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Selection and evaluation of black short grain breeding lines 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 tropical indica black rice, Riceberry and temperate japonica white rice, Akitakomachi was performed by pedigree selection with maker-assisted selection (MAS). The three candidate lines showed highly favourable 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 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 programme can create black short grain rice variety adapted to a tropical environment, similar to japonica-type rice.

Introduction

Japanese food has been widespread in Thailand for more than three decades. The number of Japanese restaurants increased dramatically in Thailand from 2274 to 5751 restaurants over 10 years (2013–2023). As a result, Thailand has the largest Japanese restaurant business in Asia (Jetro Thailand, 2023). In addition, 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 pericarp. The pericarp of coloured rice grains accumulates anthocyanins. These compounds have antioxidant activity and health benefits such as reduction in the risk of cancer and obesity (Yamuangmorn and Prom-u-Thai, 2021). 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 (Machon *et al.*, 2021; Xia *et al.*, 2021). In addition, black rice is high in fibre, vitamins B and E, iron (Fe), thiamine, magnesium (Mg), niacin and phosphorous (P) (Zhang *et al.*, 2004; Mbanjo *et al.*, 2020). Therefore, black rice is becoming popular among rice consumers and dieticians due to its high nutritive and medicinal value (Kong *et al.*, 2008). However, most 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 yield still need to be improved (Maeda *et al.*, 2014). Therefore, many researchers have tried to take advantage of the premium short grain rice variety namely Koshihikari's good eating quality (Kobayashi *et al.*, 2018), while adding a black coloured pericarp, containing anthocyanins or tannins (both healthful nutraceuticals), respectively (Maeda *et al.*, 2014). However, the cultivation of Koshihikari is very limited in tropical climates and results in a low yield and low cooking quality (Kobayashi *et al.*, 2018). Therefore, the japonica rice variety Akitakomachi is more suitable than other japonica rice varieties for growth in the northern parts of Thailand due to its resistance to hot weather (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). Thus, Akitakomachi can be used in short grain breeding programmes in tropical climates.

Recently, the premium long grain black rice variety 'Riceberry' has become a registered rice variety in Thailand; it is a cross-bred variety obtained from Jao Hom Nin, a local indica non-glutinous black rice, and Khoa Dawk Mali 105 (premium fragrant rice). Researchers initially developed Riceberry with the aim of boosting the nutritional value, fragrance and taste of rice. In addition, Riceberry is resistant to blast disease, which is a major disease in Thailand (Vanavichit, 2021). Therefore, Riceberry is the most popular black rice variety and is known for its health-promoting properties.

The main genetic factors controlling stickiness and hardness of cooked rice grains in japonica rice is the wx^b gene, which results in moderate amylose content (Shao *et al.*, 2020; Hori *et al.*, 2021). In addition, Miura *et al.* (2018) reported that $SSIIa^J$ is found in japonica rice that reduce starch synthase activity, resulting in an increase in amylopectin short chains and reduces gelatinization temperature when compared with $SSIIa^I$ that is found in indica rice. Furthermore, the *GS3* gene appears to exert the most control grain length (Li *et al.*, 2018). All the short and medium grains genotyped carry the C-allele and all the long and extra-long grains carry the A-allele (Calingacion *et al.*, 2014).

Thus, the present research focused on the improvement of a 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 maker-assisted selection (MAS) of cooking quality.

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 5 years were 26.8/23.1°C, and the mean maximum/minimum temperatures over the 5 years were 31.1/20.2°C. The mean relative humidity during the day/night over the 5 years was 72.1/87.8% RH, and the mean total rainfall over the 5 years was 1497 mm (Fig. S1). The rice plants in every generation were seeded in a field nursery. After 30 days, the rice seedlings were transplanted into breeding plots. The soil in the Phan District consisted of 1.56% organic matter, 0.07% total nitrogen, 26.7 mg/kg available phosphorus, 75.5 mg/kg exchangeable potassium, 629.0 mg/kg exchangeable calcium and 76.5 mg/kg exchangeable Magnesium and had a pH of 5.40. Additionally, basal fertilizer was applied 15 days 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 days) at a rate of 57.5 kg N/ha. Other management practices were in accordance with conventional approaches for high-yield japonica rice cultivation.

Breeding schemes

An indica black long grain, Riceberry was used as the female parent, and a japonica white short grain, Akitakomachi was used as the male parent. 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 (Fig. 1). Grain colour and japonica grain shape were used as criteria for phenotypic selection (Juliano and Villareal, 1993). 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 to March 2020 (DS19/20). The experiment was conducted in a randomized complete block design (RCBD), with three replications. The plot size for each treatment was 2.5 × 2.5 m (6.25 m^2), with a spacing of 25 × 25 cm. After that, the validation of candidate lines (F_7) was conducted in the second yield trial in wet season from June to September 2020 (WS20). The RCBD with three replications was applied, and the plot size for each treatment was 2.5 m × 3.5 m (8.75 m^2), with a spacing of 25 × 25 cm.

