzylaminopurine and 1-naphthaleneacetic acid treatments. *Plant Physiol* 137:1250–1260
- Jiménez VM, Bangerth F (2001) Hormonal status of maize initial explants and of the embryogenic and non-embryogenic callus cultures derived from them as related to morphogenesis in vitro. *Plant Sci* 160:247–257
- Kim YO, Kim JS, Kang H (2005) Cold-inducible zinc finger-containing glycine-rich RNA-binding protein contributes to the enhancement of freezing tolerance in *Arabidopsis thaliana*. *Plant J* 42:890–900
- Ledwoń A, Gaj MD (2009) *LEAFY COTYLEDON2* gene expression and auxin treatment in relation to embryogenic capacity of *Arabidopsis* somatic cells. *Plant Cell Rep* 28:1677–1688
- Lin HC, Morcillo F, Dussert S, Tranchant-Dubreuil C, Tregear JW, Tranbarger TJ (2009) Transcriptome analysis during somatic embryogenesis of the tropical monocot *Elaeis guineensis*: evidence for conserved gene functions in early development. *Plant Mol Biol* 70:173–192
- 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
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the  $2^{-\Delta\Delta CT}$  method. *Methods* 25:402–408
- Malerba M, Contran N, Tonelli M, Crosti P, Cerana R (2008) Role of nitric oxide in actin depolymerization and programmed cell death induced by fusicoccin in sycamore (*Acer pseudoplatanus*) cultured cells. *Physiol Plant* 133:449–457
- Maraschin SF, Priester W, Spaink HP, Wang M (2005) Androgenetic switch: an example of plant embryogenesis from the male gametophyte perspective. *J Exp Bot* 56:1711–1726
- 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
- Massonneau A, Coronado MJ, Audrana A, Bagniewska A, Mòl R, Testillano PS, Goralskid G, Dumas C, Riusueño MC, Matthys-Rochon E (2005) Multicellular structures developing during maize microspore culture express endosperm and embryogenic genes and show different embryogenic potentialities. *Eur J Cell Bio* 84:663–675
- Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci* 7:405–410
- Moiseyev GP, Fedoreyeva LI, Zhuravlev YN, Yasnetskaya E, Jekel PA, Beintema JJ (1997) Primary structures of two ribonucleases from ginseng calluses. New members of the PR-10 family of intracellular pathogenesis-related plant proteins. *FEBS Lett* 407:207–210
- Müller K, Job C, Belghazi M, Job D, Leubner-Metzger G (2010) Proteomics reveal tissue-specific features of the cress (*Lepidium sativum* L.) endosperm cap proteome and its hormone-induced changes during seed germination. *Proteomics* 10:406–416
- Mur LA, Sturgess FJ, Farrell GG, Draper J (2004) The AoPR10 promoter and certain endogenous *PR10* genes respond to oxidative signals in *Arabidopsis*. *Mol Plant Pathol* 5:435–451
- Natarajan S, Xu C, Caperna T, Garrett W (2005) Comparison of protein solubilization methods suitable for proteomic analysis of soybean seed proteins. *Anal Biochem* 342:214–220
- Ogas J, Kaufmann S, Henderson J, Somerville C (1999) *PICKLE* is a CHD3 chromatin-remodeling factor that regulates the transition from embryogenic to vegetative development in *Arabidopsis*. *Proc Natl Acad Sci USA* 96:13839–13844
- Pan ZY, Guan R, Zhu SP, Deng XX (2009) Proteomic analysis of somatic embryogenesis in valencia sweet orange (*Citrus sinensis* Osbeck). *Plant Cell Rep* 28:281–289
- Pérez-Núñez MT, Souza R, Sáenz L, Chan JL, Zúñiga-Aguilar JJ, Oropeza C (2009) Detection of a *SERK*-like gene in coconut and analysis of its expression during the formation of embryogenic callus and somatic embryos. *Plant Cell Rep* 28:11–19
- Ptak A, El Tahchy A, Wyzgolik G, Henry M, Laurain Mattar D (2010) Effects of ethylene on somatic embryogenesis and galanthamine content in *Leucojum aestivum* L. cultures. *Plant Cell Tissue Organ Cult* 102:61–67
- Qi YC, Wang FF, Zhang H, Liu WQ (2010) Overexpression of *Suadea SalsaS-adenosylmethionine synthetase* gene promotes salt tolerance in transgenic tobacco. *Acta Physiol Plant* 32:263–269
- Rabl J, Leibundgut M, Ataíde SF, Haag A, Ban N (2011) Crystal structure of the eukaryotic 40S ribosomal subunit in complex with initiation factor 1. *Science* 331:730–736
- Ravanel S, Gakière B, Job D, Douce R (1998) The specific features of methionine biosynthesis and metabolism in plants. *Proc Natl Acad Sci USA* 95:7805–7812

- Renault H, Roussel V, El Amrani A, Arzel M, Renault D, Bouchereau A, Deleu C (2010) The *Arabidopsis* pop2-1 mutant reveals the involvement of GABA transaminase in salt stress tolerance. *BMC Plant Biol* 10:20
- Schlögl PS, Santos ALW, Vieira LN, Floh EIS, Guerra MP (2012) Gene expression during early somatic embryogenesis in Brazilian pine (*Araucaria angustifolia* (Bert) O. Ktze). *Plant Cell Tissue Organ Cult* 108:173–180
