Scientia Horticulturae 229 (2018) 65–76

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Research Paper

Insights into the conversion potential of *Theobroma cacao* L. somatic embryos using quantitative proteomic analysis

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ARTICLE INFO

Keywords:
Micropropagation
Somatic embryogenesis
Cacao
Embryo quality
Label-free proteomics

ABSTRACT

Somatic embryogenesis (SE) has been routinely used as mass micropropagation technique, and as a model system for investigating the structural, physiological, and molecular events occurring during somatic embryo development. Successful *in vitro* SE is related to the quality and yield of somatic embryos obtained. In cacao (*Theobroma cacao* L.) SE, the efficiency of somatic embryo production has improved with secondary SE. However, the low number of somatic embryos able to conversion into viable plantlets is still low. Then, two morphological types of normal mature somatic embryos can be identified during cacao secondary SE. The first type shows white appearance and high conversion potential (75%), while the second type shows translucent appearance and exhibit low conversion potential (15%). In order to investigate the proteins that can be associated to conversion potential in cacao somatic embryos, the mass spectrometry HDMS^E proteomic approach was used. At least 60 proteins showed differences in abundance levels in cacao white somatic embryos, when compared to translucent. An increased abundance of Beta-glucosidase, NAD(P)-linked oxidoreductase and Electron transfer flavoprotein proteins were observed in white somatic embryos. Moreover, in translucent somatic embryos were observed an increased abundance of Cytochrome P450 and Pathogenesis-related proteins. Using white somatic embryos as a model, we suggest that carbohydrate metabolism process and the redox regulation are involved in the control/regulation of somatic embryo quality. These new findings may improve cacao SE protocol, as well as the understanding of the role of pivotal metabolic pathways associated to this *in vitro* morphogenetic route.

1. Introduction

Cacao (*Theobroma cacao* L.) is a tropical plant, which play a relevant role in both the stability of tropical ecosystems and in the economy of millions of small-holder farmers (Araújo et al., 2011). Cacao beans are rich source of polyphenols and represent the main raw material for the multi-billion-dollar chocolate industry (Maximova et al., 2014).

Plant propagation through somatic embryogenesis (SE) is an effective method to large-scale clonal propagation (Jin et al., 2014; Li et al., 1998), which can be incorporated into breeding programs. Cacao SE is well characterized (Alemanno et al., 1997; Li et al., 1998; Maximova et al., 2008; Maximova et al., 2002), and somatic embryo-derived plants have been tested under field conditions, revealing similar growth patterns to those from plants propagated by seeds (Maximova et al., 2008).

Secondary SE protocol for efficient somatic embryos production have been established (Minyaka et al., 2008). However, somatic embryos still exhibit very low conversion potential into plantlets, which is

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http://dx.doi.org/10.1016/j.scienta.2017.10.005
Received 21 July 2017; Received in revised form 2 October 2017; Accepted 3 October 2017
Available online 05 November 2017

a limitation for the commercial application of this technique.

Considering that somatic embryo developmental patterns are similar to zygotic embryos in terms of morphology, biochemistry, desiccation tolerance, and germination (Alemanno et al., 1997), several studies have been carried out comparing cacao zygotic and somatic embryos, using zygotic embryos as a reference model (Niemenak et al., 2015; Noah et al., 2013).

Conceptually, developmental stages of somatic and zygotic embryo are divided into two main metabolic stages: the first is a morphogenetic stage that is characterized by cell division and the onset of cell differentiation; the second is a metabolic stage or maturation phase that is characterized by biochemical activities, which involves the accumulation of major storage products and the preparation for desiccation, dormancy, and germination/conversion (Leljak-Levanić et al., 2004; von Arnold et al., 2002; Harada et al., 2010). In this last phase, somatic embryos achieve both morphological and physiological maturity, which, guarantee satisfactory post-embryonic performance. Therefore, the conversion potential is considered to be programmed during embryo maturation (Dodeman et al., 1997).

The early conversion steps in somatic embryos depends both on activation of enzymatic system that mobilizes nutrient elements, and the storage compounds accumulated during maturation phase (Stasolla and Yeung, 2003). Insufficient accumulation of storage compounds and enzymatic imbalances in somatic embryos were earlier suspected to cause poor conversion potential in cacao (Alemanno et al., 1997).

Previous studies of comparative proteomic analysis of somatic and zygotic embryos in cacao showed that the most important difference between the two types of embryos is related with carbohydrate metabolism. Thus, zygotic embryos display a high glycolytic activity while somatic embryos showed the important increased of TCA (tricarboxylic acid) cycle proteins, which is related with intensive aerobic/respiration pathway activity (Noah et al., 2013). On the other hand, it was observed high expression of stress-/defense- related proteins in somatic embryos, the authors suggest that they are resilient to the stress imposed by *in vitro* culture (Niemenak et al., 2015).

Despite the similarities between these two types of embryogenesis, some key differences exist, and, zygotic embryos may be nourished via phloem and simultaneously development of a normal endosperm tissue. In SE, embryos are dependent on exogenous carbohydrate supply and morphological stages occurs without the surrounding embryo sac and the simultaneous development of a normal endosperm tissue. In addition, one marked difference between somatic and zygotic embryos is the availability of storage compounds such as carbohydrates, lipids and proteins (Rode et al., 2012).

During cacao SE it is possible to recognize two different types of normal mature somatic embryos with well-defined and developed hypocotyl and cotyledons: the white somatic embryos type which show enhanced conversion potential, and translucent embryos type that show limited conversion potential (Li et al., 1998). Previous studies performed by our research group with EET 103 and EET 111 cacao genotypes, showed that white somatic embryos conversion rate was 90% and 76% respectively, while translucent embryos conversion rate was 17.8% and 15%. In addition, the proportions of two somatic embryos types during SE is about 50% in EET 103 cacao genotype, despite this is genotype-dependent feature (data no show).

Thus, in the present study we used a proteomic approach, involving 2D-nanoESI-HDMS^E technology, in order to compare normal somatic embryos with white and translucent morphological appearances in cacao at the equivalent developmental stage (cotyledonary-staged).

2. Material and methods

2.1. Plant material

The present study was performed with the cacao genotype “EET 103”, which is classified into the ‘Nacional’ genetic group. This genotype is known for its high productivity and is considerate as “Cacao de Aroma” fine flavor (Seguine and Meinhardt, 2014).

2.2. Somatic embryogenesis

Secondary SE was obtained from cotyledons of somatic embryos previously established *in vitro* as described by Maximova et al. (2002), using cacao genotype EET 103. All culture media were composed by DKW (Phytotechnology Lab, Overland Park, KS, USA) basal salts, as described by Driver and Kuniyuki (1984) The embryo development (ED) culture medium was supplemented with $MgSO_4$, as described by Minyaka et al. (2008).

Cotyledons from mature somatic embryos were excised and subcultured in SCG (secondary callus growth) culture medium for 14 days. This culture medium was supplemented with DKW vitamins, 20 g L^{-1} glucose, 9 µM 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma-Aldrich), 1.2 µM kinetin (Kin; Sigma-Aldrich) and 0.2% (w/v) Phytagel® (Sigma-Aldrich, St. Louis, MO, USA). Cultures from SCG culture medium were transferred to ED culture medium plant growth regulators-free, and subcultured every 21 days. After 45 days in culture, normal cotyledonary somatic embryos were classified into two types: white and translucent (Fig. 1). The weight of a normal somatic embryo was about 40–50 mg. There was no significant difference between weight of white and translucent somatic embryos (data not shown). Three independent experiments were carried out with three biological replicates. Samples (500 mg, about 12 normal somatic embryos) of two types of mature somatic embryos per biological replicate were frozen in liquid nitrogen and stored at −80 °C for 2 months until protein extraction.

2.3. Proteomic analyses

2.3.1. Total protein extraction

Proteins extractions for each somatic embryo type were carried out in biological triplicate (500 mg) according to Carpentier et al. (2005),

Fig. 1. Somatic embryogenesis of cacao genotype EET 103. (A) Somatic embryos. (B) White somatic embryo at cotyledonary stage. (C) Translucent somatic embryo at cotyledonary stage (bar = 2.0 mm).

with modifications. Samples collected were frozen in liquid nitrogen, pulverized in a bead mill and subsequently transferred to clear 10-mL micro tubes containing 5 mL of extraction buffer (50 mM Tris-HCl pH 8.5, 5 mM EDTA, 100 mM KCl, 2% (v/v) β-mercaptoethanol, 30% w/v sucrose, and 1 mM PMSF) and 5 mL of buffer-saturated phenol (pH 7.8). The extracts were homogenized by vortexing for 15 min and incubated on ice for 30 min followed by centrifugation at 12,000 × *g* for 30 min at 4 °C.

The phenolic phase was recovered and homogenized with 5 mL of extraction buffer by vortexing for 15 min followed by centrifugation at 12,000 × *g* for 30 min at 4 °C. The phenol phase was collected and proteins were precipitated with 5 mL of 100 mM ammonium acetate in methanol at −20 °C overnight. The resulting protein pellet was washed three times with cold 100 mM ammonium acetate and twice with cold acetone. Finally, the proteins were solubilized in 0.3 mL of solubilization buffer (7 M Urea; 2 M Tiourea; 2% IPG buffer; 3% CHAPS; 1.5% DTT) by mild vortexing and stored at −20 °C until proteomic analyses. The protein concentration was estimated using the 2-D Quant Kit (GE Healthcare) using bovine serum albumin (BSA, GE Healthcare) as a standard.

2.3.2. Protein digestion

The samples were prepared as described by Reis et al. (2016), and desalted on Vivaspin® 500 (polyethersulfone (PES) membrane, 5000 molecular weight cut-off; GE Healthcare, Little Chalfont, UK). Briefly, the membranes were saturated with 50 mM ammonium bicarbonate (Sigma-Aldrich) at pH 8.5 and centrifuged at 15,000*g* for 20 min at 8 °C. This procedure was repeated three times. Finally, 50 µL of sample was left on the membrane, collected and used for tripsin digestion.

Trypsin protein digestion was carried out as described by Calderan-Rodrigues et al. (2014). For each 50 µL of sample, 25 µL of 0.2% (v/v) RapiGest® (Waters, Milford, CT, USA) was added, vortexed for 5 s and heated in an Eppendorf Thermomixer® Comfort device at 80 °C for 15 min. Then, 2.5 µL of 100 mM dithiothreitol (DTT) was added and placed in the thermomixer at 60 °C for 30 min. The tubes were placed on ice (30 s), and 2.5 µL of 300 mM iodoacetamide (IAA) was added, followed by vortexing for 5 s and incubation in the dark for 30 min at room temperature. The digestion was carried out by adding 20 µL of trypsin solution (50 ng μL^{-1}; V5111, Promega, Madison, WI, USA) prepared in 50 mM NH_4HCO_3 pH 8.5. The samples were placed in a thermomixer at 37 °C overnight. For RapiGest precipitation, 10 µL of 5% (v/v) trifluoroacetic acid (TFA, Sigma-Aldrich) was added and vortexed for 5 s incubated at 37 °C for 90 min (without shaking) and centrifuged at 4000 × *g* for 30 min at 8 °C. Finally, 100 µL of supernatant was collected and transferred to the Total Recovery Vial (Waters, USA) for proteomics analysis.

