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ORIGINAL PAPER

Fine mapping of a major quantitative trait locus, *qLG-9*, that controls seed longevity in rice (*Oryza sativa* L.)

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Abstract

Key message **We fine-mapped a quantitative trait locus, *qLG-9*, for seed longevity detected between Japonica-type and Indica-type cultivars. *qLG-9* was mapped in a 30-kb interval of the Nipponbare genome sequence.**

Abstract A quantitative trait locus, *qLG-9*, for seed longevity in rice has previously been detected on chromosome 9 by using backcross inbred lines derived from a cross between Japonica-type (Nipponbare) and Indica-type (Kasalath) cultivars. In the present study, the chromosomal location of *qLG-9* was precisely determined by fine-scale mapping. Firstly, allelic difference in *qLG-9* was verified by QTL analysis of an F_2 population derived from a cross between Nipponbare and NKSL-1, in which a segment of Kasalath chromosome 9 was substituted in Nipponbare genetic background. Then, we selected F_2 plants in which recombination had occurred near *qLG-9* and performed F_3 progeny testing on these plants to determine the genotype classes of *qLG-9*. Eventually, *qLG-9* was mapped in a 30-kb interval (defined by two markers, CAPSb and CHPa12) of the Nipponbare genome sequence. This allowed us to nominate positional candidate genes of *qLG-9*. Additionally, we developed near-isogenic lines (NIL) for

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qLG-9 by marker-assisted selection. *qLG-9* NIL showed significantly higher seed longevity than isogenic control of Nipponbare. These results will facilitate cloning of the gene(s) underlying *qLG-9* as well as marker-assisted transfer of desirable genes for seed longevity improvement in rice.

Introduction

Seed longevity is defined as the length of time a seed remains viable. Seeds undergo an aging process that leads eventually to complete loss of viability. In rice, seed longevity affects the regeneration cycle of accessions stored in gene banks, the risks associated with hybrid seed production and management, and seedling vigor (Xue et al. 2008). Aged, low-viability seeds show low germination activity and late development of coleoptiles and leaves (Yamauchi and Winn 1996), resulting in poor seedling establishment. Plants from aged seeds show delayed flowering, fewer panicles and filled grains, and lower grain yield than those from high-viability seeds of the same variety (Siddique et al. 1988). These disadvantages of aged seeds cause serious problems such as yield reduction in rice paddy fields, particularly for direct seeding cropping systems (Seshu et al. 1988). In direct seeding, healthy seedling establishment is important because the germinating seeds are exposed to competition with other elements of the soil biological complex (microorganisms, animals, weeds) (Yamauchi and Winn 1996). Therefore, cultivars that can maintain seed viability for a longer time during seed storage are advantageous, especially in a direct seeding rice crop system, in which good seedling establishment is the most critical factor to success for production.

Seed longevity varies among rice cultivars (Chang 1991; Ellis et al. 1992; Rao and Jackson 1996). Chang (1991) tested the germination rates of one Japonica-type and two Indica-type cultivars every year during storage at 2 °C for 28 years. The germination rate of the Japonica-type cultivar decreased more rapidly than that of the two Indica-type cultivars. The two Indica-type cultivars maintained their germination rate at over 90 % even after 28 years of storage, while the germination rate of the Japonica-type cultivar was less than 10 % after the same period. Ellis et al. (1992) and Rao and Jackson (1996) tested different cultivars and concluded that Indica-type seeds maintained their viability for longer than Japonica-type seeds.

In attempts to understand the genetic factors controlling these variations, quantitative trait loci (QTLs) associated with seed longevity have been identified (Miura et al. 2002; Sasaki et al. 2005; Zeng et al. 2006; Xue et al. 2008; Li et al. 2012). At least 15 QTLs for seed longevity were identified in those reports, based on working with five populations derived from crosses between Nipponbare (Japonica-type) and Kasalath (Indica-type) (Miura et al. 2002), between Akihikari (Japonica-type) and Milyang23 (Indica-type) (Sasaki et al. 2005), between JX17 (Japonica-type) and ZYQ8 (Indica-type) (Zeng et al. 2006), and between Asominori (Japonica-type) and IR24 (Indica-type) (Xue et al. 2008), Koshihikari (Japonica-type) and Kasalath (Indica-type) (Li et al. 2012). Major QTLs were detected on chromosome 1 (*qRGR-1*, Xue et al. 2008), chromosome 9 (*qLG-9*, Miura et al. 2002; RC-9-2, Sasaki et al. 2005; *qSS-9*, Li et al. 2012), and chromosome 11 (*qLS-11*, Zeng et al. 2006). In these QTLs, allele from the Indica-type varieties promoted seed longevity in each population (Miura et al. 2002; Sasaki et al. 2005; Zeng et al. 2006; Xue et al. 2008; Li et al. 2012). The effects of some of these QTLs have been confirmed using chromosome segment substitution lines (CSSLs) (Miura et al. 2002; Zeng et al. 2006; Xue et al. 2008; Li et al. 2012). In our previous study, we used two CSSLs to confirm the allelic difference in *qLG-9* as a major QTL on seed longevity (Miura et al. 2002). To date, however, none of the known QTLs for seed longevity has been finely mapped and no near-isogenic line (NIL) for seed longevity has been developed in rice to prove their gene action. In addition, although many reports discuss the usefulness of seed longevity for direct seeding, there have been no confirmatory experimental studies done in practical aspect.

