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ORIGINAL PAPER

Marker-assisted breeding of a LOX-3-null rice line with improved storability and resistance to preharvest sprouting

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Abstract

Key message **Breakage of the tight linkage between rice seed *lipoxygenase-3* and easy preharvest sprouting trait led to breeding of lines with few stale flavors after long storage and desirable preharvest sprouting resistance.**

Abstract Lipoxygenase-3 (LOX-3) is involved in the production of volatile constituents in stored rice, and the development of stale flavor is delayed in LOX-3 null rice. In the process of breeding new LOX-3-null lines with long storability, we found a close association between LOX-3 and preharvest sprouting resistance. To determine whether this relationship was due to the tight linkage of two genes or the pleiotropic effect of LOX-3, we performed marker-assisted selection using a BC_3F_3 population derived from crosses between LOX-3-present/preharvest sprouting-resistant lines and LOX-3-null/preharvest susceptible lines. In one individual, a recombination event occurred 13 kb downstream of *LOX-3* (RM15750) and a significant quantitative trait locus, namely *qPHS3*, for easy preharvest sprouting trait (LOD = 10.4) was detected in an 842-kb region between RM15711 and RM15768. Using BC_3F_4 and BC_3F_5 populations, we succeeded in selecting LOX-3-absent and preharvest sprouting-resistant lines with only a 393-kb introgressed chromosome segment from the donor line for LOX-3-null at the *LOX-3* locus on chromosome 3. This result indicated that the *LOX-3* gene and the locus affecting preharvest sprouting are distinct. The selected line was named 'Hokuriku 244'. Sensory testing of rice grains with and without LOX-3 confirmed that stale flavor production in LOX-3-null rice during storage was lower than in normal LOX-3 rice. These results indicated that rice varieties with little stale flavor after long storage and preharvest sprouting resistance had been selected.

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Introduction

Because rice (*Oryza sativa*) is the staple diet of more than half of the world's population, it is important to have high storage stability to ensure the constant availability of high-quality rice. Rice grain deterioration and the development of stale flavor during storage are serious problems that reduce stored grain quality. Many researchers have suggested that lipid degradation is responsible for deterioration during grain storage (Yasumatsu and Moritaka 1964; Takano 1993; Zhou et al. 2002). Lipoxygenase (LOX) catalyzes the peroxidation

of polyunsaturated fatty acids and is thought to play an important role in the development of desirable or undesirable flavor and aroma in many plant products (Gardner 1988; Feussner and Wasternack 2002). Many compounds have been identified in raw and cooked rice or the bran fraction, and some volatile compounds, particularly hexanal, are reported to increase during storage (Yasumatsu and Moritaka 1964; Tsugita et al. 1983; Suzuki et al. 1999; Zhou et al. 2002). Because the amount of unsaturated fatty acids decreases in stored rice, Yasumatsu and Moritaka (1964) suggested that linoleic acid in rice is oxidized gradually during storage, resulting in carbonyl compounds that induce a stale flavor in rice.

LOX activity in the rice grain is localized in the bran fraction (Yamamoto et al. 1980), and one of the LOX isozymes, LOX-3, is the major isozyme component (Ida et al. 1983). Suzuki et al. (1993) identified a LOX-3-null variety, the tropical Japonica cultivar 'Daw Dam', and reported that the absence of LOX-3 is inherited as a simple recessive trait (Suzuki 1995; Suzuki et al. 1996a). Suzuki et al. (1996b) reported that the peroxidation of unsaturated fatty acids occurs at lower levels in the 'Daw Dam' bran fraction during storage than in rice varieties with LOX-3 in their seeds. Given that stale flavor production in LOX-3-null rice during storage is lower than that in normal LOX-3 rice (Suzuki et al. 1999), attempts have been made to breed new LOX-3-null lines with high storability. However, the progeny of two backcrosses of the LOX-3-null breeding line 'Daw Dam' to a Japonica variety showed high preharvest sprouting (Uehara et al. unpublished data).

Farmers growing cultivars with high preharvest sprouting often suffer severe yield loss and reduction of grain quality (Dong et al. 2003; Groos et al. 2002). Preharvest sprouting occurs under favorable temperature and humidity at maturity, and the lack of adequate seed dormancy is the major reason for preharvest sprouting. Preharvest sprouting and seed dormancy are opposed characters (Frank et al. 2005). Thus, it is important to inhibit preharvest sprouting and/or control seed dormancy while breeding many cereals. Recent progress in plant genomics has facilitated the identification of seed dormancy quantitative trait loci (QTLs) in plants such as rice (summarized in Sugimoto et al. 2009; Graeber et al. 2012). The results of these studies have revealed that the seed dormancy level is regulated by multiple genes. In addition, genes controlling seed dormancy and preharvest sprouting trait have been identified in cereal crops, e.g., rice, wheat, and maize, as well as *Arabidopsis* (Graeber et al. 2012).

In our previous study, we mapped a gene for the LOX-3-null phenotype to the long arm of chromosome 3, determined the amino acid sequences of purified seed LOX-3, and developed single nucleotide polymorphism (SNP) markers for the LOX-3 deficiency (Shirasawa et al. 2008). In the present study, we have employed these DNA markers and progeny testing using anti-LOX-3 monoclonal antibodies to develop near-isogenic lines (NILs) with the LOX-3-null allele in the background of a Japonica cultivar, 'Koshihikari', the most popular LOX-3-containing variety in Japan due to its good eating quality, through repeated backcrossing. However, there was a tight linkage relationship between the presence or absence of LOX-3 and the level of preharvest sprouting. Using DNA markers, we were able to demonstrate that the LOX-3-null allele and preharvest sprouting susceptibility are conditioned by two separate loci by breaking the tight linkage between the genes for two characteristics. We further clarified the stale flavor of stored rice with or without LOX-3 protein in seeds.

