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Proteome profiling of early seed development in *Cunninghamia lanceolata* (Lamb.) Hook

Jisen Shi[1],*, Yan Zhen[1] and Ren-Hua Zheng[2]

[1] Key Laboratory of Forest Genetics and Biotechnology (Nanjing Forestry University), Ministry of Education, Nanjing 210037, China
[2] Fujian Provincial Academic of Forestry Science, Fuzhou 350003, China

* To whom correspondence should be addressed: E-mail: jshi@njfu.edu.cn

Abstract

Knowledge of the proteome of the early gymnosperm embryo could provide important information for optimizing plant cloning procedures and for establishing platforms for research into plant development/regulation and *in vitro* transgenic studies. Compared with angiosperms, it is more difficult to induce somatic embryogenesis in gymnosperms; success in this endeavour could be increased, however, if proteomic information was available on the complex, dynamic, and multistage processes of gymnosperm embryogenesis *in vivo*. A proteomic analysis of Chinese fir seeds in six developmental stages was carried out during early embryogenesis. Proteins were extracted from seeds dissected from immature cones and separated by two-dimensional difference gel electrophoresis. Analysis with DeCyder 6.5 software revealed 136 spots that differed in kinetics of appearance. Analysis by liquid chromatography coupled to tandem mass spectrometry and MALDI-TOF mass spectrometry identified proteins represented by 71 of the spots. Functional annotation of these seed proteins revealed their involvement in programmed cell death and chromatin modification, indicating that the proteins may play a central role in determining the number of zygotic embryos generated and controlling embryo patterning and shape remodelling. The analysis also revealed other proteins involved in carbon metabolism, methionine metabolism, energy production, protein storage, synthesis and stabilization, disease/defence, the cytoskeleton, and embryo development. The comprehensive protein expression profiles generated by our study provide new insights into the complex developmental processes in the seeds of the Chinese fir.

Key words: Chinese fir, DIGE, embryogenesis, proteomics, seed development.

Introduction

Chinese fir (*Cunninghamia lanceolata* Lamb. Hook) is one of the most important coniferous evergreen tree species in terms of both industrial and commercial wood supplies in China. It has been cultivated for over 3000 years because of its desirable attributes of rapid growth, wood strength, versatility of its applications, hardiness, and high yield of timber productivity per unit. Chinese fir is the dominant tree species in southern China, with a total area of approximately 4×10^6 hm^2 occupying ~25% of man-made plantations, and planting is expanding in over 15 southern provinces (Huang *et al.*, 2005). However, the expansion of plantations has been curtailed by the inconsistent availabil-

ity of genetically improved seeds from year to year. Toward the goal of supplying large numbers of high-quality and genetically superior Chinese fir seedlings, clone propagation through somatic embryogenesis is a potentially large-scale alterative strategy for reforestation.

The immature embryo of Chinese fir is an excellent somatic embryo explant. Before starting an investigation of somatic embryogenesis, however, it is necessary to obtain basic knowledge about zygote development. Embryogenesis is a complex process governed by highly co-ordinated changes in the expression of large protein sets. Protein expression plays a central role in histodifferentiation in the

zygote and during seed development. As such, the characterization and functional analysis of proteins involved in seed development offers the possibility of gaining further information on the molecular basis of embryogenesis and histodifferentiation. Proteomics is a powerful tool to examine temporal patterns and simultaneous changes in protein accumulation during development. Although there have been many studies on the proteomics of angiosperm seed or embryo development (Gallardo *et al.*, 2001, 2003, 2007; Pawłowski, 2007), few such reports exist for gymnosperms. However, certain recent studies have reported proteomic analyses of seeds or embryos from woody plants, such as seed development in *Araucaria angustifolia* (dos Santos *et al.*, 2006), megagametophyte and zygotic embryo development in *Cupressus sempervirens* L. (Sallandrouze *et al.*, 2002), early somatic embryogenesis in *Picea glauca* (Lippert *et al.*, 2005), and zygotic embryos and somatic embryos of *Pinus strobes* (Klimaszewskaa *et al.*, 2004).

The immature embryo has been broadly used to initiate cultures for the study of somatic embryogenesis in conifers (Balbuena *et al.*, 2009). It was found that the developmental stage of the zygotic embryos used as explants could influence the successful rates of somatic embryos conversion. To obtain new insights on Chinese fir seed development and practical ways to improve somatic embryogenesis with proper microenvironmental conditions based on protein expression patterns, a two-dimensional difference gel electrophoresis (2D DIGE)-based proteomic approach was used to analyse the differentially displayed proteins in megagametophytes containing developing embryos during six important developmental phases of early embryogenesis in *C. lanceolata*. Data are presented here on the dynamics of protein accumulation patterns during seed development. Our results provide a fundamental reference for further studies on the regulation of protein expression during somatic embryogenesis.

Materials and methods

Plant materials and seed development stages

Immature cones of *C. lanceolata* (Lamb.) Hook were collected every 2 weeks from clone No. 4009 at Shao Wu seed orchards in Fujian province in south-eastern China. Cones were kept on ice and delivered to the laboratory within 48 h. Ten developing seeds were chosen randomly from the middle part of each cone, and the developmental stage of each seed was estimated according to the zygotic embryo state under a dissecting microscope. Given that most of the zygotic embryos that were observed in seeds from several cones were of a similar development stage, it was concluded that most of the seeds were at the stage that had been estimated. The developmental stages of the collected seeds were classified as follows: cleavage polyembryony, dominant embryo, columnar embryo, or early cotyledonary (Nagmani *et al.*, 2000; von Arnold *et al.*, 2000). Seeds of a similar developmental stage were placed in a vial and then frozen in liquid nitrogen and stored at –70 °C until use.

Sample preparation

Protein extracts were prepared in triplicate for each developmental stage. About 50 mg of seed material was homogenized with 0.3 ml lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM TRIS, pH 8.5) using a grinding kit (Amersham Biosciences/GE Health-

care, UK). The extracted samples were incubated at room temperature for 20 min and then centrifuged at 15 000 *g* at 4 °C for 50 min to remove insoluble material. The supernatant was cleared of residual particulates using a protein Clean-up kit (Amersham Biosciences/GE Healthcare). The resulting extract powder was solubilized in lysis buffer. After 1 h of gentle stirring at room temperature, samples were centrifuged at 15 000 *g* at room temperature for 45 min. Protein concentration was determined with the PlusOne 2-D Quant kit (Amersham Biosciences/GE Healthcare). Aliquots of protein samples were stored in 0.6 ml Eppendorf tubes at –70 °C for further analysis.

Cy dye labelling

Lyophilized protein samples (50 μg) were labelled with 400 pmol of *N*-hydroxy succinimidyl ester derivatives of cyanine dyes Cy3 or Cy5 in anhydrous dimethylformamide. A pool standard that contained equal quantities of all samples was labelled with Cy2. Labelling reactions were performed on ice for 30 min in the dark, and each reaction was terminated by adding 1 μl of 10 mM lysine (Sigma, St Louis, MO, USA). These samples were mixed, centrifuged briefly in a microcentrifuge, and then incubated on ice in the dark for 10 min. Differentially labelled samples were mixed and combined with an equal volume of 2× sample buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (w/v) DTT, and 2% (v/v) pH 4–7 Pharmalyte™] for at least 10 min on ice.

