Proteomic Analysis of *Theobroma cacao* Pod Husk

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Abstract: We performed two dimensional electrophoresis (2-DE) coupled with MS analysis on *Theobroma cacao* pod husk (fruit pericarp) to explore the proteome of this recalcitrant tissue. Using a phenol extraction/methanol-ammonium acetate precipitation method, we have obtained 2-DE images with approximately 700 protein spots detected after colloidal CBB staining. Two hundred and forty-four protein spots were analyzed by *de novo* sequencing of SPITC-derivatized tryptic peptides by MALDI-TOF/TOF MS. Applying this technique, 144 protein spots from cocoa pod husk were identified. The majority of the identified proteins were involved in metabolism and energy. Several of these proteins could be linked to pod growth and development processes.

Key words: *Theobroma cacao*, pod husk, 2-DE, MALDI-TOF/TOF MS, *de novo* sequencing

Cocoa (*Theobroma cacao* L.) is an important crop for many tropical countries and is mainly cultivated for its beans (seeds) for making chocolate. In Malaysia and other Southeast Asian countries, cocoa production is plagued by the cocoa pod borer (CPB) moth, *Conopomorpha cramerella* Snellen.¹⁾ Damage is caused by larvae that bore into the pod and feed on the placenta and pulp tissue surrounding the beans. This disrupts nutrient and water flow to developing beans and results in premature pod ripening.²⁾ The resulting immature beans clump together and become unextractable, and are low in cocoa butter (triacylglycerol) quality.^{3–5)} Current control efforts rely heavily on the use of insecticides, which are expensive and dangerous.⁶⁾

In the search for host plant resistance, many studies have been focused on the relationship of pod morphology and anatomy to CPB infestation.^{1,3,7,8)} It was found that the hardness and thickness of the sclerotic layer plays an important role in CPB resistance.³⁾ The sclerotic layer is a lignified layer of tissue in the middle of the pod husk. The harder and thicker the sclerotic layer, the more difficult it is for CPB larvae to penetrate into or out of the pod. Other non-physical elements may also contribute to CPB resistance, but no studies have yet been conducted. Nevertheless, there is still very little knowledge on many aspects of pod development, particularly at the cellular

and molecular level.

Proteomics offers an alternative and powerful method to address biological problems that cannot be dealt with adequately by genomics.⁹⁾ It has been used extensively in many plants including important crops.^{10,11)} Currently a gel-based approach using 2-DE coupled with MS is still widely used as the core technique in proteomics.^{12,13)} 2-DE gives high resolution of intact proteins from large complex protein mixtures that reveal changes in isoforms, post-translational modifications, and protein expression levels, and at a reasonable cost.^{13,14)} Furthermore, it is recommended as an excellent proteomic approach for non-model plants,¹⁵⁾ such as cocoa.

To date, only a few proteomic analyses have been reported for cocoa. Previous studies were done to investigate protein changes in beans during fruit ripening, fermentation and other processes in chocolate making.^{16,17)} Recently, protein extraction for 2-DE has been established for leaves and meristem.¹⁸⁾ There is no proteomic study, however, for pod husk, and none for any cocoa tissue combining 2-DE with MS. Pod husk is especially rich in polyphenols (particularly procyanidins) and gums (charged polysaccharides).^{19–21)} Such compounds can hamper protein isolation, 2-DE analysis, and protein identification by MS.^{14,22)} Thus, a suitable sample preparation for pod husk is needed.

Here, we report on the extraction of total proteins from cocoa pod husk for 2-DE analysis and also identification of the major proteins by MALDI-TOF/TOF MS to analyzing the pod husk proteome. For protein identification, we used *de novo* sequence analysis and cross species homology search, because the complete cocoa genome is not yet available. Sequencing was aided by simplified MALDI-TOF/TOF MS spectra obtained by *N*-terminal sulfonation of tryptic peptides. The same technique has also been used in other unsequenced organisms.^{23–26)} To the best of our knowledge, this is the first extensive report

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Abbreviations: CPB, cocoa pod borer; 2-DE, 2-dimensional electrophoresis; MALDI-TOF/TOF MS, matrix-assisted laser desorption ionization-time of flight/time of flight mass spectrometer; SPITC, 4-sulfophenyl isothiocyanate; PSD, post source decay; ACN, acetonitrile; HCCA, α -cyanohydroxycinnamic acid; TFA, trifluoroacetic acid; DTT, dithiothreitol; IAA, iodoacetamide; BPB, bromophenol blue; CBB, Coomassie Brilliant Blue; IEF, isoelectric focusing.

on proteomics of any tissue in cocoa.

MATERIALS AND METHODS

Plant materials. Three-month-old healthy pods from two clones, LAFI7 and ICS39, were obtained by hand pollination from Sabah Department of Agriculture, Quoin Hill Agriculture Research Station, Tawau, Sabah, Malaysia.

Preparation of acetone dried powder (AcDP). AcDP was prepared according to the method described by Wang *et al.*²²⁾ with modification. Beans and placenta were removed from the pod and the remaining pod husk was cut into small pieces, immediately frozen in liquid nitrogen, and lyophilized. Lyophilized pod husk (0.2 g) was wrapped with aluminum foil and crushed with pestle and mortar under liquid nitrogen. The resulting powder was additionally ground directly in liquid nitrogen into a fine powder. The pod husk powder was then resuspended in 10 mL of ice-cold acetone and vortexed for 5 min. After centrifugation ($10,000 \times g$, 5 min, 4°C), the supernatant was discarded. This step was performed three times and the resulting polyphenol-free white powder was air-dried.

Protein extraction. Protein extraction was performed according to the method described by Carpentier *et al.*¹⁴⁾ with minor modification. AcDP (0.2 g) was ground further for 5 min before resuspending in 10 mL ice-cold extraction buffer (50 mM Tris-HCl pH 8.5, 5 mM EDTA, 100 mM KCl, 2% β -mercaptoethanol, 30% sucrose) inside a pre-chilled mortar and mixed thoroughly with a pestle. To the highly viscous homogenate (due to gum), 10 mL ice-cold Tris buffered phenol (reagent-grade phenol equilibrated with 50 mM Tris-HCl, pH 8.0) was added and the homogenate mixed further for 5 min. The homogenate was then transferred to a 50 mL Falcon tube, vortexed for 15 min, and centrifuged ($10,000 \times g$, 10 min, 4°C). The top phenolic phase was re-extracted with 10 mL extraction buffer. After centrifugation ($10,000 \times g$, 10 min, 4°C), the phenolic phase was collected and 5 volumes of 100 mM ammonium acetate in methanol was added. The mixture was then incubated overnight at -20°C to precipitate protein. Protein was pelleted by centrifugation ($10,000 \times g$, 20 min, 4°C) and rinsed twice in ice-cold acetone/0.2% DTT with 60 min incubation at -20°C between the two rinsing steps. After air-drying, the protein pellet was resolubilized in 400 μ L resolubilization buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% IPG-buffer pH 3–10, 20 mM DTT). Finally, the suspension was centrifuged ($10,000 \times g$, 3 min, 20°C) and the supernatant was collected for 2-DE application. Protein concentration was determined using a 2-D Quant Kit (GE Healthcare, Hercules, UK).

2-DE. A three-hundred-microgram (300 μ g) protein sample in 450 μ L rehydration solution (resolubilization buffer with trace of BPB) was applied to a 24 cm pH 4–7 linear IPG strip (GE Healthcare) and rehydrated for 12 h. IEF was performed on the Ettan IPGphor II system (GE Healthcare) at 20°C with the following settings: current limit, 50 μ A/strip; 200 V, 15 min; 500 V, 15 min; 1 kV, 30 min; 8 kV, 1 h (gradient); 8 kV, 54325 Vh. Focused strips were then equilibrated twice in 10 mL equilibration

solution (6 M urea, 30% glycerol, 2% SDS, 0.002% BPB, 50 mM Tris pH 8.8) for 15 min. The first equilibration solution contained 1% DTT and the 2nd contained 2.5% IAA. SDS-PAGE was performed on the Ettan DALT 6 System (GE Healthcare) by using lab-cast 1 mm SDS polyacrylamide gels (12.5%) with the following settings: 10 mA/gel, 1 h; 38 mA/gel, until the BPB line reached the bottom of the glass. Proteins were visualized by colloidal CBB staining.²⁷⁾ Gels were scanned with a PharosFX Molecular Imager System (Bio-Rad Laboratories, Inc., Hercules, USA) and image analysis was performed using PDQuest 8.0.1 (Bio-Rad Laboratories, Inc.).

In-gel digestion. Protein spots were excised from colloidal CBB-stained-2-DE gels and washed with 50 mM NH_4HCO_3 , followed by destaining with 50% ACN in 50 mM NH_4HCO_3 . The gels were dehydrated by ACN and dried completely in a speed vacuum. The proteins were reduced by incubation in 50 μ L 10 mM DTT in 50 mM NH_4HCO_3 for 45 min at 56°C and subsequently alkylated by incubation in 55 mM IAA in 50 mM NH_4HCO_3 for 45 min at 30°C in darkness. The gels were washed twice with 50 mM NH_4HCO_3 and twice with 50% ACN in 50 mM NH_4HCO_3 . The gels were dehydrated again as described above. Dried gels were re-swelled by incubation in 10 μ L trypsin solution (10 ng/ μ L sequencing-grade modified porcine trypsin (Promega Corporation, Madison, USA) in 50 mM NH_4HCO_3 pH 7.8) for 45 min on ice. Excess trypsin solution was removed and gels were incubated at 37°C for 16 h after addition of 20 μ L 50 mM NH_4HCO_3 . The digestion solution was recovered and digested peptides were extracted from the gels by sonication twice with 50 μ L 50% ACN/0.1% TFA and twice with 50 μ L ACN. The digestion solution and extracts were combined and later divided into two equal aliquots. The first aliquot was further concentrated to approximately 10 μ L and desalted using C_{18} ZipTip (Millipore Corporate, Billerica, USA). The 2nd aliquot was dried completely in a speed vacuum and subjected to SPITC derivatization.

SPITC derivatization of peptides. Chemical derivatization by SPITC was performed according to the method described by Wang *et al.*²⁸⁾ A SPITC solution of 10 mg/mL SPITC was prepared in 20 mM NaHCO_3 (pH 8.2). Dried digested peptide mixtures were resolubilized in 8.5 μ L of SPITC solution and incubated at 56°C for 1 h. Reaction was stop by addition of 1 μ L 5% TFA solution. Finally, derivatized samples were desalted using C_{18} ZipTip (Millipore Corporate).

Mass spectrometry analysis. Desalted samples (0.5 μ L) were spotted onto MTP 384 target ground steel T F (Bruker Daltonics GmbH, Bremen, Germany) followed by addition of 0.5 μ L of matrix solution (10 mg/mL HCCA in 50% ACN/0.1% TFA). The mixture was allowed to air dry at room temperature. MALDI-TOF MS spectra and MALDI-TOF/TOF MS spectra were acquired in the positive reflectron mode on a Bruker Daltonics Autoflex III TOF/TOF-NN without post-ionization delayed extraction. MALDI-TOF MS Spectra were acquired with the following settings: ion source 1, 19 kV; ion source 2, 16.6 kV; lens, 8.75 kV; reflector, 21 kV; reflector 2, 9.7 kV; matrix suppression, 500 Da. Mass calibration was carried out with the peptide calibration standards (Bruker Daltonics

GmbH). MALDI-TOF/TOF MS spectra were acquired with the following settings: ion source 1, 6 kV; ion source 2, 5.3 kV; lens, 3 kV; reflector, 27 kV; reflector 2, 11.7 kV; LIFT 1, 19 kV; LIFT 2, 4.4 kV. Precursor ion selection (PCIS) was 0.65% of parent mass. No gas was applied in the collision cell. A total of 800–1200 laser shots at 100 Hz were accumulated for MALDI-TOF MS and MALDI-TOF/TOF MS spectrum on both derivatized and underivatized samples.