In this study, we performed fine-linkage mapping of *qLG-9* by using advanced backcross progeny, ultimately aiming to carry out map-based cloning of *qLG-9*. In addition to fine mapping, we developed NILs for *qLG-9* and assessed the contribution of *qLG-9* to the establishment of seedlings by seeds planted after storage.

Materials and methods

Plant materials

First, we used a small F_2 population (196 plants) derived from a cross between Nipponbare and NKSL-1, provided by the Rice Genome Resource Centre (http://www.rgrc.dna.affrc.go.jp/index.html), as described by Miura et al. (2002). In NKSL-1, the *qLG-9* region of the long-arm of chromosome 9 was substituted with a segment from Kasalath. These F_2 plants were cultivated in a paddy field at the National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, Japan, in 2001. At least 30 F_3 lines from recombinant F_2 plants were cultivated in the same paddy field in 2002. We determined the likely genotype of *qLG-9* in the F_2 plants based on the segregation of germination rate in F_4 seeds ($F_{3:4}$) that had been harvested from individual F_3 plants subjected to the aging treatment. To confirm the

candidate region at *qLG-9*, we performed substitution mapping using additional recombinant F_2 plants selected from a large F_2 population (approximately 1300 plants). F_3 lines from each additional F_2 plant were genotyped to select fixed lines and were cultivated in 2007 in a paddy field at the Experimental Farm Station, Graduate School of Life Sciences, Tohoku University, in Kashimadai, Osaki, Miyagi, Japan. To develop NILs, candidate plants were selected using marker-assisted selection (MAS) from the F_2 population and advanced generations to obtain F_4 seeds. These selected F_2, F_3 and F_4 plants were cultivated in 2005, 2006 and 2007, respectively, in the paddy field at the Experimental Farm Station in Tohoku University.

Evaluation of seed longevity

To investigate seed longevity of F_3 seeds ($F_{2:3}$) harvested from individual F_2 plants, we harvested the seeds 40 days after heading. Harvested seeds were kept at room temperature for one week and then stored for an additional week at 30 °C in a drying machine. Generally, the viability of rice seeds with high moisture contents falls rapidly at high temperatures and relative humidities (Roberts 1961). To weaken the viability and shorten the storage period, the seeds were usually stored at 30 °C under high relative humidity, as described by Ikehashi (1973). After drying, seeds were kept in airtight containers over water and placed in an incubator at 30 °C until the seed moisture content reached 15–16 %. The moisture content of the seeds was determined using a moisture tester (Riceter, Kett Electric laboratory Co. Ltd., Tokyo, Japan). Thereafter, we replaced the water with a saturated potassium chromate (K_2CrO_4) solution to maintain the seed moisture content at 15–16 % to quickly decrease viability. After storage for 14 or 16 weeks, 50 $F_{2:3}$ seeds from each F_2 plant were placed on 2 sheets of filter paper moistened with 4 ml distilled water in a 6-cm diameter petri-dish. Germinated seeds were counted after 7 days incubation at 25 °C. Germination was determined by the emergence of some part of the embryo from the lemma. For F_4 seeds ($F_{3:4}$) harvested from individual F_3 plants cultivated in Tsukuba, seeds were treated using the same method as for $F_{2:3}$ seeds, except for the storage period. Germination rates of $F_{3:4}$ seeds were investigated after storage for 5 months to evaluate seed longevity. For evaluation of seed longevity of $F_{3:4}$ and NILs seeds cultivated in Kashimadai, the method of accelerating seed aging was modified. The seeds were placed in airtight boxes in a room maintained at 30 °C. Moisture levels inside the boxes were maintained at 70 % by ventilating. As a control storage condition, seeds were stored in airtight boxes with silica gel to maintain ~15 % relative humidity. Temperature and relative humidity were measured using a data logger (Ondotori, Tech-Jam Co. Ltd., Tokyo, Japan). The germination tests were determined on the procedure described above except that incubation temperature was changed from 25 to 30 °C.

