



A comparative proteomic study of white and black glutinous rice leaves



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ABSTRACT

Background: Black glutinous rice contains remarkable levels of anthocyanins, which possess anti-oxidative properties and thus have health benefits. The accumulation of anthocyanins in grains of thirty black glutinous rice varieties was measured, and the results revealed that the accumulated anthocyanin content ranged from 0.262 to 2.539 mg/g. Black glutinous rice Br no. 19 was selected, and its leaf protein expression profile was compared with that of white glutinous rice RD 6 using 2D-PAGE, and the protein spots were then directly analyzed after proteolysis by LC-MS/MS.

Results: The proteins from the leaves of the two rice varieties were separated using 2D-PAGE and silver stained, and the spots were analyzed using Image Master 2D Platinum version 5 software. The results showed that the protein profiles of these two rice cultivars were different, with at least six protein spots that were detected only in Br no. 19. In addition, seven protein spots accumulated at higher levels in Br no. 19 than in RD 6.

Conclusion: The protein spot S1 (AP005098.4) is homologous to the Rc protein. Our results suggest that some of the proteins enriched in Br no. 19 may be involved in anthocyanin synthesis in the black glutinous rice.

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1. Introduction

Rice (*Oryza sativa* L.) is an important grain and a basic staple food for a large part of the world's population [1,2,3]. People in East and South-east Asian countries also consume glutinous rice in addition to the common white non-glutinous rice. Pigmented (red, brown, purple and black) glutinous rice in particular has gained a lot of attention as raw materials for production of commercial health food supplements due to its high phenolic, anthocyanin and antioxidant contents. Black rice, which includes several rice varieties with a long history of cultivation in Southeast Asian countries such as China, India and Thailand, derives its name from its black color [4]. In some varieties of black rice, anthocyanins are present in the stem and leaves as well as the kernels, in others only the grains are pigmented.

The health benefits of black glutinous rice have recently been reported by several investigators. Black glutinous rice has been shown to accumulate compounds such as anthocyanins [5,6] and gamma oryzanol [7,8,9,10]. Black rice also contains many vitamins and minerals, including iron, vitamin A and vitamin B, which are beneficial for overall health and the prevention of heart disease [11].

Anthocyanins are naturally occurring plant pigments that belong to the flavonoid family and are widely used for their antioxidant and pharmacological properties [12]. Reactive free radicals have been postulated to contribute to the development of chronic inflammatory

proliferative diseases (CIPDs) [13], particularly arteriosclerosis and cancer by causing oxidative damage to essential enzymes, cells and tissues [14,15]. The anthocyanins in rice act as antioxidants, which can inhibit inflammation throughout the body [16], act as anticancer agents [17,18,19,20,21], promote blood circulation, slow damage and aging of tissues, reduce cholesterol and blood sugar levels [11,22,23,24], affect pituitary gland function, inhibit gastric acid secretion and inhibit platelet aggregation [25].

Proteomics comprises a rapidly emerging set of key technologies that are being used to identify proteins and determine protein function. The rice proteomic studies that have been conducted to date have focused mainly on the changes in protein expression that are triggered by environmental factors [26,27]. Over 1100 proteins are expressed in rice leaves, as identified as spots on 2D-gels. The identified proteins have been classified into 10 functional categories, including cell structure, cell growth/division, energy metabolism, disease/defense, intracellular traffic, metabolism, protein destination and storage, signal transduction, hypothetical functions, and unknown proteins [28]. Most rice leaf proteomes were obtained from green leaves [29,30,31,32,33,34,35] however, proteomes from purple anthocyanin-containing leaves have never been reported. The proteomic profiling of plants that accumulate anthocyanins, such as grapes [36], and more specifically the mesocarp of vine-ripened grapes have helped to elucidate the biochemical and physiological changes that occur during anthocyanin accumulation and have been of paramount importance in advancing the understanding of berry development and the ripening process [37]. To gain information on the proteins involved in anthocyanin production in black

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glutinous rice, we compared the protein profiles of purple leaves of a black glutinous rice variety with those of the green leaves of a white glutinous rice using 2D-PAGE. The information obtained will be useful in further studies of the functions of proteins that are involved in the anthocyanin pathway.