To find derivatized peptide ions, MALDI-TOF MS spectra of underivatized and SPITC-derivatized samples were first compared. SPITC-derivatized peptide ions were identified by a 215 m/z increase in mass from their underivatized peptide ions. Moreover, MALDI-TOF/TOF MS spectra of SPITC-derivatized peptides were preceded by the loss of 215 m/z of SPITC and 173 m/z of sulfanilic acid.²⁵⁾ PSD of SPITC-derivatized peptides resulted in highly abundant y -ion series, and sequences were obtained by measuring the differences between adjacent fragment ion peaks of the assumed y -ions series with the assistance of FlexAnalysis 3.0 (Bruker Daltonics GmbH). Gaps in some sequences resulting from missing y -ions or low intensity ambiguous peaks were filled by possible amino acid combinations suggested by RapiDeNovo program in Biotoools 3.1 (Bruker Daltonics GmbH).²⁵⁾

Database searches. Database searches were performed by referring to Samyn *et al.*^{23,24)} *De novo*-derived peptide sequences were combined in one search query and analyzed by FASTS and MS-BLAST.^{29,30)} The FASTS algorithm was carried out using default settings, and searches were performed against the SwissProt and NCBI Refseq proteins with BLOSUM 50 as the search matrix. The MS-BLAST algorithm was performed using default settings. All mass increments of 113 Da between consecutive y -ions were designated as Leu. The FASTS and MS-BLAST search results were considered significant if the E-score was below $1e-2$ and if the resulting scores were higher than the threshold score indicated in the software, respectively. Proteins were considered as being positively identified if the 2 search algorithms yielded the same homologous protein in the first hit.

RESULTS AND DISCUSSION

Protein extraction and 2-DE.

In order to investigate the proteome of cocoa pod husk, it is necessary to establish a protocol to isolate high-quality proteins. Plants contain many compounds that can interfere with 2-DE analysis^{14,22)} and cocoa is no exception. Cocoa pod husk contains high amounts of polyphenols (especially procyanidins), charged polysaccharides (gum), cellulose, hemicellulose, lignin and potassium.^{19–21,31)} Pod husk also contains a woody sclerotic layer, which can vary greatly in thickness and hardness depending on the clone. Therefore, pod husk is a recalcitrant sample and exceptionally challenging to work with. We used a phenol extraction/ammonium acetate in methanol precipitation method, because it has proven to give good results in many other recalcitrant plants such as banana,¹⁴⁾ olive,²²⁾ cotton³²⁾ and palm date.³³⁾

Before protein extraction, we prepared AcDP by wash-

ing samples with large amounts of ice-cold acetone (0.2 g of tissue: 10 mL of acetone) to remove mainly phenolic compounds. Further washing steps using aqueous TCA and aqueous acetone as reported in Wang *et al.*²²⁾ and Pirovani *et al.*¹⁸⁾ were omitted because they resulted in a pellet that was sticky and difficult to dry. A fine powder could not be obtained by grinding this pellet, and it is necessary to obtain a fine AcDP for efficient removal of contaminants during phenol extraction. Such an observation was not reported in previous work using vegetative tissues.¹⁸⁾

To analyze 2-DE images, total proteins from clones LAFI7 and ICS39 were separated by 2-DE in the range of pI 4–7. After staining with colloidal CBB, about 700 protein spots were visualized in both clones (Fig. 1). Protein spots were sharp, without streaking, and well-distributed along pI 4.5–6.5 and mass range of 100–15 kDa. The 2-DE images obtained were also low in background. These results indicate that the phenol extraction/ammonium acetate in methanol precipitation method can be used to successfully eliminate most interfering compounds for 2-DE analysis from pod husk. In addition, the 2-DE proteome pattern of pod husk did not contain highly abundant protein spots such as RuBisCo in leaves that can reduce 2-DE dynamic resolution.

On the other hand, other methods such as TCA/acetone were not suitable for protein extraction due to co-precipitation of gums and protein. Moreover, the method described here does not require extensive washing and sonication when preparing AcDP as reported by Pirovani *et al.*,¹⁸⁾ thus shortening the extraction procedure. The 2-DE produced from total protein is comparable or even better than previously reported in vegetative tissues.¹⁸⁾ Thus we believe that our procedure could also be used for other cocoa tissues. Finally, we also showed that lyophilization of cocoa pod husk tissue did not affect 2-DE analyses, thus making tissue storage easy as well as helping to concentrate protein. This result is consistent with a report by Carpentier *et al.*³⁴⁾

Identification of proteins by *de novo* sequencing and homology search.

For organisms with unsequenced genomes, such as cocoa, one way to identify their proteins is to do *de novo* sequence analyses and cross-species homology searches.^{23,24)} Even when the genomes are known, *de novo* sequence analyses are useful because they are not affected by errors in a search databases and they can increase the confidence level of protein identification.^{35,36)} Nevertheless, application of MALDI for *de novo* sequencing is initially limited due to the low signal to noise level and spectral complexity.^{23,24)} This was solved by addition of SPITC to the N -terminal of tryptic peptides resulting in abundant sequence information-bearing y -ions in the MS/MS spectra (Fig. 2). Accordingly, unambiguous sequences can be deduced by simply measuring the differences between adjacent fragment ion peaks in a y -ion series (Fig. 2).

As an initial step toward a comprehensive cocoa pod husk proteome analysis, we excised 233 and 11 protein spots with varying spot intensities, pI , and M_r from 2-DE gels of clones LAFI7 and ICS39, respectively. Protein

to be identified due to insufficient sequence information or gaps within sequences. Similar to a previous report by Samyn *et al.*,²³⁾ this was most likely due to the low ion signal of the derivatized peptides. Furthermore, fragmentation of SPITC-derivatized peptides involves the loss of the SPITC group as the major fragmentation pathway, thus resulting in a decrease in the total y-ion series.³⁶⁾ In some spots, for example 63, 323 and 424, sufficient sequence information was obtained, but homology searches resulted in scores below the threshold. These proteins may be

Fig. 2. MALDI-TOF MS spectra of spot 323 before (a) and after derivatization with SPITC (b). MALDI-TOF/TOF MS spectrum of the non-derivatized precursor ion at m/z 1262 (c) compared with SPITC-derivatized counterpart at m/z 1477 (d).

Asterisk indicate the peptide that was successfully derivatized and identified by a 215 Da increase in precursor mass. The SPITC-derivatized spectrum started with a mass loss of 215 and 173, which are characteristic for SPITC-derivatized peptides. The complete series of y-ions lead to confident interpretation of the spectrum. a.u. indicates arbitrary unit.

unique to cocoa pod growth and development.

Classification of identified proteins.

We classified the identified proteins according to their general function based on reports by Imin *et al.*,³⁷⁾ Faurobert *et al.*,³⁸⁾ and Baba *et al.*³⁹⁾ The descriptions of the identified proteins are shown in Fig. 3. The majority of the identified proteins are involved in primary and energy metabolism. This class of proteins is abundant in most cells³⁷⁾ and functions as housekeeping proteins involved in general cellular processes.⁴⁰⁾ Proteins related to glycolysis: enolase (spots 77, 88, 95), triose-phosphate isomerase (spots 146, 225), phosphoglycerate kinase (spots 163, 165, 179, i704), Calvin cycle: RuBisCo (spot 276), and TCA cycle: malate dehydrogenase (spots 115, 187) were among those identified. Similar results were reported for developing tomato fruit³⁸⁾ and *Medicago truncatula* embryogenic culture.³⁷⁾ Within this class, three intense protein spots close in pI and M_r were identified as S-adenosylmethionine synthetase 1 (spots 96, 97, 98).

This enzyme catalyzes the biosynthesis of S-adenosylmethionine (Adomet) from methionine and ATP. In plants, Adomet functions as a precursor in the biosynthesis of the phytohormone ethylene and serves as a propylamine group donor after decarboxylation in the biosynthesis of polyamines.⁴¹⁾ Increases in polyamine levels in tomato pericarp have been associated with slow ripening due to inhibition of ethylene production by polyamines.⁴²⁾ Because cocoa fruit has non-climacteric ripening, whereby its ripening is not controlled by ethylene, the high abundance of this enzyme in pod husk may be associated with the production of polyamines. S-adenosylmethionine synthetase is also required as a cofactor for lignin biosynthesis.⁴³⁾

Plants produce a broad variety of secondary metabolic compounds such as lignins, flavonoids, and phytoalexins. These compounds are produced by the phenylpropanoid pathway and have important roles in plant physiology.⁴⁴⁾ We have identified six proteins (spots 136, 140, 173, 177, 309, i706) as members of this pathway. Two of the pro-

Table 1. Protein identification by *de novo* sequencing of SPITC-derivatized peptides from clones LAF17 and ICS39.

