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Quantitative proteomic analysis of *Araucaria angustifolia* (Bertol.) Kuntze cell lines with contrasting embryogenic potential

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ABSTRACT

GeLC–MS/MS based label free proteomic profiling was used in the large scale identification and quantification of proteins from Brazilian pine (*Araucaria angustifolia*) embryogenic cell (EC) lines that showed different propensities to form somatic embryos. Using a predicted protein sequence database that was derived from *A. angustifolia* RNA-Seq data, 2398 non-redundant proteins were identified. The $\log_2$ of the spectral count values of 858 proteins of these proteins showed a normal distribution, and were used for statistical analysis. Statistical tests indicated that 106 proteins were significantly differentially abundant between the two EC lines, and that 35 were more abundant in the responsive genotype (EC line SE1) and 71 were more abundant in the blocked genotype (EC line SE6). An increase in the abundance of proteins related to cell defense, anti-oxidative stress responses, and storage reserve deposition was observed in SE1. Moreover, in SE6 we observed an increased abundance of two proteins associated with seed development during the embryogenic cell proliferation stage, which we suggest is associated with genotypes showing a low responsiveness to embryo formation. Differences in protein abundance between the EC lines are discussed in terms of carbohydrate metabolism, cell division, defense response, gene expression, and response to reactive oxygen species.

1. Introduction

Asexual plant propagation *via* somatic embryogenesis (SE) has proven to be effective in large-scale clonal propagation, as well as for the development of stable transgenic varieties [1]. SE has also been used as an experimental system to better understand totipotency in later diverging land plants [2] and in association with cryopreservation can provide biotechnological tools to the *ex situ* conservation of endangered plant species [3,4]. Although SE protocols have been developed for a number of species [1,2,4], the limitations imposed by suboptimal conditions during *in vitro* growth of somatic embryos have restricted its use in others [5]. The establishment of a successful SE protocol requires the development of molecular markers that can be used to select cell lines with highly embryogenic potential [6,7]. Conservation strategies based on SE associated with cryopreservation are being developed for *Araucaria angustifolia*, an endangered conifer that is native to Brazil [7,8]. SE induction in *A. angustifolia* was first described in Ref. [9], and since then several reports have outlined attempts to develop a SE protocol for this species, but to date successful plant regeneration has not been possible [6,7,10].

One approach to generating such markers and to better understanding the regulation of *in vitro* embryogenesis is to use global transcript expression or protein profiling [11,12]. Indeed, proteome profiling has already been used to study somatic embryo formation and development in several plant species [2,12,13], including *A. angustifolia* [7,8]. Moreover, proteome analysis represents a promising approach for biomarker discovery, since it is now possible to identify and quantify proteins on a large scale [14,15]. However, protein identification is generally dependent on the existence of high quality DNA sequence databases, derived from genome sequences or expressed sequence tags (ESTs), which are by definition not available for non-model species [16]. However, next-generation sequencing technologies and their applications, such as RNA-sequencing (RNA-Seq), are substantially expanding the number of plant species with high coverage sequence databases, thereby improving the potential for proteomic analysis of species for which a genome sequence is not available [17]. This has the potential to pave the way for studies of the molecular events associated with embryogenesis [18]. Recently, Elbl *et al.* [10] developed an extensive *A. angustifolia* sequence dataset by *de novo* sequencing of the transcriptomes of *A. angustifolia* seeds at different stages of development, as well as embryogenic cell lines with different embryogenic potential: cell line SE1,

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which is responsive to somatic embryo development, and cell line SE6 in which somatic embryo development is blocked. This analysis provided insights into putative genetic determinants that contribute to the embryogenesis potential of cell lines, somatic embryo initiation, and differences in gene expression between the initial stages of somatic and zygotic embryogenesis.

In the current study, an *A. angustifolia* protein database, derived from RNA-Seq data, was generated in order to conduct a large scale proteomic analysis of the SE1 and SE6 embryogenic cell lines. Label-free proteome analysis, involving gel electrophoresis and liquid chromatography–tandem mass spectrometry (GeLC–MS/MS), was used to identify and quantify proteins extracted from the *A. angustifolia* cell lines. Proteins that were detected in both cell lines were annotated by Gene Ontology (GO) analysis and their annotations indicated a relationship with the GO categories 'gene expression', 'carbohydrate metabolism', 'response to reactive oxygen species', 'cell division' and 'defense response'. In addition to the potential for improving SE protocols by revealing molecular markers for early detection of cell lines with high embryogenic capacity, this study further advances the current understanding of molecular regulation during conifer embryogenesis.

2. Experimental procedures

2.1. Plant material

Two *A. angustifolia* embryogenic cell (EC) lines were used, SE1 and SE6. These cell lines (induced from zygotic embryos of different mother trees) were previously selected as described by Jo *et al.* [7], based on different responses under maturation conditions. Initially, these two cell lines were referred to as R and B cell lines [7]; however, during the transcriptome analysis of the R and B cell lines [10] they were renamed SE1 and SE6, respectively. The SE1 cell culture produces pre-cotyledonary embryos (85 ± 19 [Mean ± S.D.] embryos produced per gram fresh weight of embryonal mass) (de Oliveira LF and Floh EIS, unpublished data) in medium supplemented with osmotic agents and abscisic acid (maturation medium), which reach the early cotyledonary stage, while further development of the SE6 cell line is blocked and somatic embryos do not develop [7,10]. For proteomic analyses, the SE1 and SE6 cultures were grown for 21 days on MSG proliferation medium [10] in the dark, at 25 °C ± 1 before harvesting.

2.2. Protein database

Protein sequence database was derived from the *A. angustifolia* transcriptome datasets [10] using Transdecoder software (http://transdecoder.sourceforge.net/) from the Trinity suite, with default parameters. Partial (derived from internal, 5′ and 3′ cDNA sequences) and complete open reading frames (ORFs) were identified. The ORF annotations were assigned based on the previously annotated *Araucaria* transcriptome [10] and were coupled to the domain identification by analysis with HMMER using Pfam-B non-model species [19] in order to further characterize the predicted proteome. An overview of the *Araucaria* protein sequence database is provided in Supplemental material 1.

2.3. Protein extraction and gel electrophoresis

Proteins were extracted from EC lines frozen in liquid nitrogen, and ground to a fine powder in a pre-chilled mortar. Aliquots of 0.5 g of powder were further ground in extraction buffer (proportion 1:3 powder:buffer, *w*/*v*) comprising 50 mM Tris–HCL (pH 8.0), 2% (*w*/*v*) SDS, 1 mM phenylmethanesulfonylfluoride, 1 mM ethylenediaminetetraacetic acid and 50 mM DL-Dithiothreitol After centrifugation at 10,000 × *g* for 25 min at 25 °C, the supernatants were collected and 200 μL aliquots stored −20 °C. The protein concentrations in the supernatants were determined using the 2D Quant kit (GE Healthcare Life Sciences), following the manufacturer's instructions. Protein extracts were prepared from four biological replicates for each EC line. Prior to SDS-PAGE [20], sample aliquots containing 10 μg of proteins were mixed with an equal volume of loading buffer (0.5 M Tris [pH 6.8], 20% [*v*/*v*] glycerol, 2% [*w*/*v*] SDS, 5% [*v*/*v*] 2-mercaptoethanol and traces of bromophenol blue) and incubated for 5 min at 99 °C. Gel electrophoresis was performed using 1 mm thick 12% (*w*/*v*) acrylamide slab gels for 100 min using 10 mA constant current. After electrophoresis, gels were fixed for 1 h in a solution containing 40% (*v*/*v*) ethanol and 10% (*v*/*v*) acetic acid and stained overnight with colloidal Coomassie blue, as previously described [21].