2. Materials and methods

2.1. Plant materials

Thirty varieties of local black glutinous rice seeds (Table 1) collected from northeast Thailand during 2008 and 2009 were generously provided by Chumphae Rice Research Center, Khon Kaen and the Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand.

2.2. Total anthocyanin content (TAC)

The TAC in the grain samples of all 30 rice varieties was determined using a previously described spectrophotometric method [38]. The supernatant from a crude extract was poured into a 50 mL volumetric flask and brought up to volume at 24 mL with acidified methanol. The absorbance was measured using a UV–visible spectrophotometer at 535 nm. The TAC was calculated as follows: $TAC = A \times 288.21$, where A is the absorbance reading.

2.3. Plant growing conditions

Seeds from the Br no. 19 and the white glutinous improved cultivar RD 6 were grown hydroponically in a greenhouse as described by Gregorio et al. [39], with some modifications. The experiment was conducted in a greenhouse at the Department of Biology, Faculty of Science, Khon Kaen University, Thailand. After the seedlings were grown for 35 d in nutrient solution, the leaves were collected, frozen in liquid nitrogen and stored at -70°C .

Table 1
Characteristics of thirty varieties of local black glutinous rice.

Varieties	Color			TAC (mg/g) \pm SD
	Grains	Pericarp	Leaves	
Br no. 3	Black	Black brown	Green	0.954 \pm 0.020
Br no. 16	Brown	Black brown	Green	0.598 \pm 0.037
Br no. 18	Straw	Black brown	Green	0.437 \pm 0.024
Br no. 19	Black	Black	Purple	2.539 \pm 0.011
Br no. 20	Black	Black brown	Green	0.942 \pm 0.022
Br no. 26	Black	Black brown	Green	0.720 \pm 0.045
Br no. 27	Black	Black brown	Purple	0.415 \pm 0.057
Br no. 28	Black	Black brown	Green	1.366 \pm 0.056
Br no. 29	Black	Black	Green	1.010 \pm 0.044
Br no. 30	Straw	Black brown	Green	0.919 \pm 0.037
Br no. 32	Black	Black brown	Green	0.631 \pm 0.039
Br no. 42	Black	Black brown	Green	0.751 \pm 0.097
Br no. 44	Black	Black brown	Purple	0.663 \pm 0.061
Br no. 46	Black	Black brown	Purple	0.262 \pm 0.071
Br no. 50	Black	Black	Green	1.022 \pm 0.071
Br no. 52	Black	Black brown	Green	1.222 \pm 0.002
Br no. 53	Brown	Black brown	Green	0.752 \pm 0.031
Br no. 54	Black	Black brown	Purple	0.530 \pm 0.015
Br no. 55	Brown	Black	Green	0.526 \pm 0.063
Br no. 56	Brown	Black brown	Purple	0.683 \pm 0.056
Br no. 58	Brown	Black	Purple	0.743 \pm 0.065
Br no. 59	Straw	Black brown	Green	0.405 \pm 0.019
Br no. 63	Straw	Black	Green	1.243 \pm 0.048
Br no. 64	Black	Black	Green	0.790 \pm 0.093
Br no. 65	Black	Black	Green	0.733 \pm 0.011
Br no. 68	Black	Black	Green	0.707 \pm 0.037
Br no. 70	Black	Black brown	Green	0.419 \pm 0.030
Br no. 71	Black	Black	Green	1.101 \pm 0.037

2.4. Protein extraction

The leaves of rice cultivars RD 6 (white glutinous rice with green leaves) and Br. no. 19 (black glutinous rice with purple leaves) were ground to a fine powder in liquid nitrogen and dissolved in ice-cold double-distilled water. The homogenate was centrifuged at $13,000 \times g$ for 15 min at 4°C . The dried protein pellets were solubilized in rehydration buffer [8 M urea, 0.5% (w/v) CHAPS, 20 mM DTT, and 0.5% (v/v) IPG buffers]. The amount of protein was determined according to the Bradford method [40].

