Genetic dissection of black grain rice by the development of a near isogenic line

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Rice (*Oryza sativa* L.) can produce black grains as well as white. In black rice, the pericarp of the grain accumulates anthocyanin, which has antioxidant activity and is beneficial to human health. We developed a black rice introgression line in the genetic background of *Oryza sativa* L. 'Koshihikari', which is a leading variety in Japan. We used *Oryza sativa* L. 'Hong Xie Nuo' as the donor parent and backcrossed with 'Koshihikari' four times, resulting in a near isogenic line (NIL) for black grains. A whole genome survey of the introgression line using DNA markers suggested that three regions, on chromosomes 1, 3 and 4 are associated with black pigmentation. The locus on chromosome 3 has not been identified previously. A mapping analysis with 546 F_2 plants derived from a cross between the black rice NIL and 'Koshihikari' was evaluated. The results indicated that all three loci are essential for black pigmentation. We named these loci *Kala1*, *Kala3* and *Kala4*. The black rice NIL was evaluated for eating quality and general agronomic traits. The eating quality was greatly superior to that of 'Okunomurasaki', an existing black rice variety. The isogenicity of the black rice NIL to 'Koshihikari' was very high.

Key Words: black rice, *Kala*, 'Koshihikari', 'Hong Xie Nuo', mapping, near isogenic line, *Oryza sativa* L.

Introduction

Rice (*Oryza sativa* L.) is one of the most important cereal crops in the world. Most rice cultivars produce grains with white pericarps, but some have colored pericarps, which can be red, brown, or black (also known as purple). The pericarps of colored rice grains accumulate proanthocyanidin ("red rice") or anthocyanin ("black rice"). These proanthocyanidins and anthocyanins have antioxidant activity and their health benefits have been demonstrated not only in rice but also in soy bean, potato, and sweet potato (Hou *et al.* 2010, Jang *et al.* 2010, Kaspar *et al.* 2011, Kawasaki *et al.* 2007, Kwon *et al.* 2007, Ling *et al.* 2001, Suda *et al.* 2008). Since rice is a staple food for many consumers around the world, we expect that the introduction of colored rice could have a greater global health impact than the introduction of other foods containing proanthocyanidins and anthocyanins.

In Japan, black rice varieties have been bred with im-

provements in traits related to cultivation (for example lodging resistance and early maturing) and several cultivars have already been released (Higashi *et al.* 1997, Saka *et al.* 2007, Takita *et al.* 2001). However, black rice varieties with improved eating quality have not yet been developed. The most widely produced rice cultivar in Japan is 'Koshihikari', because of its palatability and good viscosity when boiled. One approach to developing new cultivars with superior eating quality is to develop near isogenic lines (NILs) with novel agronomic traits in the genetic background of 'Koshihikari' (Kojima *et al.* 2004, Takeuchi *et al.* 2006, Wan *et al.* 2005). An effective tool in the development of NILs is marker-assisted selection, in which DNA markers are used to identify plants carrying desired traits. Several rice varieties have been developed by marker-assisted selection in the last decade (Ebitani *et al.* 2011, Ishimaru *et al.* 2013, Ookawa *et al.* 2010, Saka *et al.* 2010, Takeuchi *et al.* 2006).

Our goal was to develop a novel rice variety with a black pericarp in the 'Koshihikari' genetic background. For this purpose we could not use marker-assisted selection, because genetic markers associated with loci responsible for

anthocyanin production in the pericarp had not been identified. Classical genetics indicated that two genes, *PURPLE PERICARP B* (*Pb*) located on chromosome 4 and *PURPLE PERICARP A* (*Pp*) located on chromosome 1, were required for black pigmentation (Causse *et al.* 1994, Yoshimura *et al.* 1997). However, these genes have not been mapped well enough to identify nearby DNA markers. Therefore, we made an initial cross between the black-grained variety 'Hong Xie Nuo' and the popular edible variety 'Koshihikari', and then used phenotype selection in backcrosses with 'Koshihikari'. After four backcrosses we performed a whole genome survey using DNA markers covering the rice genome. The results suggested that a region on chromosome 3, in addition to regions on chromosomes 1 and 4, might be associated with black pigmentation of the pericarp. This is the first report of a gene locus on chromosome 3 being associated with black pigmentation in the pericarp.

