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Proteomics of Seed Nutrition-Associated Proteins in Germinated Brown Rice in Four Thai Rice Cultivars Analyzed by GeLC-MS/MS

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Abstract

Brown rice (BR) and germinated brown rice (GBR) of *Oryza sativa* L. are popularly consumed by Asians because of their important healthy diet components. They are known to contain bioactive compounds and nutrients, such as phenolics, vitamins, fatty acids, and sugars, which help to maintain good health and reduce the incidences of various chronic diseases. The objective of this study was to investigate the effects of germination on changes of nutrition-associated proteins in 4 rice cultivars. After germination for 24 h, the changes of seed nutrition-associated proteins were examined by shotgun proteomics. The total proteins from non-germinated seeds and 24 h germinated seeds of 4 rice cultivars were extracted and analyzed by in-gel digestion coupled with tandem mass spectrometry (GeLC-MS/MS). A total phenolic content was analyzed from the crude methanol extract of those grains after germination for 0, 24, and 48 h using Folin-Ciocalteu assay. The analysis showed that seed nutritional-associated proteins, especially phenolic-associated proteins, increased after germination according to the accumulation of the total phenolic content. The expressions of 6 phenolic-associated proteins, including phenylalanine ammonia-lyase, serine carboxypeptidase-like protein, isoflavone-7-O-methyltransferase, isoflavonoid glucosyltransferase, glycosyltransferase family 61 protein and UDP-glucose flavonoid 3-O-glucosyltransferase were increased by 2.20 - 15.90 folds after germination. This study provides evidence that rice germination for 24 h has essentially influenced the increased nutritional values of BR and the phenolic biosynthetic pathway.

Keywords: Germinated brown rice, Hom Nil (HN), Phenolic compounds, Riceberry (RB), Shotgun proteomics

Introduction

Rice (*Oryza sativa* L.) in the Poaceae family is an important staple food throughout the world. In the previous decade, brown rice (BR) consumption, which contains more nutritional components than ordinary white rice, has become increasingly popular because of its health-related benefits. BR is whole grain rice without the inedible outer hull, whereas white rice is whole grain rice without hull, bran layers and embryo. BR is comprised of 7 % bran (pericarp, seed coat, nucellus, and aleurone layer), 91 % embryo and 2 % endosperm representing 29, 5 and 66 % nutritional value, respectively [1]. The bran layer is rich in various nutritional components, such as dietary fibers, starch, oils, proteins, phenolic acids, flavonoids, anthocyanins, and proanthocyanidins tocopherols, tocotrienols, g-oryzanol, phytic acid, vitamins, and minerals [1,2]. BR is not considered a staple food because of its dark appearance and hard texture [3]. To improve the texture quality, soaking the whole grain in water, allowing germination for an appropriate period, is a choice method. In addition to improving texture quality, germination also

enhances the phytochemical content, antioxidant activity, and health functions of BR [4-7]. Amino acid biosynthesis and starch hydrolysis were increased after 24 h of imbibition [8]. Moreover, it has been reported that antioxidative activity, γ-aminobutyric acid, and other bioactive compounds were enhanced after germination for 24 h [7,9]. After 48 h of germination, radicle protrusion begins making the grain less desirable for cooking due to its appearance and taste quality [8].

Many studies reported that BR and germinated brown rice (GBR) regular consumption could reduce incidences of various chronic diseases, including diabetes, obesity, cardiovascular disease, cancer, and Alzheimer's disease [4,10-15]. Moreover, BR and GBR could function as an immunomodulator since it contains the immune system enhancing compounds including phytosterols, polysaccharides, peptides, minerals and trace minerals including magnesium, selenium, zinc, vitamin E, omega-3 fatty acids and several other potent antioxidant phytonutrients [1]. Despite the germination duration, the grain color also affects seed constituents and their nutritional values [16,17]. Purple (or black) rice that abundant in anthocyanins and proanthocyanidins had the highest total phenolics, total flavonoids, and antioxidant activity among all colors (red, brown, light brown, and white) rice [16].

