Black rice cultivar from Java Island of Indonesia revealed genomic, proteomic, and anthocyanin nutritional value

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Black rice is considered to be functional food containing anthocyanins as bioactive compounds. This study examined the genomic and proteomic patterns in local black rice from Java Island, Indonesia, with attention to the mechanism of anthocyanin synthesis. Three kinds of black rice from Java Island, including black rice from East Java (BREJ), black rice from Central Java (BRCJ), and black rice from West Java (BRWJ), were studied in comparison to white rice (WREJ) and red rice (RREJ). Genomic profiling was done by simple sequence repeat (SSR) analysis, and sequencing of red coleoptile (Rc) and glycosyltransferase (GT) genes, followed by in silico analysis. Total anthocyanin was investigated by ultra-high performance liquid chromatography-diode array detector (UHPLC-DAD). The proteomic profiles were determined by liquid-chromatography and mass spectrometry of tryptic peptides. The SSR profiles showed a specific band in each black rice variant. The Rc gene exon-2 sequences were similar in the three black rice cultivars. The GT gene sequence was identified as a new variant that correlates with the purple stem, leaf, bran, and whole grain morphology seen exclusively in the BRWJ cultivar. The anthocyanin composition in Java black rice is diverse. The highest cyanidin level was seen in BRWJ and the highest level of peonidin-3-O-glucoside in BREJ. Proteomic profiling of the black rice cultivars demonstrated that the expression of proteins that might be related to the levels of anthocyanin synthesis varied. These studies conclude that the genomic, proteomic, and anthocyanins composition of Java black rice cultivars may be used the improvement of their functional nutrition values.

Keyword: anthocyanins, black rice, genomic, glycosyltransferase, proteomic

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Abbreviations: NS, anthocyanin synthase; ANR, anthocyanin reductase; bHLH, basic helix loop helix; BREJ, black rice from East Java; BRCJ, black rice from Central Java province; BRWJ, black rice from West Java province; c, coding sequence; CHS, chalcone synthase; CHI, chalcone isomerase; CTAB, hexadecyltrimethylammonium bromide; DAD, diode array detector; del, deletion; DFR, dihydroflavonol reductase; DNA, deoxyribonucleic acid; F3H, flavonone-3-hydroxylase; GT, glycosyltransferase; ins, insertion; MBW, MYB-bHLH-WD40; MS, Mass spectrometry; MS/MS, tandem mass spectrometry; MYB, myeloblastosis transcription factor; OsPAL, Oryza sativa phenylalanine ammonia lyase, OsCHS, Oryza sativa chalcone synthase, OsGT, Oryza sativa 3-glycosyltransferase; OsGST, Oryza sativa glutathione-S-transferase; OsLDOX, Oryza sativa leucoanthocyanidin oxidase; OsMT, Oryza sativa methyltransferase; OsWD40, Oryza sativa beta transducing; PAL, phenylalanine ammonia lyase; Rc, red coleoptile; RM, Rice marker; RREJ, red rice East Java province; SNPs, single nucleotide polymorphisms; SSR, Simple sequence repeat; subs, substitution; UPGMA, unweighted pair-group method with arithmetic average; UHPLC, ultra-high performance liquid chromatography; WREJ, white rice East Java province.

INTRODUCTION

Pigmented rice, including brown, red, and black rice (Oryza sativa L.) has a high nutritional value, including high amounts of amino acids and beneficial phytochemical compounds (Sivamaruthi et al., 2018; Fatchiyah et al., 2020a). For instance, previous studies have reported that bioactive compounds in pigmented rice extract have antioxidant (Anisimovien et al., 2013), anti-inflammatory (Sari et al., 2019a), anti-diabetes (Azzini et al., 2017), anti-obesity (Thompson et al., 2016; Yan & Zheng, 2017), and anti-apoptosis (Sari et al., 2020a) activities. The bioactive compounds in pigmented rice include the anthocyanins responsible for rice pigmentation (Hou et al., 2013). Anthocyanins accumulate in various rice tissues such as leaves, stems, anthers, and grains (Zheng et al., 2019; Zaidi et al., 2019; Prasad et al., 2019). Samyoor and others (Samyoor et al., 2017) identified cyanidin, cyanidin-3-O-glucoside, peonidin-3-O-glucoside, and peonidin in black-purple rice grains, while Zheng and others (Zheng et al., 2019) reported anthocyanin content in black rice leaves.

