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Proteomic analysis of Chinese kale (*B. alboglabra*) leaves during growth

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ABSTRACT

Brassica alboglabra (Chinese kale) is a vegetable extensively grown in Thailand, which has high nutritional value and useful phytochemicals. Farmers generally harvest *B. alboglabra* starting from the fifth week of growth to sell in the market. In this study, changes in protein expression during growth and development of *B. alboglabra* were investigated. Proteins were extracted from two to eight-week leaves, and a total of 334 protein spots separated by two-dimensional gel electrophoresis and selected 103 spots were digested and analyzed by using LC-MS/MS. The identified proteins could be classified into nine classes, namely proteins involved in photosynthesis and photorespiration, amino acid metabolism, carbon-compound and carbohydrate metabolism, protein metabolism, stress response, cellular communication and signal transduction, glycolysis and gluconeogenesis, unidentified and others. The highest number of proteins was the proteins involved in photosynthesis and photorespiration, presumably because leaves are the primary sites for photosynthesis and photorespiration, so there is an induction of proteins such as ribulose bisphosphate carboxylase and ribulose bisphosphate carboxylase activase. This is the first study to investigate protein expression in *B. alboglabra* leaves during growth and development. The studies provide information for protein database in this plant species.

Keywords: Proteomic; Chinese kale; *Brassica alboglabra*; Leaves; Growth.

1. Introduction

Plants are unique in possessing the ability to generate own energy from photosynthesis and carbon fixation, which eventually provide food and oxygen to the other organisms including humans (Micol, 2009). Leaves contain chlorophyll, a molecule which acts as a photoreceptor to trap sunlight for the production of oxygen and simple sugars, and has been suggested to have anti-mutagenic and anti-carcinogenic properties, as well as protective properties against chronic diseases such as coronary heart diseases, diabetes, and cataracts (Ma and Dolphin, 1999). Besides, photosynthesis provides the carbon skeleton for the plant metabolic pathways to support the plant growth and development.

The "Omics" era enables researchers to study plant development in-depth by using novel tools such as transcriptomics, proteomics, metabolomics and modelling (Hennig, 2007). Proteomic studies allow the detection and identification of proteins expressed, leading to the discovery of genes and pathways involved in stress responsiveness and tolerance (Salekdeh *et al*, 2007).

Several studies on proteomic analyses related to plant growth and development processes have been reported, including rice, soybean, wheat, barley, maize, and Arabidopsis (Nozu *et al*. 2006, Mooney *et al*. 2006, Salekdeh *et al*. 2007). Proteomics is a sensitive tool for molecular studies geared towards the identification and characterization of genes responsible for regulating key plant processes and traits. Proteomic studies of plants have been extensive, since entire genome of the reference plant species *Arabidopsis thaliana* has been sequenced. *Arabidopsis thaliana*, in the family of Brassicaceae genus, has been used as a model organism in molecular biology. Plant proteomics studied in several food crops such as rice (Goff, 2002) and wheat (Ven-

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sel *et al*. 2005), vegetables such as tomatoes (Chen, 1999) and peas (Kav, 2005), and medicinal plants such as ginseng (Lum *et al*. 2002). Proteomics also serves as a modern approach to ascertain the protein alterations due to stress or the response of tolerance to stress environment (Salekdeh *et al*. 2007).

Chinese kale or Chinese broccoli (*Brassica alboglabra*) is one of the most commonly grown leafy vegetables in the Southeast Asian region and Thailand, as it tolerates the tropical weather, in particular which makes it suitable to be grown throughout the year (Issarakraisila *et al*. 2007). Chinese kale is categorized under the *Brassica oleracea* group, the same species with broccoli and curly kale. Its edible parts consist of waxy, broad and dark green leaves and thick and crunchy stems. The slightly bitter and crunchy taste, with high nutritional value includes protein, calcium, iron, vitamins, and fiber, have made Chinese kale a favorite among the vegetables in the region. Studies have revealed that Brassica vegetables contain phytochemicals such as glucosinolates to fight against cancer (van Poppel *et al*. 1999, Talalay *et al*. 2001, La *et al*. 2009). Chinese kale is harvested when it is still immature starting from the fifth week and can be stored for about 7-10 days at 0ºC, with relative humidity 95-100% (Sukontasing, 2009).

