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ORIGINAL PAPER

Mapping and characterization of seed dormancy QTLs using chromosome segment substitution lines in rice

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Abstract Seed dormancy—the temporary failure of a viable seed to germinate under favorable conditions—is a complex characteristic influenced by many genes and environmental factors. To detect the genetic factors associated with seed dormancy in rice, we conducted a QTL analysis using chromosome segment substitution lines (CSSLs) derived from a cross between Nona Bokra (strong dormancy) and Koshihikari (weak dormancy). Comparison of the levels of seed dormancy of the CSSLs and their recurrent parent Koshihikari revealed that two chromosomal regions—on the short arms of chromosomes 1 and 6—were involved in the variation in seed dormancy. Further genetic analyses using an F_2 population derived from crosses between the CSSLs and Koshihikari confirmed the allelic differences and the chromosomal locations of three putative QTLs: *Sdr6* on chromosome 1 and *Sdr9* and *Sdr10* on chromosome 6. The Nona Bokra alleles of the three QTLs were associated with decreased germination rate. We discuss the physiological features of the CSSLs and speculate on the possible mechanisms of dormancy in light of the newly detected QTLs.

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Introduction

Seed dormancy, defined as the temporary failure of an intact viable seed to germinate under favorable conditions (Bewley 1997), is an important trait in rice breeding programs because it is associated with resistance to pre-harvest sprouting (PHS) (Bewley and Black 1982). PHS is a major constraint to rice production worldwide when long periods of hot, humid weather occur during harvesting seasons, and it leads to substantial losses of yield and quality.

In rice, PHS and seed dormancy are both complex traits that are controlled by many genes. More than 40 quantitative trait loci (QTLs) associated with seed dormancy or PHS have been identified across all the chromosomes of rice cultivars (Dong et al. 2003; Guo et al. 2004; Lin et al. 1998; Wan et al. 2005), of the wild relatives *Oryza rufipogon* and *Oryza nivara* (Cai and Morishima 2000; Lee et al. 2005; Li et al. 2006; Thomson et al. 2003), and of weedy rice (Gu et al. 2004). Although there has been substantial progress in the genetic dissection of seed dormancy, the molecular mechanism by which seed dormancy is regulated remains to be analyzed. In Arabidopsis, a major dormancy QTL, *Delay of Germination 1* (*DOG1*), has been cloned by using a map-based strategy (Bentsink et al. 2006). *DOG1* is a member of a small gene family of unknown function. Recently, in rice, a major QTL for seed dormancy, *Seed dormancy 4* (*Sdr4*) on chromosome 7, was found to encode a protein with no similarity to proteins with known functions; it is thought to be involved in domestication or adaptation of rice (Sugimoto et al. 2010).

Dormancy is controlled by plant hormones. Abscisic acid (ABA) and gibberellins are the main regulators. Brassinosteroids and ethylene are less involved (Koornneef et al. 2000). In higher plants, the level of ABA is finely

controlled by balancing of the rates of ABA biosynthesis and catabolism (Nambara and Marion-Poll 2005). In rice, Gu et al. (2010) delimited the candidate region and focused on three candidate *qSD12* genes; they assigned a functional relationship for *qSD12* in ABA accumulation. One recent report has revealed that *SD7-1/Rc*, which encodes a bHLH (basic helix-loop-helix) transcription factor, regulates seed dormancy through ABA biosynthesis and pigmentation (Gu et al. 2011). In contrast, previous research found no correlation between ABA content and the dormancy status of dry or imbibed seeds of red weedy rice (Gianinetti and Vernieri 2007). The association between ABA accumulation, ABA sensitivity, and seed dormancy is still puzzling, especially in rice, because a limited number of genes and QTLs associated with these characters have been isolated.

We describe the identification of QTLs for seed dormancy using chromosome segment substitution lines (CSSLs) derived from the cultivar Nona Bokra, which exhibits strong dormancy. We also verified the allelic differences among the QTLs by using F_2 populations derived from crosses between two CSSLs and the recurrent parent, Koshihikari, which exhibits weak dormancy. To further characterize seed dormancy in the CSSLs, we measured the levels of ABA accumulation and sensitivity in two CSSLs and the recurrent parent Koshihikari. The results suggested that CSSLs with putative QTLs on chromosomes 1 and 6 showed ABA hyperaccumulation and hypersensitivity, respectively. On the basis of our results, we discuss the possible functions of the genes at these QTLs.

