**Original paper**

**Differentiation of Four Varieties of Germinating Thai Colored Indica Rice** (*Oryza sativa* L.) by Metabolite Profiling

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A comparative metabolite profiling procedure was applied to differentiate four different Thai colored indica rice (*Oryza sativa* L.), including Hom Daeng (red), Rice Berry (purple), Hom Nin (black) and Hom Mali 105 (non-colored) over the course of germination. The samples taken during germination at 28 – 30°C with 90 – 95% relative humidity for a total of 48 h were subjected to an extraction and fractionation procedure, covering a wide spectrum of lipophilic and hydrophilic low molecular weight constituents. A clear differentiation by principal component analysis indicated that all samples represented similar pattern direction along PC1 according to metabolic changes during the germination process. A differentiation at every stage of germination was observed along PC2 for each of the varieties of colored rice. Relative quantifications of selected metabolites exhibited dynamic changes in the metabolites at different germination stages. Thai black and purple rice contained higher levels of metabolites than the red and colorless samples.

Keywords: Thai colored indica rice, germinating Thai colored rice, metabolite profiling

**Introduction**

Metabolomics is a potentially promising analytical approach for yielding massive data sets aimed at facilitating an improved understanding of the dynamic biochemical composition within plant systems (Dixon et al. 2006). Unbiased metabolite profiling has been identified as a comprehensive and exemplary analytical methodology for the simultaneous detection of heterogeneous plant matrices (Castro and Manetti 2007). It aims at extracting, detecting, identifying, and quantifying a broad spectrum of compounds to give a deeper insight into complex plant metabolism (Fiehn 2002). This technique can generate a comprehensive overview of low molecular weight compositional analysis in agricultural food crops (Davies et al. 2010). Thus, it can be considered as an effective tool to assist in improving nutrition, safety, stability, processability, and other desirable characteristics to meet current consumer requirements worldwide (Dixon et al. 2006; Iijima and Aoki 2009).

GC-based metabolite profiling methods have been employed to investigate a phenotypic diversity of food crops (Davies 2007; Matsuda et al. 2012), assess compositional variability due to the environmental impacts (Semel et al. 2007), differentiate different input systems, i.e. conventional versus organic farming (Röhlig and Engel 2010), as well as monitor the plant developmental process (Fait et al. 2006). Metabolite profiling of sprouting plant-based food has captured increasing attention in the last few years. GC-based metabolite profiling techniques were also applied to follow the natural development of plant-derived food, for example, the metabolic changes in the course of germination of rice (Shu et al. 2008), potato (Shepherd et al. 2010), barley (Frank et al. 2011) and mung beans (Na Jom et al. 2011), among which germinated rice has commonly received great attention (Wu et al. 2013). Germinated rice and its related products have been available in Chinese, Japanese, and South East Asian markets and have become

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Once removed, samples were immediately frozen in liquid nitrogen with an automatic sprinkler. The germinated seeds were removed double layers of cotton cloth and incubated in a germinating cloth and placed in a plastic basket. This basket was covered by water at 28 °C for 4 h. The samples were then distributed on double layers of cotton cloth (gray), Hom Nin (black) and Hom Mali 105 (colorless) varieties (purple), including Hom Daeng (red), Rice Berry (purple), Hom Nin (black) and Hom Mali 105 (colorless) varieties all grown at Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand during the 2012 growing season were used in this study. Not only sprouting rice, but also colored rice is currently highly consumed in and exported from Thailand and other countries in South East Asia (Dawe 2002). Therefore, the aim of this study was to further investigate and complete an understanding and a perspective of germinated rice metabolite profiling by applying a GC-based metabolomic procedure to quantify the major constituents in four different Thai colored indica rice samples.

Materials and Methods

Chemicals Internal standards (tetracosane, 5α-cholestan-3β, phenyl-β-D-glucopyranoside, p-chloro-L-phenylalanine) and retention time standards (undecane, hexadecane, tetracosane, triacontane, octatriacontane) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Reference compounds were obtained from Merck (Darmstadt, Germany) and Fluka (Taufkirchen, Germany). All other reagents and solvents were of analytical grade, distributed by Fischer Scientific (Fair Lawn, NJ, USA).

Sample materials Paddy Thai colored and colorless indica rice of *Oryza sativa* L., including Hom Daeng (red), Rice Berry (purple), Hom Nin (black) and Hom Mali 105 (colorless) varieties all grown at Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand during the 2012 growing season were used in this study.

