Proteome analysis of *Pueraria mirifica* tubers collected in different seasons

Jutarmas Jungsukcharoen¹, Daranee Chokchaichamnankit², Chantragan Srisomsap², Wichai Cherdshewasart³ and Polkit Sangvanich⁴,*

¹Faculty of Science, Program in Biotechnology, Chulalongkorn University, Bangkok, Thailand; ²Laboratory of Biochemistry, Chulabhorn Research Institute, Bangkok, Thailand; ³Faculty of Science, Department of Biology, Chulalongkorn University, Bangkok, Thailand; ⁴Faculty of Science, Department of Chemistry, Chulalongkorn University, Bangkok, Thailand

Received September 30, 2015; accepted December 15, 2015
http://dx.doi.org/10.1080/09168451.2016.1141035

*Corresponding author. Email: spolkit@chula.ac.th

**Pueraria mirifica**-derived tuberous powder has been long-term consumed in Thailand as female hormone-replacement traditional remedies. The protein profiles of tubers collected in different seasons were evaluated. Phenol extraction, 2D-PAGE, and mass spectrometry were employed for tuberous proteome analysis. Out of the 322 proteins detected, over 59% were functionally classified as being involved in metabolism. The rest proteins were involved in defense, protein synthesis, cell structure, transportation, stress, storage, and also unidentified function. The proteins were found to be differentially expressed with respect to harvest season. Importantly, chalcone isomerase, isoflavone synthase, cytochrome p450, UDP-glycosyltransferase, and isoflvanone reductase, which are all involved in the biosynthesis pathway of bioactive isoflavonoids, were most abundantly expressed in the summer-collected tubers. This is the first report on the proteomic patterns in *P. mirifica* tubers in relevant with seasonal variation. The study enlights the understanding of variance isoflavonoid production in *P. mirifica* tubers.

Key words: *Pueraria mirifica*; Isoflavonoid producing enzymes; plant proteomics; secondary metabolite

*Pueraria mirifica* or “White Kwao-Krua,” is a traditional Thai medicinal plant long-term used as a rejuvenating herb for post-menopausal women.¹ The key chemicals from the tubers have been tested in animals and human cells and found to exhibit estrogenic effects.² The tuberous crude extract showed a dose-dependent biphasic growth response to MCF-7 cells with strong competitive binding with estrogen at a high dose to estrogen receptors.³ The active components, including the major isoflavonoids daidzin, daidzein, genistin, genistein, and puerarin⁴ play an important role in the antioxidant⁵ and estrogenic activity of *P. mirifica* tubers.⁶ However, the dose-dependent estrogenic activity was noted to vary seasonally, where those collected in the winter and summer exhibit a higher estrogenic activity than those harvested in the rainy season.⁷

In the field of plant proteomic studies, various methods for protein extraction have been developed.⁸ Protein extraction by the phenol method is accepted as the best because a large proportion of the non-proteinaceous materials could be eliminated, some of which interfere with the subsequent protein separation (resolution) stages.⁹ For example, a proteomic reference map of soybean leaves has been established using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) as a separation technique and identified by mass spectrometry (MS) of tryptic peptides.¹⁰ The result indicated that 2D-PAGE, combined with MALDI-TOF-MS and liquid chromatography tandem mass spectrometry (LC-MS/MS), was the most sensitive and powerful technique for separation and identification of soybean leaf proteins.¹¹ Various studies of plant tuber proteomes have been performed, including that for ginger root extract,¹² cassava,¹³ curcuma,¹⁴ grape,¹⁵ potato tubers,¹⁶ and carrot root.¹⁷ In the terms of plant growth and productive factors, climate changes have been reported to be one of the key factors, including photosynthetic, air pollutants, thermal (heat and cold), osmotic (drought, salt, flooding stress), and metal stress.¹⁸ Some evidence shows high temperature and humidity stress plays important roles in inducing differentially expressed proteins in soybean.¹⁹ However, alteration in the protein profile of Legume plants in different seasons as never been reported. Low temperature stress (less than 20 °C) can cause considerable loss of agricultural yields in tropical crops including maize, rice, and chickpea.²⁰ Low temperature exposure coordinately induces the accumulation of phenylalanine ammonia lyase and chalcone synthase mRNAs only in the light condition.²¹ In addition, high temperature or heat stress causes oxidative damage that manifests in lipid peroxidation. The plant responds to heat stress by producing detoxification

© 2016 Japan Society for Bioscience, Biotechnology, and Agrochemistry
enzymes, such as glutathione S-transferase (GST), catalases, SOD, and ascorbate peroxidases for protection.\textsuperscript{21)} The optimal growth temperature for soy and other tropical legumes was found to be about 22 °C with a maximum of about 40 °C.\textsuperscript{22)}

This work aimed to set up a protein database of \textit{P. mirifica} tubers and investigate variances in the proteome expression pattern in the three seasons (winter, summer, and rainy) for the same plant cultivar in Thailand. Proteomic techniques were employed to investigate the relationship of enzymes or proteins to the secondary metabolite productions. Changes in the relative expression levels of proteins involved in secondary metabolite synthesis, and especially isoflavonoid production, were focused upon.

**Materials and methods**

**Plant material.** The tubers from at least three-year-old \textit{P. mirifica} plants were freshly collected from the same plant cultivar at least nine plants in each season at Banpong District, Ratchaburi Province (E \textdegree 99°52′/N 13°37′), Thailand in 2011. Plant cultivation was grown under the same cultivation system in each season and year. The samples were cleaned with water and stored at −80 °C.

**Determination of the optimal protein extraction method.** For each protein extraction method, initially a 1 g sample of the respective \textit{P. mirifica} tuberous tissue was pulverized to a fine powder in liquid nitrogen using a mortar and pestle. The powder was then extracted as follows.

