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DIFFERENTIALLY EXPRESSED PROTEIN PROFILES OF BARIO UPLAND AND MR219 LOWLAND RICE CULTIVARS

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Abstract: Traditional Sarawak upland rice known as Bario, exhibits different physical traits in height, flowering period, grain size and starch content compared to commercial rice cultivars. These variations may be associated with the differentially expressed proteins in the rice varieties. This study compared the differentially expressed protein profiles of upland and commercial rice cultivars using 2D-PAGE. The proteins were isolated from leave samples using PEG fractionation and quantified using Bradford assay. The Supernatant Fraction (SF) and Pellet Fraction (PF), two distinct protein fractions were visualised through 2-DE gels and analysed using 2-DE software. The 2D-PAGE analysis revealed the differentially expressed protein profiles between the rice varieties in SF and PF, respectively. Upland rice had fewer protein spots on the 2-DE gels than the MR219 rice cultivar. There were 399 protein spots in SF and 506 protein spots in PF were detected. Specifically, 102 and 129 differentially expressed proteins were found in SF and PF, with a p-value ≤ 0.05 and a fold change ≥ 2.0, respectively. From the value, an abundance of proteins was found down-regulated in the upland rice compared to the commercial rice cultivar. These proteins may contribute to the differences in physical characteristics and other biological responses in the diversity of rice.

Keywords: *Oryza sativa*, Bario rice, proteomics, differentially expressed.

Introduction

The rice production in Malaysia is primarily concentrated in the lowland area of Peninsular Malaysia, accounting for two-thirds of commercialised rice while the remaining production (including upland and lowland rice) is in Sabah and Sarawak (Firdaus *et al.*, 2020; Dorairaj & Govender, 2023). Upland rice cultivation includes traditional rice varieties and is mostly practised by rural communities for home consumption and also to add to their household income (Hanafi *et al.*, 2009; Sinton *et al.*, 2019). Bario rice cultivar, an indigenous rice crop cultivated in Bario's Sarawak highlands is recognised as one of the finest rice grains worldwide. It has a soft texture with fine elongated grains and exquisite taste and fragrance (Tan *et al.*, 2008; Wong *et al.*, 2009; Ronie *et al.*, 2022). The rice grain colour can generally distinguish four different types of Bario rice: Bario *Adan Halus* (white rice), Bario *Tuan* (brownish), Bario *Merah Sederhana* (red bran layer), and Bario *Celum* (black bran layer) (Nicholas *et al.*, 2014; Ronie *et al.*, 2022). Among these, Bario *Adan Halus* is widely commercialised due to its mild pleasant aroma, sticky texture, fine elongated grains, and exquisite taste (Wong *et al.*, 2009; Nicholas *et al.*, 2013; 2014). The research interest in upland rice has increased due to its nutritional composition, which is geared towards improving food and promoting healthy nutrition. It is reported that this rice contains higher amylose, an appropriate amount of crude fibre, and a lower carbohydrate content than wheat bread (Ronie *et al.*, 2022; 2023). Therefore, it can be used in gluten-free bread (Ronie *et al.*, 2022). A previous study on the metabolomics of the upland rice in Sabah also found a higher ratio of linoleic acid esters and glycerolipids but a decrease in

triacylglycerolipids and lyso (lysoPC) compared to the lowland rice (Ang *et al.*, 2020).

The MR219, a Malaysian *indica* rice cultivar, is the most common rice cultivated in the granary area (Talei *et al.*, 2013; Bashar *et al.*, 2014) after MR297, MR315, MR220CL2, and MR269 (Hosnan, 2023). The Malaysian Agricultural Research and Development Institute (MARDI) developed and released this variety in 2001 from the cross-breed between MR137 and MR151 (Fasahat *et al.*, 2012; Talei *et al.*, 2013). Even though more than 20 years have passed since its release, this variety still gained preference among local farmers as an extensively planted rice variety (Dorairaj & Govender, 2023). On top of that, researchers are working to enhance their research on this variety of rice by employing marker-assisted breeding in the drought improvement study. They discovered that certain combinations of *qDTYs*, affect the increase in the yield production from 903 kg to 2,523 kg per hectare (Shamsudin *et al.*, 2016). Long-grade grains, a short maturation period and high resistance to bacterial leaf blight and brown hopper (Fasahat *et al.*, 2012) with high-yielding (Ahmad *et al.*, 2023) are the advantages of this variety among other rice.