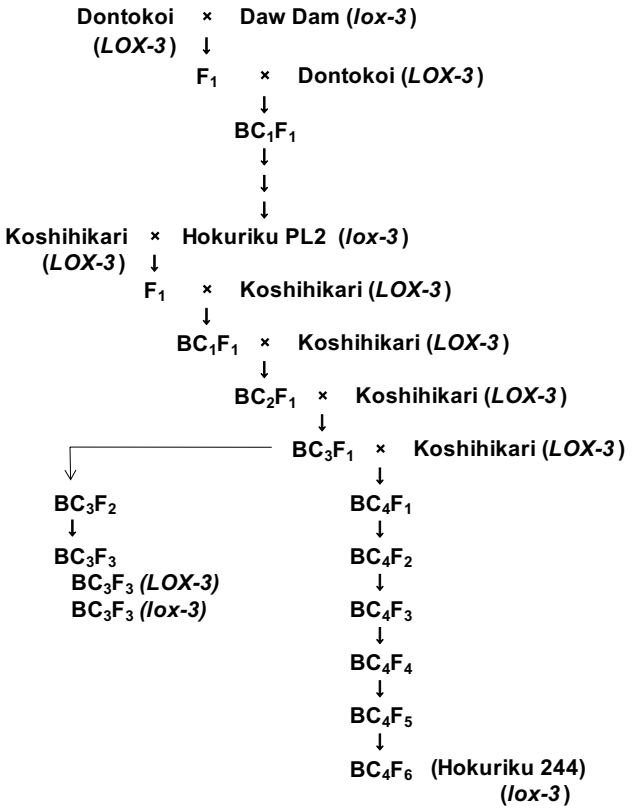

Fig. 1 Crossing scheme for the breeding of seed LOX-3-null varieties and lines. Presence or absence of the LOX-3 protein in seeds is indicated in parentheses by *LOX-3* and *lox-3*, respectively

Materials and methods

Plant materials

The rice varieties used were 'Daw Dam' and 'Hokuriku PL2', whose seeds lack LOX-3, and 'Dontokoi' and 'Koshihikari', whose seeds carry LOX-3 (Suzuki et al. 1993) (Fig. 1). 'Daw Dam' is a tropical Japonica cultivar and its agronomic characters are not suitable for cultivation in Japan

Fig. 2 Preharvest sprouting of Koshihikari (*LOX-3*), Hokuriku PL2 (*lox-3*), and Dontokoi (*LOX-3*). *The numbers in parentheses* indicate the preharvest sprouting score for each variety

(Suzuki 1995; Suzuki et al. 2000), while the other three lines are Japonica cultivars of Japan. These varieties and progenies derived from crossing wild-type varieties ('Dontokoi' and 'Koshihikari') carrying LOX-3 in seeds with varieties ('Daw Dam' and 'Hokuriku PL2') lacking LOX-3 were planted and grown in paddy fields of the Hokuriku Research Center, NARO Agricultural Research Center, Niigata, Japan and of NARO Institute of Crop Science, Ibaraki, Japan.

Protein analysis and screening of LOX-3-null lines

LOX-3 protein in seeds was detected by Western blot analyses using anti-LOX-3 monoclonal antibodies (Suzuki et al. 1993; Shirasawa et al. 2008). Identification of LOX-3-null lines was performed by progeny testing.

Evaluation of preharvest sprouting

Five to ten panicles collected from each plant in the field at 40–45 days after heading were stored for approximately 3 months in a refrigerated room and three panicles from each plant (or two panicles if plants were small) were used for preharvest sprouting evaluation. The panicles were soaked in water at 20 °C for 16 h and then transferred to wet paper towels and incubated at 30 °C with 100 % humidity for 6 days. The preharvest sprouting score was based on the percentage of germinated seeds per panicle, as described by Horiuchi (1996), and it ranged from highly resistant (2.0; one or two grains of the panicle germinate) to very sensitive (8.0; all grains of the panicle germinate and its shoot and root elongate) (Supplemental Fig. S1). As a typical example, the preharvest sprouting score of 'Koshihikari', 'Hokuriku PL2' and 'Dontokoi' was 3.0, 7.0 and 5.0, respectively (Fig. 2).

DNA and QTL analysis

Genomic DNA was isolated from leaves using the method of Murray and Thompson (1980). Simple sequence repeat (SSR) markers developed from the rice genome sequence data (International Rice Genome Sequencing Project 2005) and SNP in *LOX-3* (Shirasawa et al. 2008) were used. Polymerase chain reaction (PCR) and signal detection were performed as described by Shirasawa et al. (2008) and Suzuki et al. (2011). Genome-wide genotyping was performed with SSR markers (International Rice Genome Sequencing Project 2005) as described by Ideta et al. (2012). The average preharvest sprouting scores of each line of the BC_4F_3 population and genotyping data were employed for QTL analysis. Linkage maps were constructed with MAPMAKER/EXP 3.0 (Lincoln et al. 1993). The composite interval mapping (CIM) procedure and interval mapping in WINDOWS QTL CARTOGRAPHER v2.5 (Wang et al. 2007) were used for QTL mapping. The LOD threshold for each QTL was determined with 1000 permutation tests. For the CIM, standard CIM model and forward regression method were used.

Stale flavor evaluation

Rice grains of the BC_4F_4 and BC_4F_6 collected from an experimental field were dehulled with a Satake (Hiroshima, Japan) experimental dehuller, and their moisture content was adjusted to 14.0–15.0 %. Samples of 100 g of brown rice were stored in a polyethylene bag at 25 °C for 4 weeks. After storage, the rice was kept below −40 °C until analysis. The stored brown rice (10 g) was weighed into a Petri dish (9-cm diameter) and/or a 50-mL test tube with a screw cap. Fourteen and 12 tasters evaluated the intensity of oxidized flavor of the stored brown rice of the BC_4F_4 and BC_4F_6, respectively.

Statistical analysis for allelic effects of LOX-3 in the preharvest sprouting

Data were analyzed by analysis of variance. When statistically significant effects of lines on specific traits were found, differences among these traits were tested using protected least square differences (post hoc test).

Results

Close association between LOX-3-null and preharvest sprouting susceptibility

To breed good flavor and quality of rice grains after long storage, we backcrossed 'Daw Dam' twice to rice variety 'Dontokoi'. By progeny testing with anti-LOX-3 monoclonal antibodies, in 2000, we developed 'Hokuriku