2.5. Two-dimensional PAGE

The proteins from each sample (5 μg) were separated by 2D-PAGE as described by Berkelman and Stenstedt [41]. Isoelectric focusing gel electrophoresis (IEF) was conducted at 20°C using an IPG phor™ IEF System and a DryStrip kit (Amersham Biosciences, Uppsala, Sweden). Each of the 7-cm IPG strips (pH 3–10, non-linear) was rehydrated with 125 μL of rehydration buffer for 13 h, and the protein sample was then loaded onto the strips. The isoelectric focusing was performed in 5 steps at 150 V for 2 h, 300 V for 30 min, 1000 V for 30 min, 5000 V for 1.20 h and 5000 V for 25 min. The focused strips were equilibrated twice for 30 min in 10 mL equilibration buffer [50 mM Tris HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 100 mg DTT] with gentle shaking. During the second equilibration, 250 mg iodoacetamide was used instead of DTT.

The second-dimension separation was performed by SDS-PAGE (10% total monomer, with 2.6% crosslinker) using a PROTEAN II Multi Cell (Bio-Rad, Hercules, USA). The focused strips were transferred to the tops of the gels, and the two slabs were electrophoresed simultaneously with an initial current of 10 mA for 10 min and then at 25 mA until the tracking dye reached the bottom of the gel. The protein spots were visualized by staining with silver nitrate. The isoelectric point (pI) values were determined automatically using Image Master 2D Platinum version 5 software (Amersham Biosciences, Uppsala, Sweden), and the relative molecular weight (MW) of each protein spot was calculated. All 2D protein gel analyses were performed at least three times.

2.6. Chromatography coupled with tandem mass spectrometry

The protein spots were excised from the gels and analyzed after proteolysis by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) at the National Center for Genetic engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency, Thailand. External calibration was performed, and the data were collected in linear mode. The empirical peptide mass values were matched with the theoretical digested mass and database sequence information using Mascot (<http://www.matrixscience.com>).

3. Results

3.1. Total anthocyanin content

Thirty varieties of local black glutinous rice seeds collected from northeast Thailand during the years 2008 and 2009 were collected and characterized for the color of grains, pericarp and leaves (Table 1). The color of grains for black glutinous rice varieties were straw color in four varieties, brown color in six varieties and black color in nineteen varieties. The colors of pericarps for black glutinous rice varieties were black color in ten varieties and black brown color in twenty varieties. The colors of leaves for black glutinous rice varieties were green in seven varieties and purple in twenty three varieties. The contents of anthocyanins in brown rice grains of thirty varieties of local black glutinous rice were measured and the results revealed that

the accumulated anthocyanin ranged from 0.262 to 2.539 mg/g. The highest anthocyanin content was at 2.539 mg/g in Br. no. 19 (Table 1).

3.2. Comparison of protein expression profiles in black and white glutinous rice

In an attempt to understand the molecular basis of anthocyanin synthesis, 2D-PAGE was used to identify the proteins that were present exclusively in black glutinous rice. The results showed that more than 100 reproducible protein spots were detected and that the protein profiles of these two rice varieties were different (Fig. 1). A comparison of the 2D patterns of the black glutinous rice varieties Br no. 19 with the white glutinous rice varieties RD 6 showed that at least six protein spots, designated S1 to S6, were found only in Br no. 19. The MW and pI values of these proteins are as follows: 15 kDa, pI 6.2 (spot S1); 19 kDa, pI 6.5 (spot S2); 22 kDa, pI 6.4 (spot S3); 17 kDa, pI 8.6 (spot S4); 26 kDa, pI 6.4 (spot S5) and 13 kDa, pI 6.5 (spot S6). In addition, seven protein spots (M1 to M7) were found at higher levels in Br no. 19 than in RD 6: 18 kDa, pI 6.2 (spot M1); 22 kDa, pI 6.2 (spot M2); 12 kDa, pI 5.4 (spot M3); 23 kDa, pI 6.3 (spot M4); 23 kDa, pI 6.1 (spot M5); 22 kDa, pI 5.8 (spot M6) and 16 kDa pI 5.9 (spot M7). A three-dimensional view of these protein spots (spots S1–S6 and spots M1–M7) is shown in Fig. 2.