Germinated rice preparation Paddy rice was dehusked with a NW 1000 Turbo laboratory de-husker (Thongtrawee, Thailand). Subsequently, 50 g of the dehusked rice was soaked in distilled water at 28 – 30 °C for 12 h. The soaking water was changed every 4 h. The samples were then distributed on double layers of cotton cloth and placed in a plastic basket. This basket was covered by double layers of cotton cloth and incubated in a germinating chamber at 28 – 30°C, with 90 – 95% relative humidity, equipped with an automatic sprinkler. The germinated seeds were removed at the following intervals: after 0 (non-germinating), 24, and 48 h. Once removed, samples were immediately frozen in liquid nitrogen and finely ground (80 mesh) using a SR 300 rotor mill (Retsch, Haan, Germany). The powder was freeze-dried for a total of 48 h using a Heto-Holten A/S, Allerød, Denmark. The freeze-dried samples were stored at −20°C until analysis.

Extraction and fractionation procedures The extraction and fractionation of the freeze-dried rice flour was performed by a previously described protocol (Frank et al. 2007). Lipids and polar compounds were consecutively extracted from the flour. The lipids were transesterified in methanol, and subsequently separated by solid phase extraction (LiChrolut Si, Merck, Germany) into a fraction containing fatty acid methyl esters (FAME) and hydrocarbons (fraction I), and a fraction containing minor lipids, e.g. sterols and free fatty acids (fraction II). Selective hydrolysis of silylated derivatives was applied to separate the polar extract into a fraction containing silylated sugars and sugar alcohols (fraction III), and a fraction containing organic acids and amino acids (fraction IV).

Analysis and identification of constituents The four fractions obtained were analyzed by gas chromatography (GC/FID, GC/MS). Fractions II and IV were silylated with N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) before GC analysis. The injection volume was 1 μL. The GC conditions were in accordance with previously described procedures (Frank et al. 2007; Na Jom et al. 2011). The GC/FID analysis was undertaken with a 6890N GC machine equipped with a flame ionization detector (Agilent Technologies, Palo Alto, CA, USA), using a DB-1, 60 m x 0.32 mm i.d. fused silica capillary coated with a 0.25-μm film of polydimethylsiloxane (J&W Scientific, Folsom, CA). Hydrogen was employed as the carrier gas (flow 1.8 mL/min). Split injection (1:14) was performed at 250°C. The column temperature was set from 100 to 320°C (25-min hold) at 4°C/min. GC/MS analysis was undertaken with a 6890 GC machine equipped with a HP5973 mass selective detector with electron ionization (EI) ion source (Agilent Technologies, Palo Alto, CA, USA). A factorFOUR VF-1 ms, 60 m x 0.32 mm internal diameter (ID) column, coated with a 0.25 μm film of 100% polydimethylsiloxane (J&W Scientific, Folsom, CA) was used. Following a delay of 6 min in the solvent, full scan mass spectra were observed and recorded. The scan range was 40 – 700 m/z at an electron energy of 70 eV, and a source temperature of 230°C. Rice constituents were identified by comparing retention times and mass spectra with those of the authentic reference compounds, and by comparing mass spectra with the entries of the mass spectra libraries NIST11.

Statistical analysis Rice samples were analyzed in triplicate. GC-FID data were acquired and integrated with HP-ChemStation A.06.03 software (Hewlett Packard, USA). The peak height and retention time data was exported to Chrompare 1.1 (Frenzel et al. 2003) for standardization and consolidation of the data. In order to analyze the relationship among different samples containing multiple variables, principal component analysis (PCA) was
performed using XLSTAT version 2011.1.01 (Addinsoft, NY, USA). Additionally, by using the aforementioned XLSTAT, significant differences between means in the ordination space were tested by multivariate analysis of variance (MANOVA).

**Results and Discussion**

Hom Daeng (red), Rice Berry (purple), Hom Nin (black) and Hom Mali 105 (colorless) indica rice grains allowed to proceed through to germination after dehusking. The developmental stages in the course of incubation are shown in Fig. 1. The growth of seeds is accompanied by monocotyledonous morphological developments involving the initial enlargement of the rice embryos and the subsequent formations of shoots and roots. This phenomenon is in agreement with the germination progress previously described for brown rice (Shu et al. 2008). The developmental changes upon sprouting process of non-colored, red, purple, and black Thai indica rice samples were comparable. However, a distinct thicker root after 48 h of incubation could be visually observed on Thai purple and black indica rice.

