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Research Paper

Analysis of quantitative trait loci for fertility restoration in seven F_2 populations derived from sorghum F_1 hybrids bred in Japan

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To clarify the genetic mechanisms of fertility restoration in sorghum F_1 hybrids produced in Japan ('Ryokuryu', 'Hazuki', 'Haretaka', 'Natsuibuki', 'Hanaaoba', 'Akidachi' and 'Kazetachi'), we analyzed QTLs for fertility restoration using seven F_2 populations derived from those hybrids. By QTL mapping with a series of SSR markers, we detected three major QTLs for fertility restoration. These data and the results of haplotype analysis of known fertility restorer (*Rf*) genes showed that *qRf5*, corresponding to the *Rf5* locus, was the most widely used *Rf* gene for fertility restoration of sorghum F_1 hybrids among the lines tested. Other major *Rf* genes detected were *qRf8*, corresponding to *Rf1*, and *qRf2*, corresponding to *Rf2*. QTLs for grain weight also corresponded to these *Rf* loci. A minor QTL, *qRf3*, may also affect restoration of fertility. Our data show that three major *Rf*s—*Rf1*, *Rf2*, and *Rf5*—were used in F_1 hybrid sorghum production in Japan. This knowledge can be used to improve the efficiency of the F_1 sorghum breeding program.

Key Words: sorghum, Japanese cultivars, cytoplasmic-nuclear male sterility (CMS), QTL analysis, *Rf* (fertility restorer) genes.

Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is one of the most important grain crops in the world. Sorghum was often cultivated as a staple food crop in mountainous and semi-arid regions in Japan. The modern breeding of sorghum in Japan started in the 1960s. Modern sorghum cultivars has been introduced into Japan from the USA, and some Japanese sorghum cultivars are probably derived from US germplasm (Anas and Yoshida 2004). Sorghum plant size, flowering time, and yield are highly variable. Therefore, the use of heterosis in commercial F_1 hybrid cultivars is a major strategy in sorghum breeding programs (Tarumoto 1971). For example, the F_1 cultivar 'Kazetachi', derived from short early-flowering parents, flowers extremely late under long-day conditions and shows hybrid vigor in plant size (Tarumoto *et al.* 2000). Currently, sorghum F_1 hybrids are used as feed and green fodder for livestock in Japan.

F_1 hybrids are typically produced by crossing a cytoplasmic-nuclear male sterile (CMS) line as a female parent and a fertility restorer (Rf) line that restores male fertility in hybrid cultivars as a pollen donor. CMS in sorghum was discovered from the interaction of the milo (A1) cytoplasm and kafir nuclear background (Stephens and Holland 1954). This A1 cytoplasm is used in almost all female parents in commercial hybrid seed production.

A number of *Rf* genes encoding pentatricopeptide repeat (PPR) proteins have been cloned in several crops (Dahan and Mireau 2013). PPR proteins, characterized by tandem repeats of a degenerate 35-amino-acid motif, comprise a large family of modular RNA-binding proteins that regulate gene expression primarily in mitochondria (Manna 2015). By RNA cleavage, RNA destabilization, or translation inhibition, they specifically suppress the expression of mitochondrial transcripts, causing sterility (Dahan and Mireau 2013). *Rf* genes encoding PPR proteins were cloned first from *Petunia* (Bentolila *et al.* 2002) and then from radish (*Raphanus sativus* L.) (Brown *et al.* 2003, Desloire *et al.* 2003, Koizuka *et al.* 2003). *Rf* genes encoding PPR proteins for two genetically independent CMS systems in rice were cloned (Kazama and Toriyama 2003, Komori *et al.* 2004).

In sorghum, the major *Rf* genes *Rf1–Rf5* have been reported. In the case of A1 cytoplasm, *Rf1* is located on chromosome (Chr) 8, *Rf2* on Chr 2, and *Rf5* on Chr 5 (Klein *et al.* 2005, Jordan *et al.* 2010, 2011). *Rf1* and *Rf2* belong to the PPR gene family (Klein *et al.* 2005, Jordan *et*

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al. 2010). The *Rf5* locus was delimited to a ~584-kb region of Chr 5; among 70 predicted genes in this region, 7 encode PPR proteins (Jordan *et al.* 2011). *Rf3* and *Rf4* can restores male sterility of A3 cytoplasm (Tang *et al.* 1998, Tang and Pring 2003). A3 is a less common cytoplasm, which appears to reduce grain yield as a pleiotropic effect (Moran and Rooney 2003) and is therefore less likely to have been used in Japan for commercial hybrid seed production. For effective use and selection of crop genetic resources in breeding programs, a population should be characterized genetically; however, the origin and pedigree history of sorghum cultivars grown in Japan are not well known (Anas and Yoshida 2004). After the production of 'NakeiMS3', a CMS female parent that harbors genes for the bloomless trait and brown mid-rib (Tarumoto *et al.* 1993), a series of F_1 hybrids were produced, such as 'Hazuki', 'Akidachi', 'Suzukaze', 'Hanaaoba', and 'Natsutarou' (Kiyosawa 2015). However, information on the genetic background and genetic diversity of CMS and *Rf* lines used to produce sorghum F_1 hybrids has not been reported.