- Sghaier-Hammami B, Drira N, Jorrín-Novo JV (2009) Comparative 2-DE proteomic analysis of date palm (*Phoenix dactylifera* L.) somatic and zygotic embryos. *Proteomics* 73:161–177
- Shohaeh AM, Ali MB, Hahn EJ (2007) Glutathione metabolism and antioxidant responses during *Eleutherococcus senticosus* somatic embryo development in a bioreactor. *Plant Cell Tissue Organ Cult* 89:121–129
- Steiner N, Santa-Catarina C, Guerra MP, Cutri L, Dornelas MC, Floh EIS (2012) A gymnosperm homolog of *SOMATIC EMBRYO-GENESIS RECEPTOR-LIKE KINASE-1* (*SERK1*) is expressed during somatic embryogenesis. *Plant Cell Tissue Organ Cult* 109:41–50
- Stone SL, Braybrook SA, Paula S, Kwon LW, Meuser J, Pelletier J, Hsieh TF, Fischer RL, Goldberg B, Harada JJ (2008) *Arabidopsis* *LEAFY COTYLEDON2* induces maturation traits and auxin activity: implications for somatic embryogenesis. *Proc Natl Acad Sci USA* 105:3151–3156
- Su YH, Zhao XY, Liu YB, Zhang CL, O'Neill SD, Zhang XS (2009) Auxin-induced *WUS* expression is essential for embryogenic stem cell renewal during somatic embryogenesis in *Arabidopsis*. *Plant J* 59:448–460
- Surabhi GK, Reddy AM, Jyothsnakumari G, Sudhakar C (2008) Modulation of key enzymes of nitrogen metabolism in two genotypes of mulberry (*Morus alba* L.) with differential sensitivity to salt stress. *Environ Exp Bot* 64:171–179
- Sweetlove LJ, Heazlewood JL, Herald V, Holtzapffel R, Day DA, Leaver CJ, Millar AH (2002) The impact of oxidative stress on *Arabidopsis* mitochondria. *Plant J* 32:891–904
- Tan EC, Karsani SA, Foo GT, Wong SM, Rahman NA, Khalid N, Othman S, Yusof R (2012) Proteomic analysis of cell suspension cultures of *Boesenbergia rotunda* induced by phenylalanine: identification of proteins involved in flavonoid and phenylpropanoid biosynthesis pathways. *Plant Cell Tissue Organ Cult* 111:219–229
- Terrier N, Glissant D, Grimplet J, Barriau F, Abbal P, Couture C, Ageorges A, Atanassova R, Léon C, Renaudin JP, Dedaldecamp F, Romieu C, Delrot S, Hamdi S (2005) Isogene specific oligo arrays reveal multifaceted changes in gene expression during grape berry (*Vitis vinifera* L.) development. *Planta* 222:832–847
- van Eldik GJ, Wingens M, Ruiter RK, Van Herpen MMA, Schrauwen JAM, Wullems GJ (1996) Molecular analysis of a pistil-specific gene expressed in the stigma and stylar cortex of *Solanum tuberosum*. *Plant Mol Biol* 30:171–176
- Vierstra RD (2009) The ubiquitin-26S proteasome system at the nexus of plant biology. *Nat Rev Mol Cell Biol* 10:385–397
- Visarada KBR, Sailaja M, Sarma NP (2002) Effect of callus induction media on morphology of embryogenic calli in rice genotypes. *Bio Life Sci* 45:495–502
- Voges D, Zwickl P, Baumeister W (1999) The 26S proteasome: a molecular machine designed for controlled proteolysis. *Ann Rev Biochem* 68:1015–1068
- Wang J, Weng ZX, Cheng CL, Liu H, Liang WY, Jiang JM, Chen W (2010) Identification and analysis of differentially expressed proteins during cotyledon embryo stage in longan. *Sci Hortic* 126:426–433
- Wilkinson B, Gilbert HF (2004) Protein disulfide isomerase. *BBA-Protein Proteom* 1699:35–44
- Williams EG, Maheswaran G (1986) Somatic embryogenesis: factors influencing coordinated behaviour of cells as an embryogenic group. *Ann Bot* 57:443–462
- Winkelmann T, Heintz D, Dorsseleer AV, Serek M, Braun HP (2006) Proteomic analyses of somatic and zygotic embryos of *Cyclamen persicum* Mill. reveal new insights into seed and germination physiology. *Planta* 224:508–519
- Wu XM, Li FG, Zhang CJ, Liu CL, Zhang XY (2009) Differential gene expression of cotton cultivar CCRI24 during somatic embryogenesis. *J Plant Physiol* 166:1275–1283
- Yin L, Tao Y, Zhao K, Shao J, Li XB, Liu GZ, Liu SQ, Zhu LH (2007) Proteomic and transcriptomic analysis of rice mature seed-derived callus differentiation. *Proteomics* 7:755–768
- Zhang JW, Ma HQ, Chen S, Ji M, Perl A, Kovacs L, Chen SW (2009) Stress response proteins' differential expression in embryogenic and non-embryogenic callus of *Vitis vinifera* L. cv. *Cabernet Sauvignon*—A proteomic approach. *Plant Sci* 177:103–113
- Zhang SZ, Liu XG, Lin YA, Xie GN, Fu FL, Liu HL, Wang J, Gao SB, Lan H, Rong TZ (2010) Characterization of a *ZmSERK* gene and its relationship to somatic embryogenesis in a maize culture. *Plant Cell Tissue Organ Cult* 105:29–37
- Zimmerman JL (1993) Somatic embryogenesis: a model for early development in higher plant. *Plant Cell* 5:1411–1423