Fluorescent two-dimensional electrophoresis analysis

For two-dimensional electrophoresis, proteins were solubilized in a rehydrating solution containing 8 M urea, 2% CHAPS, 20 mM DTT, a trace of bromophenol blue, and 0.5% (v/v) immobilized pH gradient (IPG) buffer (pH 4–7) (Amersham Biosciences/GE Healthcare) in the dark. IPG strips (24 cm, linear pH 4–7, Amersham Biosciences/GE Healthcare) were rehydrated overnight with 450 μl rehydrating solution containing 150 μg proteins. Rehydration and isoelectric focusing were carried out at 20 °C using an IPGphor III (Amersham Biosciences/GE Healthcare), with the following settings: 30 V for 6 h, step; 60 V for 6 h, step; 200 V for 1 h, step; 500 V for 1 h, step; 1000 V for 1 h, step; 4000 V for 1 h, step; 8000 V for 30 min, grad; 8000 V for 6 h, step. After isoelectric focusing, proteins were chemically reduced by incubating the IPG strips with 1% (w/v) DTT and then alkylated with 4% (w/v) iodoacetamide in 10 ml equilibration buffer [6 M urea, 30% (w/v) glycerol, 2% SDS, and 50 mM TRIS-HCl] with gentle shaking for 15 min. After equilibration, the IPG strips were blotted with filter paper for 1 min to remove excess equilibration solution, and gently rinsed for 1 min using SDS-PAGE running buffer (250 mM TRIS pH 8.3, 1.92 M glycine, and 1% SDS). The IPG strips were then transferred to 12.5% SDS-PAGE gels with low fluorescence glass plates for second-dimension electrophoresis with the Ettan Dalt twelve gel system (Amersham Biosciences/GE Healthcare) using SDS-PAGE running buffer. After the sealed agarose cooled down, the second-dimension SDS-PAGE was carried out at 1 W gel^{-1} for 16 h at 20 °C until the dye front reached the bottom of the gel.

Image and data analysis

Gel images were scanned with a Typhoon Trio Variable Mode Imager (Amersham Biosciences/GE Healthcare). Cy2 images were scanned using a 488 nm laser and an emission filter of 520 nm/band pass 40. Cy3 images were scanned using a 532 nm laser and an emission filter of 580 nm/band pass 30. Cy5 images were collected using a 633 nm laser and an emission filter of 670 nm/band pass 30. Prescan was carried out with a pixel size of 500 μm to adjust the photomultiplier tube voltage to give a maximum pixel value of between 60 000 and 80 000 to avoid image saturation. All gels were then scanned using a pixel size of 100 μm to obtain high-resolution images. The images were cropped with ImageQuant TL 2005 software (Amersham Biosciences/GE Healthcare) prior to

DeCyder™ 6.5 analysis (Amersham Biosciences/GE Healthcare). The differential in-gel analysis function of DeCyder™ 6.5 was used to co-detect and differentially quantify the protein spots in each gel, which included a Cy2-labelled pooled standard and Cy3/Cy5-labelled samples. Variations between samples, as determined by DeCyder™ 6.5, enabled gel to gel matching of spots according to the internal standard, allowing quantitative comparisons of proteins differentially expressed across multiple gels. The Batch Processor of DeCyder™ 6.5 linked both differential and biological variation analysis to perform all analyses automatically without user intervention, and spot intensities were quantified based on an estimated 3000 spots per gel. To match spots across gels accurately, landmarks were set. One-way analysis of variance was used to reveal proteins that were differentially expressed ($P < 0.01$) among six samples (i.e., stages 1, 2, 3, 4, 5, 6; see the Results and discussion for stage description). A statistical analysis was performed on triplicate measurements for each stage. Hierarchical clustering analysis was performed on the standardized log abundance of spot volumes using Gene Cluster software 3.0 with centred correlation and the average linkage procedure. Phylogenetic trees were constructed/visualized using TreeView software (http://rana.lbl.gov/downloads/TreeView/TreeView_vers_1_60.exe).

Protein identification by mass spectrometry analysis

Pick gels were prepared with sufficient amounts of protein (150 μg) and stained with mass spectrometry (MS)-compatible silver. Spots of interest were located on each gel by comparison with the 2-D DIGE protein pattern. Spots were excised manually from the gels, washed with double-distilled water and then transferred to siliconized Eppendorf tubes. The excised gel pieces were destained and dehydrated with acetonitrile. The proteins were chemically reduced in 10 mM DTT/50 mM NH_4HCO_3 for 1 h at 56 °C and alkylated in 55 mM iodoacetamide/50 mM NH_4HCO_3 for 1 h at room temperature. The gel pieces were washed several times in 50 mM NH_4HCO_3 followed by dehydration with acetonitrile and dried via centrifugation in a Speed-Vac centrifuge. The proteins were digested with trypsin (10 ng μl^{-1} Promega, Madison, WI, USA) at 37 °C for 16–18 h. The digestion reaction was stopped by the addition of 2 ml 10% trifluoroacetic acid, and the digestion products were desalted with a Millipore ZipTip C18 column. The resulting peptide mixtures were analysed by MS.

The resulting peptide mixtures were dissolved in 0.1% formic acid and separated on a capillary chromatography column connected directly to the source of the linear ion trap LC-MS/MS (LTQ; Thermo Finnigan, San Jose, CA, USA). Each sample was loaded on a C18 trap column (id 150 μM, length 150 mm, Agilent Technologies, Wilmington, DE, USA) for concentration and desalting using an acetonitrile gradient. The mass spectrometer was operated in positive ion mode with a capillary temperature of 170 °C. All data were acquired with the mass spectrometer operating in automatic data-dependent switching mode. Zoom scans were performed on the ten most intense ions to determine the charge state prior to MS/MS analysis.

For analysis via matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS, digestion products were desalted with a Millipore ZipTip C18 column. The MALDI-TOF MS was operated in the positive ion delayed extraction reflector mode for highest resolution and mass accuracy. Peptides were ionized with a 337 nm laser, and spectra were acquired at 19 kV acceleration potential with optimized parameters.

Database search

Peptide mass fingerprinting data were interpreted using Mascot (http://www.matrixscience.com) in the non-redundant database of the National Center for Biotechnology Information (NCBI). Taxonomic assignments were based on data available for Pinaceae in that non-redundant database. Our criterion for positive peptide mass fingerprinting was that at least four peptides must be matched.

Monoisotopic masses were used, and a mass tolerance of ±100 ppm was allowed. The maximum number of missed cleavages was set at one. Complete carbamidomethylation of cysteines and variable oxidation of methionines (Met) were assumed.