In this paper we describe the development of a black rice NIL in the 'Koshihikari' genetic background. The line has three introgression fragments from the black rice donor parent 'Hong Xie Nuo'. Furthermore, we performed a genetic analysis of black pigmentation in the pericarp and examined various eating quality and agronomic traits of the black rice NIL.

Materials and Methods

Plant materials and development of the NIL

The process we used to produce the black rice NIL is shown in Fig. 1. 'Koshihikari' (with white grains) was
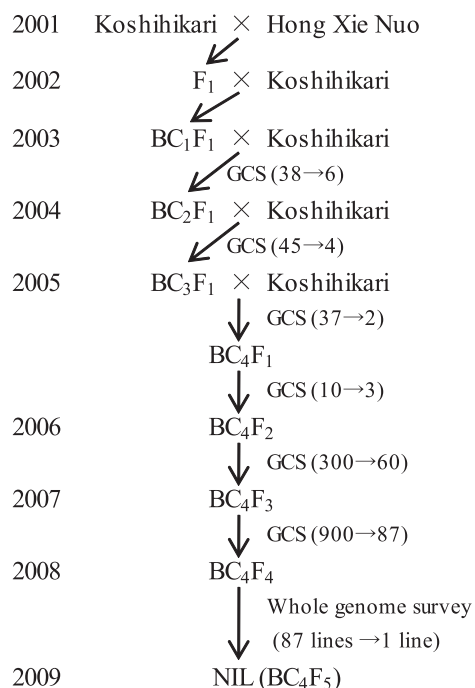
2001	Koshihikari $\times$ Hong Xie Nuo
2002	$F_1 \times$ Koshihikari
2003	$BC_1F_1 \times$ Koshihikari
	GCS (38→6)
2004	$BC_2F_1 \times$ Koshihikari
	GCS (45→4)
2005	$BC_3F_1 \times$ Koshihikari
	GCS (37→2)
	BC_4F_1
	GCS (10→3)
2006	BC_4F_2
	GCS (300→60)
2007	BC_4F_3
	GCS (900→87)
2008	BC_4F_4
	Whole genome survey
	(87 lines →1 line)
2009	NIL (BC_4F_5)

Fig. 1. Breeding of the black rice NIL. GCS: grain color selection. Numbers in parentheses indicate the total number of plants in each generation and the number selected for backcrossing or selfing.

crossed as a female parent with 'Hong Xie Nuo' (black grains); the grain colors are shown in Supplemental Fig. 1. A resultant F_1 plant was backcrossed to 'Koshihikari' to produce the BC_1 line. Each of 38 BC_1F_1 plants was both selfed and backcrossed with 'Koshihikari' the following summer (2003). Grain color selection (GCS) was used to identify which BC_1F_1 progeny would be used in further crosses. The self progeny of the BC_1F_1 plants were grown in the autumn and those with colored pericarps identified. Six BC_1F_1 lines were selected, and a total of 45 BC_2F_1 seeds were harvested from these lines. The same GCS method was used to select the BC_2F_1, BC_3F_1 and BC_4F_1 plants whose progeny were used in further crosses (see Fig. 1). Three of 10 BC_4F_1 plants produced self-progeny with colored grains, and the BC_4F_2 seeds from these plants were harvested. A total of 300 BC_4F_2 plants were raised, and of these, 60 produced colored grains. In the same way, 87 lines were selected from 900 BC_4F_3 plants. The BC_4F_4 plants derived from these 87 BC_4F_3 plants were raised, and a single BC_4F_4 line (no. 1 in Supplemental Fig. 2) was selected as the black rice NIL based on its phenotype and the results of a whole genome survey with 130 simple sequence repeat (SSR) markers (see below). The black rice NIL was self-pollinated to get BC_4F_5 seeds. The 87 BC_4F_4 lines were also used for the fine mapping of a locus on chromosome 3 that we found to be associated with black pigmentation in the pericarp.