Plant-omics and systems biology are advancing to integrate multi-omics data towards plant biology research. The gel-based proteomics technique using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is still the preferred protein analysis tool. It is a straightforward technique based on a single protein spot, separated through its isoelectric point and molecular weight. The proteins between samples are compared through the 2-DE analysis software prior to mass spectrometry identification. Various studies have successfully determined the differentially expressed proteins targeting a specific factor due to diseases, microbial interactions, or environmental stress. The differentiation of 24 expressed proteins was found in response to *Ganoderma* spp. inoculation by 2-D gel distinguished the functions of the proteins in photosynthesis, signalling, stress, and metabolism regulation (Al-Obaidi *et al.*, 2016).

Another study on wheat (*Triticum durum*) found 36 protein spots showing significant changes towards drought response between the control and stressed samples (Caruso *et al.*, 2009). Similar research regarding stress-related proteins between healthy and unhealthy tissues of *Piper nigrum* found 25 and 41 differentially expressed proteins in the root and stem samples, respectively (Hamdin *et al.*, 2020). Apart from that, an investigation also conducted on *Metroxylan sagu*, a slow-growing plant which produces the highest edible starch, discovered 19 out of 34 proteins have higher expression in non-trunking sago plant that was contributing majorly to its metabolic pathways (Hussain *et al.*, 2020).

Meanwhile, a study on rice proteome provides information on protein complement to the genome after post-translational regulation and the correlation between mRNA and protein abundance (Rakwal & Agrawal, 2003). With the availability of genome sequences, the identified protein might play a significant role as an important biomarker in genome annotation and rice development research. For example, a previous study run using SDS-PAGE and Experion™260 from several varieties of colouring rice suggested that some protein bands were found only in specific rice varieties and may become potential biochemical markers (Sari *et al.*, 2019). Most recent studies on Bario have focused on the nutritional and physiochemical composition of different types of Bario rice. However, no prior research has been done using the proteomics approach towards the Bario rice cultivar. The study of proteins creates opportunities to understand and generate more knowledge on functional aspects of the plant in biological processes, enabling us to relate with the characteristics of the rice plant. Therefore, this study aims to compare the differentially expressed protein profiles between Bario upland and MR219 lowland rice cultivars using 2D-PAGE, which contributes to the nutritional composition of the rice, growth condition, or physiology.

Materials and Methods

Plant Materials

The first flag leaf from two different rice cultivars used in this study were Bario upland (*Adan Halus*-white) rice and MR219 lowland rice. The rice seeds were germinated in the laboratory and cultivated in the greenhouse with a scheduled application of fertilisers and pesticides. After 10 to 14 days of flowering period, the leave samples were collected from the three biological samples of each cultivar. The leaves were sprayed with 70% ethanol and wiped with tissues to remove any contaminant, then stored at −80°C until used.

Protein Extraction Method

The proteins were extracted based on Nakamura *et al.* (2012) and Alam *et al.* (2013), with some modifications. The leaf sample was ground until finely powdered in liquid nitrogen using mortar and pestle. About 2 g of the powdered sample was mixed in an 8 ml ratio 1:4 with ice-cold extraction buffer [8 mM Mg Cl_2, 50 mM imidazole-HCl (pH 7.4), 12.5% v/v glycerol, 50 mM β-Mercaptoethanol and 1 mM PMSF] by vortexing for a minute. Then, the mixture was sonicated for five minutes at the temperature below 8°C to 12°C and was vortexed again for one minute. The mixture was centrifuged at 10,000 g for 20 minutes at 4°C. The supernatant was filtered through a double-layered Miracloth into a 15 mL Falcon tube. The filtered supernatant was added to a final concentration of 15% (w/v) PEG 4000 from 50% (w/v) PEG stock solution. The solution was inverted several times to mix and placed on ice for 30 minutes. The fraction protein was obtained through centrifugation at 8,500 rpm for 20 minutes at 4°C. The supernatant was pipetted out into a new tube and designated as a PEG supernatant fraction while the pellet was labelled as a PEG pellet fraction.