MS/MS spectra data were searched against the available data for Pinaceae in NCBI using the SEQUEST algorithm as part of the BIOWORKS software suite. Search criteria of SEQUEST queries were as follows: Charge +1, Xcorr ⩾1.9; Charge +2, Xcorr ⩾2.2; Charge +3, Xcorr ⩾3.75; DeICN ⩾0.1 (Xcorr: SEQUEST cross-correlation value; DeICN: delta correlation value). The search parameters were based on a precursor-ion mass tolerance of 2 Da and a fragment ion tolerance of 1.0 Da with consideration for Met oxidation and cysteine carbamidomethylation. The estimated experimental Mr/pI was applied to increase the confidence of identification. Assignments annotated as 'unknown' were based on sequence similarity as assessed by BLASTP (www.ncbi.nlm.nih.gov/BLAST/).

Results and discussion

DIGE comparison

2-D DIGE was used to compare changes in the proteome of Chinese fir seeds during development, which spanned six important developmental phases during early embryogenesis: the cleavage polyembryony-stage seed (stages 1, 2, and 3), dominant embryo-stage seed (stages 4), columnar embryo-stage seed (stage 5), and early cotyledonary-stage seed (stage 6) (Fig. 1). After 2-D DIGE, Typhoon scanning indicated that each gel had approximately 2500 protein spots. The spots between gels were matched by DeCyder™ 6.5 software based on an internal standard. All gel images were matched against a master gel image, allowing comparison of protein abundances across the stages of seed development. The abundance of 136 proteins varied significantly ($P < 0.01$, one-way analysis of variance) during seed development (Fig. 2), and these proteins were subsequently analysed by LC MS/MS and MALDI-TOF. Table 1 presents a list of proteins identified by MS analysis, which were functionally categorized according to the classification reported for *Arabidopsis* genes (Bevan *et al.*, 1998). Unknown or hypothetical proteins were assigned based on sequence similarity as assessed by BLASTP (www.ncbi.nlm.nih.gov/BLAST/). Twenty-two proteins were assigned as conifer proteins, 27 proteins were identified as the *Arabidopsis thaliana* homologues, the remaining proteins were classified as homologues of proteins from other plants. It was recently reported that gymnosperms harbour genes, having sequences very similar to genes that regulate angiosperm embryogenesis genes. Moreover, approximately 72% of pine embryo ESTs could be detected in the *A. thaliana* proteome (Cairney and Pullman, 2007). A comparison of the proteins identified in Chinese fir with those of pine (as deduced from cDNA libraries) that have been implicated in embryogenesis and assigned according to angiosperm homologues, listed in Cairney *et al.* (2006) revealed only a few identical proteins. Differentially expressed proteins were subjected to hierarchical clustering based on our defined six stages of seed development. These proteins could be grouped into three categories as follows: one class of proteins whose levels increased between stages 5 and 6, a second class of proteins whose levels increased between stages 3 and 6, and a third class of proteins whose
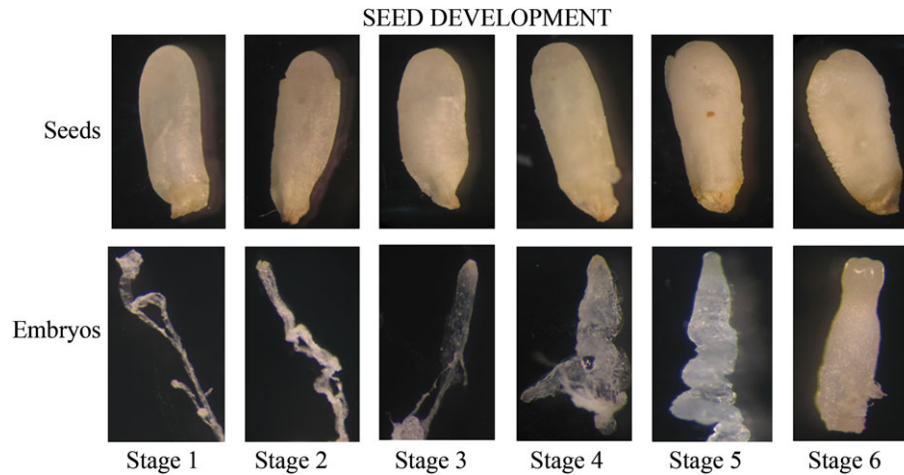
Fig. 1. Dissected characteristic zygotic embryo stages of the Chinese fir during seed development. Stages 1, 2, and 3: cleavage polyembryony stage; stage 4: dominant embryo stage; stage 5: columnar embryo stage; stage 6: early cotyledon stage.
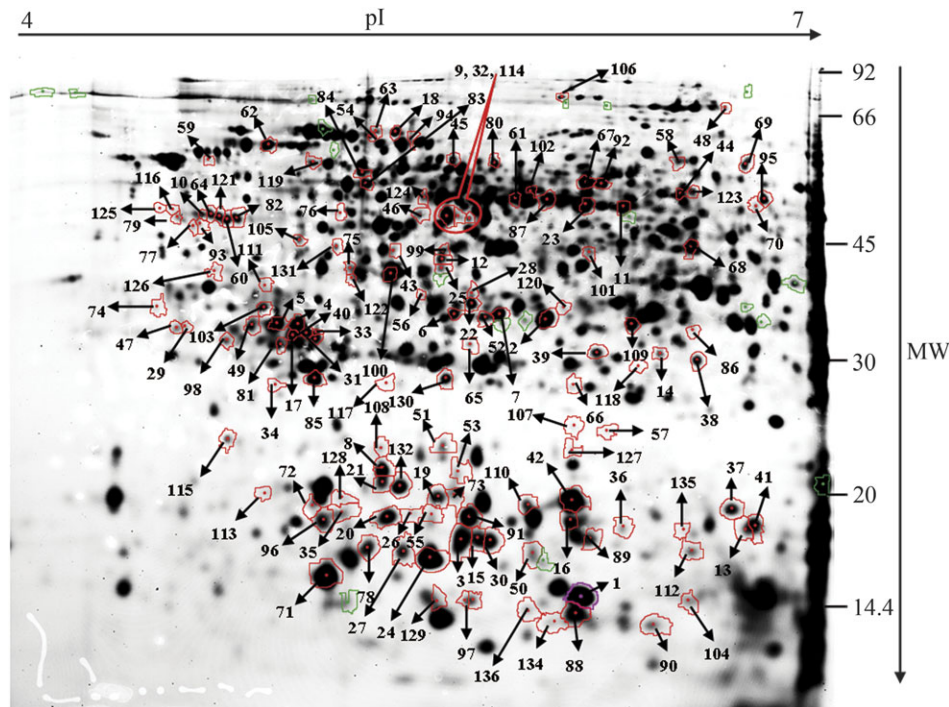
Fig. 2. Analytical 2-D DIGE gel of Cy2-labelled mixed-sample developing seeds. The marked spots show 136 proteins that were differentially expressed ($P <0.01$; one-way analysis of variance). MW: apparent molecular mass.

levels decreased during seed development (Fig. 3). The functions of some of the differently expressed proteins are discussed below.