The black rice NIL was crossed with 'Koshihikari' and 542 F_2 progeny were used as a mapping population to examine the contributions of the three loci (on chromosomes 1, 3 and 4) toward black pigmentation in the pericarp.

In order to evaluate the eating quality of the black rice NIL, we compared eating quality-related traits between the NIL, 'Koshihikari', and *Oryza sativa* L. 'Okunomurasaki', which is a widely cultivated black rice variety in Japan.

Evaluation of phenotypes

Plant materials were cultivated in a paddy field at Toyama Prefectural Agricultural, Forestry & Fisheries Research Center (TAFFRC). Grain color was classified visually into three types: black, brown and white (Fig. 2). The brown color could be further classified into three subgroups: brown, partial brown, and light brown; however for genetic analyses only the three main categories were used. Twenty plants in each of the 87 lines were evaluated for grain color.

Analyses of protein content, amylose content, and

| Black | Brown | White |

Fig. 2. Grain color classification. Three colors, black, brown, and white, were used in the genetic analyses. However, the brown category could be sub-classified into three colors: brown, partial brown, and light brown.

pasting properties of the rice flours, (traits related to eating quality) were carried out according to Yamamoto *et al.* (1996). The pasting properties were investigated using a Rapid Visco Analyser (Newport Scientific, Warriewood, NSW, Australia). Two viscosity parameters, breakdown viscosity and consistency viscosity, were obtained from the pasting curve. Twenty panels were used to evaluate eating quality. Rice grains milled to 95% of their weight were used for sensory evaluation. 'Okunomurasaki' was used as a blind control and the quality of the NIL was compared with that of the control. Eating qualities, including an overall evaluation, stickiness, hardness, and glossiness, were classified into nine levels (–2: especially bad to +2: excellent). Antioxidant activity was evaluated using the chemiluminescence method of Kimura *et al.* (1999). The isogenicity of the black rice NIL to 'Koshihikari' was evaluated using several agricultural traits including heading date, culm length, panicle length, panicle number, and grain yield.

DNA marker analysis

A total of 130 SSR markers covering all 12 chromosomes were used for mapping genotypes in the 87 BC_4F_4 lines (Supplemental Fig. 2). These SSR markers were developed by McCouch *et al.* (2002) or the International Rice Genome Sequencing Project (2005). Genomic DNA was extracted from leaves using the CTAB method (Murray and Thompson 1980) and used in the polymerase chain reaction (PCR). PCR amplification was performed in a 10 μl reaction volume containing 10–100 ng genomic DNA, 0.2 μM primers, 1 x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP and 0.1 μl Taq DNA polymerase (5 U/μl). Thermal cycling consisted of an initial denaturation at 95°C for 2 min followed by 35 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 3 min. To detect polymorphisms, the amplified products were electrophoresed on 3% agarose gels.

For the analysis of the 542 F_2 progeny from the cross between the black rice NIL and 'Koshihikari', genomic DNA was extracted from leaves using the single-step protocol (Thomson and Henry 1995). A 1.5 μl aliquot of this DNA extract was used as the template for PCR amplification. Thermal cycling was carried out as described above. For this analysis, the genotypes of the three loci on chromosomes 1, 3, and 4 were determined using the SSR markers RM8129, RM15191, and RM2441, respectively.

Results

Development of the black rice NIL

After crossing 'Koshihikari' (white grains) with 'Hong Xie Nuo' (black grains) and then backcrossing to 'Koshihikari' (see Methods and Fig. 1), we evaluated the genotypes and grain colors of 87 BC_4F_4 lines (Supplemental Fig. 2). Based on an analysis with 130 SSR markers, we found that all lines had a homozygous segment derived from 'Hong Xie Nuo' on chromosome 4 and produced
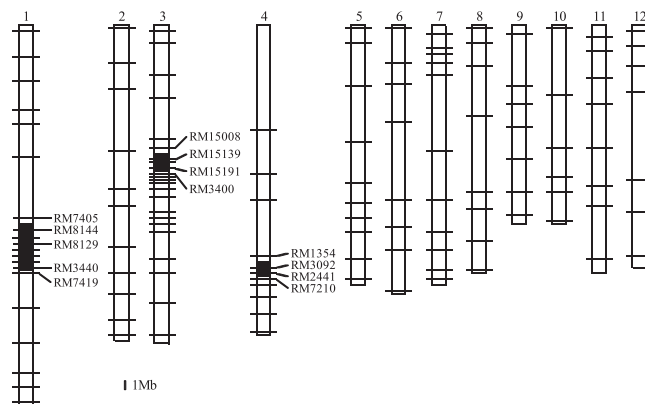