Although many studies have reported an increase in nutritional components in GBR relative to BR [5,6], studies on how the proteins or metabolic pathways are changed during germination are rare. Proteomics is a global analysis technique that allows us to comprehensively analyze crop proteins and understand the functions of the genes in plant growth and development, as well as plant responses to abiotic and biotic stresses [18,19]. Proteomic studies on rice seed germination mostly target the seed developmental processes and do not explain how nutritional components increase in GBR [8,20,21]. Therefore, we aimed to study the effects of germination on changes in nutrition-associated proteins in rice grain using proteome analysis. Here, we used GeLC-MS/MS shotgun proteomics to unveil, which phytochemical biosynthetic pathways were prominently enhanced after germination. Comparative proteomic profiling of 4 different Thai brown rice cultivars; Khao Dawk Mali 105 (KDML), Pathumthani 1 (PT1), Hom Nil (HN), and Riceberry (RB), were analyzed before and after germination for 24 h. We also compared the total phenolic content (TPC) of the colorless (KDML and PT1) and the pigmented (HN and RB) grains of rice after germination for 24 h and 48 h and discussed the relevance of proteome changes in the overall alteration of phenolic biosynthetic pathways in the germinating seeds of Thai brown grains of rice.

Materials and methods

Plant materials

Seeds of indica rice (*Oryza sativa* L. ssp. *indica*) cultivars KDML, PT1, HN, and RB were obtained from the Pathumthani Rice Research Center, Rice Research Institute, Department of Agriculture, Ministry of Agriculture and Cooperative, Pathumthani, Thailand, and the Rice Science Center and Rice Gene Discovery Unit, Kamphaeng Saen, Nakhon Pathom, Thailand. The pigmented rice seeds (HN and RB) have black-purple pericarp, whereas the pericarps of KDML and PT1 are colorless. Germinated seeds were prepared as described by previous studies with slight modification [7,31]. One gram of BR seeds was soaked in 10 mL sterile water for 6 h before being germinated in the dark room for 0 (control), 24, and 48 h at room temperature. Rice grains were germinated on wet paper tissue in 3 independent glass petri dishes (20 seeds per plate). Water was removed and seed samples (3 biological replicates) were dried with blown air at room temperature for 10 min and kept at −20 °C for proteomic and TPC analysis.

GeLC-MS/MS shotgun proteomics

Total proteins were extracted from non-germinated seeds (control) and 24 h-germinated seeds of KDML, PT1, HN and RB (3 biological replicates). About five seeds from each cultivar treatment were ground in a mortar and pestle, and 100 mg of rice powder were used to extract the total proteins by trichloroacetic acid/acetone precipitation method [7,22,23]. The protein concentrations were determined by Lowry protein assay using BSA as the protein standard [24]. Twenty micrograms of the protein from each sample was pre-fractionated by 12.5 % SDS-PAGE using an Atto mini slab gel (AE-6530, Atto, Japan). Electrophoresis was performed in 1X SDS electrophoresis buffer (25 mM Tris-HCl pH 8.3, 192

mM glycine, 0.1 % SDS) until the tracking dye reached the bottom of the gel and the gel was stained with Coomassie Blue. Gels were cut into 13 pieces according to their molecular weights (14.1 - 97 kDa), estimated using molecular weight marker proteins (Amersham Biosciences, Buckinghamshire, UK). The polypeptides in each gel piece were digested with trypsin using an in-house method developed by Proteomics Laboratory, Genome Institute, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Thailand [25]. The digested peptides (about 5 - 20 amino acids) were then injected into an Ultimate 3000 LC System (Dionex, Sunnyvale, CA) coupled to an ESI-Ion Trap MS, HCT Ultra PTM Discovery System (Bruker, Rheinstetten, Germany) with electrospray at a flow rate of 300 nL min^{-1} to a nanocolumn (Acclaim PepMap 100 C18, 3 μm, 100Å, 75 μm id × 150 mm) (2 technical replicates). A solvent gradient: solvent A (0.1 % formic acid in water) and 80 % of solvent B (0.1 % formic acid in 80 % acetonitrile): was run in 40 min. The resulting mass spectra were exported for protein identification and analysis of relative protein content.