2.4. In-gel trypsin digestion

Prior to protein digestion, the gel lane corresponding to each biological replicate was excised from the gel and divided into five equally sized segments. These were further cut into approximately 1 mm cubes with a clean scalpel, which were then transferred into 1.5 mL sterile polypropylene tubes for gel trypsin digestion, as described by Shevchenko *et al.* [22]. Protein digestion was performed by the addition of 200 μL (5 ng/μL) of sequencing grade porcine trypsin (Promega, Madison, WI) in digestion solution (100 mM ammonium bicarbonate/10% acetonitrile [ACN]). Samples were digested overnight at 37 °C and gel pieces were then saturated with 400 μL of extraction buffer (5% formic acid [FA]/ACN [1:2, *v*/*v*]) and incubated for 30 min at 37 °C. Supernatants were collected and then dried in a vacuum centrifuge and stored at −80 °C until used for LC–MS/MS analyses.

2.5. LC–MS/MS analyses

The trypsinized protein samples were dissolved in 0.1% FA and subjected to online nanoflow liquid chromatography tandem mass spectrometry (LC–MS/MS) using a Q-Exactive quadrupole Orbitrap mass spectrometer (Thermo Scientific, Germany). The peptides were fractionated using the EASY-nLC 1000 system (Thermo Scientific, Denmark) and a reverse phase analytical column (10 cm, 75 μm ID, 120 Å, C18-AQ, Thermo Scientific). The flow rate was set to 200 nL/min, and peptides were separated with a linear ACN gradient from 5 to 35% for 45 min followed by a linear increase to 80% ACN over a period of 5 min. After 5 min at 80% ACN, column re-equilibration was achieved by a reduction to 5% ACN for 3 min. Nanospray peptide ionization was carried out using a voltage equal to 2 kV. The MS/MS scans were acquired in data dependent mode. Survey scans (*m*/*z* 400–2000) were acquired with the resolution adjusted to 70,000 FWHM (full width at half maximum). The 10 most intense ions with a charge state ≥2 were isolated and fragmented in the collision cell by higher-energy collisional dissociation (HCD) at 35 eV. Analyses of MS/MS scans were performed with FWHM set at 35,000. The dynamic exclusion parameter was set to 60 s.

2.6. Identification and quantification of proteins

Raw MS data were processed using MaxQuant 1.4.1.2 software [23], supported by the Andromeda search engine [24] for peptide identification. MS/MS spectra were searched using Andromeda against a concatenated database consisting of the 48,246 EST sequences in the *A. angustifolia* transcriptome database [10] in both forward and reverse orientations, supplemented with frequently observed contaminants. MS/MS peak lists were filtered to leave the 10 peaks with highest abundance per 100 Da intervals with mass tolerances of 10 ppm for the precursor ions and 0.02 Da for the fragment ions. Cysteine carbamidomethylation (Cys + 57.021464 Da) was used as a fixed modification and oxidized methionine (+15.994915 Da) as a variable modification. Up to two missing cleavages were allowed and a minimum of two peptides were required for protein identification. Peptide matches were assembled into protein groups using the Occam's razor principle,

whereby a peptide is assigned to the protein group that most plausibly explains its existence [25]. The peptide and protein false discovery rate (FDR) was set to <1%. For label free quantification analysis, "multiplicity" was set to one. The feature "match between runs" was enabled, using a retention time window of 1 min and an alignment time window of 20 min [26]. Protein quantification was based on the MaxLFQ [26], using both unique and razor peptides and a minimal of two LFQ ratio counts for valid quantification. In other words, a requirement of at least two peptide features between runs was applied to report valid protein group quantification. Averaged label free quantification (LFQ) intensity values were used to calculate protein ratios between experiments.

2.7. Functional annotation and classification

Gene Ontology (GO) annotation, to assign putative molecular function, biological process and cellular component terms, was performed based on BLASTp results using Blast2GO software with an annotation cut-off of 10^{-6} (www.blast2go.com) [27]. In addition, annotation using the KEGG database (www.genome.jp/kegg/) was performed in order to obtain protein domain information. GO term enrichment analysis was carried out using TopGO [28] in order to identify GO classes that were overrepresented among the differentially abundant proteins (FDR < 0.01).

2.8. Statistical analysis

Only the spectral count values of proteins present in all four biological replicates were considered in the differential protein abundance analyses. The $\log_2$ of the spectral count values were subjected to a Shapiro–Wilk test, and samples with a normal distribution were tested using an F-test of equality of variances following a Student's t-test (for samples with equal variance) or Welch t-test (unequal variance). The p values were adjusted to control the FDR at a cut-off of 0.01 as previously described [29]. F-test, Student's t-test, Welch t-test, and p value corrections were carried out using the R package multtest [30].

3. Results

Using GeLC–MS/MS based label free proteomics, a total of 100,945 MS/MS spectra were generated. Proteins were subsequently identified by searching against the *A. angustifolia* transdecoder-predicted protein database using MaxQuant software, resulting in the identification of 2398 non-redundant proteins, which were quantified at a maximum of 1% FDR (confidence interval ≥ 95%).

Gene Ontology (GO) analysis of the non-redundant proteins from cell lines SE1 and SE6 (Fig. 1) resulted in the annotation of 1386 and 1297 proteins, respectively (threshold of 10%). In addition, 1413 and 1326 PFAM annotations were recorded for the two cell lines and for both cell lines, the 'binding' and 'transporter activity' terms were the most represented in the 'molecular function' category. In addition, 'organelle' and 'membrane', followed by 'macromolecular complex', had the highest number of proteins in the 'cellular component' category for both lines, while the 'cellular process' and 'single-organism process' were the most populated groups in the 'biological process' category.

Of the 2398 non-redundant identified proteins, 1277 were detected in fewer than four biological replicates and, according to the strict criteria used in this study, these were not included in subsequent analyses. In addition to 17 proteins that were exclusively detected in either SE1 or SE6, 1104 were detected in all four biological replicates and there were used in the analysis of differentially expressed genes (Supplemental material 2). Of these, 858 showed a normal distribution (Shapiro-Wilk test), and were further subjected to an F-test of equality of variances following a Student's t-test (for samples with equal variance) or a Welch t-test (for samples with unequal variance). These statistical tests indicated that 106 proteins showed significantly ($p < 0.01$) different abundance between the two embryonic cell (EC) lines, and that 35 were up-regulated in SE1, and 71 in SE6 (Supplemental material 3).

