Rice (Oryza sativa L.) breeding for novel black short grain associated with yield, cooking quality, high nutrition and antioxidant potential derived from indica black rice x japonica white rice

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Rice (*Oryza sativa* L.) breeding for novel black short grain associated with yield, cooking quality, high nutrition and antioxidant potential derived from indica black rice x japonica white rice

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Abstract

The combination of the trend of Japanese food consumption with the health benefits of black rice is in high demand for rice consumers in Thailand. For this challenge, incorporation of desirable traits from temperate japonica white rice, Akitakomachi and tropical indica black rice, Riceberry was performed by pedigree selection with maker assisted selection (MAS) and these desirable traits have been presented in breeding progenies. The three candidate lines showed highly favorable agronomic characteristics and a high grain yield, with short grains and good cooking quality, similar to japonica rice, in a tropical climate. In addition, these lines showed black coloration of the pericarp, indicating high nutritional value (including Fe, Zn, Vit B6, Vit E and folate levels) and phytochemical, antioxidant and antidiabetic activities, similar to those of the Riceberry parent. In terms of the sensory testing of unpolished rice, two breeding lines (69-1-1 and 72-4-3) showed higher scores than their parents. However, only 69-1-1 was identified as japonica type according to its genetic background. Therefore, this breeding program, involving the crossing of a temperate japonica white rice with a tropical indica black rice, can create novel black short grain rice variety adapted to a tropical environment, similar to japonica-type rice.

Keywords: Rice (*Oryza sativa* L.), Black short grain, Nutrition, Cooking quality, Antioxidant

Introduction

Japanese food has been widespread in Thailand for more than three decades. The Japan External Trade Organization Bangkok (2020) reported that the number of Japanese restaurants increased dramatically in Thailand from 1,307 to 4,094 restaurants over ten years (2010-2020), demonstrating the high market demand for japonica rice in Thailand. As a result, Thailand has the largest Japanese restaurant business in Asian. However, the cultivation of temperate japonica varieties in tropical regions such as Thailand is very limited because these varieties are sensitive to high temperatures. Thus, in countries where japonica varieties do not traditionally grow, consumers need to pay a high price for this commodity (Kang 2010).

Recently, growing consumer interest in health-promoting food products has generated a substantial market for rice with higher nutritional value (Mbanjo et al. 2020). Thus, individuals who eat rice prefer unpolished rice that has a black- or red-colored pericarp. The pericarp of colored rice grains accumulates proanthocyanidins (“red rice”) or anthocyanins (“black rice”). These compounds have antioxidant activity and health benefits (Maeda et al. 2014). Anthocyanins are the flavonoid pigments of black rice and are a source of antioxidants that have the ability to inhibit the formation or to reduce the concentrations of reactive, cell-damaging free radicals (Adom and Liu 2002). In addition, black rice is high in fiber, vitamins B and E, iron, thiamine, magnesium, niacin and phosphorous (Qiu et al. 1993; Zhang et al. 2004). Thus, black rice is becoming popular among rice consumers and dieticians due to its high nutritive and medicinal value. Black rice can be found in several varieties and has a long history of cultivation in Asian countries such as China, India and Thailand (Kong et al. 2008), but most of black rice varieties have been identified in indica-type rice.

Due to the high demand for Japanese food and healthy functional foods, many short grain black rice varieties have been bred with improved agronomical traits in Japan, but their eating quality and yields still need to be improved (Saka et al. 2007; Takita et al. 2001; Maeda et al. 2014). In general, consumers who eat japonica rice prefer cooked rice with a short grain and a sticky and chewy texture and dislike rice with a dry and crisp texture. In fact, high stickiness is one of the most important features of japonica rice (Wada et al. 2008; Hossain et al. 2009). At present, the premium short grain rice variety and the most widely produced variety in Japan is Koshihikari (Kobayashi et al. 2018). Therefore, many researchers have tried to take advantage of Koshihikari's good eating quality while adding a black- or red-colored pericarp (Maeda et al. 2014; Yamaguchi et al. 2015), containing anthocyanins or tannins (both healthful nutraceuticals), respectively.

The cultivation of temperate japonica varieties, such as Koshihikari, is very limited in tropical climates and results in low yields and low cooking quality (Kang 2010); however, japonica rice varieties, Akitakomachi is more suitable than other japonica rice varieties for growth in northern parts of Thailand due to their resistance to hot weather (Warinrak 2013; Seemanon et al 2015). In addition, Akitakomachi has been developed from Koshihikari and is being gradually accepted by consumers, continuously maintaining the fourth position in Japan. (Kobayashi et al. 2018). Therefore, it can be used in short grain breeding programs in tropical climates.

Thailand occupies the ninth position in black rice cultivation worldwide (Ichikawa et al. 2001; Sompong et al. 2011). Recently, the premium long grain black rice variety “Riceberry” has become a registered rice variety in Thailand. Researchers initially developed Riceberry with the aim of boosting the nutritional value, fragrance and taste of rice (Vanavichit 2021). Therefore, Riceberry is the most popular black rice variety and is known for its health-promoting properties. In clinical studies, Riceberry has been found to be enriched in water-soluble antioxidants, particularly anthocyanins and lipid-soluble antioxidants, such as carotenoids, gamma oryzanol, and vitamin E. All nutritive properties of Riceberry are contained in its rice bran, with only a small fraction accumulating in its endosperm (Leardkamolkarn et al. 2011; Prangthip et al. 2013).

The successes of rice breeders at incorporating desirable genetic traits from indica rice into japonica rice for breeding program have been limited. However, combining the desired characteristics from indica and japonica rice should give an elite offspring in breeding program (Karla 2020). Thus, the present research focused on the improvement of a novel black short grain rice (japonica-like) variety that is suitable for growth in tropical regions to produce high yield and has increased nutritional quality, with improved micronutrient and antioxidant contents. The genetic combination of temperate japonica white rice, Akitakamachi and tropical indica black rice, Riceberry should be introgressed into the progenies that performed by pedigree selection associated with MAS.

Materials and Methods

Rice growth conditions

This experiment was conducted from 2015 to 2020 at Tana Grain Polish, Ltd., Phan District, Chiang Rai Province (19°35'09.4" N, 99°44'42.7" E, 413 m above sea level). Weather parameters, including the air temperature, relative humidity, and the amount of rain in the field, were measured every 3 h each year (2016-2020) by a data logger (WatchDog 2000 Series Micro Stations, Spectrum Technologies, Inc., USA). The mean day/night temperatures over the five years were 26.8/23.1°C, and the mean maximum/minimum temperatures over the five years were 31.1/20.2°C. The mean relative humidity during the day/night over the five years was 72.1/87.8% RH, and the average rainfall over the five years was 4.3 mm (Fig. S1). The rice plants in every generation were seeded in a field nursery. After 30 d, the rice seedlings were transplanted into breeding plots. The soil in the Phan District consisted of 1.56% organic matter, 0.07% total N, 26.70 mg/kg available (avail) P, 75.54 mg/kg exchangeable (exch) K, 629.0 mg/kg exchangeable Ca, and 76.50 mg/kg exchangeable Mg and had a pH of 5.40. Additionally, basal fertilizer was applied 15 d after planting at rates of 33.7 kg N/ha (diammonium phosphate) and 41.3 kg of P_2O_5/ha. The second split of fertilizer was applied at the booting stage (65 d) at a rate of 57.5 kg N/ha (Rice Department, 2020). Other management practices were in accordance with conventional approaches for high-yield japonica rice cultivation.

Breeding schemes

An indica black long grain, Riceberry plant was used as the female parent, and a japonica white short grain, Akitakomachi plant was used as the male parent. The breeding program is shown in Fig. 1. The two parents were crossed to obtain F_1 seeds, and the progeny were then selfed and selected from F_2 until F_7 by the pedigree method. Grain color and japonica grain shape (Juliano and Villareal 1993; Codex Alimentarius Commission 1990) were used as criteria for phenotypic selection. In F_6, the selected lines were grown for the first yield trial with parents and control varieties in the dry season from December 2019 – March 2020 (DS19/20). The experiment was conducted as an RCBD, with three replications. The plot size for each treatment was 2.5 x 2.5 m (6.25 m^2), with a spacing of 25 x 25 cm. After that, the validation of candidate lines (F_7) was conducted in the second yield trial wet season from June – September 2020 (WS20). The RCBD with three replications was applied, and the plot size for each treatment was 2.5 m x 3.5 m (8.75 m^2), with a spacing of 25 x 25 cm. The grain yield, grain quality, cooking quality, nutrition, phytochemical, antioxidation and enzymatic analysis were evaluated in WS20.