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 analyse the single-nucleotide polymorphisms (SNPs) in each sample. 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 ($SSIIa^J$), short grain (*GS3*) and blast resistance (*Pi-ta*; TBGI453598) (Table S1). Polymerase chain reaction (PCR) was performed on Hydrocycler™ (LGC, Serial No. 2165-3564-129, Middlesex, UK) water bath-based thermal cyclers using the following thermal cycling profile, starting with the following thermocycler touch-up PCR cycle (Table S2). In addition, all Kompetitive Allele Specific PCR (KASP) assay genotyping was performed using the LGC SNP line system following standard KASP protocols (LGC Group, 2020).

Determination of yield and yield components

The agronomic traits examined included the days to 50% flowering, plant height, number of tillers per plant, number of panicles per plant, panicle length, seed set rate, 1000-grain weight and grain yield. The grain yield in each plot was determined per harvested area of 6.25 or 8.75 m^2. The grain moisture was adjusted to 14% and then extrapolated to units of kg/ha.

Grain and cooking quality assessments

The paddy grains were dehulled using a mini-polisher. Three physical grain qualities including grain length, grain width and the grain length to width ratios of both paddy grain and

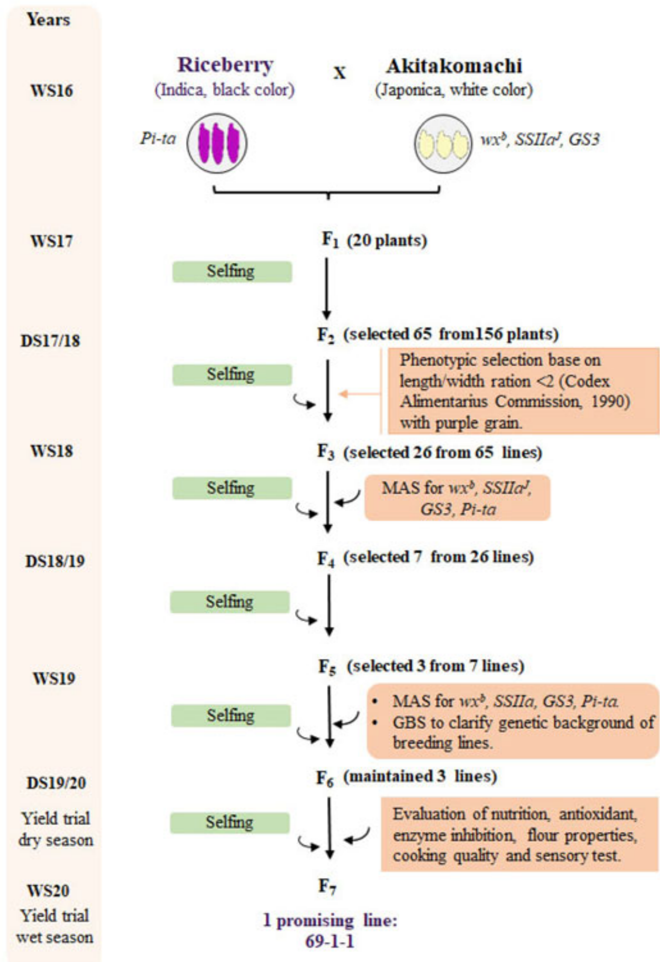

Figure 1. Scheme of breeding programmes for black short grain rice derived from Riceberry × Akitakomachi from WS16–WS20 in Phan district, Chiang Rai Province.

unpolished grain were measured using a two-decimal-point digital Vernier calliper. Three chemical grain qualities, including amylose was determined based on Juliano (1985). Protein was calculated by estimating nitrogen content with the Kjedahl method (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 and the pasting properties of the rice flours were determined according to the method described by Juliano (1985) using a Rapid Visco Analyser (RVA, Model 4-D, Newport Scientific, Australia).

Evaluation of the sensory qualities of cooked rice

The rice cooking procedure followed the method of Saichompoo *et al.* (2021). The unpolished rice samples were cooked using a rice cooker (Sharp model KS-ZT18, Thailand). Seven panellists 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). The overall quality was the sum of the scores for all the attributes.

Evaluation of nutritional and phytochemical contents

The iron, zinc, vitamin E (α-tocopherol), vitamin B6 and folic acid contents in brown rice were analysed by the Institute of Nutrition, Mahidol University, Thailand, which followed the protocol of Association of Official Agricultural Chemists (AOAC) (Juliano, 1985). The anthocyanin content in pericarp grains was measured according to the procedures described by Rahman *et al.* (2015).