Protein quantitation, identification and bioinformatics

Mass spectra of peptides in each sample were analyzed and quantified using the DeCyder MS 2.0 Differential Analysis software (GE Healthcare Europe GmbH, Freiburg, Germany) [26,27]. The peptides' sequences were then transferred into a Mascot search engine (Matrix Science, London, UK) for protein identifications [28]. The biological functions of the non-redundant proteins were defined by searching through the InterPro database (http://www.ebi.ac.uk/interpro). The expressions of protein after germination were represented as fold change. The biological functions of the identified proteins were analyzed by Software Tool for Researching Annotations of Proteins (STRAP) version 1.5 (Cardiovascular Proteomics Center, Boston University School of Medicine, Boston, MA) [29]. Lists of the increased proteins in germinated seeds from four rice cultivars were analyzed by jvenn: an interactive Venn diagram viewer [30]. The interactions of chemicals and proteins involved in phenolic compound biosynthetic pathways were analyzed by STITCH 4.0 (http://stitch.embl.de).

Analysis of total phenolic content

Sample extraction was slightly modified from Sutharut and Sudarat [31] as described by Maksup and co-workers [7]. Briefly, ground seed samples (0.5 g) were extracted with 1 mL of methanol. Samples were vortexed for 30 s and incubated at 60 °C in a water bath for 20 min. The mixture was centrifuged at 15,115×g for 10 min and the supernatant was transferred to a fresh tube before the total volume was adjusted to 5 mL. The extracts were used to determine the total phenolic content (TPC) using the Folin-Ciocalteu assay [7,32] using a standard curve of gallic acid (0 - 0.3 g L^{-1}, $R^2 = 0.99$) to quantify the TPC. The results were expressed as g Gallic Acid Equivalent (GAE) per kg rice sample. All samples were analyzed in triplicate and the mean values were analyzed using SPSS software (SPSS for Windows; SPSS Inc. Chicago. Illinois, USA) with Duncan's multiple range tests ($p \leq 0.05$).

Results and discussion

Proteome profiles in non-germinating and germinating rice seeds are different

In this study, we examined the seed proteomes of four Thai rice cultivars. KDML and PT1, the high-end export products of Thailand, are white grain fragrant grains of rice that produce soft-textured cooked grain. Whereas, HN and RB are the black-purple grains of rice that have been reported to exhibit high antioxidant activity, high TPC [33,34] and anti-cancer activity [35]. Bran extract of RB can relieve gentamicin-induced hepatotoxicity by reducing oxidative stress, inflammation and apoptosis in rats [36]. Moreover, the pigmented rice HN can be used as a light meal and food colorants in food industries [37]. In the proteomic analysis, 369 proteins with significantly distinct expression patterns among rice seed samples were detected using GeLC-MS/MS shotgun proteomics [7,23] and identified using the Mascot software [28]. Those proteins were categorized based on their biological functions (GO: biological process) using STRAP version 1.5 [29]. The results showed that BR and GBR have different proteomic profiling and biological functions (**Figure 1**). This suggested that seed proteomes were changed after germination.

