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ORIGINAL ARTICLE

Proteome study of somatic embryogenesis in *Nothapodytes nimmoniana* (J. Graham) Mabberly

Tasiu Isah[1]

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Abstract

Somatic embryogenesis (SE) is the most suitable biotechnological tool for the rapid clonal propagation of endangered woody plants, but many bottlenecks limit understanding its molecular and physiological processes in *Nothapodytes nimmoniana*. Combinations of two-dimensional electrophoresis (2-DE) and mass spectrometry (MaSp) were used to study proteomic expression changes during SE of the forest tree. Callus was induced from mature seed embryos, and embryogenic callus (EC) obtained at very low frequency after about 6 month culture. Globular embryos were induced from the seed embryo-derived EC and the subsequent stages of the SE. Analysis of the extracted proteins from globular, heart/torpedo-shaped, and maturing embryo stages resolved in the 2-DE gels showed increased protein expression across developmental stages of the somatic embryos. The mass spectrometric analysis with database search aided identification of 55 out of 100 and 54 selected protein spots. Identified proteins classified by the cellular role which they perform are involved in aspects of stress responses, energy metabolism, carbon fixation, secondary metabolism, and other metabolic functions, while three proteins are of unknown cellular role. The putative role of the expressed proteins during SE provided insight into the physiology of somatic embryo development in *N. nimmoniana*.

Keywords *Nothapodytes nimmoniana* · Camptothecin · Somatic embryogenesis · Proteomics · Two-dimensional electrophoresis · Mass spectrometry

Abbreviations

SE	Somatic embryogenesis
EC	Embryogenic callus
2-DE	Two-dimensional electrophoresis
MS	Murashige and Skoog medium
2,4-D	2,4-Dichlorophenoxy acetic acid
NAA	Naphthalene acetic acid
IAA	Indole 3-acetic acid
BAP	Benzylaminopurine
GA_3	Gibberellic acid
DTT	Dithiothreitol
IEF	Isoelectric focusing
NCBInr	National Center for Biotechnology
MaSp	Mass spectrometry
NAD^+	Nicotinamide adenine dinucleotide
ROS	Reactive oxygen species
CPT	Camptothecin
GE	Globular embryos
TH	Heart/torpedo-shaped embryos
ME	Mature/maturing embryos
HPTLC	High-performance thin-layer chromatography

Introduction

Somatic embryogenesis (SE) is an in vitro developmental program through which somatic cells generate embryogenic cells that can undergo morphological and biochemical changes to produce somatic embryos capable of regenerating plant (Zimmerman 1993; Quiroz-Figueroa et al. 2006). In dicotyledonary plants, the program involves globular, heart, torpedo-shaped, and cotyledonary stages (Zimmerman 1993). In the early years, studies on the SE of forest trees were on physiological aspects with view to optimize culture conditions for mass propagation and commercial

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✉ Tasiu Isah
taasmore@yahoo.co.uk

[1] Department of Botany, School of Chemical and Life Sciences, Hamdard University, New Delhi 110 062, India

applications. However, efforts have shifted towards understanding the molecular aspects during the last few decades (Isah 2016a). In the recent, emphasis is on describing the program at molecular levels through studying the biological processes that occur during its developmental stages by the use of molecular approaches (Abbasi et al. 2016; Isah 2016a). Proteomic expression changes are among the employed molecular approaches that have advanced the understandings of the structure, genetic organization, and evolution of plant genome (Manjasetty et al. 2012; Agrawal et al. 2013; Gantait et al. 2014). It provides insight into physiological and biochemical changes associated with somatic embryo development as a reflection of molecular changes involved, and have applications in optimizing culture conditions for mass production at commercial scale (Iraqi and Tremblay 2001a; Chen and Harmond 2006; Agrawal et al. 2013).

Nothapodytes nimmoniana is an endangered forest tree and most convenient source for the large-scale isolation of camptothecin (CPT) due to the higher yield over the other plant sources of the alkaloid. In recent years, there has been an upsurge in demand of the chemical by the pharmaceutical industry. This led to an indiscriminate exploitation of *N. nimmoniana* forest for isolation of the anticancer alkaloid to meet demand of the chemical in production of its analog drugs (Padmanabha et al. 2006; Suhas et al. 2007; Ramesha et al. 2008). Considering the state of the exploitation for isolation of CPT and the need for 7–8 years for its seedlings to reach reproductive stage, biotechnological approaches offer alternative strategy for mass production of the clones (Isah and Mujib 2015a, b; Ali et al. 2016; Prakash et al. 2016; Isah 2015a, 2017; Isah and Umar 2018; Isah et al. 2018). The approach could be employed in the scale-up production of CPT, so as to mitigate indiscriminate harvest of endangered natural population for isolation of the alkaloid (Isah and Mujib 2015a, b). SE is the most suitable biotechnological tool having application in the large-scale production to meet demand of the chemical by the pharmaceutical industry (Lutz et al. 1985; Gupta et al. 1991; Mujib et al. 2014, 2016). However, response to the production of the embryos in the in vitro cultures of *N. nimmoniana* is poor. Earlier studies have employed the molecular approach of proteomic expression changes in studying physiological metabolic changes associated with the development of in vitro embryos through the various stages in woody plants, with applications in optimizing culture conditions for mass production at commercial scale (Lippert et al. 2005; Pan et al. 2009; Noah et al. 2013). However, the reported studies on the SE in *N. nimmoniana* are few (Fulzele and Satdive 2003; Khadke and Kuvalekar 2013) despite potential application which it offers in the scale-up production of CPT. In this study, to gain physiological molecular insight about the somatic embryo development in *N. nimmoniana*, two-dimensional electrophoresis (2-DE) and mass spectrometry (MaSp) were employed to study proteomic expression changes during the SE. It is the first molecular and proteomic study of SE in the species. Among bottlenecks in understanding the molecular, physiological, developmental processes in *N. nimmoniana* and related species having poorly known genomic data in the bioinformatics databases are the deficiency in genomic information, as encountered in the present study. The results obtained have provided insight into metabolic changes involved in the in vitro embryogenesis in *N. nimmoniana* across the various developmental stages.

Materials and methods

Cultures establishment and conditions

Authenticated seed samples were obtained from Sanjivini herbals Tamil Nadu India, mature seed embryos excised from the seeds using the strategy reported earlier (Isah and Mujib 2015c). However, only seed embryo-derived callus cultures from single seed source-derived tissue were used for the proteomic expression study. Callus was induced from the isolated seed embryo explants on solid Murashige and Skoog (1962) medium (MS) amended with 2,4-dichlorophenoxy acetic acid (2,4-D) + benzylaminopurine (BAP) (13.56 + 2.22 µM). The induced callus cultures were maintained on medium amended with 2,4-D + BAP (9.04 + 4.44 µM) through regular subculture after every 3–4 weeks for up to 6 months before embryogenic tissue was produced. Globular embryos were induced from the EC when transferred to MS medium amended with BAP + 2,4-D (4.44, 6.66 or 8.88 + 2.26 µM), or plant growth regulators (PGRs)-free. The induced embryos progressed through heart-torpedo-shaped stages when transferred to medium supplemented with BAP (2.22 µM) followed by the early stage maturation on medium amended with gibberellic acid (GA_3) (5.20 µM).

In the present study, the culture medium contained 3% sucrose and media pH adjusted to 5.6–5.8 using 1N NaOH or HCl. The media were solidified with 8% Agar (Agar Agar Microbiology Mumbai, India) before autoclaved at 121 °C for 21 min. All of the cultures were maintained under the culture room conditions of 25 ± 2 °C temperatures, 50–70% relative humidity, and 16 h photoperiod provided by cool fluorescent tubes (Phillip India) having illuminations of 40 W, 50 µmol m^{-2} s^{-1} PFD. For protein extraction, somatic embryos at the heart/torpedo-shaped stages were taken as single embryo mass sample as the stages proved difficult to distinguish while separating samples for the embryos at globular and maturation stages.

Alkaloid extraction and high-performance thin-layer chromatographic analysis

CPT extraction was carried out by the use of microwave extraction method reported by Fulzele and Satdive (2005), while high-performance thin-layer chromatographic (HPTLC) analysis by the modified method of Kulkarni et al. (2010). Briefly, 20 µl of the dried embryo tissue samples extracts (globular, heart/torpedo-shaped, and maturing embryos) were loaded on an HPTLC plate (Merck silica gels) using an automated sample loading system, and then allowed to dry. The loaded samples were developed in a glass chamber saturated with ethyl acetate: toluene (7:3) mobile phase solvent system. The obtained chromatographs were scanned using Camag 3 densitometric scanner and the peaks, peak area, and Rf of the spots recorded. A pure reference sample (CPT standard) was procured from Sigma-Aldrich (CAS No. 7689-03-4) and standard solution prepared by dissolving CPT powder in chloroform:methanol (4:1) solvent mixture. Fluorescence was recorded at 366 nm and Rf of the CPT standard evaluated. CPT yield of the embryos at the various stages of development was evaluated from a standard curve of the CPT reference standard drawn from five dilutions of the prepared standard stock solution after developed in a chromatographic chamber. The alkaloid was identified by the Rf values of 8.1–82, absorbance in the UV region of 366 nm, and in comparison with the standard curve having linear regression line equation $Y = 0.0018x + 7.2184$.