DNA marker analysis

We used restriction fragment length polymorphism (RFLP) markers, cleaved amplified polymorphic sequence (CAPS) markers (Konieczny and Ausubel 1993), and sequence tag site (STS) markers to determine the genotype of each F_2 plant. Probes for RFLP analysis were selected using a high-density genetic map (Harushima et al. 1998). Southern blotting and hybridization were performed according to Kurata et al. (1994). CAPS and STS markers were chosen from 332 PCR-based genetic markers (http://rgp.dna.affrc.go.jp/publicdata/caps/index.html). PCR and electrophoresis conditions for all markers were the same as previously described (Takeuchi et al. 2003).

For QTL analysis, the DNA of F_2 plant was extracted from leaves by using the cetyltrimethylammonium bromide method (Murray and Thompson 1980). We determined the genotypes of 196 F_2 plants for 10 RFLP markers and 1 STS marker located on the target chromosomal region.

For fine mapping, we determined the genotype of *qLG-9* in F_2 plants based on segregation patterns in the germination rate of $F_{3:4}$ seeds. We selected 28 recombinant plants between R79 and R10143 from 196 F_2 plants. Additional original primers were designed using the genome sequence of the Nipponbare variety established by the Rice Genome Research Program (http://rgp.dna.affrc.go.jp/cgi-bin/statusdb/status.pl); these primers are listed in Supplementary Table 1. PCR conditions and electrophoresis conditions for all markers except for CHPa1 and CHPa12 were the same as previously described (Obara et al. 2004). The polymorphism of recombinants at CHPa1 and CHPa12 was determined using a BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and a DNA sequencer (ABI PRISM 310 Genetic Analyzer, Applied Biosystems).

For substitution mapping, we selected additional recombinant F_2 plants from a large population. DNA was extracted from young seedlings using the simplified method described by Obara et al. (2004). The primers used for substitution mapping were the same as those used for fine mapping. For developing NILs, DNA was extracted using the simplified method as in substitution mapping. The background genotypes of NILs were confirmed using markers C107 on chromosome 4, RM4691 and R1963 on chromosome 5, E4443 on chromosome 8, and RM24173, RM24179, and RM24182 on chromosome 9. These markers were chosen from 332 PCR-based genetic markers (http://rgp.dna.affrc.go.jp/publicdata/caps/index.html) or simple sequence repeat markers (www.gramene.org).

Linkage map construction and QTL analysis

Based on the genotype data of F_2 plants, we generated a linkage map using MAPMAKER/EXP 3.0 with the Kosambi function (Lander et al. 1987). The QTL analysis was performed using composite interval mapping with the QTL Cartographer version 2.5, with model 6 in forward and backward methods (Wang et al. 2007). Significant thresholds of LOD score (6.4 and 13.3 for germination rates after 14 and 16 weeks of storage, respectively) were determined using the 1,000-permutation test at the 5 % significance level.

Evaluating level of seedling establishment ability

The method used to evaluate the emergence of seedlings from soil at low temperatures has been described previously (Yamaguchi et al. 2007). Seeds were stored for one month at 30 °C and 70 % relative humidity to accelerate their aging. Aged seeds were soaked for 5 days at 13–15 °C to synchronize germination, and then incubated at 30 °C to initiate germination. We selected 20 germinated seeds from the same developmental stage, at which all the seeds exhibited similar developmental characteristics. Pots were filled with a mixture of gley and andosol soils obtained from paddy fields, air-dried and screened using 2.0-mm mesh. The germinated seeds were sown in pots at a depth of 1.0 cm and placed into an incubator (KOITO S-206A, Koito Manufacturing Co. Ltd., Tokyo, Japan) for 14 days at an average temperature of 15.4 °C under natural light through muffled glass. There were three replicates. The seedlings that showed a coleoptile or first leaf were counted as established during incubation.