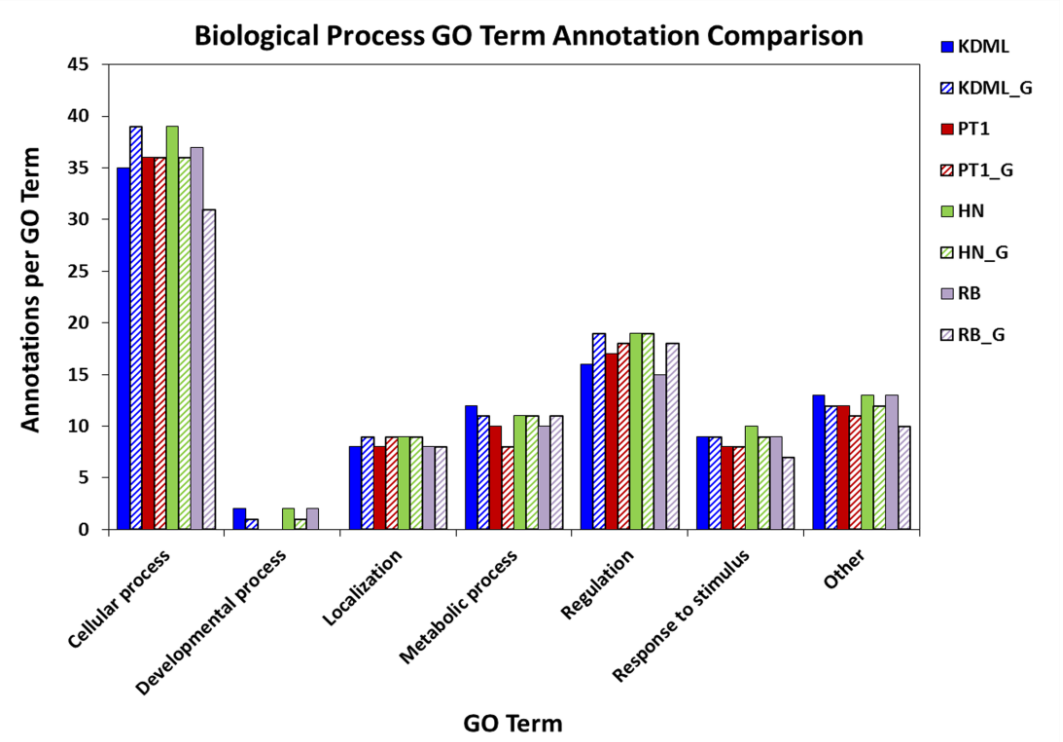

Figure 1 Comparative biological functions of the identified proteins in non-germinated and germinated brown rice seeds analyzed by Software Tool for Researching Annotations of Proteins (STRAP) version 1.5. Non-germinated seeds of Khao Dawk Mali 105 (KDML), germinated seeds of Khao Dawk Mali 105 (KDML_G), non-germinated seeds of Pathumthani 1 (PT1), germinated seeds of Pathumthani 1 (PT1_G), non-germinated seeds of Hom Nil (HN), germinated seeds of Hom Nil (HN_G), non-germinated seeds of Riceberry (RB), germinated seeds of Riceberry (RB_G).

Seed nutrition-associated proteins were increased after germination

Proteome analysis showed that among 369 identified proteins, 341 proteins (92.41 %) were up-regulated in KDML, PT1, HN and/or RB grains after germination for 24 h (**Figure 2**). This is consistent with the findings of Maksup *et al*. (2018) which reported an increase in protein content in 24 h germinated seeds of KDML and MD rices [7]. Several of the up-regulated proteins are associated with nutrition (**Table 1**). Six phenolic-associated proteins prominently increased by 2.20 - 15.90 folds after germination for 24 h. In germinated seeds of KDML, isoflavone-7-O-methyltransferase (IOMT) and glycosyltransferase family 61 protein (GT) were up-regulated by 16.2 and 2.52 folds, respectively. Isoflavonoid glucosyltransferase (IFGT), GT, phenylalanine ammonia-lyase (PAL) and serine carboxypeptidase-like protein (SCPL) were 15.90, 15.36, 3.68 and 3.64-fold increases in germinated seeds of PT1. Only GT increased in germinated seeds of HN (10.43 folds), while SCPL and UDP-glucose flavonoid 3-O-glucosyltransferase (UF3GT) increased by 7.24 and 2.20-folds in the germinated seeds of RB. PAL is a key enzyme that catalyzes the initial step in the phenylpropanoid biosynthetic pathways [38,39]. SCPL has been reported to be involved in the synthesis of the sinapoylated anthocyanins in Arabidopsis [40] and UF3GT controls the last step of anthocyanin biosynthesis [38]. IOMT, IFGT and GT plays an important role in isoflavonoid and flavonoid biosynthesis [41-43]. Notably, GT also plays a key role in arabinoxylan biosynthesis, which serves as a large reservoir of phenolic acids, especially ferulic acid [44]. Arabinoxylan fiber is one of the dietary that has various health benefits, such as reducing the glycemic index by direct inhibition of α-glucosidase activity, thus helping to alleviate the

symptom of type II diabetes [45-47]. In addition, arabinoxylans are shown to have antioxidant activity because of the presence of phenolic moieties in their molecular structures [48]. Maksup *et al*. [7] reported an up-regulation of GT in KDML after 24 h of germination. In this study, the expression of GT increased by 2.52, 15.36, and 10.43 folds in KDML, PT1 and HN, respectively (**Table 1**). The changes in this protein suggest that arabinoxylan fiber, phenolic acids and antioxidant activity may increase after germination.