For the PEG supernatant fraction, it was precipitated at −20°C overnight in four volumes (4x) of 100% acetone. Meanwhile, for the PEG pellet fraction, four volumes of 0.1 M ammonium acetate in methanol were added to the tube, mixed vigorously, and precipitated at −20°C overnight.

For the PEG supernatant fraction, the pellet was obtained by centrifugation (10,000 g, 20 minutes, 4°C). The supernatant was discarded, and the pellet was re-suspended in the extraction buffer and vortexed to mix homogeneously. An equal volume of buffered phenol and 0.7 M of sucrose were added to the solution. Next, the sample solution was vortexed vigorously until all the sucrose dissolved, followed by centrifugation (10,000 g, 20 minutes, 4°C). The upper phenol layer was pipetted and transferred into a 50 mL Falcon tube. Four volumes of 0.1 M ammonium acetate in methanol were added to precipitate at −20°C overnight. The precipitate was centrifuged (8,500 rpm, 30 minutes, 4°C), with the supernatant discarded and the pellet was washed three times with 0.1 M ammonium acetate and centrifugated at intervals. The pellet was air dried for approximately five minutes, solubilised in buffer (7 M urea and 2 M thiourea) and kept at −20°C until further use.

PEG pellet fraction was obtained by centrifugation (8,500 rpm, 20 minutes, 4°C). The pellet obtained was washed three times with 0.1 M ammonium acetate and centrifuged at intervals, with the supernatant discarded. The pellet was air dried for approximately 5 minutes, solubilised in buffer, and kept at −20°C until further use. Step-by-step procedures for protein extraction were simplified and shown in Figure 1.

Protein Quantification

The total protein concentration was determined by the standard Bradford protein assay (Bradford, 1976; Kruger, 2002). The standard graph was performed with several concentrations of 1:100, 1:80, 1:60, 1:40, and 1:20, using Bovine serum albumin (BSA). Meanwhile, the sample was mixed in several dilutions of 100x, 50x, and 20x, then incubated for at least five