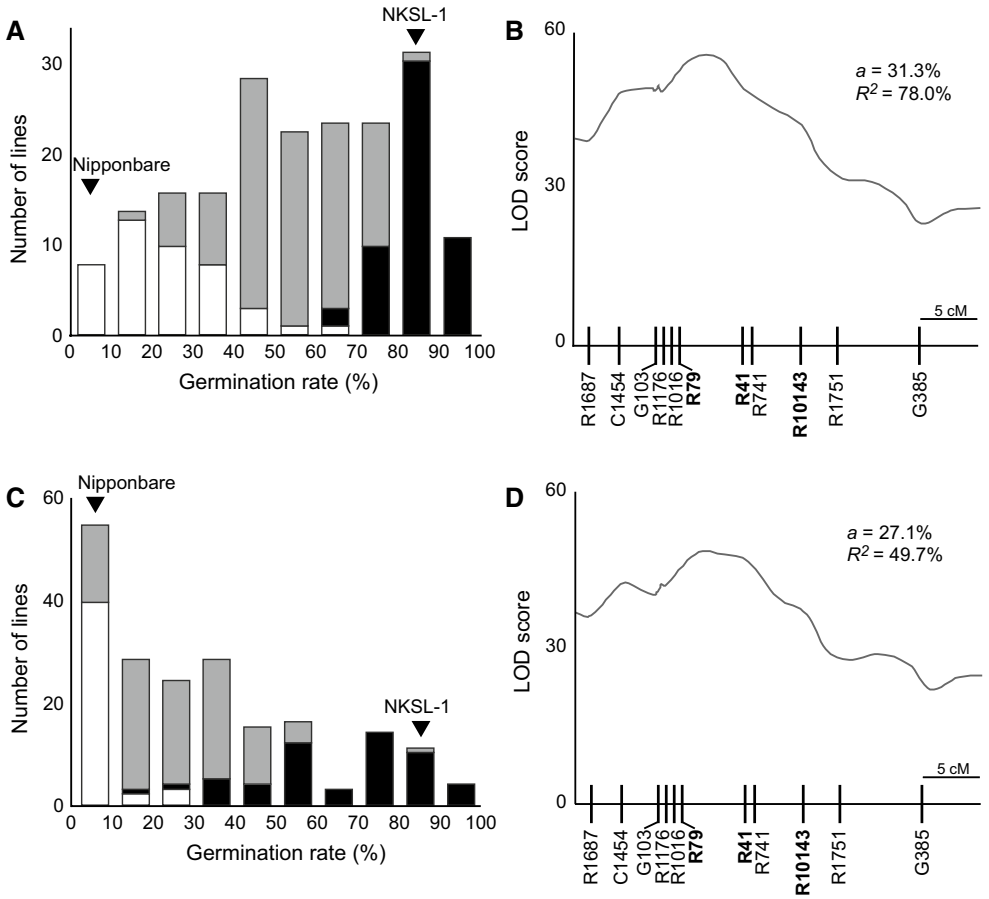


Fig. 1 Frequency distribution of germination rate and quantitative trait locus likelihood curves of the LOD score of germination rates in F_2 plants derived from Nipponbare and NKSL-1 after aging treatment. Frequency distribution of germination rates of seeds after 14 (**a**) or 16 (**c**) weeks' storage. The *arrow* indicates the mean value of the parental variety and line, Nipponbare and NKSL-1. The genotype determined using RFLP marker R79 is represented by *white bars* (homozygous for the Nipponbare allele), *shaded bars* (heterozygous), or *black bars* (homozygous for the Kasalath allele). QTL likelihood curves of the LOD score of seed germination rates after 14 (**b**) and 16 (**d**) weeks' storage for chromosome 9 in the F_2 population. The additive effect of the Kasalath allele (*a*) and the percentage of variance it accounts for (R^2) are indicated

Table 1 Genotypes of F_2 plants for 13 DNA markers on chromosome 9 and the germination rates of $F_{3:4}$ seeds after 5 months of storage

No.	Markers and genotype													Germination rate (%)					
	R79	CAPSa	SCARa	SCARb	dCAPSa	CAPSc	CAPSb	CHPa1	CHPa12	SCARc	R41	R742	R10143	<20	<40	<60	<80	<100	Estimated genotype of *qLG-9*[b]
01F2-190	H	K	K	K	K	K	K	K	K	K	K	K	K		3	4	13	12	K
01F2-67	H	H	K	K	K	K	K	K	K	K	K	K	K				10	22	K
01F2-51	N	N	H	H	H	H	H	H	H	H	H	H	K	6	9	6	6	6	H
01F2-55	N	N	N	H	H	H	H	H	H	H	H	H	H	11	2	9	9	1	H
01F2-60	H	H	H	H	H	K	K	K	K	K	K	K	K			1	3	29	K
01F2-26	N	N	N	N	N	N	H	H	H	H	H	H	H	17	10	2	3	1	H
01F2-5	N	N	N	N	N	N	N	H	H	H	H	H	H	6	5	12	3	6	H
01F2-21	N	N	N	N	N	N	N	N	H	H	H	H	H	23	7	1			N
01F2-64	N	N	N	N	N	N	N	N	N	N	H	H	H	33					N
01F2-172	H	H	H	H	H	H	H	H	H	H	N	N	N	12	6	8	3	4	H
01F2-47	H	H	H	H	H	H	H	H	H	H	H	K	K	5	12	10	5	2	H
01F2-56	N	N	N	N	N	N	N	N	N	N	N	H	H	33					N
Nipponbare	N	N	N	N	N	N	N	N	N	N	N	N	N	10					N (control)
NKSL-1	K	K	K	K	K	K	K	K	K	K	K	K	K			7	3		K (control)