3.3. Protein identification by mass spectrometry

The protein spots that were excised from the gels were analyzed after proteolysis by LC–MS/MS. The calibration was external, and the data were collected in linear mode. The empirical peptide mass values were matched with the theoretical digested mass and database sequence information by a Mascot search. The results showed that the closest homologues of the six proteins found only in Br no. 19 were a hypothetical protein [*O. sativa* Japonica Group] (spot S1), NBS-LRR-like protein A [*O. sativa* Indica Group] (spot S2), a putative isoleucine-tRNA ligase [*O. sativa* Japonica Group] (spot S3), a hydroxyproline-rich glycoprotein-like protein [*O. sativa* Japonica Group] (spot S4), a hypothetical protein [*O. sativa* Japonica Group] (spot S5) and hypothetical protein OsJ_04608 [*O. sativa* Japonica Group] (spot S6). In addition, the closest homologues of the proteins that accumulated at higher levels in Br no. 19 than in RD 6 were hypothetical protein OsJ_02230 [*O. sativa* Japonica Group] (spot M1), a hypothetical protein [*O. sativa* Japonica Group] (spot M2), OSJNBa0052021.10 [*O. sativa* Japonica Group] (spot M3), hypothetical protein OsL_12770 [*O. sativa* Japonica Group] (spot M4), a hypothetical protein [*O. sativa* Japonica Group] (spot M5) and OSJNBa0052021.10 [*O. sativa* Japonica Group] (spot M6) (Table 2).

4. Discussion

Grains of 30 varieties of local black glutinous rice collected from the northeastern region of Thailand during the years 2008 and 2009 were characterized. It was found that grains of 30 varieties of local black glutinous rice differed significantly in their TAC. The values of grain TAC were observed to range from 0.262 to 2.539 mg/g. The highest value of TAC of 2.539 mg/g was observed for Br no. 19. In our study, Br no. 19 was found to contain cyaniding 3-glucoside, as suggested by OD₅₃₅. It has been reported that the majority of black rice plants contain anthocyanidin-3-glucosides as the major anthocyanin, which functions as the major antioxidant compound [6,38,42,43] and strong superoxide radical scavenging activity [44,45,46]. Since Br no. 19 showed the highest value of TAC and no TAC was detected in RD6, these two rice varieties were therefore selected to study and compare the proteomics of green rice and purple rice to understand what proteins exist in purple rice other than those found in green rice and which are likely responding to anthocyanin synthesis to explain anthocyanin synthesis in black grains. Unfortunately, the proteomics of green rice and purple rice could not be compared due to the fact that clear protein spots on 2D-gel could not be achieved since rice rains used in this study were fully developed, in which a number of genes and proteins were inactive. Thus, leaves were employed for proteomic study since leaves of these two rice varieties were obviously different in their color. Anthocyanin synthesis in rice grains and leaves is believed to be controlled by different genes. However, it has been well-recognized that different genes can encode the same protein. With this, it is possible to employ the leaf proteomics to explain anthocyanin synthesis in black grains since our results showed that the S1 protein spot obtained from leaves of Br no. 1 was homologous to Rc protein in rice pericarp.