Fig. 3. Graphical representation of the NIL genotype. Black and white regions indicate regions homozygous for 'Hong Xie Nuo' alleles and 'Koshihikari' alleles, respectively. Horizontal bars indicate the positions of 130 SSR markers. The maps are scaled on the basis of physical distances.

colored grains. Lines that also had homozygous regions derived from 'Hong Xie Nuo' on both chromosomes 1 and 3 produced black grains. We selected one line (no. 1 in Supplemental Fig. 2) that produced only black grains and that was mainly homozygous for 'Koshihikari' alleles. This line had three chromosome segments derived from 'Hong Xie Nuo' on chromosomes 1, 3 and 4 in the genetic background of 'Koshihikari'. Based on the Rice Annotation Project Database (RAP-DB, http://rapdb.dna.affrc.go.jp/), the physical sizes of these segments were 5.8 Mbp (between the markers RM7405 and RM7419) on chromosome 1, 3.0 Mbp (between RM15008 and RM3400) on chromosome 3, and 2.3 Mbp (between RM1354 and RM7210) on chromosome 4 (Fig. 3). The pigmented tissues in this black rice NIL were only the pericarp and the stigma; other tissues, including the leaf blade, sheath, ligule, stem node, and apiculus showed normal green coloring.

Validation of three loci for black pigmentation

To understand the contribution of each of the three chromosomal segments toward black pigmentation of the pericarp, we analyzed an F_2 population (542 plants) derived from a cross between the black rice NIL and 'Koshihikari'. The population segregated into a colored group (397 plants) and a white group (145 plants), and the segregation ratio showed a good fit to the 3 : 1 ratio typical of a gene with one dominant and one recessive allele ($\chi^2 = 0.89$, P > 0.05). Among the 397 colored plants, the genotypes for RM8129 (a marker within the chromosome 1 segment) segregated 105 ('Hong Xie Nuo' homozygous) : 178 (heterozygous) : 114 ('Koshihikari' homozygous) and this fitted the 1 : 2 : 1 segregation pattern ($\chi^2 = 4.64$, P > 0.05, Table 1). Similarly, the genotypes for RM15191 (within the chromosome 3 segment) segregated 82 : 200 : 115, and this also fitted the 1 : 2 : 1 segregation pattern ($\chi^2 = 5.51$, P > 0.05, Table 1). On the other hand, the genotypes for RM2441 (in the chromosome 4 segment) segregated 116 : 274 : 7, and this

Table 1. Segregation of grain colors in an F_2 population derived from a cross between the black rice NIL and 'Koshihikari'

Marker Name (Chromosome)	Colored (Black and Brown)					White				
	No. of genotypes[a]				χ^2 [b] (1 : 2 : 1)	No. of genotypes[a]				χ^2 [b] (1 : 2 : 1)
	Kk	H	Ks	Total		Kk	H	Ks	Total	
RM8129 (1)	105	178	114	397	4.64	29	75	41	145	2.16
RM15191 (3)	82	200	115	397	5.51	26	83	36	145	4.42
RM2441 (4)	116	274	7	397	117***	0	1	144	145	427***

[a] Genotypes Kk, Ks, and H are homozygous for the 'Hong Xie Nuo' allele, homozygous for the 'Koshihikari' allele, and heterozygous, respectively.
[b] ***: $P < 0.001$.