Despite its' popularity and nutritional value, proteomics of Chinese kale has not yet been established. Therefore this study of the protein alterations in *Brassica alboglabra* leaves at two to eight weeks of growth provides a proteomic database on this plant species and allows comparison of the protein changes over time.

2. Material and methods

2.1 Plant materials

The seeds for the *B. alboglabra* were obtained from a local seed company. The seeds for the *B. alboglabra* were grown in a pot of 8 cm in diameter filled with decomposed soil under the open environment at Chulabhorn Research Institute compound. The plants were supplied with organic and chemical fertilizers only on day 0. The plants were harvested in the morning prior to extraction, and the leaves were cut and weighed before subjecting to protein extraction.

2.2 Time course study

The plants were grown with no other treatment except water and harvested on the second, third, fourth, fifth, sixth, seventh, and eighth weeks of growth. Photographs were taken at every point of time. Plants were harvested for leaves for protein analysis and identification. The time course study was performed twice. The protein analysis from leaves were subjected onto two-dimensional electrophoresis 3-5 sets.

2.3 TCA/ acetone precipitation

0.1 g plant samples were ground in liquid nitrogen and resuspended in 1 mL solution of 10% TCA and 0.07% DTT in cold acetone. The samples were incubated at 20^0C for 1 hour, followed by centrifugation at 14 000 rpm for 10 minutes. The supernatant was discarded, and the pellet was resuspended in 1 mL of 0.07% DTT in cold acetone, incubated and centrifuged as previously described. This step was repeated twice. Then, the pellet was vacuum-dried for 30 minutes and resuspended in 500 µL of rehydration buffer consisted of 8 M urea, 2% CHAPS, 40 mM DTT, 0.2% ampholytes 3-10, and 2 mM tributylphosphine (TBP). The samples were then vigorously vortexed for 5 minutes and incubated at 4 ^{0}C overnight. The next day, the samples were centrifuged as previously described. The supernatant was collected for protein determination by Bradford assay.

2.4 Two-dimensional electrophoresis

The two-dimensional electrophoresis was performed using Immobiline™ DryStrip pH 3-10 non-linear, 7 cm (GE Healthcare). The 125 µL solution containing 150 µg of protein and rehydration buffer was loaded and kept at room temperature overnight. The first IEF was conducted at 7,000 Vhrs, 55 µA/strip and run for 6 hours. Prior to the second dimension SDS-PAGE gels, the strips were soaked with equilibration buffer 1 (0.5 M Tris-HCl pH 6.8, 6 M urea, 30% glycerol, 1% SDS, and 1% DTT) with gentle agitation at room temperature for 10 minutes followed by soaking in equilibration buffer 2 (0.5 M Tris-HCl pH 6.8, 6 M urea, 30% glycerol, 1% SDS, and 2.5% iodoacetamide). The IPG strips were embedded within molten agarose directly on top of a 1 mm 12.5 % SDS-PAGE gel. The SDS-PAGE was performed at 12 mA for 3 hours.

2.5 Protein staining with Coomassie blue R-250

The gels were stained with 0.1% Coomassie blue R-250, 40% methanol and 10% acetic acid overnight, and then destained with 40% methanol and 5% acetic acid for 3 hours, followed by 10% of methanol and 5% acetic acid for 2 hours.

2.6 Gel Scanning and Image analyses

The gels were scanned by an ImageScanner II (GE Healthcare, Uppsala, Sweden). The images were analysed with the Image Master 2D Platinum 6.0 (GE Healthcare) by matching and comparing the different percent volume (% Volume) of protein spots. A master gel was selected from the gel with the highest number of spots. Significant change refers to ≥2 fold change of percent volume. Descriptive data were analyzed using Graphpad Prism 5 Software to obtain Means ± SD.

2.7 Protein identification by LC/MS/MS

The spots from the 2-DE gels were excised with a clean scalpel into 1 mm X 1 mm cubes and transferred to a 0.5 ml microfuge tube followed by washing 2 times with RO water. The samples underwent the steps of destaining with 0.1M NH_4HCO_3/50% acetonitrile, reduction of disulphide bonds with 0.1M NH_4HCO_3/10mM DTT/1mM EDTA, alkylation with 100mM iodoacetamide/0.1M NH_4HCO_3 and digestion with trypsin. The LC/MS/MS analyses were carried out using