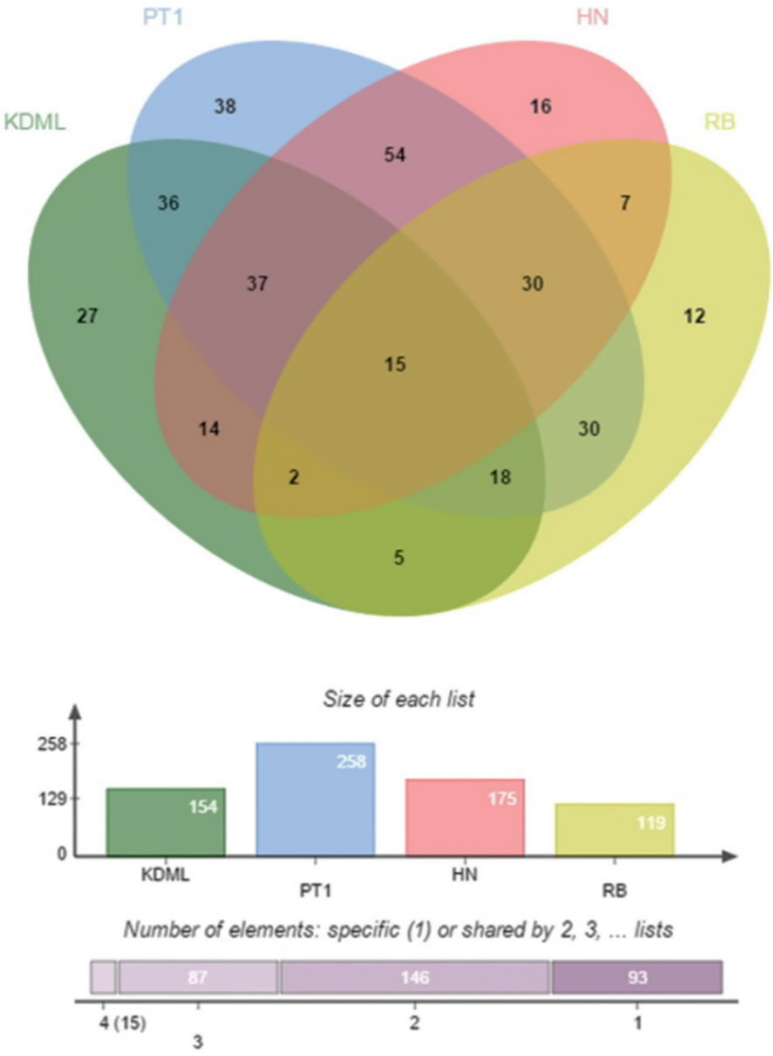

Figure 2 Venn diagram indicating overlapping and unique proteins that increased after germination for 24 h in four different Thai brown rices; Khao Dawk Mali 105 (KDML), Pathumthani 1 (PT1), Hom Nil (HN), Riceberry (RB). Total up-regulated proteins = 341 proteins. Bold numbers represent the total number of proteins identified from the four different samples analyzed by jvenn: an interactive Venn diagram viewer.

Table 1 Seed nutritional-associated proteins in germinating seeds of Thai brown rices.

Protein ID	Accession	Function	Fold change of protein expression			
			KDML	PT1	HN	RB
Phenolic-associated proteins						
Phenylalanine ammonia-lyase (PAL)	gi|657400008	Flavonoid and anthocyanin biosynthesis	-2.02	**3.68**	-1.53	-0.06
Serine carboxypeptidase-like protein (SCPL)	gi|657372174	Biosynthesis of sinapoylated anthocyanins	-7.79	**3.64**	0.63	**7.24**
Isoflavone7-O-methyltransferase (IOMT)	gi|357502395	Isoflavone biosynthesis	**16.20**	-8.20	-0.70	0.00
Isoflavonoid glucosyltransferase (IFGT)	gi|358348234	Isoflavonoid biosynthesis	-4.47	**15.90**	-14.58	1.68
Glycosyltransferase family 61 protein (GT)	gi|657383872	Flavonoid biosynthesis	**2.52**	**15.36**	**10.43**	0.00
UDP-glucose flavonoid 3-O-glucosyltransferase (UF3GT)	gi|657398130	Anthocyanin biosynthesis	1.00	-2.64	-0.15	**2.20**
Other seed nutritional-associated proteins						
3,4-dihydroxy-2-butanone 4-phosphate synthase	gi|657400075	Biosynthesis of riboflavin (vitamin B2)	**7.18**	-9.65	-0.95	-0.78
Lipase	gi|657405566	Lipids hydrolysis	**2.64**	-15.27	-2.42	-0.58
3-ketoacyl carrier synthase III	gi|657400458	Fatty-acid biosynthesis	-10.79	**2.93**	-2.69	-16.05
Cytochrome P450 family 709 protein	gi|657379610	Biosynthesis of terpenoids	-9.83	0.00	1.64	-5.41
Purple acid phosphatase	gi|657394296	Purple color (oxidised form)	-0.16	-2.27	0.00	**3.46**
Glutelin (Os02g0453600)	gi|113536300	Glutelin, seed storage protein	**2.38**	-18.70	0.54	**5.37**
Sucrose synthase	gi|657398021	Sucrose synthesis	0.03	**6.33**	0.75	1.81
Glutathione S-transferase	gi|657379669	Antioxidant	-8.76	1.37	**3.22**	1.37