Table 2. Association of *Kala1* and *Kala3* genotypes with grain color in plants possessing colored grains

Genotypes[a]			Number of plants	
Kala4	*Kala1*	*Kala3*	Black	Brown
Kk	Kk	Kk	7	0
		Ks	0	10
	Ks	Kk	0	7
		Ks	0	8

[a] Genotypes Kk and Ks are homozygous for the 'Hong Xie Nuo' allele and the 'Koshihikari' allele, respectively.

clearly did not fit the 1 : 2 : 1 segregation pattern ($\chi^2 = 117.0$, $P < 0.001$, Table 1). In the white group of 145 plants, the genotypes for RM8129 and RM15191 each segregated to fit the 1 : 2 : 1 segregation pattern but the genotypes for RM2441 did not. Thus, only RM2441 showed segregation distortion. We concluded that an allele derived from 'Hong Xie Nuo' on chromosome 4 is necessary for pericarp pigmentation.

Of the 397 colored plants, 32 had chromosomal regions that were homozygous for either the 'Hong Xie Nuo' alleles or the 'Koshihikari' alleles at the three chromosomal regions. These 32 plants were classified on the basis of genotypes and grain colors (Table 2). Only plants that had homozygous 'Hong Xie Nuo' segments on chromosomes 1, 3 and 4 produced black grains. Plants that had 'Koshihikari' segments on either chromosome 1 or chromosome 3 produced only brown grains (Table 2). Thus it was revealed that the 'Hong Xie Nuo' alleles on chromosomes 1 and 3 are necessary for black pigmentation of the rice pericarp.

Based on the above results, we confirmed that three loci on chromosomes 1, 3 and 4 together confer black pigmentation on the rice pericarp. We named these loci <u>K</u>ey gene for <u>bl</u>ack <u>co</u>loration by <u>a</u>nthocyanin accumulation on chromosome 1, 3, and 4 (*Kala1*, *Kala3* and *Kala4*). Based on the lengths of the substituted 'Hong Xie Nuo' chromosomal segments, *Kala1*, *Kala3*, and *Kala4* mapped between RM7405 and RM7419 on chromosome 1, between RM15008 and RM3400 on chromosome 3, and between RM1354 and RM7210 on chromosome 4, respectively.

Fine mapping of Kala3

Among the 87 BC$_4$F$_4$, lines the lengths of the substituted segments on chromosome 3 varied (see no. 1, 15, 22, 31, 32, 48, 82 and 83 in Supplemental Fig. 2). These lines were
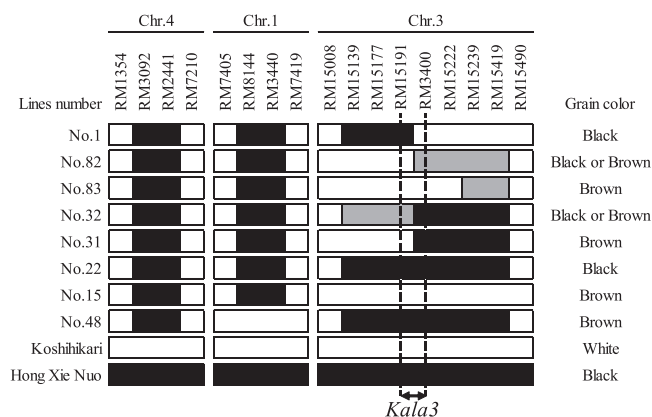

Fig. 4. Fine mapping of *Kala3*. The black, white and gray regions indicate regions homozygous for 'Hong Xie Nuo', homozygous for 'Koshihikari', and heterozygous, respectively.

used for substitution mapping of *Kala3*. The results indicated that *Kala3* is located in a region of 516 kbp between RM15191 and RM3400 (Fig. 4).

Grain color phenotypes conferred by various combinations of Kala1, Kala3, and Kala4

We identified individual plants among the mapping F_2 population that were homozygous for either the 'Koshihikari' or the 'Hong Xie Nuo' alleles at each of the three loci, *Kala1*, *Kala3*, and *Kala4*. As indicated above, only plants that carried the 'Hong Xie Nuo' alleles at all three loci produced black grains (Fig. 5). Brown grains of various shades were produced by plants with 'Hong Xie Nuo' alleles at *Kala4* and 'Koshihikari' alleles at one or both of the other two loci: with 'Koshihikari' alleles at *Kala1* and *Kala3*, the grains were light brown; with 'Koshihikari' alleles only at *Kala3*, the grains were partial brown; and with 'Koshihikari' alleles only at *Kala1*, the grains were brown. In plants that carried 'Koshihikari' alleles at *Kala4*, the grains were white, regardless of the genotypes at the other two loci (Fig. 5).