Samples of rice taken in the course of the germination were subjected to a metabolite profiling procedure as previously described for the investigations of colored rice (Frank et al. 2012). The procedure resulted in four fractions containing respectively: fatty acid methyl esters and hydrocarbons (fraction I); free fatty acids, alcohols, tocopherols and sterols (fraction II); sugars and sugar alcohols (fraction III); and acids, amino acids and amines (fraction IV). The fractions of non-polar and polar compounds were analyzed by capillary gas chromatography. The resulting peak heights and corresponding retention times were standardized by means of Chrompare, a software tool developed for comparative analysis of metabolite profiling data (Frenzel et al. 2003). Statistics on the consolidated data were conducted using a principal component analysis (PCA).

The combined four fractions PCA score plot of the seeds showed the differentiation of four Thai indica rice samples (Fig. 2A). The results, therefore, clarified the influence of the differences in varieties between Thai colored indica rice on their metabolite profiles. A comparable result was also found in another metabolite profiling study in colored rice grain (Frank et al. 2012). In addition, the plot revealed Thai purple and black indica rice positioned nearby to each other. These observed results could be attributed to the dark colored characteristic of both types of rice.

Fig. 2B shows the scores plots of the four combined fractions obtained for the data on germinating Thai non-colored and colored indica rice at different stages of the germination process. The metabolic changes were reflected by time-dependent shifts of the scores among the various rice samples owing to the impact of germination by the separation of the respective clusters along the first two principal components PC1 and PC2. All four Thai rice samples showed a similar pronounced pattern direction with the U-shape depending on the time of germination. In addition, a clear clustering due to the coloration of Thai rice samples was observed. Difference in germination time was represented along PC1, from left to right. The represent of Thai colored rice varieties was indicated along PC2 (Fig. 2B). Moreover, a cluster of Thai purple and black indica rice was located in the nearby score pattern. The distance between Thai red and colorless indica rice examined during sprouting was less pronounced than the distance between Thai black and colorless indica rice (Fig. 2B). These differentiations accord with the metabolomics of colored rice grains, not having undergone germination (Frank et al. 2012). Similar results were also reported in metabolite profiling of germinating brown rice seeds (Shu et al. 2008). Analysis of the corresponding loadings of selected peaks based on reference standard identification in fraction I-IV, polar metabolites were found to be major contributors to the separation along the first principal component, whereas non-polar metabolites were less responsible for the dissociation, thus implying that more noticeable changes might be observed for the polar metabolites during Thai rice sprouting (Fig. 3). As the results in the PCA indicated, free fatty acids and fatty alcohols in the sprouting Thai rice samples were found to be predominantly decreased. On the other hand, polar metabolites primarily exhibited increased levels over the course of the germination process (Fig. 3). The observation was discussed in the similar way by metabolite profiling studies of
Fig. 2. Principal component analysis (PCA) of standardized GC/FID metabolite profiling data of the combined fractions I-IV of (A) dehusked indica rice grains and (B) samples in the course of germination. (◆ non-color (Hom Mali 105), ■ red (Hom Daeng), ▲ purple (Rice Berry), ● black (Hom Nin))

Fig. 3. Loading graphs of standardized GC/FID metabolite profiling data from the non-polar fractions (A) and the polar fractions (B).
germinating rice seeds (Shu et al. 2008), malting barley (Frank et al. 2011), and sprouting mungbean (Na Jom et al. 2011). The dynamic changes among the germinating Thai rice samples in the selected non-polar and polar metabolite profiles identified based on the reference standards were illustrated by means of a heat plot (Fig. 4), followed by the relative quantitative analysis obtained from the different compound classes in the course of germination process (Fig. 5). Results from the heat plot clearly show that Thai black indica rice contained the highest content of most low molecular weight constituents at every stage of germination among the four Thai rice varieties tested (Fig. 4).