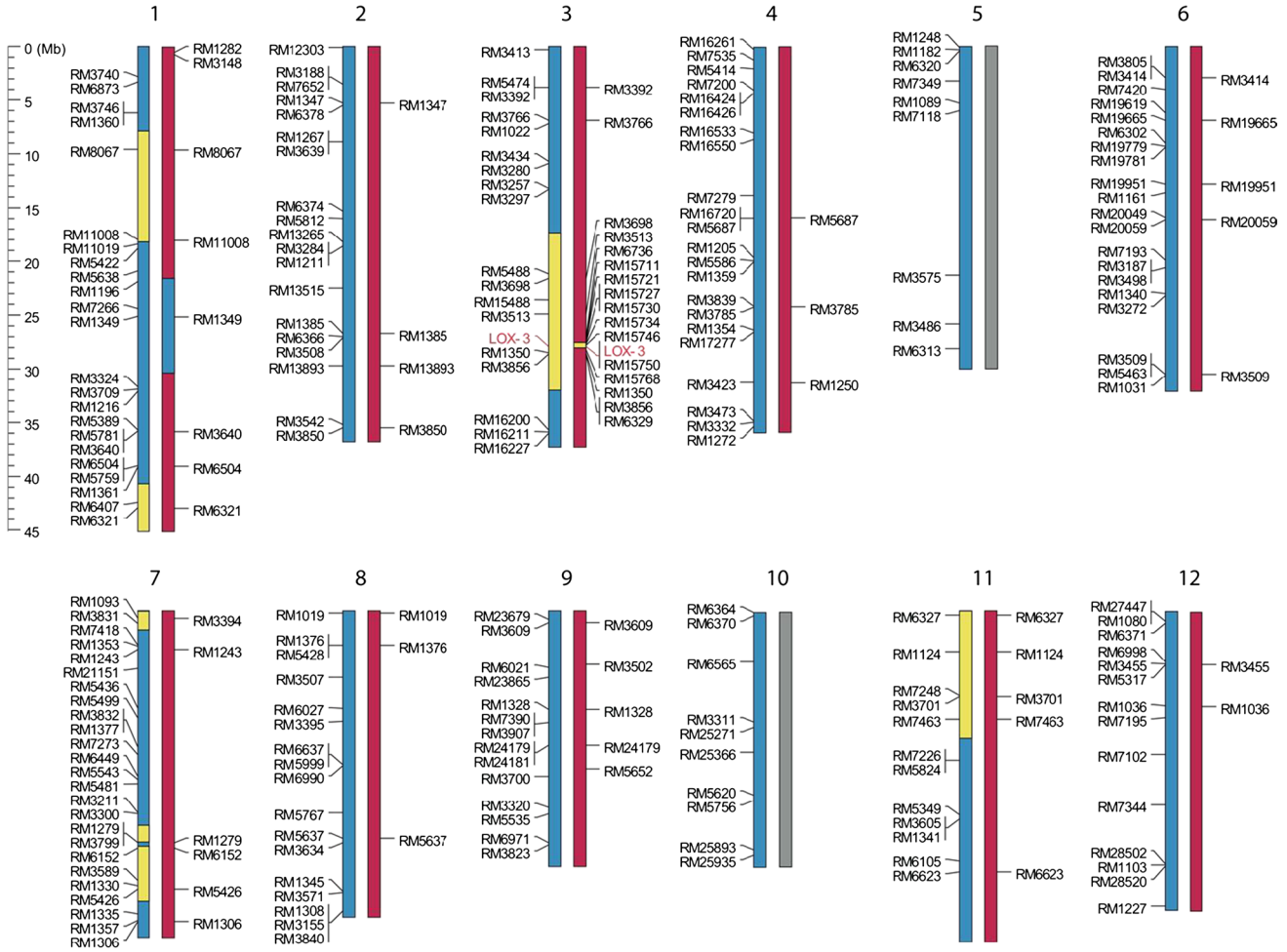

Fig. 3 Graphical genotypes of 'Hokuriku PL2' (*left*) and 'Hokuriku 244' (*right*). Polymorphic marker loci between 'Daw Dam' and 'Dontokoi' and between 'Hokuriku PL2' and 'Koshihikari' are shown on the *left* and *right* sides of the chromosomes, respectively. *Yellow*, *blue*, and *red* indicate chromosome segments derived from 'Daw Dam', 'Dontokoi', and 'Koshihikari', respectively. *Gray* indicates monomorphic regions derived from either 'Dontokoi' or 'Koshihikari' (color figure online)

Table 1 Allele effects of 'Koshihikari', the heterozygote, and 'Hokuriku PL2' in *LOX-3* on the preharvest sprouting characteristics of BC_4F_5 individuals

Variety/line	Alleles of *LOX-3*	Panicle numbers evaluated for preharvest sprouting	Preharvest sprouting score	
			Mean value	SE
Koshihikari	Koshihikari	89	3.71	0.64
Hokuriku PL2	Hokuriku PL2	89	6.93	0.29
BC4F5 line	Koshihikari	247	3.60	0.64
BC4F5 line	Heterozygous	589	3.51	0.64
BC4F5 line	Hokuriku PL2	320	3.56	0.66

BC_4F_5 progeny (381) homozygous for *LOX-3* or *lox-3* or heterozygous alleles were grown and evaluated for preharvest sprouting

PL2', which does not contain LOX-3 in its seeds (Fig. 1). Genome-wide genotyping indicated that 'Hokuriku PL2' had six chromosomal segments derived from 'Daw Dam' in the 'Dontokoi' genetic background (Fig. 3). 'Hokuriku PL2' is a variety with eating quality as good as that of the most popular LOX-3-containing variety in Japan, 'Koshihikari'; however, it sprouts extremely readily in the field (Fig. 2; Table 1). Given that 'Koshihikari' shows a relatively high level of preharvest sprouting resistance in field evaluations, we wished to introduce the sprouting

Table 2 Agronomic characters of 'Koshihikari' and BC_3F_3 NILs with and without LOX-3 in seeds

Variety/lines	LOX-3	Heading date (month . day)	Culm length (cm)	Panicle length (cm)	Panicle number (number/m^2)	Brown rice yield (Kg/a)	1000-grain weight (g)	Preharvest sprouting score	Eating quality
Koshihikari	+	8.08	92.9	19.3	329	62.1	21.9	4.0	−0.56
05-7610-1[a]	+	8.09	88.2	19.1	365	65.8	22.5	3.0	−0.32
05-7610-5	+	8.09	89.8	19.2	374	61.6	21.8	4.5	−0.50
05-7610-11	−	8.07	93.2	20.0	283	63.5	22.6	8.0	−0.52
05-7610-23	−	8.08	94.0	19.9	272	66.3	22.8	6.5	−0.36

Eating quality was compared with that of 'Koshihikari' in blind trials. Positive values represent higher and negative values represent lower eating quality. Data were average results of two iteration in 2006

[a] '05-7610-1', '05-7610-5', '05-7610-11', and '05-7610-23' are BC_3F_3 NILs derived from 'Hokuriku PL2' backcrossed to 'Koshihikari', as shown in Fig. 1. 'Koshihikari', '05-7610-1', and '05-7610-5' contained LOX-3 protein in their seeds, and '05-7610-11' and '05-7610-23' did not

resistance of 'Koshihikari' into the sprouting-susceptible cultivar 'Hokuriku PL2'. Accordingly, the F_1 progeny of 'Koshihikari'/'Hokuriku PL2' was backcrossed three times to 'Koshihikari' as the recurrent parent to improve the sprouting characteristics of 'Hokuriku PL2' and to breed NILs that lacked LOX-3 while also being resistant to sprouting (Fig. 1).