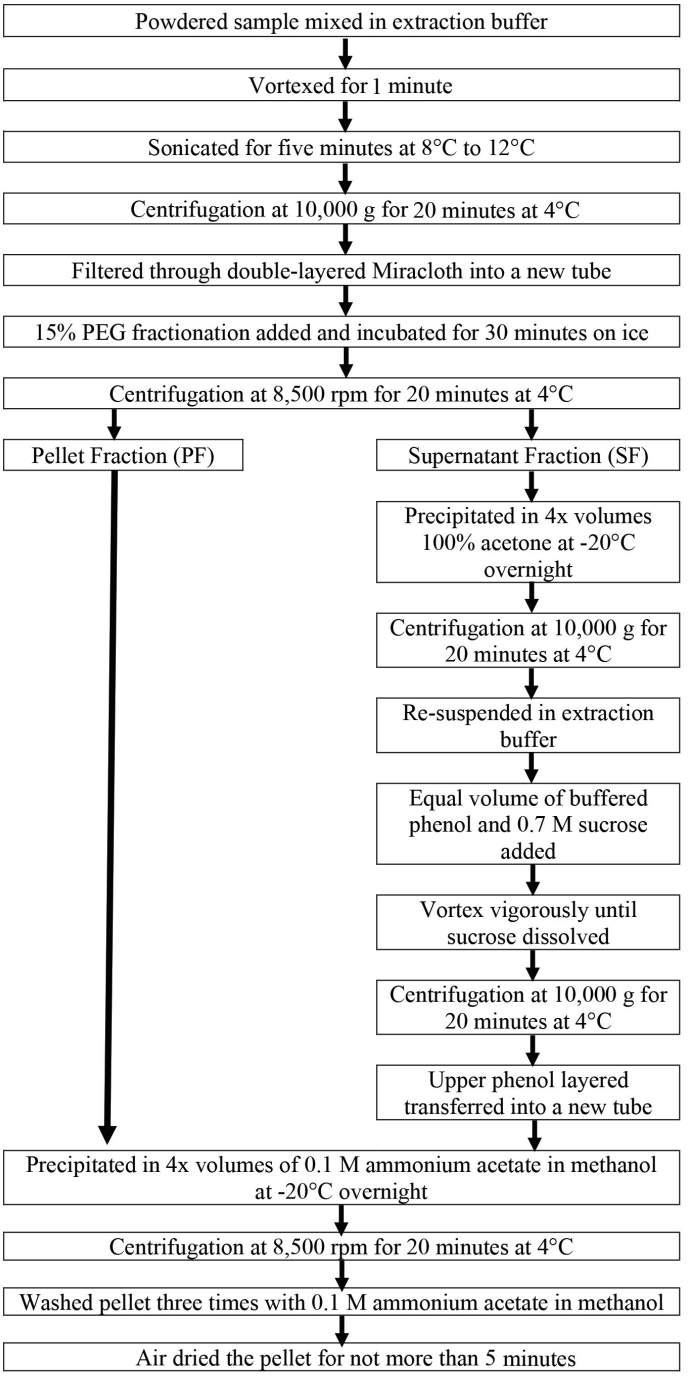

Figure 1: Simplified step-by-step protein extraction method

minutes after adding the Bradford reagent. The absorbance value was measured at 595 nm using a spectrophotometer within an hour.

Protein Visualisation

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis, SDS-PAGE, was used to first visualise the protein based on its molecular weight. The standard procedure was applied using 12% separating gel (30% acrylamide/bis, 1.5 M Tris-HCl pH 8.8, distilled deionised water, 10% SDS, TEMED, 10% APS) and 4% stacking gel (30% acrylamide/bis, 0.5 M Tris-HCl pH 6.8, distilled deionised water, 10% SDS, TEMED, 10% APS) (Laemmli, 1970). The protein marker, 9–245 kDa in sizes (PM2610-Enhanced 3-colour High Range Protein Marker, SMOBIO) and protein samples with 15 µg concentration were loaded into the well and run at 100 V. The gel was developed using the Coomassie-G blue staining method (Chong *et al.*, 2022) and documented using NuGenius/NuGenius+ Gel documentation system (SYNGENE).

Next, the proteins (solubilised in IEF buffer: 7M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 0.2%ampholytes and ultrapure water) were separated through 2D-PAGE. Protean IEF Cell (Bio-Rad, USA) was used in the first-dimensional separation, using 11 cm IPG strips (pH 3.0-10.0, nonlinear, Bio-Rad, USA). The standard protocol involved Step 1: 250 V, linear, 20 minutes; Step 2: 8,000 V, linear, 2.5 h; Step 3: 8,000 V, rapid, 26,000 Volt-hour; and Step 4: 750 V hold. The mineral oil on the focused IPG strips was removed by blotting it on Kimwipe tissues a few times. Then, it was immediately equilibrated in equilibration buffer I (375 mM Tris-HCl, pH 8.8, 6 M urea, 20% glycerol, 2% SDS, 2% DTT) and equilibration buffer II (375 mM Tris-HCl, pH 8.8, 6 M urea, 20% glycerol, 2% SDS, 2.5% iodoacetamide) for 15 minutes subsequently. Then, the equilibrated strip was run in the midi-tank SDS-PAGE gel system (Laemmli, 1970) and stained with Coomassie-G blue staining method (Chong *et al.*, 2022). Lastly, the gel was documented as mentioned previously.

Protein Analysis

The Progenesis Samespots software was used to document and analyse the 2-DE gel images. The total protein spots were calculated and the differentially expressed proteins (DEPs) with p-value < 0.05 and fold change ≥ 2.0 were categorised into up-regulated and down-regulated proteins.