Protein extraction

Extraction of proteins from the globular, heart/torpedo-shaped, and maturing embryo tissue samples was carried out in triplicate sets through the use of phenol method reported by Isaacson et al. (2006). Briefly, 2 g of each of the samples were ground into fine powder in liquid nitrogen and the resultant powder suspended in 10 ml extraction buffer (pH 7.5) that contained 50 mM HEPES, 2% β-mercaptoethanol, 700 mM sucrose, 1 mM PMSF, 50 mM ethylenediaminetetraacetic acid (EDTA) and 100 mM KCl, and 15 ml phenol was added. The resultant solution was mixed in cold room rocker for 30 min at 4 °C and then centrifuged at 3000×*g* for 10 min at 4 °C. Upper phenolic phase was recovered in separate tube and 15 ml ice-cold 0.1 M ammonium acetate added to the contents before incubated at − 20 °C overnight for precipitation. The obtained proteins were pelleted by centrifugation at 6000×*g* for 15 min at 4 °C and the resultant pellet washed with methanol, followed by two rounds with acetone. The pellet was dissolved in methanol and then centrifuged at 3000×*g* and − 20 °C for 10 min after each of the wash steps. This was followed by drying in solubilization buffer that contained 2 M thiourea, 7 M urea, 4% CHAPS and 50 mM dithiothreitol (DTT). Resultant proteins in the samples were quantified using bovine serum albumin as standard (Bradford 1976).

Two-dimensional gel electrophoresis

The 2-DE was carried out by the method of O'Farrell (1975). Briefly, immobiline dry strip gels (17 cm, pH 3–10; Bio-rad) were rehydrated at 20 °C for 14 h in 200 µl of sample that contained 250 µg proteins. Isoelectric focusing was carried out in PROTEAN IEF apparatus (Bio-Rad). The voltages applied were 250 V for 1 h, 500 V for 1 h, 1000 V for 2 h, 2000 V for 2 h followed by a linear increase of 8000 V for 18 h and 500 V for 1 h. The strips were then subjected to reduction in buffer that contained 50 mM Tris (pH 8.8), 8 M urea, 20% glycerol, 2% SDS, and 130 mM DTT. This was followed by alkylation for 15 min in buffer that contained Tris (pH 8.8), 8 M urea, 20% glycerol, 2% SDS, and 135 mM iodoacetamide. The 2-DE was carried out in a Dodeca cell (Bio-rad PROTEAN Plus) to separate the focused proteins using 12% SDS at constant voltage of 200 V. Obtained 2-DE gels were stained with colloidal Coomassie brilliant blue dye followed by destain with milli-Q water. The experiments were performed in triplicate sets using the extracted protein samples. Resultant gels were scanned with Epson Scanner (GE Healthcare Bioscience Pvt Ltd., New Delhi, India) before stored in 10% acetic acid until further analysis.

Gel image analysis and peptide extraction

The 2-DE gel images were analyzed with the image processing software (2D Platinum). Pattern of the spot distribution within and between sample groups was studied by visualizing the 3D structure of each of the spots to identify proteins and non-proteins spots; non-protein spots were eliminated from the gels, while protein spots retained. To figure the significant differences between the sampled groups, spot intensity of the gel images bearing the protein spots was subjected to the one-way analysis of variance and abundance estimated from volume percentage. The spots were normalized within replicate sample groups, and gels matched through the match set. Only spots that showed significant and reproducible biological changes between replicate gels of the various embryonic stages with a fold change ≥ 1.5 and p value of 0.05 or less were considered differentially expressed and selected for the MaSp analysis. Selected spots were excised from the gels manually using a sharp blade and transferred into centrifuge tube individually. Excised gels were then rinsed with Milli-Q water three times and chopped into smaller pieces within the centrifuge tube. The gel pieces were destained with 40 mM ammonium bicarbonate followed by 40 and 100% acetonitrile. This was followed by rehydration with 5 mM DTT in ammonium bicarbonate and alkylation with 20 mM iodoacetamide in 40 mM ammonium

bicarbonate. In-gel digestion of the reduced spots was carried out using 12.5 ng/ml trypsin (modified sequencing grade trypsin–Promega, USA) in 50 mM ammonium bicarbonate by incubating the digested gels in an ice bath for 45 min. A volume of ammonium bicarbonate was added to the pieces and incubated overnight at 37 °C. The obtained peptides were extracted from gel pieces by incubation with formic acid (5%) for 10 min at 37 °C followed by the use of extraction buffer before the final extraction in 100% acetonitrile. The supernatant obtained (from the three steps) was pooled together and dried down by spinning the tubes in speed vac and the resultant residue stored at − 20 °C until MaSp analysis.

Mass spectrometry, database search, and functional categorization of the identified proteins

Peptides extracted from each of the spots were individually mixed with volume of saturated matrix solution, i.e 10 mg/ml α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich), in 50% acetonitrile (Merck)/0.1% trifluoroacetic acid (Sigma-Aldrich). It was then spotted on silver MALDI target plate and dried at room temperature. Mass spectral data of the sampled spots were obtained with MALDI MS-ABI Sciex 7000 TOF/TOF system (Framingham Massachusetts, USA) calibrated in positive reflective mode with an accelerating voltage of about 20 kv for MaSp mode. Molecular ions that displayed common signal were submitted to the MaSp/MaSp analysis using specific mode precursor ion fragment at laser frequency of 50 Hz. Resultant MaSp/MaSp spectra were acquired in positive reflective mode following collision-induced dissociation and the data analyzed with Flex Analysis 2.4 data explorer 4.5. Peptides MaSp/MaSp were analyzed using Mascot (Matrix Science, London, UK) with the parameters: product ion mass tolerance of 150 ppm, monoisotopic mass value, one mixed cleavage, and fragment mass tolerance of ± 0.3 Da with methionine oxidation and an iodoacetamide derivative of cysteine specified as variable and fixed modifications. The Mascot was set up to search the national center for biotechnology (NCBInr) database (http://www.ncbi.nlm.nih.gov/protein) with the parameters: taxonomy; Viridiplantae, assuming the use of digestion enzyme trypsin and variable modifications with up to two missed cleavages. Only significant hits defined through the MASCOT probability analysis ($p < 0.05$) and peptides identified with individual ion scores greater than 40 were considered as true identity. Putative cellular functional categorization of the identified protein spots was performed by the use of Kyoto encyclopedia of genes and genomes (KEGG) which is available (http://www.genome.jp/kegg/kegg2.html) combined with the Uniprot database (http://www.uniprot.org/proteomes/).

Results and discussion

SE offers alternative biotechnological strategy for the rapid clonal production of endangered forest trees that produce anticancer molecule CPT. However, the program occurs at low frequency in the in vitro cultures of woody plants, and limited information is known about the prevailing molecular and physiological processes (Guan et al. 2016). During its developmental stages, molecular changes are involved in the production and proliferation of embryo mass and understanding them is key to improvement of in vitro embryogenesis in *N. nimmoniana*. In the recent, there is increasing consideration towards improving culture conditions to optimize production of the embryos. In this study, to obtain somatic embryos, callus was induced from isolated mature seed embryos explant on solid MS medium amended with 2,4-D + BAP (12.06 + 4.44 µM) within 2–9 weeks culture (Fig. 1a–c). The regular subculture of the callus on the solid medium supplemented with 2,4-D + BAP (9.04 + 4.44 µM) after every 3–4 weeks for up to 6 months resulted in the formation of embryogenic tissue (Fig. 1c–e) at very low frequency. Initially, the callus cultures cultivated on the PGRs combinations became slightly friable and whitish, followed by compact and yellowish-brown appearance within the culture duration. From the EC, globular embryos were induced when the callus cultures were transferred to medium amended with BAP + 2,4-D (4.44, 6.66 or 8.88 + 2.26 µM), or without PGRs within 3–5 weeks culture (Fig. 1f, g). Nodular features were observed on the EC, which differentiated into globular embryos on medium amended with BAP (2.22 µM) at high frequency after 3 weeks culture over the other concentrations tested. The embryos formed in clusters of nodular aggregates at periphery of the EC showed slow development, appeared earlier on BAP + NAA (8.88 + 2.69 µM)-supplemented medium than the other PGRs tested. The induced globular embryos did not progress to the other stages when maintained on the induction medium, but, upon transferred to medium amended with BAP (2.22 µM), it passed through heart/torpedo-shaped stages within 4–7 weeks culture (Fig. 1h, i). This suggests that a reduction in BAP concentration is essential for the development of globular embryos to the other stages in *N. nimmoniana*. The produced embryos showed the early stage maturation on medium supplemented with GA_3 (5.20 µM) within 3–5 weeks culture (Fig. 1j), but the majority did not show adequate maturation for conversion into plantlets. In the present study, because the somatic embryos at the embryo maturation stage (cotyledonary stage) showed a lack of physiological maturity, they are referred to as "maturing embryos."