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 1:

$$Y = 0.0083x + 0.0511, \quad R^2 = 0.999 \tag{1}$$

In addition, the total phenolic content was evaluated using Folin–Ciocalteu reagent. The total phenolic content was calculated from the calibration curve of gallic acid equivalents (GAE) according to the formula 2:

$Y = 0.0094x + 0.0028, \quad R^2 = 0.998$ (2)

Antioxidant and enzymatic assays

The inhibitory effect of rice grain water extracts on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging was determined following the method of Boskou *et al.* (2006). In addition, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging was investigated using the method of Hsu *et al.* (2011).

The α-amylase inhibition assay was performed following the method of Kwon *et al.* (2006). The α-glucosidase inhibition assay was performed following the method of Ahmad *et al.* (2011). The per cent inhibition of free radical scavenging and enzymatic activities was calculated from the absorbance data using the formula: inhibition (%) = [(Abscontrol – Abstest)/Abscontrol] × 100.

Pericarp pigment colour 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 colour of the rice pericarp, 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.

Statistical analysis

All the data were analysed using R program version 3.6.1 to test the significance of the results in terms of agronomic traits, cooking quality and nutrition value. The means were separated using Duncan's test at alpha levels of 0.05. Chi-square (χ^2) was used to analyse the relationship of the segregation ratio of grain colour in the F_2 generation. In addition, correlation analysis (r^2) was used to evaluate colour 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

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grain variety ‘Akitakomachi’, the seed pericarps of 85, 34 and 37 plants were classified as showing black, brown and white coloration, respectively (Fig. 2(*a*)). Therefore, the colour 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 colour intensity. Conversely, the paddy grain shape of the F_2 population segregated according to a normal distribution, as shown in Fig. 2(*b*) and (*c*). The paddy grain shapes of F_2 progenies ranged from 6.60 to 11.00 mm in grain length and 2.60 to 3.90 mm in grain width, respectively. Therefore, 65 of 156 F_3 seeds that had short grains with black colour 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, seven lines were selected, and their growth was continued to obtain the F_5 generation.

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 seven 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* (Table S3).

Phylogenetic relationships of the F_4 selected lines

In the F_4 generation, the seven selected lines were analysed by GBS for their genetic backgrounds together with their parents and control varieties. The phylogenetic tree was divided into two groups (Fig. 3). Group I contained one selected line (69-1-1) together with Akitakomachi, Sasanishiki, Koshihikari, DOA1 and DOA2. 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 other indica varieties together with six selected lines. However, grain shape of 69-3-4, 72-4-1, 72-4-2, 72-4-3 and 72-4-9 were identified as 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).