[a] The genotype of the DNA markers are represented by N (Nipponbare homozygous), K (Kasalath homozygous), and H (heterozygous), respectively

[b] The genotype of *qLG-9* of each F_2 plant was estimated on the basis of frequency distributions for germination rate

Results

QTL analysis of *qLG-9* in the F_2 population

We examined the germination rates of $F_{2:3}$ seeds that were harvested from individual F_2 plants and then stored for 14 or 16 weeks. The germination rates in $F_{2:3}$ seeds showed continuous distributions (Fig. 1a, c). The range of seed germination rates was between 4 and 100 % after 14 weeks storage (Fig. 1a), and between 0 and 96 % after 16 weeks (Fig. 1c). One major QTL was detected in the interval between R79 and R41 in both storage conditions (Fig. 1b, d). After 14 or 16 weeks storage, the additive effects of the Kasalath allele at the QTL were 31.3 and 27.1 %, respectively, and the phenotypic variation explained by the QTL was 78.0 and 49.7 % in the QTL analysis, respectively (Fig. 1b, d). From these results, the existence of *qLG-9* was confirmed in the interval between R79 and R41 on chromosome 9.

Defining a candidate genomic region for *qLG-9*

To delimit a candidate genomic region for *qLG-9*, we selected 28 plants from a total of 196 F_2 plants; the selected plants had a recombination event between R79 and R10143 (Fig. 1). At least 30 F_3 lines from each selected F_2 plant were cultivated. After harvesting and aging treatment, 50 F_4 seeds ($F_{3:4}$) harvested from individual F_3 plants were used for a germination test. We determined the *qLG-9* genotype in each selected F_2 plant from segregation patterns of the germination rate of $F_{3:4}$ seeds. We classified the segregation behaviour of germination rate into three patterns: those with fixed low germination rates; those with fixed high germination rates; and those segregating from low to high germination rates (Table 1). These three segregation patterns were taken to correspond to the three genotype classes at *qLG-9*: homozygous for the Nipponbare allele, homozygous for the Kasalath allele, and heterozygous (Table 1). *qLG-9* co-segregated with CHPa1 as a single Mendelian factor and mapped in the interval between the DNA markers CAPSb and CHPa12 (Table 1). To obtain more detailed evidence for determining the position of *qLG-9*, we also performed substitution mapping. We selected recombinant plants between CAPSb and SCARc from a large F_2 population (approximately 1,300 plants). Fixed F_3 lines from each selected F_2 plant were then cultivated. Fifty $F_{3:4}$ seeds harvested from individual F_3 plants were used for the germination test after the aging treatment. The germination rates of 03F2-145-F3, -39-F3B, -241-F3A and -165-F3A were all 0 %, the same level of germination as Nipponbare; 03F2-43-F3A, -69-F3B, -44-F3A and -225-F3B showed higher germination rate after storage (Fig. 2a). These results strongly indicate that *qLG-9* co-segregated with