Proteomic expression study is among emerging biotechnological approaches having application in studies aimed

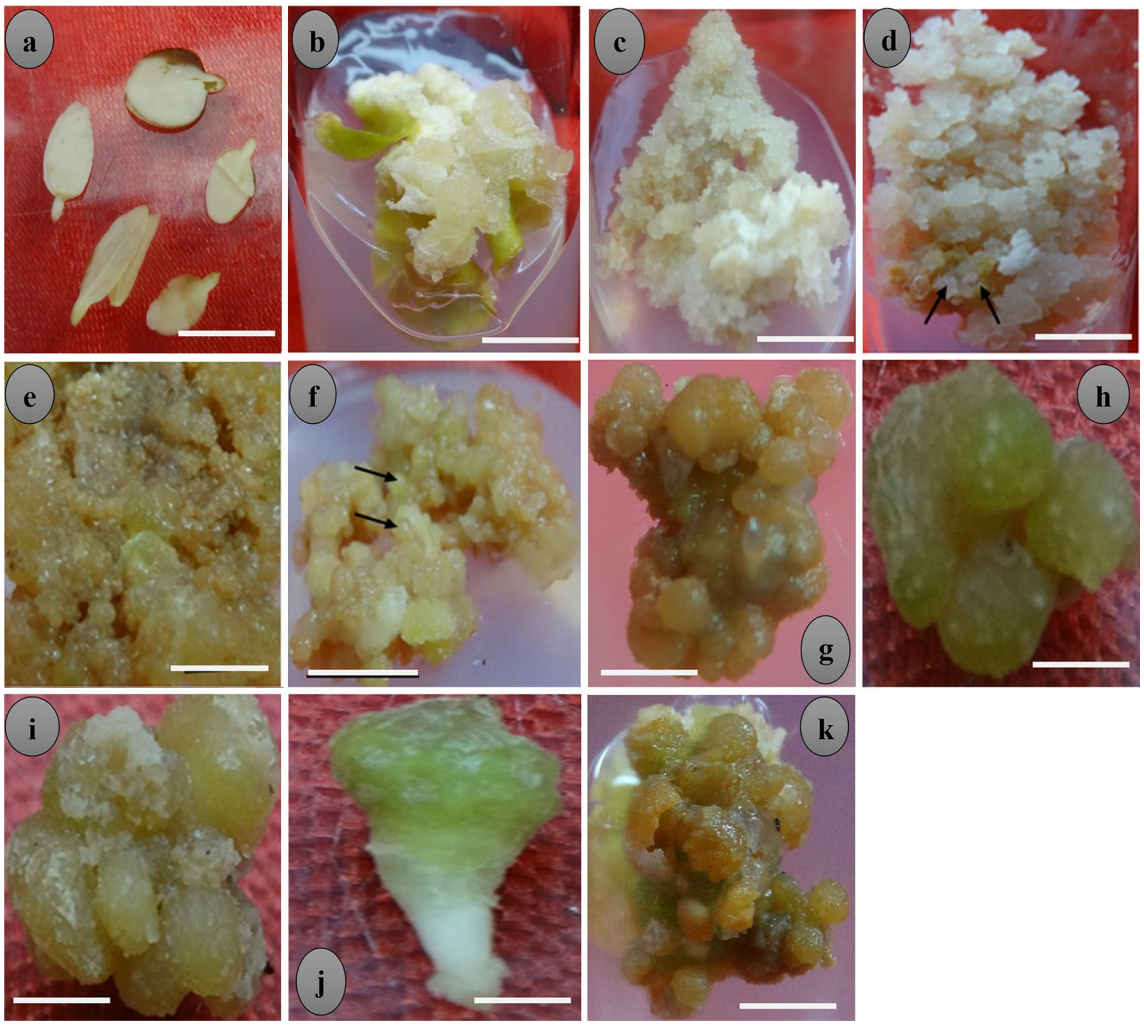


Fig. 1 **a** Isolated aseptic mature seed embryos explant, **b** cultured mature seed embryo explant on semi-solid MS medium added with 2,4-D + BAP (13.56 + 2.22 µM) showing callus induction, **c** proliferation of the callus induced from the seed embryos explant, **d** proliferated callus transition to the embryogenic callus (EC) after about 5–6 months culture, **e** obtained embryogenic tissue, **f** induction of globular embryos from the EC (arrowheads) on MS medium added with BAP (4.44 µM), **g** globular embryos induced on BAP (8.88 µM) amended medium, **h, i** heart/torpedo-shaped embryos obtained on medium added with BAP (2.22 µM), **j** maturing embryos on medium added with BAP (2.22 µM), and **k** maturing embryos showing the lack of physiological maturation on MS medium amended with GA_3 (5.20 µM). *Scale bar* (**a–d, f** and **k** = 1.0 cm, **e** and **g** = 0.7 cm, **h–j** = 0.45 cm)

at understanding physiological processes during in vitro embryogenesis in higher plants (Manjasetty et al. 2012). It could provide molecular insight into changes involved in the in vitro embryo development in endangered woody plants, with application in optimizing culture condition(s) for mass propagation and commercial application. To get some insight into the physiological changes involved in somatic embryo development in *N. nimmoniana*, proteins were extracted from the embryo tissue samples and 2-DE performed showed visible and reproducible various spots' distributions across developmental stages of the somatic embryos (Fig. 2). Analysis of the obtained 2-DE gel images resulted in the selection of 154 of the resolved protein spots that showed over 1.5 fold change in expression levels during at least one of the embryogenesis stages (GE, HT, and ME), out of which MaSp/MaSp combined with database homology search aided identification of 55 (electronic supplementary data). Because *N. nimmoniana* genome is not sequenced and limited bioinformatics data are available about related species in the NCBInr database, the homology search performed resulted in low identification success rate. In this study, the poor identification success rate was attributed to the low mascot score obtained with many of the processed proteins spots, particularly with the analyzed peptides matches/mass spectra of weak intensity which, in the overall, imply uncertainty about identity of the spots (Table 1; Fig. 3). This is a common problem in the proteomic study of plants that do not (or

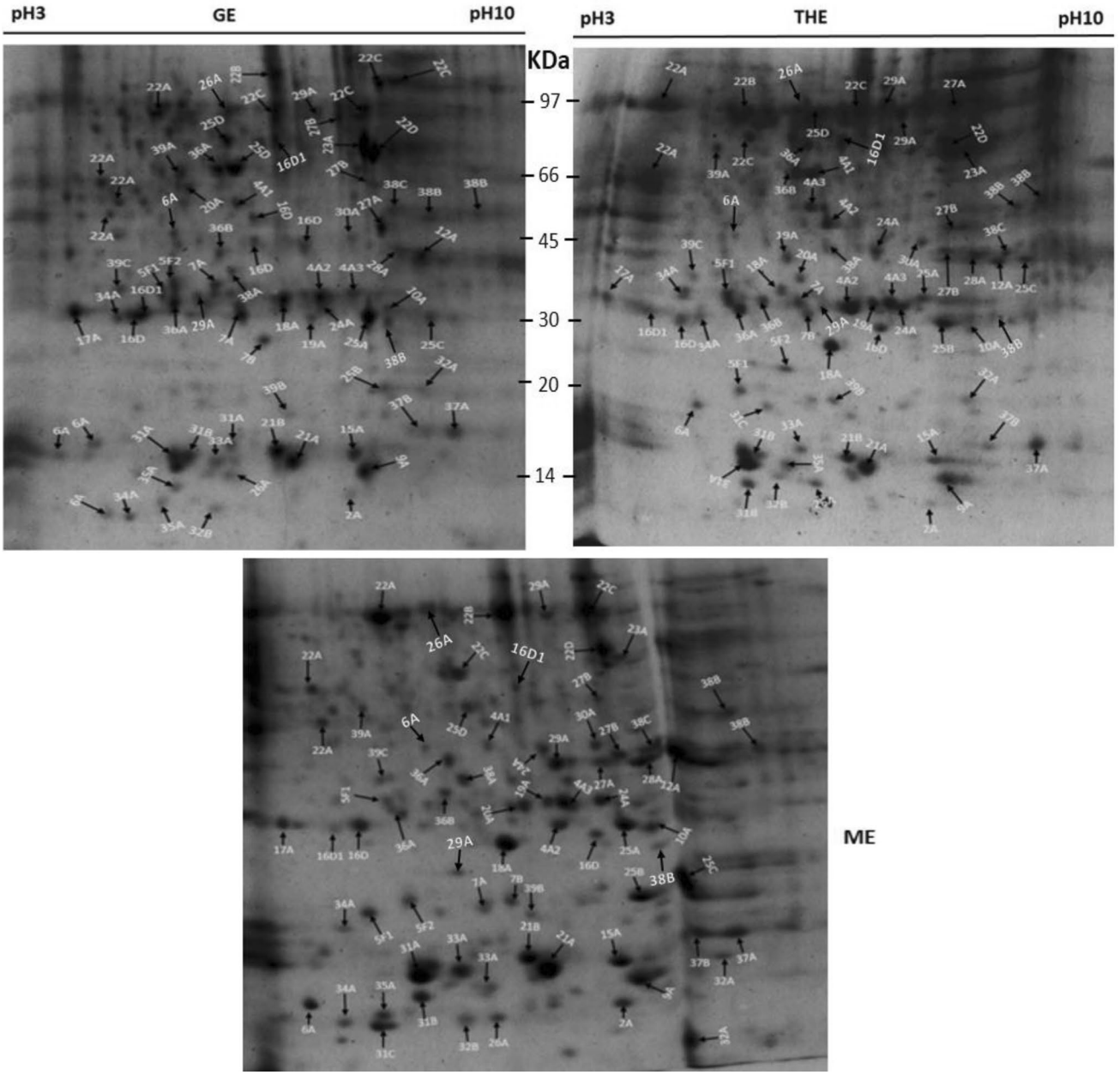


Fig. 2 Representative two-dimensional electrophoresis map is showing the resolution of the protein spots identified by mass spectrometry. *GE* Globular embryos, *TH* heart/torpedo-shaped embryos, *ME* maturing embryos

its related species) have much of genomics information in the bioinformatics databases. The identified protein spots are indicated on the representative 2D map of Fig. 2 and Table 1 and electronic supplementary data file. In some of the encountered cases, more than one protein spots were found to correspond to the same enzyme, but different isomers as, for instance, in superoxide dismutase (SOD), glyceraldehyde-3-phosphate dehydrogenase, enolase, α-tubulin, ascorbate peroxidase, dehydrins, and late embryogenesis abundant (LEA) proteins. The identified proteins based on the cellular functional categorization are involved in the aspects of stress responses (35%), energy metabolism (25%), carbon fixation (9%), secondary metabolism (6%), and other cellular and metabolic processes (20%), while 5% of the proteins are of unclassified cellular role (Fig. 3). In this study, the largest group of the identified proteins are those involved in stress response, energy metabolism, and other cellular metabolic functions, while low number of proteins involved in photosynthesis and secondary metabolism was identified. This indicates the higher role of cellular metabolic processes during SE in *N. nimmoniana*. An interesting aspect of the results is