**Acetone precipitation.** The respective powdered samples were extracted with 5 mL acetone containing 100 mL/L TCA and 10 mL/L dithiothreitol (DTT). The samples were kept at −20 °C for 2 h (overnight, if necessary) and then centrifuged at 4,000 \textit{g}_{max} at 4 °C for 20 min to collect the precipitated protein. The pellets were washed twice with acetone containing 1 mL/L DTT, incubated at −20 °C for 2 h and then centrifuged as above. The pellet was harvested, dried by inverting on Kimwipe for 15 min at room temperature, and stored at −80 °C.

**Tris-HCl extraction.** The respective powdered sample was extracted in 5 mL of extraction media (0.175 M Tris-HCl, pH 8.8, 50 g/L sodium dodecyl sulfate (SDS), 15% (v/v) glycerol, 0.3 M DTT)\textsuperscript{19) by grinding in the mortar for 30 s. The ground homogenate was then filtered through the two layers of miracloth, collecting the filtrate in a 50 mL Falcon tube and then the protein was precipitated by the addition of four volumes of ice cold acetone, briefly vortexing and then leaving overnight at −20 °C. The precipitate was harvested by centrifugation as above; the pellet was washed twice in 15–20 mL of cold 800 mL/L (v/v) acetone, and finally dried as above and stored at −80 °C.

**Phenol extraction methods A and B.** The method was slightly modified from the previous described method\textsuperscript{23)} because \textit{P. mirifica} tubers have some compound(s) that cause interference. The extraction buffer of method A comprised of 0.7 M sucrose, 0.5 M Tris, 30 mM HCl, 0.1 M KCl, and 20 mL/L β-mercaptoethanol, and extraction buffer B was the same except with the addition of 2 mM phenylmethanesulfonyl fluoride (PMSF) and 50 mM ethylenediaminetetraacetic acid (EDTA) as protease inhibitors.

The respective powdered sample was added to 3 mL of ice-cold extraction buffer (A or B), mixed by vortexing, kept at 4 °C for at least 30 min, and then centrifuged as above. The supernatant was transferred to a new tube and kept at 4 °C, while the pellet was resuspended in fresh extraction buffer and extracted as above. The pooled supernatants were added with an equal volume of water-saturated phenol, mixed vigorously, and kept on ice for 1 h. The solution was then centrifuged as above and the upper phenol phase was transferred to a new tube and back-extracted with an equal volume of 1:1 (v/v) ratio extraction media: phenol, and then centrifuged at 8,000 \textit{g}_{max}, 4 °C for 10 min. The phenol phase was harvested and added to that from the previous extraction. Proteins were then precipitated from the pooled phenol phase by the addition of five volumes of 0.1 M ammonium acetate in methanol and stored overnight at −20 °C. The solution was centrifuged as above, the pellet dissolved immediately in 1 volume of cold water with sonication for 3 min, and then precipitated by the addition of nine volumes of cold acetone and stored at −20 °C for at least 4 h prior to being centrifuged as above to recover the precipitated protein. The protein pellet was dried and stored at −80 °C until use.

**Protein quantification.** A standard protein curve was established using bovine serum albumin (BSA) at concentrations of 0, 0.2, 0.3, 0.5, 0.7, and 1.0 mg/mL, in the Bradford assay as reported\textsuperscript{23)} and measuring the absorbance at 595 nm against a blank of 0 mg/mL BSA. All determinations were performed in triplicate and the average values were used to establish the standard curve, slope, and \textit{R}^2 values. Extracts were evaluated in the same manner and the protein concentration estimated from that for the BSA calibration curve.

**Protein resolution.** 2D-PAGE analysis of the proteome.

**First dimension IEF.** The protein sample (50 μg) from the respective phenol extraction was dissolved in lysis buffer, mixed with 75 μL rehydration buffer, and subsequently loaded onto immobilized pH gradient strips of a non-linear pH 3–10 gradient. Rehydration and subsequent IEF were conducted using the Ettan IPGphor III IEF system (Amersham Biosciences, USA). Rehydration was performed overnight in a strip holder with Drystrip Cover Fluid (GE Health Care). The IEF for first dimension was run as follows: (i) step and hold at 300 V for 0.2 kVh, (ii) gradient 1,000 V...
for 0.3 kVh, (iii) gradient 5,000 V for 4.5 kVh, and (iv) step and hold at 5,000 V for 2.0 kVh.

Second dimension (SDS-PAGE). The strip was equilibrated with 1.5 mL equilibration buffer (50 mM Tris pH 8.8, 6 M urea, 300 mL/L glycerol, 20 g/L SDS, 10 g/L DTT, and a trace amount of bromophenol blue) for 20 min. The strip was placed in fresh 1.5 mL equilibration buffer, except with 25 mL/L iodoacetamide (IAA) instead of DTT, for another 20 min and then loaded on the SDS-PAGE (140 g/L acrylamide resolving gel) second dimension (11 x 11 cm) and separated using a PROTEAN II XI multi-cell (BioRad, USA). Three replicated gels were run for any P. mirmica sample. The analyzed gels were stained with Colloidal Coomassie Brilliant Blue G-250.

Image analysis. The analyzed gels were scanned using Labscan 5.0 and analyzed by the aid of Image-Master 2D Platinum 6.0 software. The 2D-PAGE analysis was independently performed and compared in triplicate numbers while the consistent spots (good appearance among different gels) were reported for statistical analysis. The paired t-test was performed for comparison of data. Only spots showing consistently significant differences (± over 1.5-fold, p < 0.05) were selected for further MS analysis.

In-gel digestion with trypsin. Each protein spot on the gel was cut out and washed twice with 100 μL distilled water. Then 50 μL of 0.1 M NH₄HCO₃/500 mL/L acetonitrile (ACN) was added, vortexed, and incubated at 30 °C for 20 min to destain the dye. The gel was dried using a speed-vac (LABCONCO 7812014), and rehydrated in 50 μL 0.1 M NH₄HCO₃/10 mM DTT/1 mM EDTA for 45 min at 60 °C. The solution was then discarded and replaced with 50 μL of 100 mM IAA/0.1 M NH₄HCO₃ and incubated for 30 min in the dark to promote alkylation. The solution was then discarded and rinsed three times with 0.05 M Tris-HCl pH 8.5/100 mL/L ACN/1 mM CaCl₂. The gel was subsequently dried using a speed-vac ready for the LC-MS/MS preparation.