Eating quality, antioxidant activity, and agricultural traits of the black rice NIL

Traits related to eating quality, including protein content, amylose content, breakdown viscosity, and consistency viscosity were compared between the black rice NIL, 'Koshihikari', and 'Okunomurasaki'. No significant differences were detected in any of the traits, however in each

Grain color	Black	Brown			White			

Genotypes	*Kala4*	■	■	■	■	□	□	□	□
	Kala1	■	□	■	□	■	■	■	□
	Kala3	■	■	□	□	■	■	□	□

Fig. 5. Patterns of the grain color defined by three loci, *Kala1*, *Kala3*, and *Kala4*. The purple and white boxes indicate 'Hong Xie Nuo' alleles and 'Koshihikari' alleles, respectively.

Table 3. Eating qualities and related traits in the black rice NIL

Varieties	Grain color	Protein content (%)	Amylose content (%)	Pasting properties		Eating qualities[a]			
				Breakdown viscosity (RVU)	Consistency viscosity (RVU)	Overall evaluation	Stickness	Hardness	Glossiness
Black rice NIL	Black	5.9	14.3	165	104	0.34**	0.28	0.14	0.22**
						0.17**	0.05	−0.20*	0.44**
'Koshihikari'	White	5.7	14.1	198	115	–	–	–	–
'Okunomurasaki'	Black	6.3	14.5	118	131	0.09	0.08	0.03	−0.04
						0.08	0.01	0.05	0.04

[a] The upper and lower values for eating qualities were obtained in 2010 and 2011, respectively. * and ** indicate that the differences between 2010 and 2011 were significant at P < 0.05 and P < 0.01, respectively.
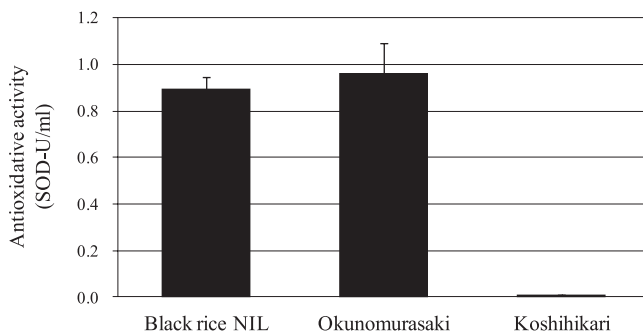

Fig. 6. Comparison of antioxidative activities between the black rice NIL, 'Koshihikari', and 'Okunomurasaki'.

case the black rice NIL showed a value that was intermediate between those of 'Koshihikari' and 'Okunomurasaki' (Table 3). The eating qualities of the black rice NIL, in particular the overall evaluation and the glossiness, were greatly superior to those of 'Okunomurasaki'. However, it was not possible to compare the eating qualities of the black rice varieties with those of 'Koshihikari' because of flavor differences. The antioxidant activity of the black rice NIL was much higher than that of 'Koshihikari' and similar to that of 'Okunomurasaki' (Fig. 6). Most of the agricultural traits of the black rice NIL were comparable with those of 'Koshihikari' (Supplemental Table 1). The black rice NIL headed two days later than 'Koshihikari' and had slightly lower yields in both 1000-grain weights and gross yield per acre.

Table 4. Possible relationships between three loci, *Kala1*, *Kala3*, and *Kala4*, and previously reported loci and genes

Locus	Possible allelic locus (Reference)	Cloned or possible gene (Reference)[a]
Kala1	*Pp* (Wang and Shu 2007)	
	Rd (Furukawa *et al.* 2006)	*DFR* (Furukawa *et al.* 2006)
	A (Nagao and Takahashi 1963)	
Kala3	*P* (Nagao and Takahashi 1963)	*MYB* (Saitoh *et al.* 2004) (Gao *et al.* 2011)
Kala4	*Pb* (Wang and Shu 2007)	
	Pl (Kinoshita and Maekawa 1986)	*bHLH* (Sakamoto *et al.* 2001)
	C (Nagao and Takahashi 1963)	

[a] The cloned gene *DFR* occurs at the *Rd* locus, and the cloned gene *bHLH* gene occurs at the *Pl* locus. In this study we infer that *P* is a *MYB* gene.