Materials and methods

Plant materials

Nona Bokra, the donor of the CSSLs that we used, exhibits extremely late heading under natural field conditions in summer in Tsukuba, Japan. Using short-day (SD) equipment, we, therefore, grew Nona Bokra and Koshihikari under SD conditions (9 h light, 15 h dark) until the harvest of the mature seeds. The SD equipment was set up in the field; the cover of the apparatus, which was able to shut out sunlight, automatically closed at 1700 hours and opened at 0800 hours.

To identify the chromosomal regions controlling seed dormancy, we used CSSLs derived from a previously developed cross between Koshihikari (weak dormancy) and Nona Bokra (strong dormancy) (Takai et al. 2007). Forty-four CSSLs and their background control, Koshihikari, were grown in a paddy field at the National Institute of Agrobiological Sciences (NIAS), in Tsukuba, in 2008 to evaluate the levels of seed dormancy. To validate allelic differences in seed dormancy at putative QTLs, we produced F_2 populations derived from the crosses between each of the two CSSLs (SL501 and SL519) and Koshihikari. Eighty-seven or ninety-one plants, respectively, in each F_2 population were grown in 2009 in the experimental field at NIAS and were used for QTL analysis of seed dormancy. In our investigation of the genes encoding NCED (9-*cis*-epoxycarotenoid dioxygenase), we reduced the size of the substituted genome by selecting one line, #6095, from the progeny of F_2 plants derived from a cross between SL502 and Koshihikari; in this line, recombination occurred between RM3740 and RM10149. Occasionally, CSSLs have additional small donor segments outside the target chromosome segment. In SL502 (and #6095) and SL519, none of these additional segments was detected. SL501 had small segment substitutions: one located on the end of the long arm of chromosome 6 and the other on the end of the short arm of chromosome 12 (Takai et al. 2007).

For our physiological analysis, we used seeds harvested from plants in a greenhouse at 5 weeks after heading (WAH). The growth conditions in the greenhouse were as follows: plants were initially grown under long-day conditions (14 h light, 10 h dark) under metal halide lamps. Then, 2 months after planting, a change was made to SD conditions (9 h light, 15 h dark) to promote heading. Under these conditions the heading dates of Koshihikari, SL502, and SL519 were synchronized to minimize any effects of heading date on seed dormancy.

Seed germination test

The degree of seed dormancy was measured as the germination rate at seed maturity. Heading date was determined as the time of protrusion of each panicle from the leaf sheath. To maximize the uniformity of conditions after the ripening period, panicles collected from plants in the field at 6 and 8 WAH (in the case of CSSLs) or 4 to 8 WAH (in the case of parental cultivars) were wrapped in paper towels and dipped in water. Panicles harvested at 8 WAH and stored for 3 months at room temperature were used to obtain after-ripened (AR) seeds, which were used for germination tests. The panicles were incubated in the dark at 30°C for 1 week, and the number of seeds that germinated was counted and expressed as a percentage of the total number of seeds on each panicle. Average germination rates were calculated by using five panicles from each line. Germination rates were compared with that of the recurrent parent, Koshihikari, by using the version of Dunnett's multiple-comparison procedure provided with the JMP 7.0 software (http://www.jmp.com/). To test the seeds harvested from the greenhouse-grown plants, 30 seeds from each plant, were dipped in water and the germination rates were scored every day after imbibition.

This experiment was repeated three times, and these results represent the mean value for the three experiments.

DNA extraction and simple sequence repeat (SSR) marker analysis

Genomic DNAs were extracted as follows: leaves (<2.5 cm long) were homogenized in 40 μL of 0.5 M NaOH with stainless-steel beads, then neutralized with 160 μL of 1 M Tris–HCl (pH 9.0). The supernatants were isolated by centrifugation (3,100*g*). A fivefold dilution of the supernatant was used for the PCR reaction. To obtain SSR markers showing polymorphism between Koshihikari and Nona Bokra, we surveyed SSR motifs on the target chromosome regions (IRGSP 2005); the resultant informative SSR markers were used for genotyping of F_2 plants. Amplification was performed for initial denaturation (5 min at 94°C) and 35 cycles (1 min at 94°C, 1 min at 55°C, and 1 min at 72°C), followed by final extension for 7 min at 72°C. The amplified products were electrophoresed on 3% agarose gel to detect polymorphisms.