LC-MS/MS analysis and protein identification. The nano LC-MS/MS analysis was performed using a capillary LC system coupled with a Micro-TOF Q11 mass spectrometer (Bruker, Germany) and equipped with a Z-spray ion-source working in nanoelectrospray mode. EASY-Column (2 cm, 100 x 5 μm id. C18-A1) was used as the trap precolumn and an EASY-Column (10 cm, 75 x 3 μm id, C18-A2) was used as the analytical column. The tryptic peptides were concentrated and desalted. Eluent A and B were 1 mL/L formic acid in either (A) 49:1 (v/v) water: ACN or (B) 1 mL/L formic acid in ACN. The respective tryptic gel digest sample (6 μL) was injected into the nano LC and separation was performed using a (v/v) ratio A:B gradient of 9:1 (0 min), 11:9 (20 min) and 1:19 (20.10 min), all at a flow rate of 50 nL/min, and subsequently 1:19 (20.20–25.00 min) at a flow rate of 80 nL/min. The MS/MS data were converted into mgf files and analyzed by the mascot search engine (http://www.matrixscience.com). The search parameters were defined as follows: Database, Swiss-Prot; taxonomy, Viridiplantae (Green Plants); enzyme, trypsin; one missed cleavage allowed. Peptide and fragment mass tolerance values were set at 1.2 and 0.6 Da, respectively. Proteins with a predicted molecular weight and pI consistent with the gel region, with at least one peptide exceeding the score threshold (p < 0.05) and a protein score ≥ 17 were considered as positively identified.

Statistical analysis. Statistical analyses were performed using IBM SPSS statistics version 21 software. Independent sample t-test was used to compare the means between variants. The correlation between isoflavonoid contents and protein density was analyzed by Pearson correlation and Duncan analysis of variance, accepting significance at the level of p < 0.05.

Results and discussion
Evaluation of the protein extraction method
The P. mirmica tubers were ground and then extracted for protein by the TCA-acetone precipitation, Tris-HCl extraction and phenol extraction method A and B. From the physical properties of the extracted proteins, the color of the enriched protein samples from the TCA-acetone and Tris-HCl methods was light brown, suggesting the presence of visible contaminants. In contrast, the color of the enriched protein samples obtained from the phenol extraction methods was white. Some protein bands in the samples derived from the Tris-HCl and TCA-acetone extractions were absent following SDS-PAGE (data not shown). In contrast, the phenol extraction method resulted in a greater abundance of proteins and more clearly separated bands (Fig. 1). The phenol extraction method has been applied previously for proteomic analysis in many legume plants including horse gram (Macrotyloma uniflorum), rajmah (Phaseolus vulgaris), mah (Vigna mungo), chickpea (Cicer arietinum), masoor (Lens culinaris), and mungbean; as well as in non-legume plants, for example, rice (Oryza sativa), wheat (Triticum aestivum), bajra (Pennisetum glaucum), and mustard (Brassica juncea). Phenol extraction is typically the most
efficient protein extraction method for recalcitrant plants to eliminate interfering compounds, such as cell wall components, storage polysaccharides, lipids, and phenolic compounds. It also results in the highest quality 2D-gel resolution with less background and less vertical streaking. The addition of PMSF and EDTA to the extraction buffer (method B) was to minimize the appearance of degradable proteins, as seen from the greater number of much more clear resolved protein bands when using method B than method A (Fig. 1). Thus, the phenol extraction method B was deemed to be the suitable protein extraction technique for *P. mirifica* tubers and was applied here after in this study.

**Protein profile of *P. mirifica* tubers**

The optimal tuber protein concentration for 2D-PAGE was found to be 50 μg (data not shown), which exhibited the most effective separation, in terms of the highest number of resolved spots compared to that of the higher (80–100 μg) or lower (30 μg) concentrations. An example 2D-PAGE resolved sample (tubers harvested in the winter season), loaded at 50 μg total protein is shown in Fig. 2, where the numbered spots were excised and identified by tryptic digestion and LC/MS/MS analysis. From a total of 322 protein spots, 169 protein species were identified. For example, spots No. 30, 31, 32, and 145 were characterized as pathogenesis-related protein (spot No. 30 and 145), GSTU6 (spot No. 31) and UGT (spot No. 32). The 322 identified tuber proteins from all three seasons were categorized according to their gene ontology (GO) into eight functional classes; cell structure, defense, metabolism, photosynthesis, protein synthesis, stress response, transportation, and unknown function (Fig. 3), which exhibited various proteome patterns in each season (Fig. 4). Unidentified proteins inferred include a number of peptides with unidentified function in...
There were 59% of the GO identified proteins functionally involved in metabolic pathways, and the majority of these were involved in carbohydrate metabolism; glycolysis, tricarboxylic acid cycle, and oxidative pentose phosphate pathway. The GO metabolism and protein synthesis functional groups were upregulated in summer-, whereas, the defense and transport protein functional groups were most upregulated in the winter-collected tubers (Fig. 3).

Some of the identified proteins were located at a similar molecular weight with various pI, for example, spots No. 78, 104, 122, 123, 125, and 153, which were identified as fructose-bisphosphate aldolase (spot No. 78 and 104), aconitate hydratase 1-like (spot No. 122), 5-methyltetrahydropteroyltriglutamate homocysteinemethyltransferase-like isoform 1 (spot No. 123), methionine synthase (spot No. 125), and alpha-amylase (spot No. 153). Moreover, the same protein may appear in multiple spots due to the presence of isoforms resulted from post-translational modifications, such as glycosylation and phosphorylation, or conformational changes including cleavage of the N- or C-terminal with exopeptidases.