Our preliminary experiments indicated that either there is a tight linkage of *LOX-3* and a preharvest sprouting locus or the *LOX-3* gene has a pleiotropic effect. To clarify this, we produced four BC_3F_3-NILs with and without LOX-3 in seeds in 2006 (Fig. 1). The agronomic characteristics of these NILs, including their heading date, were almost identical (Table 2). Two BC_3F_3-NILs with LOX-3 in the seeds (05-7610-1 and 05-7610-5) were resistant to sprouting, whereas two BC_3F_3-NILs without LOX-3 (05-7610-11 and 05-7610-23) readily underwent preharvest sprouting (Table 2). In addition to endogenous genetic factors, seed dormancy and preharvest sprouting are controlled by environmental conditions such as temperature during the ripening period and degree of maturity (Takahashi 1997; Sugimoto et al. 2009), and such environmental conditions can introduce errors into preharvest sprouting assays. However, because the heading dates of the BC_3F_3 individuals were almost identical (within 2 days), the effect of maturation temperature on the differences was excluded. As the results suggested linkage genetic rather than environmental basis for the preharvest sprouting, we crossed one of the LOX-3-heterozygous BC_3F_1 NILs with 'Koshihikari', developed BC_4F_1 and BC_4F_2 generations, and proceeded with further breeding for low preharvest sprouting.

Linkage analysis of LOX-3 deficiency and level of sprouting

Before the development of BC_3F_3-NILs, the LOX-3-null line was screened by progeny testing of its mature seeds using anti-LOX-3 monoclonal antibodies, and this method required much time and effort (Suzuki 1995; Suzuki et al. 1993, 1996a). Accordingly, in the previous study (Shirasawa et al. 2008), we first mapped the seed LOX-3 gene to an approximately 1.5-Mb interval between two SSR markers, RM6736 and RM6329, identified *LOX-3*, and developed SNP markers for LOX-3 deficiency (Fig. 4a). Thus, we screened LOX-3-null lines of BC_4F_2 individuals using DNA markers in addition to the progeny test. From 58 BC_4F_2 progeny that were generated, 22 progeny heterozygous at the *LOX-3*, RM6736, and RM6329 loci were selected for further analysis (Fig. 4b).

BC_4F_3 families, each consisting of 38 progeny derived from one of the 22 BC_4F_2 selected individuals, were generated in 2007. All samples were subjected to DNA marker-assisted selection to obtain recombinants between LOX-3 and either RM6736 or RM6329 (Fig. 4b). Of the BC_4F_2 families composed of 836 individuals, 135 were selected as recombinants between the two SSR markers. The numbers of homozygous 'Koshihikari' alleles, heterozygous alleles, and homozygous 'Hokuriku PL2' alleles for RM6736 and RM6329 were 39:59:37 and 34:70:31, respectively. Subsequent genotyping of the 135 individuals with the *LOX-3* SNP marker showed a segregation ratio of 34:70:31. The number of recombinants between *LOX-3* and RM6736 was 66 and that between *LOX-3* and RM6329 was 70.

The preharvest sprouting scores of the 135 BC_4F_3 individuals ranged from 2.9 to 7.0, with an average score of 4.9 (Fig. 5a). The scores of individuals harboring homozygous 'Koshihikari' alleles, heterozygous alleles, and homozygous 'Hokuriku PL2' alleles of *LOX-3* ranged from 2.9 to 5.6 (4.3 in average), from 3.5 to 6.8 (5.0 in average), and from 3.5 to 7.0 (5.5 in average), respectively, suggesting that *LOX-3* and the preharvest sprouting gene are tightly linked rather than identical.

To identify the precise position of the preharvest sprouting locus and separate the LOX-3-null and sprouting susceptibility characters, additional DNA markers were developed between RM6736 and RM6329 (Fig. 4b).

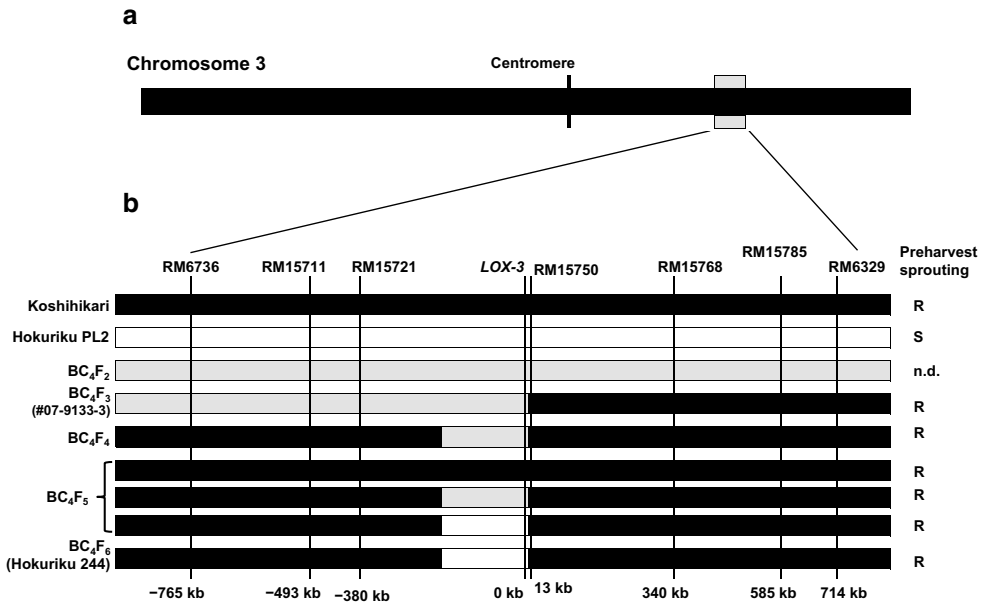

Fig. 4 Genomic structure around *LOX-3* on chromosome 3. **a** Chromosome 3 structure. *LOX-3* region (1480 kb) sandwiched between RM6736 and RM6329 is shown with a *gray box*, **b** genomic structure around *LOX-3* region of Koshihikari, Hokuriku PL2, BC_4F_2, BC_4F_3, BC_4F_4, BC_4F_5 and BC_4F_6 individuals. *Black and gray bars* indicate Koshihikari and heterozygous genotypes in SSR markers and *LOX-3*, respectively. *Numeric values* near SSR markers denote distances (in kb) from *LOX-3*