Results

Protein Profiles by SDS-PAGE

The SDS-PAGE gels in Figure 2 show the protein separation between the leaves of the MR219 and Bario rice cultivar. The proteins were extracted into two different fractions: The Supernatant Fraction (SF) and Pellet Fraction (PF). In each cultivar, a different protein profile was observed, with more proteins being able to separate in SF than PF. The distinct protein separation ranges from ~45 kDa to ~140 kDa between SF and PF in the MR219 sample. Additionally, several proteins ranging from ~20 kDa to ~35 kDa revealed significant protein profiles in both Bario and MR219 samples. Nevertheless, some proteins were able to separate after PEG fractionation in SF as indicated at the z-arrow in Figure 2 (B).

In comparison between the Bario and MR219 samples, some of the protein bands were distinctly expressed in intensity. A protein band (y-arrow) was almost non-existent and only slightly present in Bario compared to MR219. Plus, several proteins were unable to separate at ~25 kDa to 30 kDa (s-bracket) in Bario too. In contrast, another protein band (x-arrow) showed higher intensity in Bario than in MR219.

Differential Expression of Protein Profiles by 2D-PAGE

Figure 3 shows the representative images of 2D-PAGE gels from MR219 and Bario rice samples. Significant differences in protein profiles were observed between the two samples in both SF and PF, respectively. Proteins were extensively scattered, particularly at a range of

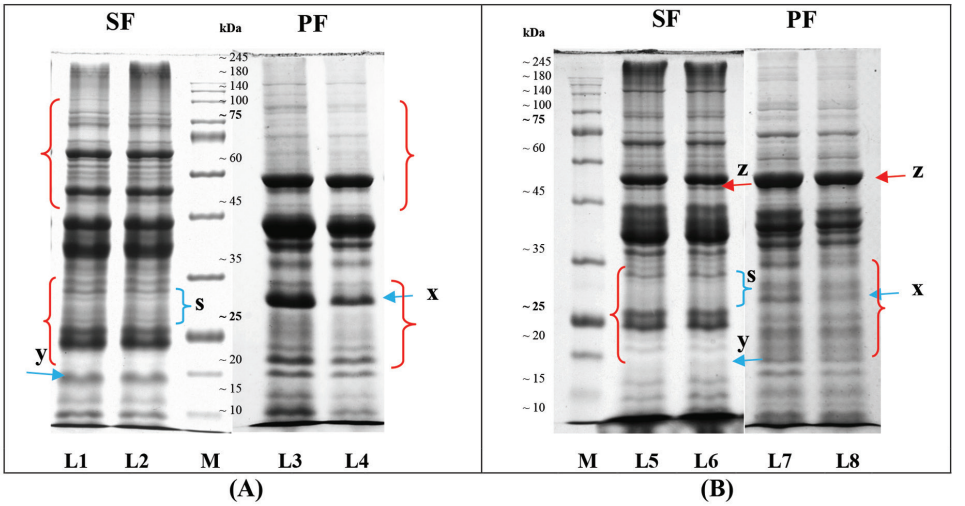

Figure 2: SDS-PAGE of MR219 rice cultivar (A) and Bario rice cultivar (B) with 15 µg protein samples loaded in each lane. [M: Protein marker, L1 & L2: MR219 Supernatant Fraction (SF), L3 & L4: MR219 Pellet Fraction (PF), L5 & L6: Bario Supernatant Fraction (SF), L7 & L8: Bario Pellet Fraction (PF)]