Our aim was to clarify the genetic property of the restoration for CMS cytoplasm used in sorghum F_1 hybrids produced in Japan by using F_2 populations. We analyzed the QTLs for fertility restoration and reported QTL mapping of major *Rf* loci in seven lines of Japanese sorghum F_1 hybrids using a series of informative SSR markers (Yonemaru *et al.* 2009); we have also clarified the inheritance of the *Rf* loci in Japanese cultivars.

Materials and Methods

Plant materials and measurements of traits related to restoring fertility

Sorghum F_1 hybrid lines (also named Tozanko lines) 'Ryokuryu', 'Hazuki', 'Haretaka', 'Natsuibuki', 'Hanaaoba', 'Akidachi', and 'Kazetachi' were bred at the Nagano Animal Industry Experiment Station (137°98′E, 36°1′N, 771 m above sea level). The experimental materials consisted of seven sets of F_2 populations of these lines. The F_1 hybrids and their parents (male-sterile lines, restorer lines) and the number of F_2 individuals are listed in **Table 1**. For experiments I to VI, the parent lines and F_2 populations were planted at the Nagano Animal Industry Experiment Station in 2009, 2011, and 2017. Experiment VII was performed in a greenhouse under short-day conditions in 2013 with two plants per 1/5000-a Wagner pot.

Each shoot of F_2 plants was bagged at the heading date and harvested after the full maturity of all plants. In experiment I, fertile plants were scored as 1 and sterile plants as 0; the fertility (%) of each panicle of main stem was measured in experiments II to VII. Panicle length (cm) and grain weight (g) of each panicle of main stem were measured.

Genotyping and QTL analysis of F_2 populations

Genomic DNA was extracted from a leaf of each F_2 plant and its parents. Among published simple sequence repeat (SSR) markers (Yonemaru *et al.* 2009), 604 markers showing polymorphism between parents of each of the seven F_2 populations were selected for genotyping and constructing a linkage map (**Supplemental Table 1**, **Fig. 1**). The SSR markers were genotyped as described previously (Takai *et al.* 2012, Yonemaru *et al.* 2015). QTL analysis was performed as described previously (Takai *et al.* 2012), except that the confidence interval for each QTL was 2-LOD instead of 1-LOD.

Next-generation DNA sequencing of the restorer lines and their relatives, SNP detection and hierarchical clustering

We performed short-read Illumina resequencing of seven restorer lines—'JN290', 'JN43', 'SDS7444', 'Chohin237.Daikoukaku' (Daikoukaku), 'JN503', 'JN358', and 'Chohin232.74LH3213' (74LH3213)—and three relatives—'F6-3A-5', 'Senkinshiro', and 'JN107'. These data were deposited in the DDBJ Sequence Read Archive under the accession number DRA008887. Low-quality bases and adapters in each read were trimmed in Trimmomatic software (Bolger *et al.* 2014). Trimmed reads were mapped to the reference genome sequence of *S. bicolor* (Paterson *et al.* 2009) (v. 3.1 DOE-JGI, http://phytozome.jgi.doe.gov/) in BWA software with default settings (Li and Durbin 2009). Only uniquely mapped reads with a mapping quality score of ≥20 were sorted and indexed in SAMtools software (Li *et al.* 2009). To improve the raw alignments around insertion and deletion mutations, local re-

Table 1. Sorghum F_2 populations derived from F_1 cultivars used in this study

Experiment	Japanese name	English name	Regional name	Parental lines		Analyzed population		Year	Condition
				Male sterile line	Restorer line	Generation	No. of individuals		
I	緑竜	Ryokuryu	Tozanko 24	AMP-21	JN290	F_2	92	2009	Field
II	葉月	Hazuki	Tozanko 21	NakeiMS-3A	JN43	F_2	91	2011	Field
III	晴高	Haretaka	Tozanko 20	(954149)A	SDS7444	F_2	88	2011	Field
IV	ナツイブキ	Natsuibuki	Tozanko 15	MS175.(932233)A	Chohin237.Daikoukaku	F_2	92	2011	Field
V	華青葉	Hanaaoba	Tozanko 34	NakeiMS-3A	JN503	F_2	94	2011	Field
VI	秋立	Akidachi	Tozanko 22	NakeiMS-3A	JN358	F_2	188	2017	Field
VII	風立	Kazetachi	Tozanko 11	MS138.(932233)A	Chohin232.74LH3213	F_2	147	2013	Greenhouse (SD)