Using Blast2GO, these proteins were classified into five main functional categories: 'carbohydrate metabolic process', 'cell division', 'defense response', 'gene expression', and 'response to reactive oxygen species (ROS)'. The distribution of proteins in the different functional classes, the associated p-value, E.C. number and domains are listed in Tables 1 (SE1) and 2 (SE6). Finally, a functional enrichment analysis of the differentially abundant proteins using TopGO ("elim" approach in the Kolmogorov–Smirnov test) suggested a significant enrichment ($p < 0.05$) of proteins in the 'response to ROS' category in the SE1 culture (Fig. 2).

The thirteen proteins that were exclusively detected in SE1 were collectively associated with five functional categories, while the four proteins exclusively detected in SE6 were affiliated with 'carbohydrate metabolic process' and 'gene expression' (Table 3). A search for proteins with highly significant difference in abundance (at least two fold with a $p < 0.01$) in the blocked EC line (SE6) compared to the SE1 line (Fig. 3) revealed a glucan endo-β-glucosidase-like protein, a desiccation-related protein (pcc 13–62-like) and an aspartic proteinase (nepenthesin-1-like protein).

4. Discussion

4.1. A. angustifolia somatic embryogenesis

As with embryogenic cell lines from other conifer species, those from *A. angustifolia* can be identified during their proliferation phase by a characteristic translucent and mucilaginous appearance, and by the presence of stage I somatic embryos [31]. However, early SE formation does not assure embryo maturation in all genotypes [3,31], which is experimentally challenging for species such as *A. angustifolia*, where complete embryo formation can take between six and seven months [7]. The development of molecular markers to allow early detection of EC lines that are responsive to factors that promote maturation is therefore highly desirable, and would be of great value in the optimization of the artificial growth medium during embryo formation. In the present study, a comparative label free proteomic analysis was performed to investigate the differences between embryogenic cell lines that are responsive (SE1), or blocked (SE6), to somatic embryo formation [7]. The potential biological significance and associated biochemical functions during embryogenesis of proteins that were exclusively identified in either cell line, or that were differentially abundant between the cell lines, are discussed in the light of the five GO functional classes that were most represented: 'gene expression', 'carbohydrate metabolic process', 'response to reactive oxygen species', 'cell division', and 'defense response'.

4.2. Response to reactive oxygen species (ROS)

In plant cells, the equilibrium between ROS generation and decomposition depends on the actions of enzymatic and non-enzymatic components of the antioxidative system [32]. The maintenance of an equilibrium between the pro-oxidant and antioxidant systems, which produce and scavenge ROS, is considered to be important during cell differentiation processes, including SE [33,34,35]. As is the case with animals, the initial stages of embryogenesis in plants are characterized by intense metabolic activity, high cell division rates, and increased ROS production [34,36]. In *A. angustifolia* it has been observed that SE1 cell lines produce higher levels of endogenous ROS compared to SE6 cell lines [7]. Using Blast2GO to identify functional categories associated with differences in protein abundance, the 'response to ROS' category was found to be enriched in SE1 (Fig. 2). We observed a distinct set of enzymes among the most abundant proteins and exclusively detected associated with 'response to ROS', including a glutathione S-transferase

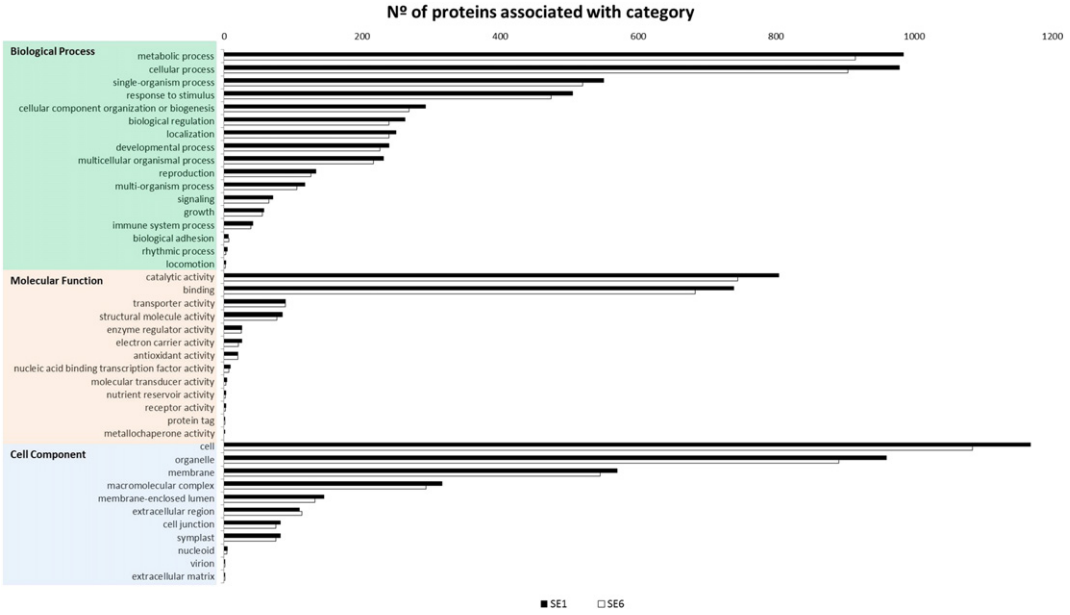

Fig. 1. Number of proteins associated with different GO categories (level 2) in the *A. angustifolia* SE1 (1386 proteins) and SE6 (1297 proteins) cell lines.

(GST) (Table 3), a protein that is involved in many different stress responses in plants [37]. GSTs ensure proper somatic embryo development by protecting against byproducts of oxidative stress, in a process where glutathione is conjugated to a variety of substrates [37]. A reduced glutathione pool during early somatic embryo development, promotes cell proliferation and increases the frequency of both gymnosperm and angiosperm somatic embryos [38]. In studies of *A. angustifolia* and *Picea glauca*, experimental manipulation of the glutathione redox state by medium culture supplementation with reduced glutathione (GSH) or glutathione disulfide (GSSG) in EC cultures was shown to enhance the number of early somatic embryos [39,40], and GST isoforms have also been described in the EC line proteomes of *Vitis vinifera* [41], *Persea americana* Mill. [42], and *Theobroma cacao* L [43].

During induction and development of somatic embryos, the conjugation of glutathione by GSTs influences signaling pathways related to the hormones auxin and ethylene [38,43]. 1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase), a key enzyme in ethylene biosynthesis, is more abundant in SE1 cell lines than SE6 (Table 1), and changes in the glutathione redox state are thought to increase ethylene production through the activation of ACC oxidase [44,45]. Ethylene has also been found to be positively correlated with the development of somatic embryos in *Picea mariana* [46], *Pinus sylvestris* L. [47], and *Medicago sativa* [48], and the SE1 line has been shown to produce much larger amounts of ethylene during the proliferation phase than SE6 line [7]. Our proteomic analysis is congruent with this result and suggests a role for ethylene during *A. angustifolia* early SE.