a capillary LC system (Waters) coupled to a Q-TOF mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray ion-source working in the nanoelectrospray mode. Glu-fibrinopeptide was used to calibrate the instrument in MS/MS mode. The tryptic peptides were concentrated and desalted on a 75 µm ID X 150 mm C18 PepMap column (LC Packings, Amsterdam, Netherlands). Eluents A and B were 0.1 % formic acid in 97% water, 3% acetonitrile and 0.1% formic acid in 97% acetonitrile respectively. Six µl sample was injected into the nanoLC system, and separation was performed using the following gradient: 0 min 7% B, 35 min 50% B, 45 min 80% B, 49 min 80% B, 50 min 7% B, 60 min 7% B. The database search from SWISS-PROT and NCBI was performed with ProteinLynx,. The Mascot search tool available on the Matrix Science site screening (http://www.matrixscience.com) was used for some proteins which were not available in previous database. Protein function was obtained from UniProtKB website.

3. Results and Discussion

In this study, *B. alboglabra* seeds were grown and plants were harvested from two weeks until eight weeks. The growth and development were observed from the first week (seedling) to eighth week. There were changes in the growth observed from the seedling to the eighth week as shown in Figure 1. At the seedling stage, plants possessed only cotyledons and true leaves were observed starting from the second week of harvest. The leaves began maturing at the third week of growth with waxy, dark green surface appearances observed, while the stems gradually became hard throughout the time course. The cotyledons began to wilt at the seventh week of growth.

Plants from two to eight weeks were harvested in the morning, leaves were cut and proteins were extracted for 2-DE analyses. In the first dimension, proteins (150 µg) were separated by using IEF strips of pH 3-10, followed by the second dimension of 12.5 % SDS PAGE and stained with Coomassie blue. The gels of the second to the eighth week leaves were scanned and shown in Figure 2. From the second to the eighth week of 2-DE protein patterns, the lowest number of proteins was observed in the second week and began to increase towards the sixth week and decreased at the seventh and eighth weeks. This may indicate increase of protein expression during the growth and development of the *B. alboglabra* from the second to the sixth week and decline starting the seventh week. There may also be morphological changes of the leaves which became thicker, so that protein extraction was more difficult.

Since the sixth week gel gave the highest number of spots, the database for *B. alboglabra* leaves was established by using the protein pattern at the sixth week, as shown in Figure 3. A total of 103 protein spots were detected by using Image Master Software analysis. Protein spots were cut, digested and analysed by LC/MS/MS for protein identification. Peptides were searched by using the protein database search tool which includes ProteinLynx, SWISS-PROT, NCBI and Mascot. Proteins identified in *B. alboglabra* leaves are listed as Table in Supplementary material.

The 2-DE protein patterns from leaves at the second to the eighth week were matched by using Image Master Software and compared with the protein map from the sixth week leaves. The proteins from the second to the eighth week were classified into different functional classes as shown in Figure 4.

Protein functions were identified by using UniProtKB, and classified into nine classes consisting of photosynthesis and photorespiration, amino acid metabolism, carbon-compound and carbohydrate metabolism, protein metabolism, stress response, cellular communication and signal transduction, glycolysis and gluconeogenesis, unidentified and other

Figure 1. The development and growth of B. alboglabra observed at one to eight weeks. A: 1 week; B: 2 weeks; C: 3 weeks; D: 4 weeks; E: 5 weeks; F: 6 weeks; G: 7 weeks; H: 8 weeks using the same scale. The scale referred is 1 inch.

unknown proteins. The unidentified proteins refer to proteins with other functions and unknown refer to proteins with unknown function.

From the graph, similar patterns were observed in all classes with the lowest number of protein spots in the second week and increasing gradually to the sixth week, which had the highest number of protein spots, presumably due to the metabolism needed to support growth and development of the plants. The number of protein spots decreased starting the seventh week, possibly due to the plants becoming thicker making protein harder to extract, as they reached senescent stage.

The photosynthesis and photorespiration class showed the highest number of proteins in the leaves, followed by unknown proteins, protein metabolism, glycolysis and gluconeogenesis, stress response, amino acid metabolism, carbon compound and carbohydrate metabolism, cellular communication and signal transduction and unclassified proteins.

Proteins involved in photosynthesis and photorespiration were mainly oxygen evolving proteins, photosystem proteins, ribulose bisphosphate carboxylase/oxygenase and rubisco activase. These proteins play vital roles in photosynthesis and photorespiration required to provide energy and substrates for other pathways.