Role of programmed cell death in gymnosperm seed development

Programmed cell death (PCD) is a part of the mechanism that controls the number and type(s) of cells at a certain location. The archegonium of developing gymnosperm seeds contains a number of embryos, but only one embryo in the polyembryonic seed usually develops and survives. PCD is the major mechanism that eliminates the subordinate embryos in

a polyembryonic seed (Filonova *et al.*, 2002). The suspensor, which promotes continuous growth of the embryo and thus is necessary for early embryonic development, begins to degenerate at the early cotyledon stage. This degeneration process is a classic example of developmental PCD (Lombardi *et al.*, 2007). Consistent with a PCD process, two putative PCD-related proteins (spot 57 and spot 74) were down-regulated during seed development, coincident with the elimination of subordinate embryos and of suspensor degeneration. One of these proteins is a cysteine protease (spot 74); notably, in animals, PCD is executed by the caspase family of cysteine proteases. Plants do not have similar caspase homologues but rather express a phylogenetically

Table 1. Proteins identified in developing seeds by LC-MS/MS and MALDI-TOF and functional category according to classification reported for *Arabidopsis* genes

Expression pattern: the *y*-axis is standardized Log abundance; the *x*-axis is the stages of development that have been defined in the manuscript.

	Spot number	Accession number	One-way ANOVA P value	Species	Theoretical MW/pI	Experimental MW/pI	Expression pattern
01 Metabolism							
Probable fructose-*bis*phosphate aldolase 1*[b]	22	sp|Q9SJU4	2.5E-05	*Arabidopsis thaliana*	42.9/6.18	37.1/5.7	
S-adenosylmethionine synthetase*	46	gb|AAG17036	1.2 E-04	*Pinus contorta*	43.4/5.55	48.9/5.51	
S-adenosyl-L-homocysteine hydrolase 2*[b]	61	ref|NP_189023	4.2 E-04	*Arabidopsis thaliana*	53.2/5.94	51.7/5.58	
Glyceraldehyde-3-phosphate dehydrogenase[b]	65	sp|P34924	6.5 E-04	*Pinus sylvestris*	36.5/6.67	30.0/5.69	
Similar to AMP binding/acetate-coa ligase*[b]	71	ref|NP_188316	0.0011	*Arabidopsis thaliana*	62.4/6.9	15.7/5.18	
Similar to cysteine synthase[b] *	86	gb|ABQ88339	0.002	*Arabidopsis thaliana*	34.5/5.5	34.1/6.49	
Similar to cysteine synthase[b]	6	ref|NP_181903	2.50E-06	*Arabidopsis thaliana*	34.5/5.5	34.5/5.63	
Phosphoenolpyruvate carboxylase[b]	97	sp|P51063	0.0034	*Picea abies*	109.5/5.94	13.9/6.07	
Enolase[b]	102	emb|CAB96173	0.0039	*Spinacia oleracea*	48.2/5.49	62.5/5.48	
ENO1_HEVBR Enolase 1[b]	103	sp|Q9LEJ0	0.0039	*Hevea brasiliensis*	47.8/5.57	47.6/4.72	
Glycoside hydrolase starch-binding domain-containing protein[b]	110	NP_850989	0.0053	*Arabidopsis thaliana*	68.4/5.1	52.8/5.9	
Similar to light-harvesting CAB*[b]	15	ref|NP_174286	1.6E-05	*Arabidopsis thaliana*	13.2/9.6	17.7/5.72	
Similar to light-harvesting CAB*[b]	21	ref|NP_174286	2.5E-05	*Arabidopsis thaliana*	13.2/9.6	21.1/5.38	

Table 1. *Continued*
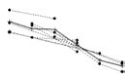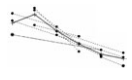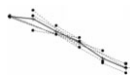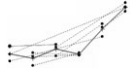
	Spot number	Accession number	One-way ANOVA P value	Species	Theoretical MW/pI	Experimental MW/pI	Expression pattern
Light-independent protochlorophyllide reductase subunit B[b]	68	sp|Q85X76	7.5E-04	*Picea sitchensis*	57.7/5.96	44.3/6.48	
Similar to light harvesting CAB1* [b]	69	ref|NP_174286	9.5E-04	*Arabidopsis thaliana*	13.2/9.6	57.5/6.68	
Similar to CAB1 (chlorophyll *a/b*-binding protein1* [b]	75	ref|NP_174286	0.001	*Arabidopsis thaliana*	13.2/9.6	41.46/5.26	
Similar to CAB1* [b]	80	ref|NP_174286	0.0014	*Arabidopsis thaliana*	13.2/9.6	57.8/5.78	
GDP-fucose protein-*O*-fucosyltransferase [b]	67	emb|CAE30295	5.9 E-04	*Pinus taeda*	50.7/9.38	54.4/6.11	
02 Energy							
Acetate kinase[a]	12	ref|XP_001694505	8.3E-06	*Chlamydomonas reinhardtii*	46.1/7.64	42.7/5.59	
Similar to ATP synthase beta chain 2 mitochondrial* [b]	83	ref|NP_568204	0.0015	*Arabidopsis thaliana*	60.4/6.01	54.1/5.32	
Similar to oxidoreductase, zinc-binding dehydrogenase family protein* [b]	25	ref|NP_173786	3.3E-05	*Arabidopsis thaliana*	33.7/5.49	41.4/5.59	
Cytochrome P450 71D9[a]	23	sp|O81971	3.0E-05	*Glycine max*	56.3/8.99	50.7/6.1	
04 Transcription							
Similar to ELM2 domain-containing protein/PHD finger family protein* [b]	66	gb|AAO64869	6.8 E-04	*Arabidopsis thaliana*	28.1/6.6	28.7/6.06	
Similar to maternal effect embryo arrest 51* [b] (MEE51)	92	ref|NP_192313.3	0.0023	*Arabidopsis s thaliana*	34.7/5.84	54.1/6.16	
05 Protein synthesis							
Translation elongation factor-1 alpha[b]	70	emb|CAC27139	9.8E-04	*Picea abies*	49.1/9.06	50.3/6.71	
Translation elongation factor-1 alpha[b]	95	emb|CAC27139	0.0028	*Picea abies*	49.1/9.06	51.5/6.74	