Proteins involved in secondary metabolites synthesis and isoflavonoid biosynthesis pathway

Plant secondary metabolites are biosynthesized in specific pathways, the regulation of which is strongly susceptible to environmental changes, as well as cultivar genetics, biotic (for example insects, pathogenic microorganisms, and herbivores), and abiotic (temperature, light, UV, high condition of nutrients, higher latitude, drought etc.) stress signals. The proteomic data herein proteomic data revealed diverse proteins that are likely to be involved in secondary metabolite biosynthesis are present in the tubers (Fig. 3).

GST was found as one of the abundant protein in the tubers. In general, GSTs detoxify toxins by conjugation with reduced glutathione (GSH). The toxin-SG conjugate is subsequently transported into the vacuole by ABC transporters prior to proteolytic processing. However, the disulfide containing flavoenzyme GSTU6 (spot No. 30) is required for transport of flavonoid to the vacuole. GSTs appear to be involved in the intracellular binding and stabilization of flavonoids, rather than in catalyzing glutathionylation. Glutathione reductase (GR) (spots No. 15–139), also flavoenzyme, converts oxidized glutathione (GSSG) – GSH and thus maintains a high GSH/GSSG ratio under various abiotic stresses, subject to substrate availability.

For the synthesis of isoflavonoids, the major key enzymes found were chalcone flavonone isomerase (CHI) (spot No. 46), isoflavone synthase (IFS) (spot No. 49), UGT (spot No. 32), and isoflavone reductase (IFR) (spots No. 13, 73, and 136). The role of found enzymes in isoflavonoid biosynthesis is shown in Fig. 5. The key enzyme CHI converts isoliquiritigenin to liquiritigenin and p-coumaroyl-Co-A to naringenin, while the cytochrome p450 monoxygenase (IFS) converts liquiritigenin to daidzein and naringenin to genistein. UGT is involved in defense via sugar conjugation to aid increase the molecular stability (by protecting a reactive nucleophilic group) and water solubility, and is also involved in glycosylation of daidzein and genistein to daidzin and genistin, respectively. Whereas, IFR catalyzes the reduction of the heterocyclic ring of isoflavone to form isoflavanone, an
The intermediate molecule within the isoflavonoid biosynthesis is the aglycone, which is further modified by enzymes such as UDP-glycosyltransferase (UGT) to produce aglycones.

The comparison between the levels of isoflavonoid compounds and the proteins/enzymes in *P. mirifica* tubers is shown in Table 1. The most abundant glycosidic isoflavonoids (puerarin, daidzin, and genistin) were reported to be upregulated in *P. mirifica* tubers in summer.35) Such a study is consistent with enzymes of the isoflavonoid biosynthesis pathway being expressed in the tuber proteome that were correlated with cell structure, three of them are involved in actin polymerization: actin 101 (spot No. 83), actin depolymerizing factor (ADF) (spot No.101 and 102), and actin related proteins and isoforms at low temperatures.36) 3-Ketoacyl-CoA synthase (spot No. 2) was expressed in tubers collected from the three seasons but was present in lowest amount in the rainy season-collected samples. This protein is involved in the biosynthesis of cuticular wax and suberin that help protect the plant tuber from dehydration by forming water transport-limiting barriers.37) Thus, in the rainy season the relative humidity and water content might initiate less necessary for protection from a waxy cuticle and thus lower amounts of

| Name                          | Winter          | Summer          | Rainy season    |
|-------------------------------|-----------------|-----------------|-----------------|
| **Isoflavonoids content (mg kg⁻¹ powder)** |                 |                 |                 |
| Puerarin                      | 146.3 ± 30.4ᵃ     | 2284.1 ± 878.0ᵇ | 144.8 ± 60.3ᵃ     |
| Daidzin                       | 279.1 ± 63.4ᵃᵇ   | 1782.0 ± 435.5ᵇ | 134.0 ± 32.5ᵃ     |
| Genistin                      | 193.9 ± 37.6ᵃᵇ   | 668.8 ± 111.0ᵃᵇ | 108.1 ± 29.0ᵃ     |
| Daidzein                      | 339.2 ± 36.0ᵇ     | 202.5 ± 79.2ᵃ   | 472.1 ± 334.5ᵃ    |
| Genistein                     | 250.8 ± 39.2ᵃᵇ   | 35.4 ± 17.0⁰    | 132.9 ± 32.0ᵃ     |
| Total                         | 1261.3 ± 117.1    | 4972.7 ± 1256.9 | 991.9 ± 423.9    |

**Total protein density**

| Name                          | Winter          | Summer          | Rainy season    |
|-------------------------------|-----------------|-----------------|-----------------|
| UDP-glycosyltransferase (UGT) | 11.3 ± 0.3ᶜ     | 62.2 ± 0.2ᵃᵈ   | 31.8 ± 0.5ᶜ     |
| Chalcone flavonone isomerase (CHI) | 3.0 ± 0.1ᵇ     | 0.9 ± 0.1ᵃᵇ   | 1.7 ± 0.0ᵃ     |
| Cytochrome p450 monoxygenase (IFR) | 2.8 ± 0.1ᵃ     | 3.6 ± 0.1ᶜ     | 3.4 ± 0.0ᵇ     |
| Isoflavone reductase (IFR)    | 6.1 ± 0.3ᵇ      | 1.5 ± 0.1ᵇ     | 2.1 ± 0.1ᵃ     |
| Total                         | 23.2 ± 2.0      | 68.3 ± 15.0    | 39.1 ± 7.3     |

Notes: Data are shown as the mean ± 1 SEM, derived from five independent assays. Means not sharing a common letter (superscripts a,b,c) within the same column are significantly different (P < 0.05) as determined by Duncan’s multiple range tests. *P. mirifica* tuber samples expressed significant-correlation between individual and total protein density and between individual isoflavonoid/protein and total isoflavonoid/tootal protein contents: *P (P < 0.01), **P (P < 0.05) as determined by Pearson correlation analysis.
epicuticular wax should accumulate. Therefore, we hypothesize that the tuber might produce less wax during the rainy season.