Anthocyanins are influenced by several environmental factors and regulated by genetic variability and transcription factors (Chin et al., 2016; Zheng et al., 2019). Genes related to anthocyanins’ production that have been well studied include structural genes encoding the enzymes: phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), anthocyanin synthase (AN3), flavonol-3-hydroxylase (F3H), dihydroflavonol reductase (DFR), and anthocyanin reductase (ANR) (Chen et al., 2013). Furthermore, these anthocyanin synthesis genes are regulated by several transcription factors, such as MYB, MYC, R, and C-S-A (Himi & Taketa, 2015).
**Red coleoptile** (*Re*) is a gene encoding a basic helix loop helix (bHLH) transcription factor protein mapped on chromosome 7 (Xu et al., 2015). The *Re* gene is associated with the rice domestication process. The *Re* gene has seven exons, and a mutation in *Re* exon 7 causes a frameshift in the open reading frame, thereby producing a dysfunctional bHLH protein to switch off anthocyanin gene expression in white rice (Furukawa et al., 2006; Sweeney et al., 2006; Zhu et al., 2019). However, mutations and variations in other exons of the *Re* gene are less well characterized. In their functional forms, bHLH proteins with WD40 and MYB form an MBW (MYB, bHLH, and WD40) complex to activate anthocyanin gene expression, including that of DFR, L-4R, AN3, ANR, and glycosyltransferase (*GT*) (Albert et al., 2014).

The *GT* gene encodes the glycosyltransferase protein that transfers sugar residues to anthocyanidins (Chen et al., 2016). Glycosyltransferases constitute a large group of proteins, which have several functions in plant metabolism and physiology (Cao et al., 2008). The *GT* genes contribute to rice anthocyanin biosynthesis (Moon et al., 2013), hormone inactivation (Luangphas et al., 2013), structural polysaccharide formation, and anthocyanin biosynthesis (Sun et al., 2016; Wang et al., 2018; Liu et al., 2020). In the anthocyanin synthesis, glycosyltransferases add a sugar group onto carbon number three of anthocyanidins. Glycosylation stabilizes anthocyanins, allows them to be stored in the vacuole, and increases anthocyanin solubility in water (Wang et al., 2018). The glycosyltransferase enzymes have been widely studied in many plants. Nevertheless, the genes that encode anthocyanin glycosyltransferases in rice have not been described.

Genomic variability also influences the anthocyanin content in rice and correlates with rice cultivars. Genomic variability could be assessed by targeting simple sequence repeats (SSR), which present some advantages over other markers. The SSR markers are highly suitable for genetic diversity analysis with high reproducibility (Chen et al., 2013). SSR markers were used to effectively assess the genomic variability of African cultivated and wild-type rice (Chen et al., 2017). Both of these rice populations showed SSR allele polymorphisms that separated them well. Park and others (Park et al., 2019) used sixteen selective SSR primers to evaluate several black-purplish and red rice cultivars’ genetic diversity. They identified several clades that were associated with the wild-type rice genomic variability using SSR markers and partial proteomic profiles in pigmented rice from Java Island.

**Materials and Methods**

The white rice cultivar Mentik Wangi (WREJ), red rice cultivar Mentik (RREJ), black rice cultivar NTT local (N790) (BREJ) from Malang, East Java, red rice cultivar Melik from Semarang, Central Java (BRCJ), and black rice Toraja local from Sukabumi, West Java (BRWJ) were used in this study. Peonidin (≥97% purity, HPLC grade, Cat. 0906 S), cyanidin (≥96% purity, HPLC grade, Cat. 0909 S), cyanidin-3-O-glucoside (≥96% purity, HPLC grade, Cat. 0915 S), and peonidin-3-O-glucoside (≥95% purity, HPLC grade, Cat. 0929 S) were acquired from Extrasynthese (France). The ethical clearance committee of Brawijaya University approved all experiments in this study, with approval number 896-KEP-UB.