Table 1. *Continued*

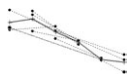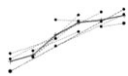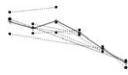|	Spot number	Accession number	One-way ANOVA P value	Species	Theoretical MW/pI	Experimental MW/pI	Expression pattern
Similar to eukaryotic translation initiation factor*[b]	91	NP_172848.1	0.0023	*Arabidopsis thaliana*	17.3/5.46	18.9/5.69	
Similar to 40S ribosomal protein S20*[b]	90	AT5G62300	0.0022	*Arabidopsis thaliana*	13.7/9.55	14.4/6.35	
Rubber elongation factor [b]	100	gb|ABW37164	0.0039	*Hevea brasiliensis*	14.7/5.04	54.1/6.16	
06 Protein destination and storage							
Legumin-like storage protein[b]	1	gb|AAA68981	1.00E-07	*Picea sitchensis*	57.0/6.84	14.7/6.09	
Legumin-like storage protein	2	gb|AAA68981	1.00E-07	*Picea sitchensis*	57.0/6.84	35.2/ 5.96	
Legumin-like storage protein	3	gb|AAA68981	1.00E-07	*Picea sitchensis*	57.0/ 6.84	34.5/5.0	
Seed storage protein	11	gb|AAB01560	6.7E-06	*Picea glauca*	19.4/7.57	50.3/6.24	
Small heat-shock protein	8	emb|CAC81962	3.60 E-06	*Picea glauca*	23.4/8.74	22.1/5.38	
High molecular weight heat shock protein	18	gb|AAF34134	1.6E-05	*Malus×domestica*	71.5/5.17	63.7/5.42	
17.5 kDa class I heat shock protein[a]	19	sp|P04793	1.8E-05	*Glycine max*	17.5/5.33	20.2/5.58	
Similar to 17.8 kDa class I heat shock protein*[b]	26	ref|NP_172220.1	3.5E-05	*Arabidopsis thaliana*	19.9/5.45	19.1/5.48	
hsp70 (AA 6–651)[a]	60	emb|CAA31663	4.2 E-04	*Petunia×hybrida*	71.1/5.07	48.3/4.82	
Similar to putative hsp70*[b]	63	gb|AAT39165	4.7E-04	*Oryza sativa*	71.5/5.18	63.9/5.35	

Table 1. *Continued*

	Spot number	Accession number	One-way ANOVA *P* value	Species	Theoretical MW/pI	Experimental MW/pI	Expression pattern
Similar to putative hsp70* [b]	64	emb|CAB72128	5.9E-04	*Cucumis sativus*	44.4/4.8	49.1/4.76	
Similar to low molecular weight heat-shock protein* [b]	73	emb|CAA63571	0.0011	*Pseudotsuga menziesii*	18.2/5.73	20.3/5.63	
Similar to heat shock protein 70* [b]	77	emb|CAB72128	0.0013	*Cucumis sativus*	44.4/4.8	47.4/4.7	
Similar to putative hsp70* [b]	79	gb|AAT39165	0.0014	*Oryza sativa*	71.5/5.18	48.7/4.64	
Similar to chaperonin, putative* [b]	84	NP_187956	0.0015	*Arabidopsis thaliana*	65.5/5.69	56.1/5.3	
Heat shock protein 17.0	89	gb|AAB01561	0.002	*Picea glauca*	17.1/5.76	17.7/6.12	
Protein SHEPHERD	82	sp|Q9STX5	0.0015	*Arabidopsis thaliana*	94.2/4.94	48.6/4.86	
FBK93_ARATH Putative F-box/Kelch-repeat protein[b]	112	sp|O49488	0.0054	*Arabidopsis thaliana*	33.4/6.66	17.0/6.49	
09 Cell structure AF172094_1 actin[b]	14	gb|AAF03692	1.3E-05	*Picea rubens*	41.6/5.3	31.7/6.37	
10 Signal transduction Similar to 14-3-3-like protein GF14 nu* [b]	5	gb|ABK21293	1.2E-06	*Arabidopsis thaliana*	29.7/4.87	34.8/5.00	
Similar to RANGAP2* [b]	62	ref|NP_197433	4.7E-04	*Arabidopsis thaliana*	60.2/4.68	66.0/4.97	
Similar to zinc-binding protein, putative/protein kinase C inhibitor* [b]	78	ref|NP_567038	0.0014	*Arabidopsis thaliana*	14.3/5.61	17.2/5.32	

Table 1. *Continued*
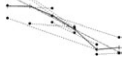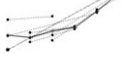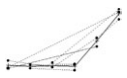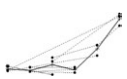
	Spot number	Accession number	One-way ANOVA P value	Species	Theoretical MW/pI	Experimental MW/pI	Expression pattern
Similar to phosphoprotein phosphatase*	127	gb|AAQ65125	0.01	*Arabidopsis thaliana*	16.3/9.32	20.4/4.96	
11 Disease/defence							
Peroxidase C2 precursor [a]	13	sp|P17179	1.3E-05	*Armoracia rusticana*	38.5/8.7	18.2/6.68	
PR10-1.13[b]	24	gb|AAW21972	3.30E-05	*Pinus monticola*	17.8/5.3	16.7/5.55	
Superoxide dismutase [Fe][b]	29	ref|XP_001690591	4.60E-05	*Chlamydomonas reinhardtii*	25.9/9.34	17.0/5.45	
Putative intracellular pathogenesis-related protein [b]	50	gb|ABA54799	1.9 E-04	*Picea abies*	11.2/4.95	16.9/5.91	
Ascorbate peroxidase[b]	85	gb|AAR32786	0.0018	*Pinus pinaster*	27.3/5.4	29.3/5.13	
Similar to monodehydroascorbate reductase* [b]	87	ref|NP_190856	0.002	*Arabidopsis thaliana*	47.3/6.03	51.4/5.97	
11.03 Cell death							
Poly [ADP-ribose] polymerase 1[a]	57	sp|Q9ZSV1	2.8E-04	*Zea mays*	111.5/8.74	38.1/5.52	
Similar to cysteine proteinase (RD21A)/thiol protease* [b]	74	AT1G47128	0.0011	*Arabidopsis thaliana*	49.9/4.72	36.7/4.57	
Transglutaminase-like protein[b]	4	XP_001698218	1.2E-06	*Chlamydomonas reinhardtii*	36.5/5.91	34.7/5.07	
12 Unclear classification							
Unigene V from Maize Genome Project *Zea mays* cDNA, mRNA sequence[a]	17	gi|26558665	1.6E-05	*Zea mays*	33.6/5.06	22.4/9.93	
ORF115[b]	20	ref|NP_042497	2.1E-05	*Pinus thunbergii*	13.9/6.83	18.9/5.39	
ORF45g[b]	72	ref|YP_001152231	0.0011	*Pinus koraiensis*	54.2/9.23	19.7/5.13	

Table 1. *Continued*

	Spot number	Accession number	One-way ANOVA P value	Species	Theoretical MW/pI	Experimental MW/pI	Expression pattern
Transposons							
Putative retroelement[b]	107	gb|AAK53841	0.0036	*Oryza sativa*	22.4/5.35	33.0/4.82	
Unknown protein							
Unknown[a]	7	gb|ABK93540	2.5E-06	*Populus trichocarpa*	20.2/9356	35.9/5.8	
Unknown	9	gb|EDQ51452	6.4E-06	*Picea sitchensis*	13.9/9.83	48.8/5.61	
Hypothetical protein[a]	49	emb|CAN62646	1.7 E-04	*Vitis vinifera*	53.6/7.67	34.6/4.91	
Hypothetical protein[a]	56	emb|CAN63338	2.7E-04	*Vitis vinifera*	14.6/6.66	38.1/5.52	
Unknown[b]	88	gb|ABK22457	0.002	*Picea sitchensis*	10.9/5.85	13.9/6.07	
Unknown[b]	81	gb|ABK26383	0.0015	*Picea sitchensis*	19.6/5.83	32.6/5.01	

[a] Identified by MALDI-TOF.
[b] Identified by LC-MS/MS.
* Identified as a hypothetical or unknown protein, based on sequence similarity as assessed by BLASTP (www.ncbi.nlm.nih.gov/BLAST/) and annotated as having a cellular function similar to that of the protein indicated under Accession number.