**Defense.** In terms of defense response, resistance proteins are expressed when the plant is infected by pathogens, such as viruses, bacteria, fungi, and nematodes, enabling the plant to protect itself by synthesizing antimicrobial secondary compounds and subsequently inducing defense responses. Pathogen- and wound-inducible antifungal protein CBP20 precursor (spot No. 23), pathogenesis-related protein 1 and PR10.61 (spot No. 145) exhibit antifungal activity against *Trichoderma viride* and *Fusarium solani* by inducing cell lysis of the germ tubes and/or growth inhibition. Leaf rust resistance protein Lr10 (spot No. S14) protects against fungal pathogens that cause leaf rust diseases, and these have been reported to occur in the late summer or early fall but not at other times of the year. It correlates with the expression of Leaf rust resistance protein Lr10 seen in this study in the winter-collected tubers.

The NBS-LRR type disease resistance protein (spot No. 48) is known to induce a series of plant defense responses, such as the activation of an oxidative burst, calcium and ion fluxes, mitogen-associated protein kinase cascade, induction of pathogenesis-related genes, and the hypersensitive response. Hence, the upregulated proteins in the rainy season-collected tubers may be relevant to the better condition of temperature and humidity during the rainy season for most plant pathogens of the grown *P. mirifica*.

The CuZn-SOD (spots No. 26, 27, 101, and 144) rapidly catalyzes the conversion of superoxide to hydrogen peroxide and molecular oxygen within a cell and thus reduces the oxidative damage to different cellular components. This enzyme has also been found in various plant tubers, such as *Curcuma*; potato; and *Stemonoides tuberosa*.

**Metabolism.** Carbohydrate metabolism plays an important role within plant tubers during dormancy and sprouting because it is necessary for starch/sugar storage in dormancy and energy production in sprouting. Furthermore, the respiration pathway and the formation of root/shoot meristem also required energy. Fructose-biphosphate aldolase (spot No. 72) is involved in carbohydrate metabolism, such as glycolysis, gluconeogenesis, and the Calvin cycle, where it catalyzes fructose 1,6-biphosphate to dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP). In this study, fructose-biphosphate aldolase was mainly expressed in winter-collected tubers at 10.3 and 8.9-fold higher than in the summer and rainy season-collected tubers, respectively. Likewise, fructose-1,6-bisphosphatase (spot 30), which converts fructose-1,6-bisphosphate to fructose 6-phosphate, was expressed at the highest level in *P. mirifica* in winter-collected tubers (Table 2), as was triose phosphate isomerase (spot No. 49), which interconverts DHAP and GAP. Alpha mannosidase (spot No. 127), involved in glycan biosynthesis and degradation, was also found at the highest expression level in the winter-collected tubers, while fructokinase (spot No. S10), a phosphotransferase of fructose, was only expressed in the winter-collected tubers.

There should be certain reasons to support the abundant expression of metabolism-related proteins in winter-collected samples. Firstly, it might result from the osmotic adjustments within the cells during winter. The accumulation of carbohydrates and some solutes changes the osmotic potential of the cell and, therefore, decreases the difference in water potential between the ice formed in the apoplastic space and the solution within the cell. However, the situation in Thailand seems impossible because the temperature is not cold enough to initiate an ice formed. Secondly, certain proteins might be involved in plant dormancy or sprouting, for complex carbohydrate anabolism in the tuber during dormancy and then their catabolism during sprouting, as previously reported or the rhizomes of *Curcuma longa*. Thirdly, it is possible that the plant was relied on these enzymes to produce ATP for shedding their leaves during winter. Moreover, fructokinase is also found in plants grown at low temperatures, and contributes to starch metabolism, stem and root growth and seed development.

Some certain proteins such as ribulose bisphosphate carboxylase (RuBisCO; spot No. 33) expressed lower levels during winter. RuBisCO which is found to be abundantly expressed in the summer and rainy season (Table 2), is involved in the Calvin cycle. Some other proteins such as deoxyxuridine triphosphatase (dUTPase; EC 3.6.1.23; spot S1 in Fig. 4), which converts dUTP to deoxyxuridine monophosphate and pyrophosphate and is essential for plant sprouting, expressed at higher levels during summer. dUTPase was reported to be expressed 1 week before visible sprouting occurred and its expression could be used as a marker for the end of potato tuber dormancy.
## Table 2: Ascribed GO functions of the identified proteins in *P. mirifica* tubers, as analyzed by LC-MS/MS, and their relative seasonal abundance compared to tubers harvested in the winter.