2.3.3. Mass spectrometry analysis

A nanoAcquity UPLC connected to a Synapt G2-Si HDMS mass spectrometer (Waters) was used for ESI-LC–MS/MS analysis. Peptide mixtures were separated by liquid chromatography using 1 µL of digested samples in scouting runs. Normalization among samples was based on total ion counts. The peptide mixture was first loaded into a nanoAcquity UPLC 5 µm C18 trap column (180 µm × 20 mm) and then into a nanoAcquity HSS T3 1.8 µm analytical reversed-phase column (100 µm × 100 mm) at 600 nL min^{-1}, with a column temperature of 60 °C.

For peptide elution, the binary gradient consisted of water (Tedia, Fairfield, Ohio, USA) and 0.1% formic acid (Sigma-Aldrich, St. Louis, MO, USA) as mobile phase A, and acetonitrile (Sigma-Aldrich) and 0.1% formic acid as mobile phase B. Gradient elution started at 7% B up to 40% B in 90.09 min and from 40% B to 85% B until 94.09 min, maintained at 85% until 98.09 min, then decreased to 7% B until 100.09 min and maintained at 7% B to the end at 108.09 min.

Mass spectrometry was performed in positive and resolution mode, 35,000 FWMH, and the transfer collision energy ramped from 19 V to 45 V in high-energy mode; cone and capillary voltages of 30 V and 2800 V, respectively; and a source temperature of 70 °C. In TOF parameters, the scan time was set to 0.5 s in continuum mode with a mass range of 50–2000 Da. The human [Glu1]-fibrinopeptide B (Sigma-Aldrich) was used as an external calibrant. Data-independent acquisition (DIA) scanning with added specificity and selectivity of a non-linear 'T-wave' ion mobility device was performed ($HDMS^E$) (Heringer et al., 2015).

2.3.4. Proteomics data analysis

Progenesis QI for Proteomics Software v.2.0 (Nonlinear Dynamics, Newcastle, UK) was used to process the MSE data. The analysis was performed following parameters: one missed cleavage, minimum fragment ion per peptide equal to 1, minimum fragment ion per protein equal to three, minimum peptide per protein equal to 1, fixed modifications of carbamidomethyl (C) and variable modifications of oxidation (M) and phosphoryl (STY) groups, and a default false discovery rate (FDR) value at a 4% maximum, a score greater than five, and maximum mass errors of 10 ppm. A protein databank from *Theobroma cacao* was used, obtained from UniProt database (http://www.uniprot.org/taxonomy/3641). Label-free relative quantitative analyses were performed by the ratio of protein ion counts among contrasting samples. After the Progenesis analysis and to ensure the quality of results, only proteins present in 3 of 3 runs and with coefficients of variation less than 0.3 were selected. Proteins common to all treatments were filtered based on a fold change of log2 determined by the overall coefficient of variance for all quantified proteins across all replicates. Proteins differentially abundant were classified as up-regulated when log1.2 was 2 or greater and as down-regulated when log1.2 was −2 or less. Functional annotation based on protein gene ontology was performed using the Blast2Go software v3.0 PRO (Conesa et al., 2005) and UniProtKB (www.uniprot.org) databases. The subcellular localization of the proteins was predicted through TargetP and UniprotKB databases.

3. Results and discussion

Cacao SE represents an important method for clonal propagation and provides an *in vitro* experimental system for studying embryo development (Maximova et al., 2005). However, many cacao genotypes are recalcitrant to SE (Minyaka et al., 2008), being the embryo conversion the most inefficient step of this process (Traore et al., 2003).

As previously presented, in cacao is possible to observe two different morphological types of somatic embryos with contrasting conversion potential. (Alemanno et al., 1997; Pila, 2013). Thus, the white appearance in cacao somatic embryos is a highly desirable feature, which allow the early detection of somatic embryos with satisfactory post-embryonic performance. In Persian walnut, which also presents these different somatic embryos phenotypes, it were identified two protein bands (20 and 11.7 kDa) in white somatic embryo that were absent in translucent somatic embryos, which could be considered as a marker for normal maturation of somatic embryos (Jariteh et al., 2015).

Comparative label free proteomic analysis between white and translucent somatic embryos revealed qualitative and quantitative differences between samples. Protein data from white somatic embryos were contrasted against protein data from translucent somatic embryos. The prior list of differentially abundant proteins for white and translucent embryos presented a total of 1667 identified proteins. From the total proteins, 1339 were present in both samples and across all replicates. Proteins present in both samples were grouped according differences in abundance levels of at least 2-fold. Therefore, 25 proteins from white somatic embryos were up-regulated in relation to translucent somatic embryos, whereas 35 proteins from white somatic embryos were down-regulated when compared to translucent somatic embryos.

Beta-glucosidase (4.78 fold), NAD(P)-linked oxidoreductase (3,5

Table 1
List of proteins associated at main gen ontology (GO) functional categories and proteins with differences in abundances levels in cacao white somatic embryos and translucent somatic embryos.

Seq Name	Peptide count	Unique peptides	Confidence score	Description	Biological process	Relative abundance (white)	Relative abundance (translucent)	Ratio (W/T)	Tag expression*
Carbohydrate metabolic process									
A0A061ENV0	10	2	85.17	Beta-glucosidase 44 OS = Theobroma cacao GN = TCM_019272 PE = 3 SV = 1	Carbohydrate metabolic process	29532.59	6175.32	4.78	up
A0A061G3B6	2	2	12.06	3,4-dihydroxy-2-butanone kinase, putative OS = Theobroma cacao GN = TCM_013222 PE = 4 SV = 1	Metabolic process, phosphorylation	29532.59	6175.33	2.54	up
A0A061DYK6	4	2	33.83	Fructose-bisphosphate aldolase OS = Theobroma cacao GN = TCM_006785 PE = 3 SV = 1	Glycolytic process	1975.87	805.23	2.45	up
A0A061DST1	17	9	152.41	Sucrose synthase OS = Theobroma cacao GN = TCM_004698 PE = 3 SV = 1	Seed maturation, starch metabolic process, sucrose metabolic process	53754.75	23318.41	2.31	up
Oxidation-reduction process									
A0A061EMC5	1	1	5.87	NAD(P)-linked oxidoreductase superfamily protein, putative OS = Theobroma cacao GN = TCM_020708 PE = 4 SV = 1	Energy metabolism	2835.77	809.51	3.5	up
A0A061ECK6	3	1	28.13	Oxidoreductase, zinc-binding dehydrogenase family protein isoform 2 (Fragment) OS = Theobroma cacao GN = TCM_016876 PE = 4 SV = 1	Oxidation-reduction process	2031.49	814.44	2.49	up
A0A061FF21	2	1	16.75	Cytochrome P450, family 706, subfamily A, polypeptide 6, putative OS = Theobroma cacao GN = TCM_034411 PE = 3 SV = 1	Oxidation-reduction process	114.15	1443.66	12.65	down
S1SIP6	2	1	11.18	Cytochrome p450 79a2, putative OS = Theobroma cacao GN = TCM_046361 PE = 4 SV = 1	Oxidation-reduction process	8172.48	70744.30	8.66	down
A0A061DPK2	11	9	135.01	Geraniol dehydrogenase 1 OS = Theobroma cacao GN = TCM_000950 PE = 3 SV = 1	Oxidation-reduction process	52194.40	189685.50	3.63	down
A0A061F232	16	3	170.48	NAD(P)-linked oxidoreductase superfamily protein OS = Theobroma cacao GN = TCM_026371 PE = 4 SV = 1	Energy metabolism	3798.23	12365.15	3.26	down
A0A061G198	3	2	18.44	NADH-ubiquinone oxidoreductase-related OS = Theobroma cacao GN = TCM_015272 PE = 4 SV = 1	Oxidation-reduction process	616.01	1797.81	2.92	down
Response to stimulus									
A0A061EIR4	2	1	20.33	RAB GTPase A5E isoform 1 OS = Theobroma cacao GN = TCM_020064 PE = 3 SV = 1	Protein transport	655.66	207.98	3.15	up
A0A061DHX	2	1	11.53	RAB GTPase H1E isoform 2 (Fragment) OS = Theobroma cacao GN = TCM_001112 PE = 3 SV = 1	Protein transport, small GTPase mediated signal transduction	1552.87	6463.03	4.16	down
A0A061EWI0	7	1	55.7	Pathogenesis-related protein 10.5 OS = Theobroma cacao GN = TCM_021284 PE = 3 SV = 1	Stress response, defense response, response to biotic stimulus	9581.34	30795.06	3.21	down
A0A061FUN2	4	4	35.62	Eukaryotic aspartyl protease family protein OS = Theobroma cacao GN = TCM_012281 PE = 4 SV = 1	Response to water deprivation, systemic acquired resistance, response to abscisic acid	4271.64	11191.31	2.62	down
Protein not assigned with the selected functional groups									
A0A061FDC9	2	2	11.63	Electron transfer flavoprotein alpha isoform 1 OS = Theobroma cacao GN = TCM_030993 PE = 4 SV = 1	Energy metabolism	1837.10	197.35	9.31	up
A0A061E8P3	3	3	21.33	Nucleoporin GLE1, putative isoform 1 OS = Theobroma cacao GN = TCM_010631 PE = 4 SV = 1	Seed development, embryo sac egg cell differentiation, mitotic recombination	11575.89	1326.44	8.73	up
A0A061DYA8	4	2	28.31	PLC-like phosphodiesterases superfamily protein OS = Theobroma cacao GN = TCM_006664 PE = 4 SV = 1	Lipid metabolic process	10003.48	1753.13	5.71	up
A0A061GGL7	2	2	12.35	Nudix hydrolase isoform 1 OS = Theobroma cacao GN = TCM_027573 PE = 4 SV = 1	Metabolic process	1999.69	355.64	5.62	up
A0A061E0D3	3	2	22.88	5′-methylthioadenosine/S-adenosylhomocysteine nucleosidase 1 isoform 1 OS = Theobroma cacao GN = TCM_006702 PE = 4 SV = 1	Metabolic proecess, nucleoside metabolic process	2445.41	559.38	4.37	up
A0A061FVE9	2	1	16.11	CCHC-type integrase, putative OS = Theobroma cacao GN = TCM_013205 PE = 4 SV = 1	Metabolic process	767.25	194.01	3.95	up