**Genomic analysis of pigmented rice from Java Island**

Genomic DNA was extracted from 20-day-old seedling leaves of five cultivars of pigmented rice by the hexadecyltrimethylammonium bromide (CTAB) method (Patchiyah et al., 2011). DNA concentration was measured with a NanoDrop spectrophotometer (ND-1000, NanoDrop Inc., USA). DNA quality was checked by running it on a 0.8% agarose gel in 1× TBE buffer (Tris base, boric acid, and EDTA pH 8.3) at 100 V for 30 min, and then the gel was observed on a UV transilluminator. PCR was conducted to analyze the genomic variability of pigmented rice. The set of primers used for the SSR was from Chen and others (Chen et al., 2017), while the *Re* gene (KX549256) and the *GT* gene (XM015777298.2) primers were designed to bind at specific sites (Table S1). Both of these rice cultivars showed SSR allele polymorphisms that separated them well. Park and others (Park et al., 2019) used sixteen selective SSR primers to evaluate several black-purplish and red rice cultivars’ genetic diversity. The primer sequences and their product sizes are shown in Table 1. The PCR reaction mix consisted of 25 µL of 2× GoTag™ Green Master Mix (Promega, Cat.M712), 0.2 µM of each primer, 50 ng/µL genomic DNA, and deionized water. The PCR program was set to 95°C for 30 s, 51–57°C for 30 s, and extension at 72°C for 45 s, for 35 cycles. The *Re* and *GT* genes were separated by electrophoresis using 1.5% agarose gels, while the SSR profile was done using 5% polyacrylamide non-denaturing gels. All DNA gels were stained with ethidium bromide and then visualized on a UV transilluminator (BioRad, Cat. No 161-0433).

**Total anthocyanin extraction, purification and analysis using UHPLC-DAD of pigmented rice from Java Island**

The pigmented rice grains were ground to a fine powder and extracted with 0.1% HCl in methanol (Huang & Lai, 2016). Anthocyanin extracts were concentrated on a rotary evaporator (Heidolph, Germany) at 40°C and cooled to 4°C. Total anthocyanin extracts were partially purified using Sephadex® LH-20 chromatography in methanol followed by silica gel column chromatography (Zhang et al., 2018). The silica gel was...
eluted with a gradient of 20-100% of ethyl acetate in n-hexane followed by 30–100% methanol in dichloromethane. Pigmented rice fractions were separated by thin-layer chromatography (TLC) with n-butanol:acetic acid:water (3:1:1) as mobile phase and silica gel 60 F254 plates (Merek; Cat. No. 1.05554.0007) as stationary phase (Priya, et al., 2013). Based on the presence of anthocyanin spots on the TLC plates, fraction 6 of RREJ (RREJ F6), fraction 5 of BREJ (BREJ F5), fraction 9 of BRCJ (BRCJ F9), and fraction 15 of BRWJ (BRWJ F15) from the silica gel column chromatography were analyzed using ultra-high-performance liquid chromatography with a diode array detector (UHPLC-DAD, Agilent 1100 series). Two microliters of 0.5 mg/ml filtered fractions were injected into the UHPLC and separated through a Zorbax SB-C18 2.1×150 mm 1.8-micron column (Agilent, USA; Part Number:5188-5328). Solution A (0.2% formic acid) and solution B (acetonitrile) were used as mobile phases. The flow rate was 0.2 ml/min, and the gradient began in 100% A, then 0–2 min 0–5% B; 2–13 min 5–50% B; 13–14 min 50–70% B; 14-16 min 70–100% B and 16–25 min 100% B. Anthocyanin peaks were detected by diode array detector (DAD) (1260 Infinity II Diode Array Detector HS, Agilent) at the wavelength 520 nm.

Extraction and determination of total protein from pigmented rice from Java Island

Total protein was extracted from the ground rice seeds in Tris-HCl buffer followed by 10% trichloroacetic acid (TCA)/acetone precipitation (Wang et al., 2016). Approximately 500 mg of pigmented rice powder was extracted with lysis buffer solution (20 mM Tris-HCl pH 8.0, 2% NP-40, 1 mM of EDTA) and centrifuged at 10,000×g, 15 min. The supernatant was mixed with 10% TCA in cold acetone and incubated overnight at −20°C. The insoluble protein was washed with cold acetone five times and air-dried. The dried proteins were re-suspended with 0.5% SDS in Tris-HCl buffer, and the protein concentration was measured by the Lowry method with bovine serum albumin as a protein standard (Shen, 2019).