| Spot No. | Accession number | Description | MW/pI | Score | Winter | Summer | Rainy | Comparison between winter (fold) |
|----------|------------------|-------------|-------|-------|--------|--------|-------|-------------------------------|
| **Cell structure** | | | | | | | | |
| 2 | gi|356535919 | 3-ketoacyl-CoA synthase 11-like [*Glycine max*] | 58.04/9.40 | 21 | 0.10 | +1.20 | −1.70 |
| 83 | gi|356585878 | Actin-101-like [*Glycine max*] | 41.60/5.31 | 867 | 0.28 | −1.98 | +1.94 |
| 102 | gi|84028521 | Actin depolymerizing factor-like protein [*Arachis hypogaea*] | 16.08/6.15 | 152 | 1.95 | −6.70 | −3.70 |
| **Defense** | | | | | | | | |
| 23 | gi|7547630 | Pathogen- and wound-inducible antifungal protein CBP20 precursor [*Nicotiana tabacum*] | 22.15/8.38 | 49 | 1.36 | −1.38 | −1.10 |
| 27 | gi|356539366 | Superoxide dismutase [Cu-Zn], chloroplastic-like [*Glycine max*] | 20.88/6.03 | 38 | 0.24 | +5.74 | +3.90 |
| 48 | gi|15237409 | TIR-NBS-LRR class disease resistance protein [*Arabidopsis thaliana*] | 137.39/7.71 | 26 | 0.19 | +1.21 | −1.27 |
| 145 | gi|130829 | Pathogenesis-related protein 1 (PvPR1) | 16.52/4.83 | 264 | 16.32 | +1.32 | +1.26 |
| **Metabolism** | | | | | | | | |
| 30 | gi|255088257 | Fructose-1,6-bisphosphatase [*Micromonas sp. RCC299*] | 39.47/5.79 | 64 | 5.13 | −4.59 | −3.31 |
| 32 | gi|387135324 | UDP-glycosyltransferase 1 [Linum usitatissimum] (UGT) | 52.81/5.83 | 63 | 1.14 | +5.44 | +2.82 |
| 33 | gi|131933 | Ribulose bisphosphate carboxylase; RuBisCO large subunit | 20.88/6.03 | 28 | 0.38 | +6.19 | +3.79 |
| 49 | gi|15237409 | TIR-NBS-LRR class disease resistance protein [*Arabidopsis thaliana*] | 137.39/7.71 | 26 | 0.19 | +1.21 | −1.27 |
| 127 | gi|35670259 | Lysosomal alpha-mannosidase-like [*Glycine max*] | 115.53/5.84 | 639 | 0.26 | −5.30 | −3.77 |
| 136 | gi|4731376 | Isoflavone reductase homolog Bet v 6.0101 (*Betula pendula*) (IFR) | 33.13/7.82 | 65 | 0.10 | −2.24 | −2.50 |
| **Protein synthesis** | | | | | | | | |
| 3 | gi|356496249 | Proteasome subunit alpha type-4-like isofrom 1 [*Glycine max*] | 27.29/5.96 | 270 | 0.35 | +1.59 | +1.13 |
| 8 | gi|356516563 | Elongation factor 1-delta-like [*Glycine max*] | 25.02/4.42 | 113 | 0.11 | −1.09 | −2.77 |
| 43 | gi|345450064 | Ribosomal protein S4 [*Delosperma napiforme*] | 20.06/10.33 | 30 | 0.14 | +1.36 | +1.36 |
| **Storage** | | | | | | | | |
| 69 | gi|156630208 | Avenin-like proteins [*Amblyopyrum muticum*] | 32.58/7.83 | 52 | 0.32 | −1.81 | −2.33 |
| **Stress** | | | | | | | | |
| 31 | gi|357140899 | Glutathione S-transferase GSTU6-like [*Brachypodium distachyon*] | 25.79/6.12 | 50 | 13.53 | −2.06 | −1.89 |
| 91 | gi|356595973 | Glutathione reductase, cytosolic-like [*Glycine max*] | 53.96/5.63 | 866 | 0.18 | −1.18 | −1.25 |
| 93 | gi|356767411 | Chaperonin CPN60–2, mitochondrial-like isoform 1 [*Glycine max*] | 16.52/4.83 | 755 | 0.19 | −2.94 | −2.89 |
| 97 | gi|38325811 | Heat shock protein 70–1 [*Nicotiana tabacum*] | 70.97/5.02 | 197 | 1.48 | −2.21 | −1.90 |
| **Transportation** | | | | | | | | |
| 52 | gi|384252728 | E set domain-containing protein [*Coccomyxa subellipsoidea C-169*] | 70.25/8.30 | 27 | 0.22 | −1.04 | −1.54 |
| 109 | gi|356543209 | Patellin-5-like [*Glycine max*] | 69.52/4.87 | 592 | 1.14 | −1.08 | +1.09 |
| 146 | gi|308803158 | ABC transporter, putative (ISS) [*Ostreococcus tauri*] | 71.11/7.83 | 45 | 0.58 | −1.43 | −2.27 |
| **Unknown** | | | | | | | | |
| 41 | gi|351734454 | Uncharacterized protein LOC100006148 [*Glycine max*] | 24.53/6.82 | 411 | 0.25 | −2.18 | −2.88 |
| 55 | gi|242071029 | Hypothetical protein SORBIDRAFT_05g018540 [*Sorghum bicolor*] | 179.12/8.41 | 21 | 0.19 | −2.09 | −3.88 |

+ = higher (fold); = lower (fold) than that in winter.
Protein synthesis. The total 30 of the identified *P. mirifica* tuber proteins were classified by GO annotation to be involved in protein synthesis (Table 2), mostly belong to the proteosome subunit alpha type. The protein exhibits ability to cleave peptide bonds with Arg, Phe, Tyr, Leu, and Glu adjacent to the leaving group at a neutral or slightly basic pH. Two ribosomal proteins, S4 (spot No. 43) and 60S (spots No. S12 and S13), were found and are known to be involved in peptide bond formation and prevention of hydrolysis of the premature polypeptide, thus provide a binding site for G-protein factors (assists initiation, elongation, and termination) and associate in protein folding. Elongation factor 1 (spots No. 4, 7, and 8) was also found in the tuber proteome and is known to be involved in protein synthesis at the ribosome. Serine hydroxymethyltransferase (spots No. S6, S12, and S13) catalyzes the reversible, simultaneous conversion of L-serine to glycine and tetrahydrofolate to 5,10-methylenetetrahydrofolate (hydrolysis).[^50]

Storage. Two storage proteins, avenin-like protein (spot No. 69) and glutelin type-A 2-like (spot No. S10) were detected. Avenin-like protein is a member of the small family of storage proteins in wheat,[^51] while glutelin type-A 2-like was mainly found in rice grains,[^52] and only expressed in winter (Table 2).

Stress response. Abiotic stress (such as salinity, drought, chilling, heavily metal) is one of the limiting factors for plant growth and production. Under stress conditions, reactive oxygen species (ROS) are overproduced to protect the cells from oxidative damage. In terms of the ascribed stress response proteins, 24 proteins were identified in the *P. mirifica* tuber proteome in which GST was the most found abundant protein. The enzyme functions in stress tolerance enroll in cell signaling.[^28] The second most abundant protein in *P. mirifica* tubers was GR (spots No. 15, 91, and 139) which is known to play a significant role in protecting cells against ROS and its reaction products-accrued potential anomalies. Both GST and GR play a potential role in detoxification of xenobiotic and endogenous compounds.[^51][^53]

Heat stress increases membrane damage and impairs metabolic functions by enhancing the risk of improper protein folding and denaturation of intracellular protein and membrane complexes,[^54] and so plants need to activate proper defense systems to survive under heat stress.[^18] One of the key factors for heat tolerance is the induction of heat shock proteins. Heat shock proteins and chaperonin also play a role in stress response by preventing aggregation and stabilizing non-native unfolded proteins or insoluble proteins under environmental stresses.[^14] In this study, heat shock proteins (spots No. 97, 103, and 115) and chaperonin (spots No. 93, 113, and 114) were found to be expressed at the highest level in the winter-collected tubers, while the underground temperature is more stable than in other seasons. Therefore, the plant may possibly transfer these proteins from the leaves to the tubers for storage.