(*continued on next page*)

Table 1 (*continued*)

Seq Name	Peptide count	Unique peptides	Confidence score	Description	Biological process	Relative abundance (white)	Relative abundance (translucent)	Ratio (W/T)	Tag expression*
A0A061G4G8	2	2	16.61	Tobamovirus multiplication 2A isoform 1 OS = Theobroma cacao GN = TCM_015779 PE = 4 SV = 1	Viral replication complex formation and maintenance	7142.17	1926.18	3.71	up
A0A061GZE2	3	3	17.04	Pyrimidine 2 isoform 2 OS = Theobroma cacao GN = TCM_040900 PE = 4 SV = 1	Metabolic process	5284.31	1452.04	3.64	up
A0A061EUN5	1	1	5.98	Pentatricopeptide (PPR) repeat-containing protein, putative isoform 1 OS = Theobroma cacao GN = TCM_022424 PE = 4 SV = 1		2927.88	808.59	3.62	up
A0A061GDF7	1	1	5.71	DNA binding protein, putative OS = Theobroma cacao GN = TCM_029637 PE = 4 SV = 1	Genetic information processing	2461.75	5691.06	2.31	down
A0A061FMP5	3	3	20.69	Uncharacterized protein isoform 1 OS = Theobroma cacao GN = TCM_042648 PE = 4 SV = 1		9807.04	22853.55	2.33	down
A0A061G8J4	4	1	31.11	Uncharacterized protein isoform 1 OS = Theobroma cacao GN = TCM_026904 PE = 4 SV = 1		778.31	1816.73	2.33	down
A0A061G5Y3	3	3	17.14	Hydroxymethylglutaryl-CoA synthase/HMG-CoA synthase/3-hydroxy-3-methylglutaryl coenzyme A synthase OS = Theobroma cacao GN = TCM_016163 PE = 4 SV = 1	Isoprenoid biosynthetic process	4622.52	11038.51	2.39	down
A0A061H049	6	2	59.6	Ribosomal L29 family protein OS = Theobroma cacao GN = TCM_041920 PE = 3 SV = 1	Translation	5695.28	13680.78	2.4	down
A0A061FUV3	2	2	11.75	Pathogenesis-related thaumatin superfamily protein OS = Theobroma cacao GN = TCM_012750 PE = 4 SV = 1	Stress response	5444.51	13496.39	2.48	down
A0A061DVN6	1	1	6.04	RNA-binding family protein OS = Theobroma cacao GN = TCM_005932 PE = 4 SV = 1	Photosynthetic electron transport in photosystem	12371.17	30729.27	2.48	down
A0A061GBZ8	13	12	101.33	Receptor-like protein kinase-related family protein OS = Theobroma cacao GN = TCM_029030 PE = 4 SV = 1	Phosphorylation	21252.37	52820.09	2.49	down
A0A061E6K3	1	1	5.41	Papain family cysteine protease OS = Theobroma cacao GN = TCM_006819 PE = 3 SV = 1	Proteolysis	246.55	613.90	2.49	down
A0A061E105	5	4	38.48	UDP-glycosyltransferase 73B4, putative OS = Theobroma cacao GN = TCM_007099 PE = 4 SV = 1	Carbohydrate metabolic process	17437.05	44619.83	2.56	down
A0A061DYN7	19	2	106.54	Coatomer subunit alpha OS = Theobroma cacao GN = TCM_006812 PE = 4 SV = 1	Intracellular protein transport	1570.53	4022.06	2.56	down
A0A061FHY0	3	1	22.67	Long chain acyl-CoA synthetase 9 isoform 1 OS = Theobroma cacao GN = TCM_035112 PE = 4 SV = 1		10172.22	26464.09	2.6	down
A0A061GPW5	1	1	6.04	Voltage dependent anion channel 4 OS = Theobroma cacao GN = TCM_039252 PE = 4 SV = 1	Regulation of anion transmembrane transport	202.23	531.28	2.63	down
A0A061GR28	14	1	126.93	Tubulin alpha-5 OS = Theobroma cacao GN = TCM_040014 PE = 3 SV = 1	Microtubule-based process, protein polymerization	695.34	1864.69	2.68	down
A0A061EXA3	4	2	35.23	Transport protein particle (TRAPP) component isoform 1 OS = Theobroma cacao GN = TCM_024391 PE = 4 SV = 1	Vesicle-mediated transport	2171.10	5889.62	2.71	down
A0A061ELW2	7	5	54.89	Pseudouridine synthase/archaeosine transglycosylase-like family protein OS = Theobroma cacao GN = TCM_020727 PE = 4 SV = 1	Sulfate assimilation	8263.86	22504.46	2.72	down
A0A061EQ03	4	1	32.52	Nucleic acid-binding proteins superfamily, putative isoform 2 OS = Theobroma cacao GN = TCM_021358 PE = 4 SV = 1	Genetic information processing	212.45	603.04	2.84	down
A0A061FTX7	3	2	19.54	Actin binding-like protein OS = Theobroma cacao GN = TCM_012244 PE = 4 SV = 1		3477.42	10590.96	3.05	down
A0A061ECX9	12	4	104.35	Phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent OS = Theobroma cacao GN = TCM_016784 PE = 4 SV = 1	Glucose catabolic process	2564.34	8421.97	3.28	down
A0A061FK70	1	1	5.95	Mitogen-activated protein kinase kinase kinase 21, putative OS = Theobroma cacao GN = TCM_036235 PE = 3 SV = 1	Phosphorylation	11485.45	39847.83	3.47	down
A0A061FP97	13	2	75.79	Phenylalanine ammonialyase OS = Theobroma cacao GN = TCM_043179 PE = 3 SV = 1		4311.64	17041.67	3.95	down
A0A061GQP5	8	1	62.63	Plasma membrane ATPase 4 isoform 2 OS = Theobroma cacao GN = TCM_039126 PE = 3 SV = 1	ATP biosynthetic proces	2121.72	9833.35	4.63	down

(*continued on next page*)

Table 1 (*continued*)

Seq Name	Peptide count	Unique peptides	Confidence score	Description	Biological process	Relative abundance (white)	Relative abundance (translucent)	Ratio (W/T)	Tag expression*
A0A061FP20	1	1	6.24	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein, putative OS = Theobroma cacao GN = TCM_043741 PE = 4 SV = 1	Lipid metabolic process	330.44	1891.50	5.72	down
A0A061EL22	8	1	68.58	Eukaryotic aspartyl protease family protein isoform 2 OS = Theobroma cacao GN = TCM_020529 PE = 4 SV = 1	Proteolysis	1392.43	8180.30	5.87	down
A0A061DP55	2	1	18.35	Uncharacterized protein OS = Theobroma cacao GN = TCM_002872 PE = 4 SV = 1		11425.98	78321.39	6.85	down
A0A061EQB8	8	1	47.6	Eukaryotic translation initiation factor 2 gamma subunit, GAMMA isoform 1 OS = Theobroma cacao GN = TCM_021231 PE = 4 SV = 1	Genetic information processing	25.02	448.90	17.94	down

*(up) Ratio = proportion between relative abundance of white/relative abundance of translucent somatic embryos.
(down) Ratio = proportion between relative abundance of translucent/relative abundance of white somatic embryos.

fold) and Electron transfer flavoprotein (9.31 fold) proteins were the proteins with highly significant difference in abundance in white somatic embryos compared to the translucent. In contrast, two subunits of Cytochrome P450 (12.65 and 8.66 fold) and Pathogenesis-related proteins (3.61 fold) proteins were the proteins with highly significant difference in abundance in translucent somatic embryos compared to white (Table 1).

Protein groups with differences in abundance levels were associated with GO categories on Level 2. GO functional categories indicated that proteins associated with “metabolic process”, “single-organism process”, “cellular process”, “localization”, “signaling”, “regulation biological process”, “response to stimulus” and “biological regulation” were annotated according to “biological processes” category. “Catalytic activity”, “binding”, “structural molecular activity” and “electron carrier activity” were the most representative categories annotated according to “molecular function” category. In addition, “cell part”, “membrane”, “cell”, “membrane part”, “organelle part”, “macromolecular complex” and “supramolecular fiber” were the main annotated categories according to “cellular component” category (Fig. 2).

The up- and down-regulated proteins showed a similar number of proteins into the groups related to “biological processes” and “molecular function” categories, while the up-regulated proteins in white somatic embryos were the most populated into “supramolecular fiber” group, which is classified into “cellular component” category.

The “supramolecular fiber” is the fibrillar network outside the outer periclinal walls, which is known as the extracellular matrix surface network (ECMSN) that is characteristic for SE (Šamaj et al., 2005). During zygotic and SE, the proteins associated with this network have remarkable physiology functions, attributable to signal transduction, formation of tensions influencing cell shape, cell proliferation, cell differentiation and regulation of cell–cell (apoplastic) and cell to cell (symplastic) information flow (Bobák et al., 2004; van Engelen and de Vries, 1992). The detailed significance of this ECMSN proteins is still under study, but it has been suggested that these proteins participate during embryo development (Chapman et al., 2000). Several studies have been showed that ECMSN proteins can be considered a specific molecular marker for embryogenic competence and they also play an important role in intracellular and intercellular signaling, and control the cell and division and cell expansion during embryogenic development (Bobák et al., 2004; Dubois et al., 1992; Pilarska et al., 2014).

The high number of proteins into “supramolecular fiber” group offer speculative insights that related to ECMSN proteins with suitable somatic embryos development in cacao. Nevertheless, understanding the regulation and participation of these ECMSN proteins in this process requires significant efforts.

Functional classification based on protein Gene Ontology (GO) was performed. Protein annotation was carried out according to biological processes (Table 1). Some proteins were associated with various biological processes. In the present study, a comparative label free proteomic analysis was performed in order to investigate the differences in conversion ability between white and translucent normal somatic embryos. The biological significance associated to biochemical functions of proteins that were differentially abundant between the two types of normal somatic embryos, are discussed in the terms of the 3 main functional GO categories: “carbohydrate metabolic process”, “oxidation-reduction process” and “response to stimulus”.

3.1. Carbohydrate metabolic process

Proteins associated to carbohydrate metabolism (Table 1) were significantly up-regulated in white somatic embryos when compared to translucent somatic embryos. Within this functional category, enzymes involved in sucrose metabolism (sucrose synthase, 3.21-fold) and in energy metabolism (beta-glucosidase, 4.78-fold), fructose-bisphosphate aldolase, 3.45-fold and 3,4-dihydroxy-2-butanone kinase, 2.54-fold were identified.