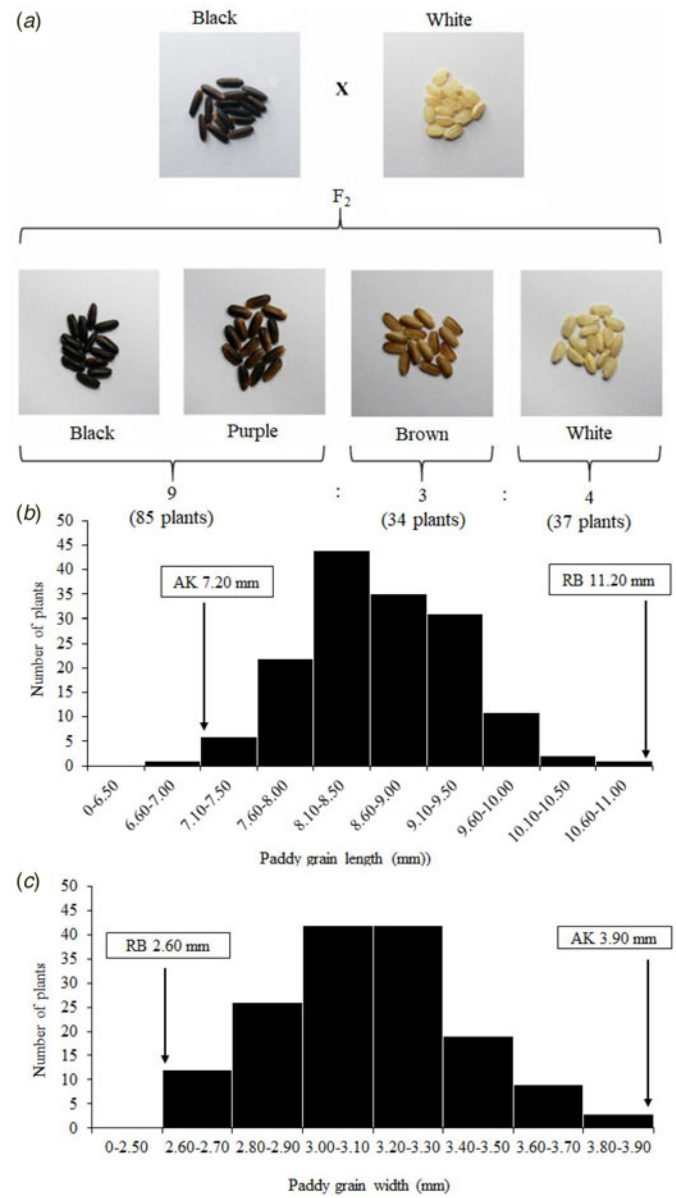

Figure 2. F_2 segregation of (a) grain colour, (b) grain length and (c) grain width compared with Akitakomachi and Riceberry.

Evaluations of agronomic traits and grain yield

The yield trial experiment was conducted during a dry season 2019/2020 (DS19/20) (F_6) and a wet season 2020 (WS20) (F_7). The plant types of the candidate lines with their parents are shown in Fig. 4(*a*) and (*b*). The three candidate lines (69-1-1, 72-4-3 and 72-4-9) 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.

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. In addition, the numbers of tillers and panicles of 69-1-1 was higher than the other lines/varieties. However, the grain yield of 69-1-1 was not significantly different from that of Riceberry (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. 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).

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). 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

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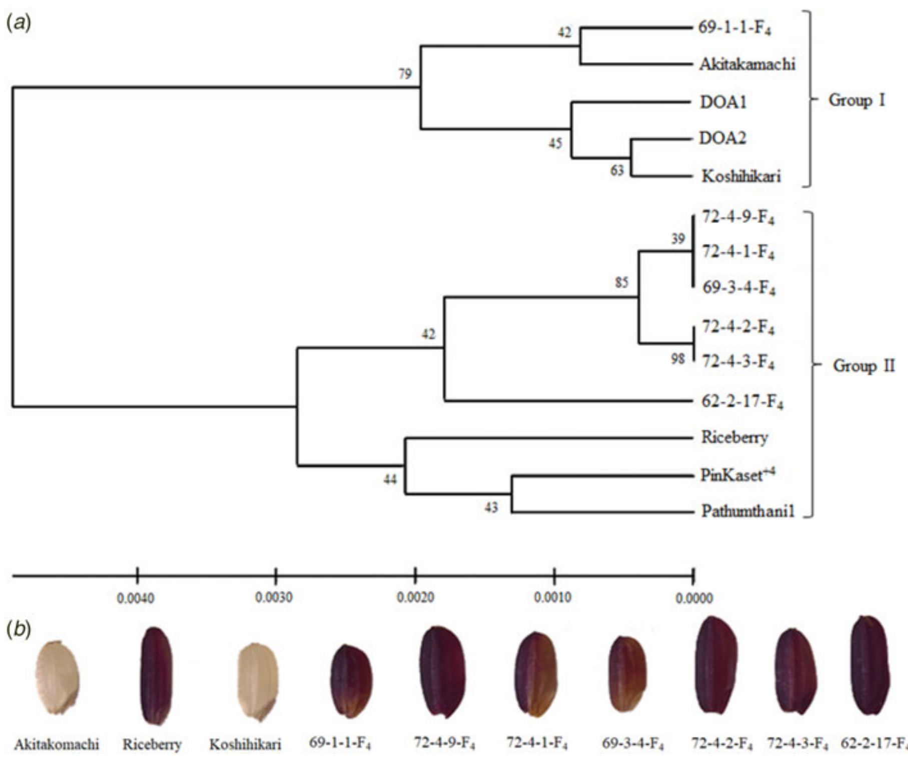

Figure 3. Phylogenetic tree (a) of the breeding lines in F_4 and controlled varieties based on genotyping by sequencing. The phylogenetic tree was classified into two groups, and the numbers at the nodes indicate the percentages obtained with 1000 bootstrap replicates. The unpolished grains (b) of seven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

and Koshihikari in both seasons (Table 1 and Fig. 4(*c*)–(*e*)). Therefore, in terms of grain yield, grain colour and grain shape, 69-1-1 was identified as a promising black short grain line.

Colour intensity

The colour intensity on the rice pericarp differed among the candidate lines and their parents, as shown in Table 2 and Fig. 4(*d*)–(*f*). The 72-4-3 and 72-4-9 were not significantly different from Riceberry and had the most intense colour, while 69-1-1 was slightly brighter than the other candidate lines and Riceberry.

Sensory test and cooking quality

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 (Fig. 5).

The three candidate lines and Riceberry had the longest cooking times (23–25 min), while Akitakomachi (white colour 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 3).