Transportation. There were 17 identified proteins in *P. mirifica* tuber proteome that were classified as functionally GO annotated and were most likely to be involved in transportation. They were mostly E-set domain containing proteins (spots No. 52, 53, and 90), patellin-5 (spots No. 74, 85, 109, 139, and S7) and ABC transporters (spots No. 146, S2, and S8). The E-set containing and patellin-5 proteins are responsible for ion transportation, whereas the ABC transporters translocate larger substrates across cell membranes.[^55]

Unknown function proteins. However, there were 21 unidentified proteins or hypothetical GO-annotated functional classification, such as uncharacterized protein LOC100306148 (spot No. 41) and hypothetical protein SORBIDRAFT_05g018540(spot No. 55).

Conclusion

The proteomic study of *P. mirifica* tubers was enlightening into key proteins or enzymes related to isoflavonoid synthesis and transformation, including glycosylation/deglycosylation of isoflavonoids in *P. mirifica* tubers. This study revealed an interesting potential proteome variance pattern among different season-collected plant samples. The identified proteins were successively classified by GO functional classification into eight classes in which proteins involve in metabolism, which mostly comprised of carbohydrate metabolism, becoming the largest class, especially in the summer-collected tubers. For the proteins involved in plant secondary metabolites, the seasonal changes in isoflavonoid contents were correlated with changes in the expression level of isoflavonoid synthesis-associated

### Table 3. The correlation of isoflavonoid and protein involved in isoflavonoid biosynthetic pathway in *P. mirifica* tuber.

| Protein name                  | Quality | Quantity | Related with | Evaluation | Quality | Quantity |
|------------------------------|---------|----------|--------------|------------|---------|----------|
| Glycoside synthesis protein  | UGT     | High     | Puerarin     | *          | High    | Medium   |
| Aglycoside synthesis protein | IFR     | Very low | Daidzin      | *          | Low     | Low      |
| Non-glycoside synthesis protein | CHI    | Low      | Genistein    | *          | Low     | Very low |
proteins. Some of the proteins involved in isoflavonoid biosynthesis were found in the tuber proteome, including CHL, IFR, UGT, and cytochrome p450 (IFS). This study will lead to the answer of how the plant tubers produced proteins that are necessary for the production and accumulated of tuberous secondary metabolites, especially isoflavonoids. Most important, not only the tuberous tissue specific regulation of the relevant enzymes but also the harvesting season of the tubers may lead to a successive harvest of isoflavonoid-rich tubers from *P. mirifica* for dietary supplement or pharmaceutical product raw materials.

**Author contribution**

Conceived and designed the experiments: Wichai Cherdshewasart, Jutarmas Jungskukharoen, Daranee Chokchaichammankit, Chantragan Srisomsap and Polkit Sangvanich. Performed the experiments: Jutarmas Jungskukharoen. Analyzed the data: Jutarmas Jungskukharoen, Chantragan Srisomsap and Polkit Sangvanich. Contributed reagents/materials/analysis tools: Jutarmas Jungskukharoen and Daranee Chokchaichammankit. Wrote the paper: Wichai Cherdshewasart, Jutarmas Jungskukharoen, Daranee Chokchaichammankit, Chantragan Srisomsap and Polkit Sangvanich.

**Acknowledgment**

We acknowledge the Laboratory of Biochemistry, Chulabhorn Research Institute, for facilities.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Funding**

This work was supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission [grant number FW 0663]; The Thailand Research Fund [grant number DBG 5180025]; The Science for Locale Project under Chulalongkorn University Centenary Academic Development Plan [grant number 2008–2012].

**Supplemental material**

Supplemental material for this article can be accessed at [http://dx.doi.org/10.1080/09168451.2016.1141035](http://dx.doi.org/10.1080/09168451.2016.1141035).

**References**

[1] Suntara A. The remedy pamphlet of Kwao Krua tuber of Luang Anusarnsuntara kromkarnphiset. Chiang Mai, Thailand: Chiang Mai Upatiponga Press; 1931.

[2] Jones HE, Pope GS. A study of the action of miroestrol and other oestrogens on the reproductive tract of the immature female mouse. J. Endocrinol. 1960;20:229–235.

[3] Cherdshewasart W, Cheewasopit W, Picha P. The differential anti-proliferation effect of white (*Pueraria mirifica*), red (*Butea superba*), and black (*Mucuna collitettii*) Kwao Krua plants on the growth of MCF-7 cells. J. Etnopharmacol. 2004;93:255–260.

[4] Cherdshewasart W, Sujit W. Correlation of antioxidant activity and major isoflavonoid contents of the phytoestrogen-rich *Pueraria mirifica* and *Pueraria lobata* tubers. Phytomedicine. 2008;15:38–43.

[5] Cherdshewasart W, Traisup V, Picha P. Determination of the estrogenic activity of wild phytoestrogen-rich *Pueraria mirifica* by MCF-7 proliferation assay. J. Reprod. Dev. 2008;54:63–67.

[6] Cherdshewasart W, Kitsamai Y, Malaiwijitpon D. Evaluation of the estrogenic activity of the wild *Pueraria mirifica* by vaginal cornification assay. J. Reprod. Dev. 2007;53:385–393.

[7] Cherdshewasart W, Srivatcharachukul S, Malaiwijitpon D. Variance of estrogenic activity of the phytoestrogen-rich plant. Maturitas. 2008;61:350–357.