Spot ^a	Accession Number ^a	Protein ^c	PM ^d	AA ^e	Organism	E-score ^f	Exp. Mr/pI ^g	Theo. Mr/pI ^h	Class ⁱ
(a) LAF17									
11	gil113363	Alcohol dehydrogenase 1	2/3	26	<i>Petunia × hybrida</i>	4.7e-03	57.0/6.8	41.6/6.2	ME
15	gil2494175	Glutamate decarboxylase 2	2/2	25	<i>Arabidopsis thaliana</i>	7.8e-06	57.2/6.6	56.1/5.3	ME
17	gil6015065	Elongation factor 2	1/1	16	<i>Beta vulgaris</i>	3.5e-09	100.7/6.3	93.9/5.9	PSP
18	gil8134568	5-Methyltetrahydropteroyltriglutamate	6/7	76	<i>Mesembryanthemum crystallinum</i>	3.3e-42	84.0/6.4	84.8/5.9	ME
25	gil3914360	Phospholipase D α 1	3/3	33	<i>Pimpinella brachycarpa</i>	4.6e-05	57.5/6.5	91.7/5.7	ME
31	gil51701894	Tubulin β-1 chain	1/1	12	<i>Encephalitozoon cuniculi</i>	9.9e-04	54.9/6.5	49.1/4.9	CS
35	gil3914449	Protease regulatory subunit 7	4/5	58	<i>Prunus persica</i>	6.7e-34	50.2/6.4	47.6/6.4	PSP
48	gil2499489	Pyrophosphate-fructose 6-phosphate 1-phosphotransferase subunit	3/3	32	<i>Ricinus communis</i>	6.3e-15	60.3/6.3	60.1/6.2	ME
49	gil10720307	T-complex protein 1 subunit ζ	2/2	31	<i>Schizosaccharomyces pombe</i>	8.1e-08	61.9/6.2	58.5/5.9	PSP
50	gil6225542	Ketol-acid reductoisomerase, chloroplastic	3/3	42	<i>Pisum sativum</i>	7.1e-20	58.8/6.2	57.5/5.9	ME
53	gil2501356	Transketolase, chloroplastic	2/2	22	<i>Solanum tuberosum</i>	6.2e-13	75.0/6.1	72.9/5.5	ME
57	gil2501356	Transketolase, chloroplastic	2/2	22	<i>Solanum tuberosum</i>	1.3e-12	75.1/6.0	72.9/5.5	ME
60	gil2499489	Pyrophosphate-fructose 6-phosphate 1-phosphotransferase subunit	2/4	36	<i>Ricinus communis</i>	9.1e-10	61.6/6.1	60.1/6.2	ME
61	gil2499489	Pyrophosphate-fructose 6-phosphate 1-phosphotransferase subunit	5/5	51	<i>Ricinus communis</i>	6.1e-35	59.8/6.1	60.1/6.2	ME
62	gil25452981	Glucose-6-phosphate-dehydrogenase	4/4	44	<i>Arabidopsis thaliana</i>	1.8e-24	58.5/6.1	59.2/8.4	ME
64	gil54038948	Phosphoribosylaminoimidazolecarboxamide formyltransferase	1/2	23	<i>Streptococcus agalactiae</i>	6.4e-07	59.8/6.1	56.1/4.9	ME
65	gil6225542	ketol-acid reductoisomerase chloroplastic	1/1	19	<i>Spinacia oleracea</i>	5.0e-06	59.7/6.0	57.8/5.9	ME
67	gil135535	T-complex protein 1 α subunit	2/3	38	<i>Arabidopsis thaliana</i>	3.5e-13	60.7/6.0	59.2/5.9	PSP
68	gil146286092	Coatomer subunit δ	1/3	29	<i>Arabidopsis thaliana</i>	7.4e-06	59.0/6.0	57.7/5.6	MT
69	gil3914394	2,3-biphosphoglycerate-independent phosphoglycerate mutase	4/4	52	<i>Mesembryanthemum crystallinum</i>	7.1e-26	61.8/5.9	61.2/5.4	ME
70	gil75115272	ketol-acid reductoisomerase	3/3	37	<i>Spinacia oleracea</i>	9.3e-21	58.2/5.9	57.4/5.5	ME
71	gil543865	ATP synthase subunit α, mitochondria	2/3	33	<i>Beta vulgaris</i>	2.8e-13	56.7/5.9	54.9/6.0	MT
72	gil266346	ketol-acid reductoisomerase	2/4	46	<i>Spinacia oleracea</i>	4.8e-10	56.9/5.8	56.9/5.6	ME
74	gil74996549	T-complex protein 1 subunit β	2/3	34	<i>Arabidopsis thaliana</i>	2.5e-03	57.7/5.8	58.0/5.8	PSP
76	gil1172858	Ribulose-1,5-biphosphate carboxylase large chain	4/4	42	<i>Cibotium barometz</i>	8.4e-30	52.3/6.3	45.9/6.2	ME
77	gil14423687	Enolase 2	4/4	51	<i>Hevea brasiliensis</i>	5.6e-16	52.1/6.2	47.9/5.9	ME
81	gil543865	ATP synthase subunit α	8/8	80	<i>Beta vulgaris</i>	1.6e-58	53.1/6.1	54.9/6.0	MT
86	gil6094242	Putative selenium-binding protein	3/4	59	<i>Arabidopsis thaliana</i>	1.0e-22	50.0/5.9	54.1/5.4	DS
87	gil543865	ATP synthase subunit α, mitochondrial	2/4	49	<i>Beta vulgaris</i>	1.1e-12	53.4/5.9	54.9/6.0	MT
88	gil14423687	Enolase 2	4/5	51	<i>Hevea brasiliensis</i>	2.6e-19	50.5/5.9	47.9/5.9	ME
92	gil25453205	Peroxidase 12	4/5	64	<i>Arabidopsis thaliana</i>	8.6e-19	48.3/5.8	36.1/8.4	DS
93	gil115646	α-S1-casein	3/6	54	<i>Bos taurus</i>	4.5e-20	49.5/5.8	23.0/4.9	U
94	gil12585489	UDP-glucose pyrophosphorylase	3/3	43	<i>Musa acuminata</i>	4.0e-35	49.5/5.8	47.9/5.6	ME
95	gil1169534	Enolase	4/6	77	<i>Ricinus communis</i>	5.3e-32	50.1/5.7	47.9/5.6	ME
96	gil400246	S-adenosylmethionine synthetase I	2/4	53	<i>Petroselinum crispum</i>	9.4e-19	46.5/5.8	25.7/7.9	ME
97	gil400246	S-adenosylmethionine synthetase I	3/3	34	<i>Petroselinum crispum</i>	2.3e-27	45.5/5.8	25.7/7.9	ME
98	gil400246	S-adenosylmethionine synthetase I	3/4	56	<i>Ostreococcus lucimarin</i>	6.9e-25	45.6/5.8	25.7/7.9	ME
99	gil68052019	Biotin carboxylase	2/6	54	<i>Populus euphratica</i>	1.3e-06	49.9/6.2	42.9/6.3	ME
102	gil20141399	3-oxoacyl-[acyl-carrier-protein] synthase	3/7	69	<i>Arabidopsis thaliana</i>	2.7e-17	47.7/6.3	45.1/6.2	ME
103	gil73919692	GDP-D-mannose 3,5-epimerase	4/6	62	<i>Arabidopsis thaliana</i>	6.9e-26	46.3/6.2	42.8/5.9	ME
108	gil189044723	Elongation factor Tu 2	2/3	37	<i>Alkaliphilus metalliredigens</i>	1.1e-16	44.2/6.3	43.7/5.0	PSP
110	gil122064253	Isocitrate dehydrogenase [NAD] regulatory subunit 1	3/3	30	<i>Arabidopsis thaliana</i>	1.4e-12	44.1/6.4	36.9/6.4	ME
115	gil126896	Malate dehydrogenase, mitochondrial	3/6	67	<i>Citrullus lanatus</i>	3.3e-13	40.2/6.3	33.2/6.3	ME
116	gil1168493	Arginase	6/6	56	<i>Arabidopsis thaliana</i>	1.5e-31	40.2/6.3	37.3/6.1	ME
126	gil1352461	IN2-2 protein	2/5	59	<i>Zea mays</i>	7.8e-16	41.6/6.6	33.8/8.9	U
135	gil51701846	26S proteasome non-ATPase regulatory subunit 14	3/4	27	<i>Arabidopsis thaliana</i>	3.1e-10	35.2/6.6	34.3/6.3	PSP
136	gil1708422	Isoflavone reductase homolog	2/3	32	<i>Solanum tuberosum</i>	9.6e-13	37.3/6.5	33.9/6.1	SM
139	gil75337884	Annexin D1	3/6	66	<i>Arabidopsis thaliana</i>	1.9e-13	34.2/6.5	36.2/5.2	ST
140	gil1708421	Isoflavone reductase homolog IRL	3/3	31	<i>Zea mays</i>	1.4e-15	34.9/6.4	32.9/5.7	SM
146	gil1351279	Triose-phosphate isomerase, cytosolic	3/3	36	<i>Petunia × hybrida</i>	6.0e-21	26.7/6.8	27.1/5.5	ME
151	gil75331395	Probable calcium-binding protein CML49	1/3	29	<i>Arabidopsis thaliana</i>	8.5e-06	29.6/6.4	35.3/6.4	U
152	gil75331395	Probable calcium-binding protein CML49	2/2	21	<i>Arabidopsis thaliana</i>	1.2e-12	30.3/6.3	33.3/6.3	U
161	gil121345	Glutamine synthetase PR-2	2/2	29	<i>Phaseolus vulgaris</i>	9.6e-13	41.8/6.2	39.2/5.4	ME
163	gil2499498	Phosphoglycerate kinase, cytosolic	3/5	64	<i>Nicotiana tabacum</i>	5.1e-26	43.7/6.0	42.4/5.7	ME
165	gil2499499	Phosphoglycerate kinase, cytosolic	3/3	44	<i>Arabidopsis thaliana</i>	5.3e-30	40.7/6.0	42.4/5.8	ME
171	gil229891797	α-1,4-glucan-protein synthase [UDP-forming]	1/2	19	<i>Phoenix dactylifera</i>	8.8e-08	41.4/5.9	68.5/7.8	CS
173	gil30173108	Leucoanthocyanidin dioxygenase	1/1	17	<i>Arabidopsis thaliana</i>	7.0e-08	44.5/5.9	40.4/5.2	SM
175	gil75163801	L,L-Diaminopimelate aminotransferase	7/8	37	<i>Arabidopsis thaliana</i>	3.9e-59	43.4/5.8	46.5/6.4	ME
177	gil1345655	Cinnamyl alcohol dehydrogenase 4	2/2	27	<i>Arabidopsis thaliana</i>	3.9e-13	43.4/5.7	39.1/5.3	SM
178	gil119194	Elongation factor Tu, chloroplastic	5/5	59	<i>Arabidopsis thaliana</i>	3.2e-34	43.7/5.7	44.7/5.3	PSP
179	gil12644295	Phosphoglycerate kinase, chloroplastic	2/2	27	<i>Arabidopsis thaliana</i>	6.4e-17	43.4/5.6	42.6/5.0	ME
180	gil231502	Actin-85C	2/3	26	<i>Solanum tuberosum</i>	9.3e-13	43.4/5.5	21.7/5.9	CS
181	gil231503	Actin-85C	3/4	37	<i>Solanum tuberosum</i>	1.1e-19	43.9/5.5	21.7/5.9	CS
187	gil75313643	Malate dehydrogenase, chloroplastic	2/2	23	<i>Arabidopsis thaliana</i>	2.2e-06	36.3/6.2	34.1/5.7	ME
193	gil74683233	Protein transport SEC13	1/3	32	<i>Filobasidiella neoformans</i>	2.3e-05	31.4/6.0	36.8/5.8	MT
198	gil74996477	Probable 3-hydroxyisobutyrate dehydrogenase	2/3	30	<i>Dictyostelium discoideum</i>	1.2e-06	31.8/5.9	33.9/8.8	ME
199	gil51316816	Photosystem I assembly protein Ycf4	1/2	26	<i>Adiantum capillus-veneris</i>	7.6e-03	29.7/5.9	20.8/9.3	ME
205	gil1351963	L-Ascorbate peroxidase, cytosolic	4/4	45	<i>Pisum sativum</i>	9.2e-22	27.4/5.9	27.1/5.5	DS
210	gil1717752	Tropinone reductase 1	3/3	46	<i>Datura stramonium</i>	2.2e-12	28.5/6.3	29.6/6.1	SM
220	gil225446793	Glutathione S-transferase 3	2/3	25	<i>Vitis vinifera</i>	2.6e-05	24.9/6.2	25.0/5.8	DS
223	gil75334429	GSH-dependent dehydroascorbate reductase 1	2/3	31	<i>Arabidopsis thaliana</i>	4.9e-07	25.2/6.0	23.6/5.6	ME
224	gil17380179	20S proteasome β subunit D, type 2	4/6	53	<i>Arabidopsis thaliana</i>	4.0e-26	23.2/6.0	22.5/6.0	PSP