Polymorphism analysis of the parental lines was performed with 44 SSR markers (International Rice Genome Sequencing Project 2005). Among the 44 SSR markers, five (RM15711, RM15721, RM15750, RM15768, and RM15785) showed polymorphism between the parental lines and mapped in the expected order. Using the preharvest sprouting scores of 192 BC_4F_3, QTL analysis was performed (Figs. 4b, 5a). Between RM15711 and RM15768, an interval which contains the *LOX-3* locus, a significant QTL peak (LOD = 10.4) was detected (threshold of LOD was 2.1 determined from the permutation tests) (Supplemental Fig. 2A). This locus accounted for 20.7 % of the phenotypic variance in the BC_4F_3 individuals, with the 'Koshihikari' allele showing a negative additive effect (preharvest sprouting score of −0.54). In the interval mapping, a significant QTL was detected in this region (Supplemental Fig. 2B).

In one BC_4F_3 progeny, #07-9133-3, recombination occurred in the interval between *LOX-3* and the SSR marker RM15750 (Fig. 4b). The distance between *LOX-3* and RM15750 was only 13 kb. This #07-9133-3 individual was heterozygous from RM6736 to *LOX-3* and homozygous for the 'Koshihikari' allele from RM15750 to RM6329. The preharvest sprouting score of #07-9133-3 was 3.7, whereas those of 'Koshihikari', 'Dontokoi', and 'Hokuriku PL2' were 3.4, 5.3, and 7.0, respectively.

Generating progeny from #07-9133-3 was expected to break the tight linkage between the seed LOX-3-null phenotype and easy preharvest sprouting characteristics. Accordingly, 786 BC_4F_4 individuals derived from the #07-9133-3 BC_4F_3 parent were grown and genotyped for RM15711, RM15721, *LOX-3*, and RM15750, yielding 14 recombinants between RM15721 and *LOX-3*. These progeny were homozygous for the 'Koshihikari' allele at RM15721 and heterozygous for the *LOX-3* allele. Evaluation of preharvest sprouting of the 786 progeny led to the selection of four progeny showing higher preharvest sprouting resistance (Fig. 5b). These were homozygous for the 'Koshihikari' allele at RM15721 and RM15750 and heterozygous at *LOX-3*; that is, only a 350-kb region near LOX-3 was heterozygous and other genomic regions carried the 'Koshihikari' genotype.

To confirm the effect of 'Hokuriku PL2' allele of LOX-3 on preharvest sprouting characteristics, progeny of these four BC_4F_4 individuals were grown and analyzed as above. Of 381 BC_4F_5 progeny, 81 were homozygous for 'Koshihikari' at *LOX-3*, 195 were heterozygous at *LOX-3*, and 105 were homozygous for 'Hokuriku PL2' at *LOX-3*. These progeny were evaluated for preharvest sprouting. As shown in Table 1 and Fig. 5c, the degrees of preharvest sprouting among these BC_4F_5 progeny harboring three genotypes at *LOX-3* were not significantly different, indicating that the tight linkage between rice seed LOX-3-null phenotypes and easy preharvest sprouting characteristics had been broken. This was confirmed by testing the BC_4F_6 progeny in the following season and the LOX-3-null BC_4F_6 individuals with high sprouting resistance were designated as 'Hokuriku 244'. 'Hokuriku 244' had almost the same

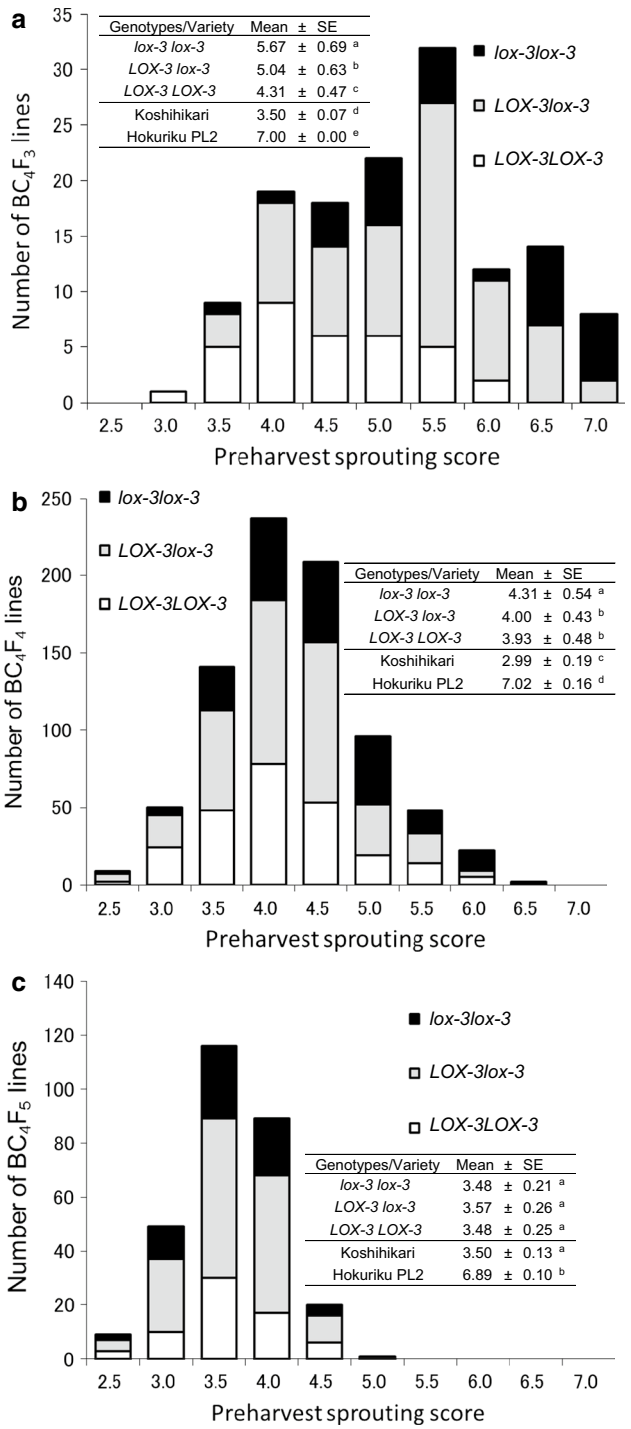

Fig. 5 Histogram of preharvest sprouting scores of the BC_4F_3, the BC_4F_4, and the BC_4F_5population. **a** Histogram of preharvest sprouting scores of the BC_4F_3 population based on 135 individuals (Supplemental Fig. 2), **b** histogram of preharvest sprouting scores of the BC_4F_4 population based on 814 individuals, **c** histogram of preharvest sprouting scores of the BC_4F_5 population based on 517 individuals