The selected protein spots in *B. alboglabra* leaves from the second to the eighth week of protein expressions are shown in Figure 5. The five listed proteins were involved in photosynthesis and photorespiration, namely oxygen evolving enhancer protein 2 chloroplastic, photosystem II protein, ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, ribulose-1,5 bisphosphate activase and rubisco activase. These proteins showed increasing patterns from second to the sixth weeks and gradually decrease from the seventh to the eighth week.

4. Discussion

The protein identification of *B. alboglabra* has not yet been established, and this study would provide a baseline for the assessment of plant stress, tolerance and changes during plant growth and development. The vegetable, like other higher plants, possesses many unique pathways which include photosynthesis to provide energy for its growth (Bryce, 2003). Plant proteomics has been intensely studied since the availability of the full 2-DE mapping of the reference plant, *Arabidopsis thaliana*. Since then, progress has been made towards identifying and cataloguing proteins from various plants which include rice, soybean, barley and maize corresponding to their developmental processes (Salekdeh *et al.* 2007). There is no reported proteomic study of *B. alboglabra*, so far. In this study, the protein database was established as a reference gel from sixth week *B. alboglabra* leaves.

In the time course study, the seeds were grown on soil supplied with organic and chemical fertilizers with no other treatment except for water and harvested from two to eight weeks. The plants were harvested in the morning to minimize the possibility of protein changes. The proteins from leaves

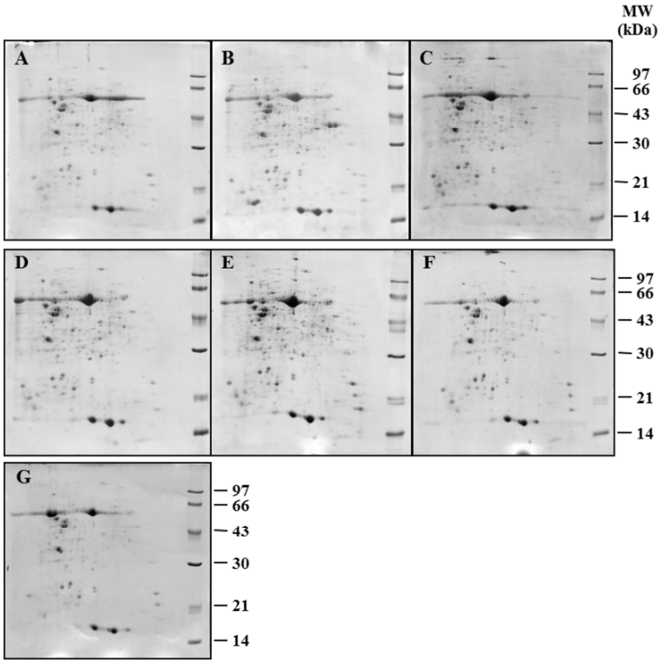

Figure 2. Two-dimensional PAGE of B. alboglabra (A-F) at different stages of growth. A: 2 weeks; B: 3 weeks; C: 4 weeks; D: 5 weeks; E: 6 weeks; F: 7 weeks; G: 8 weeks. Proteins (150 µg) were separated by using non-linear IEF strips of pH 3-10, followed by 12.5 % SDS PAGE and stained with Coomassie blue.