After matching the empirical peptide mass values with the theoretical digested values and database sequence information, we found that the closest homologues of proteins found only in Br no. 19 were mainly hypothetical proteins. Protein Spot S1 (AP005098.4) is homologous to the Rc protein. Nagao and Takahashi [47] reported that Rc is a domestication-related gene required to produce a red pericarp in rice; the gene encodes a basic helix–loop–helix (bHLH) protein and controls the anthocyanin activator, purple node, lop-leaved dwarf, and recessive long empty glume traits. It is also reported that the Rc and Rd genes are involved in pro-anthocyanidin synthesis in the rice pericarp [48]. The Rc gene is expressed in both red-grained and white-grained rice, but it is expressed as a shortened transcript in the white varieties [49,50]. NBS-LRR-like protein A (AY518220.1, Spot No. S2) (<http://www.ncbi.nlm.nih.gov>) is a protein present in the *O. sativa* Indica Group. It has been shown to be expressed at the transcript level, and the protein is

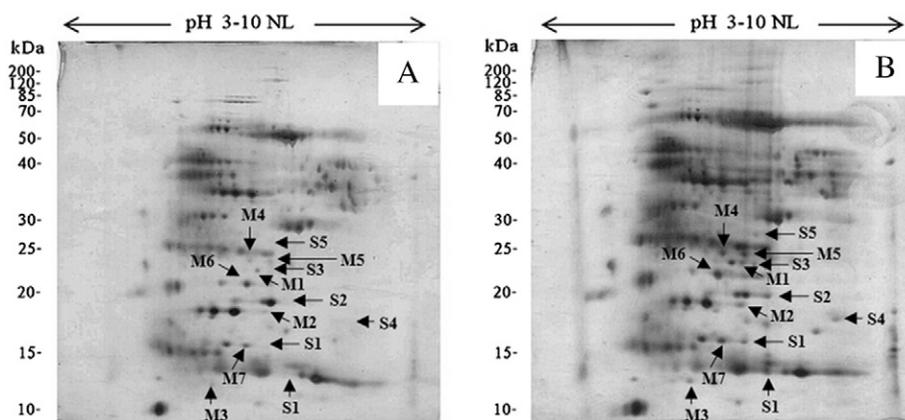


Fig. 1. Silver-stained two-dimensional protein patterns of leaves of rice seedlings cvs. RD 6 (A) and Br no. 19 (B). First-dimensional separation was performed using Immobiline Dry Strip 3–10 NL, 7-cm run on Ettan IPG phor IEF System. Second-dimensional separation was performed using a 10% polyacrylamide gel on Bio-Rad mini vertical system (S1–S6; protein spots were found only in Br no. 19, M1–M7; protein spots accumulated at higher levels in Br no. 19 than RD 6).