In the SE1 culture, we observed that L-ascorbate peroxidase (APX) and dehydroascorbate reductase (DHAR), two enzyme components of the ascorbate-glutathione cycle, were more abundant than in SE6 (Table 1). The presence of DHAR is essential for ascorbate regeneration through the recycling of monodehydroascorbate, which in turn affects cell responsiveness and tolerance of ROS [49]. In *Arabidopsis thaliana*, changes in the endogenous ascorbate redox status, obtained through genetic manipulation, accelerate cell proliferation during the induction phase and improve the maturation of somatic embryos [50]. APX is thought to be involved in the reducing the amount of hydrogen peroxide available for cell wall peroxidases that cross-linking cell wall components, and so APX action can be associated with increasing cell wall elasticity during plant embryogenesis [38]. We note that several other studies have identified high abundance of APX in embryogenic cultures and zygotic embryos [42,51,52,43].

In *Larix principis-rup-prechtii* Mayr [12] and *Larix* × *eurolepis* [54] luminal-binding proteins are highly abundant in embryogenic cultures, which in turn may affect stress adaptive processes related to cell division and embryogenic competence acquisition. In the SE6 culture, a luminal-binding protein was found to be more abundant than in SE1, although the presence of this protein at more abundance does not seem to contribute to embryo development in SE6. In summary, as observed in other plant species [36,42], the generation of ROS may be necessary for *A. angustifolia* embryogenesis [7]. The results of the proteomic analysis performed here suggest that compared with SE6, cell line SE1 contains a more extensive enzymatic apparatus to regulate cellular redox homeostasis and ROS-induced signal transduction during embryogenesis.

4.3. Defense response

High levels of stress and/or high non-physiologically relevant concentrations of growth regulators have been used for genetic reprogramming and the induction of embryogenesis in cell cultures [55]. Thus the levels of stress tolerance exhibited by individual cell lines may influence both embryogenic cell viability and metabolism, as well as their adaptation to environmental conditions during *in vitro* culturing [12,36]. The presence of higher levels of defense associated proteins may reflect a response to stressful *in vitro* conditions, or the proteins may play a non-defensive role, generating signal molecules for embryo development [56]. Transcriptomic analysis of SE1 showed elevated expression of genes associated with defense response [10]. Furthermore, embryogenic cell lines of *Picea abies* [57] showed an induction of defense-responsive genes during callus proliferation. However, in proteome studies, an accumulation of proteins related to cell defense, including so-called 'pathogenesis related' (PR) proteins, is not always associated with embryogenic cell lines [2,41].

In our study, two chitinases were more abundant in SE1 than in SE6 (Table 1). Besides acting as hydrolases that degrade fungal cell walls during pathogen infection, chitinases have also been attributed with non-defensive roles in plant development, including somatic embryogenesis [58]. In *P. abies* [59], *A. angustifolia* [31], and *L. principis-rup-prechtii* Mayr [12] the presence of class IV chitinase isoforms was associated with embryogenic cell lines that had high embryogenic potential. It has been suggested that members of this class of chitinases may cleave the glycosidic bonds of the glucosamine and N-acetyl-glucosamine residues on arabinogalactan

Table 1
List of identified proteins by GeLC–MS/MS with a significantly different abundance ($p < 0.01$) in SE1 when compared to SE6. Proteins are grouped by selected biological function (GO annotation). A full list of the proteins identified can be found in the Supplemental material 4.

Unigenes		Proteins	PEP[a]	Peptide number	Fold change[b]	p-Value	Domain
Gene expression							
1	comp45204_c0_seq2	Argonaute 4	3.19E−132	14	−1.29	7.19E−04	PF02170.17
2	comp54333_c0_seq1	Fructose-bisphosphate aldolase	0.00E + 00	17	−1.64	2.52E−03	PF00274.14
3	comp54003_c0_seq1	Chaperone protein 1	1.36E−252	24	−1.65	8.24E−12	PF00004.24
4	comp29363_c0_seq1	Cell division control protein 48-	6.17E−277	25	−1.24	4.15E−06	PF00004.24
5	comp36359_c0_seq1	Cell division control protein 48c	9.70E−221	10	−1.66	4.42E−04	PF00004.24
Carbohydrate metabolic process							
1	comp54487_c0_seq1	Beta-adaptin-like protein c	1.97E−150	20	−0.38	4.18E−03	PF01602.15
2	comp24410_c0_seq1	L-ascorbate peroxidase	8.03E−27	6	−1.21	9.10E−03	PF00141.18
3	comp41457_c0_seq1	Dehydroascorbate reductase	1.10E−32	6	−1.61	3.94E−07	PF02798.15
4	comp39983_c0_seq1	Methyltransferase pmt9	1.92E−80	10	−1.39	4.74E−09	PF03141.11
5	comp50675_c0_seq1	Subtilisin-like protease	0.00E+00	13	−1.46	2.42E−09	PF00082.17
6	comp46886_c0_seq8	Patellin-4	4.50E−127	18	−1.44	2.35E−09	PF00650.15
7	comp54333_c0_seq1	Fructose-bisphosphate aldolase	0.00E+00	17	−1.64	2.52E−03	PF00274.14
8	comp16467_c0_seq1	Pyruvate kinase	1.84E−286	15	−1.11	1.35E−08	PF00224.16
9	comp24690_c0_seq1	1-aminocyclopropane-1-carboxylate oxidase	1.09E−74	10	−1.12	9.67E−03	PF03171.15
10	comp36931_c0_seq1	Adenosine kinase 2	1.15E−138	16	−1.56	1.53E−06	PF00294.19
11	comp53281_c0_seq2	Phosphoenolpyruvate carboxylase	1.23E−122	25	−0.96	1.04E−06	PF00311.12
12	comp23747_c0_seq1	Tubulin alpha-3 chain	0.00E+00	15	−0.68	1.05E−03	PF00091.20
13	comp28543_c0_seq1	Vesicle-associated membrane protein 722	7.95E−38	5	−1.05	5.58E−11	PF00957.16
14	comp50603_c0_seq1	Ap-1 complex subunit gamma-1	1.96E−64	9	−0.46	1.99E−04	PF01602.15
15	comp29363_c0_seq1	Cell division control protein 48	6.17E−277	25	−1.24	4.15E−06	PF00004.24
16	comp36359_c0_seq1	Cell division control protein 48c	9.70E−221	10	−1.66	4.42E−04	PF00004.24
17	comp45204_c0_seq2	Argonaute 4	3.19E−132	14	−1.29	7.19E−04	PF02170.17
Response to reactive oxygen species							
1	comp24410_c0_seq1	L-ascorbate peroxidase	8.03E−27	6	−1.21	9.10E−03	PF00141.18
2	comp41457_c0_seq1	Dehydroascorbate reductase	1.10E−32	6	−1.61	3.94E−07	PF02798.15
3	comp16160_c0_seq1	Dnaj heat shock protein	7.08E−22	5	−1.26	0.00E+00	PF00226.26
4	comp45318_c0_seq1	Chaperonin 60 subunit beta 1	0.00E+00	28	−1.04	1.38E−04	PF00118.19
5	comp54003_c0_seq1	Chaperone protein 1	1.36E−252	24	−1.65	8.24E−12	PF00004.24
6	comp24690_c0_seq1	1-aminocyclopropane-1-carboxylate oxidase	1.09E−74	10	−1.12	9.67E−03	PF03171.15
Cell division							
1	comp29363_c0_seq1	Cell division control protein 48	6.17E−277	25	−1.24	4.15E−06	PF00004.24
2	comp36359_c0_seq1	Cell division control protein 48c	9.70E−221	10	−1.66	4.42E−04	PF00004.24
3	comp28543_c0_seq1	Vesicle-associated membrane protein 722	7.95E−38	5	−1.05	5.58E−11	PF00957.16
4	comp45204_c0_seq2	Argonaute 4	3.19E−132	14	−1.29	7.19E−04	PF02170.17
Defense response							
1	comp24690_c0_seq1	1-aminocyclopropane-1-carboxylate oxidase	1.09E−74	10	−1.12	9.67E−03	PF03171.15
2	comp29363_c0_seq1	Cell division control protein 48	6.17E−277	25	−1.24	4.15E−06	PF00004.24
3	comp36359_c0_seq1	Cell division control protein 48c	9.70E−221	10	−1.66	4.42E−04	PF00004.24
4	comp54843_c0_seq1	Plasma-membrane associated cation-binding protein 1	6.12E−51	7	−1.05	2.54E−11	PF05558.7
5	comp45204_c0_seq2	Argonaute 4	3.19E−132	14	−1.29	7.19E−04	PF02170.17
6	comp28863_c0_seq1	Aldehyde dehydrogenase 2b7	2.40E−123	11	−1.81	3.76E−06	PF00171.17
7	comp45318_c0_seq1	Chaperonin 60 subunit beta 1	0.00E+00	28	−1.04	1.38E−04	PF00118.19
8	comp42558_c0_seq1	Class IV chitinase	6.37E−155	6	−1.82	6.76E−07	PF00182.14
9	comp54056_c0_seq9	Class IV chitinase	0.00E+00	14	−1.06	3.16E−03	PF00182.14