agronomic characteristics, yield, and eating quality as 'Koshihikari' (Table 3). In addition, genome-wide genotyping of 'Hokuriku 244' and 'Koshihikari' indicated that the majority of the Hokuriku 244 genome is derived from 'Koshihikari' with the exception of the *LOX-3* locus (chromosome 3) and a small segment of chromosome 1, the latter of which is from 'Dontokoi' not 'Daw Dam' (Fig. 3). However, it should be noted that the origins of chromosome 5 and 10 in 'Hokuriku 244' were not determined due to the lack of polymorphic markers, but are likely to be from the Japonica-type cultivars 'Koshihikari' or 'Dontokoi' (Fig. 3).

Sensory evaluation of stored brown rice with and without LOX-3

To evaluate the extent of stale flavor in rice grains with and without LOX-3, brown rice was stored and stale flavor of the stored rice was evaluated by 12 tasters and 14, respectively. All but one panel judged that the stale flavor of LOX-3-null grains from the BC_4F_4 and BC_4F_6 populations was significantly weaker than that of 'Koshihikari' grains with LOX-3 in the seeds (Table 4). Those results are in accordance with the finding that lower levels of volatile compounds and peroxidation products of unsaturated fatty acids are produced in LOX-3-null varieties during storage than in varieties with normal LOX-3 activity in seeds (Suzuki et al. 1996b, 1999).

Discussion

We previously suggested that LOX-3 is involved in the production of volatile constituents in stored rice and that the development of stale flavor is delayed in LOX-3-null rice (Suzuki et al. 1996b, 1999). Given that the LOX-3-null character would be a favorable phenotype for brown rice storage, we have bred rice varieties with good flavor and quality of rice grains after prolonged storage. First, we bred the seed LOX-3-null variety 'Hokuriku PL2' by crossing 'Daw Dam' twice with the rice variety 'Dontokoi' (Fig. 1). 'Hokuriku PL2' is a variety with eating quality as good as that of the most popular LOX-3-containing variety 'Koshihikari'; however, it is extremely susceptible to preharvest sprouting (Fig. 2; Table 1). We accordingly backcrossed 'Hokuriku PL2' to 'Koshihikari' four successive times; however, the resulting BC_3F_3 individuals without LOX-3 in seeds (i.e., LOX-3-null) were also extremely sprouting-susceptible, whereas the progeny with LOX-3 were resistant (Table 2). These results indicated that either the LOX-3 locus was tightly linked to a preharvest sprouting locus or that LOX-3-null has a pleiotropic effect on preharvest sprouting susceptibility.

It is difficult to determine from linkage studies whether different traits are controlled by two or more linked genes or one gene with pleiotropic effects. To breed novel rice

Table 3 Agronomic characters of 'Hokuriku 244' without LOX-3 in seeds

Variety/lines	LOX-3	Heading date (month . day)	Culm length (cm)	Panicle length (cm)	Panicle number (number/m^2)	Brown rice yield (Kg/a)	1000-grain weight (g)	Preharvest sprouting score	Eating quality
Koshihikari	+	8.06	93.0	19.2	360	60.1	22.1	3.1	0.8
Hokuriku 244	–	8.06	93.0	19.8	352	60.4	22.5	3.7	0.9

Agronomic characters of 'Hokuriku 244' without LOX-3 in seeds were compared with those of 'Koshihikari' with LOX-3. Eating quality was compared with that of 'Koshihikari' in blind trials. Positive values represent higher eating quality, while negative values represent lower eating quality. Data were the average of the 4 years of 2009–2012

Table 4 Evaluation of stale flavor in stored rice grains with and without LOX-3

Year of analysis	Number of panels judged as stronger stale flavor			Total	X^2 (1:1)	*P*
	Koshihikari	LOX-3 null strain	No decision			
2009	13	0	1	14	13.0	0.000
2011	12	0	0	12	13.0	0.000

Brown rice was harvested from 'Koshihikari' and BC_4F_4 or BC_4F_6 populations (Fig. 1). 'Koshihikari' seeds contain LOX-3; however, brown rice from BC_4F_4 or BC_4F_6 populations did not. In 2009, 'Koshihikari' and a BC_4F_4 population were harvested, and in 2011, 'Koshihikari' and a BC_4F_6 population were harvested. After storage, stale flavor of the stored brown rice was evaluated by tasting panels. The BC_4F_6 population is the same as 'Hokuriku 244'

varieties with good storage quality and resistance to sprouting, we identified *LOX-3* and developed SNP markers for LOX-3 deficiency (Shirasawa et al. 2008). In the present study, we developed novel rice lines with high resistance to preharvest sprouting in addition to LOX-3-null seeds using DNA marker-assisted selection. Although QTL analysis can define only a chromosomal region controlling a target trait and cannot be used to map single Mendelian factors, in this study, we showed that the gene responsible for sprouting susceptibility is not identical to *LOX-3* (*Os03g0700400*) but is tightly linked with the *LOX-3* locus (Fig. 5, Supplemental Fig. 2; Table 1), meaning that sprouting susceptibility is not a pleiotropic effect of *LOX-3*.

The preharvest sprouting locus linked to *LOX-3* was mapped between the SSR markers RM15711 and RM15768 on the long arm of chromosome 3. Preharvest sprouting is associated with seed dormancy and germination activities (Graeber et al. 2012), and many QTLs are reported in cereal crops and *Arabidopsis* (Sugimoto et al. 2009; Graeber et al. 2012). However, none of the previously reported preharvest sprouting QTLs on chromosome 3 map near the LOX-3 gene, thus indicating that we have identified a novel QTL, and named it *qPHS3* standing for "QTL for preharvest sprouting on chromosome 3" in accordance with the guidance of McCouch and CGSNL (2008).