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 3). 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.

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%). However, the protein contents of all lines/varieties in this study were within ranges previously reported for rice (5–8%) (Table 3).

The results of the nutrition analysis are shown in Table 2. 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.

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 2).

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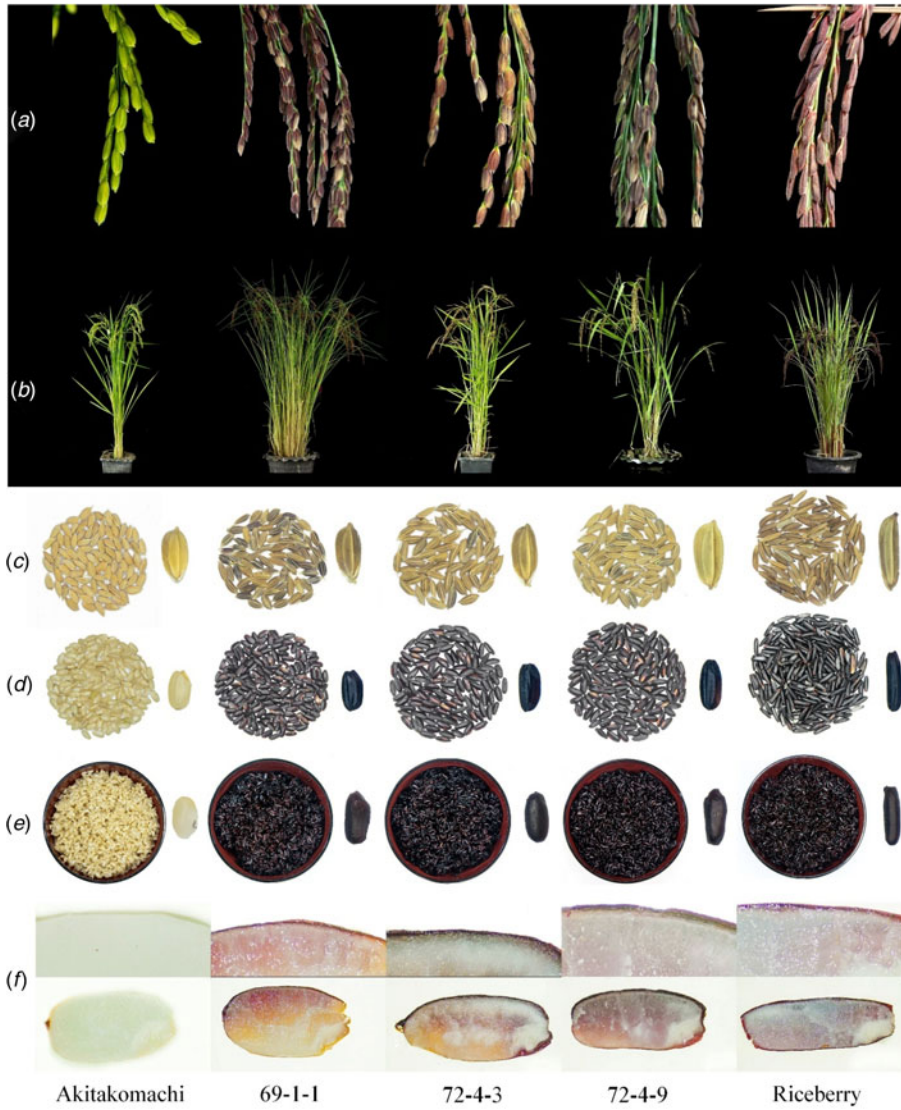

Figure 4. Plant type (a and b), paddy grain (c), unpolished grain (d), cooked grain (e) and longitudinal section of unpolished grain (f) of the three candidate lines in the F_7 generation compared with their parents in WS20.

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%). 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 (Table 2).

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 2).

When considering the correlation analysis (Fig. 6), it was found that the intensity of grain colour (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 colour in pericarp and grain size

The colour segregation analysis in F_2 progenies from Riceberry (black colour) × Akitakomachi (white colour) showed the same results as many researchers (Rahman *et al.*, 2013; Maeda *et al.*, 2014; Lee *et al.*, 2018) reported that the character of black pigment is controlled by two complementary dominant genes, *Pb* and *Pp*, with recessive epistasis (9:3:4). The black pigment gene of rice is perfectly dominant, and its activity is higher than the parent with black pericarp pigment (Maeda *et al.*, 2014; Kristamtini *et al.*, 2019). However, in the black and brown colour groups, there was also variation in colour intensity. This may suggest that the colour of the rice pericarp is controlled by other genes in the pathway of anthocyanin synthesis (Xia *et al.*, 2021). However, the purple pigmentation in the pericarp is an easily observable and selective feature that does not require MAS.