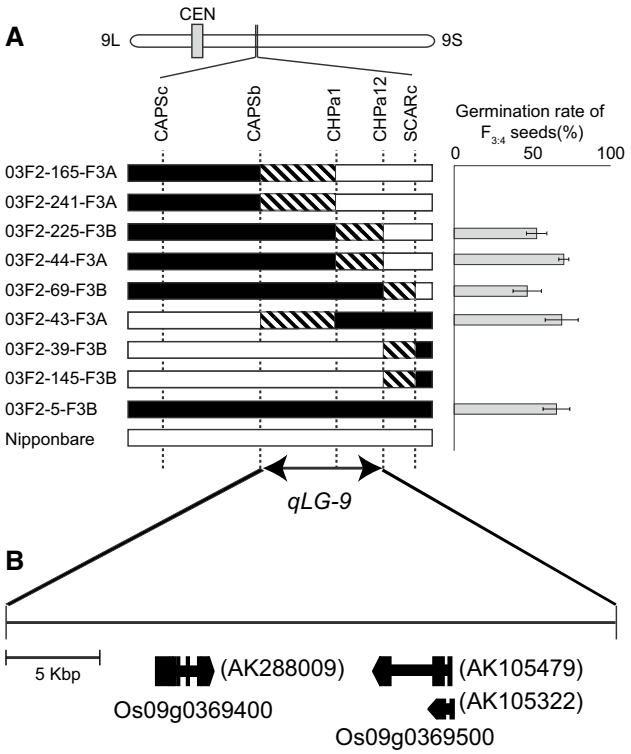
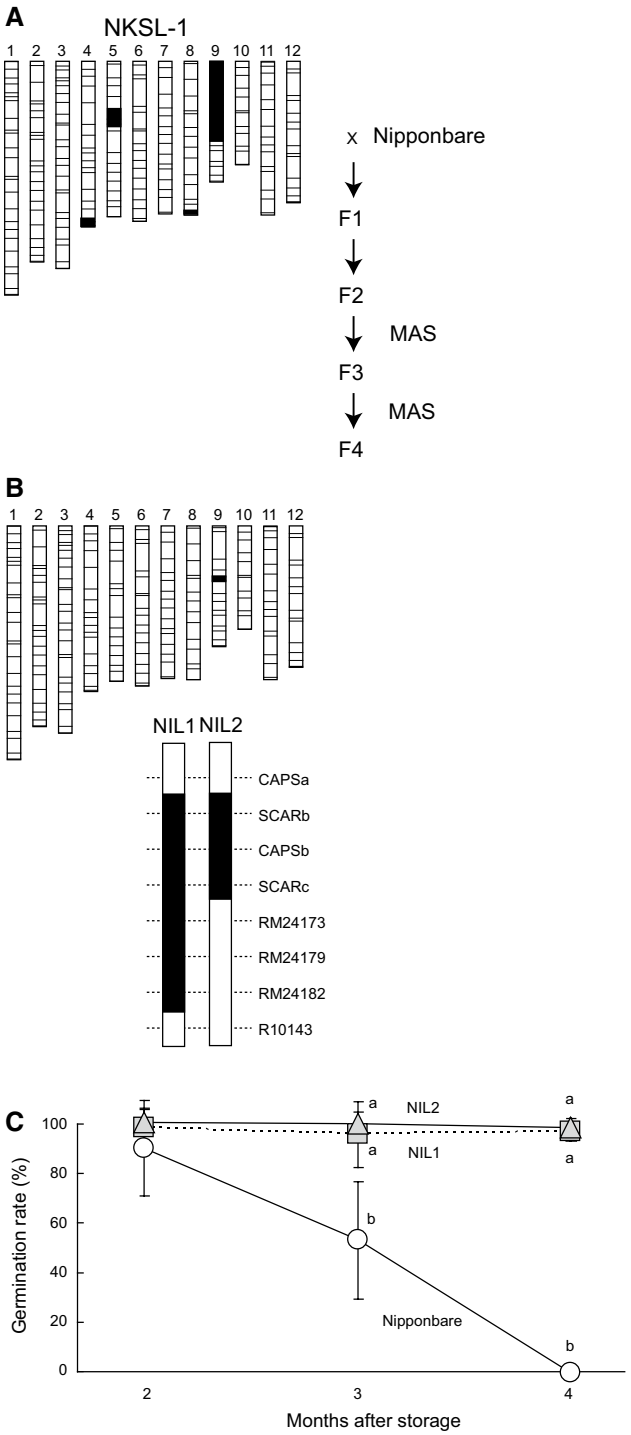


Fig. 2 The location of *qLG-9* and candidate genomic region. **a** Graphical genotypes of the F_3 plants and germination rate of $F_{3:4}$ seeds. *Horizontal columns* indicate the genotype of each F_3 line. *Black* and *white bars* indicate homozygous for Kasalath and Nipponbare, respectively. *Hatched box* indicates the recombination region. The *dotted vertical line* indicates the position of each DNA marker. *Arrows* indicate the region of *qLG-9*. Mean values of germination rates are shown and *error bars* indicate standard errors ($n = 6$). **b** Candidate genes indicated by gene ID (http://rapdb.dna.affrc.go.jp/)

CHPa1 and was located in the interval between CAPSb and CHPa12. Based on the Nipponbare genome sequence in the Rice Annotation Project Database (2014), this interval is approximately 30 kb (Fig. 2b). In this region, two genes are annotated, one encoding trehalose-6-phosphate phosphatase (TPP) (Os09g0369400), and the other encoding an unknown protein (Os09g0369500) (Fig. 3c).

Evaluation of effect of *qLG-9* on seed longevity and seedling establishment ability

We developed two NILs for *qLG-9* in which different sized segments of Kasalath were introgressed (Fig. 3). The germination rates of NIL-1 and NIL-2 were significantly higher than that of Nipponbare for seeds stored 3 or 4 months at 30 °C and 70 % relative humidity (Fig. 3c), indicating, these lines have greater seed longevities than Nipponbare. We also investigated the level of seedling establishment for the NIL-2 when its seeds were planted in a low temperature condition after storage. Seeds were