Phylogenetic analysis based on genotyping by sequencing (GBS)

A phylogenetic analysis was performed among breeding lines (F_4), parents and control varieties. The gDNA from the leaves was isolated according to the DNeasy Plant Mini Kit (Qiagen) protocol. The gDNA was then sequenced on an Illumina HiSeq X by Novogene AIT, Singapore. The Bowtie 2 program was subsequently used to align the nucleotides (Langmead and Salzberg 2012), and the GATK program was used to analyze the single-nucleotide polymorphisms (SNPs) in each sample (McKenna et al. 2010). Finally, the nucleotide sequences from the candidate lines and control varieties were used to construct a phylogenetic tree using the MEGA X program.

Screening SNP markers by KASP genotyping technology

The screening of MAS in breeding lines was conducted in F_3 and F_4 using SNP markers, including markers for starch (*wx*^{b}), gelatinization temperature (*SSIIa*), short grain (*GS3*) and blast resistance (*Pi-ta*; TBGI453598) (Table S1). All KASP assay genotyping was performed using the LGC SNP line system following standard KASP protocols (LGC Group 2016).

Agronomic trait collection in yield trials

Agronomic traits were collected, including days to 100% flowering, number of tillers per plant, number of panicles per plant, plant height, 1000 grain weight and grain yield. The days to flowering were recorded when 100% of the individual plants in each plot flowered. The number of tillers per plant, number of panicles per plant and plant height were measured at maturity. The grain yield in each plot was determined per harvested area of 6.25 or 8.75 m^2. The grain yield moisture was adjusted to 14% and then extrapolated to kg per ha. After threshing, the grains were weighed to obtain a 1000 grain weight.

Grain and cooking quality assessments

The dried grains were stored at room temperature for one month prior to grain quality trait evaluation. The paddy grains were dehulled using a mini-polisher. Three physical grain qualities, namely, grain length, grain width and the grain length to width ratios of both paddy grain and unpolished grain, were measured using a two-decimal-point digital Vernier caliper. Three chemical grain qualities, including amylose (AC) was determined based on the colorimetric procedure as described by Juliano (1985). Protein (PC) was calculated by estimating nitrogen content with the Kjedahl method and then using a conversion factor of 5.95 to calculate PC in the rice sample (AOAC 2000). Fat was determined using the soxhet distillation method which involved repeated fat extractions with petroleum ether (AOAC 2000).

The cooking times of the unpolished rice samples were determined according to the method described by Juliano (1985). The pasting properties of the rice flours were evaluated according to the AACC method (AACC 2000). Rice flour (3 g) was suspended in water (25 g) in a canister, and the viscosity changes were measured using a Rapid Visco Analyzer (RVA, Model 4-D, Newport Scientific, Australia).

Evaluation of the sensory qualities of cooked rice

The rice cooking procedure by Xu et al. (2018) was applied. Unpolished rice samples (500 g, 14% moisture content) were washed three times with distilled water and soaked for 12 h. Distilled water was added to the rice samples to obtain an optimum rice:water weight ratio (1.0:1.4). The samples were cooked using the preset cooking setting of a rice cooker (Sharp model KS-ZT18, Thailand). Seven panelists who had been well trained in the principles and concepts of descriptive sensory analysis participated in the sensory quality evaluation. The sensory items included smell (score 1–5), appearance (score 1–5), stickiness (score 1–5), softness (score 1–5) and taste (score 1–5). A comprehensive assessment was performed based on the above factors. Using a relative scale, the panelists gave a score for each attribute compared with the reference sample attributes, and the overall quality was the sum of the scores for all the attributes.

Pericarp thickness and pigment color analysis

The pigment intensity of unpolished rice was measured with a colorimeter meter (model CR-300, Minolta, Japan). L*, a* and b* values were calculated to determine the color of the rice pericarp of each line/variety, where ‘L*’ indicates the degree of lightness or darkness (L* = 0 indicates perfect black and L* = 100 indicates most perfect white; hue chart); ‘a*’ indicates the degree of redness (+) and greenness (−); and ‘b*’ indicates the degree of yellowness (+) and blueness (Lamberts et al. 2006).

The pericarp thickness was measured in each line/variety. The unpolished rice grains were soaked in water for 24 h and dissected into longitudinal sections using a razor blade. The dissected grain was placed on agar in a Petri dish and observed under a light stereoscope (Leica model EZ4, Switzerland). The pericarp thickness was measured at 5 points on the pericarp using the program GIMP 2.10.12.

Evaluation of nutritional and phytochemical contents

Nutritional values, including iron, zinc vitamin E (α-tocopherol), vitamin B6 and folic acid contents, were analyzed by the Institute of Nutrition, Mahidol University, Thailand, which followed the protocol of AOAC (2000). The anthocyanin content in pericarp grains was measured according to the procedures described by Rahman et al. (2015). The absorbance was measured at 535 nm using a spectrophotometer (SpectraMax M2 Multilabel Microplate Reader).

The total flavonoid content was determined following the method of Djeridane et al. (2006). The total flavonoid content was calculated from the calibration curve of rutin equivalents (RUE) according to the formula $Y = 0.0083x + 0.0511$, $R^2 = 0.999$. In addition, the total phenolic content was evaluated using Folin-Ciocalteu reagent (Kähkönen et al. 1999). The total phenolic content was calculated from the calibration curve of gallic acid equivalents (GAE) according to the formula $Y = 0.0094x + 0.0028$, $R^2 = 0.998$.

Antioxidant and enzymatic assays

The inhibitory effect of rice grain water extracts on DPPH radical scavenging was determined following the method of Boskou et al. (2006). In addition, the antioxidant properties of rice grain water extracts on ABTS radical scavenging were investigated using the method of Hsu et al. (2011).

The α-amylase inhibition assay was performed following the method of Kwon et al. (2006), and the α-glucosidase inhibition assay was performed following the method of Ahmad et al. (2011). The percent inhibition of free radical scavenging and enzymatic activities was calculated from the absorbance data using the formula: inhibition (%) = $[(\text{Abs}_{\text{control}}-\text{Abs}_{\text{test}})/\text{Abs}_{\text{control}}] \times 100$.

Statistical analysis

All the data were analyzed using R program version 3.6.1 (R Core Team 2014) to test the significance of the results in terms of agronomic traits and cooking quality. The means were separated using Duncan’s test at alpha levels of 0.01 and 0.05. If there was a significant difference among the experiments for a given parameter, then the values from all of the experiments for that parameter were used to obtain the mean and standard error. Chi-square ($\chi2$) was used to analyze the relationship of the segregation ratio of grain color in the F_2 generation. In addition, correlation analysis (*r*) was used to evaluate color intensity, nutritional and phytochemical values, and antioxidation and antidiabetic activities.

Results

Breeding results during early generations

Among 156 F_2 plants obtained from a cross between the indica black long grain variety "Riceberry" and the japonica white short grain variety "Akitakomachi", the seed pericarps of 85, 34 and 37 plants were classified as showing black, brown and white coloration, respectively (Fig. 2a and 2S). Therefore, the color of the pericarp was identified as a qualitative trait with a segregation ratio of 9 black:3 brown:4 white ($\chi 2 = 2.62$, $P>0.05$). The Mendelian ratio 9:3:4 fit to recessive epistasis of two gene interactions. However, the black seed group consisted of two types, black and purple, depending on the degree of color intensity. Conversely, the paddy grain shape of the F_2 population segregated according to a normal distribution, as shown in Fig. 2b and c. The paddy grain shapes of Akitakomachi (length/width 7.20/3.90 mm) and Riceberry (length/width 11.20/2.60 mm) were clearly identified as the shortest and longest grain phenotypes, respectively, whereas the paddy grain shapes of their F_2 progenies ranged from 6.60-11.00 mm in grain length and 2.60-3.90 mm in grain width, respectively. Therefore, 65 of 156 F_3 seeds that had short grains with black color were selected and grown as F_3 plants. After that, 26 of the F_4 seeds were selected according to the same criteria to grow F_4 plants as the family lines. In this generation, 7 lines were selected, and their growth was continued to obtain the F_5 generation (Fig. 1).

In addition, MAS for cooking quality and blast resistance (wx^b, *SSIIa*, *GS3* and *Pi-ta*) was used to detect the target genes/QTLs at the seedling stage in F_3 and F_4. The results indicated that 17 of 26 plants in F_3 were successfully fixed in terms of the homozygosity of all target genes. Moreover, the homozygosity of these genes was confirmed again in the 7 selected lines of the F_4 generation. When considering these genes in the parents, it was found that Akitakomachi was homozygous for wx^b, *SSIIa*, *GS3* and *Pi-ta*, while Riceberry was homozygous for wx^a and *Pi-ta*.