Note: Proteins that the expression was up-regulated more than 2-folds are shown in bold.

Other seed nutritional-associated proteins such as 3, 4-dihydroxy-2-butanone 4 phosphate synthase, lipase, 3-ketoacyl carrier synthase III, cytochrome P450 family 709 protein, purple acid phosphatase, glutelin, sucrose synthase and glutathione S-transferase, are shown in **Table 1**. They play important roles in the biosynthesis of vitamin B2 [49], fatty acids [50], terpenoids [51], sucrose, and seed storage

proteins [52]. Glutelin is one of rice seed storage proteins that exhibits better digestibility and antioxidant activity than total protein and prolamin [52]. Thus, the observed germination enhancement of glutelin content in KDML and RB may improve the antioxidant capacity in germinated seeds of these cultivars. An increase in abundance of the purple acid phosphatase enzyme was observed in germinated RB. As this enzyme turns purple in its oxidized state [53] it may, at least in part, contribute to the purple color of the RB grain.

Interactions of chemicals and proteins involved in phenolic compounds biosynthetic pathways

The analysis by STITCH 4.0 unveiled the lists of proteins and chemicals that are probably associated with our interesting proteins. Stronger associations are represented in thicker lines. Protein-protein interactions are blue, whereas chemical-protein interactions in green, and interactions between chemicals in red. In this study, proteins and chemicals that interact with the identified proteins, PAL, SCPL, GT and UF3GT, were investigated as displayed in **Figure 3**. Many proteins including anthocyanin 5-O-glucosyltransferase (AT4G14090), dihydroflavonol 4-reductase (DFR), cinnamate-4-hydroxylase (C4H), 4-coumarate:CoA ligase 1 (4CL1), arogenate dehydratase 3 (PD1) and arogenate dehydratase (ADT1, ADT2, ADT5 and ADT6), and phenolic compounds such as anthocyanin, isoflavone, cinnamic acid, and flavonol have been reported to interact with PAL and UF3GT (**Figure 3**). These proteins function as enzymes that directly or indirectly participate in the phenolic compound biosynthetic pathways. Based on this analysis, SCPL and GT did not directly participate in the phenolic compound biosynthetic pathways and there are no reports about the linkage to IOMT and IFGT. Thus, this is the first to report that SCPL, GT, IOMT and IFGT are involved in phenolic biosynthesis in GBR.