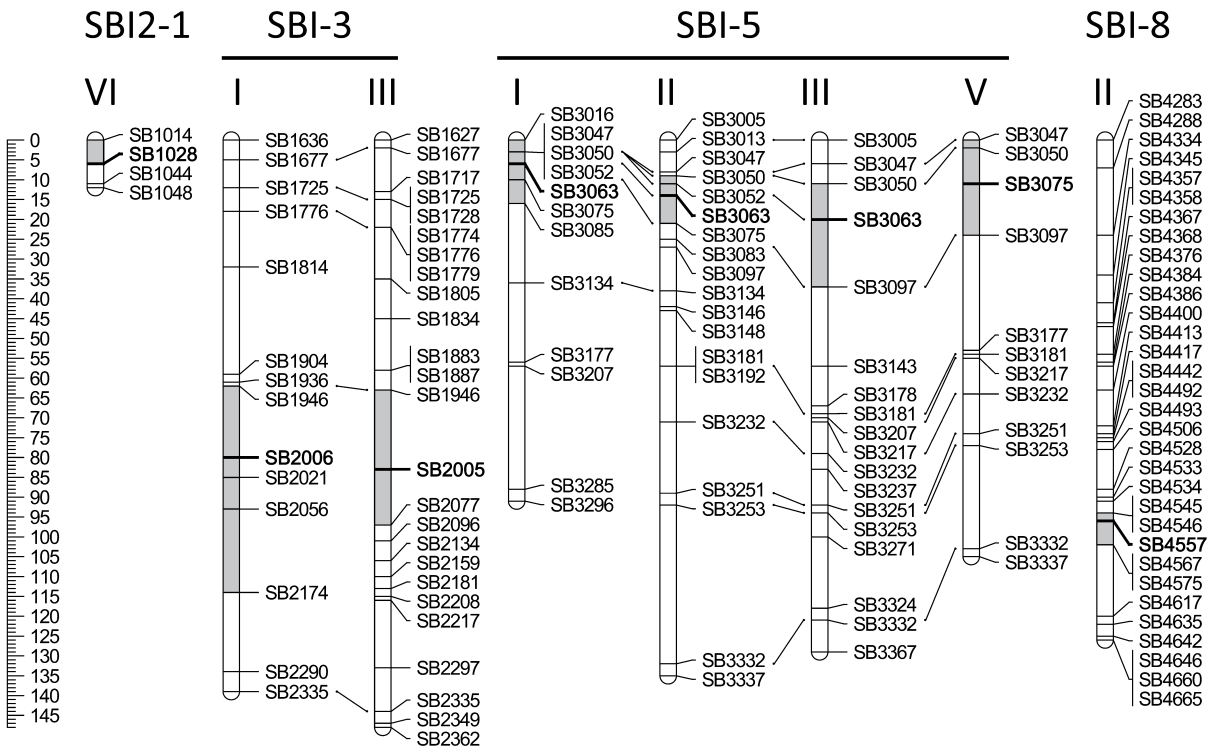

Fig. 1. Genetic maps and QTLs for restoration of fertility in five F_2 populations of sorghum. Roman numerals above each map indicates experimental populations (**Table 1**). "SBI" with Arabic numbers indicate linkage groups. QTL regions (2-LOD interval) are shown as gray boxes, and nearest markers are in bold. Detailed information on each QTL is provided in **Table 3**.

alignments were performed in GATK software (DePristo *et al.* 2011; https://software.broadinstitute.org/gatk/). PCR duplicates were removed in Picard software (http://picard.sourceforge.net). Non-homozygous and low-depth (<3-fold) SNP variants were removed by GATK software and the remaining variants were used to predict haplotypes of the candidate genes in the detected QTL regions. Hierarchical clustering of the haplotypes of the gene loci *Rf1*, *Rf2*, *Rf5*, *Dw1* (Yamaguchi *et al.* 2016), *sbGhd7* (*Ma6*) (Murphy *et al.* 2014), and *sbPHYB* (*Ma3*) (Childs *et al.* 1997) in the restorer lines and their relatives was based on SNP variants in the adjacent 200-kb sequences and was performed using the weighted neighbor-joining method with simple matching coefficients implemented in DARwin software (http://darwin.cirad.fr/darwin) (Perrier and Jacquemoud-Collet 2006).