[8] Sheoran JS, Ross ARS, Olson DH, et al. Compatibility of plant protein extraction methods with mass spectrometry for proteome analysis. Plant Sci. 2009;176:99–104.

[9] Natarajan S, Xu C, Caperna TJ, et al. Comparison of protein solubilization methods suitable for proteomic analysis of soybean seed proteins. Anal. Biochem. 2005;342:214–220.

[10] Natarajan SS, Xu C, Bae H, et al. Characterization of storage proteins in wild (*Glycine soja*) and cultivated (*Glycine max*) soybean seeds using proteomic analysis. J. Agric. Food. Chem. 2006;54:3114–3120.

[11] Komatsu S, Ahsan N. Soybean proteomes and its application to functional analysis. J. Proteomics. 2009;72:325–336.

[12] Fa shadow. A study of the action of miroestrol and other oestrogens on the reproductive tract of the immature female mouse. J. Endocrinol. 1960;20:229–235.

[13] Owiti J, Grossmann J, Gehrig P, et al. iTRAQ-based analysis of the osaava root proteome reveals pathways associated with post-harvest physiological deterioration. Plant J. 2011;67:145–156.

[14] Chokchaichammankit D, Subhasitanont P, Paricharttanakul NM, et al. Proteomic alteration during dormant period of *Curcuma longa* rhizomes. J. Proteomics Bioinf. 2009;02:380–387.

[15] Niu N, Cao Y, Duan W, et al. Proteomic analysis of grape berry skin responding to sunlight exclusion. J. Plant Physiol. 2013;170:748–757.

[16] Yu JW, Choi JS, Upadhaya CP, et al. Dynamic proteomic profile of potato tuber during its in vitro development. Plant Sci. 2012;195:1–9.

[17] Louarn S, Nawrocki A, Edelenbos M, et al. The influence of the fungal pathogen *Mycosporospora acerina* on the proteome and polycyclotenes and 6-methoxymellein in organic and conventionalically cultivated carrots (*Daucus carota*) during post harvest storage. J. Proteomics. 2012;75:962–977.

[18] Hashiguchi A, Ahsan N, Komatsu S. Proteomics application of crops in the context of climatic changes. Food Res. Int. 2010;43:1803–1813.

[19] Wang L, Ma H, Song L, et al. comparative proteomics analysis reveals the mechanism of pre-harvest seed deterioration of soybean under high temperature and humidity stress. J. Proteomics. 2012;75:2109–2127.

[20] Thakur P, Kumar S, Malik JA, et al. Cold stress effects on reproductive development in grain crops: an overview. Environ. Exp. Bot. 2010;67:429–443.

[21] Timperio AM, Egidi MG, Zolla L. Proteomics applied on plant abiotic stresses: role of heat shock proteins (HSP). J. Proteomics. 2008;71:391–411.

[22] Nieuwelink J. AD10E cultivation of soya and other legumes. Wageningen: Agromissa; 2005.

[23] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 1976;72:248–254.

[24] Bhardwaj J, Yadav SK. A common protein extraction protocol for proteomic analysis: horse gram a case study. Am. J. Agric. Biol. Sci. 2013;8:293–301.

[25] Thillement H. Plant proteomics: methods and protocols. Totowa (NJ): Humana Press; 2007.
Ponstein AS, Lee kJ. Post-translational modifications and their biological functions: proteomic analysis and systematic approaches. J. Biochem. Mol. Biol. 2004;37:35–44.

Pavarini DP, Pavarini SP, Niehues M, et al. Exogenous influences on plant secondary metabolite levels. Anim. Feed Sci. Technol. 2012;176:5–16.

Dixon DP, Lapthorn A, Edwards R. Plant glutathione transferases. Genome Biol. 2002;3:REVIEWS3004.

Cummins I, Cole DJ, Edwards R. A role for glutathione transferases functioning as glutathione peroxidases in resistance to multiple herbicides in black-grass. Plant J. 1999;18:285–292.

Rao AV, Reddy A. Sulfur assimilation and abiotic stress in plants. In: Khan N, Singh S, Umar S, editors. Berlin: Heidelberg: Springer; 2008. p. 111–147.

Yu O, Shi J, Hession AO, et al. Metabolic engineering to increase isoflavone biosynthesis in soybean seed. Phytochemistry. 2003;63:753–763.

Shimada N, Akashi T, Aoki T, et al. Induction of isoflavonoid pathway in the model legume Lotus japonicus: molecular characterization of enzymes involved in phytoalexin biosynthesis. Plant Sci. 2000;160:37–47.

Versteyss BG, Töth J. Keeping uracil out of DNA: physiological role, structure and catalytic mechanism of dUTPases. Acc. Chem. Res. 2009;42:97–106.

Gachon CM, Langlois-Meurinne M, Saindrenan P. Plant secondary metabolism glycosyltransferases: the emerging functional analysis. Trends Plant Sci. 2005;10:542–549.

Cherdshewasart W, Sriwatcharuk S. Major isoflavonoid contents of the 1-year-cultivated phytoestrogen-rich herb, pueraia mirifica. Biosci. Biotechnol. Biochem. 2007;71:2527–2533.

Ouellet F, Carpentier E, Cope MJ, et al. Regulation of a wheat actin-depolymerizing factor during cold acclimation. Plant Physiol. 2001;125:360–368.

Schreiber L. Effect of temperature on cuticular transpiration of isolated cuticular membranes and leaf discs. J. Exp. Bot. 2001;52:1893–1900.

Martin GB, Begdanove AJ, Sessa G. Understanding the functions of plant disease resistance proteins. Annu. Rev. Plant Biol. 2003;54:23–61.

Heath MC. Nonhost resistance and nonspecific plant defenses. Curr. Opin. Plant Biol. 2000;3:315–319.

Ponstein AS, Bres-Vloemans SA, Sela-Buurlage MB, et al. A novel pathogen – and wound-inducible tobacco (Nicotiana tabacum) protein with antifungal activity. Plant Physiol. 1994;104:109–118.