Tryptic digestion and analysis using Impact II UHR-TOF LC-MS

Five micrograms of crude protein were reduced using 5 mM dithiothreitol (DTT) in 10 mM ammonium bicarbonate at 60°C for an hour and alkylated using 15 mM iodoacetamide (IAA) in 10 mM ammonium bicarbonate at room temperature for 45 min in the dark. Then, the samples were digested with sequencing grade trypsin (Promega, Germany) at the protein to enzyme ratio of 1:25 for 4 hours at 37°C. The peptides were dried at 30°C under vacuum and analyzed on an Impact II UHR-TOF MS System (Bruker Daltonics Ltd., Germany). Pigmented rice peptides were enriched on a C18, 5 µm 100 Å (Thermo Scientific, UK), Pepmap 100, 5 mm × 300 µm i.d., µ-precolumn and separated on an analytical column (75 µm i.d. × 15 cm) packed with Acclaim PepMap RSLC C18 2 µm 100 Å, nanoViper (Thermo Scientific, UK). The gradient system of 5–55% B over 30 min, with solvent A (0.1% formic acid) and solvent B (0.1% formic acid in 80% acetonitrile) was used to elute peptides. The flow rate was 0.3 µl/min. Mass spectra (MS) and MS/MS spectra were acquired in the positive-ion mode (m/z) = 150–2200 with 1.6 kV of Captive Spray (Compass 1.9 for TOF Series software, Bruker Daltonics).

Proteins quantification and identification

Mass spectra of peptides were analyzed with MaxQuant 1.6.3.3 software associated with the Andromeda search engine (Tyanova et al., 2015). The peptide search parameters were a maximum of three missed cleavages, 0.07 Da and 0.006 Da as first and main search tolerances, 30 as threshold intensity, trypsin as digesting enzyme, carbamidomethylation of cysteines as a fixed modification, and the oxidation of methionine and acetylation of the protein N-terminus as variable modifications. To identify the proteins, peptides with a minimum of 7 amino acids and at least one unique peptide were required.

Data analysis

The diagram of total protein in pigmented rice from Java Island was constructed with the Jvenn tool (http://jvenn.toulouse.inra.fr/app/index.html) and presented as a Venn diagram. A heat map was generated using the online heat-mapper software (http://heatmapper.ca), and the biological functions of each protein were predicted using the protein informatics resource (PIR) database (https://proteinfamilyresource.org/). Anthocyanin contents were reported as mean ± standard deviation. All of the experiments were conducted in triplicate and analyzed using either one-way analysis of variance (ANOVA) for cyanidin-3-O-glucoside among three black rice cultivars, or t-test for other anthocyanins between BREJ F5 and BRWJ F15. A p-value of <0.05 was considered to be statistically significant. All genomic data were scored with the values 0 for conserved band/sequence, 1 for similarity >variability, and 2 for similarity<variability (Supplementary Data, Table S2). Phylogenetic analyses were done using the Multi-Variate Statistical Package (MVSP) with UPGMA (Unweight pair group method with arithmetic averages) similarity coefficient.

RESULTS

Genomic characterization of local black rice from Java Island, Indonesia

In order to evaluate the genomic profiles of three black rice cultivars, the simple sequence repeat (SSR) patterns were assessed based on five percent polyacrylamide gel electrophoresis. A preliminary study identified nine sets of SSR primers that showed polymorphic bands in black rice samples. A total of 74 SSR alleles were identified using nine SSR markers across the five cultivars of rice from Java island, 29 of which were polymorphic (Fig. 1a). The SSR markers produced 100–800 bp bands, and specific bands were seen in three black rice cultivars, as shown in Fig. 1a. We found specific bands in all black rice samples, including RM318 and RM224 markers (both of which are 200 bp) in BRWJ, RM202 at around 350 bp in BREJ, and RM251 at around 500 bp in BRCJ and 800 bp in BREJ. A 100 bp RM1369 band was observed in BRCJ and BRWJ, and the 120 bp and 200 bp bands from RM223 appeared in the three cultivars of black rice. RM6364 showed a specific band at around 300–400 bp that was only identified in BRWJ, and 200–300 bp bands that were detected in BREJ and BRCJ.