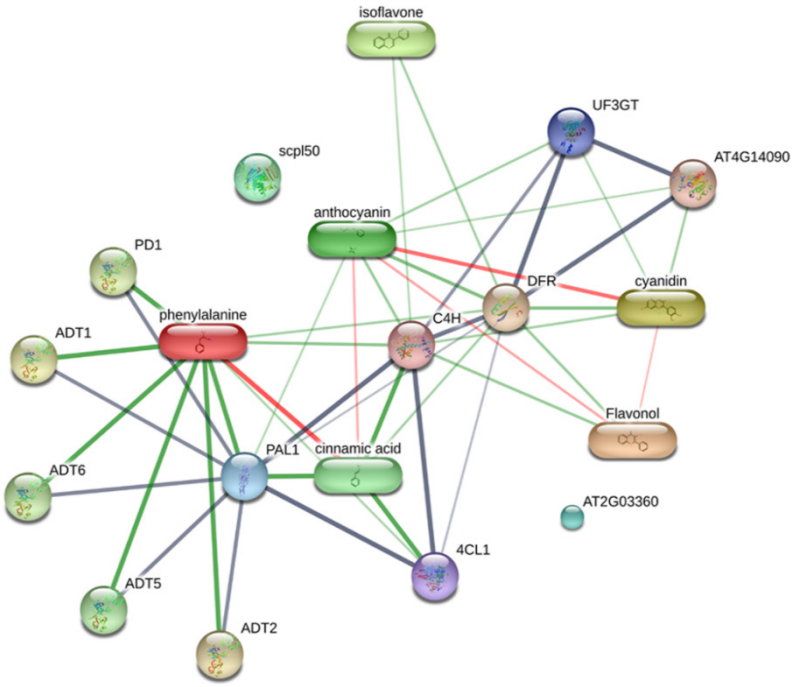

Figure 3 The interactions of chemicals and proteins involved in phenolic compound biosynthetic pathways analyzed by STITCH 4.0 (http://stitch.embl.de). Stronger associations are represented by thicker lines. Protein-protein interactions are shown in blue, chemical-protein interactions in green, and interactions between chemicals in red. Abbreviations: PAL1, phenylalanine ammonia-lyase; UF3GT, UDP-glucose:flavonoid 3-O-glucosyltransferase; scpl50, serine carboxypeptidase-like 50; AT2G03360, glycosyltransferase family 61 protein.

Total phenolic contents were increased in germinated brown rices

In the previous decade, the consumption of GBR extensively rose since it could provide a steadier supply of bioactive compounds for maintaining health and preventing complications in several diseases than BR [4,9,54]. Many publications report that bioactive compounds increase after germination [5,6,7,9]; however, there is a lack of detailed information concerning germination effects on these compounds. In this study, the TPC analyzed from the crude methanol extract of Thai brown grains of rice; PT1, HN and RB, significantly ascended after germination for 24 and 48 h (**Figure 4**). Notably, the TPC of HN significantly increased after germination for 24 and 48 h by 1.22 and 2.16 folds, respectively. Moreover, the TPC of RB significantly arose by 1.17 and 1.66 folds after germination for 24 and 48 h (**Figure 4**). The increase of phenolic content in GBR corresponding to the up-regulation of 6 phenolic-associated proteins (**Table 1**) indicated that these proteins might assume duties in the phenolic biosynthesis of GBR.