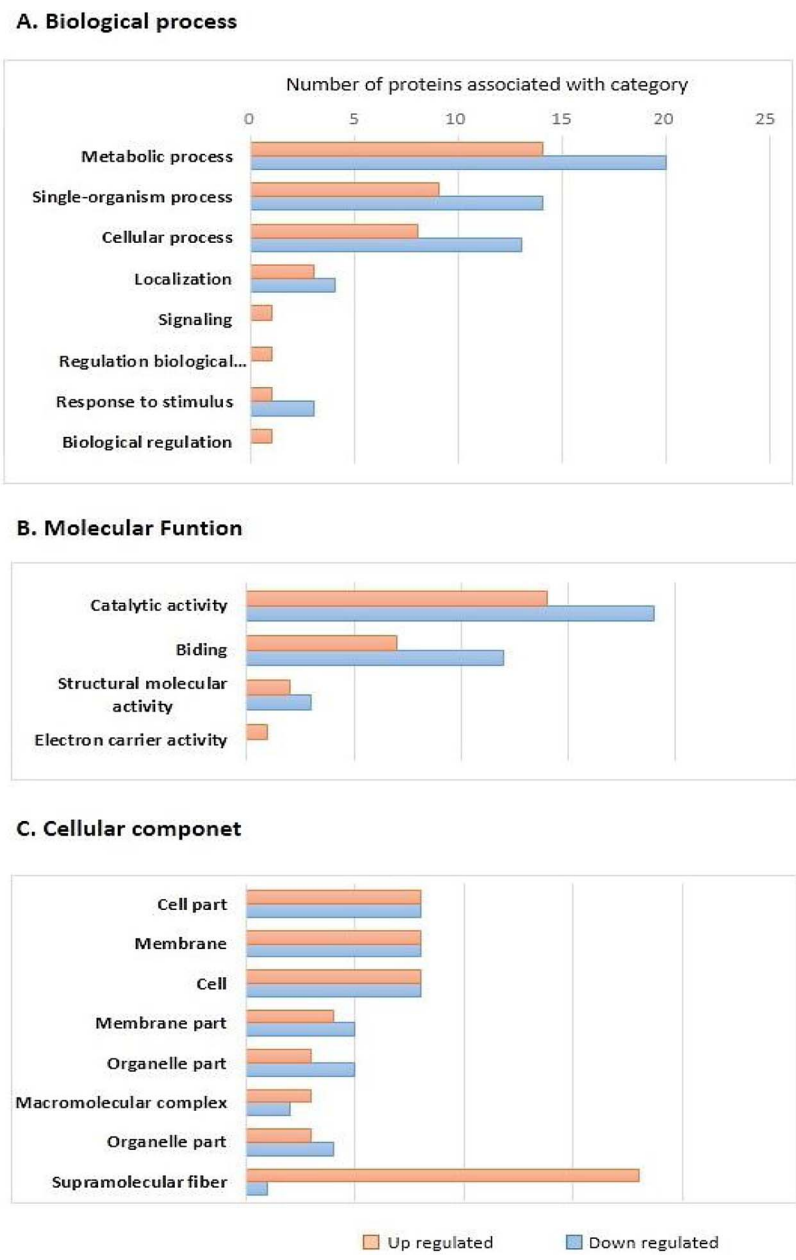

Fig. 2. Functional classification of proteins with difference in abundance level in cacao somatic embryos by Blast2GO software based on universal gene ontology (GO) annotation terms. Up-regulated and down-regulated proteins in white somatic embryos compared to translucent somatic embryos. The proteins were related to at least one annotation term within the GO category: (A) Biological process. (B) Molecular Function. (C) Cellular component. The histograms represent the number of proteins associated to level 2 GO categories.

Sucrose can be incorporated into the cell metabolism only after the hydrolysis (Tognetti et al., 2013); the first step is the cleavage of the glycosidic bond by either sucrose synthase (SuSy) or invertase (Inv) resulting in the hexoses production. These hexoses are essential energy sources for the deposition of different storage products and tissue growth (Rolletschek et al., 2004).

Somatic embryo development is a complex multi-step process which demand high energy (Dinakar et al., 2012; Iraqi and Tremblay, 2001; Konrádová et al., 2002; Lipavská et al., 2000). During early stages, establishment and growth of embryo structures prevails (Hakman, 1993) while later stages, such as maturation, are characterized by deposition of storage compounds (Iraqi et al., 2005; Konrádová et al., 2002; Lipavská and Konrádová, 2004; Verdier and Thompson, 2008). Thus, a transition from metabolic to storage status has been proved to be accompanied by changes in activities of sucrose metabolism enzymes (Iraqi, 2001). Metabolic status is mainly characterized by high Inv activities (Konrádová et al., 2002; Winter and Huber, 2000), while storage status is characterized by SuSy activity (Businge et al., 2013; Déjardin et al., 1997a,b; Yaseen et al., 2013). In addition to a function in starch biosynthesis, SuSy is also correlated with cell wall synthesis (Delmer and Amor, 1995; Koch, 2004; Tognetti et al., 2013) and play a role in the respiratory pathway (Ishizaki et al., 2006).

In the present study, SuSy proteins were up-regulated in white somatic embryos (2.31 fold) in comparison to translucent somatic embryos. Previous, comparative proteomics analysis of cacao somatic and zygotic embryogenesis showed that SuSy enzyme was expressed in lower abundance in somatic embryos (Noah et al., 2013). This fact was ascribed to a possible disturbance in carbohydrate metabolism, resulting in irregularities in storage compound and cell wall metabolism. However, these results are possibly a consequence of culture conditions. Corroborating our results, SuSy had a prominent role in the transition from metabolic to storage status in Norway spruce. Therefore, these authors concluded that low Inv activity accompanied by high SuSy activity could be considered as an indicator of embryo quality (Konrádová et al., 2002).

Likewise, studies in Spruce embryo development showed that an imbalance on Inv and SuSy activities by replacing sucrose by glucose and fructose in the maturation medium, resulted in an alteration of

storage accumulation and, consequently, in a reduction of somatic embryo production and germination capacity (Iraqi, 2001). These findings suggest that a high SuSy activity result in to a suitable starch accumulation in somatic embryo. Consequently, the deposited starch in the mature somatic embryo will expected to provide energy to the embryo metabolism during conversion and thereby enhance plantlets vigor.

In the present work, specific enzymes for glycolysis (beta-glucosidase (4.78-fold), fructose-bisphosphate aldolase (3.45-fold) and 3,4-dihydroxy-2-butanone kinase (2.54-fold)) were up-regulated in white somatic embryos. In a similar way, Noah et al. (2013) and Niemenak et al. (2015) found that proteins involved in metabolic processes, including glycolysis were expressed in zygotic and somatic embryos of cacao, suggesting that glycolysis enzymes activity could be associated with the energy demand required for embryo development in the storage phase.

Beta-glucosidase, one of the enzymes responsible for glycolysis, was up-regulated in white somatic embryos. This enzyme plays important roles in plant physiology, including the conversion of plant growth regulators such as cytokinin, gibberellin and auxin, into an active form (Brzobohaty et al., 1993) as well as the activation of chemical defense compounds (Jones et al., 2006; Halkier and Gershenzon, 2006; Suzuki et al., 2006). Fructose-bisphosphate aldolase was also found up-regulated in white somatic embryos. This enzyme plays different roles in the processes of plant cell proliferation, growth, development, photosynthesis and stress resistance (Konishi et al., 2004; Lu et al., 2012; Zeng et al., 2013).

These results suggest a possible positive correlation between the conversion potential of cacao somatic embryos with enzymes related with carbohydrate metabolic process and as a result, the storage compounds accumulation. In somatic embryos of Persian walnut, it was observed that the white somatic embryos accumulate a great number of starch grains in epidermal cells, and the translucent lack of them (Jariteh et al., 2015). Thus, we can suggest that an increase in specific proteins in the somatic embryos could result in an increase in metabolic proteins (enzymes) accompanying the storage compounds accumulation, which guarantees the conversion ability. However, more studies need to be performed to test this hypothesis.

3.2. Oxidation-reduction process

Recent evidences revealed that cellular signaling pathways are regulated by the intracellular redox state (Kamata and Hirata, 1999). The plant cell apparently has redox sensors, which detect deviations from redox homeostasis prior to the development of major imbalances (Dietz and Scheibe, 2004). These redox changes affect growth and development through signaling networks due to the reprogramming of the transcriptome, proteome and metabolome (Dietz, 2014; Go and Jones, 2013). The model for redox homeostasis depends on the reactive oxygen species (ROS) activity which, acts as a metabolic interface for signals derived from metabolism and the environment (Dietz, 2003; Foyer and Noctor, 2005). In SE, it has been shown that redox state represents a key metabolic switch which triggers the induction phase, by means of the regulation of embryogenic competence (Dos Santos et al., 2016; Go and Jones, 2013; Kamata and Hirata, 1999; Mohamed and Stasolla, 2015; Stasolla, 2010; Vieira et al., 2012) and embryo development (Pullman et al., 2015; Stasolla et al., 2004).

In the present study, the white somatic embryos proteins associated with redox process category (Table 1) were both up- and down-regulated, although these groups were represented by different proteins. On one hand, the up-regulated proteins were represented byNAD(P)-linked oxidoreductase superfamily protein (2.49-fold) and oxidoreductase, zinc-binding dehydrogenase (3.5-fold). On the other hand, the down-regulated proteins were represented by NADH-ubiquinone oxidoreductase-related (2.92 fold), NAD(P)-linked oxidoreductase superfamily protein (3.26-fold), Geraniol dehydrogenase 1 (3.63-fold), Cytochrome p450 79a2, putative (8.66 fold) and Cytochrome P450, family 706, subfamily A, polypeptide 6, putative (12.5 fold).

These results revealed the simultaneous presence of both oxidized and reduced forms of electron carriers in somatic embryos. Enzymes such NAD(P)-linked oxidoreductase, NADH-ubiquinone oxidoreductase-related and oxidoreductase, zinc-binding dehydrogenase are continuously produced not only as products of various metabolic processes but also in response to specific signals from culture conditions (Downs and Heckathorn, 1998; Gäbler et al., 1994; Ishizaki et al., 2006; Kamata and Hirata, 1999; Kocsy et al., 2013). This enzymes-set has also been found present in different somatic embryos developmental stages in other species (Lindemann and Luckner, 1997; Morel et al., 2014), and the equilibrium promote the optimal growth and development (Kocsy et al., 2013). Accordingly, the imbalance of these enzymes can affect both redox state of antioxidants and ROS formation, which, through a redox signaling pathway, leads to the metabolism reprogramming of many compounds, including sulphur, nitrogen and carbohydrates containing organic compounds (Kocsy et al., 2013).