The nucleotide sequences of the Rc and GT genes showed specific variations in the three black rice cultivars from Java Island. The Rc gene segment from exons 1–2 was amplified to give 990 bp fragment (Fig. 1b-c).
All three cultivars of black rice had a c.1797A>T substitution in the Rc gene exon 1. Moreover, other mutations were found in Rc gene exon 1 in BRCJ, namely, a c.1781C>T substitution and a c.1782delC deletion, while a 1800G>A substitution was detected in BRWJ. These particular mutations were not found in black rice hull from Cambodia (gene accession KX549109.1), and showed to be specific for the black rice cultivars from Java Island.
Java island. The \( R_c \) gene exon 2 in the three black rice cultivars showed similar sequences, not found in white rice. The \( G_T \) gene consisted of one exon of 1693 bp, as shown in Fig. 1d. A partial sequence of the \( G_T \) gene covering 624 bp that encoded an N-terminal region of the glycosyltransferase protein was compared for the black, red, and white rice cultivars (Fig. 1d–e).

We found some mutations in the \( B_R W J \) \( G_T \) gene compared to the other cultivars, which included substitutions c.130G>C, c.135C>A, c.144C>A, c.174C>T, c.264A>T, c.297C>A, c.366C>T, c.377G>A, c.499G>A, a c.162delA deletion and the insertions c.150_150insT, c.207_208insA, and c.695insG. The mutations observed in the \( B_R W J \) \( G_T \) gene might be unique to this black rice cultivar. This study found several DNA polymorphisms in rice plants from Java island occurring at 305, 328, 454, 479, 543, and 667 bp compared to the database sequence for black rice \( japonica \). This sequence variation may be specific for the rice from Java Island.

The genetic relationship between the three black rice cultivars compared to red rice and white rice is illustrated as a phylogenetic tree in Fig. 1e.

According to the SSR patterns and \( R_c \) and \( G_T \) gene sequences, two black rice cultivars, \( B_R W J \) and \( B_R C J \), clustered in one group with a similarity coefficient of 0.935, while black rice cultivar \( B_R E J \) clustered with red rice and white rice in another group, with a similarity coefficient of 0.936. Genomic variability analysis showed that \( B_R W J \) had a high \( G_T \) gene sequence variability, and in addition, \( B_R W J \) differed in color and morphological traits. \( B_R W J \) has purple color in the stem, auricle, leaf blade, bran, and whole grain (Fig. 1g). In comparison, \( B_R E J \) and \( B_R C J \) are purple only in whole grains, primarily in the bran.

### Anthocyanin compositions in local Javanese black rice

The black rice anthocyanins profile was analyzed with UHPLC-DAD and the chromatogram of absorbance at 520 nm is shown in Fig. 2. Cyanidin-3-O-glucoside was identified in the various fractions of all three cultivars of black rice including F5 in \( B_R E J \), F9 in \( B_R C J \), and F15 in \( B_R W J \) (Fig. 2a–2c). In \( B_R E J \) F5 and \( B_R W J \) F15, cyanidin, peonidin, and peonidin-3-O-glucoside were found. Interestingly, cyanidin expression was higher in \( B_R W J \) F15 than in other cultivars, and peonidin-3-O-glucoside expression was higher in \( B_R E J \) F5 than in other cultivars.

### Proteomic profiling of local black rice from Java island

When the rice seed proteins were extracted and trypsin – digested, and the resulting peptides analyzed with Impact II UHR-TOF LC-MS, a total of 3434 proteins were identified in the pigmented rice from Java island. The 2316 proteins were conserved in all the three black rice cultivars from Java island. A Venn diagram shown in Fig. 3a compares the identified proteins in three black rice from Java Island, red rice and white rice (Fig. 3a). Among the black rice cultivars, the most proteins were identified in \( B_R E J \), followed by \( B_R W J \) and \( B_R C J \). A group of proteins was identified only in a certain rice
cultivar, including 166 in BREJ, 72 in BRCJ, and 88 in BRWJ. Figure 3b shows the numbers of common proteins between different cultivars. Out of 2124 proteins in pigmented rice, black rice BRWJ had 166 proteins in common with BRCJ, and 314 proteins in common with BREJ. Black rice BREJ and BRCJ had 266 proteins in common. In comparison BRWJ had 15 proteins in common with RREJ, and 10 with WREJ.