Results

Analysis of the fertility restoration trait in seven F_2 populations

F_2 plants with ≤5% fertility were considered sterile, and the rest as fertile (**Table 2**). In experiments I, IV, and V, the fertile-to-sterile ratio matched 3:1 in the chi-squared test, indicating the presence of a single dominant *Rf* gene in the male parents. In experiment II, the segregation ratio was consistent with 15:1 rather than 3:1 (chi-squared test: $\chi^2 = 1.00$, $P = 0.32$), suggesting the presence of two independent *Rf* genes in the male parent, 'JN43'. In experiment III, the ratio was 5:3, inconsistent with the single dominant gene hypothesis. In experiment VI, $P < 0.05$ in the chi-squared test, indicating that fertility might not be controlled by a single dominant gene. In experiment VII, the ratio was consistent with 15:1 ($\chi^2 = 3.12$, $P = 0.07$) or 63:1 ($\chi^2 = 1.28$, $P = 0.28$) but not with 3:1 ($\chi^2 = 38.9$, $P < 0.01$), suggesting the presence of two or three independent *Rf* genes in the restorer line '74LH3213'.

Linkage map construction and QTL identification

We genotyped each of the seven F_2 populations and constructed genetic maps on the basis of a total of 604 markers covering the 10 sorghum chromosomes (**Supplemental Table 1**). A major QTL for restoration of fertility on Chr.5 (*qRf5*) corresponding to *Rf5* was detected in experiments I–III and V (**Fig. 1**, **Table 3**). An additional major QTL on Chr.8, *qRf8*, was detected in experiment II and corresponded to *Rf1*. In experiment VI, one major QTL, *qRf2*, corresponding to *Rf2*, was detected on Chr.2. In experiments I and III, a minor QTL, *qRf3*, was detected on Chr.3. In experiments IV and VII, no significant QTLs were detected by QTL analysis.

We also analyzed QTLs for heading date, panicle length, and grain weight (**Table 3**), which may influence fertility. Two QTLs for heading date were detected: *qHd1* in experiments I, VI, and VII corresponded to *sbPHYB*, and *qHd6-1* in experiment I corresponded to *sbGhd7*. Two QTLs for

Table 2. Segregation of seed fertility in F_2 populations derived from F_1 cultivars

Experiment	No. of individuals			Chi-squared test for		
	Total	Fertile	Sterile	3:1	15:1	63:1
I	83	68	15	$\chi^2 = 2.12$, $P = 0.14$	$\chi^2 = 19.8$, $P < 0.01$	$\chi^2 = 147$, $P < 0.01$
II	91	83	8	$\chi^2 = 12.75$, $P < 0.01$	$\chi^2 = 1.00$, $P = 0.32$	$\chi^2 = 30.9$, $P < 0.01$
III	88	55	33	$\chi^2 = 7.33$, $P < 0.01$	$\chi^2 = 147$, $P < 0.01$	$\chi^2 = 739$, $P < 0.01$
IV	79	55	24	$\chi^2 = 1.22$, $P = 0.27$	$\chi^2 = 78.5$, $P < 0.01$	$\chi^2 = 427$, $P < 0.01$
V	94	63	31	$\chi^2 = 3.19$, $P = 0.07$	$\chi^2 = 115$, $P < 0.01$	$\chi^2 = 603$, $P < 0.01$
VI	170	139	31	$\chi^2 = 4.15$, $P < 0.05$	$\chi^2 = 41.7$, $P < 0.01$	$\chi^2 = 307$, $P < 0.01$
VII	147	143	4	$\chi^2 = 38.9$, $P < 0.01$	$\chi^2 = 3.12$, $P = 0.07$	$\chi^2 = 1.28$, $P = 0.28$

panicle length were detected: *qPl1* in experiment VI corresponded to *sbPHYB*, and *qPl9* in experiment V corresponded to *Dw1*. Three QTLs for grain weight were detected: *qGw5* in experiments I and III–V was located in the same region as *Rf5*, *qGw2* in experiment VI was located in the same region as *Rf2*, and *qGw1-2* in experiment VI corresponded to *sbPHYB*.

Haplotype analysis of candidate genes in QTL regions

We performed haplotype analysis of approximately 200-kb regions adjacent to *Rf1*, *Rf2*, *Rf5* (**Fig. 2**), *Dw1*, *sbGhd7*, and *sbPHYB* (**Supplemental Fig. 1**). These regions had from 1695 (*sbGhd7*) to 3634 (*Rf5*) SNPs; using this information, we estimated the allele types of the region in restorer male parents and their relatives. In experiments I–V, *qRf5* and *qGw5* in the same region as *Rf5*, was detected and the restorer lines 'JN290', 'JN43', 'Daikoukaku', and 'JN503' were clustered in the same clade in the haplotype analysis of *Rf5*. 'JN43' was clustered with 'JN107' and 'F6-3A-5' in the haplotype analysis of *Rf1*. The restorer line 'JN358' was clustered with '74LH3213' and 'F6-3A-5' in the haplotype analysis of *Rf2*. The restorer lines 'JN290', 'JN358', and '74LH3213' were clustered in the same clades in the haplotype analysis of both *SbPHYB* and *SbGhd7*. 'JN503' was clustered with 'JN290', used in the analysis of *Dw1*.