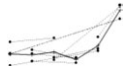distant family of cysteine proteinases (Bozhkov *et al.*, 2005). In the early stages of seed development in *Brassica napus*, a cysteine proteinase gene was found to be expressed only in the inner integument as it undergoes PCD (Wan *et al.*, 2002). That cysteine protease, which is expressed in the early stage in Chinese fir seed development, mediates PCD during embryonic patterning, and thus constitutes a functional link between PCD and embryogenesis in plants (Bozhkov *et al.*, 2005). Another PCD protein is poly [ADP-ribose] polymerase 1 (PARP-1, spot 57), which is also down-regulated during Chinese fir seed development; PARP-1 is a nuclear enzyme that plays a critical role in genomic stability by mediating the DNA damage surveillance network, and it is also a mediator of cell death (Yu *et al.*, 2002). In some animal cell types, PARP may play a role in controlling cell death through NAD depletion. In plants, the function of

PARP is less understood. The activation and specific cleavage of PARP are essential for PCD progression induced by heat shock in tobacco suspension cells (Tian *et al.*, 2000). In gymnosperm seed development, another PCD process involves the degeneration of a narrow, unicellular layer that lines the corrosion cavity of the female gametophyte, making room for the actively growing embryo later in seed development (Filonova *et al.*, 2002). In the present study, one PCD-related protein (spot 4, a transglutaminase) accumulated in the columnar embryo stage and early cotyledonary stage, in which the embryo is growing rapidly. Transglutaminases are responsible for the formation of apoptotic bodies in animal PCD. Transglutaminases also function as GTPases and thus can act as effectors in the prevention of PCD (Serafini-Fracassini *et al.*, 2002). Little information is available regarding the involvement of transglutaminase in
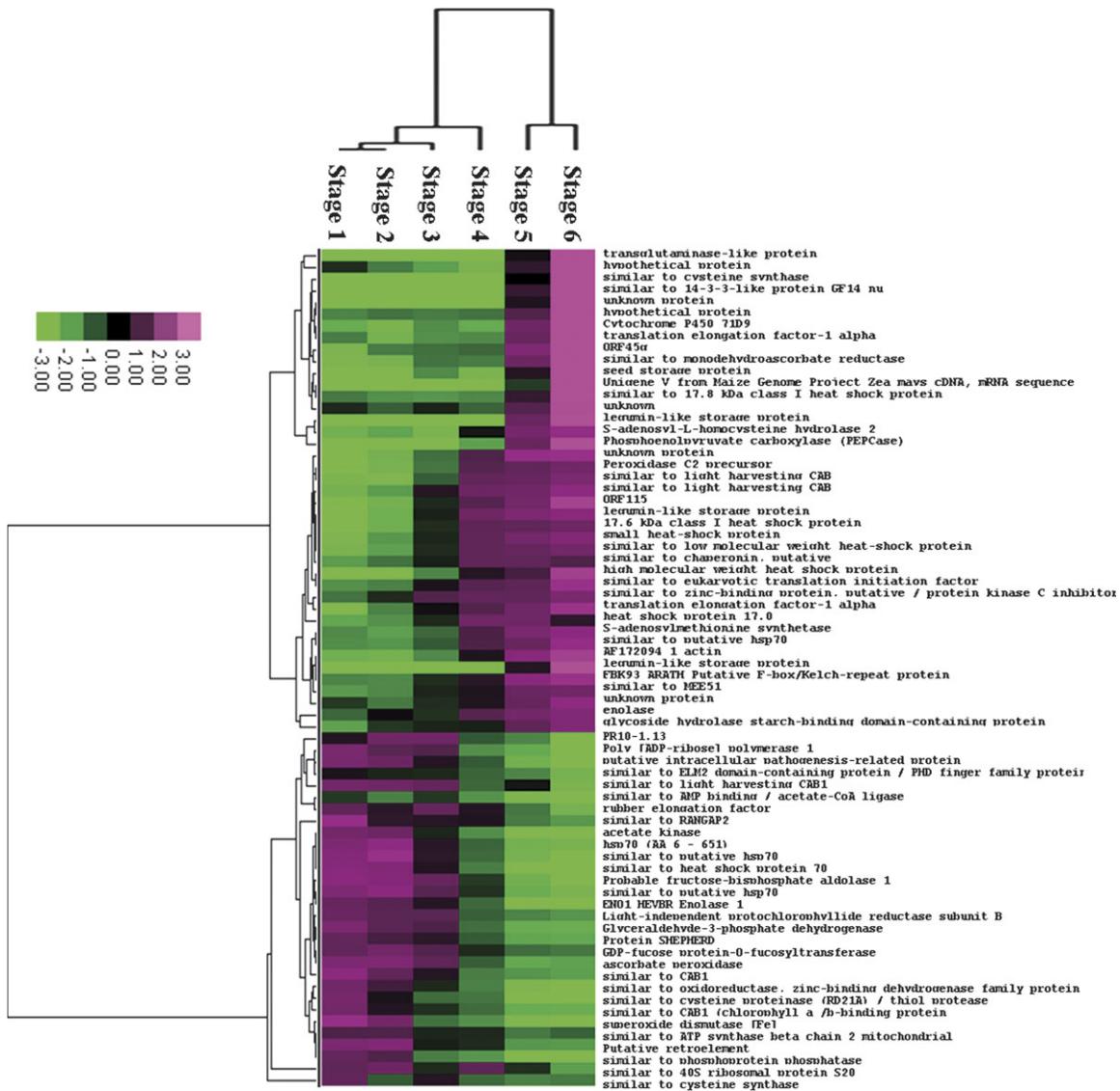
Fig. 3. Hierarchical clustering of the differentially expressed proteins identified in this study. Clustering was based on the protein expression levels across stages 1, 2, 3, 4, 5, and 6 of seed development (the grading represents the ratios of protein expression levels).

plant PCD. Recently, however, a transglutaminase was found to participate in PCD and regulate flower life span in the tobacco corolla (Della Mea *et al.*, 2007).