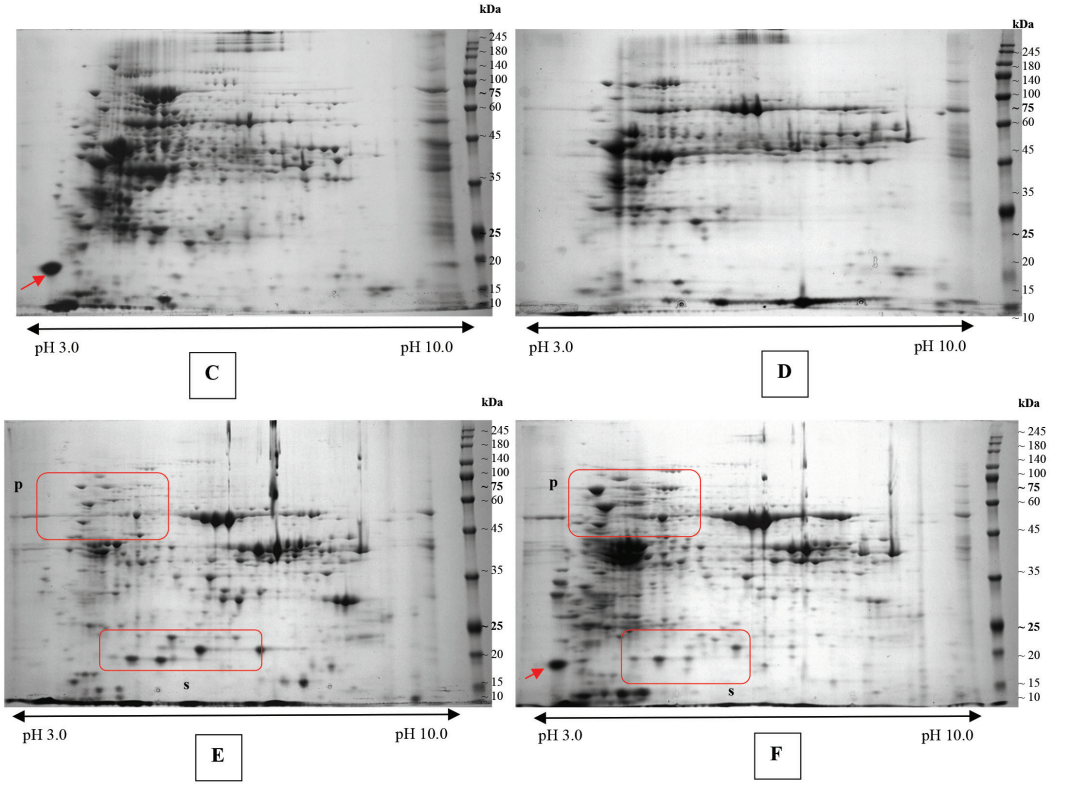

Figure 3: Representative image of differentially expressed proteins (DEPs) by 2D-PAGE from MR219 and Bario rice cultivar, loaded with a total of 300 µg concentration. [C: MR219 Supernatant Fraction (SF), D: Bario Supernatant Fraction (SF), E: MR219 Pellet Fraction (PF), F: Bario Pellet Fraction (PF)]

pH 3.0 to pH 6.0 in the SF samples. Meanwhile, the proteins in PF samples were mainly concentrated at pH 6.0 to pH 9.0. Both SF and PF included the total proteins in different pH ranges extracted from the leaf samples.

Interestingly, the Bario SF lacked many proteins compared to the MR219 SF. In other words, the majority of proteins were down-regulated in the Bario upland rice. On the other hand, a protein supposedly detected in Bario SF was discovered in Bario PF (indicated by an arrow). Despite that, it is uncertain if the anticipated protein is similar to the MR219 SF protein without performing protein identification since the extraction steps involved the same parameter. Besides that, the protein dispersion between MR219 PF and Bario PF is almost comparable. The obvious difference was in the intensity of the protein spots. For example, the proteins in the p-box were more intense in Bario PF than MR219 PF. In contrast, the proteins in the s-box were more intense in MR219 PF rather than Bario PF. It is also apparent that several proteins were found only in MR219 lowland rice and not in Bario upland rice (refer to the proteins in the box).

Analysis of Proteins by 2-DE Software

The gel images were loaded into the 2-DE analysis software and all the protein spots were evaluated according to the SF group and PF group. Based on the Progenesis Samespots programme analysis, the PF group (506 protein spots) had calculated more proteins than the SF group (399 protein spots). Further analysis was conducted utilising the proteins with Anova p-value ≤ 0.05, which found 247 and 212 proteins in the SF and PF groups, respectively. There were 99 (SF group) and 129 (PF group) differentially expressed proteins (DEPs) with a fold change ≥ 2.0 and p-value ≤ 0.05, then categorised into up-regulated and down-regulated proteins. From the SF group, there were 47 up-regulated and 52 down-regulated proteins in the Bario sample. Meanwhile, 63 up-regulated and 66 down-regulated proteins were detected in the Bario upland rice PF group.