The major fatty acid methyl esters (FAME) in fraction I resulting from transesterification of the crude lipid extract indicate the rice triglycerides. By means of the dehusked grain, Thai black indica rice showed the highest levels of FAME. These results are consistent with the previous report, in which black rice varieties typically contain higher amount of lipid than other varieties (Frank et al. 2011; Sompong et al. 2001; Yoshida et al. 2010). Within the first 24 h of incubation, changes of most FAME contents were slightly increased. However, after 24 h all four Thai rice samples yielded the maximum level of FAME and the quantity remained constant afterward (Fig. 5A). The major FAMEs were found to achieve the highest levels after 24 to 48 h during malting of barley (Frank et al. 2011) and sprouting of mung beans (Na Jom et al. 2011). However, it should be noted that germinating conditions have an influence on the degree of lipid degradation such as moisture, temperature, and incubation time. In addition, the time-dependent alterations for the representative hydrocarbons are shown in Fig. 5A. Overall, changes of these compounds during germination were relatively small, except squalene in Thai purple and black rice samples. Nevertheless, hydrocarbons determined by metabolite profiling of germinating brown rice significantly increased during the course of germination (Shu et al. 2008). The condition of incubation might play an important role in the change of hydrocarbon levels during sprouting of Thai rice grain as well. Interestingly, the amount of hydrocarbon in Hom Nin (black)

Fig. 4. Heat plots of the non-polar (A) and polar fractions (B) in the course of germination. Metabolite levels correspond to the color temperature. Higher temperature indicates higher levels of the respective compound. FAME: fatty acid methyl ester, ffa: free fatty acid.
variety was approximately 1.5 and 2 folds higher than that of Hom Daeng (red) and Hom Mali (colorless) variety through the germination process, respectively. The amount of hydrocarbons in black rice was reported as 2–5 folds higher than red rice (Frank et al. 2011; Yoshida et al. 2010).

The changes of the metabolites existing in fraction II, substituting free fatty acids and fatty alcohols were less pronounced. No significant trend of overall changes by those

Fig. 5. Standardized peak heights for selected compounds determined in the non-polar fractions (A) and the polar fractions (B) in the course of the germination of Thai colored indica rice samples. (◆ non-color (Hom Mali 105), ■ red (Hom Daeng), ▲ purple (Rice Berry), ● black (Hom Nin))
compounds during the germination process could be observed. Hence, these results might reflect an insufficient germination time to follow the alterations of all minor lipids in the course of sprouting. Within 24 h, free fatty acids and fatty alcohols increased, but then decreased after 24 h (Fig. 5A). The previous study reported that the concentrations of free fatty acids increased in the initial stage of the incubation, but decreased rapidly after 72 h in germinating brown rice (Shu et al. 2008). Another study also found that palmitic and linoleic acids increased in germinating brown rice Keunnun (Choi and Kim 2009). All phytosterol levels were drastically increased over the course of germination from 0 to 48 h (Fig. 5A). It was previously reported that the levels of phytosterols and tocopherols increased significantly in soybean during the 3-day germination (Shi et al. 2010); however, no significant tendency could be observed in germinated rice (Shu et al. 2008). The total amounts of free fatty acids, fatty alcohols, and sterols in black rice (Hom Nin) were approximately 3–5 times higher than those in red (Hom Daeng) and colorless (Hom Mali 105) rice through the incubation process (Fig. 5A). The 30% higher levels of free fatty acids in black rice seeds were also reported in the literature (Frank et al. 2011).

In the case of polar metabolites, the overall changes in metabolic profiles of Thai colored and non-colored indica rice seeds during the germination process were more noticeable than those of the lipid fractions. Quantities of monosaccharides (e.g. fructose, glucose) increased dramatically throughout the germination period, whereas disaccharide sucrose and trisaccharide raffinose were significantly decreased (Fig. 5B). These changes might reflect the breakdown of starch or polysaccharide, which subsequently convert to monosaccharide. The results generally correspond well with the previous metabolite profiling-based studies on germinated brown rice (Shu et al. 2008), barley (Frank et al. 2011) and mung bean (Na Jom et al. 2011). The significant increase of total sugars and reducing sugar in germinating rice after 24 h of incubation was at most 15% higher than the intact rice seeds (Moongngarm and Saetung 2010). The degradation in the amounts of raffinose oligosaccharides indicates that trisaccharides act basically as a donor for the distribution of energy supply at the beginning of germination (Da Silva Ferreira et al. 2009). In the same way as lipid changes due to germination, starch degradation and small sugar molecule accumulation in germinating colored rice are highly influenced by germination conditions (Wang et al. 2006). However, no consistent change during incubation of germinating Thai colored indica rice was observed in the contents of myo-inositol and glycerol. The highest concentration of overall sugars at every stage of sprouting was observed in Thai black and purple indica rice samples (Fig. 4 and 5B). Black rice variety was observed with higher amounts of sugars and sugar alcohols than rice and colorless variety as determined by the metabolite profiling approach (Frank et al. 2012).