Discussion

At the beginning of F_1 hybrid breeding of sorghum in Japan, CMS lines introduced from the USA were used as female parents and domestic Japanese lines as male parents. These F_1 hybrids were first aimed at seed production, and later at forage production. F_1 hybrid lines were produced in Japan by trial and error, as there was little genetic information about the CMS cytoplasm and the *Rf* genes. We tried to clarify the genetics of sorghum F_1 hybrids produced in Japan by using F_2 populations to analyze QTLs for Rf traits. To study fertility restoration, we used seven F_2 populations. Our QTL analysis detected *qRf5*, corresponding to the *Rf5* locus, in four of the seven experiments. We were considered that the restorer lines 'JN290', 'JN43', 'SDS7444', and 'JN503' have functional *Rf5*. In experiment IV, no significant QTLs for fertility restoration were detected, but the restorer line 'Daikoukaku' was clustered in the same clade as the restorer lines with *qRf5* in the haplotype analysis of *Rf5* (**Fig. 2**), suggesting that it contained a functional *Rf5* gene (**Fig. 3**).

qRf8, corresponding to the *Rf1* locus, was detected only in experiment II. It is derived from the restorer line 'JN43', which was clustered with 'JN107' and 'F6-3A-5' in the haplotype analysis of *Rf1* (**Fig. 2**). These three lines have a direct parent–child relationship (**Fig. 3**); thus, we consider 'JN107' and 'F6-3A-5' to have a functional *Rf1* gene. *qRf2*, corresponding to the *Rf2* locus, was detected in experiment VI and is derived from the restorer line 'JN358'. This line was clustered with '74LH3213' and 'F6-3A-5' in the haplotype analysis of *Rf2* (**Fig. 2**); thus, we consider '74LH3213' and/or 'F6-3A-5' to have a functional *Rf2* gene (**Fig. 3**).

In the QTL analysis of grain weight, QTLs (*qGw5* and *qGw2*) corresponding to *Rf5* and *Rf2* were detected (**Table 3**), indicating that most of the increase in grain weight can be explained by fertility in our experiments. In experiment III, the segregation ratio was 5:3; thus, the single dominant gene hypothesis was rejected, but a clear QTL (*qGw5*) corresponding to *Rf5* was detected along with *qRf5*. Similarly, in experiment IV, although no QTL for restoration of fertility was detected, *qGw5* was detected. The haplotype analysis also supports the presence of *Rf5* in the restorer line 'Daikoukaku' used in experiment IV. In experiment II, two QTLs were detected because the restorer line 'JN43' has both *Rf1* and *Rf5*.

The data from experiment VII were consistent with the presence of two or three independent dominant *Rf* genes in the restorer line '74LH3213', but no QTLs for fertility restoration were detected. Because this experiment was done in a greenhouse under short-day conditions, the fertility data may not be suitable for analysis. The haplotype analysis showed that '74LH3213' harbors at least *Rf2* (**Figs. 2**, **3**), but not *Rf1* or *Rf5*, because '74LH3213' did not cluster with 'JN43', which has functional *Rf1* and *Rf5*.

A minor QTL for fertility restoration, *qRf3*, was detected in experiments I and III. The presence of modifiers or partial fertility genes contributing to full pollen fertility restoration was indicated by a classical genetic study

Table 3. QTLs for restoration of fertility and related characteristics in seven experimental populations