Discussions

The application of 2-D PAGE in proteomics study is still relevant for understanding the expression of proteins in the various organelles and tissues caused by differences in phenotype, environmental stress, or diseases. This technique observes the changes occurring in the protein complement of tissue and subcellular compartments (Komatsu & Tanaka, 2005). The differences in protein expression between Bario and MR219 were identified using this technique.

In the present study, the Bario upland and MR219 lowland rice showed differential protein expression based on the SDS-PAGE and 2D-PAGE profiles. As proteins are directly responsible for the function and phenotypes of a cell (Xu *et al.*, 2012), the differences in protein expression give Bario rice many unique characteristics that make it one of the finest rice grains worldwide. Meanwhile, differential studies on Bario rice involving its physiochemical properties, nutritional compositions and cooking characteristics, as well as the effects of ageing towards starch, fat and protein of the rice component have been conducted (Nicholas *et al.*, 2013; 2014). A study found the ash contents were significantly lower in Bario (1.46%) than in MR219 (2.20%) (Nicholas *et al.*, 2013; 2014) while thiamine (vitamin B1) increased significantly in Bario rice varieties compared to MR219 (Nicholas *et al.*, 2014). Thiamine plays a major role in the metabolism of glucose for energy (Lonsdale, 2006). It was reported that Bario *Adan Halus* had the lowest amylose content (10.48%), which makes it firmer and stickier than MR219 (27.98%) (Nicholas *et al.*, 2013). The amylose content in Bario *Adan Halus* was 26.67%, the lowest among Bario *Tuan* (36.52%), Bario *Merah Sederhana* (32.05%), and Bario *Celum* (26.68%) (Ronie *et al.*, 2022). However, a previous investigation using parental varieties of MR219 calculated the amylose content as 23.23% (Asma Ilyani *et al.*, 2019), slightly lower than the Bario *Adan Halus*.

The unique protein expression profile of Bario rice that showed significant differences

compared to MR219 can be further developed as a protein marker system or for the identification of specific rice traits. Different rice varieties have different protein expression levels or unique characterisation of proteins to identify specific rice traits (Sari *et al.*, 2019). The specific features include morphology, physiology, and plant adaptations to growth and development. For example, a study on MR219 characterised overexpression of the stress-associated protein (SAP), which may be induced during the plant stress response and is involved in stress acclimation (Hedayati *et al.*, 2015). Besides, another study on the black rice from Java detected specific protein bands using SDS-PAGE that may related to the rice pericarp colour and could potentially become biochemical markers for future research (Sari *et al.*, 2019). These findings can be applied to gene manipulation and transformation in rice genetic engineering to develop a potential rice variety with specific characteristics such as climate acclimation, colourful rice, or even nutritious rice.

The identification of the differentially expressed proteins through mass spectrometry is being performed. Mass spectrometry is an essential tool in proteomics to study purified proteins and characterise post-translational modifications that alter the mass of a protein (Mann *et al.*, 2001). Identifying these proteins is essential not just for the development of a protein marker system and for the identification of specific rice traits but also for the determination of the roles of each protein. The proteins provide insights based on their molecular function and biological activity in cellular compartments, multiprotein complexes, and signalling pathways, which will strengthen the understanding of rice physiology, metabolism, and plant adaptations of rice.

Conclusions

In conclusion, the proteins were expressed differentially in the Bario upland and MR219 lowland rice varieties. A higher number of proteins were differentially expressed in lowland rice than in highland rice. Several unique proteins were observed in each rice variety and classified as up-regulated and down-regulated expression. Based on the differences in the protein profiles, specific or unique proteins have great potential to become protein biomarkers to distinguish the two rice varieties. These proteins will also lead to breakthroughs in the study of comparative rice proteomes, encompassing distinct rice features that differentiate upland from lowland rice and extending to rice breeding applications. Henceforward, identifying these proteins may determine their roles and contribute towards understanding rice physiology, metabolism, and plant adaptation.

Future Research Directions

This study can further improve by identifying the differentially expressed proteins using mass spectrometry and bioinformatics analysis. Therefore, a new foundation in comparative proteomics is being laid to advance the knowledge of protein function and regulation in upland and lowland rice.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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