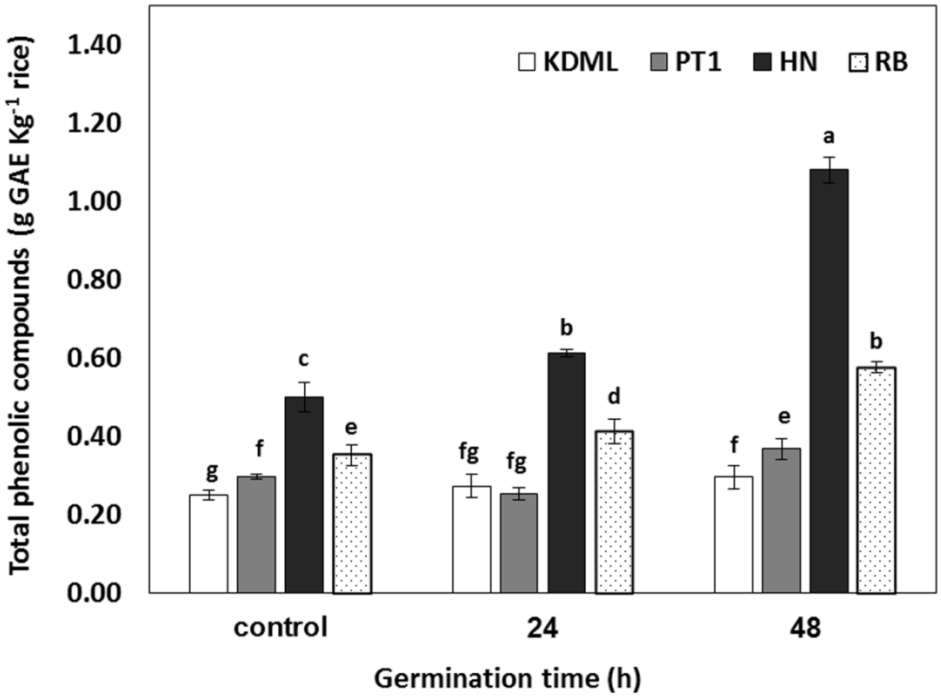

Figure 4 Total phenolic content analyzed from the crude methanol extracts of Thai brown rices; Khao Dawk Mali 105 (KDML), Pathumthani 1 (PT1), Hom Nil (HN), Riceberry (RB), after germination for 0 (control), 24 and 48 h. Bars represent the mean ± SD and the different letters indicate a significant difference ($p \leq 0.05$) analyzed by Duncan's multiple range tests.

Based on this proteome and phenolic compounds analysis, the schematic of the phenolic compounds biosynthetic pathways in germinating seeds of Thai brown rice is proposed, as shown in **Figure 5**. The up-regulation of these 6 phenolic-associated proteins may intensify the synthesis of phenolic compounds, such as sinapoylated anthocyanins, flavones, isoflavones, flavonols, flavan-3ols, and anthocyanins in GBR (**Figure 5**). Other chemicals and proteins involving in phenolic biosynthetic pathways may also increase (**Figure 3**). Therefore, the results suggest that the biosynthetic pathways of phenolic compounds were prominently enhanced in GBR. Moreover, the increases in TPC in seeds of germinated pigmented grains of rice, HN and RB, were significantly greater than in the colorless grains of rice, KDML and PT1. These results are similar to previous studies, which described the black-purple rice as being relatively more abundant in phenolic compounds, flavonoids, and antioxidant activity in comparison to other

pigmented-brown rices [16,55-57]. Our results are consistent with those of Na Jom and co-workers, who comprehensively analyzed metabolite profiling of four germinating Thai pigmented indica types of rice, including Hom Daeng (red), RB (purple), HN (black) and KDML (colorless) over 24 and 48 h of germination and found that the black and purple rices contained higher levels of bioactive compounds than the red and colorless rices [57]. Thus, the pigmented GBR would provide the highest health-related benefits for consumers.

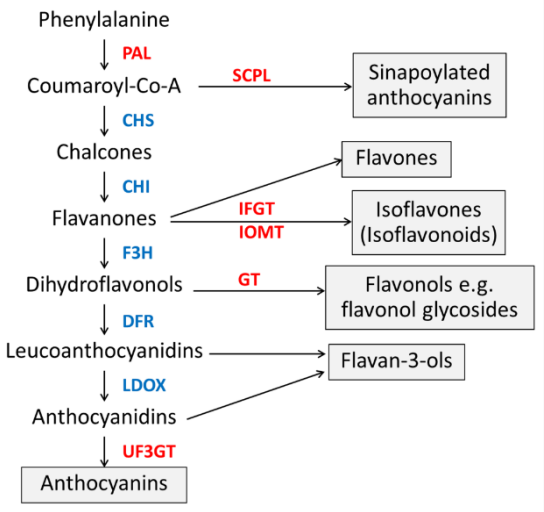

Figure 5 Scheme of the phenolic biosynthetic pathways in germinating seeds of Thai brown rices. Enzymes for each step are shown in bold; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone-3-β-hydroxylase; DFR, dihydroflavonol-4-reductase; LDOX, leucoanthocyanidin dioxygenase. End products of phenolic compounds are in gray boxes. The identified proteins in this study: PAL, Phenylalanine ammonia-lyase; SCPL, Serine carboxypeptidase-like protein; IFGT, Isoflavonoid glucosyltransferase; IOMT, Isoflavone-7-O-methyltransferase; GT, Glycosyltransferase family 61 protein; UF3GT, UDP-glucose flavonoid 3-O-glucosyltransferase are in red.

Conclusions

Germination increases phenolic-associated proteins and other seed nutritional-associated proteins as well as TPC in BR. As a consequence, the germination process can be utilized to develop the quality of BR. The biosynthetic pathways of phenolic compounds were conspicuously enhanced by germination, especially in the pigmented GBR (HN and RB). These results indicate that pigmented GBR are natural sources of phytochemicals considered very useful for the development of future functional foods and additives.

Acknowledgements

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