Another proteins widely up-regulated in translucent somatic embryos was Cytochrome P450 (12.65 fold and 8.66 fold). Cytochromes P450 represent a family metabolic enzymes, found in all kingdoms showing expressive diversity in their chemical reactions (Bolwell et al., 1994; Mizutani, 2012; Mizutani and Ohta, 2010; Schuler et al., 2006). Cytochromes P450 (P450s) are involved in essential housekeeping functions and metabolism of most phytohormones, including auxins, gibberellins, cytokinins, brassinosteroids, abscisic acid, and jasmonic acid, as well as many secondary metabolites (Werck-Reichhart et al., 2002). P450 also play a significant role in plant defense responses (Matthes et al., 2011; Schuler et al., 2006). Redox regulation is a central control element in P450 metabolic pathways, since cytochrome P450 oxygenase reactions require cytochrome P450 reductase to transfer two electrons from NADPH to their substrate (Xu et al., 2015).

Thus, it can be suggested that the up-regulation of P450 in translucent somatic embryos, when compared to white somatic embryos, might generate a redox imbalance, which often trigger an oxidative burst (De Gara et al., 2010; Dietz, 2003; Foyer and Noctor, 2005).

In *Arabidopsis*, it was suggested that the overexpression of P450 proteins was associated with seed growth (Fang et al., 2012). Although P450 proteins functions have been underreported in SE process, based in our results, it could be suggested that P450 s are closely involved in metabolic pathways that regulate cacao somatic embryo development. However, more studies are necessary to define P450 proteins functions during cacao SE.

An intensive redox activity is apparently necessary for optimal somatic embryo maturation, if enzymatic apparatus are carefully balanced. Any imbalance in redox homeostasis have crucial consequences into cell function and it may induce severe damage in the embryo development and therefore in the embryo quality. In this sense, our results suggest that white somatic embryos could contain a more adequate enzymatic apparatus to regulate cellular redox homeostasis.

3.3. Response to stimulus

The presence of higher levels of proteins associated to response to stimulus may reflect a cell response to signal molecules for embryo development and their adaptation to environmental conditions during *in vitro* culture (Jin et al., 2014; Zeng et al., 2007). The perception of/and response to these stimulus sets off various signal cascades that eventually might result in healthy somatic embryos (Fehér, 2003, 2015; Zimmerman, 1993).

In our results, we observed the expression of two subunits of RAB GTPase protein: RAB GTPase A5E isoform 1, which was up-regulated (3.15 fold), and RAB GTPase H1E isoform 2 (Fragment), which was down-regulated (4.12 fold). RAB GTPases proteins are regulators of intracellular vesicular transport and the trafficking of proteins between different organelles of the endocytic and secretory pathways (Zerial and

McBride, 2001). Membrane trafficking is required for a variety of cellular functions, such as storage-protein accumulation, cell differentiation and growth, secretion of protein and polysaccharide components of the cell wall and cell plate, and for the morphogenesis, which depends on spatial and quantitative control of cell expansion (Lycett, 2008; Rutherford and Moore, 2002).

In addition, it has been evidenced that Rab GTPases expression is modulated in response to variations in levels of phytohormones such as ethylene (Moshkov et al., 2003) and abscisic acid during plant development and germinating seedlings (Cui et al., 2013; Nishimura, 2004). In maritime pine embryogenesis, RAB GTPases were expressed in early stages of embryo development; the transcript was detected in early developmental stages of zygotic embryogenesis and the amount of transcript was progressively reduced in later stages (Gonçalves et al., 2007; Tzafrir et al., 2002).

In the present study, RAB GTPases were down-regulated in white somatic embryos when compared to translucent embryos. RAB GTPases seem to play a role during embryo development; however, the higher levels of these proteins are related with early developmental embryo stage. Thus, we can suggest that the translucent somatic embryo were unable to follow a complete developmental program, which is a requisite for embryo conversion ability.

Also in the present study, eukaryotic aspartyl protease (APs) family protein (2.62-fold) showed a similar expression pattern with pathogenesis-related (PR) proteins (3.21-fold). Both were up-regulated in translucent somatic embryos compared to white somatic embryos.

Aspartyl protease has been studied in different plant species. Nevertheless, their biological functions are not as well identified. In general, it is related with plant senescence, programmed cell death, stress responses and plant reproduction (Simões and Faro, 2004) Besides, plant aspartyl protease participation in storage protein degradation during the mobilization of reserve proteins in seed germination was proposed in wheat (Belozersky et al., 1989), rice (Asakura et al., 1997) and cacao (Voigt et al., 1997). On the other hand, the high activity of these enzymes is common in non-germinated seeds, but nevertheless in suitable concentrations their activity has been proved to be essential during the seed development and germination (Palma et al., 2002).

During *Arabidopsis* embryogenesis, an aspartic protease was identified, which play a significant role as an anti-cell-death component by processing and activating a polypeptide that functions as a survival factor (Ge et al., 2005). Comparative proteomic studies of cacao somatic and zygotic embryos revealed that, aspartic proteases were more abundant in the torpedo stage of zygotic embryos. Therefore it was suggested that this protein could be a marker of the onset of embryo maturation (Niemenak et al., 2015; Noah et al., 2013) since that aspartic protease play important role for storage protein processing during seed development (D'Hond et al., 1993; Voigt et al., 1997).

In the present study, we analyzed mature somatic embryos. However, the accumulation of aspartic protease in translucent somatic embryo might be an indication that although both somatic embryos types were collected in the same morphological developmental stage, they may not be in equivalent physiological stages. Translucent somatic embryo has accumulated aspartic protease proteins in similar patterns with early zygotic embryos.

Pathogenesis-related (PR) proteins have been identified as produced by the host plant, but induced by several pathogens (van Loon, 1985). However, recent evidences show that these proteins display additional functions, including response to environmental stress and oxidative signals (Mur et al., 2004; Sabater-Jara et al., 2014), hormones signaling (Sessa et al., 1995), role in developmental processes and enzymatic activities in secondary metabolism (Liu and Ekramoddoullah, 2006). Direct SE in *Cichorium* was accompanied by an increase in the level on PR proteins expression in the culture medium, suggesting that these proteins could be correlated with SE process (Helleboid, 2000).

In fact, plant somatic cells respond to biotic and abiotic stress factors by activating an array of defense mechanisms, which, switch their developmental program to a specific physiological state that allows the reprogramming of gene expression and therefore the acquisition of embryonic competence (Sabater-Jara et al., 2014; van Loon, 1985). Previous studies by Noah et al. (2013) and Niemenak et al. (2015) found that cacao somatic embryos were more stressed than zygotic embryos and PR proteins were expressed in higher abundance. In addition, a large number of differentially regulated genes that encode for transcription factors were related to stress responses in cacao somatic embryos when compared to zygotic embryos, suggesting that restrict cotyledon development in somatic embryos is related with gene stress responses expression (Maximova et al., 2014). Based on these results, it could be hypothesized that PR proteins expression are involved in cacao somatic embryo maturation pathways, where a subtle imbalance could lead to abnormal embryo development.

In the present study, the higher abundance of proteins as Aspartyl protease and RAB GTPases in translucent somatic embryos suggests that their developmental physiological stages correspond to immature somatic embryo. On the other hand, the presence of higher levels of stress response proteins may reflect a response to stressful *in vitro* conditions, since that an increased level of these proteins may generate signal molecules for embryo maturation and/or influence metabolism pathways.

3.4. Protein not assigned with the selected functional groups

In this work, electron transfer flavoprotein alpha isoform 1 (ETF) (9.31-fold) was a highly up-regulated protein in white somatic embryos that was not assigned in the main selected functional groups. ETF belongs to "energy metabolism" category and is associated with oxidative phosphorylation.

During oxidative phosphorylation, ETF protein serves as a specific electron acceptor for several dehydrogenases, including five acyl-CoA dehydrogenases, glutaryl-CoA and sarcosine dehydrogenase. ETF protein transfers electrons to the main mitochondrial respiratory chain via ETF-ubiquinone oxidoreductase. These redox reactions release energy, which is used to form ATP (Christie et al., 2015). In addition, ETF protein was found in plants under stress conditions and when carbohydrates as respiratory substrates are limiting. The ETF protein provides an alternate electron supply to the mitochondrial electron transport chain, either by supplying alternative substrates: protein and lipids or by promoting the metabolism of toxic products thereof, or both (Araújo et al., 2011; Buer et al., 2013; Ishizaki et al., 2006).

Thus, the higher abundance of ETF alpha isoform 1 in white somatic embryos suggest an intensive energy metabolism activity, which is induced in order to break down of the carbohydrate supply in culture medium. On the other hand, we cannot exclude the possibility that under stress conditions or when the carbohydrate supply is inadequate, the metabolism of plant cells is modified and alternative respiratory substrates are metabolized to carry out the embryo development.

4. Conclusion

In this study, we report for the first time a comparative proteomic data between two different cacao somatic embryos types with relevant differences in somatic embryo conversion potential. Cacao SE is multistep complex process where embryo induction, development and maturation depend on a series of factors encompassing salt composition, plant growth regulators, culture conditions and internal response to this stimuli. The proteomic analysis showed a large number of differentially regulated proteins between withe and translucent somatic embryos.

Considering white somatic embryos as model, we found important differences between the accumulation patterns of the proteins related to carbohydrate metabolic process, which are involved in synthesis storage compounds. The accumulation of storage product is an important event during embryo maturation and could be considered a marker for

somatic embryo quality. At the same time, stress responses and oxidation-reduction proteins were down-regulated in white somatic embryos. Congruent with these results, it was observed that the same proteins were down-regulated in later development stages of zygotic embryos, but in later somatic embryo development these proteins continued up-regulated. Based on these data, we can suggest that the cacao somatic embryos are able to develop efficient redox homeostasis system for controlling oxidative stress in order to reach the full development, which determines the conversion potential.

In addition, we hypothesized that despite the morphological differences between the two types of somatic embryos there are also relevant physiological differences. Translucent somatic embryos apparently do not reach physiological maturity, which compromises the conversion potential. This feature could be triggered by sub-optimal media composition or inadequate culture conditions. Knowledge about the differences in white somatic embryos compared to translucent somatic embryos might serve as a basis for manipulation of the culture conditions in order to increase the frequency of white somatic embryos and improve the cacao SE protocol.

Based in our results, and in order to improve the cacao SE protocol, we can suggest investigations focusing in redox homeostasis in the culture medium, through the supplementation of chemical agents with antioxidant activity, such as glutathione, and/or the interaction with different carbohydrate sources.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.scienta.2017.10.005.