Spot ^a	Accession Number ^b	Protein ^c	PM ^d	AA ^e	Organism	E-score ^f	Exp. Mr/pI ^g	Theo. Mr/pI ^h	Class. ⁱ
225	gil1360571	Triose-phosphate isomerase, cytosolic	5/5	327	<i>Coptis japonica</i>	2.4e-32	26.8/5.8	39.4/5.6	ME
226	gil22096383	Uncharacterized protein	1/1	16	<i>Arabidopsis thaliana</i>	2.6e-08	28.1/5.8	34.9/8.4	U
227	gil1172601	Probable proteasome subunit α type-4	1/1	9	<i>Schizosaccharomyces pombe</i>	2.2e-03	27.0/5.8	27.9/5.9	PSP
231	gil12643263	Chaperonin 10	2/3	30	<i>Arabidopsis thaliana</i>	1.1e-10	24.7/5.6	21.4/5.2	PSP
234	gil73921673	Allene oxide cyclase 3, chloroplastic	2/2	21	<i>Arabidopsis thaliana</i>	2.6e-04	21.9/5.6	22.4/8.7	ME
249	gil1346675	Nucleoside diphosphate kinase B	3/3	38	<i>Flaveria bidentis</i>	3.2e-27	15.0/6.5	16.1/5.9	ME
250	gil14285792	T-complex protein 1 subunit γ	2/2	21	<i>Thalassiosira weissflogii</i>	1.3e-05	67.9/6.5	61.7/5.6	PSP
251	gil109909540	Formate dehydrogenase 1, mitochondrial	6/9	93	<i>Oryza sativa</i>	3.9e-41	44.6/6.7	39.3/6.2	ME
255	gil30315948	Caffeic acid 3-O-methyltransferase	3/3	31	<i>Capsicum annuum</i>	1.8e-18	41.2/5.4	39.4/5.6	ME
256	gil231502	Actin-85C	2/2	20	<i>Solanum tuberosum</i>	7.3e-12	43.3/5.4	21.7/5.9	CS
257	gil2500522	Eukaryotic initiation factor 4A	4/4	45	<i>Zea mays</i>	1.3e-20	47.7/5.3	46.5/5.4	PSP
260	gil26391492	ATP synthase subunit β -2, mitochondrial	2/2	22	<i>Arabidopsis thaliana</i>	2.6e-06	51.5/5.4	54.2/5.4	MT
261	gil189045124	Glucose-6-phosphate isomerase	2/3	32	<i>Microcystis aeruginosa</i>	1.4e-03	55.5/5.4	58.1/5.8	ME
262	gil21759093	Betaine aldehyde dehydrogenase 1	5/5	53	<i>Zea mays</i>	8.5e-24	58.5/5.4	53.7/5.2	ME
263	gil62900360	Importin subunit α -1a	3/3	42	<i>Oryza sativa</i>	5.3e-16	58.7/5.4	57.6/5.1	MT
264	gil585451	NAD-dependent malic enzyme 62 kDa isoform, mitochondrial	3/4	57	<i>Solanum tuberosum</i>	8.8e-30	62.0/5.5	66.1/5.3	ME
265	gil401322	V-type proton ATPase catalytic subunit A	7/7	103	<i>Gossypium hirsutum</i>	3.2e-64	66.8/5.5	68.5/5.4	MT
267	gil399940	Heat shock 70 kDa protein, mitochondrial	3/3	36	<i>Phaseolus vulgaris</i>	2.2e-20	66.3/5.4	67.0/5.3	DS
276	gil2506277	RuBisCO subunit binding-protein β subunit, chloroplastic	4/4	53	<i>Pisum sativum</i>	2.2e-26	60.2/5.2	57.9/5.3	ME
277	gil190358177	ATP synthase α subunit, chloroplast	9/13	140	<i>Illicium oligandrum</i>	2.8e-76	53.9/5.2	55.4/5.4	MT
285	gil1703131	Actin 12	3/4	40	<i>Arabidopsis thaliana</i>	2.0e-24	44.1/5.3	41.8/5.4	CS
287	gil21759255	3-isopropylmalate dehydrogenase 2	3/3	25	<i>Arabidopsis thaliana</i>	4.0e-10	43.1/5.3	39.8/5.2	ME
288	gil399785	Glutamate-1-semialdehyde 2,1-aminomutase, chloroplastic	2/2	20	<i>Nicotiana tabacum</i>	1.0e-07	42.1/5.3	50.9/6.4	ME
289	gil75261432	Succinyl-CoA ligase [GDP-forming] subunit β	3/3	28	<i>Vitis vinifera</i>	1.1e-13	41.8/5.2	42.1/5.2	ME
290	gil17366963	Adenosine kinase 2	2/3	36	<i>Arabidopsis thaliana</i>	1.3e-13	40.0/5.2	37.9/5.1	ME
294	gil2493895	Cysteine synthase	3/3	34	<i>Citrullus lanatus</i>	5.4e-23	35.3/5.2	34.3/6.3	PSP
298	gil148872800	DNA-damage-repair/tolerance protein	1/1	18	<i>Oryza sativa</i>	7.9e-09	34.3/5.1	33.2/5.1	DS
299	gil18202458	α -Soluble NSF attachment protein	3/3	34	<i>Vitis vinifera</i>	1.4e-18	33.3/5.1	32.5/4.9	MT
300	gil11134054	Oxygen-evolving enhancer protein 1	3/4	50	<i>Nicotiana tabacum</i>	6.1e-21	31.5/5.2	26.7/5.1	ME
309	gil224146220	Chalcone isomerase-like protein	3/3	36	<i>Populus trichocarpa</i>	4.2e-16	25.0/5.1	23.3/5.0	SM
317	gil461461	Enoyl-[acyl-carrier-protein] reductase	2/2	18	<i>Brassica napus</i>	3.4e-08	37.8/5.1	32.9/5.4	ME
318	gil8928330	40S Ribosomal protein SA (p40)	3/3	29	<i>Daucus carota</i>	4.7e-17	43.0/5.0	32.5/4.9	PSP
320	gil225464635	Hypothetical protein	3/5	43	<i>Vitis vinifera</i>	6.6e-11	43.9/5.1	40.6/5.0	U
322	gil54036493	Tubulin α -2 chain; α -2-tubulin	6/7	80	<i>Gossypium hirsutum</i>	2.1e-38	49.1/5.1	49.5/4.9	CS
325	gil229464991	Heat shock 70 kDa protein	4/4	48	<i>Zea mays</i>	2.9e-32	69.7/5.2	70.6/5.2	DS
327	gil123592	Heat shock 70 kDa protein	3/3	37	<i>Leishmania major</i>	7.2e-22	70.2/5.1	56.5/6.5	DS
335	gil1708311	Stromal 70 kDa heat shock-related protein	4/7	72	<i>Spinacia oleracea</i>	4.3e-31	76.0/4.8	64.9/4.9	DS
339	gil123656	Chloroplast envelope membrane 70 kDa heat shock protein	3/3	34	<i>Spinacia oleracea</i>	1.6e-22	72.0/5.0	71.6/5.4	DS
349	gil11133775	Protein disulfide isomerase	2/2	21	<i>Ricinus communis</i>	5.3e-06	59.2/5.0	53.0/4.9	PSP
354	gil223635791	Transaldolase	1/3	32	<i>Capsicum annuum</i>	5.7e-06	44.9/4.8	26.3/8.6	ME
355	gil123142886	Transaldolase	3/3	34	<i>Frankia alni</i>	3.2e-10	44.7/4.8	40.0/5.8	ME
356	gil123142886	Transaldolase	3/3	34	<i>Frankia alni</i>	3.2e-10	44.6/4.9	40.0/5.8	ME
362	gil12229892	Proteasome subunit α type-1-B	3/4	48	<i>Arabidopsis thaliana</i>	8.5e-25	35.5/4.9	30.4/5.0	PSP
368	gil1345698	Chlorophyll-a/b binding protein 151	2/3	28	<i>Gossypium hirsutum</i>	1.8e-11	25.8/4.9	24.7/5.0	ME
369	gil115777	Chlorophyll-a/b binding protein, chloroplastic	1/3	29	<i>Silene latifolia</i>	2.4e-04	26.1/4.8	22.0/5.0	ME
372	gil1168191	14-3-3 protein 4	4/4	48	<i>Solanum lycopersicum</i>	5.4e-18	30.0/4.8	29.3/4.7	ST
374	gil26454610	14-3-3 protein 8	3/4	37	<i>Pseudotsuga menziesii</i>	3.0e-21	32.0/4.8	29.5/4.6	ST
376	gil12230867	14-3-3-like protein GF14 ω	5/6	62	<i>Arabidopsis thaliana</i>	1.9e-28	32.8/4.7	29.2/4.7	ST
377	gil26454609	14-3-3 protein 7	4/6	62	<i>Solanum lycopersicum</i>	3.8e-16	31.2/4.7	28.8/5.0	ST
378	gil12230867	14-3-3-like protein GF14 ω	5/5	60	<i>Arabidopsis thaliana</i>	1.1e-27	30.7/4.7	29.2/4.7	ST
385	gil75294319	14-3-3-like protein GF14-C	2/4	34	<i>Oryza sativa</i>	8.7e-09	25.0/4.7	28.8/4.8	ST
386	gil9979193	Translationally-controlled tumor protein	1/1	9	<i>Vitis vinifera</i>	2.2e-03	22.5/4.6	18.8/4.7	U
410	gil12585487	V-type H-ATPase subunit C	2/3	30	<i>Hordeum vulgare</i>	2.7e-17	42.6/5.5	40.0/6.1	MT
411	gil75146893	Bifunctional dihydroflavanol 4-reductase	3/3	34	<i>Pyrus communis</i>	5.2e-16	46.4/5.7	38.9/5.9	SM
413	gil75038951	α -S1-casein precursor	1/1	51	<i>Bubalus bubalis</i>	1.5e-03	38.7/5.8	22.8/4.8	U
414	gil6225258	Aspartate semialdehyde dehydrogenase	2/2	23	<i>Aquifex aeolicus</i>	1.8e-06	39.0/5.7	37.8/6.0	ME
421	gil14548089	Inositol-3-phosphate synthase	3/3	41	<i>Hordeum vulgare</i>	7.7e-29	53.7/5.5	56.2/5.5	ME
423	gil12585296	Phosphoglucomutase, cytoplasmic	5/6	66	<i>Pisum sativum</i>	2.5e-22	65.5/5.6	63.3/5.5	ME
427	gil75338403	ATP synthase subunit β , chloroplastic	5/5	70	<i>Ornithogalum longibracteatum</i>	2.2e-50	50.9/5.5	53.2/5.3	MT
429	gil78099761	Argininosuccinate synthase, chloroplast	3/6	82	<i>Arabidopsis thaliana</i>	1.2e-21	47.6/5.5	46.2/5.4	ME
436	gil62510584	Rab GDP dissociation inhibitor β	2/2	25	<i>Pongo abelii</i>	8.8e-13	49.4/5.7	50.7/6.1	ST
444	gil50400860	Monodehydroascorbate reductase	4/5	48	<i>Solanum lycopersicum</i>	8.9e-28	43.3/5.4	47.0/5.8	ME
451	gil8928411	β -2-tubulin	3/4	41	<i>Daucus carota</i>	6.7e-18	52.8/4.9	49.6/4.8	CS
453	gil129699	Proliferating cell nuclear antigen	1/1	8	<i>Glycine max</i>	7.3e-03	35.7/4.7	26.1/4.7	DNA
469	gil1172586	Polyphenol oxidase A1, chloroplastic	2/2	21	<i>Vicia faba</i>	4.0e-10	70.6/5.7	58.5/6.0	SM
472	gil18410026	Mevalonate diphosphate decarboxylase, putative	1/1	16	<i>Arabidopsis thaliana</i>	5.1e-04	47.7/6.4	46.1/6.0	ME
802	gil12229922	Proteasome subunit α type-6	4/4	47	<i>Oryza sativa</i>	5.0e-26	26.8/6.4	27.6/6.2	PSP
(b) ICS39									
i704	gil2499498	Phosphoglycerate kinase, cytosolic	3/5	64	<i>Nicotiana tabacum</i>	7.9e-32	43.9/6.2	42.4/5.7	ME
i706	gil224078816	Anthocyanidin reductase	2/3	25	<i>Populus trichocarpa</i>	8.5e-08	40.2/6.1	35.4/7.8	SM
i708	gil3913034	β -Amylase	2/3	29	<i>Vigna unguiculata</i>	5.8e-08	60.1/4.8	56.2/4.9	ME
i710	gil8134568	5-methyltetrahydropteroyltriglutamate	4/4	46	<i>Mesembryanthemum crystallinum</i>	1.1e-28	86.3/6.5	84.8/5.9	ME
i711	gil85700445	Probable mitochondrial-processing peptidase β subunit	4/6	68	<i>Arabidopsis thaliana</i>	2.8e-31	61.5/6.4	56.2/5.7	PSP

^aSpot name corresponds to the protein spots tagged in Fig. 1. ^bNCBI Entrez entries (<http://www.ncbi.nih.gov/Entrez/>). ^cIdentification by FASTS search. ^dNumber of matched peptides in the FASTS query. ^eTotal number of amino acid used in the query. ^fE-score from FASTS algorithm. ^gExperimental Mr (kDa) and pI calculated by analysis of the gel images with PDQuest 8.0.1 software. ^hTheoretical Mr (kDa) and pI computed from ExPASy Mr/pI calculation tool (http://au.expasy.org/tools/pi_tool.html). ⁱClassification of proteins based on general function (ME, metabolism and energy; PSP, protein synthesis and processing; MT, membrane transport; DS, defense and stress; SM, secondary metabolism; CS, cell structure; ST, signal transduction; DNA, DNA synthesis and processing; U, unclassified).

Fig. 3. Classification of proteins identified from cocoa pod husk by general functions.

tein spots were identified as leucoanthocyanidin dioxygenase (spot 173) and anthocyanidin reductase (spot i706). These two enzymes participate in the synthesis of flavan-3-ol, a monomer required for formation of condensed tannin polymers (proanthocyanidins).⁴⁵⁾ This finding is consistent with an earlier report that pod husk contains condensed tannins. Moreover, two key enzymes related to lignin biosynthesis, cinnamyl alcohol dehydrogenase (spot 177) and polyphenol oxidase (spot 469) were identified. Enzymes related to lignin biosynthesis particularly interest us because their expression levels may help to explain the differences in lignification in pods with thick/hard or thin/soft sclerotic layers. As mentioned previously, pods with thick/hard (more lignified) sclerotic layers are more tolerant to CPB than pods with thin/soft sclerotic layers. Polyphenol oxidase is also believed to have a role in resistance to insects. Cotton bollworms feeding on transgenic tomato lines over-expressing polyphenol oxidase exhibit lower weight gains and foliar consumption than insects feeding on normal plants.⁴⁶⁾

Fruit development requires two major cellular activities: cell division and cell enlargement.⁴⁷⁾ A number of identified proteins categorized in cell structure, signal transduction, and membrane transport could be linked to these processes. Four (180, 181, 256, 285) and three (31, 322, 451) spots were identified as actin and tubulin, respectively. These are the fundamental components of the cytoskeleton. Spot 256 (actin) was exceptionally highly expressed, and is probably required for cellular processes such as cytoplasmic streaming, cell shape determination, and organelle movement.⁴⁸⁾ Spot 139, identified as annexin, was also abundantly expressed. Plant annexins are ubiquitous, soluble proteins capable of Ca^{2+} -dependent and Ca^{2+} -independent binding to endomembranes and the plasma membrane.⁴⁹⁾ These multifunctional proteins have been reported to be involved in the Golgi-mediated secretion of wall polysaccharides in plant cells, suggesting their roles in regulation of cell wall extension.⁵⁰⁾ In developing tomato, annexin expression is well correlated with cell expansion.³⁸⁾

Cells also expand as a result of water intake into the vacuole during fruit development.⁵¹⁾ Vacuolar ATPase (V-ATPase) is a multimeric enzyme that contributes to the generation of proton gradients across the tonoplast for transporting substances into the vacuole against their concentration gradient.^{38,51)} In tomato, V-ATPase expression is enhanced during the cell enlargement phase.³⁸⁾ Suppression of V-ATPase in antisense-transgenic tomato results in reduced fruit growth.⁵¹⁾ In this study, we have identified two

protein spots as V-ATPase (spots 265, 410).