Table 1 Differentially expressed proteins that showed ≥ 1.5 fold change in abundance in at least globular, heart/torpedo-shaped or maturing somatic embryos of *Nothapodytes nimmoniana*

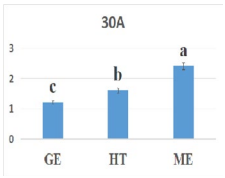
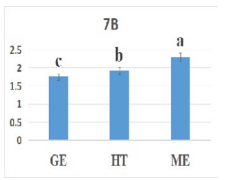
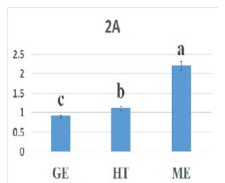
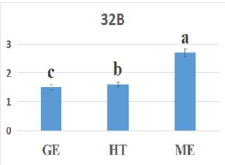
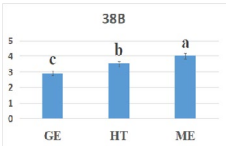
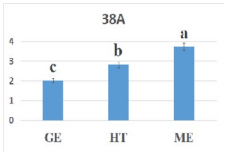
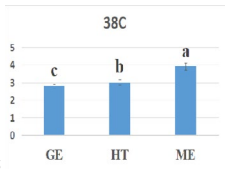
Spot	Protein/Species	Accession number	Exp. PI/Mw (KDa)	Sequence coverage (%)	Mascot score	Matched unique peptides	Relative spot volume[a] G TH M
30A	Glyceraldehyde 3-phosphate dehydrogenase/ *Arabidopsis thaliana*	gi|332191312	6.52/36.9	9	89	4	
7B	Glyceraldehyde-3-phosphate dehydrogenase/ *Glycine max*	gi|351727206	6.53/37.0	12	96	4	
2A	Cyclophilin/*Citrus sinensis*	gi|260401128	9.5/17.4	13	55	3	
32B	11S globulin precursor isoform 4/ *Sesamum indicum*	gi|81238594	4.6/51.74	9	53	3	
38B	Tryptophan decarboxylase, partial /*Ophiorrhiza prostrate*	gi|155966000	6.94/58.74	12	72	5	
38A	Strictosidine synthase/ *Nothapodytes nimmoniana*	gi|675144532	5.34/38.95	92	265	6	
38C	Secologanin synthase/ *Nothapodytes nimmoniana*	gi|675144547	6.55/44.9	89	279	5	
5F1	Alpha-tubulin/*Zea mays*	gi|452474	4.89/49.7	11	59	4	

Table 1 (continued)

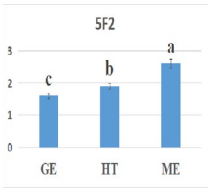
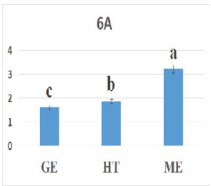
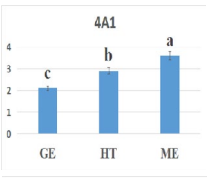
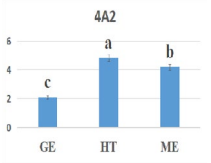
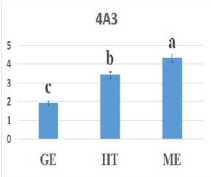
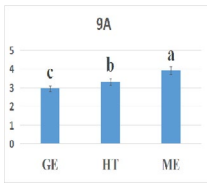
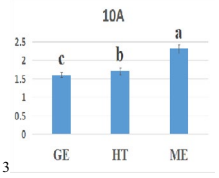
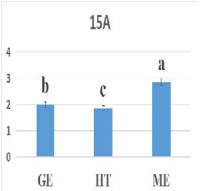
Spot	Protein/Species	Accession number	Exp. PI/Mw (KDa)	Sequence coverage (%)	Mascot score	Matched unique peptides	Relative spot volume[a] G TH M
5F2	Alpha-tubulin, partial / *Eucalyptus globulus* subsp. bicostata	gi|1101025	4.91/49.4	12	58	4	
6A	Spermidine synthase/*Coffea arabica*	gi|3242659	5.63/33.51	11	54	3	
4A1	Enolase 2/*Zea mays*	gi|162460735	5.4/49	9	76	3	
4A2	Enolase/*Solanum lycopersicum*	gi|350538295	5.5/53	12	73	4	
4A3	Enolase/*Ricinus communis*	gi|433609	5.4/45.2	10	65	3	
9A	Phosphoglycerate mutase, 2,3-bisphospho-glycerate-independent/*Theobroma cacao*	gi|508710355	6.3/68.3	14	58	5	
10A	Lactoylglutathione lyase/*Gossypium arboreum*	gi|728808180	7.6/48.2	11	52	3	
15A	Malate synthase/*Arabidopsis thaliana*	gi|332003281	6.2/62.6	10	59	4	

Table 1 (continued)

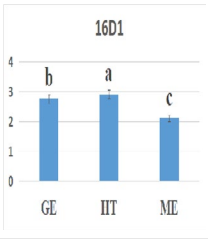
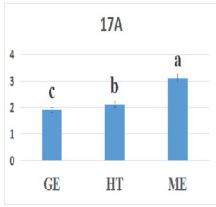
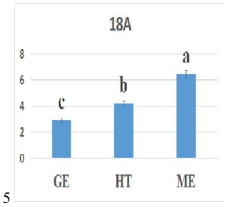
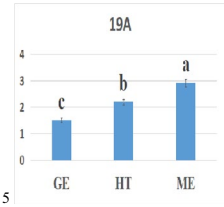
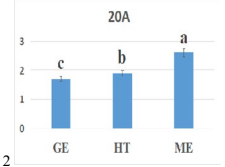
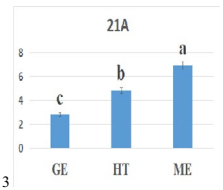
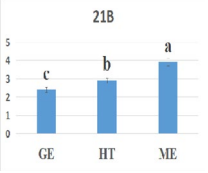
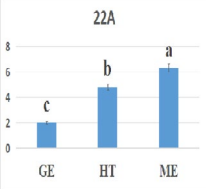
Spot	Protein/Species	Accession number	Exp. PI/Mw (KDa)	Sequence coverage (%)	Mascot score	Matched unique peptides	Relative spot volume[a] G	TH	M
16D1	NADH-ubiquinone oxidoreductase/ *Medicago truncatula*	gi|357500405	6.26/83.1	15	55	2			
17A	Auxin IAA hydrolase, partial/ *Medicago truncatula*	gi|95106145	4.95/44.9	14	63	4			
18A	ATP synthase CF1 beta subunit/ (Chloroplast)/*Pinus thunbergii*	gi|7524659	5.12/58.49	13	69	5			
19A	Pyruvate decarboxylase/*Citrus sinensis*	gi|572152997	5.92/59.7	12	54	5			
20A	Phosphoenol pyruvate carboxykinase, partial/*Santiria trimera*	gi |295041983	5.85/70.6	14	47	2			
21A	Cytosolic Ascorbate peroxidase 2/ *Solanum lycopersicum*	gi|929524247	5.93/27.4	11	55	3			
21B	Ascorbate peroxidase/*Theobroma cacao*	gi|508705797	5.96/27.5	19	84	3			
22A	Cu/Zn superoxide dismutase /*Panax ginseng*	gi |2645997	4.2/18.9	17	69	3			

Table 1 (continued)

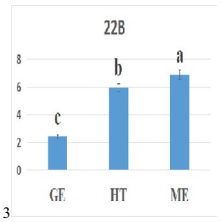
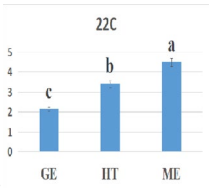
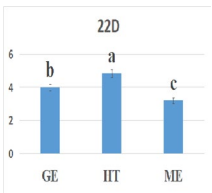
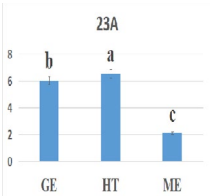
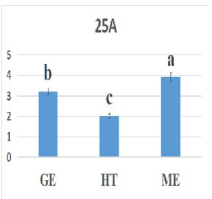
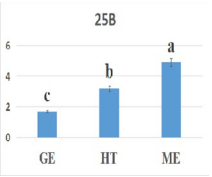
Spot	Protein/Species	Accession number	Exp. PI/Mw (KDa)	Sequence coverage (%)	Mascot score	Matched unique peptides	Relative spot volume[a] G TH M
22B	Cu/Zn superoxide dismutase/ *Hevea brasiliensis*	gi|27449246	5.37/16.34	19	74	3	
22C	Cu/Zn superoxide dismutase/ *Helianthus annuus*	gi|50978416	6.14/20.38	21	66	3	
22D	Manganese superoxide dismutase /*Vitis vinifera*	gi|526117872	6.9/29.4	17	75	4	
23A	Glutathione S-transferase/*Vitis vinifera*	gi|526117830	6.7/28.5	16	69	4	
24A	UDP-glucose pyrophosphorylase/ *Gossypium hirsutum*	gi|308445439	5.69/53.5	13	63	5	
25A	Heat shock protein 70 (Hsp 70) family protein/*Theobroma cacao*	gi|508777841	5.29/72.4	14	89	5	
25B	HSP 70 family protein/ *Arabidopsis thaliana*	gi|332190646	5.4/72.6	11	62	3	

Table 1 (continued)