Discussion

In this study involving a whole genome survey of 87 BC$_4$F$_4$ lines and a genetic analysis of 542 F$_2$ plants, we identified three loci, *Kala1*, *Kala3*, and *Kala4*, that influence grain color. Table 4 summarizes the possible relationships between these three loci and genes and loci identified in previous studies of pigment production in rice. Previous studies

of black pigmentation in the rice pericarp showed that two loci, *Pp* on chromosome 1 and *Pb* on chromosome 4, acted together to influence grain color. Grains were black in the presence of both alleles, brown in the presence of *Pb* and the absence of *Pp*, and white in the absence of *Pb* (Rahman *et al.* 2013, Wang and Shu 2007). On the other hand, we found that three loci influence black coloration of the rice grain in 'Hong Xie Nuo' (Tables 1, 2 and Supplemental Fig. 2). Like *Pb*, the 'Hong Xie Nuo' allele at *Kala4* is essential for grain color, and both alleles map to a region between RM1354 and RM7210 on chromosome 4 (Fig. 3 and Wang and Shu 2007). These results strongly suggest that *Pb* is an allele of *Kala4*.

Kala1 mapped to the region between RM7405 and RM7419 on chromosome 1. However, it is not clear whether *Pp* is an allele of *Kala1* because the *Pp* gene locus was not precisely mapped (Oryzabase, www.gramene.org). On the other hand, since the 'Hong Xie Nuo' alleles at *Kala4* and *Kala1* interact genetically in the same way that *Pb* and *Pp* do, it is possible that *Pp* is an allele of *Kala1*.

Aside from these loci on chromosomes 1 and 4, *Kala3* is the first additional gene locus that has been found to influence rice pericarp color.

Based on the pigmentation model reviewed by Koes *et al.* (2005), *dihydroflavonol reductase* (*DFR*) is a widely conserved gene that encodes an enzyme involved in both the anthocyanin and the proanthocyanidin synthetic pathways. In addition, certain MYB, basic helix-loop-helix (bHLH), and WD40 proteins are transcription factors that can activate the *DFR* gene. Since the *WD40* genes are highly conserved (reviewed by Koes *et al.* 2005), it was proposed that polymorphisms at three genetic loci, *DFR*, *MYB*, and *HLH*, may confer the deposition of anthocyanin or proanthocyanidin pigments in various plant tissues. The chromosomal region (between RM7405 and RM7419) of *Kala1* contains the *Rd* locus, which has been identified as a *DFR* gene (Furukawa *et al.* 2006). In red rice, the *Rd* gene interacts with the *Rc* gene on chromosome 7 to produce red grains (Furukawa *et al.* 2006). Similarly, *Kala1* interacts with *Kala4* in the production of colored grains. Thus it appears likely that *Kala1* is *Rd*/*DFR*, and that 'Koshihikari' has a defective allele at this locus.

The purple leaf (*Pl*) locus was detected on chromosome 4 by Kinoshita and Maekawa (1986). Sakamoto *et al.* (2001) found that the *Pl* gene encodes a bHLH protein and reported that among three *Pl* alleles (*Pl^i*, *Pl^w* and *Pl^j*), *Pl^w* confers pigmented pericarps. As we mentioned above, the *Rc* gene on chromosome 7 interacts with *Rd* on chromosome 1 in the production of red pericarps. Sweeney *et al.* (2006) revealed that the *Rc* gene encodes a *bHLH* protein. These results led us to speculate that *Kala4* may also encode a bHLH protein, and that *Kala4* may either be *Pl* or that the two genes, *Kala4* and *Pl*, might be tandemly duplicated genes with distinct tissue-specific transcription patterns. Three genes registered in the RAP-DB, *Os04t0557200-1*, *Os04t0557500-00*, and *Os04t0557800-00*, are located in the

chromosomal region containing *Kala4* and have *bHLH* domains. We are currently conducting expression analyses and complementation tests to determine which of the three is *Kala4*. If our speculations regarding *Kala1* and *Kala4* are correct, it seems likely that *Kala3* might be a *MYB* gene. The RAP-DB shows 45 genes within the region of *Kala3* identified by fine mapping of the locus (Fig. 4). Of these, *Os03t0410000-01* was annotated as similar to *OsMYB3*. Therefore, *Kala3* may be *OsMYB3*. More fine mapping and expression analyses are underway to reveal the identity of *Kala3*.