14-3-3 proteins were identified in six spots (372, 374, 376, 377, 378, 385). These proteins are ubiquitous in plants and are major regulators of plant primary metabolism and other cellular processes. For example, members of this protein family were reported to have critical roles in regulating cotton fiber elongation⁵²⁾ and starch biosynthesis in plastids.⁵³⁾ In tomato, it was reported that 14-3-3 homolog protein profiles vary with the ripening stage in fruit development, suggesting their involvement in the regulatory control of tomato fruit development.⁵⁴⁾ The many isoforms that we identified, especially the most abundant spot 385, demonstrate their importance in pod husk development.

Several proteins associated with stress and defense, DNA synthesis, or having unclassified function were identified as well. For instance, five spots representing proteasomes were identified (135, 224, 227, 362, 802). Proteasomes are known to degrade proteins, but they are also involved in various plant biological processes such as development and stress responses. In *Arabidopsis thaliana* mutants with a decrease in 26S proteasome biogenesis, cell expansion increases, whereas complete loss of 26S proteasome enhances the cellular capacity to degrade oxidized proteins.⁵⁵⁾ Intriguingly, two protein spots (93, 413) were identified as α -S1-casein. The function of this protein in pod husk is unknown.

Finally, a number of protein spots identified in this study were found to be differentially expressed between the two clones. For example, spots i704, i706, i708, i710 and i711 were unique for the clone ICS39, whereas spots 255 and 469 were more expressed in LAF17. These two clones differ in pod shape, surface texture, and especially in the thickness and hardness of the sclerotic layer. Clone LAF17 has a smoother and more waxy pod surface and has a much harder and thicker sclerotic layer—features characteristic of highly CPB-resistant clones. Spots 255 and 469 were identified as caffeic acid 3-O-methyltransferase and polyphenol oxidase, respectively, enzymes involved in lignin synthesis. Thus these two enzymes may be associated with formation of the sclerotic layer. However, differential analysis between these two clones is beyond the scope of this article. Work on differential proteomic analysis between the two clones throughout pod development is currently in progress in our lab.

As conclusions, we have established a method to extract good quality proteins from cocoa pod husk that gives well-resolved 2-DE images. Protein extraction is compatible with MALDI-TOF/TOF MS and suitable for cocoa proteomic analysis. We have identified 144 protein spots from cocoa pod husk for the first time, with many associated with pod growth and development. This study can serve as a reference for future proteomic studies of cocoa tissues.

Cocoa genome sequencing is currently in progress and scheduled to be completed in 2013. Its completion will open a new era in cocoa molecular studies, including proteomics. Because the protein sequences we discovered in this study were derived independently of any database, they could be useful for validating genes after completion of genome sequencing.

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カカオポッド果殻のプロテオミックス解析

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カカオ (*Theobroma cacao*) は、多くの熱帯地域の国の重要な作物であり、チョコレートの原材料として栽培されている。カカオにおいても害虫耐性の付与など品種改良が望まれているが、その基礎となるカカオの生理・生化学、分子遺伝学についてはほとんどわかっていない。また、現時点ではカカオのゲノム情報も明らかにされていない。われわれは、カカオポッド果殻のプロテオームを明らかにするため2次元電気泳動 (2-DE) / 質量分析を行った。カカオポッドには大量のガムが含まれているため、ポッド果殻タンパク質をフェノール抽出 / メタノール-酢酸アンモニア沈澱法により調製した。タンパク質試料を2-DEで分離し、コロイドCBBで染色したところ、2-DEゲル中において約700のタンパクスポットを検出することができた。244個のタンパクスポットについてトリプシン消化物のSPITC誘導体を調製し、MALDI-TOF/TOF MSを用いた *de novo* シークエンシングを行った。この方法により144個のカカオポッド果殻タンパク質を同定することができた。同定されたタンパク質の大部分は代謝やエネルギー生産に関与するものであった。また、ポッドの成長・分化に関わるタンパク質も検出された。

Supplementary Table 1(a). *De novo* derived peptide sequences for the identification of proteins from cocoa clone LAFI7.

Spot	Accession number	Protein	Precursor ion	Sequence	E-score	MS-Blast score
6	—	—	1315.646	SXXXLVVTDWR		
10	—	—	950.636	GYTPLFPR		
			1399.705	LKGLGLR		
11	gil113363	Alcohol dehydrogenase 1	950.624	GYTPLFPR	4.7e-03	170
			1010.652	LLAVDLNPR		
			1398.710	YFLQGLGLR		
15	gil2494175	Glutamate decarboxylase 2	1345.590	NLENCLENAR	7.8e-06	118
			1673.970	GXXXLLAQYYKXXR		
16	gil6015065	—	744.207	FFAFGR		
			969.447	GXXLLPTAR		
			1788.901	AXXPVLEFSSXXPLR		
17	gil6015065	Elongation factor 2	1786.874	AYLPVLESGFSSTLR	3.5e-09	111
18	gil8134568	5-methyltetrahydropteroyltriglutamate	734.278	LLWFR	3.3e-42	421
			902.389	WAVHSFR		
			1096.701	YLFAGVVDGR		
			1149.667	GVTAYGVDLVR		
			1482.817	AXXNVKLDEAALR		
			1540.679	DEAFFSANAAALASR		
			1658.804	YQXXGPGVYDLHSR		
21	—	—	1241.668	FXXFSGCKLR		
			1950.030	HR		
24	—	—	1443.661	ADXXEEALNLVNR		
			1530.695	YXXKSVFTTSGAAAR		
25	gil3914360	Phospholipase D α 1	1543.619	HDAHER	4.6e-05	67
			1571.767	FXXXXTFYSQR		
			2188.298	FXXKDDNXXGATXXXR		
27	—	—	1200.710	VXXSVSNPDTR		
31	gil51701894	Tubulin β -1 chain	1342.623	LNYYNEASNR	9.9e-04	68
34	—	—	1061.547	FXXLPFTAR		
			1697.949	VAAVNTAAEVAL		
35	gil3914449	Protease regulatory subunit 7	1132.671	DXXFELLAR	9.3e-34	250
			1389.818	KXXFXXAXXLESR		
			1407.553	FDDGVGGDNEVKR		
			1721.947	LVNQLDGFDR		
			1740.894	FFDEVDAALGAR		
42	—	—	888.509	VXXLDVLR		
			952.056	DXXSLVXXR		
46	—	—	1298.512	YXXFYLLHR		
			1513.634	YQPXXGGFGVXXR		
48	gil2499489	Pyrophosphate-fructose 6-phosphate 1-phosphotransferase subunit	912.476	GXXLYGFR	6.3e-15	175
			1284.396	GQSHFFGYEGR		
			1665.604	YXXNTADLYXXR		
49	gil10720307	T-complex protein 1 subunit ζ	1732.699	SXXNAGFFYSNAEQR	8.1e-08	199
			2033.069	DEAVVLGAGAFVEAAR		
50	gil6225542	Ketol-acid reductoisomerase, chloroplastic	1544.638	MVDNCSTTAR	7.1e-20	249
			1629.800	EXXSLAGHDEYLVR		
			2073.686	ELDKGLNSSFGVHQDVDR		
51	—	—	1680.920	FXXSESWLKVEGR		
			1851.909	LSHDXXAXXR		
53	gil2501356	Transketolase, chloroplastic	996.376	NPYWENR	6.2e-13	149
			1615.648	ALXXYTPESPPDATR		
57	gil2501356	Transketolase, chloroplastic	996.444	NXXWFNR	1.3e-12	146
			1615.745	ALPTYTPESPPDATR		
58	—	—	996.384	NPYWENR		
60	gil2499489	Pyrophosphate-fructose 6-phosphate 1-phosphotransferase subunit	884.394	YYHFVR	9.1e-10	124
			1284.431	GXXHFFGYEGR		
			1639.671	VLWD		
			1672.734	TXXVGFGLVLWDXXR		
61	gil2499489	Pyrophosphate-fructose 6-phosphate 1-phosphotransferase subunit	884.394	YYHFVR	6.1e-35	337
			912.452	GXXLYGFR		
			1284.511	GKSHFFGYEGR		
			1486.055	EVXXSFGFDTACR		
			1665.594	YVELTADLYPYR		
62	gil25452981	Glucose-6-phosphate-dehydrogenase	986.440	GDQKHVFR	1.8e-24	207
			1058.625	LFLPLWNR		
			1289.575	GXXFDEYGLLR		
			1890.802	YVSGSYDAAEGFQLDDR		
63	—	—	922.511	LFNLYPR		
			942.456	AFGLDDFR		
			1133.498	SLQAXXR		
			1300.471	NKFCDAAGLYR		
64	gil54038948	Phosphoribosylaminoimidazolecarboxamide formyltransferase	1254.598	YRSLVFTNVR	6.4e-07	131
			1398.826	TLHPNLHGGLLAR		
65	gil6225542	ketol-acid reductoisomerase chloroplastic	2073.885	ELDKGLNSSFGVHKDVGDR	5.0e-06	96
67	gil135535	T-complex protein 1 α subunit	1195.561	YFVEAGALAVR	3.5e-13	148
			1571.782	EXXLLDGYALNTGR		

Spot	Accession number	Protein	Precursor ion	Sequence	E-score	MS-Blast score
68	gil146286092	Coatomer subunit δ	1874.799 912.391 1665.488	EWSLLLLLDNXXXR GAELYXXR TADYLYXXR	7.4e-06	85
69	gil3914394	2,3-biphosphoglycerate-independent phosphoglycerate mutase	1875.784 1346.567 1346.567 1547.650	EWSLLLLLDNSNR TXXYLVDHAVR LXXLSGQFDQVR AXXYEDFDKFDR	7.1e-26	278
70	gil75115272	ketol-acid reductoisomerase	1776.783 1560.652 1980.746	AVGXXVDGDAVVTFNFR FVDNCSTTAR KLRLGWGSKGPAKAKNLR	9.3e-21	237
71	gil543865	ATP synthase subunit α , mitochondria	2088.084 1077.635 1537.864	ELDKGLNSSFAVHKDQVDR EDAVGNLFGR EXXPBGDVFYLHSR	2.8e-13	185
72	gil266346	ketol-acid reductoisomerase	1816.878 823.332 1449.545 1559.536	TNFQVDELGR SDLFWR ADLXXLFDTDVDR VDNCSTTAR	4.8e-10	188
73	—	—	2086.445 1087.501	ELDQGLNSSFAVHQDVDR GXXAQLASGNR		
74	gil74996549	T-complex protein 1 subunit β	1347.426 1173.458 1320.560	LVHDXXR VDELLTCAPR GXXHHVLDEAER	2.5e-03	146
75	—	—	1531.545	CXXXDLTRPXXR		
76	gil1172858	Ribulose-1,5-biphosphate carboxylase large chain	776.405 1021.642 1116.572 1187.564	VXXLQR DTDLLAAFR VAEACVQAR DNXXLLHLHR	8.4e-30	276
77	gil14423687	Enolase 2	1465.694 806.424 1491.453 1795.543	TFQGPPLGLKVER YNQLLR LGAAAVYXXR AXXXXKSTSTYEALELR	5.6e-16	158
81	gil543865	ATP synthase subunit α	1801.796 815.427 859.954 892.448	GNXXVEADVTLSDGTVAR ELLLGDR KMSLLLR LELAQYR	1.6e-58	546
84	—	—	972.418 1026.440 1210.481 1537.525 1816.705 1071.463 1271.496 1318.429 1427.514 1617.631 1684.752 1757.585 1873.740 2023.789 2189.190	VVSVDGLAR AVDSLVP LGR VVDALGVPLDGR EAFPGDV FYLHSR LTNFYTNFQVDEL R LXXKPQTD R GLTFDSGGYXXR VNNESA EGR YAEDVSSALLFGR ATLGLTQPANLEHPR VXXLGLGQSVLSPAAFR GQXXQEYWWCTER WVFPDTNSGLLVLA EGR LXXASALASGTVLRYEDNR FSTQDH AAS		
86	gil6094242	Putative selenium-binding protein	1546.585 1624.607 1702.618	GFNLQHVADGLYGR TWKCNVXXLQGHR EXXLYVTCVYTGTR	1.0e-22	294
87	gil543865	ATP synthase subunit α , mitochondrial	1739.703 1179.449 1537.436	KTLDLGDSGLLPLELR YEELQLTAQR VFYLHSR	1.1e-12	98
88	gil14423687	Enolase 2	1815.575 1871.429 806.384 1071.367	LXXFYTNFQVDELGR WVFXXTNSXXLVLA EGR YNQLLR GVMASHR	2.6e-19	224
91	—	—	1087.350 1797.619 1797.619 1036.479	AXXGVFASHR LEEELGADAVYAGASFR QSTRYEAL ELR SLAKEYGLR		
92	gil25453205	Peroxidase 12	1649.621 1717.724 1013.665 1262.551	FXXXXQPXXDYQKR FXXLSTVHYLSGXXR DLGQAAGLLR ATAFELLDNLR	8.6e-19	330
93	gil115646	α -S1-casein	1374.760 1745.773 1809.869 830.478 875.307 1104.623 1337.597 1696.902 1759.880	VVSCDLLALAAR KXXFTSDQDLYTDSR DWYLSGGPDYDVPLR AVPYPQR FASPLAR YLGYLEQLLR HLKQEDVP SER DYVDLPHS HQXXXXEVLNE	4.5e-20	131
94	gil12585489	UDP-glucose pyrophosphorylase	1312.563	VLQLETAAGAALR	4.0e-35	305