The quantities of amino acids, organic acids, and amines obtained from fraction IV were elevated continuously during the course of germination (Fig. 5B). The proteolytic cleavage of the proteins during germination led to a significant increase in the levels of amino acids. An increase of those compounds was also seen in barley during malting (Frank et al. 2011) and mung bean in the course of sprouting (Na Jom et al. 2011). Relative changes in this fraction were observed as well in germinating non-colored rice by the metabolite profiling-based methodology, in which up to +800% higher content of amino acid was obtained in germinating rice (Shu et al. 2008). Target selected amino acids including glutamic acid, serine, and asparagine revealed approximately 8, 13, and 16-fold increases after 24 h of germination, respectively. Similar results were reported in colorless germinating rice (Shu et al. 2008) and sprouting mung bean (Na Jom et al. 2011). It was established that the total glutamic acid content increases significantly in soaked rice samples (Roohinejad et al. 2011). In contrast, the amount of glutamic acid decreased (~76%) upon germination (Komatsuzaki et al. 2007) and reduction in both aspartic acid and asparagine was observed as well (Shu et al. 2008). Therefore, the conclusion to be drawn from these studies is that, changes in amino acid levels are strongly influenced by the germination condition. Different rice germination procedures might significantly affect the changes in the content of amino acids. A 6 to 47-fold rise in γ-aminobutyric acid (GABA) was obtained in germinated Thai colored indica rice materials (Fig. 5B). The previous study reported that the GABA content increased steadily and reached the maximum level of approximately 6-fold increase in germinating brown rice after 24 h of incubation, followed by a drastic decrease (Karlaadee and Suriyong 2012). A maximum increase in the GABA content of approximately 2500% was reported in soaked and germinated brown rice compared with ungerminated rice (Ohtsubo et al. 2005). However, the change of GABA content during soaking and germination is highly dependent on the rice variety and the germination condition (Wang et al. 2006; Saikusa et al. 2004). Since GABA is principally a biogenic amine of glutamic acid, an observably enhanced content of GABA might provide a distinct advantage because of its health promoting effects (Ito 2004). A comparison among germinating Thai colored indica rice materials reveals that Hom Nin (black) and Rice Berry (purple) varieties primarily contained higher levels of amino acids, organic acids, and GABA than Hom Daeng (red) and Hom Mali 105 (non-colored) varieties at every stage of germination. The recent study of metabolite profiling obtained from rice grain and black rice seed also demonstrated higher amounts of acids (130%), amino acids (70%) and GABA (360%) than red and non-colored rice grain (Frank et al. 2012).

Conclusion

In conclusion, the present study further more specifically describes the comprehensive differentiation of four germinating Thai colored indica rice materials by means of determining various
low molecular weight compounds known as the metabolite profiling approach. This applied methodology combined with a suitable statistical analysis is a powerful tool to record remarkable metabolic changes and reveal a separation among Thai colored indica rice through the germination process. According to the combination of the applied extraction and fractionation procedures, the method enables the coverage not only a wide range of polar and non-polar compounds (e.g. sugars, fatty acids) but also nutritionally relevant metabolites (e.g. tocopherol, phytosterols, γ-aminobutyric acid). Therefore, a comprehensive metabolite profiling should provide significant information that goes beyond single relevant nutritional targeted analyses, group of metabolic constituents, and differentiation of attractive germinating Thai colored indica rice based on genotype, phenotype as well as growing conditions. In the current work, we demonstrated that this technique could be used to observe the elevated amounts of several desirable plant compounds, such as sterols, sugars, amino acids, and GABA during germination of four varieties of Thai colored indica rice during the germination process. Moreover, the employed method could aid in differentiating samples not only of the dehusked grains, but also the germinating materials. Thus, our obtained results should be able to support and improve an understanding further of rice metabolomics. In order to gain insights in to the impact of environmental conditions on the metabolite profiles of differently Thai colored rice grains as well as the safety assessment of genetically modified Thai colored rice, the metabolite profiling approach could be utilized for further investigations.

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