In the reported QTLs for preharvest sprouting, five genes including candidates have been identified in cereals (Graeber et al. 2012): *ABI3* encoding for B3 transcription factor (Holdsworth et al. 2008); *VP8* for glutamate carboxypeptidase (Suzuki et al. 2008); *Sdr4* for protein of unknown function (Sugimoto et al. 2010); *qSD-7*: protein of unknown function (Gu et al. 2011); and *MFT* for phosphatidylethanolamine-binding protein (Nakamura et al. 2011). The distance between RM15711 and RM15768 is 842 kb, where 102 genes are annotated (IRGSP-1.0: Kawahara et al. 2013; Sakai et al. 2013); however, none of the predicted gene loci in this region have been reported as possible candidates (Sugimoto et al. 2009). Further genetic analysis using BC_4F_3 or BC_4F_4 progeny in which recombination occurred in the interval between RM15711 and RM15768 would contribute to identification of gene(s) responsible for the preharvest sprouting trait. The results would accelerate breeding programs for its improvement as well as to reveal molecular mechanism behind the phenomena.

Genetic resources such as native varieties and wild relatives of cultivated rice are useful sources of alleles with economic value for rice breeding programs (McCouch et al. 2012). However, these rice accessions are generally inferior to cultivated rice with respect to agriculturally important traits and may display low yield, ready shattering, late heading, long culm, weak disease and pest resistance, low-temperature sensitivity, and other unfavorable characters. In the present study, we used the variety 'Daw Dam' as a LOX-3-null genetic resource (Suzuki et al. 1993), which is the first LOX-3-null genetic resource we have discovered (Suzuki et al. 1993). We had initiated the breeding program using 'Daw Dam', and bred 'Hokuriku PL2' in 2000 (Fig. 1), even though we subsequently found out 21 LOX-3-null lines at that time (Suzuki et al. 1999,

2000). Development of cultivars lacking undesirable characters by introducing superior characters is essential for breeding new varieties with excellent performance. Use of DNA markers for breeding is a useful means of introducing desirable alleles into leading varieties in appropriate regions, given that DNA marker selection can target alleles to small genomic regions.

In this study, we identified a progeny (#07-9133-3 BC_4F_3) recombinant between *LOX-3* and RM15750 only 13 kb from *LOX*-3 (Fig. 4). Using the recombinant, we also succeeded in breeding a variety, 'Hokuriku 244', harboring the 'Koshihikari' genotype at RM15721, which is 382 kb distant from *LOX-3* on the short arm of chromosome 3. Thus, the size of any remaining genomic fragment of 'Hokuriku 244' in this progeny would be less than 393 kb on chromosome 3. In this region, 48 putative genes have been predicted (IRGSP-1.0), and some of them are highly expressed in seeds and roots (Sato et al. 2011). The agronomic phenotypes and eating quality of 'Hokuriku 244' were the same as those of 'Koshihikari' (Table 3), indicating that these 48 genes, except for *LOX-3*, do not appear to affect agronomic traits. These results also showed the utility of DNA marker breeding in breaking tight linkage between undesirable alleles and desirable alleles.

The breeding of 'Hokuriku 244', a LOX-3-null variety with good resistance to preharvest sprouting, provides a foundation for the development of cultivars with even greater storage stability. Triacylglycerol (TAG), the main lipid of rice bran, occurs in oil bodies with phospholipid membranes that are disintegrated by phospholipase D (PLD) (Takano 1993). Previously, we isolated a seed-PLD-null line, '03-s108', which showed no PLD enzymatic activities in seeds (Suzuki 2011). We identified a PLD null allele in rice and developed SNP markers for PLD deficiency (Suzuki et al. 2011). PLD serves as a trigger to initiate lipid degradation and deterioration of bran quality, and LOX-3 peroxidizes unsaturated fatty acids, which are degradation products of TAGs. One strategy we are currently pursuing is the combination of the LOX-3-null and PLD-null traits. This may reduce the oxidative deterioration of rice grains, promoting greater storage stability, and may also improve the fat quality and content of rice bran.

Author contribution statement YS: phenotyping, genotyping, and data analysis; KM, AS, HS, HO, and YU: breeding of LOX-3-null lines; TI: statistical data analysis; SH: phenotyping analysis; KS: phenotyping and genotyping analysis.

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Conflict of interest The authors declare that they have no conflict of interest.

References

Dong Y, Tsuzuki E, Kamiunten H, Terao H, Lin D, Matsuo M, Zheng Y (2003) Identification of quantitative trait loci associated with pre-harvest sprouting resistance in rice (*Oryza sativa* L.). Field Crops Res 81:133–139

Feussner I, Wasternack C (2002) The lipoxygenase pathway. Annu Rev Plant Biol 53:275–297

Frank G, Millar AA, Jacobsen JV (2005) Dormancy release, ABA and pre-harvest sprouting. Curr Opin Plant Biol 8:183–187

Gardner HW (1988) Lipoxygenase pathway in cereals. Adv Cereal Sci Technol 9:161–215

Graeber K, Nakabayashi K, Miatton E, Leubner-Metzger G, Soppe WJJ (2012) Molecular mechanism of seed dormancy. Plant Cell Environ 35:1769–1786

Groos C, Gay G, Perretant M-R, Gervais L, Bernard M, Dedryver F, Charmet G (2002) Study of the relationship between pre-harvest sprouting and grain color by quantitative trait loci analysis in a white × red grain bread-wheat cross. Theor Appl Genet 104:39–47

Gu XY, Foley ME, Horvath DP, Anderson JV, Feng J, Zhang L, Mowry CR, Ye H, Suttle JC, Kadowaki K, Chen Z (2011) Association between seed dormancy and pericarp color is controlled by a pleiotropic gene that regulates abscisic acid and flavonoid synthesis in weedy red rice. Genetics 189:1515–1524

Holdsworth MJ, Bentsink L, Soppe WJJ (2008) Molecular networks regulating *Arabidopsis* seed maturation, after-ripening, dormancy and germination. New Phytol 179:33–54

Horiuchi H (1996) Sprouting. In: Yamamoto T, Horisue N, Ikeda R (eds) Rice breeding manual. Yokendo, Tokyo, pp 147–150 (in Japanese)

Ida S, Masaki Y, Morita Y (1983) The isolation of multiple forms and product specificity of rice lipoxygenase. Agric Biol Chem 47:637–641