Linkage mapping and QTL analysis

We performed linkage mapping using version 3.0 of MAPMAKER/EXP (Lander et al. 1987), and we used the Kosambi map function to calculate genetic distances. We performed QTL analyses by using composite interval mapping, as implemented by the Zmapqtl program (model 6) provided in version 2.5 of the QTL Cartographer software (Wang et al. 2005). We used genome-wide threshold values ($\alpha = 0.05$) to detect putative QTLs on the basis of the results of 1,000 permutations.

Analysis of ABA content

Seeds (500 mg) harvested at 4 WAH from three plants for each experiment were extracted in aqueous methanol and purified independently by high-performance liquid chromatography (HPLC). Their ABA content was quantified by gas chromatography–mass spectrometry (GC–MS), as described by Iuchi et al. (2000). Average ABA content was calculated using three independent samples.

Analysis of ABA sensitivity

Germination of AR seeds of SL502 and SL519 stored for 3 months at room temperature was tested in the presence of increasing ABA concentrations (0–100 μM). Thirteen seeds of each CSSL were incubated in the dark at 30°C for 1 week, and the average seed germination rates were scored using three independent samples.

RNA preparation and real-time PCR

RNAs were extracted by using an RNeasy Plant Kit with on-column DNaseI treatment (Qiagen, Hilden, Germany). Total RNA (2.5 μg) was applied to cDNA synthesis by using a First-Strand cDNA Synthesis Kit (GE Healthcare, Fairfield, CT), in accordance with the manufacturer's instructions. cDNA was used as the template for each TaqMan PCR reaction (Applied Biosystems, Carlsbad, CA). The PCR conditions were 2 min at 50°C, then 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C. To quantify *OsNCED3* and *Act-1* mRNA transcription, we used the following specific primers: OsNCED3-69F: 5′-GCT CGG TCT CAC ACA CAC TCT CT-3′; OsNCED3-159R: 5′-CGC ACA ACA ATC TGA GCT ACA ATC-3′; OsNCED3-104 (VIC): 5′-AAA CAC ACC ACG ATC GCG GCC-3′; Actin1-132F: 5′-CCC TCG TCT GCG ATA ATG GA-3′; Actin1-197R: 5′-CCC TGG GCG CAT CAT CT-3′; and Actin1-157T(VIC): 5′-TAT GGT CAA GGC TGG GTT CGC CG-3′. For *OsNCED1*, *2*, *4*, and *5*, we used the primer sequences described by Zhu et al. (2009). For copy-number standards, we used quantified fragments of cloned cDNA. The results were presented as the means of at least three independent samples.

Results

Parental differences in seed dormancy levels

The germination rates of seed from Koshihikari and Nona Bokra were determined from 4 to 8 WAH. At 4 to 5 WAH, seeds from Koshihikari and Nona Bokra were equally dormant (Fig. 1). The germination rate of Koshihikari increased to more than 60% in the period from 5 to 6 WAH, showing that a transition to non-dormancy had occurred. In contrast, Nona Bokra retained dormancy from 4 to 8 WAH. At 8 WAH, the germination rate of Koshihikari was 96% and that of Nona Bokra was 5%.

Identification of chromosomal regions for seed dormancy

To identify the chromosomal segments involved in seed dormancy, we measured the germination rates of the 44 CSSL lines at 6 and 8 WAH (Fig. 2a). At both 6 and 8 WAH, the germination rates of the CSSLs varied widely among lines. In many cases, the germination rate was higher at 8 WAH than at 6 WAH, but some lines still exhibited low germination rates. The correlation coefficient between the two stages was fairly high ($r^2 = 0.475$), indicating that the CSSLs had similar dormancy release tendencies at 6 and 8 WAH. Several CSSLs had dormant

