



Proteomic analysis of differentially abundant proteins in the embryo and endosperm of *Givotia moluccana* (L.) Sreem

Durgeshwar Pullaiahgari¹ · Rajesh Kumar Meena¹ · Swaroopa Rani Tirupaati¹ · Padmaja Gudipalli¹

Received: 25 September 2018 / Accepted: 14 January 2019 / Published online: 19 June 2019
© The Author(s) 2019

Abstract Proteome analysis of embryo and endosperm of *Givotia moluccana*, a promising angiosperm tree species was carried out using two-dimensional electrophoresis combined with matrix-assisted laser desorption/ionization time of flight to identify differentially abundant proteins and their possible functions. The study revealed the presence of 492 protein spots in the embryo and 432 in the endosperm, and the relative abundance of 44 spots varied significantly between the two. Of the 21 functionally characterized protein spots, 14.3% were involved in metabolism and energy, 28.6% in protein destination and storage, 14.3% in defense and stress and 19% in cell growth and division. The embryo-specific protein ATP-dependent zinc metalloprotease FtsH is predicted to be involved in chloroplast biogenesis and the endosperm-specific protein hydroxyacyl-ACP dehydratase in fatty acid synthesis. These results suggested that the seeds have proteins required for germination and for stress responses.

Keywords Proteome · Embryo · Endosperm · Euphorbiaceae · *Givotia moluccana* · Forest tree · 2-DE

Introduction

The seed of a plant is a structurally and physiologically organised entity; the embryo develops into the plant, the endosperm is enriched with storage compounds to nurture the embryo during primordial growth stages, and a seed coat protects the seed from adverse conditions. Seed formation begins after a double fertilization event followed by embryogenesis with three phases of cell divisions, a maturation phase in which seed storage compounds accumulate, differentiation of the embryo and acquisition of desiccation tolerance. The embryo and endosperm differ in ploidy level and coordinately interact to regulate plant development (Chaudhury et al. 2001). Apart from providing nutrients during seed germination, the endosperm also sustains a high osmotic potential and provides mechanical strength during embryo growth (Lopes and Larkins 1993) and plant growth substances such as cytokines promote cytokinesis and signalling molecules for embryogenesis (Hong et al. 1996; van Hengel et al. 1998).

Seeds occupy immense economic and social relevance to humankind and animals (Shewry and Casey 1999). Majority of the proteins and oil consumed by humans and animals are derived from seeds. *Givotia moluccana* (L.) Sreem. (white catamaran tree; *Euphorbiaceae*) is an economically important tree with softwood that is used extensively for making toys, boxes and fancy articles. The genus *Givotia* is represented by four species that are confined to India, Sri Lanka, Madagascar and Eastern Equatorial Africa (Sreemadhavan 1975). The seeds of *G. moluccana* have been reported to be rich in oil used to

Project funding: The work was supported by DBT-CREBB (Department of Biotechnology-Centre for Research and Education in Biology and Biotechnology), DST-FIST (Department of Science and Technology-Fund for Improvement of Science and Technology Infrastructure) Level-II (Phase-II) and UGC-SAP (University Grants Commission-Special Assistance Programme) Departmental Research Support-I (DRS-I) programmes.

The online version is available at <http://www.springerlink.com>

Corresponding editor: Tao Xu.

✉ Padmaja Gudipalli
gudipallipadmaja@gmail.com

¹ Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad, Telangana 500046, India

lubricate fine machinery. In addition, its endosperm extracts have been used to improve the digestion of children by Palliyar tribes of Tamilnadu (Geetha and Vijayalakshmi 2013). The endospermic seeds of *G. moluccana* have a pronounced dormancy of 1–1.5 years with low natural regeneration. The molecular mechanisms underlying this seed dormancy have been unexplored, but understanding the mechanism may lead to improvements in propagation and regeneration. Proteomic analysis is a powerful tool for understanding such complex biological processes as seed dormancy and germination.

Two-dimensional electrophoresis (2-DE), followed by identification of particular spots through matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI–TOF–MS/MS) is a powerful technique for obtaining an overview of the differentially expressed proteins in numerous plant parts and also at different stages of plant development (Lin et al. 2003; Cánovas et al. 2004). Studies of proteome differences between embryo and endosperm in tomato, Arabidopsis, rice, wheat, maize and barley have shown changes in abundance of storage proteins, housekeeping enzymes, defense-related enzymes and proteins involved in metabolic processes (Runeberg-Roos et al. 1994; Gallardo et al. 2001; Méchin et al. 2004; Komatsu 2005; Sheoran et al. 2005; Bønsager et al. 2007; He et al. 2015). Liu et al. (2009) reported that significant groups of differentially abundant proteins in the embryo and endosperm of *Jatropha curcas* were associated with metabolism and disease/defence. The seed morphology of *Givotia* shares similarities with those of the most-studied species of *Euphorbiaceae*, *Jatropha* and *Ricinus*, but despite the economic importance of *Givotia* species, there are no reports on the proteomes in their seeds. The present study is thus aimed at analyzing the proteome of embryo and endosperm to discern differentially abundant proteins and understand their function.

Materials and methods

Plant material

Mature fruits of *G. moluccana* were collected from the Forest Research Station, Mulugu, Telangana during January–February 2016 and dried in the sun until the moisture content was lower than 10%.

Seed size and mass

The seeds were removed manually from the dried fruits with the help of secateurs, and seed coats were broken with the help of a bench-vice tool. The seed coat, embryo and endosperm were separated manually using sterile forceps

and weighed. Embryo and endosperm from freshly harvested dried fruits (< 10% moisture content) were frozen in liquid nitrogen and stored at -80°C until further use.

Protein extraction

Proteins were extracted from embryos and endosperms separately using the protocol described of Shen et al. (2003). About 100 mg each of embryo and endosperm were ground into fine powder in liquid nitrogen with 100 mg of polyvinylpyrrolidone and transferred into microcentrifuge tubes containing pre-chilled extraction buffer (50 mM Trizma base pH 7.5, 250 mM sucrose, 10 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT) and 1% Triton X-100). The homogenate was stirred at 4°C for 10 min and centrifuged at $13,000\times g$ for 30 min at 4°C . The supernatant was collected into a fresh tube and mixed with equal volumes of 30% trichloroacetic acid (v/v) and incubated at -20°C overnight, then centrifuged at $12,000\times g$ for 15 min at 4°C . The pellet was washed thrice with pre-chilled 100% acetone containing 6.5 mM DTT, then centrifuged. Finally, the pellet was vacuum-dried for 3 min at 30°C and allowed to dissolve overnight in sample buffer containing 7 M urea, 2 M thiourea, 4% w/v 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS), 2% w/v ampholytes and 6.5 mM DTT. Protein concentration was determined using the amido-black method with BSA as the standard (Henkel and Bieger 1994).