◀**Fig. 3** Graphical genotype and seed longevity of NILs. **a** The graphical genotype of NKSL-1 and development scheme of NIL for *qLG-9* by marker-assisted selection (MAS). *White bars* indicate homozygotes for the Nipponbare allele and *black bars* indicate homozygotes for the Kasalath allele. Positions of DNA markers used to determine genotypes are indicated as *thin lines*. **b** Graphical genotypes of two NILs selected. The genotypes determined using each marker is represented as *white* (homozygous for the Nipponbare allele) or *black bars* (homozygous for the Kasalath allele). **c** Germination rate of NIL-1, NIL-2 and Nipponbare using seeds stored at 30 °C and 70 % relative humidity. Mean values denoted by a common letter (*a* or *b*) are not significantly different by Tukey's test for pairwise mean comparison with alpha = 0.05. *Error bars* correspond to standard error ($n = 4$)

stored for one month at 30 °C and 70 % relative humidity to accelerate their aging, but germination rates of aged seeds were still 100 % at 30 °C with enough oxygen and water (data not shown). The seedling emergence ratios of aged seeds were not significantly different from that observed for the controls, NIL-2 without aging; however, emergence ratios observed for aged Nipponbare seeds were significantly lower than that in the controls starting at 10 days after sowing (Fig. 4). Furthermore, the seedling emergence ratios for aged NIL-2 seeds were almost the same as that for the Nipponbare controls at any stage after sowing (Fig. 4). These results suggested that NIL-2 seeds retain their seedling vigor even after aging treatment for one month. Therefore, the Kasalath *qLG-9* allele contributed to maintaining the ability of seedlings to establish in soil at low temperature through maintaining seed viability during storage.

Discussion

QTLs for seed longevity have been detected using populations derived from Indica-type and Japonica-type varieties. Several of these QTLs have been detected on chromosome 9, for example, RC9-1 and RC9-2 in the Akihikari/Milyang23 RIL population (Sasaki et al. 2005), *qSL-9* in the ZYQ8/JX17 DHL population (Zeng et al. 2006), *qRGR-9* in the Asominori/IR24 population (Xue et al. 2008) and *qSS-9* in the Koshihikari/Kasalath BIL population (Li et al. 2012). Among these, RC9-2, *qRGR-9* and *qSS-9* were detected near *qLG-9* (Fig. 5). Although it is likely that these QTLs are the same locus as *qLG-9,* it remains very difficult, based on current information, to decide whether the three QTLs are at the same locus because the other two QTLs were mapped only by QTL analysis. Additional fine mapping will be required to conclude allelic relationship among four loci.

We succeeded in mapping *qLG-9* as a single Mendelian factor in an interval of approximately 30 kb by using progeny tests. This allowed us to nominate candidate genes for *qLG-9* using the available sequence annotation database (http://rapdb.dna.affrc.go.jp/). In this region, two genes are annotated, one encoding trehalose-6-phosphate phosphatase (TPP) (Os09g0369400), and the other encoding an unknown protein (Os09g0369500) (Fig. 3c). TPPs catalyse the dephosphorylation of trehalose-6-phosphate to trehalose (Iordachescu and Imai 2008). Garg et al. (2002) reported that the regulated overexpression of *Escherichia coli* trehalose biosynthetic genes (*otsA* and *otsB*)

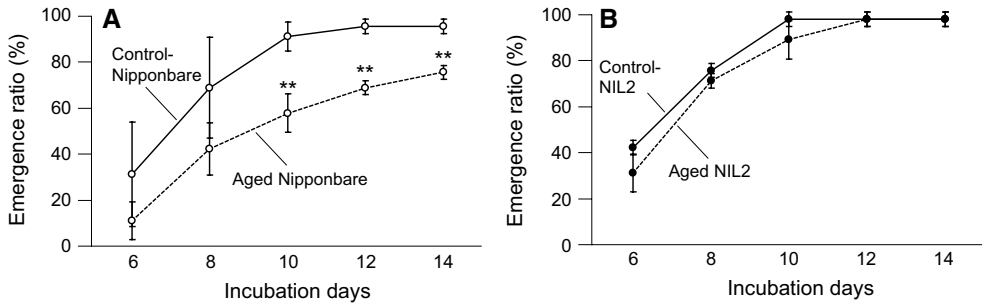


Fig. 4 Emergence of seedlings in soil at low temperature by using Nipponbare (**a**) and NIL2 (**b**) seeds after storage for a month at 30 °C and 70 % relative humidity. Mean values denoted by *asterisks* are significantly different according to a *t* test with alpha = 0.05. *Error bars* correspond to standard error ($n = 3$)