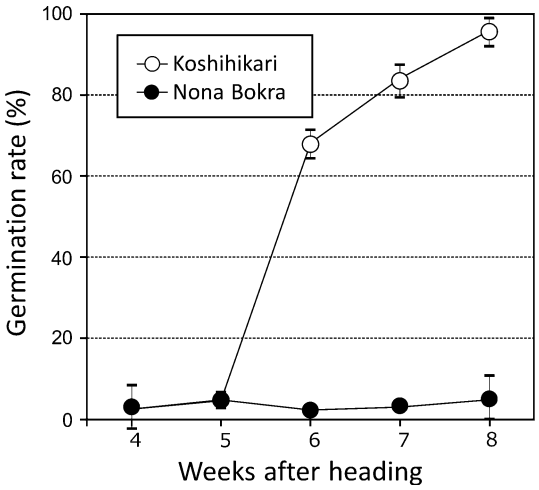

Fig. 1 Temporal changes in germination rates of Koshihikari and Nona Bokra after heading. Germination rates of Koshihikari and Nona Bokra were determined 5, 6, 7, and 8 weeks after heading. Nona Bokra and Koshihikari were grown under short-day conditions (see "Materials and methods"). Values represent mean ± standard deviation

phenotypes at both 6 and 8 WAH: the germination rates of SL501, SL502, SL503, SL506, and SL519 were consistently less than 20%, and the germination rates differed significantly ($P < 0.001$ Dunnett's test) between these CSSLs and their recurrent parents at 8 WAH (Fig. 2a). The germination rates of AR seeds that had been stored at room temperature for 3 months were nearly 100%, indicating that all of the seeds had become non-dormant and were viable (Fig. 2a; AR seeds).

We considered that the 8-WAH stage was likely appropriate for performing genetic analysis, because the variation in germination rates at 8 WAH was larger than that at 6 WAH. On the basis of the comparison of germination rates among lines with different substituted segments on the same chromosome, we focused on three chromosomal regions, on chromosomes 1, 2, and 6, which were likely to be involved in variations in germination rate (Fig. 2b). However, from the nature of the substitution mapping using the CSSLs, it was difficult to define small chromosomal regions as putative QTLs on the basis of the data we obtained. We, therefore, selected three lines (SL501, SL506, and SL519), all of which exhibited very low germination rates at 8 WAH, for the verification of allelic differences and for the QTL mapping of putative chromosome segments.

Verification of allelic differences by means of QTL analysis

To verify allele effects and define more precisely the chromosomal locations of the putative QTLs, we performed QTL analysis using three F_2 populations derived from crosses of SL501, SL506, or SL519 with Koshihikari. We observed no clear variation in germination rates in the F_2 population of SL506 × Koshihikari, and no clear QTL was detected in this population (data not shown). In contrast, a wide range of variation in germination rates was observed in the F_2 population of SL501 × Koshihikari, and one QTL with a major effect was detected in the interval between RM7278 and RM3425 on the short arm of chromosome 1 (Fig. 3a). This QTL accounted for 34% of the phenotypic variance in the F_2 plants, and the Nona Bokra allele decreased the germination rate by 19% (Fig. 3a). On the basis of the genotype at RM6902, the marker linked to the QTL, we classified the F_2 plants into three genotype classes: homozygous for the Nona Bokra or Koshihikari allele, or heterozygous (Fig. 3b). F_2 plants homozygous for the Koshihikari allele at RM6902 showed high germination rates (49–95%). In contrast, the germination rates ranged from 11 to 84% in plants homozygous for the Nona Bokra allele and from 19 to 94% in the heterozygous F_2 plants. The germination rates in plants homozygous for the Nona Bokra allele were shifted more toward the lower end of the germination range than those of the heterozygotes. These results clearly confirmed the existence of the QTL on the short arm of chromosome 1: the Nona Bokra allele at the QTL decreased the germination rate.