2-D electrophoresis and image analysis

2-DE was performed according to Rani et al. (2015). In brief, proteins were separated at 20°C in the first dimension (GE Healthcare Ettan IPGphor 3 Isoelectric focusing system) by loading 500 μg of protein sample on 18 cm immobilized pH gradient (IPG) strips with linear pH gradient of 4–7 (GE Healthcare). Before SDS-PAGE in the second dimension, the strips were equilibrated for 20 min with equilibration buffers containing 6 M urea, 30% v/v glycerol, 2% w/v SDS, 50 mM Tris-HCl pH 8.8 with 2% w/v DTT, followed by 2.5% (w/v) iodoacetamide. After equilibration, the IPG strip was separated on 12% SDS-PAGE in EttanDalt6 vertical electrophoresis system (GE Healthcare) at 20°C at 150 V and stained with Coomassie blue G-250.

Image and data analyses

The 2-DE of embryo and endosperm protein samples was performed with three biological replicates, and 2-DE gel images were obtained by scanning the gels with a Magic

Image scanner (GE Healthcare). Differentially expressed spots in 2-DE gels were analysed through ImageMaster 2D platinum software version 7.0 (GE Healthcare). Triplicate 2-DE gel images of embryo and endosperm were aligned, and spots were detected automatically. Match set classes were created, and gels were further aligned by selecting four spots as landmarks. Normalization of spots was done by considering percentage of the volume of each spot relative to the total volume of all spots in the gel. The relative change in protein abundance between embryo and endosperm was determined by comparison with embryo gels. The protein spots that differed by ≥ 1.5 -fold between embryo and endosperm were considered as more abundant and ≤ 0.6 as less abundant. This arbitrary cut-off was found to be statistically significant in proteome studies (Rani and Podile 2014). The differentially expressed protein spots were selected for further analysis after Student's *t* test ($p \leq 0.05$).

Protein identification

Spots of interest were excised from the stained 2-DE gels and digested by sequence-grade trypsin (Promega, Madison, WI, USA) as described by Rani and Podile (2014). After trypsin digestion, peptides were analyzed by MALDI-TOF mass spectrometry using a BrukerAutoflex III Smartbeam (Bruker Daltonics, Bremen, Germany). The obtained data were used in a search of the National Center for Biotechnology Information (NCBI) or Swiss-Prot databases using Mascot software (<http://www.matrixscience.com>) and BioTools 3.1 software (BrukerDaltonics, Bremen, Germany). The taxonomic category was set to Viridiplantae (green plants), and other search parameters included fixed modification of carbamidomethyl (C), variable change of oxidation (M), enzyme trypsin, peptide tolerance of 100 ppm and MS/MS tolerance of 0.4 Da. The protein identity was accepted only if the MASCOT probability was at a significant threshold level ($p \leq 0.05$), and at least two peptides matched.

Results

Seed size and mass

Seeds of *G. moluccana* are oval with a diameter of 11–16 mm and comprise embryo with two thin cotyledons that are embedded in the large endosperm (Fig. 1). The dry mass of a single seed varied from 0.6 to 1.1 g. The seeds constituted 17.7% of the fruit mass. It is composed of the seed coat, embryo and endosperm, which constituted 62.8%, 2.3% and 34.9% of the total seed mass, respectively

(Fig. 2). The seed coat comprises two layers: a hard brown epispERM with a strongly attached thin, white endopleura.

Embryo and endosperm proteome profiles

The total soluble proteins of the embryo and endosperm were profiled by 2-DE gels. Scatter plots drawn between three independent gels of the embryo and endosperm proteome revealed similar spot pattern in each experiment (Fig. 3). Coomassie blue staining followed by imaging of the gel by Image scanner revealed 492 spots in the embryo and 432 spots in the endosperm (Fig. 4). The protein spots visualized on the 2-DE gels were found in the range of 4–7 pH. Based on the protein distribution, molecular mass and pI range, the spotting pattern of embryo and endosperm proteins on the 2-DE maps had many similarities; a few protein spots differed in abundance (Fig. 5). The protein distribution between the embryo and endosperm was compared by grouping the spots horizontally (0.5 pI was considered as 1 unit, and pI ranged from 4 to 7; Fig. 6) and vertically (using known protein molecular mass markers; Fig. 7). Both histograms had high similarities in proteome distribution patterns for the embryo and endosperm (Figs. 6, 7). Most of the proteins from the embryo and endosperm were distributed similarly in the pI range from 4 to 6, comprising 70% of the total proteins (relative quantity). The protein spots of the embryo were found to be 8% less at pI of 6–6.5 and 3% higher in the pI range from 6.5 to 7 compared to endosperm proteome map (Fig. 6). Both proteomes were in the molecular mass range starting from 200 kDa to less than 30 kDa (Fig. 7) and revealed many similarities in the distribution pattern of protein spots between these tissues with respect to molecular mass. The majority of the proteins (80%) were in the range of molecular weight from 100 to 25 kDa as calculated by ImageMaster software. However, certain protein spots differed between the endosperm and embryo (Fig. 7).

With the 2-DE proteome map of the embryo as a reference, the endosperm proteome was compared. About 368 proteins (66.2%) were commonly present in both tissues, whereas 124 protein spots were found only in the embryo and 64 protein spots were explicitly present in the endosperm. Based on the relative spot volume, 44 proteins were significantly ($p \leq 0.05$) more abundant in the embryo than in the endosperm.

Protein identification

Of the 21 major differentially abundant protein spots analyzed by MALDI-TOF-MS/MS, eight spots (11, 74, 125, 128, 134, 135, 147 and 180) were significantly higher in relative volume in the endosperm than in the embryo (Figs. 5, 8) and eight (spots 27, 72, 105, 119, 132, 271, 300

Fig. 1 Morphology of different parts of *Givotia moluccana* fruit. Scale bar = 1 cm

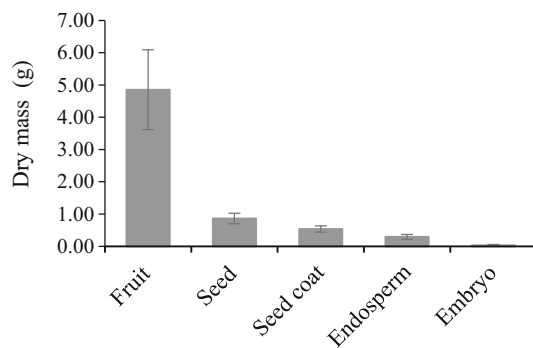


Fig. 2 Mean dry mass ($\pm$ SD) of different parts of *Givotia* fruit. Three independent experiments were done, each with three replicates

and 302) were higher in the embryo. Three protein spots (493, 512 and 514) were endosperm-specific, and 2 (414 and 427) were embryo-specific (Table 1).

The embryo-abundant protein spots were functionally classified into six classes: metabolism and energy (20%), defense and stress (20%), protein destination and storage (20%), cell growth and division (20%), and unclassified proteins (20%) (Fig. 9a). Endosperm-abundant proteins were also classified into six classes: metabolism and energy (9.1%), defense and stress (9.1%), protein destination and storage (36.3%), cell growth and division (18.2%), predicted and unknown proteins (18.2%), and unclassified proteins (9.1%) (Fig. 9b).