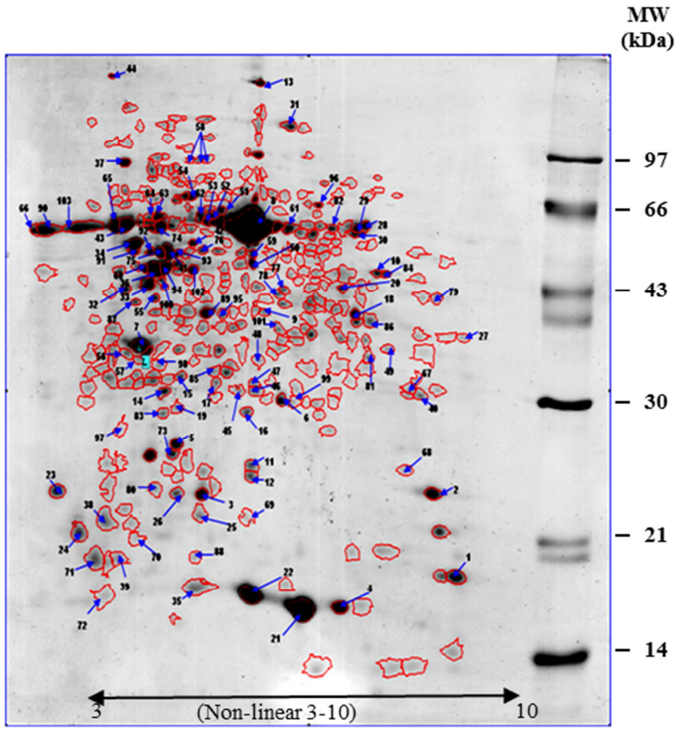

Figure 3. Protein map from B. alboglabra leaves at 6 weeks detected by using the Image Master Software. A total of 103 protein spots were identified from the database search performed with ProteinLynx screening SWISS-PROT, NCBI and the Mascot search tool available on the Matrix Science site screening.

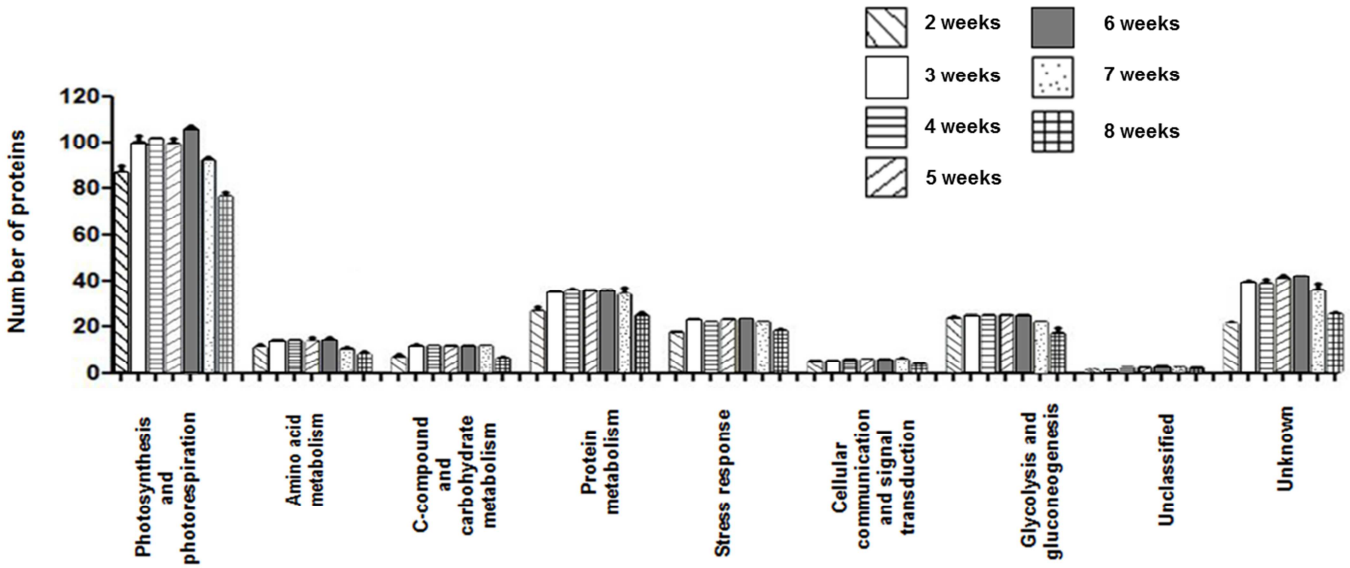

Figure 4. Protein spots of B. alboglabra leaves from two to eight weeks were grouped in nine functional classes, according to the main functions listed in UniProt (www.uniprot.org).

were identified and categorized into nine groups consisting of photosynthesis and photorespiration, amino acid metabolism, carbon-compound and carbohydrate metabolism, protein metabolism, stress response, cellular communication and signal transduction, glycolysis and gluconeogenesis, unidentified proteins and other proteins which are unclassified. The proteins showed almost similar increasing patterns from two to six weeks and began to decrease at seven weeks.