In the F_2 population of SL519 × Koshihikari, one QTL was detected in the interval between RM7488 and RM7311 on the short arm of chromosome 6 (Fig. 4a). This locus accounted for about 16% of the phenotypic variance in the F_2 plants, and the Nona Bokra allele decreased the germination rate by 21%. Furthermore, one additional QTL was detected in the interval between RM1161 and RM3498 on the long arm of chromosome 6. The presence of the Nona Bokra allele at this QTL reduced germination by 17%, and about 9% of the phenotypic variance in the F_2 plants could be explained by this QTL (Fig. 4a). On the basis of the genotype of RM5963, we classified the F_2 plants into three genotype classes: homozygous for the Nona Bokra or Koshihikari allele, or heterozygous (Fig. 4b). F_2 plants homozygous for the Koshihikari allele at RM5963 showed high germination rates, ranging from 43 to 89%, whereas those homozygous for the Nona Bokra allele had lower rates, ranging from 0 to 39%. The germination rates of heterozygous plants were intermediate between those of the two homozygous types (1–63%). From the results of this analysis using advanced F_2 progeny, we confirmed the presence of two QTLs detected in the analysis of the CSSLs and one additional QTL detected on chromosome 6. For further analysis, we designated the three QTLs as *Sdr6* (on the short arm of chromosome 1), *Sdr9* (on the short arm of chromosome 6), and *Sdr10* (on the long arm of chromosome 6).