Nagao and Takahashi (1963) demonstrated that three genes, *Chromogen* gene (*C*), *Activator* gene (*A*), and *tissue-specific regulator* gene (*P*) are required for anthocyanin pigmentation in various tissues. We propose that *Kala4*, *Kala1*, and *Kala3* might correspond to *C*, *A*, and *P*, respectively. As shown in Fig 5, only plants with segments from 'Hong Xie Nuo' on both chromosomes 4 and 1 produced dark brown grains. The color of the dark brown grains in this paper is similar to that of the grains described as medium purple, in which anthocyanin was detected, by Rahman *et al.* (2013). These results suggest that *Kala1* and *Kala4* are necessary for anthocyanin synthesis and that *Kala1* corresponds to *A*.

MYB domains have been related to tissue-specific coloration of rice in previous studies. For example, the *Colorless like regulatory gene 1* (*OsC1*) on chromosome 6, which encodes a *MYB* family transcription factor, regulates anthocyanin production in the apiculus and sheath (Gao *et al.* 2011, Saitoh *et al.* 2004). Yamaguchi *et al.* (2011) developed a novel line "Tokei 1378," which has a colored apiculus caused by expression of the *OsC1* gene from *Oryza sativa* L. 'Awaakamai' in the 'Koshihikari' background. We made a cross between our black rice NIL and Tokei 1378 and found that in the F_2 population, only plants having three substituted segments on chromosomes 4, 1 and 6 showed pigmentation in the leaf margin, sheath, and ligule (unpublished data). These results showed that the *MYB* gene functioned as a tissue-specific regulator. Together, our results suggest that *Kala3* may be *P*.

The grain color of the black rice NIL was slightly different from that of 'Hong Xie Nuo' (Fig. 5 and Supplemental Fig. 1). Thus, 'Koshihikari' may carry an additional unknown allele that is involved in pericarp pigmentation and that is not present in the 'Hong Xie Nuo' genome. Further studies are needed to identify this additional genetic factor.

Our main purpose was to breed a black rice variety with good eating quality. We used an existing black rice variety, 'Okunomurasaki', as a control and found that the black rice NIL is greatly superior to 'Okunomurasaki' in eating quality (Table 3). We want to emphasize that our black rice NIL is the first variety that has been bred to harbor both good eating quality and black grain color. Traits related to eating quality, including protein content, amylose content, breakdown viscosity, and consistency viscosity, also tended to be better in the black rice NIL than in 'Okunomurasaki': the

values for each of these traits were intermediate between those of 'Okunomurasaki' and those of 'Koshihikari'. It is not clear why the related traits in the black rice NIL were not equal to those of 'Koshihikari'. It is possible that the pigment composition in the pericarp directly influences these traits. These traits may also be influenced by genes in the substituted chromosomal regions.

The beneficial health effects due to the antioxidant activities of black rice varieties were demonstrated by Chiang *et al.* (2006), Hou *et al.* (2010), and Ling *et al.* (2001). Our black rice NIL, with high antioxidant activity and superior palatability, should provide beneficial health effects if included as a regular part of the diet. The isogenicity of the black rice NIL to 'Koshihikari' was very high apart from slight differences in heading date and yield (Supplemental Table 1). The reason for the difference in heading date remains unknown. Ji *et al.* (2012) also reported that a purple-pericarp line showed reduced yields, and they suggested that this may be due to a reduced sink size. The black rice NIL also had reduced grain thickness (data not shown). These differences may be caused by gene(s) related to sink size in the introgressed segment(s).

Acknowledgements

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