Discussion

The small embryo of *G. moluccana* has two thin cotyledons and is embedded in an enlarged and bulky endosperm. The 2-DE protein profiles of the embryo and endosperm showed high similarities in terms of distribution of protein spots. A significant similarity in the distribution of protein spots and protein types with variations in protein abundance between the mature embryo and endosperm was also

reported for *J. curcas* and tomato (Sheoran et al. 2005; Liu et al. 2009). In angiosperms, the embryo and endosperm develop after distinct fertilization events and differ in function, morphology and ploidy. The endosperm results from the fusion of two polar nuclei ($2n$) with a second sperm nucleus (n) and is triploid ($3n$), whereas the embryo is diploid ($2n$) formed by the fusion of the sperm nucleus (n) with the egg (n) (Lopes and Larkins 1993). Our results indicated that the proteomes of embryo and endosperm were highly comparable in terms of distribution of protein spots though the structures differ in ploidy and morphology.

However, 44 protein spots were differentially abundant between the embryo and endosperm. Around 368 proteins matched between the two, greater than 60% using the embryo as the reference. Unmatched proteins constituted approximately 34% of the total; 124 proteins were specific to the embryo, and 64 were specific to the endosperm. These unique protein spots suggest different functions in the embryo and endosperm.

Proteins related to metabolism and energy

Chloroplast and photosynthesis are important for germination and subsequent vigour of seedlings (Allorent et al. 2015). The purpose of seed photosynthesis is fascinating even though seeds are considered to be sink organs which acquire with storage molecules such as carbohydrates, proteins, mRNAs and other nutrients from maternal tissues (Hua et al. 2012). In the present study, cytochrome *f* (spot 27A) and tRNA (Ile)-lysine synthase (spot 271A) (TilS), involved in photosynthesis and amino acid synthesis, were more abundant in the embryo than in the endosperm. Cytochrome *f*, an intrinsic membrane-bound protein that is part of the cytochrome *b6f* complex in the photosynthetic electron transfer chain, is engaged in the transmission of electrons from the Rieske Fe-S protein to plastocyanin in the thylakoid lumen (Gray 1992). Allorent et al. (2015)

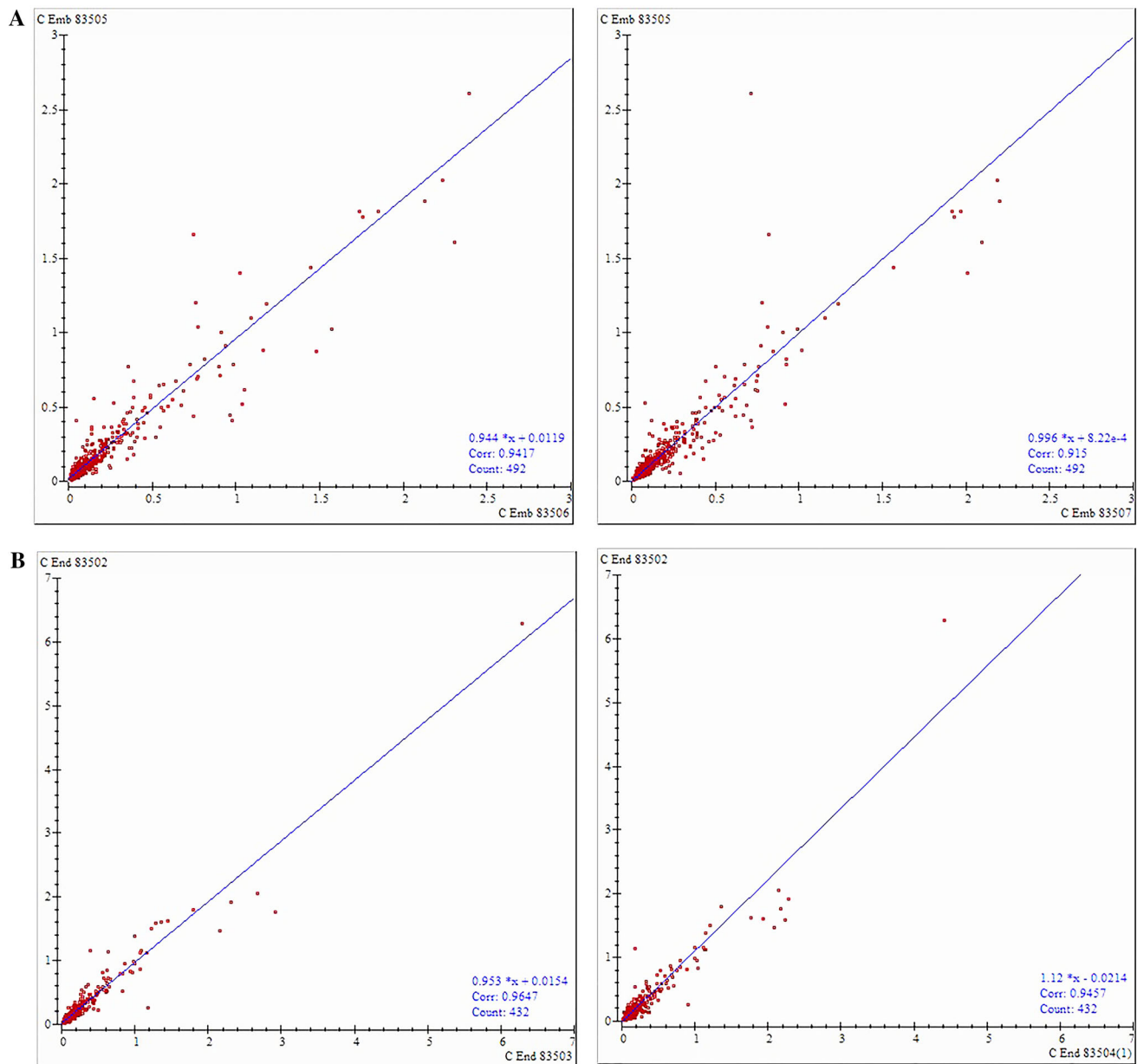


Fig. 3 Scatter plots of proteins that matched from the two 2-DE gels of (a) embryo and (b) endosperm. Plots were constructed using ImageMaster 2D Platinum software. The x-axis indicates protein

abundance from one 2-DE map, and the y-axis indicates abundance from the other

reported that *Arabidopsis thaliana* seeds lacking cytochrome b6f had poor germination. tRNA (Ile)-lysidine synthase (TilS) is involved in changing the specificity of tRNA from met to Ile. TilS binds to the tRNA ending with a purine and modifies cytidine in the first position to lysidine. Metabolic activities of cells are reactivated after the onset of germination, and the abundance of the proteins related to photosynthesis and amino acid biosynthesis is an indication that seeds, upon receiving signals for germination, can activate photosynthetic machinery to generate energy needed for plant growth.