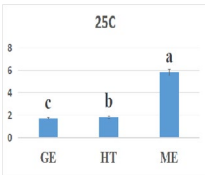
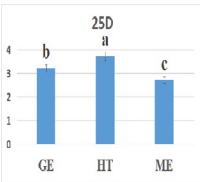
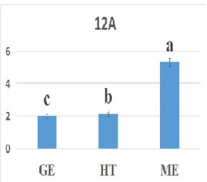
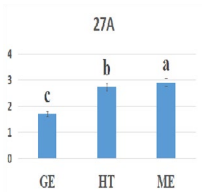
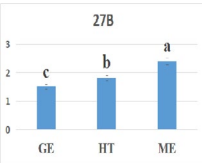
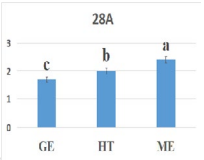
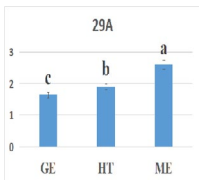
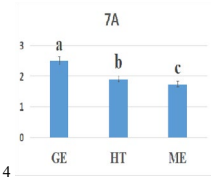
Spot	Protein/Species	Accession number	Exp. PI/Mw (KDa)	Sequence coverage (%)	Mascot score	Matched unique peptides	Relative spot volume[a] G TH M
25C	Heat shock protein 70/ *Oryza sativa* Indica Group	gi|21664287	5.32/71.9	13	58	4	
25D	HSP 23/*Medicago sativa*	gi|339792764	5.27/22.3	11	64	2	
12A	Translation initiation factor /*Medicago truncatula*	gi|657405105	7.3/46	16	58	5	
27A	Catalase/*Glycine max*	gi|358249268	6.7/56.30	14	78	4	
27B	Catalase, partial/*Prunus persica*	gi|6006609	6.8/57.40	13	53	4	
28A	Phosphoglycerate kinase/*Zea mays*	gi|226530482	6.2/43.72	9	69	3	
29A	NAD-dependent malate dehydrogenase/*Prunus humilis*	gi |937553901	6.50/38.5	14	66	3	
7A	Glutamine synthetase/ *Vitis vinifera*	gi |526117748	5.70/40.3	12	63	4	

Table 1 (continued)

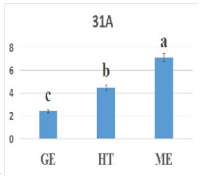
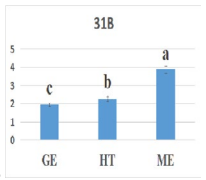
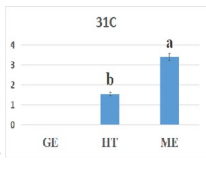
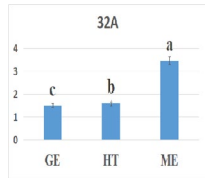
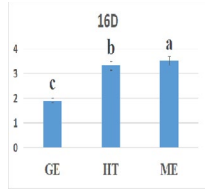
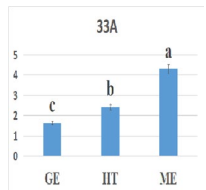
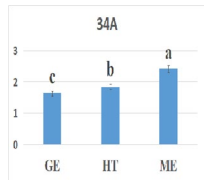
Spot	Protein/Species	Accession number	Exp. PI/Mw (KDa)	Sequence coverage (%)	Mascot score	Matched unique peptides	Relative spot volume[a] G TH M
31A	Late embryogenesis abundant domain-containing protein/*Arabidopsis thaliana*	gi|330251664	5.17 /54.19	13	59	4	
31B	Late embryogenesis abundant protein D-34/*Zea mays*	gi|226530343	5.21/32.8	16	64	3	
31C	Late embryogenesis abundant protein D-34/*Zea mays*	gi|226493450	5.18/23.4	15	62	3	
32A	Photosystem II reaction center M protein PsbM/*Medicago truncatula*	gi|355487818	9.56/29.2	19	77	2	
16D	PS II protein D1 (chloroplast)/ *Cannabis sativa*	gi|836692050	5.31/25.4	17	64	4	
33A	Chlorophyll a/b binding protein of PS II, partial/*Picea abies*	gi|1165248	5.72/29.43	21	69	3	
34A	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit /*Burchellia bubaline*	gi|1769935	4.92/19.2	12	55	4	

Table 1 (continued)

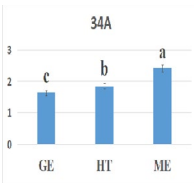
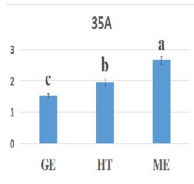
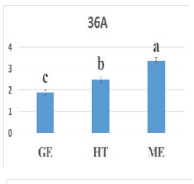
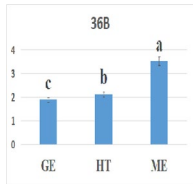
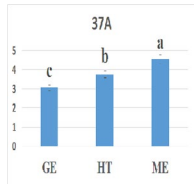
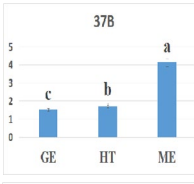
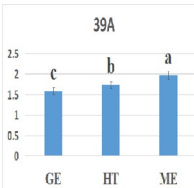
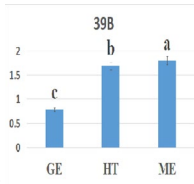
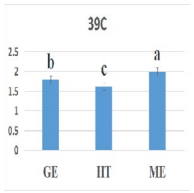
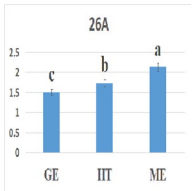
Spot	Protein/Species	Accession number	Exp. PI/Mw (KDa)	Sequence coverage (%)	Mascot score	Matched unique peptides	Relative spot volume[a] G TH M
34A	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit /*Burchellia bubaline*	gi|1769935	4.92/19.2	12	55	4	
35A	Ribulose-1,5-bisphosphate carboxylase small subunit/ *Medicago sativa*	gi|16224234	6.17/17.4	16	67	2	
36A	Dehydrin/*Vitis vinifera*	gi|526118232	5.42/19.7	18	63	2	
36B	Dehydrin/*Hordeum vulgare* subsp. vulgare	gi|6017936	5.72/29.4	16	57	5	
37A	Annexin/*Medicago truncatula*	gi|22859608	6.1/38.60	18	66	4	
37B	Annexin/*Arachis hypogaea*	gi|819731928	5.8/35.4	13	58	3	
39A	Unnamed protein product, partial /*Vitis vinifera*	gi|297744284	4.7/69.5	19	49	2	
39B	Conserved hypothetical protein /*Ricinus communis*	gi|255593332	6.1/15.50	12	44	2	

Table 1 (continued)

Spot	Protein/Species	Accession number	Exp. PI/Mw (KDa)	Sequence coverage (%)	Mascot score	Matched unique peptides	Relative spot volume[a] G TH M
39C	PREDICTED: stress-induced protein KIN2 /*Vitis vinifera*	gi|225451889	5.9/29.0	15	61	2	
26A	22.0 kDa class IV heat shock protein precursor /*Glycine max*	gi|351725595	5.42/71.38	13	47	2	

GE Globular embryos, *HT* heart/torpedo-shaped embryos, *ME* maturing embryos (DATA generated represents analysis of three replicate gels for each embryo stage)

[a]Relative spot volume intensity values are presented as mean ± standard error of the triplicate sets. The spot volumes were generated through gel images analysis using Image Master 2D Platinum Software (version 7.0, GE Healthcare Life Science New Delhi, India). Bars represent fold change of protein spot intensities in GE, TH and ME. The fold change (upregulation and down regulation) of protein spot volumes was evaluated by comparison with control (Gel having maximum number of protein spots) relative to the two embryo stages sampled

the higher expression of proteins (shown by maximum numbers of proteins spots in the 2DE gels) during embryo maturation stage (Fig. 2) and upregulation of most of the identified protein spots during the stage that, in the overall, imply higher metabolic state of the embryos.

Role of stress

Oxidative stress involving oxidative burst produces reactive oxygen species (ROS) that modulates cell division and metabolism towards SE (Zavattieri et al. 2010). Production of the ROS and scavenging enzymes plays a part in the different stages, and change in the isoforms can be used as a marker (Kairong et al. 1999; Gupta and Datta 2003). In this study, three Cu–Zn SOD isoforms (spots 22A, 22B, and 22C) that confer protection to cells against ROS-induced oxidative stress showed the upregulated expression in the maturing embryos about globular and heart/torpedo-shaped stages. An MnSOD (spot 22D) showed upregulated expression during heart/torpedo-shaped embryo stages relative to the globular, but became downregulated in the maturing embryos. The higher expression of Cu–Zn SOD isoforms during the embryo maturation implies a role in scavenging ROS generated by high metabolic state to tolerable cellular level needed for its development. In *Lycium barbarum*, activity of SODs showed the elevated levels during the early stages of the SE and was maximal at the time multicellular embryos were formed but, declined with the further development (Kairong et al. 1999). However, in Persian walnut, along with other antioxidant enzymes, it showed upregulated expression in torpedo-shaped embryos and then downregulated during the cotyledonary stage. Various cotyledonary embryos showed similar activity of the enzyme and, a Cu–Zn SOD isoform showed upregulated expression during embryo maturation (Jariteh et al. 2015). In this study, differential expression of SOD isoforms observed showed Cu–Zn as the most expressed in all the stages, while MnSOD conferred protection against ROS at higher levels during globular and heart/torpedo-shaped stage but less in the maturing embryos (spot 22D).