Phylogenetic relationships of the F_4 selected lines

In the F_4 generation, the seven selected lines were analyzed by GBS for their genetic backgrounds together with their parents and control varieties. The phylogenetic tree constructed from these lines was divided into two groups (Fig. 3). Group I contained one selected line (69-1-1) together with Akitakomachi, Sasanishiki, Koshihikari and DOA1. This group was clearly identified as a japonica type. In addition, the genetic background of Akitakomachi had closer relationships with 69-1-1. Group II contained Riceberry and the control indica varieties together with six selected lines. However, the coloration of six selected lines, 69-3-4, 72-4-1, 72-4-2, 72-4-3, 72-4-9 and 62-2-17, was clarified as black to purple. In addition, grains 69-3-4, 72-4-1, 72-4-2, 72-4-3, and 72-4-9 were identified as having short-medium grains. In contrast, only one line among the selected lines, 62-2-17, had a slender shape. Finally, the growth of three lines, 69-1-1, 72-4-3, and 72-4-9, was continued through the F_5 generation (data not shown) according to the criteria of genetic background, grain shape, grain color and agronomic traits. These three candidate lines were used for yield trial experiments in the F_6 and F_7 generations.

Evaluations of agronomic traits and grain yield

The yield trial experiment was conducted in DS19/20 (F_6) and WS20 (F_7). The plant types of the candidate lines with their parents are shown in Fig. 4a and b. The three candidate lines (69-1-1, 72-4-3 and 72-4-9) were grown along with control varieties including Akitakomachi, Koshihikari and Riceberry. The three candidate lines in F_6 (DS19/20) and F_7 (WS20) generations exhibited significant grain yield and agronomic traits among three lines and control varieties ($P<0.05$), as shown in Table 1. The days to flowering periods of the candidate lines and japonica varieties were significantly shorter than that of Riceberry in both seasons. In addition, the other agronomic traits varied among the lines/varieties and seasons.

The grain yield of 69-1-1 was the highest in both DS19/20 (7.12 kg/ha) and WS20 (6.67 kg/ha), with a significant difference when compared with the other two lines, and the japonica control varieties. However, the grain yield of 69-1-1 was not significantly different from that of Riceberry. In addition, the grain yields of 72-4-3 and 72-4-9 were lower than that of Akitakomachi in both seasons (Table 1). Hence, the grain yield of 69-1-1 showed the highest performance, similar to tropical indica, which can be grown in tropical climates, especially in Chiang Rai Province. However, the grain weight of 69-1-1 was the lowest and was significantly different from those of 72-4-3, 72-4-9, Akitakomachi and Koshihikari, while it was not significantly different from Riceberry (Table 1). Moreover, it was confirmed that the grain size of 69-1-1 was smaller than those of other lines/varieties (Table 2; Fig 4C and D).

The length-to-width ratios of unpolished grains in DS19/20, 69-1-1 and 72-4-3 were not significantly different from those in Akitakomachi and Koshihikari, and the ratios of these candidate lines were within the standard range for short grain rice (ratio<2) (Codex Alimentarius Commission, 1990). However, the length-to-width ratios of 69-1-1 and 72-7-3 in WS20 were above the standard for short grain rice (ratio=2.05), but the difference was not significant when compared with Akitakomachi and Koshihikari. Conversely, the length-to-width ratio of 72-4-9 was over the short grain standard and was significantly different from 69-1-1, 72-7-3, Akitakomachi and Koshihikari in both seasons (Table 2). Therefore, in terms of grain yield, grain color and the standard of short grain, 69-1-1 was identified as a promising black short grain line. However, the cooking and sensory qualities, nutritional value, antioxidation inhibition and antidiabetic activity of 69-1-1 were evaluated to ensure confidence before its release to the public.

Color intensity and pericarp thickness

The color intensity on the rice pericarp differed among the candidate lines and their parents, as shown in Table 3 and Fig. 4f. Lines 72-4-3 and 72-4-9 were not significantly different from Riceberry and had the most intense color, while 69-1-1 was slightly brighter than the other candidate lines and Riceberry. Conversely, the nonpigmented Akitakomachi showed the brightest color and was identified as yellow brown. When considering the pericarp thickness, the results showed that the pericarp of 69-1-1 was thinner than those of other lines/varieties, while the pericarp of Akitakomachi was the thickest (Table 3).

Sensory test and cooking quality

The sensory test of unpolished rice samples in WS20 (Fig. 5) revealed the highest overall scores for 69-1-1 (overall score=34.75) and 72-4-3 (overall score=33.00). When each sensory item was considered, it was found that 69-1-1 and 72-4-3 had high scores and differed significantly from 72-4-9 and Riceberry in terms of smell, glossiness, stickiness, elasticity, taste and texture of cold rice, respectively, while the hardness scores of 69-1-1 and 72-4-3 were lower than that of 72-4-9 and were not significantly different from that of Akitakomachi. In addition, the color scores of the three candidate lines were not significantly different from that of Riceberry (Fig. 5).

The cooking times of unpolished grains of the candidate lines and control varieties ranged from 15-25 min. The three candidate lines and Riceberry, had the longest cooking times (23-25 min), while Akitakomachi (white color grain) had a shorter cooking time (15 min). In addition, the amylose content of 69-1-1 (19.10%) was within the standard range for japonica rice (<20). However, the amylose content of 69-1-1 was significantly higher than those of Akitakomachi (17.29%) and Riceberry (17.50%) (Table 4).

The pasting temperature (PT), peak viscosity (PV), breakdown (BD), final viscosity (FV) and setback (SB) of the rice flour samples were significantly different ($P<0.05$) among the lines/varieties (Table 4). The PV, FV and SB of Akitakomachi were the highest, followed by those of the three candidate lines and Riceberry. Regarding PT values, the candidate lines had higher values than Akitakomachi but lower values than Riceberry. BD is usually related to the tendency of gelatinized starch granules to break when holding at high temperature with continuous shearing. The BDs of two candidate lines (72-4-9 and 72-4-3) were not significantly different (59.79-61.12 RVU), and those two lines had the highest BD values among the examined lines/varieties. Conversely, the BD of 69-1-1 (44.29 RVU) was the lowest among the candidate lines but higher than that of Riceberry.

The two principal components (PCA) (Fig. 6) explained a total of 91.12% of the variation. The first principal component (PC1) accounted for 54.35% of the variation and seemed to differentiate lines/varieties according to their protein content and FV and SB values, and these values were negatively correlated with the CT values. The second principal component (PC2) accounted for 36.77% of the variation and primarily explained why the fat content and PT properties of rice were negatively correlated with the amylose content and BD value. In this study, PCA showed the clustering pattern of the three groups. The two candidate lines 72-4-9 and 72-4-3 seemed to have similar properties in terms of high amylose content and BD, while both 69-1-1 and Riceberry had high fat and PT values. Akitakomachi showed a high protein content and high FV, PV, and SB property values.

Nutritional values and antioxidant and antidiabetic activities

The protein contents of the candidate lines and control varieties were significantly different ($P<0.05$). The rice flour from Akitakomachi and 69-1-1 had the highest protein content (6.87 and 6.58%) compared to the other lines/varieties, though the candidates and varieties did not differ significantly in terms of the protein content (5-8%). In addition, the protein contents of all lines/varieties in this study were within ranges previously reported for rice.

The results of the nutrition analysis are shown in Table 5. The unpolished grain of 72-4-9 had the highest total anthocyanin content (78 mg/100 g), which was significantly different from that of Riceberry (68 mg/100 g), while the anthocyanin contents of 72-4-3 and 69-1-1 were 62 and 59 mg/100 g, respectively. In addition, 69-1-1 showed the highest nutrient concentrations in two categories (Fe, and Vit B6), while 72-4-9 had the highest nutrient concentrations for Zn category. Conversely, Riceberry had the highest folic acid content (70 µg/100 g), while 69-1-1 had less folic acid (48 µg/100 g) than the other two candidate lines and Riceberry. However, when considering all parameters evaluated in the WS20 yield trial, 69-1-1 showed a higher in grain yield, low amylose (<20%), the highest score of sensory tests and higher in Fe and Vit B6 than 72-4-3 and 72-4-9.