PCD is the main mechanism by which unneeded structures are eliminated and morphology is changed during gymnosperm seed development. The involvement of PCD in the formation and development of somatic embryos in Norway spruce has also been reported (Filonova *et al.*, 2000), and the cysteine protease mcll-Pa executes PCD during Norway spruce embryogesis (Bozhkov *et al.*, 2005). The molecular mechanism by which PCD participates in gymnosperm seed development and somatic embryogenesis remains unclear, however. Based on our data for developing seeds of the Chinese fir, we speculate that there are multiple PCD programmes that maintain the proper balance between cell death and development. Except for the caspase-like protein we identified (cysteine proteinase), the PARP-1 and transglutaminase are

new candidates for PCD in gymnosperm seed development. How PCD is regulated by these proteins during seed development in Chinese fir must be investigated further.

Proteins related to embryonic development

Genomic imprinting is a phenomenon in which only a specific maternal or paternal allele is expressed in a developing embryo (Huh *et al.*, 2007). It has been shown that the maternal and paternal contributions to seed development are not equivalent due to genomic imprinting as well as the control over early seed development by the 'maternal effect' in plants (Grimanelli *et al.*, 2005). The protein 'Maternal effect embryo arrest 51' (spot 92) is involved in the progression of the embryo over time, from zygote formation to the end of seed dormancy. It was found here that this protein—a homologue of the *Arabidopsis* maternal effect embryo arrest 51 that is

responsible for the embryonic development ending in seed dormancy (the reference sequence was derived from AT4G04040)—was up-regulated during seed development. Other reports on various animal and plant species have also described the maternal effect. For example, certain maternal housekeeping proteins are translated during bovine oocyte maturation and early embryo development (Massicotte *et al.*, 2006). The maternal gene *SIN1* in the animal egg regulates expression of a diffusible morphogen required for zygotic development and is also required for embryo development in *Arabidopsis* (Golden *et al.*, 2002). Mutation in the maternal allele of the *Arabidopsis* gene *MEA* results in aborted embryos, and this mutation cannot be rescued by pollination with a paternal wild-type gene, implying that *MEA* promotes embryo development only when expressed maternally (Cairney and Pullman, 2007). There are few reports, however, on the maternal effect in gymnosperms.

Spot 66 was identified as one of the *C. lanceolata* proteins related to embryo development; this protein is similar to the ELM2 domain–containing protein/PHD finger family. The ELM2 domain is a small domain of unknown function, but it is similar in sequence to domains in Egl-27and MTA1. The ELM2 domain also may be involved in DNA binding, or perhaps may be a protein–protein interaction domain (Solari *et al.*, 1999). Both Egl-27and MTA1 have been cloned in *Caenorhabditis elegans*, and each of these proteins is in a distinct chromatin regulatory complex; these proteins are required for embryonic patterning, although they are functionally redundant (Solari *et al.*, 1999). The PHD finger, a Cys4-His-Cys3 zinc finger, is found in many regulatory proteins from plants to animals; PHD finger–containing proteins are associated with chromatin-mediated transcriptional regulation (Halbach *et al.*, 2000). Chromatin modification and large-scale remodelling events are necessary for embryo patterning during seed development, and these events control the basic body pattern from fertilization to the heart stage in angiosperm seeds (Baroux *et al.*, 2007). It was found that spot 66 was highly expressed from the cleavage polyembryony stage to the dominant embryo stage and was then down-regulated from the columnar embryo stage to the early cotyledon stage. Accordingly, it is postulated that the spot 66 protein participates in the control of embryo patterning from the cleavage polyembryony stage to the dominant embryo stage.

Met metabolism is critical for the amino acid metabolic pathway of seed filling

The Met metabolic pathway is essential for cell viability in that it provides building blocks for proteins and generates the precursor to *S*-adenosylmethionine (Ado-Met), which is the main methyl group-donating compound in cells. There have been reports that Met metabolism has an important role in seed development and germination, as illustrated by proteomic analyses of developing *Medicago truncatula* seeds (Gallardo *et al.*, 2003, 2007) and of *Arabidopsis* seed germination and priming (Gallardo *et al.*, 2001). In the present study, consistent with the high demand for protein

synthesis in seed filling, two spots involved in Met metabolism, namely, Ado-Met synthase (spot 46), and *S*-adenosyl-l-homocysteine (AdoHcy) hydrolase (spot 61), were highly expressed between stage 4 and stage 6. Two spots, 6 and 86, were found to be similar to cysteine synthase, which is responsible for the formation of cysteine from *O*-acetyl-serine and hydrogen sulphide. Cysteine is first reduced by a sulphur-containing compound and serves as the sulphur donor for Met (Chronis and Krishnan, 2003). Ado-Met synthase is a key enzyme in plant metabolism, catalysing the biosynthesis of Ado-Met from Met and ATP. Ado-Met is not only the primary methyl-group donor but also a source of amino-alkyl and amino groups used for the biosynthesis of amino acids, poly-amines, and the hormone ethylene, and it also provides the methylene group used in the biosynthesis of fatty acids that accumulate in many types of seeds (Bouvier *et al.*, 2006). AdoHcy hydrolase catalyses the production of AdoHcy, which is formed after donation of the activated methyl group of AdoMet to an acceptor. AdoHcy is the product of all Ado-Met-dependent biological transmethylation reactions and is a potent competitive inhibitor of methyltransferases that are crucial to cell growth and development (Turner *et al.*, 2000). The continuous resynthesis of Ado-Met from ATP and Met is made possible by the recycling of adenosine and homocysteine derived from AdoHcy. Thus, AdoHcy is an important intermediate in maintaining proper Ado-Met levels (Weretilnyk *et al.*, 2001). The high accumulation of Ado-Met synthase, similar to AdoHcy hydrolase, parallels the accumulation of the major seed storage proteins, and therefore these enzymes play a key role in providing the building blocks for proteins, polyamines, and ethylene during the early cotyledonary stage of seeds. In Fig. 4, a model is proposed for the involvement of Met metabolism in the various stages of seed development based on our results for the protein expression patterns observed during the development of Chinese fir seeds as well as specific features of Met biosynthesis/metabolism (Ravanel *et al.*, 1998), which indicated that the metabolism of Met and Cys is critical for storage protein synthesis during seed filling. A similar observation was made in a proteomic analysis of developing *M. truncatula* (Gallardo *et al.*, 2007).