[a] Posterior error probability.
[b] EC line SE6/SE1.

proteins (AGPs), thereby generating oligosaccharide molecules, and that plant responses to these oligosaccharides may have some features in common with the nodulation process induced by *Rhizobium* bacteria in leguminous plants [60,61].

Vicilins are important storage proteins in many conifers, and are considered to be potential markers for SE maturation [11,54]. During *A. angustifolia* zygotic embryogenesis, vicilin accumulates at the beginning of cotyledon formation [53]. Here, despite its known importance during late embryogenesis, a vicilin-like storage protein was exclusively detected during SE1 proliferation (Table 3), suggesting an involvement in processes in addition to its classical definition as a seed storage protein. In this regard, vicilins have recently been implicated as a novel class of antifungal proteins, as well as having α-amylase inhibiting activity [62,63]. However, the role of vicilin during *A. angustifolia* cell line proliferation remains to be established, as does its potential as a biomarker for early selection of cell lines with high embryogenic potential.

4.4. Carbohydrate metabolic process

Embryogenesis is a complex developmental process that is dependent on carbohydrate metabolism [43], as carbohydrates provide energy and carbon for biosynthesis, function as osmotic agents, are important in seed desiccation and cold tolerance, and act as developmental regulators controlling gene expression [64,65]. In this study, we identified 39 proteins with a significantly different abundance in SE1 and SE6 and eight that were exclusively detected in the SE1 or SE6 cultures, and which belonged to the 'carbohydrate metabolic process'. In addition, three proteins related to glycolysis (fructose-bisphosphate aldolase, pyruvate kinase and phosphoenolpyruvate carboxylase 1) were more abundant in the SE1 proteome than in SE6 (Table 1). These proteins play important roles in embryo development as energy providers, acting in cofactor regeneration and interconversion and synthesis of metabolites [15]. Glucose, fructose and sucrose are found in higher proportions in SE1 than in SE6 cultures

Table 2
List of identified proteins by GeLC–MS/MS with a significantly different abundance ($p < 0.01$) in SE6 when compared to SE1. Proteins are grouped by selected biological function (GO annotation). A full list of the proteins identified can be found in the Supplemental material 5.