To promote the SE, activity of SODs generates an intracellular accumulation of H_2O_2 which could serve as 'second cellular messenger' that induce gene expression and synthesis of proteins promoting progression to the various stages. This may occur through regulating the transcriptional level of antioxidant enzymes and superoxides (Kairong et al. 1999; Apel and Hirt 2004; Isah and Mujib 2012). The higher intracellular accumulation of the H_2O_2 induces damages to membrane lipids and other macromolecules. Catalase (CAT), ascorbate peroxidase (APX), and glutathione-S-transferase (GST) participate in regulating levels of the H_2O_2 to tolerable cellular concentration needed for the embryogenesis. Two CAT isoforms (spot 27A and 27B) showed higher expression during globular-to-embryo maturation stage, a trend similar to that observed with the SODs. This suggests an efficient role in scavenging H_2O_2 generated by the activity of SOD isoforms to sufficient cellular levels needed for the development of the in vitro embryos. A similar expression

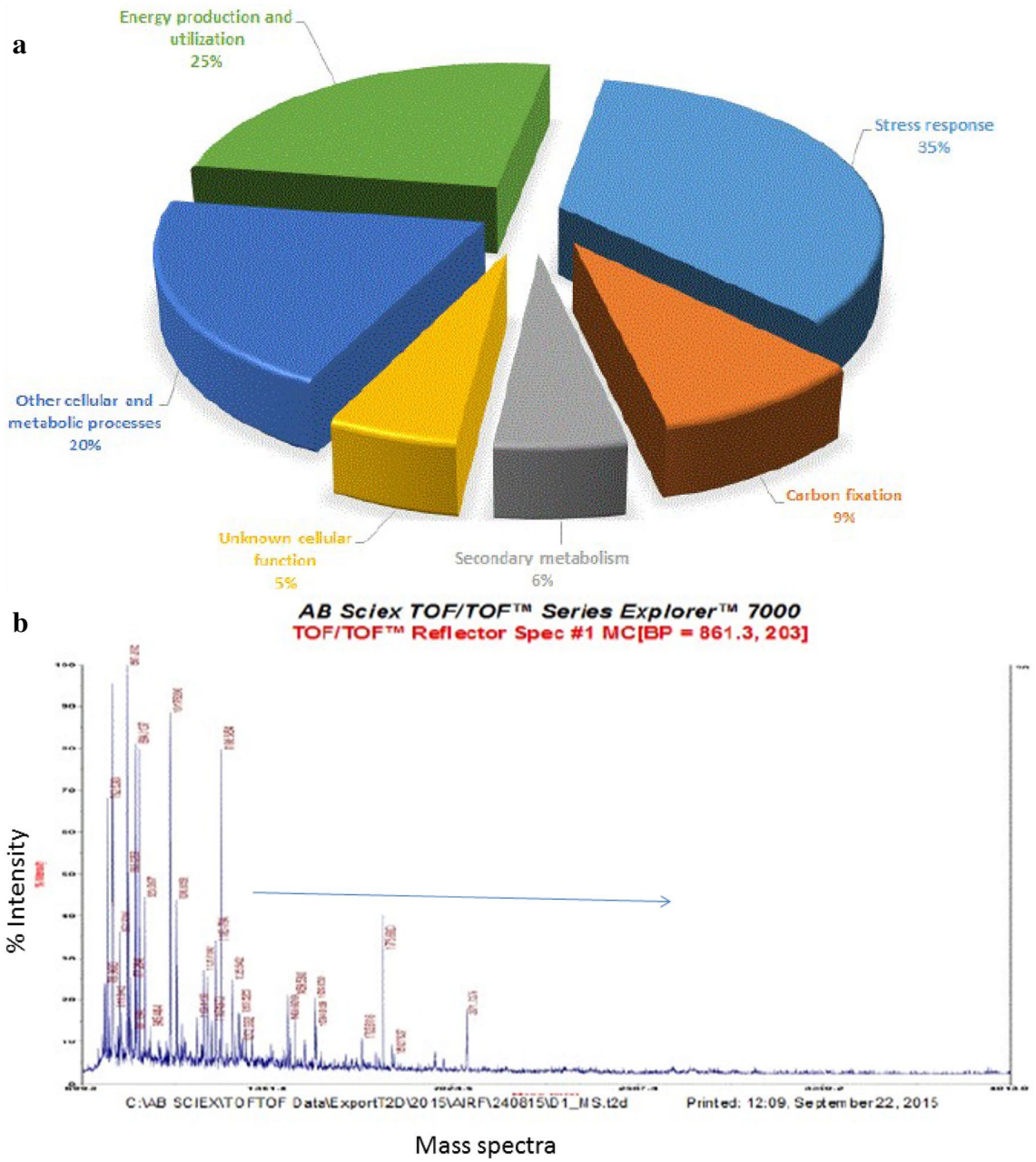


Fig. 3 **a** Putative cellular functional categorization of the expressed proteins spots (identified by mass spectrometry) from the extracted protein samples of GE, TH, and ME stages as performed using Kyoto Encyclopedia of Genes and Genomes (KEGG) which is available at (http://www.genome.jp/kegg/kegg2.html) combined with the Uniprot database (http://www.uniprot.org/proteomes/), and **b** representative mass spectra obtained by MaSp/MaSp showing one among the many protein spot that had spectra of weak intensity and that turned out low mascot score which implied uncertainty about identity of many of the processed proteins spots

pattern was shown by APX isoforms (spot 21A, 21B). In Persian walnut, differential expression of CAT isoforms was observed during the primary and secondary SE and increased in the early stages of embryo formation (Jariteh et al. 2013, 2015). However, in *Quercus suber*, upregulated expression of APX occurred across the stages with slight downregulation during intermediary stages relative to the globular embryos, and GST was proposed to play a role in ROS detoxification in the proliferating somatic embryos (Gomez-Garay et al. 2013). Glutathione-S-transferase (spot 23A) showed higher expression levels during globular and heart/torpedo-shaped embryo stages, but became remarkably downregulated in the maturing embryos. Glutathione-S-transferase was among the expressed proteins during SE in *Vitis vinifera* (Marsoni et al. 2008) and showed pronounced upregulated expression in the embryogenesis stages in

Eleutherococcus senticosus, suggesting an antioxidant role (Shohael et al. 2007). However, downregulated expression occurred in the globular and cotyledonary embryos of *Gossypium hirsutum* (Ge et al. 2014).

The stress response involved in the development of somatic embryos need a heat-shock system to play a part during the developmental reprogramming, with common elements of auxin playing a role (Zavattieri et al. 2010). Heat-shock proteins that belongs to Hsp 70 (spot 25A, B, and C) and Hsp 23 (spot 25D) categories showed higher expression in the globular and heart/torpedo-shaped stages, except spot 25A that became downregulated. This was followed by downregulation of Hsp 23 in the maturing embryos, suggesting differential expression of HSPs during somatic embryo development in *N. nimmoniana*. The higher and differential expression of the Hsps could be due to the increased and variable stress levels associated with somatic embryo development or for a role in conferring stability to other proteins during developmental stages as increased protein synthesis was observed across the stages, reflected by the number of protein spots detected in the 2-DE map in Fig. 2. This is in conformity with the role of Hsp as chaperones essential for cell recovery and function whose expression is higher during stressful condition to prevent protein denaturation (Zhu et al. 1993); Hsps were ascribed a role during SE in *Vitis vinifera* and *Picea abies* (Businge et al. 2013). The expression of chaperones and proteins involved in the somatic embryo development that includes LEA proteins (spot 31A, 31B, and 31C) having cellular roles as DNA-binding and repair, chaperone function, Ca^{2+} binding, as structural components of cytoskeleton and abiotic stress tolerance, may show changes at the developmental stages. In this study, the LEA proteins (spot 31A and 31B) were expressed in all the stages with the highest in the embryo maturation stage, while another LEA protein (spot 31C) was not expressed in the globular embryos, but, in the heart/torpedo-shaped and maturing embryos with upregulation in the later stage. The upregulated expression of the proteins could be for a role in facilitating embryo growth, maturation, and stress defense, particularly during the maturation stage. The abiotic stress of in vitro culture can induce the expression of different categories of LEA proteins, including dehydrins (spots 36A and 36B) that play a role in abiotic stress response. Many stress factors can induce their synthesis in somatic tissues, and the pattern of accumulation is under developmental control (Bomal et al. 2002; Burrieza et al. 2012). In this study, higher expression of dehydrins (spots 36A and 36B) was observed across the stages for a possible role in abiotic stress tolerance needed for the embryogenesis. This is also in conformity with the essential role of stress in embryo development through the various stages in *N. nimmoniana*. Many dehydrins were identified in the embryogenic cultures of sugarcane (Burrieza et al. 2012). A stress-inducible protein (spot 39C) showed higher expression across the stages for a possible role in reorganizing physiological and metabolic processes involved in the embryos development, and higher expression shown by enzymes involved in antioxidant defense systems.