The relative expression levels of proteins related to anthocyanin biosynthesis in different rice cultivars are shown in Fig. 3c. Several enzymes related to anthocyanin biosynthesis were more abundant in BRWJ and BREJ than in other cultivars (Fig. 3c). These included phenylalanine ammonia-lyase (PAL2), O strives flavonoid monooxygenase, UDP-Glc dehydrogenase (UDPGlc DH), 3-glycosyltransferase (Os3GT), acyltransferase, cytochrome P450, chalcone isomerase, and UDP-Glc DH. The predicted biological functions of the 2316 black rice proteins were clustered into 13 categories, including biological phase, cell proliferation, immune system process, metabolic process, multicellular organism process, rhythmic process, developmental process, response to the stimulus, biological regulation, reproduction, localization, nitrogene utilization, and cellular process. The most common functions of black rice proteins (Fig. 3d).

**DISCUSSION**

Genomic profiling of Java black rice plants revealed some specific bands in RM markers, such as RM318, RM224, RM202, RM1369, and RM6364 in case of BRWJ. Furthermore, RM202 showed a specific band in BREJ and RM251 showed specific bands in BRCJ and BREJ samples. RM223 product bands were conserved in the Java black rice cultivars. Fatimah and others (Fatimah et al., 2016) reported that some SSR markers that had high numbers of polymorphic alleles in Indonesian paddy rice were RM162, RM287, RM541, RM144, RM474, and RM171. Another study from Ladjao et al. (2019) revealed that Toraja paddy rice showed high polymorphism in RM259, RM224, RM334, and RM352 products. The different polymorphic patterns of SSR rice markers may be associated with paddy rice traits. For instance, an RM224 pattern was linked to germination rate, shoot dry weight, and shoot length, while the RM223 pattern was related to seed weight, germination rate, shoot length, and seedling early vigor (Anandan et al., 2016). Fukuta and others (Fukuta et al., 2012) mapped RM1369 on chromosome 6 and correlated it with panicle weight in new African (NERICA) rice varieties. The genomic profiling of unique alleles related to specific rice traits in Oryza glaberrima or wild type African rice revealed bands of 170–187 bp in RM250, 117 bp in RM312, and 140–
**Black Rice**

**Genomic profiles:**
- SSR profiles proved specific morphological characteristic in each black rice.
- Re gene exon 2 had similar sequence on the three black rice cultivars from Java Island.
- GT gene of BRWJ showed different sequence than others.

**Several enzyme related to anthocyanin synthesis is predicted high expression:**
- Phenylalanine ammonia lyase (PAL2)
- Chalcone isomerase (CHI)
- Flavonoid monooxygenase
- Glycosyltransferase (OsSGT)
- Acyltransferase

**Phytoc hemical Compounds:**
- High phenylalanine, flavonoids, and leucoanthocyanidin
- The variety of anthocyanins (Cyanidin, cyanidin-3-O-glucoside, peonidin, peonidin-3-O-glucoside) were established in BREJ and BRWJ

**Biological function:**
Antioxidant, Antihyperglycemia, Anti-inflammatory

**Figure 4. Summary of genomic, proteomic, and anthocyanin profiles in black rice from Java island.**

Numerical superscripts indicate the data derived from previous studies: *(Fatchiyah et al., 2020a), (Fatchiyah et al., 2020b), (Sari et al., 2020b), (Sari et al., 2019a).*

150 bp in RM223 markers (Chen et al., 2017). Karmkar and others (Karmkar et al., 2012) reported that 10 out of 22 studied SSR markers showed unique alleles in the Bengal rice cultivar. The different SSR alleles profile in Java black rice compared to other Asian rice cultivars may reflect adaptation to different environmental factors. The phylogenetic tree of five rice cultivars provided a model of the genetic relationship of these rice cultivars. The clustering of BREJ with WREJ might suggest this white rice is a product of the BREJ black rice domestication process. The domestication process, breeding activities, and landrace condition greatly impact the high similarity coefficient in genetic diversity analysis (Reig-Valiente et al., 2016). The similarity coefficient among the five rice cultivars from Java island of Indonesia might correlate with their morphological characters on BRWJ, while other anthocyanins (cyani
din, peonidin, and peonidin-3-O-glucoside) were detected in BREJ F5 and BRWJ F15. The profiles of anthocyanins in black rice from Java might correlate with their biological function. Fatchiyah and others (Fatchiyah et al., 2020a) reported that BREJ and BRWJ extracts have high total anthocyanins and antioxidant activity.