The segregation of grain size was controlled by quantitative genetics that combines grain length, grain width and grain thickness characteristics (Ponce *et al.*, 2020). Lu *et al.* (2023) reported

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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	Season	69-1-1	72-4-3	72-4-9	AK	KH	RB	*F*-test	CV%
DF (days)	DS19	89b[1/]	90b	90b	91b	91b	113a	**	3.17
	WS20	63c	72b	74b	65c	64c	107a	**	4.59
PH (cm)	DS19	98b	121a	107ab	100b	99b	94c	**	3.17
	WS20	110a	117a	108a	92b	83c	110a	**	4.59
NTP	DS19	22ab	21ab	25a	15b	21ab	27a	**	12.23
	WS20	36a	27bc	30ab	17d	21cd	27a	**	13.59
NPP	DS19	22ab	21ab	25a	15b	21ab	27a	**	8.23
	WS20	33a	25bc	29ab	17d	20cd	27abc	**	10.59
TGW (g)	DS19	22.1d	25.7c	27.4b	25.6a	29.8a	20.7d	**	4.59
	WS20	22.6c	28.1b	28.5b	30.6a	30.7a	21.8c	**	4.59
GY (t/ha)	DS19	7.12a	5.63bc	4.85c	5.93b	4.38d	7.38a	**	10.24
	WS20	6.67a	5.95bc	4.65c	6.50b	4.40cd	6.96a	**	14.85
PGL (mm)	DS19	7.23d	8.29bc	9.10ab	7.37cd	7.53cd	9.96a	**	4.59
	WS20	8.13cd	8.57bc	8.94b	7.52d	7.57d	10.52a	**	4.59
PGW (mm)	DS19	3.00c	3.49a	3.47ab	3.87a	3.75a	2.88c	**	4.59
	WS20	3.45bc	3.36bc	3.26c	3.96a	3.65b	2.50d	**	4.59
PGR (L/W)	DS19	2.28c	2.23c	2.68b	1.84d	2.08cd	3.89a	**	4.59
	WS20	2.35c	2.55bc	2.74b	1.90d	2.08d	4.22a	**	4.59
UGL (mm)	DS19	5.29c	5.76c	6.91b	5.36c	5.55c	7.71a	**	4.59
	WS20	6.01bc	6.15b	6.05b	5.51d	5.58cd	7.39a	**	4.59
UGW	DS19	2.81b	3.15a	3.13a	3.16a	3.20a	1.98c	**	4.59
	WS20	2.94a	2.88a	2.93a	3.16a	3.12a	1.96b	**	4.59
UGR (L/W)	DS19	1.88bc	1.83c	2.20b	1.70c	1.73c	3.91a	**	4.59
	WS20	2.05cd	2.07cd	2.14b	1.75cd	1.79cd	3.77a	**	4.59

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; PGL, paddy grain length; PGW, paddy grain width; PGR, paddy grain length/width ratio; UGL, unpolished grain length; UGW, unpolished grain width; UGR, unpolished grain length/width ratio.
Different letters in the same row indicate significant differences at the 0.05 level using LSD.

Table 2. 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	86.4 ± 1.2b[1/]	104 ± 3d	44.3 ± 0.1d	113 ± 4e	53.5 ± 1.1d	24.5 ± 0.5a	3.16 ± 0.25ab	6.58 ± 0.04ab	19.1 ± 0.4b
72-4-3	76.8 ± 0.1c	157 ± 2b	59.8 ± 1.8ab	152 ± 1c	54.9 ± 0.1d	24.4 ± 1.1a	2.78 ± 0.01b	6.37 ± 0.03c	21.0 ± 0.8a
72-4-9	77.6 ± 0.1c	138 ± 2c	61.1 ± 0.6a	134 ± 24d	57.2 ± 0.5c	25.2 ± 0.6a	2.21 ± 0.11c	6.23 ± 0.20cd	20.5 ± 0.2ab
Akitakomachi	76.0 ± 0.0c	195 ± 9a	47.0 ± 0.5c	242 ± 1a	85.2 ± 2.8a	15.0 ± 0.4c	2.81 ± 0.03b	6.87 ± 0.37a	17.3 ± 0.3c
Riceberry	89.9 ± 0.5a	131 ± 2c	28.0 ± 1.3e	171 ± 3b	68.6 ± 2.0b	23.0 ± 0.4ab	3.35 ± 0.01a	6.30 ± 0.75c	17.5 ± 0.2c
F-test ($P < 0.05$)	*	*	*	*	*	*	*	*	*
CV%	0.85	2.25	1.54	3.21	2.85	1.20	0.51	0.65	0.75

Different letters in the same column indicate significant differences at the 0.05 level using LSD.