Spot	Accession number	Protein	Precursor ion	Sequence	E-score	MS-Blast score
95	gil1169534	Enolase	1371.613	FFDHALGVNVPR	5.3e-32	301
			2197.660	YSNSNLELHTFNQSQYPR		
			806.456	YNKLLR		
			1071.448	AXXGVMASHR		
			1719.926	GASTGVYEALRL		
96	gil400246	S-adenosylmethionine synthetase I	1791.760	AXXPSGASTGVYEALRL	9.4e-19	201
			1795.698	LGADAVYAGASFR		
			1797.618	LEELGADAVYAGASFR		
			1141.399	TLFHLNPSGR		
			1453.515	FVLGGPHGDAGLTGR		
97	gil400246	S-adenosylmethionine synthetase I	1747.566	TSKDLYTDSR	2.3e-27	240
			1809.309	DADYLSGGPDYDVXXGR		
			979.417	TAAYGHFGR		
			1141.527	TLFHLNPSGR		
			1453.746	FVLGGPHGDAGLTGR		
98	gil400246	S-adenosylmethionine synthetase I	979.307	TAAYGHFGR	6.9e-25	256
			1141.470	TXXHLNPSGR		
			1248.418	LXXNYEEXXR		
			1394.154	SXXSPLNQVQSR		
			1453.853	FVLGGPHGDAGLTGR		
99	gil68052019	Biotin carboxylase	955.498	LLVWAXXR	1.3e-06	281
			1133.440	LLDLEDLR		
			1177.406	VHFWR		
			1342.559	LNFLGPNPDSLRL		
			1393.507	LPSGGKXXR		
100	—	—	1634.677	APSPALTXXLR	2.7e-17	204
			855.510	LEXXATPR		
			1393.641	LXXMLPSGGXXVR		
			1434.784	DASVAYKR		
			1897.856	EKLYLLSCCPCEGR		
101	—	—	830.877	AVPGYPGAR	6.9e-26	286
			917.590	EVVKWTR		
			1760.289	VLNER		
			SPT 2417	FLLYQEXXL		
			1068.519	FDASKFPTR		
102	gil20141399	3-oxoacyl-[acyl-carrier-protein] synthase	1111.516	ESXXYFAER	2.7e-17	204
			1173.525	LDLLS		
			1263.602	ADXXGVSSCLER		
			1297.641	LLAGESGLGPLDR		
			1629.821	KSFPADLR		
103	gil73919692	GDP-D-mannose 3,5-epimerase	1882.877	TKGQHGVATATV	6.9e-26	286
			896.413	DFGLECR		
			1049.382	EKAMTLNSR		
			1045.216	SSLYFCR		
			1424.869	VVGTPQAPVQLGSLR		
105	—	—	1570.953	LXXTQGGFLASHLAR	1.1e-16	169
			1671.583	LDECVEGVLR		
			1219.600	GDXXYLYTGR		
			1250.662	YLEPPHLLR		
			1273.762	TLFCTSLLYR		
107	—	—	1380.720	LLLR	1.4e-12	145
			851.476	EHLRLR		
			1010.523	LLAVDLNPR		
			1681.861	LLDQGGAGDNVGLLR		
			1787.949	ATAHVEYEXXKR		
110	gil122064253	Isocitrate dehydrogenase [NAD] regulatory subunit 1	1217.650	HLKFPSFWR	1.4e-12	145
			1280.601	LWGLFLESCR		
			1423.788	YAFEYAYLNNR		
			811.406	AXXYDER		
			1155.660	LXXANSXXLR		
115	gil126896	Malate dehydrogenase, mitochondrial	1219.767	LFRTTLDVVR	3.3e-13	114
			1574.816	ARQLFRTTLDVVR		
			2025.776	ALLYYS		
			3038.312	WEKLGQALEGSDVLLPAGV		
			896.468	LMEGGYAR		
116	gil1168493	Arginase	954.704	RLLQVGL	1.5e-31	376
			1035.437	YSHASPFAR		
			1100.589	VLDASLTLLR		
			1158.365	FREQYEMR		
			1527.785	VLTDVGDVAVKELR		
117	—	—	1271.751	HLAVFVEXXPR	7.8e-16	156
			925.531	ELLTFFR		
			950.637	GYTPLFPR		
			991.524	DHCTFVGR		
			1010.567	LLAVDLNPR		
126	gil1352461	IN2-2 protein	1310.505	DVEAHLNPFRL	7.8e-16	156
			1367.628	YLGLSEASASTLR		
			1436.618	LXXGLVAYSPLGR		

Spot	Accession number	Protein	Precursor ion	Sequence	E-score	MS-Blast score
127	—		1436.618	TLSNDDXXR		
			1621.745	LXXCVDLGLRQHR		
			1209.330	VXXDFGDFRR		
			1677.667	GAXXNTTV		
133	—		1094.531	AYNDDDLLR		
134	—		1126.420	EXXXXFALVR		
			1260.318	YXXEYLSR		
			1429.453	FFXXXFGNDXXR		
			819.413	VVLDAFR		
			961.249	WTDGLTLR		
135	gil51701846	26S proteasome non-ATPase regulatory subunit 14	1300.408	ALNYR	3.1e-10	146
			1658.389	GFLGQER		
			1096.635	AGHPTFVLLR		
			1429.639	FFPSEFGNDVDR		
			1706.028	AVXXNVLLSLR		
139	gil75337884	Annexin D1	1114.533	SSYELFNVR	1.9e-13	357
			1254.128	DHGDTSGD		
			1344.735	ALVLFILDPAER		
			1425.587	SLDKELSSDFER		
			1617.870	LXXKHYNDEEVL		
			1844.028	VXXYLFELACTR		
			997.604	GHXXFALVR		
			1260.615	YTTVEEYLSR		
			1429.725	FFPSEFGNDVDR		
146	gil1351279	Triose-phosphate isomerase, cytosolic	954.440	FFVGGN	6.0e-21	218
			1628.846	WLQANVSPEVAASTR		
			1763.837	VXXXXXQAQEVHSELR		
			1100.470	GXXGELQQDR		
			1117.479	CVLLEGSSPR		
			1347.475	LTSCFR		
			1039.544	VNAVAPSLLR		
			1347.475	LLXXLTSCFR		
			1441.487	HLACEWAGDNLR		
			1562.830	GXXHALVEELAXXKR		
			1895.053	ASGPGLVLLSSLAGLTXXR		
			1039.539	VNAVAPSLLR		
			1372.642	ALSSYNQSFSLR		
151	gil75331395	Probable calcium-binding protein CML49	1895.043	VLLSSLA	8.5e-06	83
			1372.597	ALSSYNKSFSLR		
			1660.782	VFYSLQNWR		
			899.559	TVAGALVLR		
			1498.745	SLGLSNYDLFLTR		
			1926.997	KXXVNKLETHPYXXR		
			1447.938	NXXLSCDXXFR		
			1012.444	FQGENFDR		
			1116.568	NLDGNLDSL		
			1254.584	FQWNFDXXR		
			1418.684	AXXESVPANSFLR		
			1536.716	YGLSEASPETLTR		
			1610.599	HQEHLAAYWXXER		
161	gil121345	Glutamine synthetase PR-2	1803.909	HETADLNSFSWGVXXR	9.6e-13	151
			981.422	VLLSSHLGR		
			1072.571	YXXAGPLVPR		
			1206.750	VLLSSHLGKPR		
			1808.115	LXXLXXGGVLLLENVR		
			2075.899	VDLNVPLDDNFNLTDTR		
			1072.769	YSLKPLVPR		
			1806.800	LXXLXXGGVLLLENVR		
			2074.957	VDLNVPLDDNFNLTDTR		
170	—		800.459	ELAAAGLR		
			863.343	GXXQLNR		
			839.482	GYFPSLR		
			1501.480	VPEGFDYELYNR		
			915.565	GKTPLFPR		
171	gil229891797	α -1,4-glucan-protein synthase [UDP-forming]	2459.183	WXXTPSDYTEVTSEYAR	7.0e-08	110
172	—		1306.746	VLACFSNNLLR		
173	gil30173108	Leucoanthocyanidin dioxygenase	1790.903	DTFSWRANR		
174	—		1950.511	ADXXLFNVGNXXNEVXXR		
			870.462	YAGFTGVR		
			1059.424	YANLEYMR		
			1075.438	YGQLENVPR		
			1363.642	LQAGYLFPEVAR		
			1442.692	NAXXVWVHFXXR		
			1813.387	YTXDGFDPSTVAR		
			1986.874	THLVTPGSGFGPGGEXXLR		
			2052.824	TDLLFFCSXXNPTGAAATR		
			909.483	NCSLLFR		
			1100.590	LLSATEPTLR		

Spot	Accession number	Protein	Precursor ion	Sequence	E-score	MS-Blast score
177	gil1345655	Cinnamyl alcohol dehydrogenase 4	1572.873	VLSGTDLTXXR	3.9e-13	149
			1074.517	HFGLMGSGLR		
			1868.429	FTVGELVGVCVVGCCR		
178	gil119194	Elongation factor Tu, chloroplastic	1173.671	TXXTVDLVGLR	3.2e-34	339
			1435.686	KYDELDAAPEER		
			1703.585	AGDNVGLLLR		
			1810.609	GXXLNTATVEYETENR	6.4e-17	117
179	gil12644295	Phosphoglycerate, chloroplastic	1919.792	FXXACEKXXR		
			989.531	FSLAPLVSR		
			2062.916	ADXXVPLDDNQNLTD DTR	9.3e-13	177
180	gil231502	Actin-85C	945.480	AVFPSLVGR		
			1132.486	GYSFTTTAER		
			1747.788	VLT LKER	1.1e-19	209
181	gil231503	Actin-85C	945.591	AVFPSLVGR		
			976.572	AXXAGDDAPR		
			1130.729	GYSFTTTAER	—	—
182	—	—	1384.556	SFTTTAER		
183	—	—	1610.760	HLAAYW		
			642.503	HAFDR	—	—
			1012.547	FKGENFDR		
			1116.584	NLDGNLDSL		
			1704.943	LDATGVLVDGTPEYVR	—	—
184	—	—	1469.889	ATVFAHETFGAYR		
			855.568	SPAQLSPR		
			1019.678	LXXLFEVAR	—	—
			1037.660	FSELEKER		
185	—	—	1147.717	KXXLFEVAR		
			784.438	VXXLGLGR	—	—
			827.466	GVLLNVGR		
			1550.750	SCPLSYYSR		
186	—	—	899.442	DTQPGLLR	—	—
			1227.684	HLESLEGAESR		
			1449.837	VAELYPEYNVPR		
187	gil75313643	Malate dehydrogenase, chloroplastic	1219.841	LFRTTLDVVR	2.2e-06	90
			1276.779	GVNVVVLPA G VPR		
			800.368	VYLLHR		
188	—	—	1007.000	VDLSSSPFR	—	—
189	—	—	862.594	VLA FEAGR		
			1027.566	VNTLSAGPLR		
			1260.704	YXXVEEYLSR	—	—
			1429.852	FFPSEFGNDVDR		
191	—	—	869.511	GTFLEFR		
			1289.679	YLSGVNLPVDGR	—	—
			1322.653	SLSVELGEYGLR		
			1389.612	HELFEXXR		
			1559.823	VXXLTGGASGLGESTAR	—	—
192	—	—	1071.484	NLEEWQR		
			1147.603	GFTGGLNYR		
193	gil74683233	Protein transport SEC13	964.310	ADNWDTSR	2.3e-05	159
			1383.606	VLHDFNTXXWR		
			1432.585	FXXLLASCSYDGR		
194	—	—	819.374	LAWATSQR	—	—
			1026.520	VDLPELQGR		
			1229.610	LGCQSPVEALR		
196	—	—	1641.761	YVNTGASWVTGAFNR	—	—
197	—	—	905.437	FLHAVYR		
			1034.523	KVDRCNSR		
198	gil74996477	Probable 3-hydroxyisobutyrate dehydrogenase	854.534	LFNCSSAR	1.2e-06	119
			1092.507	YXXFFQMR		
			1545.726	LXXDSSTLDXXTSR		
199	gil51316816	Photosystem I assembly protein Ycf4	1049.496	ALVSLEYVR	7.6e-03	68
			1962.071	SXXYVKVXXXQSLPLTR		
			990.479	NCAPLFLR		
205	gil1351963	L-ascorbate peroxidase, cytosolic	1258.613	EDZPHPPPEGR	9.2e-22	276
			1243.534	CYPTVSEEYR		
			1846.879	KXXLAHGANDGLDAVR		
208	—	—	1032.488	VXXELTLAR	—	—
209	—	—	1895.926	AKLQEVLLSSLAXXTXXR		
			1467.674	YLACEWAGDNLNR		
210	gil1717752	Tropinone reductase 1	1562.735	GXXALVEELAGLGAR	2.2e-12	192
			1878.954	ANTGSVLLSSLAGLLXXR		
			1036.371	VLDLYEER		
211	—	—	943.436	FYNDQTR	—	—
215	—	—	1059.375	KALKHYGSR		
			1134.402	YDGFVYTNR		
			2286.889	FXXMWGNSYYSALESGFSDR	—	—
216	—	—	943.375	FYNDQTR		
			994.453	VLDVYETVR		