The maturation phase in oil-seed plants is exemplified by the differentiation of a photosynthetic stage in which photosynthesis may play a direct role in lipid storage accumulation (Eastmond et al. 1996; Goffman et al. 2005). Hydroxyacyl-ACP dehydratase (spot 493D) (HAD), related to mitochondrial fatty acid synthesis, was specifically present in the endosperm and participates in the synthesis of unsaturated fatty acids and involved in the dehydration of long and short chain hydroxyl acyl-ACPS (Kastaniotis et al. 2004). HAD also plays a key role in mitochondrial function and morphology in yeast and crucial for promoting photorespiration in plants (Guan et al. 2017).

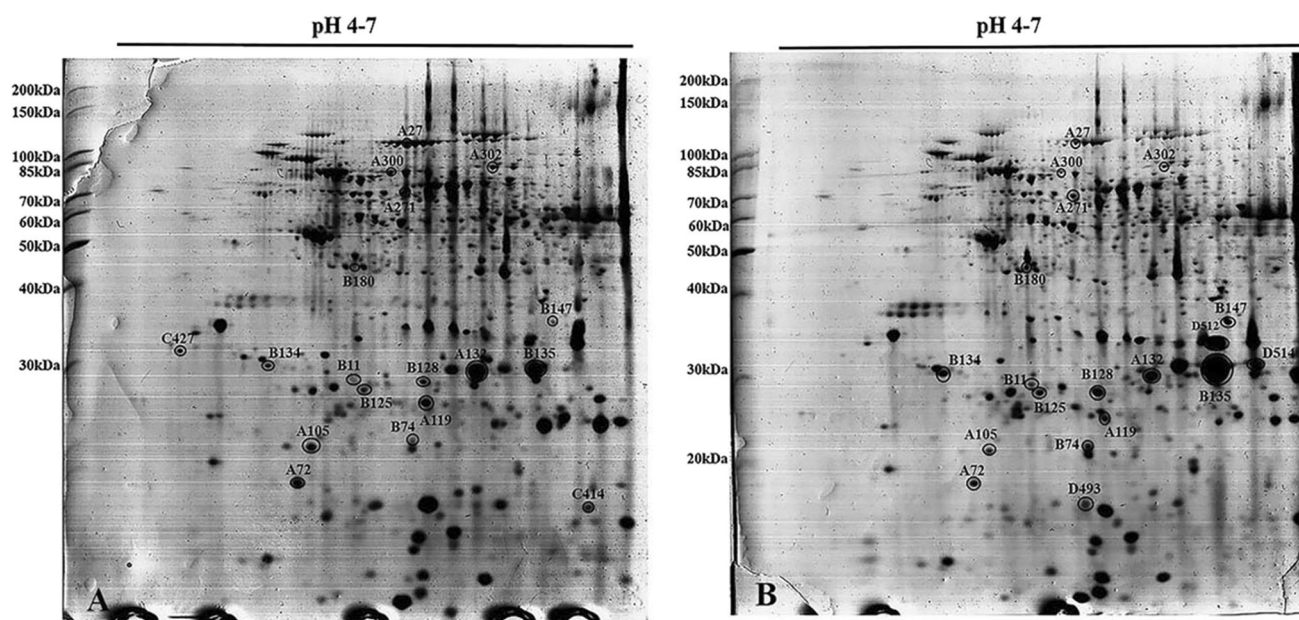


Fig. 4 2-DE maps of (a) embryo and (b) endosperm. Circles indicate significantly abundant in embryo (A) and endosperm (B) or specific to embryo (C) and endosperm (D)

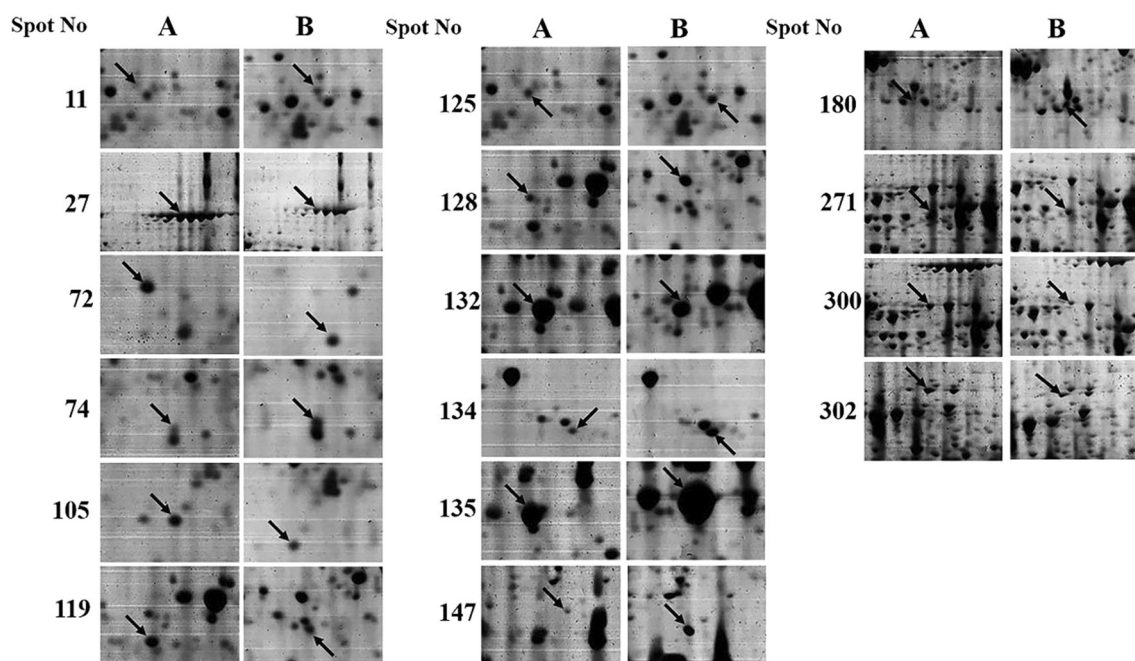


Fig. 5 Enlarged area showing differences in abundance of 16 representative protein spots in (a) embryo and (b) endosperm

Defence- and stress-related proteins

Two protein spots identified as phenylalanine ammonia lyase (PAL) (spot 72A) and glutathione *S*-transferase DHAR2-like (GST) (spot 119a) were abundant in the embryo compared to the endosperm. On the other hand, isoflavone reductase-like protein (IFR) (spot 147B) was abundant in the endosperm. PAL catalyzes the first rate-

limiting step in the phenylpropanoid pathway and protects the plant from several biotic and abiotic stresses in plants. The products of the phenylpropanoid pathway have antioxidative properties, thereby reducing the oxidative damage from reactive oxygen species (ROS). Seeds are exposed to ROS from seed development and germination (El-Maarouf-Bouteau and Bailly 2008). GST protects the cells from oxidative damage, dehydration, herbicides and

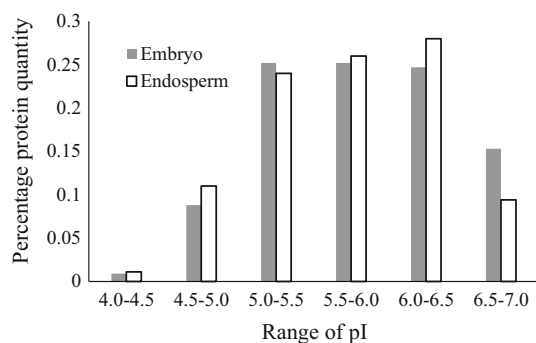


Fig. 6 Distribution of protein abundance in embryo and endosperm with respect to pI