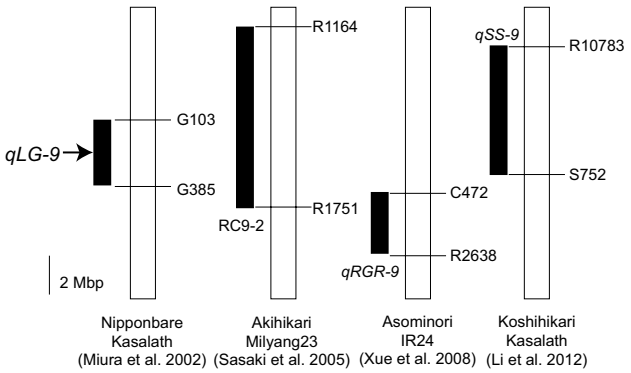


Fig. 5 Comparison between 3 rice populations of the regions on chromosome 9 with QTLs implicated in seed longevity. *Black bars* indicate the region where QTLs for seed longevity were detected in each population. Position of each marker indicated is based on its physical position in the Nipponbare genome sequence (http://rapdb.dna.affrc.go.jp/)

can be used to manipulate abiotic stress tolerance in rice. The transgenic rice plants accumulated trehalose at levels 3–10 times that of the nontransgenic controls. Compared with nontransgenic rice, several independent transgenic lines exhibited sustained plant growth, less photo-oxidative damage, and a more favorable mineral balance under drought-stress conditions. In addition, trehalose accumulation in *Ubi1*::*TPSP* plants resulted in increased tolerance to drought (Jang et al. 2003). In rice seed, it has also been noted that there is a strong correlation between seed longevity and desiccation tolerance (Ellis and Hong 1994). The idea that desiccation tolerance plays a significant role in maintaining seed longevity is supported by a study using abscisic acid-insensitive mutants in *Arabidopsis* (Clerkx et al. 2004). Based on previous observations, the gene encoding TPP may be a plausible candidate gene for *qLG-9*. Comparison of the Nipponbare and Kasalath sequences show that there are three single nucleotide polymorphisms in the candidate promoter region; the coding region of TPP did not show any polymorphism (data not shown). Further gene expression analyses and complementation tests are required to unequivocally identify the genes involved in seed longevity.

NIL-2 seeds maintained their viability during storage under accelerated aging conditions for a longer time than the Nipponbare seeds (Fig. 3). The Kasalath allele of *qLG-9* contributed to the maintenance of seed viability during storage. Since aged low-viability seeds establish seedlings poorly in the paddy field (Yamauchi and Winn 1996), the Kasalath allele of *qLG-9* is considered to be one of the gene sources for the improvement of seedling establishment ability after storage. Therefore, we compared the emergence ratio of seedlings between Nipponbare and an NIL-2 by using seeds that had undergone aging treatment. We performed seedling establishment assays in the soil and under low temperature of around 15 °C, to mimic actual paddy field conditions. Seedling establishment under low temperature condition is the most important for irrigated dry-season crops in the tropics or for wet-season crops in temperate areas because the use of deep water leads to good weed suppression at crop establishment (McKenzie et al. 1994). We found that the emergence ratios of seedlings from an NIL-2 for *qLG-9* were significantly higher than that of Nipponbare by using seeds after aging treatment s, but were similar to the emergence ratios of Nipponbare seedlings stored under control conditions (Fig. 4). NIL maintained their seed viability even under accelerated aging conditions when compared to Nipponbare, and consequently their seedling vigor and establishment were retained. Yamauchi and Winn (1996) reported that aged seeds lost their seedling vigor and establishment in anaerobic soil and suggested that varieties tolerant to aging stress would be useful for breeding rice varieties for direct seeding. Therefore, the tightly linked chromosomal markers of *qLG-9* identified here will be useful in breeding programs to develop optimal cultivars with improved seed longevity for direct seeding of rice.

QTLs for seed longevity have been detected in monocot crops in addition to rice, such as barley (Nagel et al. 2009) and wheat (Landjeva et al. 2010). The region of chromosome 9 in which *qLG-9* is located is homologous to the barley chromosome 5H (Nagel et al. 2009) and wheat chromosome 5DL (Landjeva et al. 2010), based on colinearity information (Stein et al. 2007). Interestingly, QTLs for seed longevity have been detected in chromosomes 5H and 5DL; these have been designated as *QLng.IPK-5H.1* and *QLi-fingp.ipk-5D*, respectively. Therefore, these QTLs for seed longevity in barley and wheat may be related to *qLG-9* of rice. Sequence information for the candidate region identified in this study will be useful in obtaining linkage maps for seed longevity in these other monocot crops.

Author contribution statement K.S., Y.T., K.M., M.Y. and T.S. designed research; K.S., Y.T., K.M., T. Yamaguchi, and T.A. performed research; K.S. and Y.T. analyzed data; K.S., M.Y. and T.S. wrote the paper; T.E., A.H. and T. Yamaya gave technical support and conceptual advice.

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

Ethical standard The experiments comply with the current laws of the country in which they were performed.

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