Candidate line 72-4-9 showed the highest contents of flavonoids and phenolics (71.65±1.79 mg RUE/100 g extract and 176.45±16.30 mg GAE/100 g extract, respectively), followed by 69-1-1 (61.03±1.14 mg RUE/100 g extract and 141.88±6.62 mg GAE/100 g extract, respectively). Moreover, 72-4-3 also showed higher concentrations of flavonoids and phenolics than Riceberry and Akitakomachi (Table 6).

The results of antioxidant activity assays showed that the candidate line 72-4-9 presented the strongest inhibition of DPPH (11.99%) among the lines/varieties, while 69-1-1 (8.13%) and 72-4-3 (8.24%) had an inhibitory effect on DPPH similar to that of Riceberry (7.04%). Conversely, Akitakomachi did not show the antioxidant property of DPPH radical scavenging. In terms of ABTS radical scavenging inhibition, all candidate lines, 69-1-1, 72-4-3 and 72-4-9, showed strong inhibitory activities of more than 80% and a similar inhibitory effect on ABTS to Riceberry. In contrast, the water extract of Akitakomachi showed weaker ABTS inhibition than the other extracts (Table 6).

The α-amylase activity assay showed that the water extracts of Riceberry and 72-4-9 (13.08% and 11.10%) had higher capacity to inhibit α-amylase than the two other candidate lines and Akitakomachi. In the case of α-glucosidase inhibition, it was found that all candidate lines (51.52%-60.93%) showed stronger inhibitory activity than Riceberry and Akitakomachi (Table 6).

When considering the correlation analysis (Fig. 7), it was found that the intensity of grain color (L*) was highly negatively correlated with anthocyanin, zinc, flavonoid, and phenolic levels and antioxidation activities (DPPH, ABTS). Conversely, the anthocyanin content was highly positively correlated with zinc, folic acid, flavonoid, and phenolic levels and α-amylase and antioxidation activities.

Discussion

The segregation of color in pericarp and grain size

In this research, color segregation analysis in F_2 progenies from Riceberry (black color) x Akitakomachi (white color) showed the same results as many researchers reported that the character of black pigment is controlled by two complementary dominant genes, *Pb* and *Pp*, with recessive epistasis (9:3:4) (Lee et al. 2018; Kristamtini et al. 2019). However, in the black and brown color groups, there was also variation in color intensity. This may suggest that the color of the rice pericarp is controlled by other genes in the pathway of anthocyanin synthesis (Yamuangmorn and Prom-u-Thai 2021).

The segregation of grain size was controlled by quantitative genetics that combines grain length, grain width, and grain thickness characteristics (Ponce et al. 2020). Li et al. (2018) reported that the size of rice grains is coordinately controlled by cell proliferation and cell expansion in the spikelet hull and identified several quantitative trait loci and a number of genes as key grain size regulators. However, the *GRAIN SIZE3* (*GS3*) gene was the first molecularly characterized QTL for grain size and is used as the major QTL to identify grain length differences between indica and japonica types (Fan et al. 2006, Saichompoo et al. 2021). Thus, *GS3* could be used to identify grain size in this breeding program.

Agronomic and environmental factors

Japonica rice is suitable for cultivation between 53° N and 36° S latitudes at lower temperatures and under longer days than in tropical areas (Khush 1997). Moreover, japonica rice can produce grain yields in temperate regions higher than that in tropical regions due to the photoperiod sensitivity and spikelet sterility caused by high temperatures (Kobayashi et al. 2018; Lee et al. 2018; Yoshida 1983). In this research, the breeding lines were bred at 19° N and 99° E latitude under 26.8/23.1°C mean day/night temperatures over five years. The 69-1-1 showed a high grain yield similar to that of Riceberry, although the flowering day was the same as those of temperate japonica control varieties. Therefore, rice plants genetically belonging to temperate japonica varieties can be bred to adapt to tropical region by introgressing indica genetically into breeding lines (Negrao et al. 2008).

Grain and cooking quality

Amylose is one of the components of rice starch that greatly affects cooking and eating qualities. Low-amylose rice generally becomes soft and sticky after cooking (Okadome et al. 1999). Amylose content between 15–20% is identified as low amylose rice and contain nearly all temperate japonica varieties (Juliano 1971). In this study, an amylose content of less than 20% among the candidate lines was found only in 69-1-1 (19.10%) and the unpolished grain size ratio of this line is within the standard for japonica grain shape (ratio<2) (Codex Alimentarius Commission 1990). However, the grain weights of the three breeding lines were lower than those of japonica white rice because anthocyanin deposition in the pericarp of black rice reduced the photosynthetic rate (Rahman et al. 2015).

The sensory quality of cooked rice is an important factor in determining its market price, as well as consumer acceptance and breeding efforts to improve rice grain quality (Xu et al. 2018). In this study, the 69-1-1 and 72-4-3 had higher scores for overall sensory quality which were lower protein content (6.23-6.58%) than the mean of unpolished rice grain (8%) (Mahender et al. 2016). In addition, both lines showed low hardness and high stickiness scores in the sensory analysis. The results were consistent with the findings of Xu et al. (2018), who suggested that the overall sensory quality is negatively correlated with protein content and positively correlated with hardness and stickiness. In addition, the stickiness after cooking is due to the low amylose content (Juliano 1985) and the wx^b gene that is critical gene to control amylose content (Shao et al. 2020) was presented in the candidate lines. Thus, the cooking and eating quality characteristics of breeding lines were derived from both indica and japonica parents which contained wx^b and *SSIIa* genes.

The pasting properties of rice flour are related to the cooking and eating quality of rice. The differences in pasting properties among rice varieties in this research could be attributed to differences in the amounts of amylose, lipids, and branch chain-length distribution of amylopectin present in rice starch (Singh et al. 2006). Due to the lipid content, the paste viscosity of 69-1-1 was found to be lower than that of the other lines. Starches with a high lipid content can form a rigid network of structures in granules due to amylose-lipid complexation, causing a restriction of granule swelling and resulting in a higher pasting temperature (Becker et al. 2001). Moreover, the formation of amylose-lipid complexes may have prevented amylose from leaching out, resulting in a reduction in the hot paste viscosity (Richardson et al. 2004).

The pasting and chemical properties of Akitakomachi were quite different from those of the breeding lines. This observation might be explained by a characteristic difference between japonica- and indica-type rice in terms of the different branch-chain lengths of their amylopectin molecules. The amylopectin of japonica rice has a larger proportion of short-branch chains than that of indica rice (Kang et al. 2006), and short-branch chains in amylopectin behave in a manner similar to amylose by restricting starch swelling, resulting in high PV, FV and SB values (Jane et al. 1999). Overall, the differences in pasting properties among different genotypes are attributed to protein, lipid and amylose contents, granule rigidity, and starch crystallinity. Therefore, pasting profiles associated with chemical content will provide a baseline for the selection of cooking and eating quality grain for further development. In addition, the pasting properties of 69-1-1 was closer with indica parent than those of japonica parent.

Nutritional value and antioxidant and antidiabetic activities

The anthocyanins content in black rice pericarp also varies depending on the rice cultivars (Jiamyangyuen et al. 2017; Ji et al. 2012). Kushwaha et al. (2020) reported that temperate climate could favor the rise in grain's anthocyanin content than the warm climate. In addition, the increase in the color intensity was increase the total anthocyanin content (Mackon et al. 2021). Moreover, the color intensity and pericarp thickness had affected to anthocyanin content in rice pericarp. Therefore, the breeding procedure for anthocyanin content from indica black rice x japonica white rice by phenotypic selection using color intensity was successful in obtaining new breeding lines that had similar total anthocyanin contents to the black-grained parent.

Most of the nutrients found in rice grain accumulate in the outer aleurone layer and the embryo (Mbanjo et al. 2020). The standardization of unpolished rice grains has resulted in ranges of 1.4-5.2 mg/100 g of iron, 1.9-2.8 mg/100 g of zinc, 0.80-2.50 mg/100 g of vitamin E, 0.50-0.70 mg/100 g of vitamin B6 and 16-20 µg/100 g of folate (Juliano 2016). Therefore, the three candidate lines in this study were within the standard levels. In addition, the correlation analysis in this study confirmed the results of Gao (2011) who reported that the color intensity was positively correlated with iron, zinc and folic acid contents. Other studies have suggested that pigmented rice contains higher levels of iron and zinc than white grain (Hurtada et al. 2018; Shao et al. 2018).

Total flavonoids and phenolics are natural bioactive compounds in plants that can indicate their potential for use as therapeutic agents and in controlling the quality of medicinal sources. They can also function as free radical-scavenging and reducing agents. (Ghasemzadeh and Ghasemzadeh 2011; Tungmunnithum et al. 2018). In this research, the contents of phenolics, flavonoids, and anthocyanins were negatively correlated with L* color intensity values, in accord with previous findings (Shao et al. 2018). However, the total antioxidant activity of black rice bran was correlated with the contents of total anthocyanins, total phenolics and total flavonoids (Zhang et al. 2010; Goufo and Trindade 2014), which were determined in the same way as in this research.