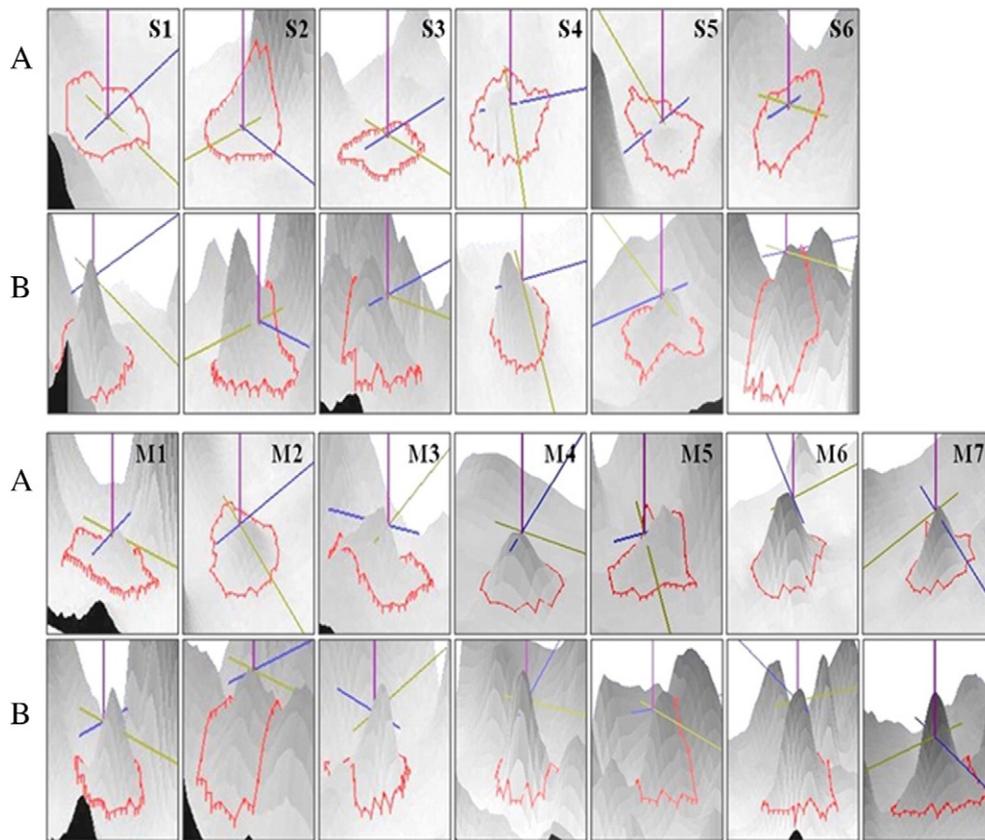


Fig. 2. Three-dimensional view of protein spots (S1–S6; protein spots were found only in Br no. 19, M1–M7; protein spots in Br no. 19 accumulated at higher levels than in RD 6) obtained from 2D-PAGE analysis of protein extracted from leaves of rice seedlings cvs.: RD 6 and Br no. 19 (A and B, respectively). The image was analyzed using Image Master 2D Platinum version 5 software (Amersham Biosciences, Uppsala, Sweden).

Table 2
Identification of proteins found specifically (S1 to S6) in black glutinous rice leaves (Br no. 19) and present at higher intensity (M1 to M7) in leaves of black glutinous rice (Br no. 19) than white glutinous rice (cv. RD 6).

Spot no.	<i>pI</i>	Mw	Peptide	Homologous protein	Score	Accession no.
S1	6.2	15	M.PTYGSR.R	Hypothetical protein [<i>O. sativa Japonica Group</i>]	21	AP005098.4
S2	6.5	19	K.EALSPR.S K.GKEALSPR.S	NBS-LRR-like protein A [<i>O. sativa Indica Group</i>]	39	AY518220.1
S3	6.4	22	R.SGTPLIYR.A	Putative isoleucine-tRNA ligase [<i>O. sativa Japonica Group</i>]	14	AP003568.3
S4	8.6	17	R.TIAEALALR.K	Hydroxyproline-rich glycoprotein-like [<i>O. sativa Japonica Group</i>]	16	AP005573.3
S5	6.4	26	M.VNGGGLNAQR.Q	Hypothetical protein [<i>O. sativa Japonica Group</i>]	20	AC103551.7
S6	6.5	13	R.WRIIYR.R R.IIYRRGR.R	Hypothetical protein OsJ_04608 [<i>O. sativa Japonica Group</i>]	27	CM000138.1
M1	6.2	22	R.VVYGGPWK.H	Hypothetical protein OsJ_02230 [<i>O. sativa Japonica Group</i>]	17	CM000138.1
M2	6.2	18	K.AVARGVGGAR.T	Hypothetical protein [<i>O. sativa Japonica Group</i>]	15	AP004654.3
M3	5.4	12	K.KAKEAMKK.G	OSJNBa0052021.10 [<i>O. sativa Japonica Group</i>]	30	AL606590.3
M4	6.3	23	K.ILSSRVISRK.V	Hypothetical protein Osl_12770 [<i>O. sativa Japonica Group</i>]	19	CM000128.1
M5	6.1	23	R.VALPSRDGR.S	Hypothetical protein [<i>O. sativa Japonica Group</i>]	18	AC091680.7
M6	5.8	21	K.AVARGVGGAR.T	Hypothetical protein [<i>O. sativa Japonica Group</i>]	11	AP004654.3
M7	5.9	15	K.KAKEAMKK.G	OSJNBa0052021.10 [<i>O. sativa Japonica Group</i>]	29	AL606590.3

Spot no. = spots were ordered exactly as in Fig. 1.

pI = theoretical isoelectric point of protein.