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

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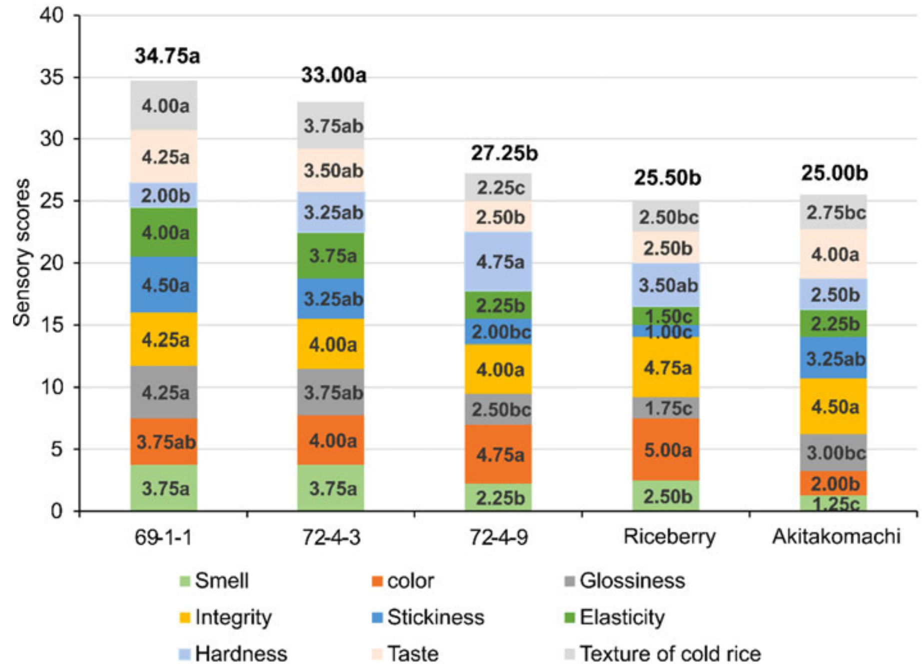

Figure 5. Sensory test results of the unpolished grains of the three candidate lines in the F_7 generation compared with their parents in WS20.

Table 3. Nutrition concentration, phytochemical contents, antioxidant inhibitory effects and enzyme inhibitory activities of the three candidate lines compared with their parents in WS20

Lines/varieties	69-1-1	72-4-3	72-4-9	AK	RB	*F*-test	CV%
Iron (mg/100 g)	1.69a[1/]	1.39b	1.19c	1.02d	1.61a	**	4.23
Zinc (mg/100 g)	1.92b	1.88b	2.15a	1.38c	1.99b	**	3.89
Vitamin E (mg/100 g)	1.49a	1.48a	1.43ab	0.41c	1.34b	**	4.18
Vitamin B6 (mg/100 g)	0.23a	0.17b	0.17b	0.15bc	0.15bc	**	3.52
Folic acid (µg/100 g)	48c	52b	54b	41d	70a	**	4.01
Anthocyanin (mg/100 g)	59bc	62b	78a	0e	68b	**	5.96
Flavonoids (mg RU/100 g extract)	61.0b	51.1c	71.7a	1.05e	31.6d	**	2.67
Phenolics (mg GA/100 g extract)	1428b	1240c	176a	4.08e	89.0d	**	9.12
DPPH (%)	8.13b	8.24b	12.0a	0.00c	7.04b	**	14.70
ABTS (%)	86.6a	87.2a	86.7a	38.7b	86.7a	**	7.42
α-amylase (%)	3.35b	2.99b	11.1a	3.86b	13.08a	**	8.66
α-glucosidase (%)	51.5a	56.8a	60.9a	33.6b	29.0b	**	25.52
Pigment intensity (L^*)	10.4b	9.70bc	8.30bc	46.7a	8.50bc	**	10.71
Pigment intensity (a^*)	8.50b	7.60b	7.10bc	10.6a	7.05bc	**	9.35
Pigment intensity (b^*)	8.05b	5.45b	5.25b	22.6a	6.00a	**	14.15

AK, Akitakomachi; RB, Riceberry.
Different letters in the same row indicate significant differences at the 0.05 level using LSD.

between indica and japonica types (Fan *et al.*, 2006). Thus, *GS3* could be used as MAS to identify grain size in this breeding programme.