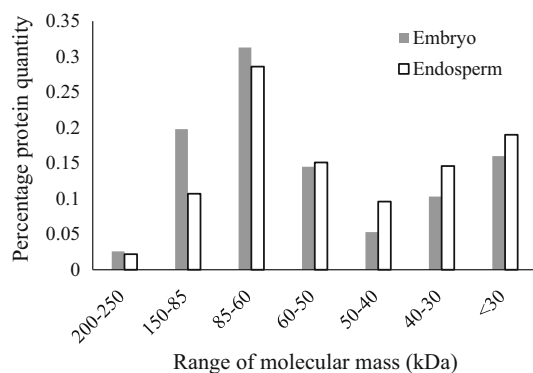


Fig. 7 Distribution of protein abundance in embryo and endosperm with respect to molecular mass

toxic substances. They are involved in fusion of non-ribosomally synthesized reduced glutathione to various toxic molecules and hydrophobic electrophiles. DHAR-like GSTs are unique and hold a cysteine molecule in the active site, catalyze the reduction of dehydroascorbate to ascorbate (Dixon et al. 2002). IFRs are a major group of phytoalexins derived from the phenylpropanoid pathway involved in isoflavonoid biosynthesis and help protect

against phytopathogens (Kim et al. 2008). IFR levels increase in *Ginkgo biloba* seeds exposed to UV-B, and after treatment with abscisic acid (ABA) and salicylic acid (SA) (Cheng et al. 2013). The high abundance of stress-related proteins in the embryo and endosperm of *Givotia* indicates the potential ability to withstand biotic and abiotic factors like high temperature, desiccation tolerance along with regulating the plant growth.

Protein destination and storage

Many seeds can withstand harsh environmental conditions and produce a well-developed plant in suitable conditions. The evolution of flexible mechanisms allows the seeds to protect themselves from stress; failure of this process can lead to death of the new seedling. One such mechanism protects proteins from dysfunction due to environmental stress. Chaperones hold the responsibility of preserving the proteins in their functionally active conformations. In seeds in the present study, chaperonin CPN60-2 (spot 74B) and aspartic proteinase A1 (AP-spot 125B and 134B) were more abundant in the endosperm while HSP70-HSP-90 organising protein 3-like (HOP3) (302A) was abundant in the embryo. These proteins are involved in protein assembly, folding and stability, and proteolysis (Lindquist and Craig 1988). Chaperonin CPN60-2, a mitochondrial chaperonin HSP60 that functions in an ATP-dependent manner, acts in the import and assembly of mitochondrial proteins (Wang et al. 2004). Under abiotic stress, CPN60 promotes refolding and assembly of misfolded or unfolded proteins in the mitochondrial matrix and the proper development of chloroplasts and subsequently embryo and seedling growth (Apuya et al. 2001).

HOP3 is a molecular cochaperone that protects the plant from both the biotic and abiotic stresses. Most of the pathogenesis-related proteins and the proteins associated

Fig. 8 Mean relative spot volume ($\pm$ SD) of the abundant proteins in the embryo and endosperm

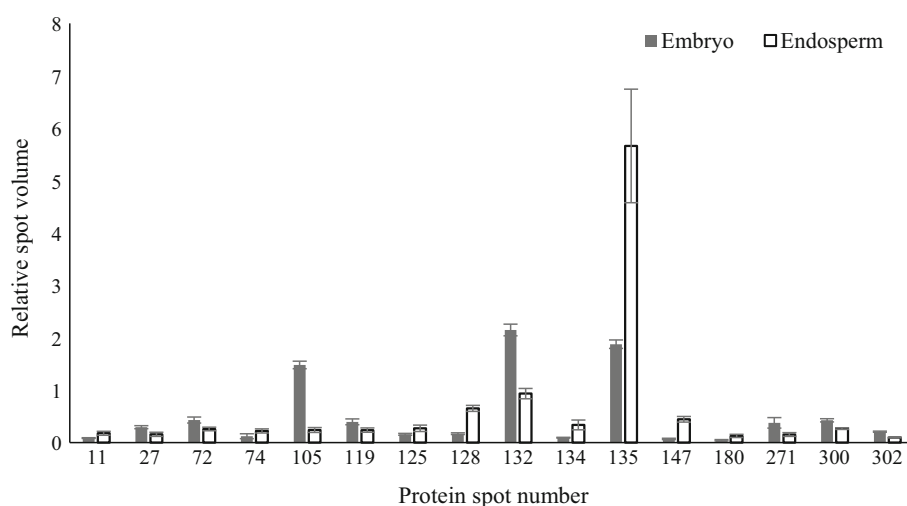


Table 1 Analysis of embryo and endosperm spots with differential protein abundance using MALDI TOF-MS/MS

Spot no.	Protein	Organism	Mt/pl	SC (%)	Accession	Matched peptides
11 ^B	Unknown protein	<i>Bathycoccus prasinus</i>	28.8/ 5.42	10.94	gil612385270	R.SPNEQV AHAR.V R.EEKYVDELTH TAK.K K.GRSTVQSTREMEK.F K.QQHGTTTRKVD MESQK.R K.TQCENAEKQQHGTTRK.V R.IPYDMQLKQV LANGK.K M.QNRNTFSWVKDQMSR.F K.ASSDWVMDSMGK.G K.VFTAMCNGQIIDS LLECLK.E K.SGIIDPLK.V K.SVAAGMNAMD LR.R R.ASWARNYA AKDIK.F K.IGGASEAEVGEKKDR.V K.GDYVDMVKSGIIDPLK.V K.VDLEMLGSCCKVTVSK.D R.RGITLAVDAVVTNLKS.R.A R.LYGIQQQAAR.E M.DSSKPMNGSAGGSPVGDER.K K.LYHLEVALGHFKK.W K.AAVGAPNVLGDCPFSQR.A
27 ^A	Cytochrome <i>f</i>	<i>Ceratophyllum demersum</i>	111.5/ 5.7	9	CYF_CERDE	
72 ^A	Phenylalanine ammonia-lyase	<i>Nicotiana tabacum</i>	14.42/ 5.24	22	PAL2_TOBAC	
74 ^B	Chaperonin CPN60-2, mitochondrial	<i>Eucalyptus grandis</i>	22/5.42	11.82	gil702441615	
105 ^A	CASP-like protein 2U10	<i>Selaginella moellendorffii</i>	22.8/ 5.20	13	CSPL6_SELML	
119 ^A	Glutathione <i>S</i> -transferase DHAR2-like	<i>Nicotiana tabacum</i>	25.6/ 5.85	14	NP_001312985.1	
125 ^B	Aspartic proteinase A1-like	<i>Fragaria vesca</i> subsp. <i>vesca</i>	27.6/ 5.43	4	XP_004287958.1	K.EPVFSFWLNR.N K.CYFSVACFFH SK.Y K.FFVGGNWK.C K.VIACIGETLEQR.E
128 ^B	Hypothetical protein SETIT_002630 mg	<i>Setaria italica</i>	27.6/5.8	7	KQL07672.1	
132 ^A	Gibberellin 3-beta-dioxygenase 2-2	<i>Triticum aestivum</i>	30/6.29	8	G3O22_WHEAT	R.VEAAIAGMFALPASEK.M K.AGHDIRHFCA VMEEFHR.E K.EPVFSWFNR.N K.NQEFIEATREPSITFLVAK.F K.NGKPADIHYGTGAISGYFSQDHSV.K.V M.AASGLKVPEMALR.V R.VLSTTGSSCHPPK.H K.AGHPTEFLVR.E R.FFPSEFGNDVDR.V R.FFPSEFGNDVDRVH AVEPAK.T K.GDHTNFEIEPSFGVEASELYPDVK.Y
134 ^B	Aspartic proteinase 1	<i>Castanea mollissima</i>	30.45/ 4.91	10	ACX55829.1	
135 ^B	CASP-like protein 2D1	<i>Sorghum bicolor</i>	30/6.41	14	CSPLA_SORBI	
147 ^B	Isoflavone reductase homolog	<i>Theobroma cacao</i>	37.3/ 6.56	17	XP_007042155.1	