Unigenes		Proteins	PEP[a]	Peptide number	Fold change[b]	p-Value	Domain
Gene expression							
1	comp43035_c0_seq2	Replication factor c subunit 3	4.71E−15	4	0.46	7.33E−03	PF00004.24
2	comp53684_c0_seq6	Ran GTPase-activating protein 1	3.46E−43	10	0.35	6.75E−03	PF13504.1
3	comp53991_c0_seq1	Sucrose synthase 1	0.00E+00	51	0.74	7.79E−03	PF00534.15
4	comp43599_c0_seq1	E3 ubiquitin-protein ligase orthrus 2	1.61E−61	10	0.88	2.26E−09	PF00097.20
5	comp54712_c0_seq1	U2 small nuclear ribonucleoprotein b	1.31E−52	4	0.72	5.72E−08	PF00076.17
6	comp44201_c0_seq1	RNAse l inhibitor protein 2	1.69E−79	12	1.01	2.80E−03	PF00005.22
7	comp44434_c0_seq1	26s proteasome non-ATPase regulatory subunit 9	0.00E+00	16	0.58	8.05E−03	PF01399.22
8	comp37301_c0_seq3	Glycine-rich RNA-binding protein 7	3.27E−33	7	1.02	5.76E−04	PF00076.17
9	comp39858_c0_seq1	RNA-binding protein rz-1b	2.10E−93	5	0.64	5.92E−03	PF00076.17
10	comp50632_c0_seq2	Zinc finger nuclease 3	8.88E−87	7	1.10	1.35E−06	PF00642.19
11	comp28395_c0_seq1	Elongation factor 1-beta	2.52E−104	11	0.80	1.95E−03	PF00736.14
12	comp44666_c0_seq1	Splicing factor snu114-like protein gfa1	0.00E+00	37	0.51	1.33E−04	PF00009.22
13	comp42797_c1_seq1	Translational initiation factor 4a-1	0.00E+00	20	0.47	2.95E−03	PF00270.24
14	comp15799_c0_seq1	Endomembrane family protein 70	7.07E−80	9	1.93	2.78E−06	PF02990.11
Carbohydrate metabolic process							
1	comp44080_c0_seq1	6-phosphogluconate decarboxylating	1.37E−204	24	0.48	2.04E−03	PF00393.14
2	comp40127_c0_seq1	Glucan endo- -beta-glucosidase-like	3.57E−199	12	2.05	7.59E−08	PF00332.13
3	comp44864_c0_seq1	Reversably-glycosylated protein 5	1.13E−241	21	1.12	1.20E−07	PF03214.8
4	comp53268_c0_seq1	Udp-glucuronic acid decarboxylase	7.84E−83	11	0.72	4.50E−05	PF01073.14
5	comp44286_c0_seq1	Phosphoglycerate mutase family protein	2.78E−70	12	1.26	2.47E−03	PF00300.17
6	comp53991_c0_seq1	Sucrose synthase 1	0.00E+00	51	0.74	7.79E−03	PF00534.15
7	comp29844_c0_seq1	Galactose mutarotase-like superfamily protein isoform 1	2.90E−56	9	1.33	3.76E−06	PF01263.15
8	comp50769_c0_seq1	Glycosyl hydrolase 9c3	3.62E−09	3	1.59	5.33E−03	PF00759.14
9	comp52297_c0_seq3	Alpha-l-fucosidase 2	1.75E−51	11	1.10	1.52E−05	PF14498.1
10	comp53493_c0_seq5	Glycosyl hydrolase family protein	1.59E−03	2	1.51	0.00E+00	PF00933.16
11	comp54175_c1_seq1	Glycosyl hydrolase family 38 protein	2.96E−228	13	1.54	0.00E+00	PF01074.17
12	comp44369_c0_seq1	Gamma carbonic anhydrase 3	2.85E−83	14	0.42	2.36E−04	PF00132.19
13	comp36732_c0_seq1	Gamma carbonic anhydrase-like 2	2.35E−96	8	0.72	1.28E−03	
14	comp37301_c0_seq3	Glycine-rich RNA-binding protein 7	3.27E−33	7	1.02	5.76E−04	PF00076.17
15	comp42599_c0_seq2	Mitochondrial hso70 2	1.63E−285	22	1.80	0.00E+00	PF00012.15
16	comp42797_c1_seq1	Translational initiation factor 4a-1	0.00E+00	20	0.47	2.95E−03	PF00270.24
17	comp44510_c0_seq1	V-type proton ATPase subunit e	3.08E−116	15	1.07	1.27E−03	PF01991.13
18	comp45679_c0_seq1	Luminal-binding protein 2	0.00E+00	36	0.45	7.69E−03	PF00012.15
19	comp54426_c0_seq1	Mitochondrial hso70 2	0.00E+00	37	0.84	0.00E+00	PF00012.15
20	comp54777_c0_seq1	Chaperonin 60E subunit beta 1	2.81E−234	27	0.41	3.17E−03	PF00118.19
21	comp44778_c0_seq1	Thiazole biosynthetic enzyme	5.04E−113	11	1.46	0.00E+00	PF00890.19
22	comp54227_c0_seq6	Hat dimerization domain-containing protein	5.47E−113	20	0.80	4.18E−03	PF01370.16
Response to reactive oxygen species							
1	comp44666_c0_seq1	Splicing factor snu114-like protein gfa1	0.00E+00	37	0.51	1.33E−04	PF00009.22
2	comp42599_c0_seq2	Mitochondrial hso70 2	1.63E−285	22	1.80	0.00E+00	PF00012.15
3	comp44434_c0_seq1	26s proteasome non-ATPase regulatory subunit 9	0.00E+00	16	0.58	8.05E−03	PF01399.22
4	comp45679_c0_seq1	Luminal-binding protein 2	0.00E+00	36	0.45	7.69E−03	PF00012.15
5	comp54426_c0_seq1	Mitochondrial hso70 2	0.00E+00	37	0.84	0.00E+00	PF00012.15
6	comp54777_c0_seq1	Chaperonin 60 subunit beta 1	2.81E−234	27	0.41	3.17E−03	PF00118.19
Cell division							
1	comp46792_c0_seq1	Dynamin-related protein 3b	1.60E−97	17	0.63	1.42E−05	PF00350.18
2	comp43599_c0_seq1	E3 ubiquitin–protein ligase orthrus 2	1.61E−61	10	0.88	2.26E−09	PF00097.20
3	comp53684_c0_seq6	Ran gtpase-activating protein 1	3.46E−43	10	0.35	6.75E−03	PF13504.1
4	comp53991_c0_seq1	Sucrose synthase 1	0.00E+00	51	0.74	7.79E−03	PF00534.15
Defense response							
1	comp43035_c0_seq2	Replication factor c subunit 3	4.71E−15	4	0.46	7.33E−03	PF00004.24
2	comp36389_c0_seq1	Ubiquinol-cytochrome c reductase iron-sulfur subunit	4.55E−71	11	0.68	0.00E+00	PF00355.21
3	comp39656_c0_seq1	Sec7-like guanine nucleotide exchange family protein	1.16E−126	32	1.35	7.59E−05	PF01369.15
4	comp44286_c0_seq1	Phosphoglycerate mutase family protein	2.78E−70	12	1.26	2.47E−03	PF00300.17
5	comp44510_c0_seq1	V-type proton ATPase subunit e	3.08E−116	15	1.07	1.27E−03	PF01991.13
6	comp44778_c0_seq1	Thiazole biosynthetic enzyme	5.04E−113	11	1.46	0.00E+00	PF00890.19
7	comp45679_c0_seq1	Luminal-binding protein 2	0.00E+00	36	0.45	7.69E−03	PF00012.15
8	comp47253_c0_seq1	NAD-binding rossmann-fold superfamily protein	8.79E−46	6	1.40	1.19E−05	PF00106.20
9	comp52406_c0_seq2	Aspartic proteinase nepenthesin-1	1.35E−80	9	2.79	0.00E+00	PF00026.18
10	comp52433_c0_seq1	Sec7-like guanine nucleotide exchange family protein	1.43E−231	40	1.29	3.85E−03	PF01369.15
11	comp50410_c0_seq4	Alcohol dehydrogenase-like 6	2.25E−26	5	1.87	5.42E−06	PF00107.21
12	comp53991_c0_seq1	Sucrose synthase 1	0.00E+00	51	0.74	7.79E−03	PF00534.15
13	comp37301_c0_seq3	Glycine-rich RNA-binding protein 7	3.27E−33	7	1.02	5.76E−04	PF00076.17
14	comp39858_c0_seq1	RNA-binding protein rz-1b	2.10E−93	5	0.64	5.92E−03	PF00076.17
15	comp54777_c0_seq1	Chaperonin 60 subunit beta 1	2.81E−234	27	0.41	3.17E−03	PF00118.19
16	comp53493_c0_seq5	Glycosyl hydrolase family protein	1.59E−03	2	1.51	0.00E+00	PF00933.16

[a] Posterior error probability.
[b] EC line SE6/SE1.