Extracts of black rice grain have been shown to effectively inhibit the activities of endogenous α-amylase and α-glucosidase, thereby inhibiting the conversion of starch to glucose in the small intestine, which acts as a source of resistant starch utilized by the gut microbiota in the colon (Boue et al. 2016; Chiou et al. 2018). All black and red bran extracts inhibit α-glucosidase activity; however, only red rice bran extracts inhibit α-amylase activity (Boue et al. 2016). In this study, α-glucosidase had the greatest correlation with anthocyanin content (Yao et al. 2010; Bae et al. 2017). However, α-amylase activity was not correlated with anthocyanin content that was different result from those of Wongsa et al. (2019).

Conclusion

A breeding program for obtaining black color, short grain with high nutrition and phytochemical can provide a novel rice variety for growth in Thailand that combines tropical adaptability with high yield and high nutritional value from tropical indica rice and short grain with good cooking and eating quality from temperate japonica rice. Finally, the promising 69-1-1 line has undergone short grain rice breeding with the same yield as indica rice and resistance to blast disease together with high anthocyanin content and good cooking and eating quality according to Japanese food standards. In addition, it has already been registered as a new short grain rice variety in Thailand named – Fuji-Murasaki (Murasaki means purple color).

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Table 1 Agronomic traits of the three candidate short grain lines compared with commercial varieties and their parents in DS19/20 (F_6) and WS20 (F_7).

Lines/Varieties	DF (days)		PH (cm)		NTP		NPP		TGW (g)		GY (t/ha)	
	DS19/20	WS20	DS19/20	WS20	DS19/20	WS20	DS19/20	WS20	DS19/20	WS20	DS19/20	WS20
69-1-1	89b[1/]	63c	98b	110a	22ab	36a	22ab	33a	22.13d	22.60c	7.12a	6.67a
72-4-3	90b	72b	121a	117a	21ab	27bc	21ab	25bc	25.73c	28.13b	5.63bc	5.95bc
72-4-9	90b	74b	107ab	108a	25a	30ab	25a	29ab	27.37b	28.50b	4.85c	4.65c
Akitakomachi	91b	65c	100b	92b	15b	17d	15b	17d	25.57a	30.60a	5.93b	6.50b
Koshihikari	91b	64c	99b	83c	21ab	21cd	21ab	20cd	29.83a	30.67a	4.38d	4.40cd
Riceberry	113a	107a	94c	110a	27a	27bc	27a	27abc	20.73d	21.77c	7.38a	6.96a
F-test			**	**	**	**	*	**	**	**	**	**
(P<0.01)	**	**										
CV%	3.17	4.59	3.17	4.59	2.23	4.59	2.23	4.59	4.59	4.59	5.24	4.85

[1/] Different letters in the same column indicate significant differences at the 0.05 level using LSD.

DF=Days to flower, PH=Plant height, NTP=Number of tillers per plant, NPP=Number of panicles per plant, TGW=1000 grain weight, GY=Grain yield

Table 2 Grain length, grain width and grain length/width ratio of paddy and unpolished grain of the three candidate short grain lines compared with commercial varieties and their parents in DS19/20 (F_6) and WS20 (F_7).

Lines/Varieties	Paddy grain						Unpolished grain					
	Length (mm)		Width (mm)		Ratio (length/width)		Length (mm)		Width (mm)		Ratio (length/width)	
	DS19/20	WS20	DS19/20	WS20	DS19/20	WS20	DS19/20	WS20	DS19/20	WS20	DS19/20	WS20
69-1-1	7.23d	8.13cd	3.00c	3.45bc	2.28c	2.35c	5.29c	6.01bc	2.81b	2.94a	1.88bc	2.05cd
72-4-3	8.29bc	8.57bc	3.49a	3.36bc	2.23c	2.55bc	5.76c	6.15b	3.15a	2.88a	1.83c	2.07cd
72-4-9	9.10ab	8.94b	3.47ab	3.26c	2.68b	2.74b	6.91b	6.05b	3.13a	2.93a	2.20b	2.14b
Akitakomachi	7.37cd	7.52d	3.87a	3.96a	1.84d	1.90d	5.36c	5.51d	3.16a	3.16a	1.70c	1.75cd
Koshihikari	7.53cd	7.57d	3.75a	3.65b	2.08cd	2.08d	5.55c	5.58cd	3.20a	3.12a	1.73c	1.79cd
Riceberry	9.96a	10.52a	2.88c	2.50d	3.89a	4.22a	7.71a	7.39a	1.98c	1.96b	3.91a	3.77a
F-test ($P<0.01$)	**	**	**	**	**	**	**	**	**	**	**	**
CV%	4.59	4.59	4.59	4.59	4.59	4.59	4.59	4.59	4.59	4.59	4.59	4.59

[1/] Different letters in the same column indicate significant differences at the 0.05 level using LSD.

Table 3 Pericarp thickness, pigmentation intensity and pericarp color of the three candidate lines compared with their parents in WS20.

Lines/Varieties	Pericarp thickness (mm)	Pigmentation intensity			Pericarp color
		L^*	a^*	b^*	
69-1-1	0.020c[1/]	10.35b	8.50b	8.05b	Dark purple

72-4-3	0.035b	9.70bc	7.60b	5.45b	Dark purple
72-4-9	0.037b	8.30bc	7.10bc	5.25b	Dark purple
Akitakomachi	0.052a	46.65a	10.55a	22.60a	Yellow brown
Riceberry	0.033b	8.50bc	7.05bc	6.00a	Dark purple
F-test ($P<0.01$)	**	**	**	**	
CV%	15.89	10.71	9.35	14.15	

[1/] Different letters in the same column indicate significant differences at the 0.05 level using LSD.

Table 4 Rice cooking quality including flour properties and chemical compositions of the three candidate lines compared with their parents in WS20.

Lines/Varieties	Pasting temperature (°C)	Viscosity (RVU) (Mean ± SD)				Cooking time (min)	Fat (%)	Protein (%)	Amylose (%)
		Peak viscosity	Breakdown	Final viscosity	Setback				
69-1-1	86.45 ± 1.20b[1/]	104.17 ± 2.95d	44.29±0.06d	113.42±4.12e	53.54±1.12d	24.50±0.45a	3.16 ± 0.25ab	6.58 ± 0.04ab	19.10 ± 0.40b
72-4-3	76.77 ± 0.11c	156.71 ± 2.42b	59.79±1.82ab	151.80±0.53c	54.87±0.06d	24.45±1.05a	2.78± 0.01b	6.37 ± 0.03c	21.02 ± 0.76a
72-4-9	77.55 ± 0.14c	137.75 ± 2.36c	61.12±0.64a	133.84±2.24d	57.20±0.53c	25.20±0.55a	2.21 ± 0.11c	6.23 ± 0.20cd	20.51 ± 0.19ab
Akitakomachi	76.02 ± 0.04c	194.67 ± 8.72a	47.00±0.47c	241.88±0.77a	85.21±2.77a	15.00±0.38c	2.81 ± 0.03b	6.87 ± 0.37a	17.29 ± 0.25c
Riceberry	89.92 ± 0.53a	130.84 ± 1.65c	28.00±1.30e	170.88±3.12b	68.58±2.00b	23.00±0.41ab	3.35 ± 0.01a	6.30 ± 0.75c	17.50 ± 0.21c
F-test ($P<0.05$)	*	*	*	*	*	*	*	*	*

CV%	0.85	2.25	1.54	3.21	2.85	1.20	0.51	0.65	0.75

[1/] Different letters in the same column indicate significant differences at the 0.05 level using LSD.

Table 5 Nutrition concentration of the three candidate lines compared with their parents in WS20.

Lines/Varieties	Iron (mg/100 g)	Zinc (mg/100 g)	Vitamin E (mg/100 g)	Vitamin B6 (mg/100 g)	Folic acid (µg/100 g)	Anthocyanin (mg/100 g)
69-1-1	1.69a[1/]	1.92b	1.49a	0.23a	48c	59bc
72-4-3	1.39b	1.88b	1.48a	0.17b	52b	62b
72-4-9	1.19c	2.15a	1.43ab	0.17b	54b	78a
Akitakomachi	1.02d	1.38c	0.41c	0.15bc	41d	0e
Riceberry	1.61a	1.99b	1.34b	0.15bc	70a	68b
F-test (*P*<0.01)	**	**	**	**	**	**

CV%	4.23	3.89	4.18	3.52	4.01	5.96

[1/] Different letters in the same column indicate significant differences at the 0.05 level using LSD.