Table 1 continued

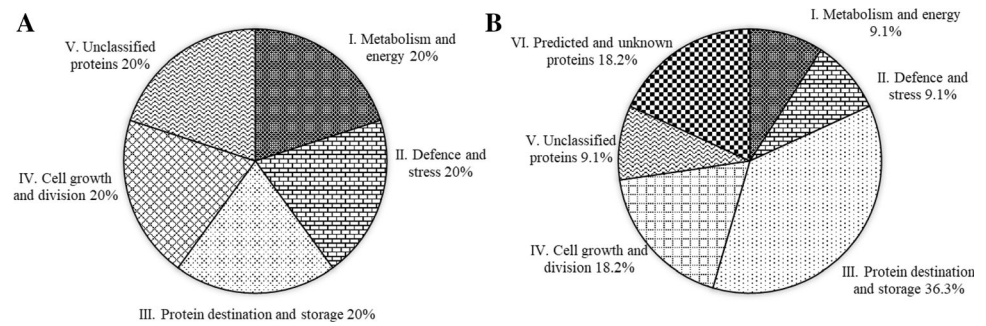
Spot no.	Protein	Organism	Mt/pl	SC (%)	Accession	Matched peptides
180 ^B	Actin	<i>Helianthus annuus</i>	46.15/ 5.42	48.5	gi 9082317 41934 297	K.AGFAGDDAPR.A K.IKVVAPPER.K K.GYALPHAILR.L R.HTGVMVGMGQK.D R.GYMFTTTAER.E R.AVFPSIVGRPR.H K.DAYVGEAQSKR.G K.GEYDESGPSIVHR.K K.IWHHTFYNELR.V K.GEYDESGPSIVHRK.C K.NYELPDGQVITIGAER.F K.LAYVALDYEQELETAK.S R.VAPEEHPVLLTEAPLNPK.A K.SSSSVEKNYELPDGQVITIGAER.F
271 ^A	tRNA(1le)-lysine synthase, chloroplastic	<i>Zygnema circumcarinatum</i>	71.87/ 5.85	5	TILS_ZYGCR	R.KLNSMLTIR.M K.LDFELLRAVPLAIQR.R
300 ^A	Hypothetical protein EUGRSUZ_J03127	<i>Eucalyptus grandis</i>	87.14/ 5.34	11	KCW53915.1	R.TTPSYVAFTDTER.L R.ARFEELNMDLFR.R K.ATAGDTHLGGEDFDNR.L K.NAVITVPA YFNDSQR.Q K.IDKSQVHDVVLVGGSTR.I K.HFTQAISLAFSNHVLYSNR.S K.DQRVMQALGVLLN.V
302 ^A	Hsp70-Hsp90 organizing protein 3-like	<i>Jatropha curcas</i>	127/6.2	6	XP_012087126.1	R.GVLIVGER.G R.LEENLKR.L K.WPKFRPK.F K.ELNGEKER.L R.LLQTVSFLLR.A R.VTAVAAPAAEVLEKK.E
414 ^C	ATP-dependent zinc metalloproteaseFtsH	<i>Morus notabilis</i>	10.3/ 6.74	3.6	gi 703120875	K.DAPQNGIK.R MRASHIR.T R.ASSEGAPENFGVR.R K.FLHEVEKDATR.A R.MKIDAYYEEMLK.S K.ISTISMHYSHFNARK.D
427 ^C	PREDICTED: uncharacterized protein LOC106761318	<i>Vigna radiata</i> var. <i>radiata</i>	30.43/ 4.36	37.8	gi 950971575	

Table 1 continued

Spot no.	Protein	Organism	Mt/pI	SC (%)	Accession	Matched peptides
493 ^D	Hydroxyacyl-ACP dehydratase	<i>Jatropha curcas</i>	16.8/ 5.83	185	gi 924434384	K.RFGIAK.M R.FGIAKMEGK.A R.FPFLLVDR.V R.DNFFAGIDK.V R.SHEVYTLQKDK.K R.DNFFAGIDKVR.F R.VIEYTPGVSVAIK.N K.YPAYPTVMDINQIR.E K.YPAYPTVMDINQIR.E R.EILPHRFPLLVDR.V R.ALRVASR.A R.SLANGDVER.A R.VSASRAGSFSSR.Y R.SLANGDVERAVR.E R.ATPLELRIAYR.A R.ALESVRANGIER.S R.AVREIISHFR.R R.TWNAFAGDAMCAVASASFR.G R.CCRNGVVSR.S K.RNISSSMKALR.N R.SMTVGYATSYSR.S R.EEQIEIDEAKEK.K R.SMTVGYATSYSRSSIR.N R.DIFMYRGDTPFCSEECR.E
512 ^D	Predicted protein	<i>Micromonas pusilla</i> CCMP1545	32.8/ 6.64	96	gi 303271347	
514 ^D	Uncharacterized protein LOC107628799	<i>Arachis ipaensis</i>	30/6.85	82	gi 102146958 19494	

Mt/pI theoretical molecular mass and isoelectric point of the proteins, SC sequence coverage. Superscripts by spot numbers: A, embryo-abundant; B, endosperm-abundant; C, embryo-specific; D, endosperm-specific

Fig. 9 Classification of identified proteins in (a) embryo and (b) endosperm according to their notated functions



with heat stress such as HSP70 cognates induce binding proteins in the endoplasmic reticulum, which guides them in proper synthesis, folding, assembly and transport (Yángüez et al. 2013). In this study, APs were abundant in the endosperm. A1 APs are the most-studied plant proteinases involved in proteolysis and protein processing in various plant organs, and are believed to play a role in plant senescence, stress responses, programmed cell death (PCD) and reproduction (Simoes and Faro 2004). Proteolysis and mobilization of storage proteins, globulins and gliadins are induced during rice and wheat seed germination. Chen and Foolad (1997) hypothesized that APs might be involved in the synthesis of new proteins from nucellar cell death proteins for embryo and endosperm development as part of the nucellar cells degeneration during PCD in barley after pollination.