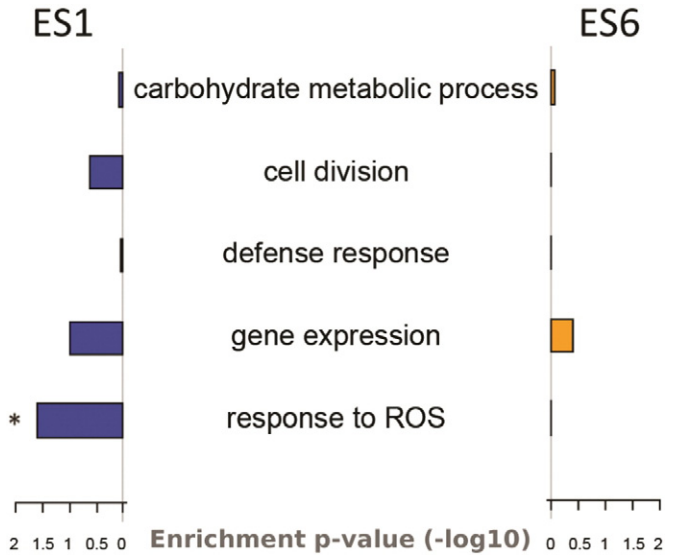

Fig. 2. GO enrichment analysis of differentially abundant proteins of SE1 and SE6 belonging to five functional classes. *Significantly different ($p < 0.05$).

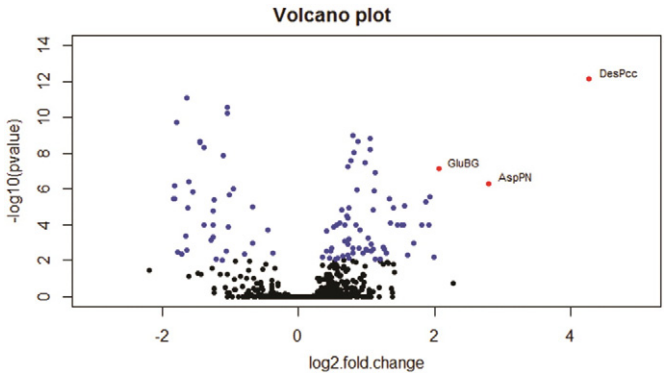

Fig. 3. Volcano plot constructed from spectral counts. The plot shows how much and how significant the identified proteins were differentially abundant between the SE1 and SE6 cell lines. Three proteins in SE6, with the most significantly altered abundance ($p < 0.01$ and $>\log_2$ fold change), are highlighted labeled with names and shown in red (GluBG — glucan endo-beta-glucosidase-like, DesPcc — desiccation-related protein pcc 13–62-like, and AspPN — aspartic proteinase nepenthesin-1-like). Proteins that showed a significant difference in abundance ($p < 0.01$) are represented by blue dots and proteins showed no change ($p < 0.01$) are represented by black dots.

(Navarro *et al.* unpublished results), and the presence of proteins associated with glycolysis and the TCA cycle in SE1, reinforces the idea of a high energy demand during somatic embryo development [7,43,66,67].

Invertase and sucrose synthase are enzymes involved in sucrose assimilation, and the latter either catalyzes a similar invertase reaction (hydrolyzing sucrose into glucose and fructose) or converts sucrose and UDP into fructose and UDP-glucose [68,69]. During the proliferation of *A. angustifolia* embryogenic cells, sucrose is rapidly hydrolyzed to glucose and fructose, suggesting high invertase activity [70], and indeed we detected invertase activity in both cell lines, although we observed no statistical difference in abundance (data not shown). The abundance of sucrose synthase 1 protein was higher in the SE6 culture than in the SE1 cell line, suggesting that for this cell line sucrose contributes to cell proliferation, since the abundance of sucrose synthase is associated with the biosynthesis of UDP-glucose, which may in turn influence cell wall polysaccharide biosynthesis [71] and starch accumulation [69].

Phosphoglucan phosphatase chloroplastic-like, one of the enzymes responsible for starch phosphorylation [72], was exclusively detected in the SE1 proteome, suggesting that starch hydrolysis is more extensive in this cell line. We also observed that the SE6 culture expressed two family 81 glycosyl hydrolases: a beta-glucan-binding protein was only detected in the SE6 sample, while a glucan endo-1,3-β-glucosidase-like protein was present in both, but more abundant in SE6. Overexpression of a glucan endo-1,3-beta-glucosidase-like protein in *A. thaliana* has been shown to result in an increase in callose (1,3-beta-glucan) concentration around plasmodesmata (Pd) [73], reducing molecular diffusion from cell to cell, a flux interruption that is typically observed in physiological defense reactions to biotic and abiotic stresses [74]. In *Cichorium*, cell isolation by callose deposition is beneficial for acquisition of embryogenic competence [75,76]. However, in the SE6 culture, the

Table 3
List of proteins exclusively found in *A. angustifolia* cell line SE1 or SE6 identified by GeLC–MS/MS. Proteins are grouped by selected biological function (GO annotation).

Cell line	Unigenes	Proteins	PEP[a]	Peptide number	Domain
	Defense response				
	comp16875_c0_seq1	Gtp binding protein	4.18E−28	2	PF00071.17
	comp40836_c0_seq1	Dsba oxidoreductase family protein	1.63E−132	4	PF01323.15
	comp43742_c0_seq1	Lipoxygenase 5	3.62E−32	4	PF00305.14
	comp53332_c0_seq8	Glutathione S-transferase	2.18E−42	3	PF00043.20
	comp53656_c1_seq4	Vicilin-like storage protein	9.20E−04	2	PF00190.17
	Carbohydrate metabolic process				
	comp35940_c0_seq1	Phosphoglucan phosphatase chloroplastic-like	7.37E−14	5	PF00782.15
	comp43742_c0_seq1	Lipoxygenase 5	3.62E−32	4	PF00305.14
SE1	comp46866_c0_seq3	Prolyl endopeptidase-like	2.20E−12	4	PF00326.16
	comp53320_c0_seq2	Aspartic proteinase nepenthesin-1-like	2.69E−37	5	PF00026.18
	comp37618_c0_seq1	2-oxoglutarate mitochondrial-like	6.76E−52	5	PF02779.19
	Cell division				
	comp36284_c0_seq1	Ras-related protein rab11a-like	6.41E−25	4	PF00025.16
	Another terms				
	comp40448_c0_seq1	Metalloprotease m41 isoform 2	4.69E−13	4	PF00004.24
	comp45385_c0_seq1	Gdsl esterase lipase	1.36E−47	5	PF00657.17
	comp47363_c0_seq2	Urease accessory protein g	5.15E−11	3	PF02492.14
	comp53320_c0_seq2	Aspartic proteinase nepenthesin-1-like	2.69E−37	5	PF00026.18
	Carbohydrate metabolic process				
	comp30354_c0_seq1	Cell division protein ftsz homolog chloroplastic-like	7.94E−37	3	PF00091.20, PF12327.3
	comp51507_c0_seq1	Heterogeneous nuclear ribonucleoprotein 1-like	5.06E−30	2	PF00076.17, PF13893.1, PF14259.1
SE6	comp53533_c0_seq5	Beta-glucan-binding protein	1.46E−23	6	PF03639.8
	Gene expression				
	comp39686_c0_seq1	Gmp synthase	4.98E−12	5	PF00117.23, PF00733.16, PF00958.17, PF02540.12, PF07722.8

[a] Posterior error probability.

deposition of callose in the cell wall (Navarro *et al.* unpublished results) may be a result of defense response activation, since the isolation caused by callose seems not to contribute to the embryogenic potential.