Table 6 Phytochemical contents, antioxidant inhibitory effects and enzyme inhibitory activities of the three candidate lines compared with their parents in WS20.

Lines/Varieties	Phytochemical contents		Antioxidants inhibitory effects (%)		Enzymes inhibitory activities (%)	
	Flavonoids (mg RU/100 g extract)	Phenolics (mg GA/100 g extract)	DPPH	ABTS	α-amylase	α-glucosidase
69-1-1	61.03±1.14b[1/]	141.88±9.62b	8.13±3.91b	86.59±0.39a	3.35±0.22b	51.52±5.41a
72-4-3	51.13±1.25c	123.50±3.06c	8.24±3.97b	87.18±0.60a	2.99±0.23b	56.76±9.77a
72-4-9	71.65±1.79a	176.45±16.30a	11.99±2.89a	86.72±0.25a	11.10±1.38a	60.93±4.66a
Akitakomachi	1.05±0.58e	4.08±0.24e	0.00±0.00c	38.69±5.02b	3.86±0.75b	33.57±8.86b
Riceberry	31.56±0.51d	89.02±10.41d	7.04±3.21b	86.72±0.34a	13.08±0.61a	28.99±5.04b
F-test (P<0.05)	*	*	*	*	*	*
CV%	2.67	9.12	14.70	7.42	8.66	25.52

[1/] Different letters in the same column indicate significant difference at the 0.05 level using LSD.

Table S1 Gene-based/linked markers used for foreground selection for blast resistance and cooking quality for validation in the breeding lines.

Target Traits	**Gene**	**Chr.**	**Marker name**	**SNP/Indel**	**Homozygous target SNP/Indel**	**LGC code[1]**	**Aliquot ID[1]**
Amylose content	*wx*^{b}	6	wx_5UTR_G/T	G/T	T:T	002–0052.1	1107690034
Gelatinization temperature	*SSIIa*	6	ALK_ex8_SNP_A/G	A/G	A:A	002–0049.1	1103441167
Short grain	*GS3*	3	GS3	C/A	C:C	002-0755.1	1132528814
Blast resistance	*Pi-ta*	11	TBGI453598	T/C	T:T	002–0821.1	1140749274

[1] LGC group, 2015

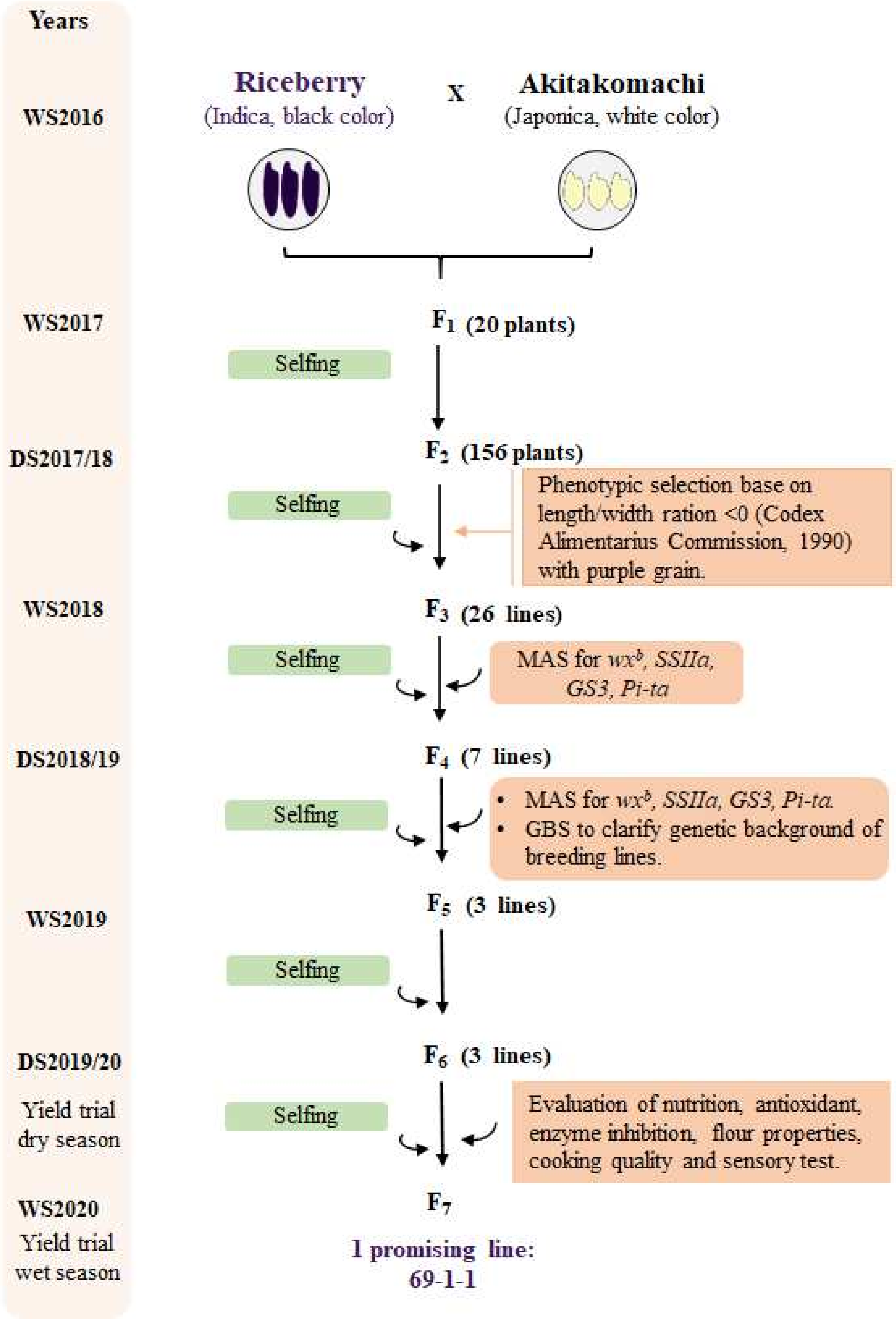

Figure 1 Scheme of the breeding program for black short grain rice derived from Akitakomachi x Riceberry from WS16 to WS21 in the Phan District, Chiang Rai Province, Thailand.

WS, Wet season; DS, Dry season; MAS, Marker-assisted selection; GBS, Genotype by sequencing