Ideta O, Kono I, Takeuchi Y, Hirabayashi H, Hirayama M, Ohta H, Sato H, Ando I, Kato H, Nemoto H, Yano M, Imbe T, Yamasaki M, Yoshida T (2012) Diversity and relationships between coefficient of parentage and genetic distance estimated by SSR markers in Japanese rice cultivars. Breed Res 14:106–113

International Rice Genome Sequencing Project (2005) The map-based sequence of the rice genome. Nature 436:793–800

Kawahara Y, de la Bastide M, Hamilton JP, Kanamori H, McCombie WR, Ouyang S, Schwartz DC, Tanaka T, Wu J, Zhou S, Childs KL, Davidson RM, Lin H, Quesada-Ocampo L, Vaillancourt B, Sakai H, Lee SS, Kim J, Numa H, Itoh T, Buell CR, Matsumoto T (2013) Improvement of the *Oryza sativa* Nipponbare reference genome using next generation sequence and optical map data. Rice 6:4

Lincoln SE, Daly MJ, Lander ES (1993) Constructing genetic linkage maps with MAPMAKER, version 3: a tutorial and reference manual. Whitehead Institute for Biomedical Research, Cambridge

McCouch SR, CGSNL (Committee on Gene Symbolization, Nomenclature and Linkage, Rice Genetics Cooperative) (2008) Gene nomenclature system for rice. Rice 1:72–84

McCouch SR, McNally KL, Wang W, Hamilton RS (2012) Genomics of gene banks: a case study in rice. Am J Bot 99:407–423

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8:4321–4325

Nakamura S, Abe F, Kawahigashi H, Nakazono K, Tagiri A, Matsumoto T, Utsugi S, Ogawa T, Handa H, Ishida H, Mori M, Kawaura K, Ogihara Y, Miura H (2011) A wheat homolog of MOTHER OF FT AND TFL1 acts in the regulation of germination. Plant Cell 23:3215–3229

Sakai H, Lee SS, Tanaka T, Numa H, Kim J, Kawahara Y, Wakimoto H, Yang CC, Iwamoto M, Abe T, Yamada Y, Muto A, Inokuchi H, Ikemura T, Matsumoto T, Sasaki T, Itoh T (2013) Rice Annotation Project Database (RAP-DB): an integrative and interactive database for rice genomics. Plant Cell Physiol 54:e6

Sato Y, Antonio BA, Namiki N, Takehisa H, Minami H, Kamatsuki K, Sugimoto K, Shimizu Y, Hirochika H, Nagamura Y (2011) RiceXPro: a platform for monitoring gene expression in japonica rice grown under natural field conditions. Nucleic Acids Res 39:D1141–D1148

Shirasawa K, Takeuchi Y, Ebitani T, Suzuki Y (2008) Identification of gene for rice (*Oryza sativa*) seed lipoxygenase-3 involved in the generation of stale flavor and development of SNP markers for lipoxygenase-3 deficiency. Breed Sci 58:169–176

Sugimoto K, Marzougi S, Yano M (2009) Genetic control of seed dormancy in rice. Gamma Field Symp 48:53–60

Sugimoto K, Takeuchi Y, Ebana K, Miyao A, Hirochika H, Hara N, Ishiyama K, Kobayashi M, Ban Y, Hattori T, Yano M (2010) Molecular cloning of *Sdr4*, a regulator involved in seed dormancy and domestication of rice. Proc Natl Acad Sci USA 107:5792–5797

Suzuki Y (1995) Screening and mode of inheritance of a rice variety lacking lipoxygenase-3. Gamma Field Symp 33:51–62

Suzuki Y (2011) Isolation and characterization of a rice (*Oryza sativa* L.) Mutant deficient in seed phospholipase D, an enzyme involved in the degradation of oil-body membranes. Crop Sci 51:567–573

Suzuki Y, Nagamine T, Kobayashi A, Ohtsubo K (1993) Detection of a new rice variety lacking lipoxygenase-3 by monoclonal antibodies. Japan J Breed 43:405–409

Suzuki Y, Nagamine T, Okuno K (1996a) Genetic analysis of a null-allele for lipoxygenase-3 in rice seeds. Euphytica 91:99–101

Suzuki Y, Yasui T, Matsukura U, Terao J (1996b) Oxidative stability of bran lipids from rice variety [*Oryza sativa* (L.)] lacking lipoxygenase-3 in seeds. J Agric Food Chem 44:3479–3483

Suzuki Y, Ise K, Li C, Honda I, Iwai Y, Matsukura U (1999) Volatile components in stored rice [*Oryza sativa* (L.)] of varieties with and without lipoxygenase-3 in seeds. J Agric Food Chem 47:1119–1124

Suzuki Y, Ise K, Nagamine T (2000) Geographical variation of the gene (*lox-3 (t)*), causing lipoxygenase-3 deficiency in Asian rice varieties. Rice Genet Newslett 17:13–14

Suzuki M, Latshaw S, Sato Y, Settles AM, Koch KE, Hannah LC, Kojima M, Sakakibara H, McCarty DR (2008) The maize *viviparous8* locus, encoding a putative ALTERED MERISTEM PROGRAM1-like peptidase, regulates abscisic acid accumulation and coordinates embryo and endosperm development. Plant Physiol 146:1193–1206

Suzuki Y, Takeuchi Y, Shirasawa K (2011) Identification of a seed phospholipase D null allee in rice (*Oryza sativa* L.) and development of SNP markers for phospholipase D deficiency. Crop Sci 51:2113–2118

Takahashi N (1997) Inheritance of seed germination and dormancy. In: Science of the rice plant. 3. Genetics. Food and Agriculture Policy Research Center, Tokyo, pp 348–359

Takano K (1993) Advances in cereal chemistry and technology in Japan. Cereal Foods World 38:695–698

Tsugita T, Ohta T, Kato H (1983) Cooking flavor and texture of rice stored under different conditions. Agric Biol Chem 47:543–549

Wang S, Basten C, Zeng Z (2007) Windows QTL cartographer 2.5. Department of Statistics, North Carolina State University, Raleigh

Yamamoto A, Fujii Y, Yasumoto K, Mitsuda H (1980) Product specificity of rice germ lipoxygenase. Lipids 15:1–5

Yasumatsu K, Moritaka S (1964) Fatty acid compositions of rice lipid and their changes during storage. Agric Biol Chem 28:257–264

Zhou Z, Robards K, Helliwella S, Blanchard C (2002) Ageing of stored rice: changes in chemical and physical attributes. J Cereal Sci 35:65–78