Energy metabolism

Many genes that are expressed during somatic embryo development are involved in regulating developmental processes, and involve the biosynthesis of compounds, cell division, elongation, and differentiation. The most pressing need of cells during the processes and proper function is energy and metabolic intermediates that are provided by glycolysis and tricarboxylic acid cycle (TCA). In this study, enzymes involved in glycolysis and TCA cycle were the second most expressed for their possible role in providing cellular energy needed for metabolic processes associated with somatic embryo development in *N. nimmoniana*. The most expressed glycolytic enzyme is enolase with three identified proteins spots (4A1, 4A2, and 4A3). Enolase converts 2-phosphoglycerate to phosphoenolpyruvate in the glycolytic pathway, while, during gluconeogenesis, it is important in starch accumulating seeds. Accumulation of its transcripts was found maximal in the torpedo stage embryos of *Arabidopsis* (Andriotis et al. 2010) and was regarded mature embryo marker in *Picea glauca* (Lippert et al. 2005). Glyceraldehyde 3-phosphate dehydrogenase (spot 30A, 7B) that converts glyceraldehyde 3-phosphate into glycerate 1, 3-bisphosphate plays a role in ROS signaling in plants, while phosphoglycerate kinase (spot 28A) catalyzes reversible transfer of phosphate group from 1,3-bisphosphoglycerate to adenosine diphosphate to produce 3-phosphoglycerate and adenosine triphosphate, besides an ascribed role as an α-cofactor. In this study, the two enzymes showed upregulated expression in the maturing embryos about the other stages. Phosphoglycerate kinase was among the expressed proteins during secondary SE in cassava (Baba et al. 2008). The 3-phosphoglycerate generated by the activity of phosphoglycerate kinase is transformed to 2-phosphoglycerate through 2,3-bisphosphoglycerate intermediates involving internal transfer of phosphate group in reaction catalyzed by phosphoglycerate mutase (spot 9A). Although the cellular role of phosphoglycerate mutase in plants is unelucidated, it was proposed to be involved in defense response (Shin et al. 2009). Phosphoglycerate mutase was among embryo-specific proteins expressed during SE in *Cyclamen persicum* (Lyngved et al. 2008), suggesting a possible role in embryo development in *N. nimmoniana*. Pyruvate decarboxylase involved in the decarboxylation of pyruvate generated by glycolytic pathway into acetaldehyde and CO_2 in cytoplasm and mitochondria of plant cells (spot 19A), which showed higher expression across embryogenesis stages with

the most in the heart/torpedo-shaped to maturing embryo stages, suggesting more of role in the development of mature embryos than others. In the cotyledonary somatic embryos of cotton, its downregulation was involved in reduced respiration, and occurred due to the low energy production, and for nutrient storage needed for future germination (Ge et al. 2014). However, during SE of *Quercus suber*, it showed the upregulated expression between proliferating, cotyledonary to mature embryo stages (Gomez-Garay et al. 2013). Malate synthase (spot 15A) that catalyzes condensation and later hydrolysis of acetyl-Co enzyme A and glyoxylate to form malate and CoA showed a little change in globular and heart/torpedo-shaped stage, but upregulated in the maturing embryos. During the early stage of castor bean seed maturation, it was among the expressed at higher level, but downregulated in mature seeds, suggesting a role in embryo maturation (Gonzalez 1990). Its role in *N. nimmoniana* somatic embryo development is likely for facilitating embryo maturation as most of the embryonic tissues showed only early stage maturation. It could also be for a role in somatic embryo development through energy production as enzymes involved in energy production showed upregulated expression during embryo maturation. The NAD-dependent malate dehydrogenase (spot 29A) that catalyzes the oxidation of malate to oxaloacetate by reducing nicotinamide adenine dinucleotide (NAD^+) to NADH in many metabolic pathways that includes TCA cycle and gluconeogenesis showed upregulated expression in maturing embryos about other SE stages in *N.nimmoniana* (spot 29A). It was ascribed a critical role in embryonic development and heterotrophic metabolism in *Arabidopsis* (Beeler et al. 2014), and was among energy production enzymes expressed at higher level during SE in date palm (Sghaier-Hammamia et al. 2009). The NADH–ubiquinone oxidoreductase (spot 16D1) that plays a role in oxidative phosphorylation which provides high energy needed for cellular processes showed upregulation in its expression during heart/torpedo-shaped embryo stages about the globular but, downregulated in maturing embryos. This suggests its higher role in embryogenesis during heart/torpedo-shaped stages, besides the primary role in energy production. It also suggests that oxidative phosphorylation provides less energy needed for metabolic activities involved in embryo maturation in *N. nimmoniana*, but higher in the heart/torpedo-shaped stages. Its expression was observed in the embryogenic cultures of *Medicago truncatula* (Imin et al. 2004) and during embryo maturation in Larch (Teyssier et al. 2014). Chloroplast ATP synthase 1 β subunit (spot 18A), another enzyme that plays a role in cell energy production through synthesis of ATP at the expense of electrochemical proton gradient formed by light-dependent electron flow in the mitochondrial thylakoid membrane, was among the higher expressed proteins in all the stages. This is in conformity with the increased cellular demand for energy needed for high metabolic processes during somatic embryo development and upregulated expression shown by enzymes involved in energy production. Its higher expression suggests high chloroplastic ATP generation needed for metabolic process- associated somatic embryo development in *N. nimmoniana*.

Cellular function of nucleotide sugar precursor synthesis needed for cell wall biogenesis is performed by UDP-glucose pyrophosphorylase (spot 24A), besides its role in plant growth and development through participation in carbohydrates biosynthesis regulation (Winter and Huber 2000). The proteins showed higher expression with the advance in embryogenesis stages, suggesting possible role in somatic embryo development through carbohydrate metabolism needed to build cell wall polymers essential for cell division. The high expression had been observed in the somatic embryos of *C. persicum* (Rode et al. 2012) and in polyethylene glycol-treated mature embryos of *C. papaya* (de Moura Vale et al. 2014). Phosphoenolpyruvate carboxykinase (spot 20A) showed higher expression across the embryogenesis stages for the possible embryo-specific role which it plays in carbohydrate metabolism. Its expression in the embryos also suggests a likely role in converting glycerol 3-phosphate product of photosynthesis to starch for storage in the chloroplast or glucose and sucrose as upregulated expression of photosynthetic enzymes and UDP-glucose pyrophosphorylase was observed. In soybean, during the induction of globular embryos, transcripts of a gene encoding phosphoenolpyruvate carboxykinase were among the expressed (Thibaud-Nissen et al. 2003).

During embryo maturation, metabolic activities including respiration are slowed to prepare embryos for quiescence, and lack of the maturation is a feature of recalcitrant seeds. The similarity is also found in somatic embryos, although, not at comparable physiological level to the in vivo embryo maturation (Iraqi and Tremblay 2001b). In this study, upregulated expression of energy metabolism and antioxidant enzymes were observed during later stages of somatic embryo development, indicating their possible higher metabolic state. In *Araucaria augustifolia*, Balbuena et al. (2009) attributed higher expression of enzymes involved in energy metabolism during late stages of zygotic embryogenesis to shift from stress-associated metabolism to make molecular oxygen available as the final hydrogen acceptor needed to increase the levels of respiration in mature seeds. However, during secondary SE of cassava, higher expression of enzymes involved in energy production was attributed to the higher metabolic state essential for maintaining intense cell division in the developing embryos (Baba et al. 2008). In this study, high expression of antioxidant and energy metabolism enzymes observed could be attributed to the higher metabolic state of the embryos which coupled with the ROS generation from metabolic processes needed for

proper development possibly played a role in lack of physiological maturation and conversion of most maturing embryos to plantlets (Fig. 1k).

Other cellular and metabolic functions

The development of somatic embryos involves dedifferentiating somatic cells into embryogenic through the induction and expression stages involving molecular and physiological changes regulated by the expressed genes. The changes are based on the influence of expressed gene products that determine the growth and development of cultures, including polyamines which play a role in growth and development through their role in cell division and morphogenesis. Spermidine synthase (spot 6A), a key enzyme involved in the biosynthesis of polyamines whose activity is regulated at transcriptional and translational level and the activity correlates with the cellular levels of polyamines (Martin-Tanguy 2001), showed upregulated expression in the maturing embryos about the other stages, and was among the higher expressed proteins in the embryos. Spermidine was reported to play a significant role in promoting embryogenic differentiation in the in vitro cultures (Silveira et al. 2006) and correlated increase in the polyamine levels promoted somatic embryo development (Minocha et al. 2004). Their continual biosynthesis is an essential part of increased metabolic state during differentiation and development of somatic embryos. Annexins (spot 37A and 37B), which are multigene super-family of cytosolic calcium-dependent membrane binding proteins that play a role in cellular functions such as membrane cytoskeleton interaction, calcium channel activity and signal transduction, exocytosis, and endocytosis fundamental to cellular processes as secretion and repair, growth, and differentiation (Gerke and Moss 2002), showed upregulated expression in the embryo maturation stage with one of the spots (37B) expressed at higher levels over the other (37A), suggesting that their differential expression is involved in SE in *N. nimmoniana*. Alpha-tubulin (spot 5F1 and 5F2) that plays a role in cell division and elongation through organelles and daughter chromosome segregation during mitotic division essential for morphogenesis showed transient upregulated expression across embryogenesis stages for possible role in cell division and elongation. Alpha-tubulin was among the expressed proteins during SE in *Picea glauca* (Lippert et al. 2005) and secondary SE in *Manihot esculentum* (Baba et al. 2008). In addition, the cellular processes involved in somatic embryo development such as glycolysis and ROS production generate toxic electrophilic methylglyoxal whose reaction with the intracellular macromolecules that include protein and nucleic acid can lead to their inactivation. Lactoylglutathione lyase (spot 10A) involved in their detoxification showed higher expression across embryogenesis stages in similar way to enzymes involved in glycolytic pathway and antioxidant defense. Translation initiation factor (spot 12A) that binds to small ribosomal subunit during initiation of translation in protein synthesis by helping stabilizing the formation of functional ribosome around a start codon to give translation initiation a regulatory mechanism showed higher expression across embryogenesis stages, particularly during the embryo maturation. This could be due to the increased cell protein synthesis needed for embryo development and maturation that involves the covalent bonding of synthesized amino acids facilitated by peptidyl-prolyl isomerase proteins such as cyclophilins. Recent findings have implicated many cellular functions to the cyclophilins, including cell division, regulation of transcription, playing part in photosynthesis, as molecular chaperones, signaling molecules, and cellular stress tolerance (Kumari et al. 2013). In this study, cyclophilins showed upregulated expression (spot 2A) in the maturing embryos, suggesting their role in embryo development and stress tolerance needed for SE. Glutamine synthetase (spot 7A) that plays an essential role in nitrogen metabolism by catalyzing condensation of ammonia and glutamate to form glutamine showed downregulated expression between globular and maturing embryo stages, suggesting decreased nitrogen metabolism during embryogenesis in *N. nimmoniana*. In carrot, accumulation of its isoforms, transcripts, and activity was the highest during globular embryo stage, but declined over later stages of somatic and zygotic embryogenesis, suggesting similar nitrogen assimilation pattern in the embryos (Higashi et al. 1998). However, accumulated expression occurred during the early stages of cotyledonary embryo development in Brazilian pine (Balbuena et al. 2009), while an intense expression was observed in somatic than zygotic embryos of date palm (Sghaier-Hammamia et al. 2009).