Spot	Accession number	Protein	Precursor ion	Sequence	E-score	MS-Blast score
217	—	—	1641.775	TTANQDXV		
219	—	—	975.466	NHLQPNPR		
			1113.755	VVTALTLLKR		
			1151.596	LXXLHSVAER		
			1989.031	LFQDRLLQD		
220	gil225446793	Glutathione S-transferase 3	1036.471	VLDLYEER	2.6e-05	112
			1215.412	GEHKD		
			2397.464	TQDXXYLLYESR		
222	—	—	1022.480	TWSTPFGAR		
			1804.707	AXXPDLGDCXXCQR		
223	gil75334429	GSH-dependent dehydroascorbate reductase 1	1081.478	LYQFEXXR	4.9e-07	78
			1307.779	NLSDQPR		
			1804.543	AXXAXXVLGDCPFCQR		
224	gil17380179	20S proteasome β subunit D, type 2	821.393	CLMELR	4.0e-26	281
			830.524	GELATALR		
			873.429	LVDKDGAR		
			1010.468	NVALYQFR		
			1075.429	AASWSXXR		
			1518.753	NXXPLTTAAANFTR		
225	gil1360571	Triose-phosphate isomerase, cytosolic	807.298	FFVGGNXXR	2.4e-32	327
			1117.306	DNVGAGVAASTR		
			1438.551	ALLNESNEFVSSR		
			1589.620	SDFHVAAQNCWVR		
			1708.670	VXXPAQAKEVHCELRL		
226	gil22096383	Uncharacterized protein	1858.893	ELLVGKDDQLQTETRL	2.6e-08	103
227	gil1172601	Probable proteasome subunit α type-4	1007.424	TTLFSPEGR	2.2e-03	64
228	—	—	1258.663	EDKPHPPXXR		
229	—	—	857.483	LLAWAQR		
			1487.657	SXXXFVASD		
230	—	—	938.207	EFLEXXR		
231	gil12643263	Chaperonin 10	970.626	DLKPLGGDR	1.1e-10	161
			1149.740	YTSKPLGDR		
			1166.747	GSDGTNYLALR		
232	—	—	931.491	KAEQYHR		
			1552.296	TQLVR		
			1150.820	EXXXXTQLVR		
233	—	—	1542.813	FLTLDDG		
234	gil73921673	Allene oxide cyclase 3, chloroplastic	1034.587	DRGSPAYLR	2.6e-04	149
			1554.853	VQELYVYELNER		
249	gil1346675	Nucleoside diphosphate kinase B	913.588	GLVGELLGR	3.2e-27	245
			1370.616	NVLHGSDSVESAR		
			1567.770	LLGATNPAESAPGTLR		
250	gil14285792	T-complex protein 1 subunit γ	1132.550	SXXPTVLCR	1.3e-05	130
			1344.610	TLAQNCGVNVLRL		
251	gil109909540	Formate dehydrogenase 1, mitochondrial	868.308	GMFDKDR	3.9e-41	448
			955.587	GVLLVNNAR		
			1062.548	EWLAPYYR		
			1174.688	LSXXTLDAQLR		
			1492.629	FVGCVEKVXXR		
			1602.570	NXXFVGCVGEGALGLR		
			1689.597	LKXXNKNLLYHDLR		
			2328.332	TXXLSGTTLD		
			3004.513	KYLVTDDE		
252	—	—	1012.551	LLQEGSXXR		
			1148.775	HXXSHQWPR		
			1179.561	KLTAGR		
255	gil30315948	Caffeic acid 3-O-methyltransferase	924.480	FNKVFNR	1.8e-18	198
			1014.520	TLPDGKVER		
			1715.588	AXXXAFEYHGTDPRL		
256	gil231502	Actin-85C	976.416	AXXAGDDAPR	7.3e-12	146
			1132.465	GYSFTTTAER		
257	gil2500522	Eukaryotic initiation factor 4A	976.595	GXXLNFVTR	1.3e-20	181
			1114.662	VLLTTDLLAR		
			1518.943	LXXSGVHVVGTXXR		
			1826.750	YGFEQPSALKQR		
258	—	—	965.441	KVLTYYR		
			1017.406	SVYTGDDYR		
			1148.448	SXXLFNLESLR		
			1494.672	EVEALNNVHAGVSR		
260	gil26391492	ATP synthase subunit β -2, mitochondrial	1118.613	HYLPLHR	2.6e-06	125
			1492.808	FTKKANSEVSALLGR		
261	gil189045124	Glucose-6-phosphate isomerase	917.554	LAMKWLR	1.4e-03	173
			1458.792	EXXTVTVKEVTPLR		
			1515.668	GXXZHAYLQKLR		
262	gil21759093	Betaine aldehyde dehydrogenase 1	1009.454	LLVHEXXR	8.5e-24	304
			1129.338	KLLLDGEWR		
			1175.441	LSDPLEEGC		
			1230.485	GQDWALASGAVR		

Spot	Accession number	Protein	Precursor ion	Sequence	E-score	MS-Blast score
263	gil62900360	Importin subunit α -1a	1913.763	KVTKYVSDEPWGWYR	5.3e-16	260
			1369.611	NATWTLNFCR		
			1590.773	SXXEEVLQAGVVPR		
			2502.173	KXXCDLLNCXXR		
264	gil585451	NAD-dependent malic enzyme 62 kDa isoform, mitochondrial	1500.850	LDLYVAAAGLNPR	8.8e-30	311
			1595.921	LVVAGAGSAGLGVLNAAR		
			1693.716	LXXDXXYLGLQQHR		
			2829.320	VGDNLLFAXXR		
265	gil401322	V-type proton ATPase catalytic subunit A	1216.634	FDKDFLYLR	3.2e-64	562
			1255.724	LAADTPLLTGQR		
			1335.677	VGHNNLLGELLR		
			1675.658	YNSDAVVYVGCWR		
			1733.799	EXXLYTGLTLAEYFR		
			2052.816	LXXFEDSEKESEGYVVR		
			2691.582	NXXXXTNKVYEETAGLTVNDXXLR		
			1018.464	KXXATYGR		
266	—	—	1139.584	SLFDFELLR	2.2e-20	235
			2005.704	ALHRAQYR		
			1419.773	DVDEVLL		
			1564.686	AVLTPPAYFNDAQR		
267	gil399940	Heat shock 70 kDa protein, mitochondrial	1955.632	EAVAFGAALQGGLLR	2.2e-26	296
			1229.615	LAALKAPGFWR		
			1280.737	VVAAGANPVLLTR		
			1679.779	EAAEZDYEXXR		
277	gil190358177	ATP synthase α subunit, chloroplast	1819.818	DXXTLVGDGSTQGAVNKR	2.8e-76	656
			815.407	ELLXXR		
			822.369	LEQYNR		
			876.434	KFSLLLR		
			904.628	KMEVELR		
			1030.530	ADELSNLLR		
			1093.515	DRLEKYNR		
			1252.711	LLESPAPGLLSR		
			1266.670	ALAKPLDGR		
			1266.670	SPAPGLLSR		
			1416.836	LAQLPVSEAYLGR		
			1522.767	ASSVAQVVTTFZER		
			1553.641	EAYPGDVFYLSR		
			1612.961	LVNTGTVLQVGDGLAR		
			1132.570	GYSFTTTAER		
			1198.730	TTTAER		
285	gil1703131	Actin 12	1445.531	ESGPSLVHR	2.0e-24	230
			1962.000	SXXLPDGQVLTGAER		
			805.444	VAFELAR		
			1337.718	YFGQPR		
287	gil21759255	3-isopropylmalate dehydrogenase 2	2064.762	NTEVYATHEXXR	4.0e-10	112
			1180.535	KLTAGR		
			1286.604	LLGGGLPVGAYGGR		
			801.491	SKLLAGGR		
288	gil399785	Glutamate-1-semialdehyde 2,1-aminomutase, chloroplastic	1182.432	LNFDNNAFR	1.1e-13	160
			1185.522	TAPPLLLACR		
			1332.549	DXXAAAAMGLFGPR		
			1351.677	AXXYASNVLLQR		
289	gil75261432	Succinyl-CoA ligase [GDP-forming] subunit β	1903.657	DFVFGNETEAR	1.3e-13	153
			1335.694	AXXXAANVLLKR		
			1410.611	SLVANLSAAN		
			861.517	YKVGAXXR		
290	gil17366963	Adenosine kinase 2	1411.764	LLT	1.3e-13	153
			1359.545	AFGAELVLTDPAR		
			1427.825	YLSVLFESVVR		
			2071.175	ESAVLSGGQ		
291	—	—	964.539	EYEALALR	7.9e-09	107
			2024.803	YDXXXG DYLFTPAGDVHR		
			1206.600	ALEYEELAR		
			1257.596	GDVVALTNALER		
293	—	—	1413.562	YQELDPTFSGTR	1.4e-18	218
			958.473	NAPPEFQR		
			1456.602	SFPAFATTGDDATR		
			1759.772	DXXYAAVTVQL		
294	gil2493895	Cysteine synthase; CSase; O-acetylserin	2253.742	KLVASGPESFGDFLVXXYR	6.1e-21	208
			1107.511	DTLDFVER		
			1314.647	GQDLSVVAGLEAR		
			852.395	WYLGGR		
300	gil11134054	Oxygen-evolving enhancer protein 1	1393.612	GSQYGVQLESAR	4.2e-16	157
			1632.741	GXXTTLTSLANALSAR		
			960.498	ALLPDLYR		
			971.347	FGASWFTR		
308	—	—	1393.694	GXXYGVKLESAR	4.2e-16	157
			947.520	TALQVPYR		
309	gil224146220	Chalcone isomerase-like protein			4.2e-16	157
310	—	—			4.2e-16	157
316	—	—			4.2e-16	157