Mw = molecular weight of identified protein.

Peptide = peptide matched with the identified protein in mass analyses.

Score = score of Mascot search Results.

Accession no. = GenBank accession number of proteins.

proposed to selectively and non-covalently interact with adenosine 5'-diphosphate (ADP). A putative isoleucine-tRNA ligase (AP003568.3, Spot No. S3) is present in the *O. sativa* Japonica Group [51] and is found in other cereals [52]. The function of this putative protein is inferred from its homology to other members of the class I aminoacyl-tRNA synthetase family. This putative isoleucine-tRNA ligase is predicted to be an aminoacyl-tRNA synthetase, which activates an amino acid for translation by forming an aminoacyladenylate intermediate and then linking the activated amino acid to the corresponding tRNA molecule (amino acid-tRNA or aminoacyl-tRNA). In general, a specific aminoacyl-tRNA synthase is available for each amino acid, and the enzyme functions include ATP binding, aminoacyl-tRNA editing activity and isoleucine-tRNA ligase activity. Hydroxyproline-rich glycoprotein-like (AP005573.3, Spot No. S4), a protein present in the *O. sativa* Japonica Group, hydrolyses peptide bonds in a polypeptide chain by a mechanism in which the sulphhydryl group of a cysteine residue at the active center acts as a nucleophile [49,53]. Hypothetical protein (AC103551.7, Spot No. S5) is an *O. sativa* chromosome 3 BAC OSJNBb0058G04 genomic sequence and is present in the *O. sativa* Japonica Group (<http://www.ncbi.nlm.nih.gov>). A hypothetical protein is a protein whose existence has been predicted but for which there is no experimental evidence that it is expressed *in vivo*. The function of a hypothetical protein can also be predicted by domain homology searches with various confidence levels. Hypothetical protein OsJ_04608 (CM000138.1, Spot No. S6) is present in the *O. sativa* Japonica Group [54].

The seven homologous proteins in Br no. 19 that accumulated at higher levels than in RD 6 included the following: hypothetical protein OsJ_02230 (CM000138.1, Spot No. M1); a hypothetical protein (AP004654.3, Spot No. M2) present in the *O. sativa* Japonica Group [49]; OSJNBa0052O21.10 (AL606590.3, Spot No. M3) present in the *O. sativa* Japonica Group and encoded on rice chromosome 4 [55]; hypothetical protein OsI_12770 (CM000128.1, Spot No. M4) present in the *O. sativa* Japonica Group [49]; a hypothetical protein (AC091680.7, Spot No. M5) present in the *O. sativa* Japonica Group and encoded on rice chloroplast DNA [56]; a hypothetical protein (AP004654.3, Spot No. M6) present in the *O. sativa* Japonica Group [50]; and OSJNBa0052O21.10 (AL606590.3, Spot No. M7) present in the *O. sativa* Japonica Group and encoded on rice chromosome 4 [55]. OSJNBa0052O21.10 is predicted to have a transferase activity, transferring hexosyl groups from one compound (donor) to another (acceptor). However, the homologues of the other proteins found to be enriched in black glutinous rice cultivar Br no. 19 (protein spots No. S2–S6 and M1–M7) have unidentified functions.

Our results demonstrate that at least six proteins are found only in the leaves of Br no. 19 and that seven proteins were up-regulated in Br no. 19 compared to RD 6; these differentially expressed protein spots were identified using 2D-PAGE and LC-MS/MS. We found that the molecular weight and pI values of the proteins in this study were similar to those of many other proteins in the database. Thus, our results identified several previously undescribed proteins that may be associated with the characteristics of black glutinous rice or related to anthocyanin synthesis in black glutinous rice.

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Conflicts of interest

None.

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