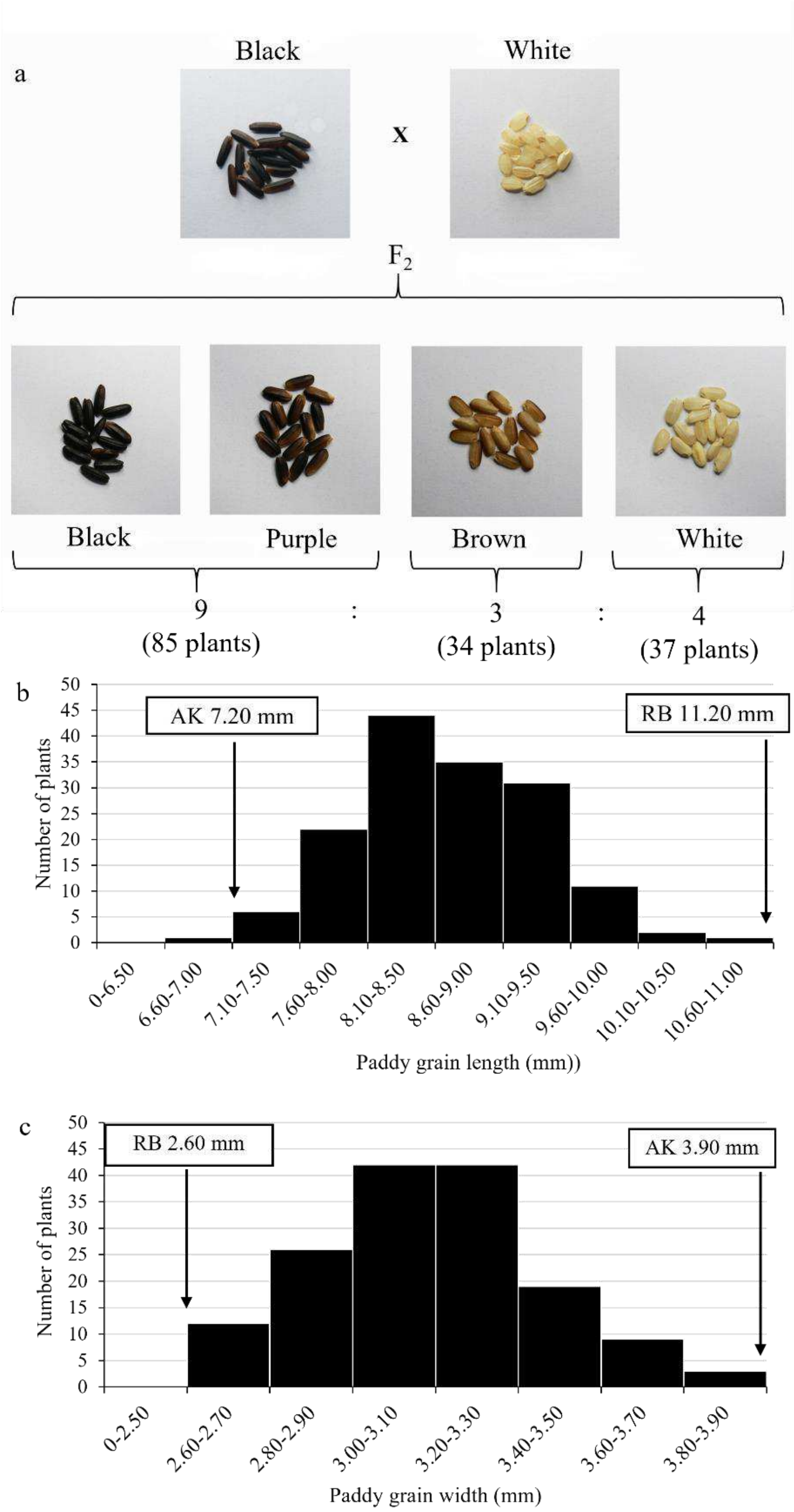

Figure 2 F$_2$ segregation of (a) grain color, (b) grain length and (c) grain width compared with Akitakomachi and Riceberry.

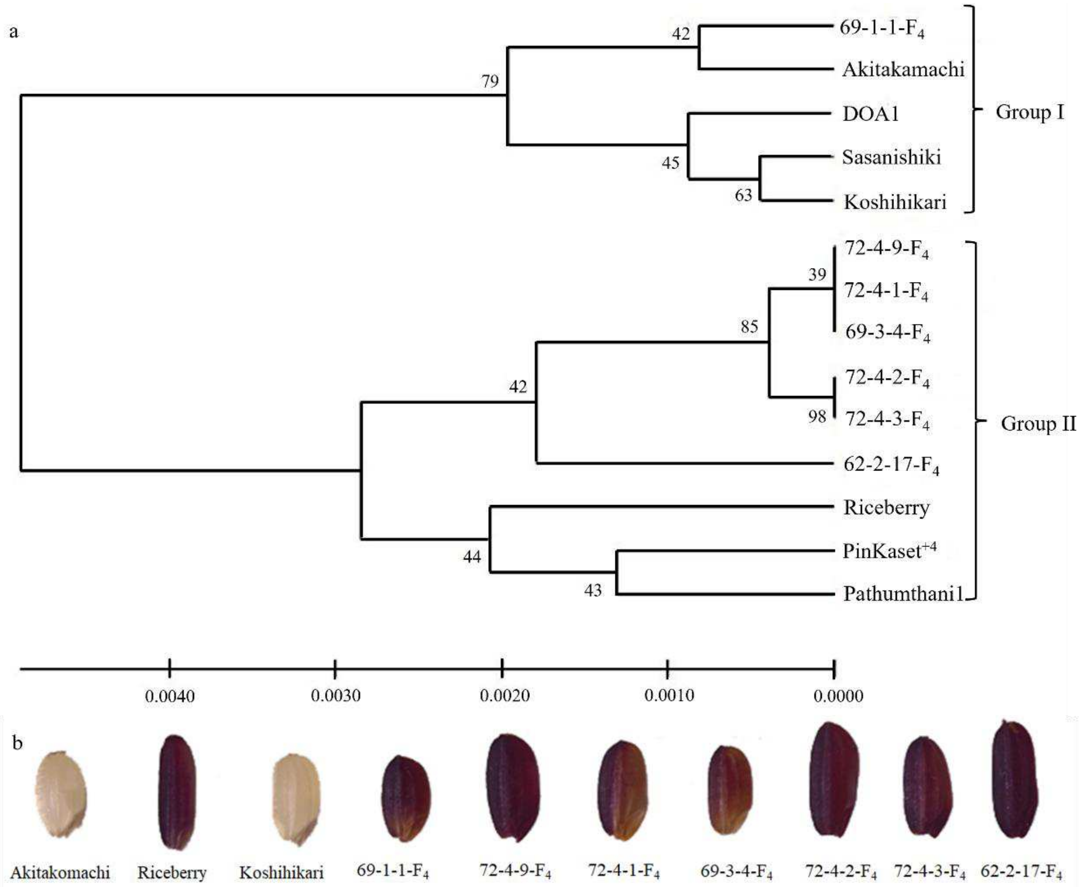

Figure 3 Phylogenetic tree (a) of the breeding lines in F_4 and controlled varieties based on genotyping by sequencing. The phylogenetic tree was classified into two groups, and the numbers at the nodes indicate the percentages obtained with 1000 bootstrap replicates. The unpolished grains (b) of seven selected lines in F_4 compared with their parents and Koshihikari.

Figure 4 Plant type (a and b), paddy grain (c), unpolished grain (d), cooked grain (e) and longitudinal section of unpolished grain (f) of the three candidate lines in the F_7 generation compared with their parents in WS20.

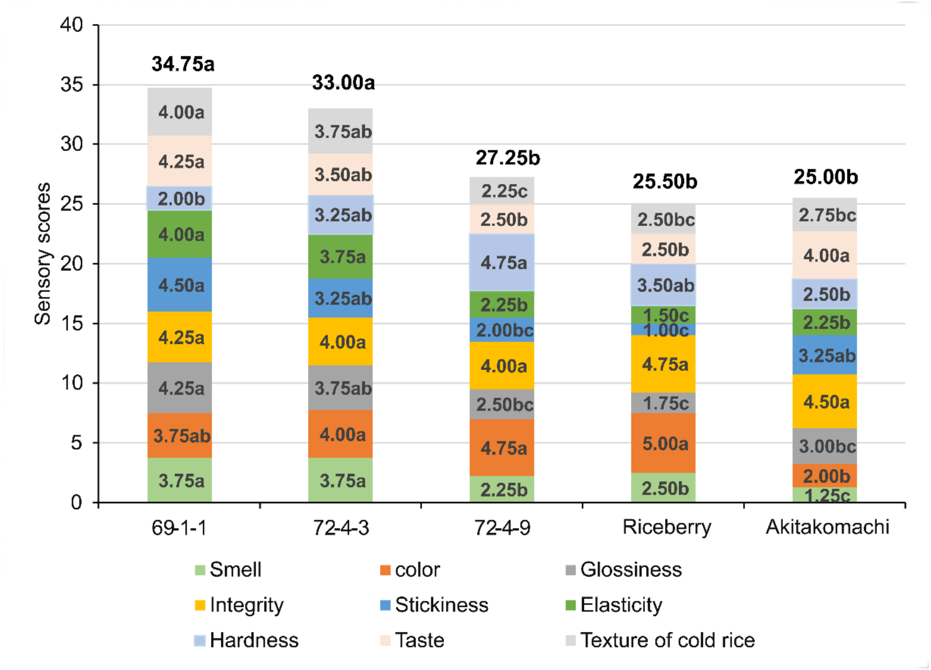

Figure 5 Sensory test results of the unpolished grains of the three candidate lines in the F_7 generation compared with their parents in WS20.