References

Alemanno, L., Berthouly, M., Michaux-Ferriere, N., 1997. A comparison between Theobroma cacao L. zygotic embryogenesis and somatic embryogenesis from floral explants. Vitr. Cell. Dev. Biol. Plant 33, 163–172. http://dx.doi.org/10.1007/s11627-997-0016-8.

Araújo, W.L., Tohge, T., Ishizaki, K., Leaver, C.J., Fernie, A.R., 2011. Protein degradation – an alternative respiratory substrate for stressed plants. Trends Plant Sci. 16, 489–498. http://dx.doi.org/10.1016/j.tplants.2011.05.008.

Asakura, T., Watanabe, H., Abe, K., Arai, S., 1997. Oryzasin as an aspartic proteinase occurring in rice seeds: purification, characterization, and application to milk clotting. J. Agric. Food Chem. 45, 1070–1075. http://dx.doi.org/10.1021/jf960582x.

Belozersky, M.A., Sarbakanova, S.T., Dunaevsky, Y.E., 1989. Aspartic proteinase from wheat seeds: isolation, properties and action on gliadin. Planta 177, 321–326. http://dx.doi.org/10.1007/BF00403589.

Bobák, M., Šamaj, J., Hlinková, E., Hlavačka, A., Ovečka, M., 2004. Extracellular matrix in early stages of direct somatic embryogenesis in leaves of drosera spathulata. Biol. Plant. 47, 161–166. http://dx.doi.org/10.1023/B:BIOP.0000022245.64929.8b.

Bolwell, G.P., Bozak, K., Zimmerlin, A., 1994. Plant cytochrome P450. Phytochemistry 37, 1491–1506. http://dx.doi.org/10.1016/S0031-9422(00)89567-9.

Brzobohaty, B., Moore, I., Kristoffersen, P., Bako, L., Campos, N., Schell, J., Palme, K., 1993. Release of active cytokinin by a beta-glucosidase localized to the maize root meristem. Science 262, 1051–1054. http://dx.doi.org/10.1126/science.8235622.

Buer, C.S., Kordbacheh, F., Truong, T.T., Hocart, C.H., Djordjevic, M.A., 2013. Alteration of flavonoid accumulation patterns in transparent testa mutants disturbs auxin transport, gravity responses, and imparts long-term effects on root and shoot architecture. Planta 238, 171–189. http://dx.doi.org/10.1007/s00425-013-1883-3.

Businge, E., Bygdell, J., Wingsle, G., Moritz, T., Egertsdotter, U., 2013. The effect of carbohydrates and osmoticum on storage reserve accumulation and germination of Norway spruce somatic embryos. Physiol. Plant. 149, 273–285. http://dx.doi.org/10.1111/ppl.12039.

Calderan-Rodrigues, M.J., Jamet, E., Bonassi, M.B.C.R., Guidetti-Gonzalez, S., Begossi, A.C., Setem, L.V., Franceschini, L.M., Fonseca, J.G., Labate, C.A., 2014. Cell wall proteomics of sugarcane cell suspension cultures. Proteomics 14, 738–749. http://dx.doi.org/10.1002/pmic.201300132.

Carpentier, S.C., Witters, E., Laukens, K., Deckers, P., Swennen, R., Panis, B., 2005. Preparation of protein extracts from recalcitrant plant tissues: an evaluation of different methods for two-dimensional gel electrophoresis analysis. Proteomics 5, 2497–2507. http://dx.doi.org/10.1002/pmic.200401222.

Chapman, A., Helleboid, S., Blervacq, A.S., Vasseur, J., Hilbert, J.L., 2000. Removal of the fibrillar network surrounding Cichorium somatic embryos using cytoskeleton inhibitors: analysis of proteic components. Plant Sci. 150, 103–114. http://dx.doi.org/10.1016/S0168-9452(99)00185-5.

Christie, J.M., Blackwood, L., Petersen, J., Sullivan, S., 2015. Plant flavoprotein photoreceptors. Plant Cell Physiol. 56, 401–413. http://dx.doi.org/10.1093/pcp/pcu196.

Conesa, A., Götz, S., García-Gómez, J.M., Terol, J., Talón, M., Robles, M., 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21, 3674–3676. http://dx.doi.org/10.1093/bioinformatics/bti610.

Cui, S., Fukao, Y., Mano, S., Yamada, K., Hayashi, M., Nishimura, M., 2013. Proteomic analysis reveals that the rab GTPase RabE1c is involved in the degradation of the peroxisomal protein receptor PEX7 (Peroxin 7). J. Biol. Chem. 288, 6014–6023. http://dx.doi.org/10.1074/jbc.M112.438143.

Déjardin, A., Rochat, C., Maugenest, S., Boutin, J.-P., 1997a. Purification, characterization and physiological role of sucrose synthase in the pea seed coat (Pisum sativum L.). Planta 201, 128–137. http://dx.doi.org/10.1007/BF01007697.

Déjardin, A., Rochat, C., Wuilleme, S., Boutin, J.P., 1997b. Contribution of sucrose synthase, ADP-glucose pyrophosphorylase and starch synthase to starch synthesis in developing pea seeds. Plant Cell Environ. 20, 1421–1430. http://dx.doi.org/10.1046/j.1365-3040.1997.d01-32.x.

D'Hond, K., Bosch, D., Van Damme, J., Goethals, M., Vandekerckhove, J., Krebbers, E., 1993. An aspartic proteinase present in seeds cleaves Arabidopsis 2 S albumin precursors in vitro. J. Biol. Chem. 268, 20884–20891.

Delmer, D.P., Amor, Y., 1995. Cellulose biosynthesis. Plant Cell 7, 987–1000. http://dx.doi.org/10.1146/annurev.pp.38.060187.001355.

De Gara, L., Locato, V., Dipierro, S., de Pinto, M.C., 2010. Redox homeostasis in plants. The challenge of living with endogenous oxygen production. Respir. Physiol. Neurobiol. 173, S13–S19. http://dx.doi.org/10.1016/j.resp.2010.02.007.

Dietz, K.-J., Scheibe, R., 2004. Redox regulation: an introduction. Physiol. Plant. 120, 1–3. http://dx.doi.org/10.1111/j.0031-9317.2004.0277.x.

Dietz, K.-J., 2003. Redox homeostasis in plant cells. Int. Rev. Cytol. 228, 141–193.

Dietz, K.-J., 2014. Redox regulation of transcription factors in plant stress acclimation and development. Antioxid. Redox Signal. 0, 1–17. http://dx.doi.org/10.1089/ars.2013.5672.

Dinakar, C., Djilianov, D., Bartels, D., 2012. Photosynthesis in desiccation tolerant plants: energy metabolism and antioxidative stress defense. Plant Sci. 182, 29–41. http://dx.doi.org/10.1016/j.plantsci.2011.01.018.

Dodeman, V.L., Ducreux, G., Kreis, M., 1997. Zygotic embryogenesis versus somatic embryogenesis. J. Exp. Bot. 48, 1493–1509. http://dx.doi.org/10.1093/jxb/48.8.1493.

Dos Santos, A.L.W., Elbl, P., Navarro, B.V., de Oliveira, L.F., Salvato, F., Balbuena, T.S., Floh, E.I.S., 2016. Quantitative proteomic analysis of Araucaria angustifolia (Bertol.) Kuntze cell lines with contrasting embryogenic potential. J. Proteomics 130, 180–189. http://dx.doi.org/10.1016/j.jprot.2015.09.027.

Downs, C.A., Heckathorn, S.A., 1998. The mitochondrial small heat-shock protein protects NADH:ubiquinone oxidoreductase of the electron transport chain during heat stress in plants. FEBS Lett. 430, 246–250. http://dx.doi.org/10.1016/S0014-5793(98)00669-3.

Driver, J.A., Kuniyuki, A.H., 1984. In vitro propagation of Paradox Walnut root stock. HortSci. Publ. Am. Soc. Hortic. Sci. 18, 506–509.

Dubois, T., Dubois, J., Guedira, M., Dlop, A.L., Vasseur, J., 1992. Sem characterization of an extracellular matrix around somatic proembryos in roots of cichorium. Ann. Bot. 70, 119–124. http://dx.doi.org/10.1093/oxfordjournals.aob.a088447.

Fang, W., Wang, Z., Cui, R., Li, J., Li, Y., 2012. Maternal control of seed size by EOD3/CYP78A6 in *Arabidopsis thaliana*. Plant J. 70, 929–939. http://dx.doi.org/10.1111/j.1365-313X.2012.04907.x.

Fehér, A., 2003. Transition of somatic plant cells to an embryogenic state. Plant Cell Tissue Organ Cult. 74, 201–228. http://dx.doi.org/10.1023/A:1024033216561.

Fehér, A., 2015. Somatic embryogenesis — Stress-induced remodeling of plant cell fate. Biochim. Biophys. Acta Gene Regul. Mech. 1849, 385–402. http://dx.doi.org/10.1016/j.bbagrm.2014.07.005.

Foyer, C.H., Noctor, G., 2005. Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. Plant Cell 17, 1866–1875. http://dx.doi.org/10.1105/tpc.105.033589.

Gäbler, L., Herz, U., Brennicke, A., Grohmann, L., Liddell, A., Leaver, C.J., Schröder, W., 1994. The 42.5 kDa subunit of the NADH: Ubiquinone oxidoreductase (complex I) in higher plants is encoded by the mitochondrial nad7 gene. Mol. Gen. Genet. 244, 33–40. http://dx.doi.org/10.1007/BF00280184.

Ge, X., Dietrich, C., Matsuno, M., Li, G., Berg, H., Xia, Y., 2005. An Arabidopsis aspartic protease functions as an anti-cell-death component in reproduction and embryogenesis. EMBO Rep. 6, 282–288. http://dx.doi.org/10.1038/sj.embor.7400357.

Go, Y.M., Jones, D.P., 2013. The redox proteome. J. Biol. Chem. 288, 26512–26520. http://dx.doi.org/10.1074/jbc.R113.464131.

Gonçalves, S., Cairney, J., Rodríguez, M.P., Cánovas, F., Oliveira, M., Miguel, C., 2007. PpRab1, a Rab GTPase from maritime pine is differentially expressed during embryogenesis. Mol. Genet. Genom. 278, 273–282. http://dx.doi.org/10.1007/s00438-007-0247-8.

Halkier, B.A., Gershenzon, J., 2006. Biology and biochemistry of glucosinolates. Annu. Rev. Plant Biol. 57, 303–333. http://dx.doi.org/10.1146/annurev.arplant.57.032905.105228.

Hakman, I., 1993. Embryology in Norway spruce (Picea abies). An analysis of the composition of seed storage proteins and deposition of storage reserves during seed development and somatic embryogenesis. Physiol. Plant. 87, 148–159. http://dx.doi.org/10.1111/j.1399-3054.1993. tb00137.x.