Syncytin gene is the OsGST gene inactivated in white-pericarp in rice (Sweeney et al., 2006; Maeda et al., 2014; Zhu et al., 2019). Another anthocyanin synthesis gene is the GT gene that encodes a glycosyltransferase that can add sugar to stabilize the anthocyanin structure. Some black rice GT gene mutations might be related to specific morphological characters on BRWJ, which has purple leaves, blades, stems, and grains. Li and others (Li et al., 2017a) stated that overexpression of a GT gene implied higher anthocyanin contents in *Arabidopsis thaliana*. However, GT gene overexpression decreased glycosyltransferase activity and reduced color intensity in *Rosa rugosa* (Sui et al., 2019).

In the present study, proteomic data identified some transferases in black rice, including proteins with putative methyltransferase, acyltransferase, and glycosyltransferase activities. These transferases may contribute to anthocyanin modifications (Sasaki et al., 2014; Provenzano et al., 2014; Li et al., 2017b; Wang et al., 2018; Sui et al., 2019). We found some proteins related to anthocyanin synthesis with the higher expression in black rice than white rice. Phenylalanine is an amino acid precursor for secondary metabolite synthesis in plants, including that of flavonoids and anthocyanins (Cheng et al., 2014). Phenylalanine is processed to leucoanthocyanidin, which is oxidized to cyanidin by leucoanthocyanidin oxidase (LDOX) (Poustka et al., 2007). Fatchiyah and others (Fatchiyah et al., 2020a) described that black rice has higher phenylalanine, flavonoids, and leucoanthocyanidin than red and white rice. Cyanidin is glycosylated to cyanidin-3-O-glucoside by glycosyltransferase (OsSGT), while it is methylated to peonidin by a methyltransferase (Cheng et al., 2014; Provenzano et al., 2014; Olivas-Aguirre et al., 2016; Peng et al., 2017; Zheng et al., 2019b). The acyltransferase in black rice transfers an acyl group to produce more complex anthocyanins (Bontpart et al., 2015). Anthocyanins and proanthocyanidins are synthesized in different pathways. Proanthocyanidins are derived from leucoanthocyanidins and cyanidin through flavan-3-ol (Olivas-Aguirre et al., 2016). Anthocyanins and proanthocyanidins are carried to the vacuole with glutathione-8-transferase (OsGST), serving as their protein transporter (Gomez et al., 2011; Chanoca et al., 2015). Cyanidin-3-O-glucoside and peonidin-3-O-glucoside are the main anthocyanins in black rice (Hou et al., 2013; Pengkumsri et al., 2015; Pedro et al., 2016). Similarly, the present study identified cyanidin 3-O-glucoside in the three kinds of black rice from Java, while other anthocyanins (cyanidin, peonidin, and peonidin-3-O-glucoside) were detected in BREJ F5 and BRWJ F15. The profiles of anthocyanins in black rice from Java might correlate with their biological function. Fatchiyah and others (Fatchiyah et al., 2020a) reported that BREJ and BRWJ extracts have high total anthocyanins and antioxidant activity. In silico study showed that peonidin-3-O-glucoside may have anti-inflammatory activity via inhibiting TNF-α receptor (Sari et al., 2019a). Cyanidin-3-O-glucoside and peonidin-3-O-glucoside are predicted to have anti-apoptosis effect by inhibiting caspase-3 (Sari et al., 2020a). In vivo and in-silico studies have proven that black rice anthocyanins act against obesity and adipogenesis (Fatchiyah et al., 2020b, Fatchiyah et al., 2020c; Sari et al., 2020b). In the current study, anthocyanins were not detected in RREJ red rice fraction containing pigment. Several studies identified malvidin (Chen et al., 2012) and proanthocyanidin, rather than anthocyanins, as the pigments found in high amounts.
in red rice (Vargas et al., 2018; Laokuldilok et al., 2011; Olivas-Aguirre et al., 2016).

**CONCLUSIONS**

Three black rice cultivars demonstrated different genomic, proteomic, and anthocyanin profiles. The SSR profiles identified specific bands in three black rice cultivars. The gene exon 2 showed a similar sequence in all black rice cultivars from Java and GT gene demonstrated some mutations and predicted a new variant of the gene in BRWJ cultivar. Black rice Anthocyanin from Java cultivars proved some biological activities and the Java black rice substantiate recommended as a functional food.

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