Experiment	Trait	QTL name	Chr.	Position[a] (Mb)	Nearest marker	Interval markers		Interval of QTL[a] (bp)	LOD score	Additive effect[b]	Dominant effect[b]	% PVE[c]	Reported QTL/gene	Estimated position of reported QTL/gene[a] (bp)
I	Restoration of fertility[d]	*qRf3*	3	57.36	SB2006	SB1946	SB2174	51,323,871–66,103,861	2.56	–0.16	0.16	0.16		
I	Restoration of fertility[d]	*qRf5*	5	2.43	SB3063	SB3016	SB3085	375,801–3,544,158	9.29	–0.24	0.35	0.53	*Rf5*	2,433,620–2,573,735
II	Restoration of fertility	*qRf5*	5	2.43	SB3063	SB3050	SB3075	1,684,645–3,209,805	9.88	–22.75	8.31	0.29	*Rf5*	2,433,620–2,573,735
II	Restoration of fertility	*qRf8*	8	57.85	SB4557	SB4545	SB4567	57,429,747–58,554,302	15.19	–28.04	10.33	0.49	*Rf1*	58,099,299–58,102,691
III	Restoration of fertility	*qRf3*	3	57.35	SB2005	SB1946	SB2077	51,323,871–60,894,978	6.1	15.6	24.8	0.00		
III	Restoration of fertility	*qRf5*	5	2.43	SB3063	SB3050	SB3097	1,684,645–4,048,253	9.1	–28.5	9.8	0.39	*Rf5*	2,433,620–2,573,735
V	Restoration of fertility	*qRf5*	5	3.21	SB3075	SB3047	SB3097	1,590,839–4,048,253	12.0	–38.5	1.3	0.48	*Rf5*	2,433,620–2,573,735
VI	Restoration of fertility	*qRf2*	2	5.66	SB1028	SB1014	SB1028	4,479,115–5,659,115	82.1	–47.0	46.6	0.00	*Rf2*	5,546,273–5,550,944
I	Heading date[e]	*qHd1*	1	72.28	SB740	SB598	SB838	63,402,876–76,639,245	6.4	6.8	5.7	0.09	*sbPHYB*	68,034,103–68,043,358
I	Heading date	*qHd6-1*	6	0.37	SB3385	SB3385	SB3395	366,321–901,452	3.8	–6.0	0.8	0.16	*sbGhd7*	697,459–700,101
II	Heading date	*qHd10*	10	22.95	SB5322	SB5313	SB5438	17,630,815–55,051,632	7.0	–2.3	–1.5	0.08		
V	Heading date	*qHd2*	2	56.52	SB1203	SB1196	SB1362	55,966,728–64,361,380	4.3	1.9	–2.3	0.27		
V	Heading date	*qHd8*	8	56.38	SB4529	SB4462	SB4557	45,178,098–57,848,959	4.0	3.3	0.7	0.09		
VI	Heading date	*qHd1*	1	69.08	SB688	SB650	SB688	66,133,950–69,075,466	30.1	14.3	11.8	0.12	*sbPHYB*	68,034,103–68,043,358
VI	Heading date	*qHd6-1*	6	0.34	SB3383	SB3377	SB3384	11,782–347,108	24.3	–14.3	9.0	0.64		
VII	Heading date	*qHd1*	1	71.08	SB723	SB628	SB745	64,762,030–72,376,022	15.1	5.2	0.9	0.27	*sbPHYB*	68,034,103–68,043,358
VII	Heading date	*qHd5*	5	4.74	SB3112	SB3050	SB3137	1,684,645–6,613,220	3.6	2.4	0.3	0.05		
VII	Heading date	*qHd6-2*	6	0.85	SB3875	SB3875	SB3916	853,972–2,831,305	17.4	–4.4	3.6	0.54		
II	Panicle length	*qPl7-1*	7	14.39	SB4018	SB4003	SB4069	8,733,235–54,376,436	5.1	1.7	1.1	0.06		
III	Panicle length	*qPl7-2*	7	57.94	SB4109	SB4050	SB4124	52,168,665–58,644,978	8.2	2.6	–0.5	0.35		
V	Panicle length	*qPl9*	9	56.81	SB5046	SB5028	SB5092	55,970,428–58,531,611	5.0	–1.6	–1.8	0.01	*Dw1*	57,038,653–57,041,166
VI	Panicle length	*qPl1*	1	69.08	SB688	SB650	SB769	66,133,950–73,639,141	14.3	3.3	3.5	0.04	*sbPHYB*	68,034,103–68,043,358
VI	Panicle length	*qPl6*	6	0.34	SB3383	SB3377	SB3384	11,782–347,108	9.8	–3.4	2.0	0.30		
I	Grain weight	*qGw1-1*	1	10.61	SB237	SB128	SB270	5,225,977–12,387,376	5.0	–10.6	–34.2	0.07		
I	Grain weight	*qGw5*	5	2.43	SB3063	SB3016	SB3085	375,801–3,544,158	5.6	–22.8	6.5	0.31	*Rf5*	2,433,620–2,573,735
II	Grain weight	*qGw4*	4	2.44	SB2455	SB2383	SB2486	252,393–3,980,485	2.6	20.7	–15.1	0.24		
III	Grain weight	*qGw5*	5	2.43	SB3063	SB3050	SB3097	1,684,645–4,048,253	9.1	–8.6	1.8	0.37	*Rf5*	2,433,620–2,573,735
IV	Grain weight	*qGw5*	5	1.59	SB3047	SB3034	SB3078	1,064,049–3,356,906	5.9	–6.6	6.9	0.33	*Rf5*	2,433,620–2,573,735
V	Grain weight	*qGw5*	5	3.21	SB3075	SB3047	SB3097	1,590,839–4,048,253	12.3	–16.0	2.6	0.47	*Rf5*	2,433,620–2,573,735
VI	Grain weight	*qGw1-2*	1	69.08	SB688	SB650	SB744	66,133,950–72,341,663	9.8	17.4	24.4	0.02	*sbPHYB*	68,034,103–68,043,358
VI	Grain weight	*qGw2*	2	5.66	SB1028	SB1014	SB1044	4,479,115–6,795,657	3.7	–21.9	21.4	0.24	*Rf2*	5,546,273–5,550,944