Developmental expression of enzymes related to carbon metabolism in seeds

The enzymes and substrates involved in carbon metabolism in seeds contribute to the synthesis of starch. Several enzymes related to carbon metabolism (spot 22, probably fructose-*bis*phosphate aldolase; spot 65, glyceraldehyde-3-phosphate dehydrogenase; spot 67, GDP-fucose protein-*O*-fucosyltransferase; spot 103, ENO1_HEVBR enolase) were down-regulated as seed development proceeded (Fig. 5). These enzymes were highly expressed in stage 1 of developing Chinese fir seeds. These proteins are probably involved in supplying carbon substrates for the synthesis of energy storage compounds under conditions that limit CO_2 fixation. The proteins 'similar to ATP synthase beta chain 2
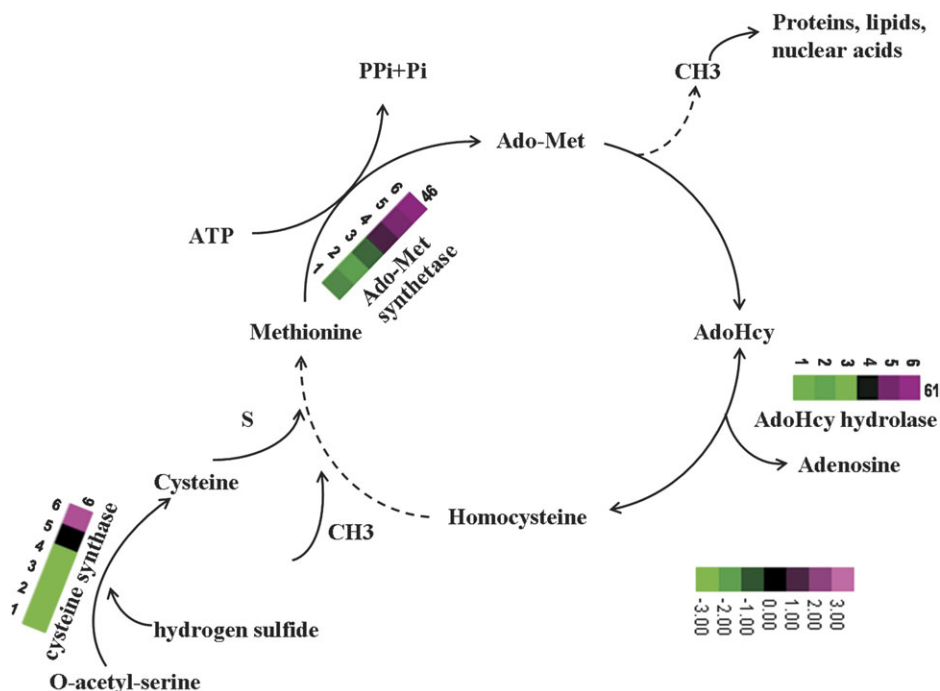
Fig. 4. Methionine metabolic pathway during Chinese fir seed filling. The proteins identified in our study as being significantly up- or down-regulated are shown with the corresponding grading.
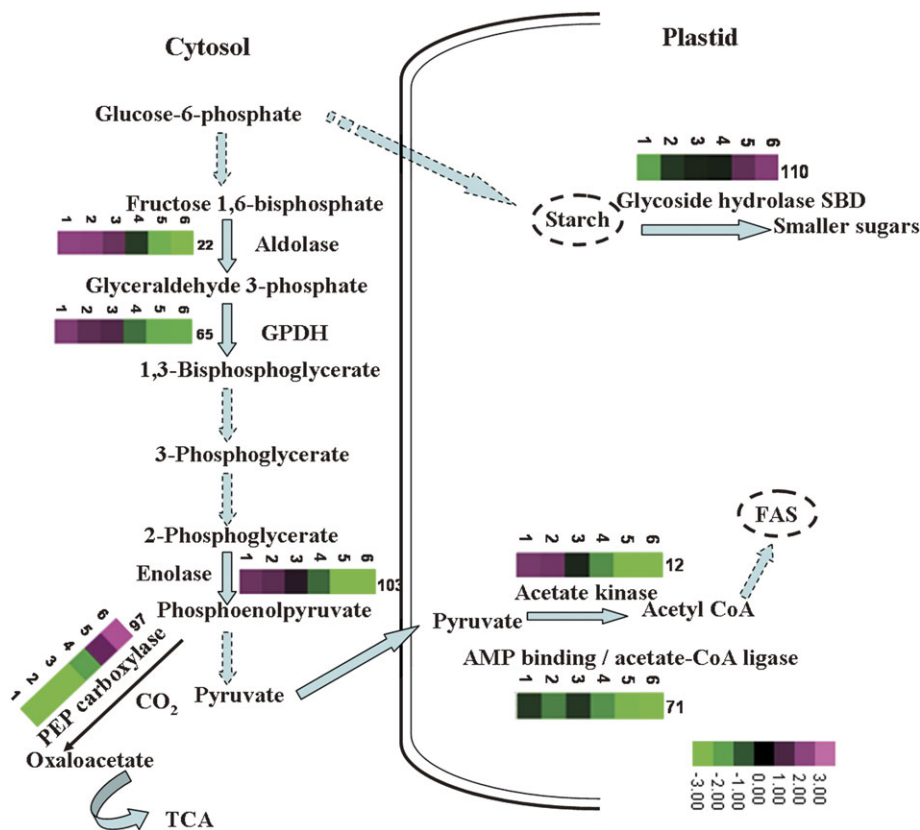
Fig. 5. Kinetics of carbon metabolism in developing seeds. Proteins identified as being significantly up- or down-regulated are shown with the corresponding grading.

mitochondrial' (spot 83) and 'similar to CAB1' (chlorophyll *a/b*-binding protein 1; spots 69, 75, 80) also mainly accumulated in stage 1. Embryonic photosynthesis has been proposed to play a role in providing ATP and NADPH for the synthesis of storage compounds during seed development (Gallardo *et al.*, 2003). Another enzyme, glycoside

hydrolase starch-binding domain-containing protein (spot 110), was up-regulated during seed development. Glycoside hydrolases catalyse the hydrolysis of glycosidic linkages to generate smaller polysaccharides/monosaccharides. The starch-binding domain belongs to the carbohydrate binding module family 20, members of which accelerate the rate of hydrolysis of insoluble starch. AMY1-SBD (α-amylase 1-starch-binding domain-containing protein) is a type of glycoside hydrolase starch-binding domain-containing protein that hydrolyses barley starch granules 15-fold faster than recombinant AMY1 at low concentration (Juge *et al.*, 2006). A similar observation was reported in developing seeds of soybean (Adams *et al.*, 1980). In stage 1, starch accumulates in transient reserves, which are later utilized in seed development. The up-regulation of AMY1-SBD is possibly responsible for starch mobilization.

Information on seed development contributes importantly to our understanding of the molecular and physiological bases of embryogenesis (Balbuena *et al.*, 2009; Silveira *et al.*, 2008). For example, the identification of the PCD proteins will be helpful in establishing a mechanistic link between PCD and embryo patterning; the proteins of highest abundance, namely legumin-like storage protein, are further used for plantlet growth. The up-regulated expression of these proteins might constitute a marker for maturation during seed development. Thus, growth-stimulating agents that induce protein expression when added to the culture medium, are necessary for successful somatic embryo maturation. Our results provide new insights into the complex processes involved in Chinese fir seed development as well as information on both the practical means of further improving somatic embryogenesis through medium manipulation and the protein markers useful for screening based on protein expression. A more spatio-temporally detailed investigation will provide a better understanding of the biological processes involved in seed and embryo development.

Acknowledgements

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