The endogenous substances that regulate somatic embryo development include auxin whose concentration plays a role during embryogenic competence acquisition and expression (Jimenez 2001). Plants use strategies to regulate its levels by hydrolysis of amide-linked conjugates that act as storage or inactive forms of the hormone. Hydrolysis of the indole-3-acetic acid (IAA)—amide conjugates—results in free IAA in reaction mediated by auxin amidohydrolase (Woodward and Bartel 2005). An auxin hydrolase (spot 17A) showed higher expression during the stages and peaked during embryo maturation, an unusual feature of late developmental stages of embryogenesis. In *Abies alba*, embryo maturation was associated with an increase in the endogenous concentration of IAA, and suppression of the hormone biosynthesis inhibited somatic embryo development (Vondrakova et al. 2011). In this study, higher expression of enzymes involved in auxin regulation observed could be explained by the lack of physiological maturation shown by the embryos as auxin is needed for the proper development of somatic embryos.

Physiological quality of the somatic embryos is determined by maturation, germination, and subsequent development into seedling which accumulation of storage proteins plays a role, and among them include 11S globulins precursor isoform 4 (spot 32B). This protein showed higher expression from globular to maturing embryo stages, suggesting role in mature embryo development. Storage proteins identified in the somatic embryos of *C. persicum* were proposed as markers of embryogenesis (Winkelmann et al. 2006).

In this study, a higher metabolism that was shown by the somatic embryos through upregulated expression of various proteins in the maturation stage further supported the results on antioxidant and energy metabolism that 'pointed' to lack of physiological maturation. During zygotic embryogenesis in *Araucaria augustifolia*, a woody species that produces recalcitrant seeds (as in *N. nimmoniana*) which are shed with high moisture, and shows little desiccation tolerance, proteins involved in metabolic processes were of high abundance in the late stages of embryos development due to the active metabolic state (Balbuena et al. 2009), which further supports the results of the study.

Carbon fixation

In plants, several proteins and other co-factors assemble to execute energy conversion reaction of photosynthesis; reaction center captures the energy of photons using pigment molecules to turn them into usable form (Isah 2015b). Once the light energy is absorbed by pigment molecule or passed to them by resonance transfer from surrounding light harvesting complex, two electrons are released into the electron transport chain. The transported electrons within the chain get raised to an excited state and their return to ground state couples to energy production. In this study, photo system (PS) II reaction center M proteins (PsbM) (spot 32A) are light-driven water: plastoquinone oxidoreductase that uses light energy for abstracting electrons from water, thus, generating oxygen and proton gradient which are later used to form ATP. The protein comprises core antenna complex that captures photons and an electron transfer chain for converting photonic excitation into charge separation. A subunit is involved in dimerization of PSII, and at the monomer–monomer meet is the only protein–protein contacts observed between PsbM subunits and lipid, the chlorophyll and carotenoids that contribute to dimerization of PSII. Photo system II protein D1 (PsbA) (spot 16D) together with the D2 reaction center heterodimer binds P680, the primary electron donor of PSII. The later electron acceptors share non-heme iron with each subunit binding pheophytin, quinone, other chlorophylls, carotenoids, and lipids, and provides most of the ligands for the Mn_4–Ca–O_5 complex cluster of oxygen-evolving complex. A Cl (− 1) ion association with the D1 and D2 is the need for oxygen evolution. Chlorophyll a/b-binding proteins of the PS II (spot 33A) associates with the chlorophylls and xanthophylls to make up part of antenna complex. The excitation energy of the PS II due to stress condition is balanced by the chlorophyll a/b-binding protein (Liu and Shen 2004; Li et al. 2004). In this study, a higher expression was shown by the components of photosynthetic apparatus making up the photo systems and chlorophyll a/b-binding protein during the developmental stages of the embryos from globular to maturation stage. Given that the embryonic tissues showed increased visible pigmentation over the stages (Fig. 1h–k), it suggests higher carbon fixation to provide carbon compounds and monomers for the synthesis of cell wall polymers, and that photosynthesis plays role in the development of the embryos in *N. nimmoniana*. It also suggests that a role is played by enzymes involved in carbon fixation to provide products used in the energy generation for embryo development given the expression of enzymes involved in carbon fixation identified in the present study (spot 34A and 35A). The large and small sub units of Ribulose—1,5-bisphosphate carboxylase/oxygenase (RubisCO) (spot 34A, 35A) showed higher expression from globular to maturing embryo stage in similar way to the other identified carbon-fixing enzymes. Higher carbon fixation in the maturing embryos facilitates synthesis of 3-carbon compounds that can be converted to the other forms of carbohydrates for reserve accumulation and respiration to provide cellular energy needed for metabolic processes. It also suggests that the embryos developed photosynthetic capacity/autotrophy during the advanced stages of development. Although enzymes involved in photosynthetic energy production showed higher expression across embryogenesis stages, particularly in the embryo maturation and as supported by the increased visible pigmentation of the embryos, the majority did not convert to plantlets, suggesting a higher metabolic state that possibly led to the lack of physiological maturation (Fig. 1k).

Secondary metabolism

Plant secondary metabolites play an essential role in physiological and biochemical processes in cells, tissues, and organs and their biosynthesis are regulated by the developmental stage adaptation and environmental conditions involved in protective response during stresses (Kliebenstein 2004; Ahuja et al. 2010; Batra and Sharma 2013). CPT is a monoterpene indole anticancer alkaloid biosynthesized through the terpenoid indole alkaloid biosynthetic pathway that involves strictosidine synthase, tryptophan decarboxylase, and secologanin synthase, and is isolated from *N. nimmoniana* in the highest yield quantity than other natural sources (Govindachari and Viswanathan 1972; Yamamoto et al. 2000; Yamazaki et al. 2003a). It is the most promising anticancer drug of plant origin, but the biosynthetic pathway

and regulatory mechanism of production are not clear (Isah 2016b). In this study, strictosidine synthase (spot 38A) catalyzed the condensation of secologanin and tryptamine to produce strictosidine in the CPT biosynthetic pathway. Tryptamine then provided indole moiety of terpenoid indole alkaloids through decarboxylation of tryptophan by tryptophan decarboxylase (spot 38B) in reaction that serves as branching point from primary metabolism to secondary metabolic pathways. Secologanin moiety was then derived from the geraniol, while geraniol 10-hydrolase hydroxylates geraniol at the C-10 position to form loganin and secologanin in reaction catalyzed by secologanin synthase (spot 38C) (Yamamoto et al. 2000; Yamazaki et al. 2003a). In the present study, the enzymes showed higher expression across the stages, in particular, secologanin synthase and tryptophan decarboxylase whose expression was relatively higher than the strictosidine synthase. Accumulation of strictosidine synthase proteins and transcripts in the stem and roots of *Ophiorrhiza pumila* correlated with the expression of CPT biosynthesis and organ development (Yamazaki et al. 2003b). Similarly, accumulation of CPT was found to be higher in the somatic embryos (Fulzele and Satdive 2003) and as supported by the results of HPTLC analysis carried out in the present study. The accumulated CPT increased between the various stages of somatic embryo development with 495 ± 0.14a, 687 ± 0.18b, and 804 ± 0.16c µg CPT/g embryo DW for the globular, heart/torpedo-shaped, and maturing embryo stages respectively. This reflects the expression of CPT biosynthesis enzymes identified in the study.

Conclusion

SE was reported in *N. nimmoniana* over a decade ago, but yet, there has been no progress on the production and application in the clonal propagation of the tree so far, despite the potentials, it offers in scale-up production of CPT. In this study, application of 2-DE and MaSp has aided understanding some physiological, metabolic processes involved in the somatic embryos development in *N. nimmoniana* at proteome expression levels through the roles of stress, energy production, and other metabolic processes by expression of the associated proteins identified. Results of the study have provided some insight into the lack of physiological maturation shown by the embryos at the maturation stage, which could be attributed to their higher metabolic state. As SE offers an alternative technology for studying the development of embryos in the in vivo, due to their genetic and physiological, developmental similarity, further research on the physiology could provide proper information on recalcitrance exhibited by *N. nimmoniana* seeds, with application in conservation of the tree to mitigate indiscriminate harvest of its endangered natural population for the anticancer alkaloid. Information from the studies could also be of use in understanding metabolism, molecular markers of the various stages, with application in optimizing culture conditions for mass propagation and commercial production of CPT, especially in a scale-up production. Given the low number of the expressed proteins involved in embryo maturation stage identified in the present study, the use of maturation-inducing agents such as polyethylene glycol could provide insight on optimizing culture condition of the embryos through enhancing embryo maturation, in particular. The low number could also be attributed to the limited bioinformatics data available in the NCBInr database (http://www.ncbi.nlm.nih.gov/protein) for the successful identification of the proteins as the number of expressed protein spots during embryo maturation stage was relatively higher compared to the other stages (Fig. 2). Studies on the role(s) of stress in the induction and expression of the somatic embryos will aid the optimization of condition culture factors for enhanced production of the in vitro embryos. This is the first molecular study of SE in *N. nimmoniana*, and could open further studies aimed at identifying culture conditions for the induction and expression of the embryogenesis, with possible application in scale-up production of CPT to mitigate indiscriminate harvest of endangered natural population for the anticancer alkaloid.

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Compliance with ethical standards

Conflict of interest Authors declare that no conflict of interest exists in the manuscript contents.

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