[a] Sorghum bicolor v3.1 DOE-JGI, http://phytozome.jgi.doe.gov/.
[b] Positive values show that female parent's allele increases values.
[c] Percentage of phenotypic variance explained.
[d] Sterile = 0; Fertile = 1.
[e] Day of the panicle bagging for self-crossing.

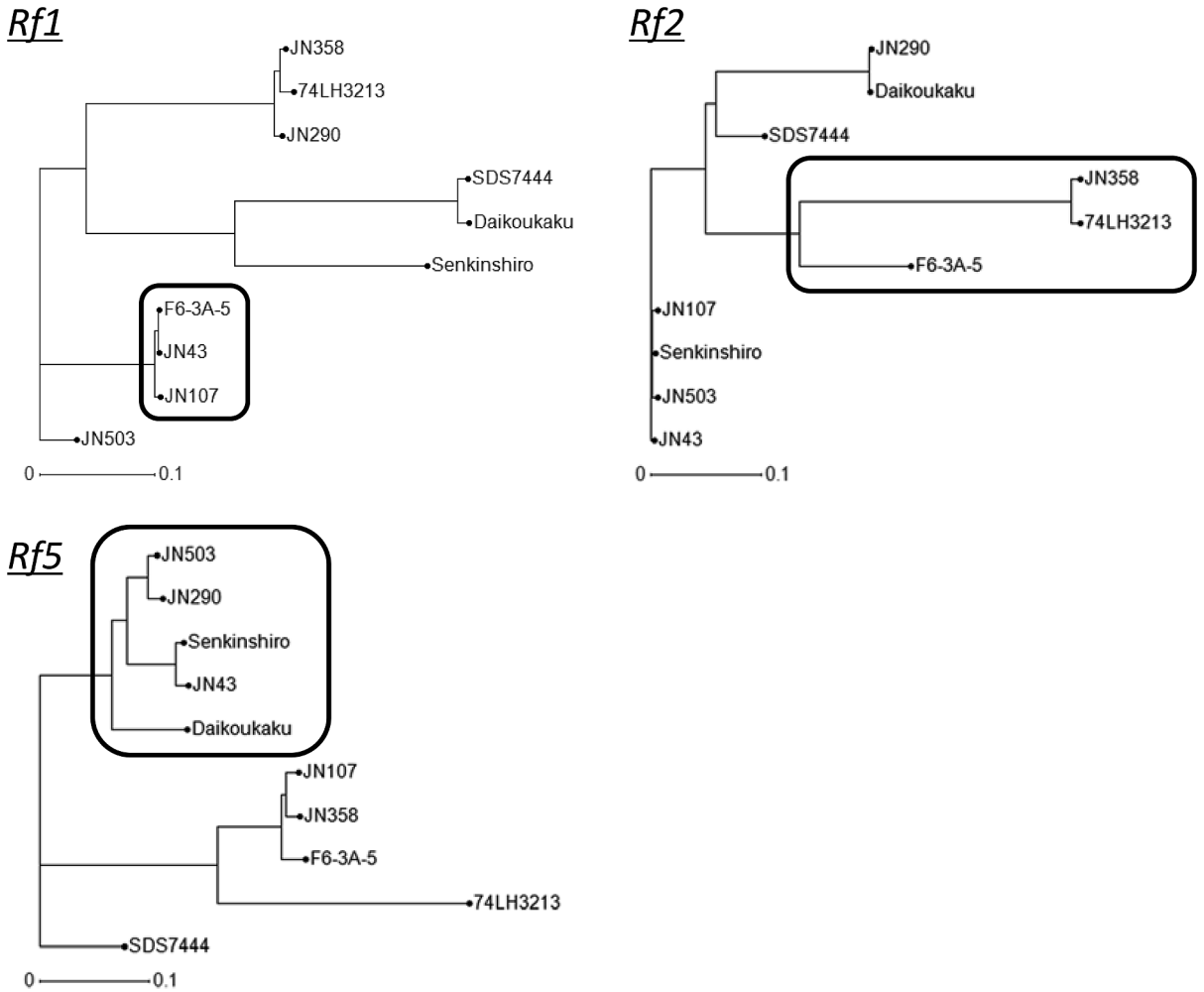

Fig. 2. Hierarchical analysis of the haplotypes in 200-kb regions adjacent to three fertility-restoring genes from 10 male parents used in this study. Boxes indicate the expected functional genes based on QTL analysis. Scale bars, distance based on the simple matching coefficient.