Spot	Accession number	Protein	Precursor ion	Sequence	E-score	MS-Blast score
317	gil461461	Enoyl-[acyl-carrier-protein] reductase	1025.428	NLYLLFSR	3.4e-08	121
			1429.588	GYXXSDLPPPEPER		
			1580.689	FHQSLPEGFYLSR		
			862.514	VLAFAEAGR		
318	gil8928330	40S Ribosomal protein SA (p40)	1027.554	VNTLSAGPLR	4.7e-17	128
			940.612	LLLLTDPR		
			1115.298	NCDYQMER		
319	—	—	1336.537	FXXHTGAHALAGR	6.6e-11	229
			1009.598	LFHS		
			1172.759	YLLHDENLR		
320	gil225464635	Hypothetical protein	828.502	EVTVKPR	6.6e-11	229
			901.475	LLPDKCR		
			951.491	NAELYADR		
			1413.774	AXXETGAALAPGESR		
321	—	—	1496.595	WEDTRR	2.1e-38	484
			866.602	LLQLHSR		
			1062.692	EEFQR		
322	gil54036493	Tubulin α -2 chain; α -2-tubulin	1007.507	EDAANFAR	2.1e-38	484
			1132.530	ELVDLCCLR		
			1299.468	QVPPCLFYR		
			1598.779	TLQFVDWC		
323	—	—	1857.795	LSNSTSVAEVFSR	2.9e-32	240
			1977.921	TVXXDAFNTFFSE		
			809.390	SLLSFSR		
			1079.533	TLVSLEYVR		
			1209.596	LTSQDSTLFLR		
			1262.555	GTLKLGYNLR		
			1313.577	YXXVTVGYSVVR		
			1484.582	NENLTFANYDKR		
			1555.610	LKQSFQTFSSAVQR		
			1662.716	LXXSGSYVDGYNVVR		
			1837.836	NWTAVVAAADTGGVSYVR		
			1947.738	PXXASAAFNVDEND		
324	—	—	941.603	VLELALKR	2.9e-32	240
			1518.742	EXXKL		
			1197.650	FELSGLPXXXR		
			1228.724	VELLANDKGNR		
325	gil229464991	Heat shock 70 kDa protein	1412.986	SWHDVVLVGGSTR	7.2e-22	228
			1487.880	TTPSYVAFTDTER		
			1197.653	FELSGLPXXXR		
			1412.723	SWHDVVLVGGSTR		
327	gil123592	Heat shock 70 kDa protein	1473.564	TTPSYVAFTDSEER	4.3e-31	362
			970.483	HLETXXXR		
			1058.569	FPAQELVR		
			1156.541	LQTELVER		
335	gil1708311	Stromal 70 kDa heat shock-related protein	1373.753	LDEVLLVGGSTR	1.6e-22	233
			1461.731	QFAAEELSAKVL		
			1566.854	AVVTVPAYFNDSQR		
			1623.937	ELCSDLLDR		
339	gil123656	Chloroplast envelope membrane 70 kDa heat shock protein	1329.706	LNFDLFR	1.6e-22	233
			1426.866	STVHDVVLVGGSTR		
			1487.769	TTPSYVAFTDTER		
			1950.843	AHGADAY		
343	—	—	1002.536	GYPTVYFR	5.3e-06	128
			1309.850	YXXTVGYXXVR		
			1002.463	GYPTVYFR		
			1421.688	SADGNLKPVEGDR		
348	gil11133775	Protein disulfide isomerase	1084.492	LXXPEALDLR	5.7e-06	69
			1199.580	VTWASFTVSR		
			1472.758	AXXSSNAYNDQFR		
			1084.667	LGTPEALDLR		
354	gil223635791	Transaldolase	1199.604	VTSVASFFVSR	3.2e-10	232
			1472.731	ALSSSNAYNDQFR		
			1084.807	LGTPEALDLR		
			1199.715	VTSVASFFVSR		
355	gil123142886	Transaldolase	1472.913	ALSSSNAYNDQFR	3.2e-10	240
			1400.766	LXXDATAQLDLR		
			840.460	PATVSLPR		
			873.517	KXXALGLR		
358	gil12229892	Proteasome subunit α type-1-B	1679.916	VDDHLGVLAGLTADGR	8.5e-25	242
			1596.526	NXXDQDVTWSPAGR		
			960.562	ALLPDLYR		
			1107.661	DTLDLFVER		
362	gil1345698	Chlorophyll-a/b binding protein 151	1055.533	ELEVLHCR	1.8e-11	135
			1325.605	NRELEVLHCR		
			1544.766	SAPASLWYGA		
			1055.390	ELEVLHCR		
366	—	—	1179.475	YEELQLTAAGR	2.4e-04	76
			1179.475	YEELQLTAAGR		

Spot	Accession number	Protein	Precursor ion	Sequence	E-score	MS-Blast score
372	gil1168191	14-3-3 protein 4	1325.433 816.458 1418.624 1418.624 1802.917	NRELEVLHCR LAEQAEER LSDVEELLSEER TVDVVEQKEESR SXXDLALAELAPTHXXR	5.4e-18	271
373	—	—	1102.555	NPGDSFPNVR		
374	gil26454610	14-3-3 protein 8	816.460 1202.704 2138.979	LAEKAER LDVELTVEER AXXTATATAEAELPPTHXXR	3.0e-21	206
376	gil12230867	14-3-3-like protein GF14 ω	1093.290 1109.538 1205.488 1418.739 1675.942 1803.833	HVAVLR GNEDHVAVLR LFQLLR LLSSLEKKEESR VSASVENEELSVEER LANAELAPTHXXR	1.9e-28	304
377	gil26454609	14-3-3 protein 7	805.443 932.491 1006.471 1205.504 1484.786 2084.894	LFVLTGR FKGDYFR EQKVYLAR LLFQLLR NLLFAGYTDWER AXXXAATSTASSDLPPPTHXXR	3.8e-16	232
378	gil12230867	14-3-3-like protein GF14 ω	922.432 1667.526 1374.750 1803.957 2247.810	FKGDYHR GNADHVSVLR LVSSLEKKEEGR SXXDLANAELAPTHXXR SAVPAPDELTVEER	1.1e-27	323
379	—	—	938.600 1125.715	YCVGLGNNR FLWNR		
380	—	—	1109.466	YNLMLWNR		
381	—	—	1061.409 1109.451 1990.430	YXXLWNR YXXMLWNR YWGQNLYE		
382	—	—	701.511 1723.860 1827.775	LHFER STYDDLQPGSLL TWLXXXXNKDD		
385	gil75294319	14-3-3-like protein GF14-C	808.399 1151.807 1685.144 1772.946	LDYLER LVSLELP AELTVEER LALADLAXXHPLR	8.7e-09	150
386	gil9979193	Translationally-controlled tumor protein	1063.475	VVDLVDTFR	2.2e-03	66
397	—	—	1791.434 1836.797 2035.930	NNSLTFR ELVEALDSENNSLTFR AHFWEALDSENNSLTFR		
410	gil12585487	V-type H-ATPase subunit C	1013.579 1457.517	ALNKR DFEYSPEAQESR	2.7e-17	172
411	gil75146893	Bifunctional dihydroflavanol 4-reductase	1035.457 1608.665 1848.254	TVDGVXXDSYLTR LLEDGYAVR LVFTSSAGTVNVEER MFTEAVETCR	5.2e-16	198
412	—	—	967.084 1419.659	ALSFSLATR DNFVSLWHFVR		
413	gil75038951	α-S1-casein precursor	1267.712	YLGYLEQLLR	1.5e-03	70
414	gil6225258	Aspartate semialdehyde dehydrogenase	1154.638 1235.568 1156.566 1613.745	NAPGVVVLDDR GXXVVDNSSAFR EXXAYLETGR KXXXAEYTAVEER	1.8e-06	118
415	—	—	1038.569 1156.481 1613.676	GVFPFSQTR EAFAYLETGR KFGTLAEYTAVEER		
416	—	—	1038.567 1156.483 1473.776	GVFPFSQTR EAFAYLETGR LAXXEXXWHAXXR		
417	—	—	1613.659 1287.704 1574.634 2127.200	QFXXLAEYTAVEER VVVLWTANTER VXXYDGEELYA DXXQKANYFGSLTQASTLR	7.7e-29	255
421	gil14548089	Inositol-3-phosphate synthase	2725.866 970.568 1018.483 1139.658 1773.891 2057.053 2057.053	TRVVFAGALLEQAER FLFEDGSR KHWAYTGR SLDFELLR DXXKPGTSXXR ALHRAQYAGR QLVTVEDXXR	2.5e-22	237
422	—	—	1666.001 1666.001 2251.547	VXXKLYLER LYGASGYLFR SSDVALLASNKLDQDR		
423	gil12585296	Phosphoglucosyltransferase, cytoplasmic	1328.509 1471.694	AHGGVSVFVGVGGER VXXALTMAEYFR	2.2e-50	447
424	—	—				
427	gil75338403	ATP synthase subunit β, chloroplastic				

Spot	Accession number	Protein	Precursor ion	Sequence	E-score	MS-Blast score
428	—		1601.609	YQGMNEPPGAR	1.2e-21	246
			2097.371	LDTGALPSVPVGGATLGR		
			2386.229	KLNVTCCEVQKLLGNRR		
			1035.452	DXXLVYAGR		
429	gil78099761	Arginosuccinate synthase, chloroplast	1083.545	DXXFPCLR	1.2e-21	246
			1041.424	YAEVYAGR		
			1693.710	GXXTPGGTLLFNAVR		
			1518.886	LXXSGVHVVVGTXXR		
430	—		1827.858	YGFEQPSALKQR	8.8e-13	162
			2417.218	KDLSSFESGQLYDQADAAGFLR		
			2074.934	PEPALELTR		
			1271.670	AXXHHTAAFLR		
431	—		1420.834	EXXQXXAWALHR	8.9e-28	283
			1334.458	YQTGLAR		
			1373.536	VXXDCATANXXGR		
			1734.700	FHLTYYNTEFNHGR		
434	gil62510584	Rab GDP dissociation inhibitor β	922.507	LYAESLAR	6.7e-18	145
			2544.244	FKNSXXLYPLYLGLGELPKAFAR		
			1177.512	KLKEEYXXR		
			1236.444	HNFSSLNDFR		
443	—		1708.669	GXXQYFTTHTDLVR	7.3e-3	64
			825.446	NLFYLR		
			1080.411	GYLFPEGAAR		
			1179.473	QLTAGR		
444	gil50400860	Monodehydroascorbate reductase	1430.145	LGGGVAAGYAAR	4.0e-10	131
			1800.535	SVDEYDYLPPFYSR		
			1030.578	SWCDLPPR		
			1146.498	FPXXLNSDLR		
451	gil8928411	β -2-tubulin	1274.615	FKXXLNSDLKR	5.1e-04	84
			1342.703	LNYYNEASGGR		
			1024.436	SEGEHYR		
			1239.545	WYLYFYER		
453	gil129699	Proliferating cell nuclear antigen	1570.636	DXXXYAHHSNXXR	5.0e-26	261
			1761.840	YXXDNTLFHLNKLKLR		
			1296.678	VAYTFDAGNPVLLAR		
			836.352	NEAAEFR		
469	gil1172586	Polyphenol oxidase A1, chloroplastic	1156.455	HLTLFSPEGR		
			1452.634	FLXXLATGFTADAR		
			1970.952	AXXXAELDEHLTALSE		

X indicates any amino acid.

Supplementary Table 1(b). *De novo* derived peptide sequences for the identification of proteins from cocoa clone ICS39.

Spot	Accession number	Protein	Precursor ion	Sequence	E-score	MS-Blast score
i700	—		1151.686	LVTVEL	7.9e-32	331
i701	—		1493.939	DSENNNS		
i702	—		1837.012	ELVEALDSENSLTFR		
			905.435	LHAVYR		
i704	gil2499498	Phosphoglycerate kinase, cytosolic	1451.772	VFELLQR	8.5e-08	128
			981.668	VLLSSHLGR		
			1072.694	YSLAGPLVPR		
			1206.864	VLLSSHLGKR		
i705	—		1807.179	LVAELPDGGVLLLENVR	5.8e-08	162
			2075.981	VDLNVPLDDNFNLTDTR		
			860.618	QSPPYLR		
			980.680	GYAVNTTVR		
i706	gil224078816	Anthocyanidin reductase	1054.552	LRLNSR	1.1e-28	291
			1209.512	VPTDFGDFRR		
			1088.505	KYDWSAYR		
			1424.875	EXXEVAWN		
i708	gil3913034	β -amylase	1996.900	SDXXAAELTAGYY	2.8e-31	282
			1233.711	KXXLEELFAR		
			1950.989	AHGADAYPFTEER		
			902.527	WAVHSFR		
i710	gil8134568	5-methyltetrahydropteroyltriglutamate	1096.645	YLFAGVVDGR		
			1697.815	AXXNVKLDEAALR		
			1658.955	YQGLGPGVYDLHSPR		
			850.451	KLLTYGR		
i711	gil85700445	Probable mitochondrial-processing peptidase β subunit	900.622	VWDVLLR		
			986.658	LTTLPDGLR		
			1284.627	GQSHFFGYEGR		
			1871.100	EHLQNYLQTHYTAPR		
i800	—		2561.760	TASQLVANEPATFTGSEVR		
			989.430	KDXXHAHR		

X indicates any amino acid.