These results suggest a possible correlation between the embryogenic potential of *A. angustifolia* cell lines with enzymes involved in energy metabolism and starch biosynthesis/degradation. In comparison to SE1, the greater abundance of enzymes related to starch accumulation in the SE6 culture suggests that energy storage is more predominant in this cell line. In contrast, the higher levels of proteins involved in the generation and acquisition of energy supplies in the SE1 cell line than the SE6 cell line may be indicative of more active metabolism, centered on obtaining important intermediates for embryonic growth and development.

4.5. Gene expression

Somatic embryogenesis involves coordinated cell-divisions that are regulated by spatiotemporal changes in the expression of a large number of genes [77]. In this study we identified differentially abundant proteins involved in different aspects of the regulation of gene expression, including proteins associated with 'DNA replication and repair', 'mRNA splicing', 'translation' and 'protein degradation'.

A putative member of the Argonaute (AGO) family was found to be more abundant in the SE1 cell line than in SE6. AGO proteins are involved in miRNA-directed gene silencing and many isoforms are required for normal body-plan formation and stem fate identity [78]. It has been reported that high expression levels of *AGO* transcripts are required for correct development of somatic and zygotic embryos in *P. glauca* and *A. angustifolia*, respectively [6,79]. We propose that the high levels of AGO protein in the SE1 cell line may represent a key factor in embryogenic potential acquisition, enabling embryogenic development under maturation conditions. A recent study demonstrated the importance of miRNAs for correct axial–basal patterning in embryos [80] and, interestingly, a putative glycine-rich RNA-binding protein 7 (GRP7) involved in transcript processing [81] was more abundant in SE6 than in SE1 (Table 2). In *A. thaliana*, this protein is responsible for pri-miRNA processing, and when overexpressed it alters miRNA composition in the cell [82]. Taken together these data suggest a misbalance in miRNA composition in the SE6 line that may lead to loss of maturation competence. Moreover, proteins involved in transcript processing, such as U2 small nuclear ribonucleoprotein B2 and splicing factor snu114-like protein gfa1 (both members of the spliceosome), are highly abundant in SE6 (Table 2). This profile is consistent with *A. angustifolia* transcriptome data, where genes involved in transcription regulation were found to be highly expressed in the non-responsive cell line [10], indicating that the transcription machinery is more active in the SE6 than in the SE1 cell line. Consistent with this proposition, RAN GTPase-activating protein, responsible for transcript trafficking from the nucleus to the cytoplasm, was found more abundant in SE6 than in SE1 (Table 2).

4.6. Cell division

Embryo development requires the coordination of cell division, expansion and differentiation [83,84]. In comparison to SE1, the transcriptome of SE6 displayed high levels of differentially expressed genes associated with transcription, translation and cell division [10]. Although, none of these categories were found enriched in SE1 and SE6 proteomes. In the SE1 cell line, two highly abundant cell division control 48 proteins were identified (CDC48b and c). The proteins function as chaperones in a variety of cellular processes, including cytokinesis, cell expansion, and somatic embryogenesis [85]. Phosphorylation of CDC48 by somatic embryogenesis receptor kinase (SERK) is essential for cell-cycle progression, and cytokinesis is mediated by targeting of the CDC48 protein to the cell plate [86,87]. *A. thaliana* AtCDC48 interacts with SERK to increase somatic embryo development [88], and the importance of SERK during somatic embryogenesis induction has been well established in both gymnosperm and angiosperm species [89,90]. In *A. angustifolia*, the high levels of CDC48 observed in SE1 cell line are accompanied by an increase in *AaSERK* transcripts [90], and might be associated with the embryogenic potential of this cell line.

Dynamin-related proteins, which were highly abundant in the SE6 cell line, are associated with endomembrane morphogenesis during mitochondrial and peroxisomal divisions [91,92]. High levels of these proteins might indicate greater metabolic rates [93] and correlate with the higher growth rate exhibited by this cell line [10]. Altogether, these results demonstrate that SE6 morphogenesis is directed towards cell proliferation instead of differentiation.

4.7. Proteins not associated with the selected functional groups

Two of the proteins that were identified as being highly abundant in SE6 were not classified in the major selected functional groups. The first belongs to the aspartic proteases (APs), a group of proteolytic enzymes that are found in a taxonomically broad range of organisms [94]. In plants, APs are found in different organs, including seeds, leaves and flowers [95]. During maturation of zygotic embryos of cacao, the accumulation of nepenthesin-aspartic proteases is considered important for storage protein processing during late stages of seed development [43]. However, only low levels of nepenthesin-aspartic proteases were detected in a proteome analysis of cacao somatic embryos [43]. The second protein that did not fall within a select functional group has sequence homology to plant desiccation-related proteins (pcc 13–62), which have been implicated in processes related to cell water availability [96]. Pcc 13–62 was first identified in the resurrection plant *Craterostigma plantagineum* [97], and is normally found in plants subjected to dehydration [98], or during late stages of seed development [99]. Despite the fact that both APs and pcc 13–62 were detected, and seem to have a role during seed development, the higher levels of these proteins during SE6 proliferation, apparently do not increase the responsiveness of this cell line to embryo formation. However, considering previous reports, we cannot exclude the possibility that APs and pcc 13–62 are important for *A. angustifolia* embryogenesis. As observed for other species [43,99], the presence of both proteins might be associated with late stages of embryo development, and not during early embryogenesis, as was the case with SE6.

5. Conclusion

We undertook a large scale identification and quantification of proteins in *A. angustifolia* cell lines using a predicted protein database derived from RNA-Seq data for MS spectral matching. The proteomic analyses confirmed the results of previous studies of *A. angustifolia* embryogenic cell lines regarding the importance of proteins related to defense responses [31], anti-oxidative stress responses [7,10,40,100], and gene expression [81] in cell lines with high embryogenic potential. The detection of vicilin and GST only in the SE1 line suggests that they have potential as molecular markers in the early selection of cell lines with responsiveness to maturation conditions. Furthermore, two proteins associated with seed development (aspartic proteases and pcc 13–62) were highly abundant during the proliferation of cell line SE6. We concluded from this that the presence of both these proteins during early embryogenesis may block the transition from proembryogenic masses to early somatic embryo formation. Besides being a valuable sequence and protein data resource for studying conifer proteomes, our data have value for the identification of proteins with potential roles in early somatic embryo formation, and for the early detection of cell lines with high embryogenic potential.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2015.09.027.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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