Agronomic and environmental factors

Japonica rice is suitable for cultivation in mid-latitude regions (between 53° N and 36° S latitudes) at lower temperatures and under longer days than in tropical areas (Khush, 1997; Zhou *et al.*, 2021). In this research, the breeding lines were bred and the yield trials were conducted at a longitude of 19 N and a latitude of 99 E under 26.8/23.1°C mean day/night temperatures and day length was not more than 12 h over 5 years. Interestingly, the 69-1-1 showed a high grain yield similar to that of indica variety ‘Riceberry’, although the flowering day was the same as those of temperate japonica varieties. Therefore, rice plants genetically

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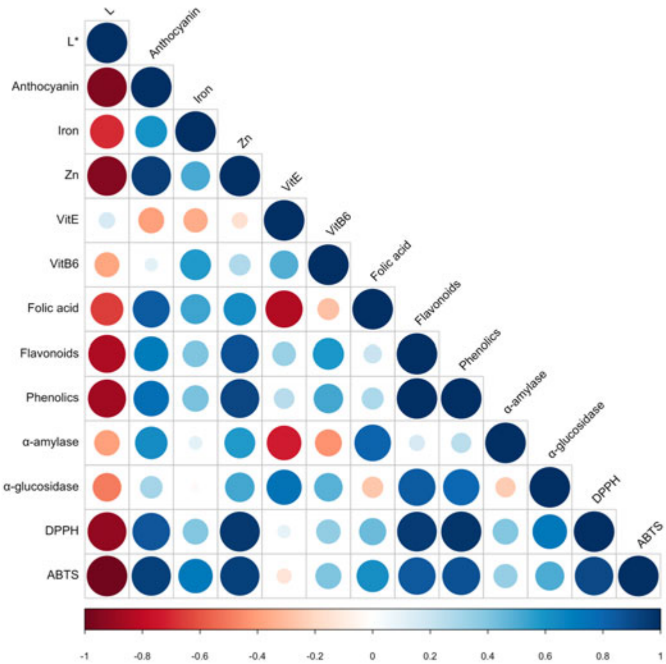

Figure 6. Correlation coefficients (*r*) among colour intensity (*L**), nutrition, phytochemical, antioxidant and antidiabetic activity. *L**, degree of lightness or darkness; Zn, zinc; VitE, vitamin E; VitB6, vitamin B6; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid).

belonging to temperate japonica varieties can be bred to adapt to tropical region by introgressing indica genetically into breeding lines (Negrao *et al.*, 2008; Saichompoo *et al.*, 2021).

Grain and cooking quality

Amylose is one of the components of rice starch that greatly affects cooking and eating qualities (Karim *et al.*, 2024). Generally, the amylose content of Japonica rice starch ranges from 0 to 20% (Luo *et al.*, 2021). In this study, an amylose content of less than 20% among the candidate lines was found only in 69-1-1 (19.10%). In addition, the unpolished grain size ratio of the promising line 69-1-1 was within the standard for japonica grain shape (ratio < 2) (Juliano and Villareal, 1993). 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} and *SSIIa* genes that are critical genes to control amylose content (Saichompoo *et al.*, 2021) were presented in the candidate lines.

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 were closer with indica parent than those of japonica parent.

Nutritional value and antioxidant and antidiabetic activities

The anthocyanin content in black rice pericarp also varies depending on the rice cultivars (Jiamyangyuen *et al.*, 2017). Kushwaha *et al.* (2020) reported that temperate climate could favour the rise in grain's anthocyanin content than the warm climate. In addition, it was also found that as the colour intensity increased, the anthocyanin content also increased (Machon *et al.*, 2021). Moreover, the colour intensity had affected the

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anthocyanin content in rice pericarp. Therefore, the breeding procedure for anthocyanin content from indica black rice x japonica white rice by phenotypic selection using colour 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 *et al.* (2012) who reported that the colour 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 (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 (Tungmunnithum *et al.*, 2018). In this research, the contents of phenolics, flavonoids and anthocyanins were negatively correlated with L^* colour intensity values, in accord with previous findings (Goufo and Trindade, 2014). However, the total antioxidant activity of black rice bran was correlated with the contents of total anthocyanins, total phenolics and total flavonoids (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 (Chiou *et al.*, 2018). All black and red bran extracts inhibit α-glucosidase activity; however, only red rice bran extracts inhibit α-amylase activity (Wongsa *et al.*, 2019). In this study, α-glucosidase had the greatest correlation with anthocyanin content. However, α-amylase activity was not correlated with anthocyanin content that was different from those of Wongsa *et al.* (2019).

Conclusion

A breeding programme for obtaining black colour, short grain with high nutritional and phytochemical values can provide a promising line for growth in tropical climate that combines tropical adaptability with high yield and high nutritional value from tropical indica rice (Riceberry) and short grain with good cooking and eating quality from temperate japonica rice (Akitakomachi). Finally, the promising 69-1-1 line has undergone short grain rice breeding with the same yield as indica black rice together with high anthocyanin content and good cooking and eating quality according to Japanese food standards.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0021859625000048

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