Harada, J.J., Belmonte, M.F., Kwong, R.W., 2010. Plant Embryogenesis (Zygotic and Somatic). Encyclopedia of Life Sciences. John Wiley & Sons, Ltd, Chichester, UK. http://dx.doi.org/10.1002/9780470015902.

Helleboid, S., 2000. Three major somatic embryogenesis related proteins in Cichorium identified as PR proteins. J. Exp. Bot. 51, 1189–1200. http://dx.doi.org/10.1093/jexbot/51.348.1189.

Heringer, A.S., Barroso, T., Macedo, A.F., Santa-Catarina, C., Souza, G.H.M.F., Floh, E.I.S., de Souza-Filho, G.A., Silveira, V., 2015. Label-free quantitative proteomics of

embryogenic and non-embryogenic callus during sugarcane somatic embryogenesis. PLoS One 10, e0127803. http://dx.doi.org/10.1371/journal.pone.0127803.
Iraqi, D., Tremblay, F.M., 2001. The role of sucrose during maturation of black spruce (Picea mariana) and white spruce (Picea glauca) somatic embryos. Physiol. Plant. 111, 381–388. http://dx.doi.org/10.1034/j.1399-3054.2001.1110316.x.
Iraqi, D., Le, Quy, Lamhamedi, V., Tremblay, M.S., 2005. Sucrose utilization during somatic embryo development in black spruce: involvement of apoplastic invertase in the tissue and of extracellular invertase in the medium. J. Plant Physiol. 162, 115–124. http://dx.doi.org/10.1016/j.jplph.2003.06.001.
Iraqi, D., 2001. Analysis of carbohydrate metabolism enzymes and cellular contents of sugars and proteins during spruce somatic embryogenesis suggests a regulatory role of exogenous sucrose in embryo development. J. Exp. Bot. 52, 2301–2311. http://dx.doi.org/10.1093/jexbot/52.365.2301.
Ishizaki, K., Schauer, N., Larson, T.R., Graham, I.A., Fernie, A.R., Leaver, C.J., 2006. The mitochondrial electron transfer flavoprotein complex is essential for survival of Arabidopsis in extended darkness. Plant J. 47, 751–760. http://dx.doi.org/10.1111/j.1365-313X.2006.02826.x.
Jariteh, M., Ebrahimzadeh, H., Niknam, V., Mirmasoumi, M., Vahdati, K., 2015. Developmental changes of protein, proline and some antioxidant enzymes activities in somatic and zygotic embryos of Persian walnut (Juglans regia L.). Plant Cell, Tissue Organ Cult. 122, 101–115. http://dx.doi.org/10.1007/s11240-015-0753-z.
Jin, F., Hu, L., Yuan, D., Xu, J., Gao, W., He, L., Yang, X., Zhang, X., 2014. Comparative transcriptome analysis between somatic embryos (SEs) and zygotic embryos in cotton: Evidence for stress response functions in SE development. Plant Biotechnol. J. 12, 161–173. http://dx.doi.org/10.1111/pbi.12123.
Jones, A.M.E., Thomas, V., Bennett, M.H., Mansfield, J., Grant, M., 2006. Modifications to the a *Arabidopsis* defense proteome occur prior to significant transcriptional change in response to inoculation with pseudomonas syringae. Plant Physiol . 142, 1603–1620. http://dx.doi.org/10.1104/pp.106.086231.
Kamata, H., Hirata, H., 1999. Redox regulation of cellular signalling. Cell. Signal. 11, 1–14. http://dx.doi.org/10.1016/S0898-6568(98)00037-0.
Koch, K., 2004. Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. Curr. Opin. Plant Biol. 7, 235–246. http://dx.doi.org/10.1016/j.pbi.2004.03.014.
Kocsy, G., Tari, I., Vankova, R., Zechmann, B., Gulyas, Z., Poor, P., Galiba, G., 2013. Redox control of plant growth and development. Plant Sci. 211, 77–91. http://dx.doi.org/10.1016/j.plantsci.2013.07.004.
Konishi, H., Yamane, H., Maeshima, M., Komatsu, S., 2004. Characterization of fructose-bisphosphate aldolase regulated by gibberellin in roots of rice seedling. Plant Mol. Biol. 56, 839–848. http://dx.doi.org/10.1007/s11103-004-5920-2.
Konrádová, H., Lipavská, H., Albrechtová, J., Vreugdenhil, D., 2002. Sucrose metabolism during somatic and zygotic embryogeneses in Norway spruce: content of soluble saccharides and localisation of key enzyme activities. J. Plant Physiol. 159, 387–396. http://dx.doi.org/10.1078/0176-1617-00624.
Leljak-Levanić, D., Bauer, N., Mihaljević, S., Jelaska, S., 2004. Changes in DNA methylation during somatic embryogenesis in Cucurbita pepo L. Plant Cell Rep. 23, 120–127. http://dx.doi.org/10.1007/s00299-004-0819-6.
Li, Z., Traore, A., Maximova, S., Guiltinan, M.J., 1998. Somatic embryogenesis and plant regeneration from floral explants of cacao (*Theobroma cacao* L.) using thidiazuron. Vitr. Cell. Dev. Biol. - Plant 34, 293–299. http://dx.doi.org/10.1007/BF02822737.
Lindemann, P., Luckner, M., 1997. Biosynthesis of pregnane derivatives in somatic embryos of Digitalis lanata. Phytochemistry 46, 507–513. http://dx.doi.org/10.1016/S0031-9422(97)00315-4.
Lipavská, H., Konrádová, H., 2004. Somatic embryogenesis in conifers: the role of carbohydrate metabolism. Vitr. Cell. Dev. Biol. Plant 40, 23–30. http://dx.doi.org/10.1079/IVP2003482.
Lipavská, H., Svobodová, H., Albrechtová, J., Kumstýřová, L., Vágner, M., Vondráková, Z., 2000. Carbohydrate status during somatic embryo maturation in Norway spruce. Vitr. Cell. Dev. Biol. Plant 36, 260–267. http://dx.doi.org/10.1007/s11627-000-0048-9.
Liu, J.-J., Ekramoddoullah, A.K.M., 2006. The family 10 of plant pathogenesis-related proteins: their structure, regulation, and function in response to biotic and abiotic stresses. Physiol. Mol. Plant Pathol. 68, 3–13. http://dx.doi.org/10.1016/j.pmpp.2006.06.004.
Lu, W., Tang, X., Huo, Y., Xu, R., Qi, S., Huang, J., Zheng, C., Wu, C., 2012. Identification and characterization of fructose 1,6-bisphosphate aldolase genes in Arabidopsis reveal a gene family with diverse responses to abiotic stresses. Gene 503, 65–74. http://dx.doi.org/10.1016/j.gene.2012.04.042.
Lycett, G., 2008. The role of Rab GTPases in cell wall metabolism. J. Exp. Bot. 59, 4061–4074. http://dx.doi.org/10.1093/jxb/ern255.
Matthes, M., Bruce, T., Chamberlain, K., Pickett, J., Napier, J., 2011. Emerging roles in plant defense for cis -jasmone-induced cytochrome P450 CYP81D11. Plant Signal. Behav. 6, 563–565. http://dx.doi.org/10.4161/psb.6.4.14915.
Maximova, S.N., Alemanno, L., Young, A., Ferriere, N., Traore, A., Guiltinan, M.J., 2002. Efficiency, genotypic variability, and cellular origin of primary and secondary somatic embryogenesis of Theobroma cacao L. Vitr. Cell. Dev. Biol. Plant 38, 252–259. http://dx.doi.org/10.1079/IVP2001257.
Maximova, S.N., Young, A., Pishak, S., Miller, C., Traore, A., Guiltinan, M.J., 2005. Integrated system for propagation of Theobroma cacao L. Protocol for Somatic Embryogenesis in Woody Plants, Series: Forestry Sciences. Springer, pp. 209–227.
Maximova, S.N., Young, A., Pishak, S., Guiltinan, M.J., 2008. Field performance of *Theobroma cacao* L. plants propagated via somatic embryogenesis. Vitr. Cell. Dev. Biol. - Plant 44, 487–493. http://dx.doi.org/10.1007/s11627-008-9130-5.
Maximova, S.N., Florez, S., Shen, X., Niemenak, N., Zhang, Y., Curtis, W., Guiltinan, M.J., 2014. Genome-wide analysis reveals divergent patterns of gene expression during zygotic and somatic embryo maturation of Theobroma cacao L., the chocolate tree. BMC Plant Biol. 14, 185. http://dx.doi.org/10.1186/1471-2229-14-185.
Minyaka, E., Niemenak, N., Fotso Sangareo, A., Ndoumou Omokolo, D., 2008. Effect of MgSO4 and K2SO4 on somatic embryo differentiation in Theobroma cacao L. Plant Cell Tissue Organ Cult. 94, 149–160. http://dx.doi.org/10.1007/s11240-008-9398-5.
Mizutani, M., 2012. Impacts of diversification of cytochrome P450 on plant metabolism. Biol. Pharm. Bull. 35, 824–832. http://dx.doi.org/10.1248/bpb.35.824.
Mizutani, M., Ohta, D., 2010. Diversification of P450 genes during land plant evolution. Annu. Rev. Plant Biol. 61, 291–315. http://dx.doi.org/10.1146/annurev-arplant-042809-112305.
Mohamed, E., Stasolla, C., 2015. Reactive oxygen and nitrogen species signaling and communication in plants. ROS Signalling in Plant Embryogenesis. pp. 196–214.
Morel, A., Teyssier, C., Trontin, J.F., Eliásova, K., Pesek, B., Beaufour, M., Morabito, D., Boizot, N., Reymond, I., Harvengt, L., Cadene, M., Corbineau, F., Vagner, M., Label, P., Lelu-Walter, M.A., 2014. Early molecular events involved in Pinus pinaster Ait. somatic embryo development under reduced water availability: transcriptomic and proteomic analyses. Physiol. Plant. 152, 184–201. http://dx.doi.org/10.1111/ppl.12158.
Moshkov, I.E., Mur, L.A.J., Novikova, G.V., Smith, A.R., Hall, M.A., 2003. Ethylene regulates monomeric GTP-binding protein gene expression and activity in arabidopsis. Plant Physiol. 131, 1705–1717. http://dx.doi.org/10.1104/pp.014035.
Mur, L.A.J., Sturgess, F.J., Farrell, G.G., Draper, J., 2004. The AoPR10 promoter and certain endogenous PR10 genes respond to oxidative signals in Arabidopsis. Mol. Plant Pathol. 5, 435–451. http://dx.doi.org/10.1111/j.1364-3703.2004.00244.x.
Niemenak, N., Kaiser, E., Maximova, S.N., Laremore, T., Guiltinan, M.J., 2015. Proteome analysis during pod, zygotic and somatic embryo maturation of Theobroma cacao. J. Plant Physiol. 180, 49–60. http://dx.doi.org/10.1016/j.jplph.2015.02.011.
Nishimura, N., 2004. Isolation and characterization of novel mutants affecting the abscisic acid sensitivity of arabidopsis germination and seedling growth. Plant Cell Physiol. 45, 1485–1499. http://dx.doi.org/10.1093/pcp/pch171.
Noah, A.M., Niemenak, N., Sunderhaus, S., Haase, C., Omokolo, D.N., Winkelmann, T., Braun, H.P., 2013. Comparative proteomic analysis of early somatic and zygotic embryogenesis in Theobroma cacao L. J. Proteomics 78, 123–133. http://dx.doi.org/10.1016/j.jprot.2012.11.007.
Palma, J.M., Sandalio, L.M., Corpas, Javier, Romero-Puertas, F., McCarthy, M.C., del Río, I., 2002. Plant proteases, protein degradation, and oxidative stress: role of peroxisomes. Plant Physiol. Biochem. 40, 521–530. http://dx.doi.org/10.1016/S0981-9428(02)01404-3.
Pila, L.A., 2013. Embriogênese somática, metilação do DNa e proteômica em quatro genótipos de Theobroma cacao L. do Equador (Dissertação de mestrado). Programa pós-graduação em Recur. genéticos Veg. Univ. Fed. St. Catarina, Bras, pp. 103.
Pilarska, M., Popielarska-Konieczna, M., Lesak, H., Kozieradzka-Kiszkurno, M., Góralski, G., Konieczny, R., Bohdanowicz, J., Kuta, E., 2014. Extracellular matrix surface network is associated with non-morphogenic calli of Helianthus tuberosus cv. Albik produced from various explants. Acta Soc. Bot. Pol. 83, 67–73. http://dx.doi.org/10.5586/asbp.2014.009.
Pullman, G.S., Zeng, X., Copeland-Kamp, B., Crockett, J., Lucrezi, J., May, S.W., Bucalo, K., 2015. Conifer somatic embryogenesis: improvements by supplementation of medium with oxidation-reduction agents. Tree Physiol. 35, 209–224. http://dx.doi.org/10.1093/treephys/tpu117.
Reis, R.S., Vale, E., de, M., Heringer, A.S., Santa-Catarina, C., Silveira, V., 2016. Putrescine induces somatic embryo development and proteomic changes in embryogenic callus of sugarcane. J. Proteomics 130, 170–179. http://dx.doi.org/10.1016/j.jprot.2015.09.029.
Rode, C., Lindhorst, K., Braun, H.P., Winkelmann, T., 2012. From callus to embryo: a proteomic view on the development and maturation of somatic embryos in Cyclamen persicum. Planta 235, 995–1011. http://dx.doi.org/10.1007/s00425-011-1554-1.
Rolletschek, H., Weschke, W., Weber, H., Wobus, U., Borisjuk, L., 2004. Energy state and its control on seed development: starch accumulation is associated with high ATP and steep oxygen gradients within barley grains. J. Exp. Bot. 55, 1351–1359. http://dx.doi.org/10.1093/jxb/erh130.
Rutherford, S., Moore, I., 2002. The arabidopsis rab GTPase family: another enigma variation. Curr. Opin. Plant Biol. 5, 518–528. http://dx.doi.org/10.1016/S1369-5266(02)00307-2.
Sabater-Jara, A.B., Almagro, L., Pedreño, M.A., 2014. Induction of extracellular defense-related proteins in suspension cultured-cells of Daucus carota elicited with cyclodextrins and methyl jasmonate. Plant Physiol. Biochem. 77, 133–139. http://dx.doi.org/10.1016/j.plaphy.2014.02.006.
Šamaj, J., Bobák, M., Blehová, A., Pret'Ová, A., 2005. Importance of cytoskeleton and cell wall in somatic embryogenesis. Plant Cell Monogr. 2, 35–50. http://dx.doi.org/10.1007/7089_024.
Schuler, M.A., Duan, H., Bilgin, M., Ali, S., 2006. *Arabidopsis* cytochrome P450s through the looking glass: a window on plant biochemistry. PhytoChem. Rev. 5, 205–237. http://dx.doi.org/10.1007/s11101-006-9035-z.
Seguine, E., Meinhardt, L., 2014. Cacao flavor through genetics — anatomy of fine flavor. Manuf. Confect. 25–30.
Sessa, G., Yang, X.Q., Raz, V., Eyal, Y., Fluhr, R., 1995. Dark induction and subcellular localization of the pathogenesis-related PRB-1b protein. Plant Mol. Biol. 28, 537–547. http://dx.doi.org/10.1007/BF00020400.
Simões, I., Faro, C., 2004. Structure and function of plant aspartic proteinases. Eur. J. Biochem. 271, 2067–2075. http://dx.doi.org/10.1111/j.1432-1033.2004.04136.x.
Suzuki, H., Takahashi, S., Watanabe, R., Fukushima, Y., Fujita, N., Noguchi, A., Yokoyama, R., Nishitani, K., Nishino, T., Nakayama, T., 2006. An Isoflavone Conjugate-hydrolyzing β-Glucosidase from the Roots of Soybean (*Glycine max*) Seedlings. J. Biol. Chem. 281, 30251–30259. http://dx.doi.org/10.1074/jbc.M605726200.
Stasolla, C., Yeung, E.C., 2003. Recent advances in conifer somatic embryogenesis:

improving somatic embryo quality, Plant Cell. Tissue Organ Cult. 74, 15–35. http://dx.doi.org/10.1023/A:1023345803336.
Stasolla, C., Belmonte, M.F., Van Zyl, L., Craig, D.L., Liu, W., Yeung, E.C., Sederoff, R.R., 2004. The effect of reduced glutathione on morphology and gene expression of white spruce (Picea glauca) somatic embryos. J. Exp. Bot. 55, 695–709. http://dx.doi.org/10.1093/jxb/erh074.
Stasolla, C., 2010. Glutathione redox regulation of in vitro embryogenesis. Plant Physiol. Biochem. 48, 319–327. http://dx.doi.org/10.1016/j.plaphy.2009.10.007.
Tognetti, J.A., Pontis, H.G., Martínez-Noël, G.M.A., 2013. Sucrose signaling in plants: a world yet to be explored. Plant Signal. Behav. 8, e23316. http://dx.doi.org/10.4161/psb.23316.
Traore, A., Maximova, S.N., Guiltinan, M.J., 2003. Micropropagation of *Theobroma cacao* L. using somatic embryo-derived plants. Vitr. Cell. Dev. Biol. - Plant 39, 332–337. http://dx.doi.org/10.1079/IVP2002409.
Tzafrir, I., McElver, J.A., Liu, C.-M., Yang, L.J., Wu, J.Q., Martinez, A., Patton, D.A., Meinke, D.W., 2002. Diversity of TITAN functions in arabidopsis seed development. Plant Physiol. 128, 38–51. http://dx.doi.org/10.1104/pp.010911.
van Engelen, F.A., de Vries, S.C., 1992. Extracellular proteins in plant embryogenesis. Trends Genet. 8, 66–70. http://dx.doi.org/10.1016/0168-9525(92)90352-5.
van Loon, L.C., 1985. Pathogenesis-related proteins. Plant Mol. Biol. 4, 111–116. http://dx.doi.org/10.1007/BF02418757.
Verdier, J., Thompson, R.D., 2008. Transcriptional regulation of storage protein synthesis during dicotyledon seed filling. Plant Cell Physiol. 49, 1263–1271. http://dx.doi.org/10.1093/pcp/pcn116.
Vieira, L., do, N., Santa-Catarina, C., de Freitas Fraga, H.P., dos Santos, A.L.W., Steinmacher, D.A., Schlogl, P.S., Silveira, V., Steiner, N., Floh, E.I.S., Guerra, M.P., 2012. Glutathione improves early somatic embryogenesis in Araucaria angustifolia (Bert) O. Kuntze by alteration in nitric oxide emission. Plant Sci. 195, 80–87. http://dx.doi.org/10.1016/j.plantsci.2012.06.011.
Voigt, G., Biehl, B., Heinrichs, H., Voigt, J., 1997. Aspartic proteinase levels in seeds of different angiosperms. Phytochemistry 44, 389–392. http://dx.doi.org/10.1016/S0031-9422(96)00485-2.
von Arnold, S., Sabyala, I., Bozhkov, P., Dyachok, J., Filonova, L., 2002. Developmental Pathways of somatic embryogenesis. Plant Cell. Tissue Organ Cult. 69, 233–249. http://dx.doi.org/10.1023/A:1015673200621.
Werck-Reichhart, D., Bak, S., Paquette, S., 2002. Cytochromes P450. Arab. B 1, e0028. http://dx.doi.org/10.1199/tab.0028.
Winter, H., Huber, S.C., 2000. Regulation of sucrose metabolism in higher plants: localization and regulation of activity of key enzymes. Crit. Rev. Biochem. Mol. Biol. 35, 253–289. http://dx.doi.org/10.1080/07352680091139178.
Xu, J., Wang, X., Guo, W., 2015. The cytochrome P450 superfamily: key players in plant development and defense. J. Integr. Agric. 14, 1673–1686. http://dx.doi.org/10.1016/S2095-3119(14)60980-1.
Yaseen, M., Ahmad, T., Sablok, G., Standardi, A., Hafiz, I.A., 2013. Review: role of carbon sources for in vitro plant growth and development. Mol. Biol. Rep. 40, 2837–2849. http://dx.doi.org/10.1007/s11033-012-2299-z.
Zeng, F., Zhang, X., Cheng, L., Hu, L., Zhu, L., Cao, J., Guo, X., 2007. A draft gene regulatory network for cellular totipotency reprogramming during plant somatic embryogenesis. Genomics 90, 620–628. http://dx.doi.org/10.1016/j.ygeno.2007.07.007.
Zeng, Y., Tan, X., Wang, B., Long, H., Xu, S., Zhu, S., Zhao, L., Chen, J., 2013. Sequence analysis and expression of one fructose-bisphosphate aldolase gene in camellia oleifera. J. Green Sci. Technol. 1, 30–36. http://dx.doi.org/10.1166/jgst.2013.1003.
Zerial, M., McBride, H., 2001. Rab proteins as membrane organizers. Nat. Rev. Mol. Cell Biol. 2, 107–117. http://dx.doi.org/10.1038/35052055.
Zimmerman, J.L., 1993. Somatic embryogenesis: a model for early development in higher plants. Plant Cell 5, 1411–1423. http://dx.doi.org/10.1105/tpc.5.10.1411.