From the functional classification, photosynthesis and photorespiration proteins were dominantly found in the leaves. The proteins involved in photosynthesis and photorespiration were mainly from rubisco family, photosystem subunits oxygen evolving proteins and ATP synthase. During the development, the stems and leaves increased in size, and therefore plants require energy to promote biosynthesis and growth by the process of photosynthesis and photorespiration. The final products of photosynthesis are sucrose and starch which will be oxidized and enter pathways of proteins and cell wall synthesis (Bryce, 2003). Rubisco is the most abundant protein found in the leaves where large amounts of Rubisco are required to support photosynthesis (Good, 2003).

Oxidative stress has been proposed to play a developmental role in plants. Heat shock proteins and chaperonins are involved in stabilizing proteins folding in different kind of stimuli (Negri *et al*., 2008). Proteins involved in amino acid metabolism includes aminotransferases play roles in transporting nitrogen during development of cotyledons of germinating seeds to expand root, shoots and leaves (Lea, 2003).

Glycolysis and gluconeogenesis are the processes of breaking down and synthesizing sucrose or reducing sugars to provide energy and carbon skeletons for plant development, respectively (Bryce, 2003). The carbon metabolism of leaves is a light-independent process for carbon fixation to produce 3

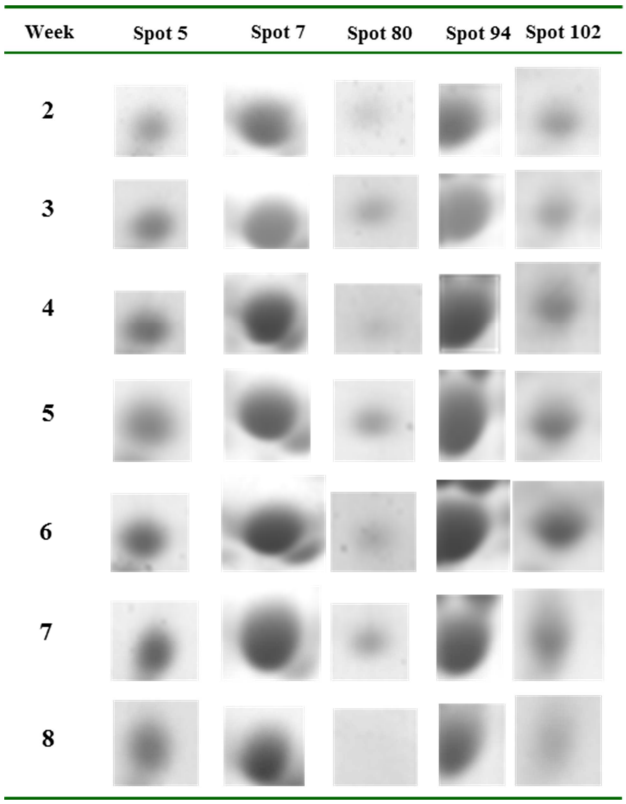

Figure 5. Five selected protein spots involved in photosynthesis and photorespiration from the second to the eighth week of B. alboglabra leaves. Spot 5 = Oxygen evolving enhancer protein 2 chloroplastic; Spot 7 = Oxygen evolving enhancer protein 1, photosystem II protein; Spot 80 = Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit; Spot 94 = Ribulose bisphosphate carboxylase/ oxygenase activase; Spot 102= Rubisco activase

carbon sugars, unlike photosynthesis product of 6 carbon sugars. The 3 carbon sugars are converted to produce larger carbohydrates (Good, 2003). The skeleton of carbohydrates is used as the carbon source and energy such as cellulose to provide support to the plant the same way chitin does to insects. The major difference between carbohydrate metabolism in plants and animal is the synthesis of cell wall in plants which composed of mixture of complex polysaccharides (Smith, 2003).

Other proteins characterized were involved in protein synthesis and transcription grouped under protein metabolism, and proteins involved in signal transduction.

5. Concluding remarks

Proteomic studies of *B. alboglabra* have not yet been reported. Since this is the first proteomic study of *B. alboglabra*, database for the leaves had been established. The proteins were classified according to their functions as in photosynthesis and photorespiration, amino acid metabolism, carbon-compound and carbohydrate metabolism, protein metabolism, stress response, cellular communication and signal transduction, glycolysis and gluconeogenesis, unidentified proteins and other proteins. Further proteomic studies in *B. alboglabra* would provide more information for protein database in this plant species.

6. Supplementary material

The Table of identified proteins in this manuscript is online available in the web page of JIOMICS.

http://www.jiomics.com/index.php/jio/rt/suppFiles/25/0

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