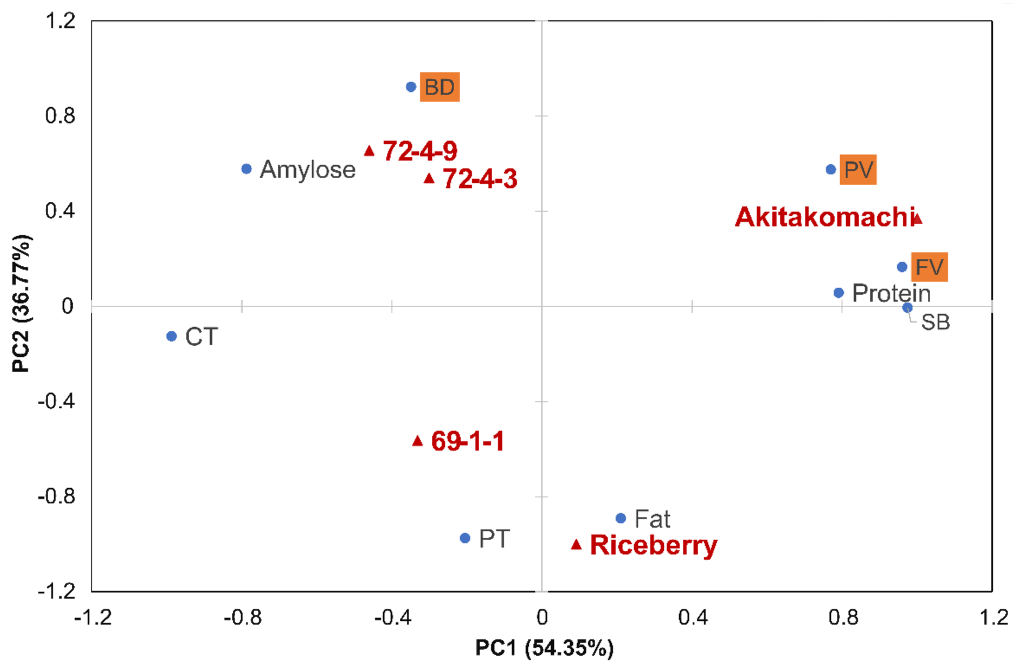

Figure 6 Biplot graph of the PC1 score versus the PC2 score for the grain quality of the three candidate lines and the control varieties. AK, Akitakomachi, RB, Riceberry, BD, Breakdown, FV, Final viscosity, PV, peak viscosity, SB, Setback, CT, Cooking time, PT, Pasting temperature.

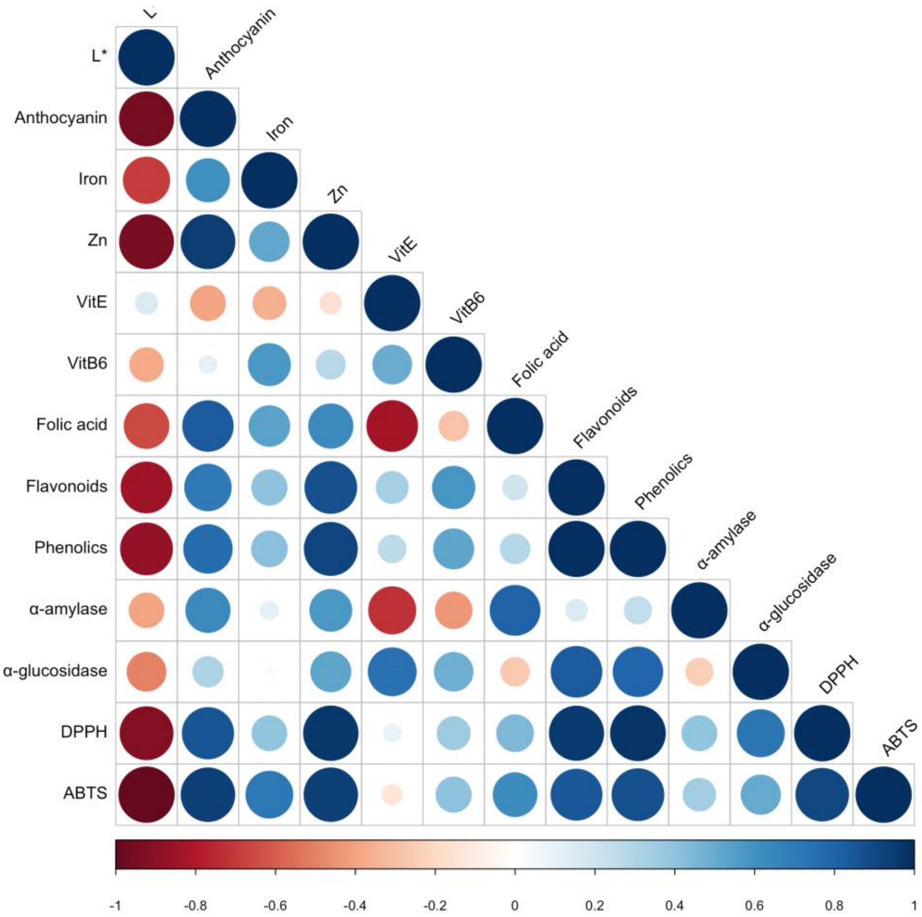

Figure 7 Correlation coefficients (*r*) among color intensity (L*), nutrition, phytochemical, antioxidant and antidiabetic activity.

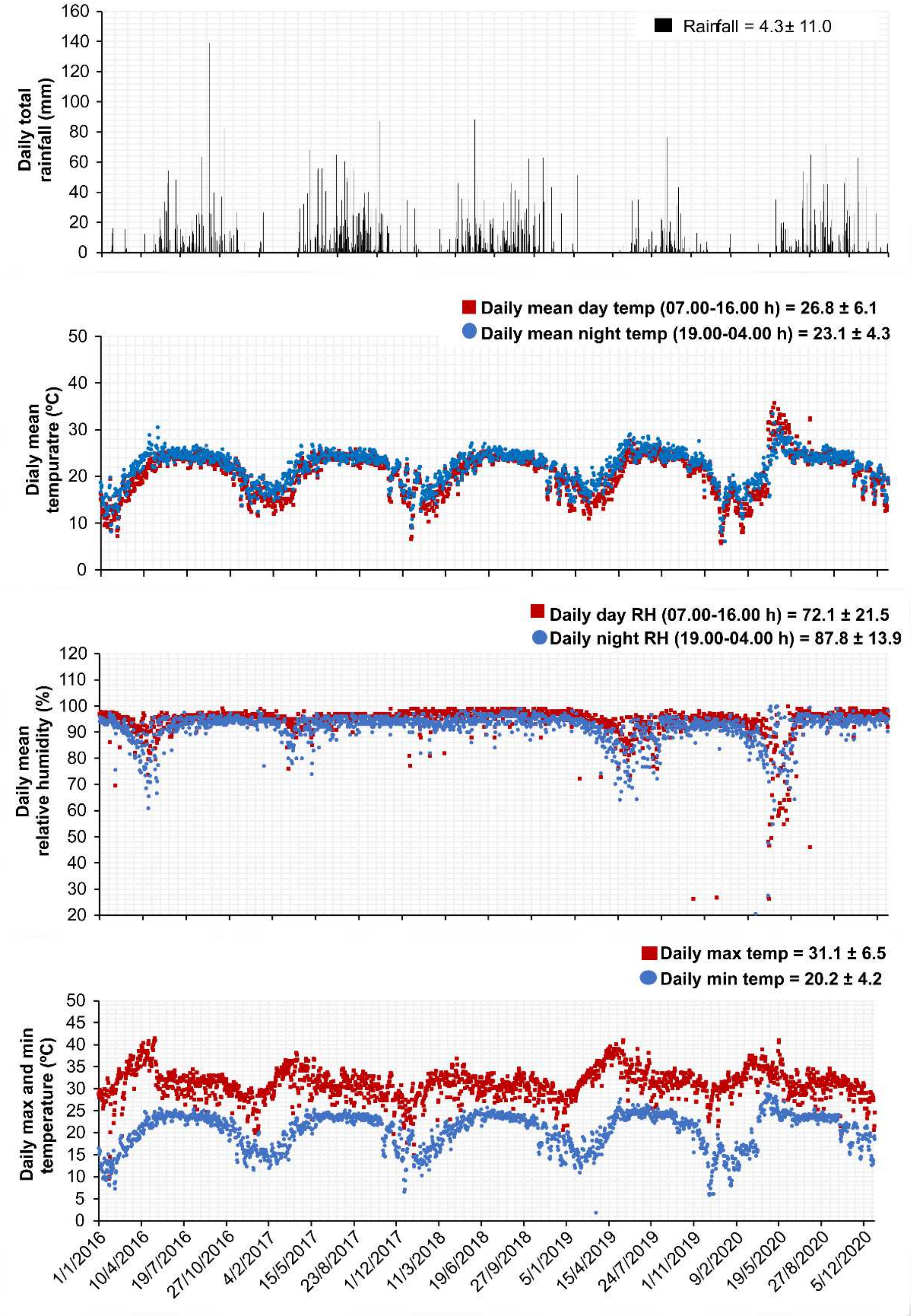

Figure 1S Weather data from WS16-WS20 in the Phan District, Chiang Rai Province.

Fig. 2S F_2 segregation of grain color derived from Riceberry x Akitakomachi.