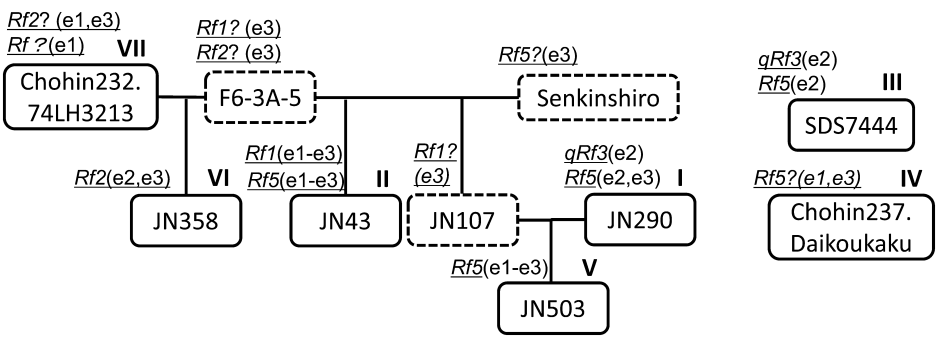

Fig. 3. Inheritance of the candidate fertility restorer genes from male parents in seven F_1 cultivars. Male restorer lines used to produce the experimental F_2 populations are shown in solid boxes and those used for haplotype analysis only are shown in dotted boxes. Connections between boxes indicate breeding lineage. "*Rf*" with Arabic numbers indicate candidate fertility restorer genes. The experimental evidences (e1, segregation test; e2, QTL analysis; e3, haplotype analysis) are shown in the parentheses; Roman numerals indicate experimental F_2 populations.

(Miller and Pickett 1964). The *qRf3* locus may be capable of partially restoring pollen fertility (additive effect –0.16 in experiment I, +15.6 in experiment III). The *qRf3* locus differed from the *Rf* locus with a minor effect on Chr. 4 reported by Jordan *et al.* (2011). In experiment I, *qRf3* originated from the restorer line 'JN290'. Thus, 'JN290' contained a major QTL, *Rf5*, and a minor locus, *qRf3*. On the other hand, in experiment III, *qRf3* originated from the female CMS parent '(954149) A' and the additive effect was relatively high (15.6). We consider that the effect of

qRf3 made the fertile-to-sterile segregation ratio 5:3, not the ideal 3:1, in experiment III. In F_1 hybrid breeding programs, this type of minor *Rf* gene must be excluded from female parent lines. In some cases, as noted by Jordan *et al.* (2010), "partial fertility is only expressed under particular environmental conditions and may pass unnoticed for some generations." Partial Rf in female parents can result in serious commercial losses in seed production. Some CMS female lines restore fertility in certain conditions (Dr. Kasuga, personal communication). Thus, DNA markers for minor *Rf*s need to be developed to eliminate such loci when new female parents for F_1 hybrids are developed.

Heading date and panicle length may affect fertility. Interestingly, we detected QTLs corresponding to *SbPHYB*, *SbGhd7*, and *Dw1*, and our haplotype analysis showed the presence of different alleles of these loci in restorer lines (**Supplemental Fig. 1**). In the haplotype analysis of both *SbPHYB* and *SbGhd7*, 'JN358', 'JN290'. and '74LH3213' were in the same clade, and these three lines have similar flowering time (data not shown).

Our QTL analysis showed that three major *Rf*s—*Rf1*, *Rf2*, and *Rf5*—were used in the F_1 hybrid breeding of sorghum in Japan, *Rf5* most frequently (**Fig. 3**). The restorer lines used in our experiments were bred from 'Kaoliang' sorghum, originally from north China and the Korean peninsula. Thus, we consider that all the three *Rf* genes were present in 'Kaoliang'. In contrast, the female CMS lines were introduced from the USA into Japan. The pedigree of CMS female lines used for F_1 hybrids is not known. Since *Rf1*, *Rf2*, and *Rf5* can restore the fertility of A1-cytoplasm lines to the same level (Jordan *et al.* 2011) and the A1 cytoplasmic-nuclear male sterility system in sorghum is used almost exclusively for the production of commercial hybrid seed (Jordan *et al.* 2010), the CMS female lines used for F_1 hybrid sorghum in Japan probably have A1 cytoplasm. Our data on the *Rf* genes in the restorer lines will be useful for the effective production of new sorghum F_1 hybrid varieties in Japan in the future.

Author Contribution Statement

AK and KG designed and performed the field experiments. JY analyzed the data and interpreted the results. AK, JY and HK wrote the paper. All authors read and reviewed the paper.

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