The embryo-specific ATP-dependent zinc metalloprotease FtsH (spot 414C) is an ATP-dependent protease associated with diverse cellular activities (AAA) that control the quality of integral membrane proteins. They are similar to bacterial FtsHs and are localized in chloroplast and mitochondria of plants. FtsHs proteases are highly conserved, consisting of three functional domains in a single polypeptide chain where the N-terminal membrane-spanning helices are exposed to ATPase of AAA superfamily and zinc-binding domain toward the stroma (Leonhard et al. 1999). Single or double mutant analysis of FtsH proteases revealed their importance in thylakoid biogenesis (Zaltsman et al. 2005). AtFtsH 1 is believed to be involved in the assembly and maintenance of PSII complexes by proteolysis of damaged D1 proteins due to photo-oxidation. A mutation in AtFtsH 4 also resulted in abnormalities in the structure of mitochondria (Rigas et al. 2009), and the functional protein is required for the dynamic assembly and stability of complex I (Kolodziejczak et al. 2007).

Cell division and growth

Embryo spots 132A, 105A and endosperm spots 135B and 180B are expected to play an essential role in cell division and growth of the plant. Gibberellin 3-beta-dioxygenase 2–2 (GA20ox) (spot 132A) play an important role in hormonal biosynthesis and root hair formation. Gibberellins (GAs) are involved in diverse functions such as seed formation, germination, stem elongation, development of leaf and trichomes, pollen maturation and induction of flowering (Daviere and Achard 2013). Chemically, they are diterpenoids, and their synthesis occurs in three phases in different compartments of the cell. The first phase occurs in plastids with the cyclization of geranylgeranyldiphosphate, followed by formation of ent-kaurene takes place. The second phase, oxidization of ent-kaurene to GA12, occurs in the endoplasmic reticulum, and the final phase in cytosol involves the formation of bioactive GAs by the action of 2-oxoglutarate-dependent dioxygenases, GA 20-oxidase (GA20ox) and GA3-oxidase (GA3ox) (Yamaguchi 2008). Gibberellin 3-beta-dioxygenase 2–2 converts the inactive GA9 and GA20 into bioactive GA1 and GA4, which are active in the regulation of shoot elongation and in floral induction in short-day conditions (Eriksson et al. 2006).

CASP-like protein 2U10 (spot 105A) was found in the embryo and CASP-like protein 2D1 (spot 135B) in the endosperm. CASP-like proteins (CASPLs) are membrane-bound proteins that are important in the regulation of cell wall component deposition in the root endodermis (Enstone et al. 2002), thereby they are crucial for root formation (Roppolo et al. 2014). CASPLs also prevent the lateral diffusion of solutes by regulating the apoplastic barrier among inter- and intraorganismal apoplasts. Though the endosperm may not be involved in the root hair formation during seed germination, the presence of CASPLs in the endosperm is unclear and unexplored. Actin (spot 180B), highly conserved and abundant in eukaryotes, is expressed throughout the life of plants and involved in developmental processes including cell division and

polarity, cell wall deposition and cell elongation (Meagher et al. 2000).

Conclusions

The proteome analysis of *G. moluccana* showed marked differences in the abundance of 44 protein spots between embryo and endosperm, and 21 were selected and identified through MALDI-TOF-MS/MS. The identified proteins are involved in cell division, root formation, hormone biosynthesis, photosynthesis, chloroplast biogenesis, fatty acid, amino acid synthesis including cellular redox homeostasis and stress responses. This study provides a foundation for further studies related to seed development and germination of this important tree species.

Acknowledgements Durgeshwar Pullaiahgari gratefully thanks the University Grants Commission (UGC) for providing a Below Basic Level (BBL) fellowship. We thank the Officials of the Mulugu Forest Research Station for allowing us to collect the seeds. Special thanks to Monica Kannan, Scientific Officer of the proteomics facility for mass spectral analysis.

Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

References

- Allorent G, Osorio S, Vu JL, Falconet D, Jouhet J, Kuntz M, Fernie AR, Lerbs-Mache S, Macherel D, Courtois F, Finazzi G (2015) Adjustments of embryonic photosynthetic activity modulate seed fitness in *Arabidopsis thaliana*. *New Phytol* 205:707–719
- Apuya NR, Yadegari R, Fischer RL, Harada JJ, Zimmerman JL, Goldberg RB (2001) The *Arabidopsis* embryo mutant schlepperless has a defect in the chaperonin-60 α gene. *Plant Physiol* 126:717–730
- Bønsager BC, Finnie C, Roepstorff P, Svensson B (2007) Spatio-temporal changes in germination and radical elongation of barley seeds tracked by proteome analysis of dissected embryo, aleurone layer, and endosperm tissues. *Proteomics* 7:4528–4540
- Cánovas FM, Dumas-Gaudot E, Recorbet G, Jorin J, Mock HP, Rossignol M (2004) Plant proteome analysis. *Proteomics* 4:285–298
- Chaudhury AM, Koltunow A, Payne T, Luo M, Tucker MR, Dennis ES, Peacock WJ (2001) Control of early seed development. *Annu Rev Cell Dev Biol* 7:677–699
- Chen F, Foolad MR (1997) Molecular organization of a gene in barley which encodes a protein similar to aspartic protease and its specific expression in nucellar cells during degeneration. *Plant Mol Biol* 35:821–831
- Cheng H, Li LL, Xu F, Wang Y, Yuan HH, Wu CH, Wang SB, Liao ZQ, Hua J, Wang YP, Cheng SY, Cao FL (2013) Expression patterns of an isoflavone reductase-like gene and its possible roles in secondary metabolism in *Ginkgo biloba*. *Plant Cell Rep* 32:637–650
- Daviere JM, Achard P (2013) Gibberellin signaling in plants. *Development* 140:1147–1151
- Dixon DP, Davis BG, Edwards R (2002) Functional divergence in the glutathione transferase superfamily in plants identification of two classes with putative functions in redox homeostasis in *Arabidopsis thaliana*. *J Biol Chem* 277:30859–30869
- Eastmond P, Kolář L, Rawsthorne S (1996) Photosynthesis by developing embryos of oilseed rape (*Brassica napus* L.). *J Exp Bot* 47:1763–1769
- El-Maarouf-Bouteau H, Bailly C (2008) Oxidative signaling in seed germination and dormancy. *Plant Signal Behav* 3:175–182
- Enstone DE, Peterson CA, Ma F (2002) Root endodermis and exodermis: structure, function, and responses to the environment. *J Plant Growth Regul* 21:335–351
- Eriksson S, Böhlenius H, Moritz T, Nilsson O (2006) GA₄ is the active gibberellin in the regulation of leafy transcription and *Arabidopsis* floral initiation. *Plant Cell* 18:2172–2181
- Gallardo K, Job C, Groot SPC, Puype M, Demol H, Vandekerckhove J, Job D (2001) Proteomic analysis of *Arabidopsis* seed germination and priming. *Plant Physiol* 126:835–848
- Geetha M, Vijayalakshmi A (2013) Pharmacognostic and phytochemical investigation of *Givotia rottleriformis* Griff. Ex Wight bark. *J Pharmacogn Phytochem* 2:130–136
- Goffman FD, Alonso AP, Schwender J, Shachar-Hill Y, Ohlrogge JB (2005) Light enables a very high efficiency of carbon storage in developing embryos of rapeseed. *Plant Physiol* 138:2269–2279
- Gray JC (1992) Cytochrome *f*: structure, function and biosynthesis. *Photosynth Res* 34:359–374
- Guan X, Okazaki Y, Lithio A, Li L, Zhao XF, Jin HA, Nettleton D, Saito K, Nikolau BJ (2017) Discovery and characterization of the 3-hydroxyacyl-ACP dehydratase component of the plant mitochondrial fatty acid synthase system. *Plant Physiol* 173:2010–2028
- He M, Zhu C, Dong K, Zhang T, Cheng ZW, Li JR, Yan YM (2015) Comparative proteome analysis of embryo and endosperm reveals central differential expression proteins involved in wheat seed germination. *BMC Plant Biol* 15:97
- Henkel AW, Bieger SC (1994) Quantification of proteins dissolved in an electrophoresis sample buffer. *Anal Biochem* 223:329–331
- Hong SK, Kitano H, Satoh H, Nagato Y (1996) How is embryo size genetically regulated in rice? *Development* 122:2051–2058
- Hua W, Li RJ, Zhan GM, Liu J, Li J, Wang XF, Liu GH, Wang HZ (2012) Maternal control of seed oil content in *Brassica napus*: the role of silique wall photosynthesis. *Plant J* 69:432–444
- Kastaniotis AJ, Autio KJ, Sormunen RT, Hiltunen JK (2004) Htd2p/Yhr067p is a yeast 3-hydroxyacyl-ACP dehydratase essential for mitochondrial function and morphology. *Mol Microbiol* 53:1407–1421
- Kim ST, Kang SY, Wang Y, Kim SG, du Hwang H, Kang KY (2008) Analysis of embryonic proteome modulation by GA and ABA from germinating rice seeds. *Proteomics* 8:3577–3587
- Kolodziejczak M, Gibala M, Urantowka A, Janska H (2007) The significance of *Arabidopsis* AAA proteases for activity and assembly/stability of mitochondrial OXPHOS complexes. *Physiol Plant* 129:135–142
- Komatsu S (2005) Rice proteome database: a step toward functional analysis of the rice genome. *Plant Mol Biol* 59:179–190
- Leonhard K, Stiegler A, Neupert W, Langer T (1999) Chaperone-like activity of the AAA domain of the yeast Yme1 AAA protease. *Nature* 398:348
- Lin D, Tabb DL, Yates JR (2003) Large-scale protein identification using mass spectrometry. *Biochim Biophys Acta Proteins Proteom* 1646:1–10

- Lindquist S, Craig EA (1988) The heat-shock proteins. *Annu Rev Genet* 22:631–677
- Liu H, Liu YJ, Yang MF, Shen SH (2009) A comparative analysis of embryo and endosperm proteome from seeds of *Jatropha curcas*. *J Integr Plant Biol* 51:850–857
- Lopes MA, Larkins BA (1993) Endosperm origin, development, and function. *Plant Cell* 5:1383–1399
- Meagher RB, McKinney EC, Kandasamy MK (2000) The significance of diversity in the plant actin gene family. In: Staiger CJ, Baluška F, Volkmann D, Barlow PW (eds) *Actin: a dynamic framework for multiple plant cell functions*. Springer, Dordrecht, pp 3–27
- Méchin V, Balliau T, Château-Joubert S, Davanture M, Langella O, Négroni L, Prioul J-L, Thévenot C, Zivy M, Damerval C (2004) A two-dimensional proteome map of maize endosperm. *Phytochemistry* 65:1609–1618
- Rani TS, Podile AR (2014) Extracellular matrix-associated proteome changes during non-host resistance in *Citrus-Xanthomonas* interactions. *Physiol Plant* 150:565–579
- Rani TS, Durgeshwar P, Podile AR (2015) Accumulation of transcription factors and cell signaling-related proteins in the nucleus during *Citrus-Xanthomonas* interaction. *J Plant Physiol* 184:20–27
- Rigas S, Daras G, Laxa M, Marathias N, Fasseas C, Sweetlove Lee J, Hatzopoulos P (2009) Role of Lon1 protease in post-germinative growth and maintenance of mitochondrial function in *Arabidopsis thaliana*. *New Phytol* 181:588–600
- Roppolo D, Boeckmann B, Pfister A, Boutet E, Rubio MC, Dénervaud-Tendon V, Vermeer JEM, Gheyselinck J, Xenarios I, Geldner N (2014) Functional and evolutionary analysis of the casparian strip membrane domain protein family. *Plant Physiol* 165:1709–1722
- Runeberg-Roos P, Kervinen J, Kovaleva V, Raikhel NV, Gal S (1994) The aspartic proteinase of barley is a vacuolar enzyme that processes probarley lectin in vitro. *Plant Physiol* 105:321–329
- Shen SH, Jing YX, Kuang TY (2003) Proteomics approach to identify wound-response related proteins from rice leaf sheath. *Proteomics* 3:527–535
- Sheoran IS, Olson DJ, Ross AR, Sawhney VK (2005) Proteome analysis of embryo and endosperm from germinating tomato seeds. *Proteomics* 5:3752–3764
- Shewry PR, Casey R (1999) Seed proteins. In: Shewry PR, Casey R (eds) *Seed proteins*. Springer, Dordrecht, pp 1–10
- Simoës I, Faro C (2004) Structure and function of plant aspartic proteinases. *Eur J Biochem* 271:2067–2075
- Sreemadhavan CP (1975) Reinstatement of an alleged nomen confusum and typification of *Givottia* Griff. (Euphorbiaceae). *Taxon* 24:695–696
- van Hengel AJ, Guzzo F, van Kammen A, de Vries SC (1998) Expression pattern of the carrot *EP3* endochitinase genes in suspension cultures and in developing seeds. *Plant Physiol* 117:43–53
- Wang WX, Vinocur B, Shoseyov O, Altman A (2004) Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. *Trends Plant Sci* 9:244–252
- Yamaguchi S (2008) Gibberellin metabolism and its regulation. *Annu Rev Plant Biol* 59:225–251
- Yángüez E, Castro-Sanz AB, Fernández-Bautista N, Oliveros JC, Castellano MM (2013) Analysis of genome-wide changes in the transcriptome of *Arabidopsis* seedlings subjected to heat stress. *PLoS ONE* 8:e71425
- Zaltsman A, Ori N, Adam Z (2005) Two types of FtsH protease subunits are required for chloroplast biogenesis and photosystem II repair in *Arabidopsis*. *Plant Cell* 17:2782–2790

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.