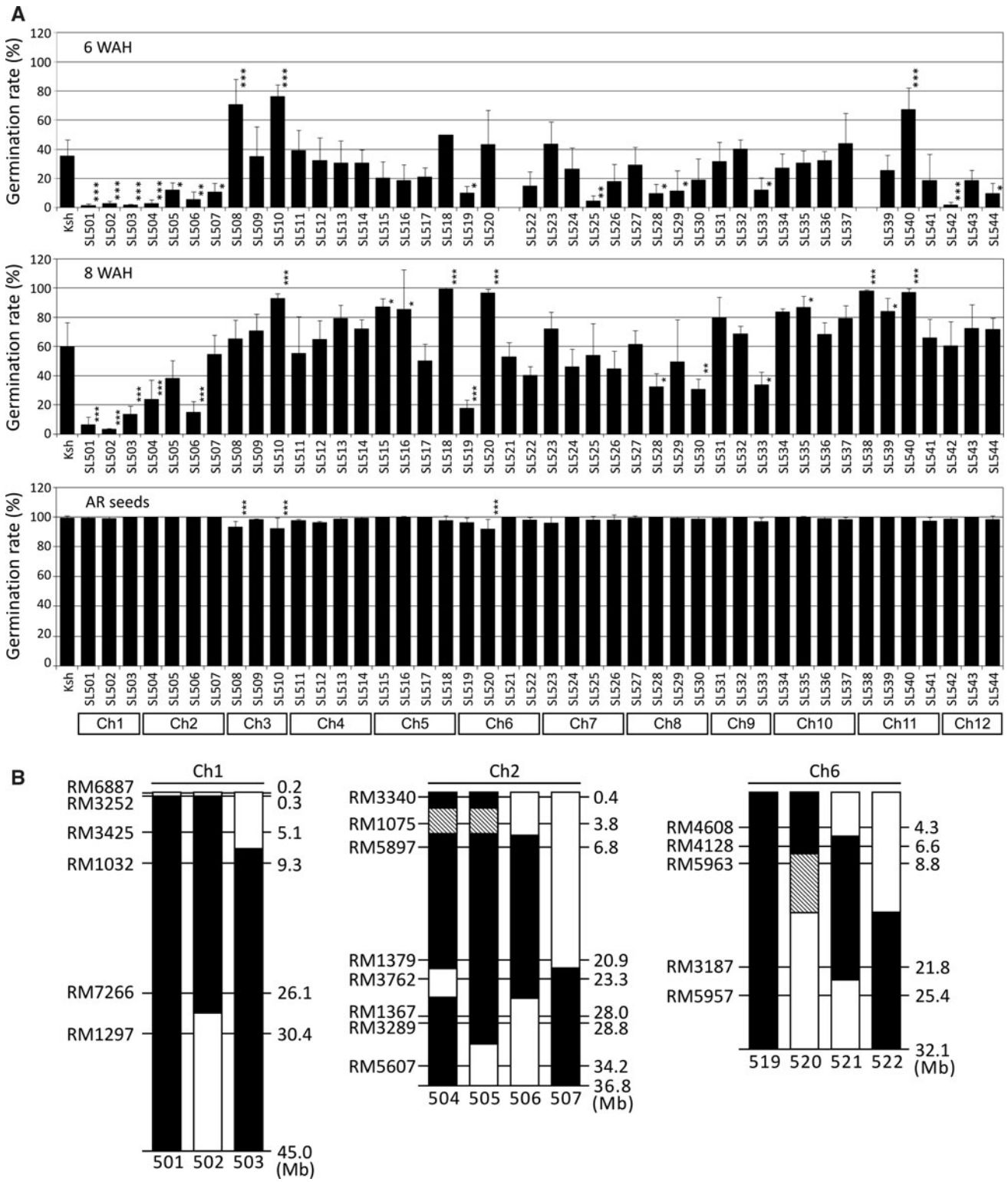

Fig. 2 Germination rates of Koshihikari and chromosome segment substitution lines (CSSLs) derived from Koshihikari × Nona Bokra. **a** Germination rates of each CSSL were determined at 6 weeks after heading (WAH) and 8 WAH and in seeds (harvested at 8 WAH) stored for 3 months at room temperature in 2008 in Tsukuba (AR seeds). Chromosome numbers on which substitution occurred in Nona Bokra are shown below the *x* axis. **b** Graphical genotypes of selected CSSLs. SSR markers are indicated to the *left* of each chromosome, and physical distances based on RAP-DB (Build 5) are shown to the *right*. *White*, *black*, and *hatched boxes* indicate Koshihikari, Nona Bokra, and heterozygous regions, respectively. *, **, and *** indicate significant difference at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, between CSSLs and the recurrent parent, Koshihikari, by Dunnett's test

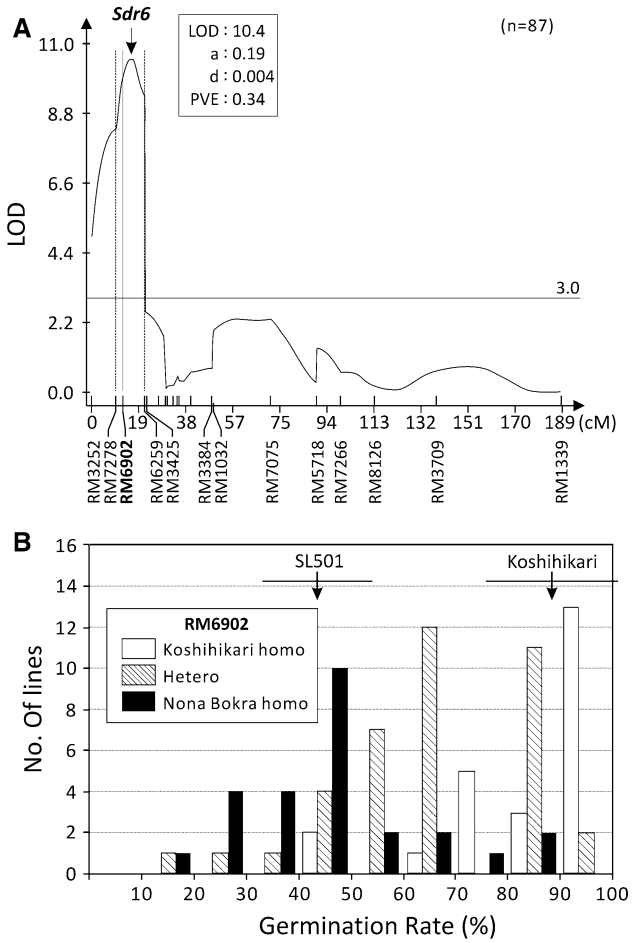

Fig. 3 Chromosomal location of the QTL on the short arm of chromosome 1, and allelic differences. **a** The *log-likelihood curve* indicates a putative QTL position on chromosome 1 in the F_2 population of Koshihikari × SL-501. *LOD* logarithm of odds, *a* additive effect, *d* dominance effect, *PVE* percentage of variance explained. **b** Frequency distribution of seed germination rates in F_2 plants showing three genotype classes of the SSR marker RM6902, which was found to be linked to the QTL. Genotypes of RM5963 are represented as *white bars* (homozygous for Koshihikari allele), *hatched bars* (heterozygous allele), and *black bars* (homozygous for Nona Bokra allele). Germination rates of seeds from F_2 populations were determined 8 weeks after heading. *Arrows* indicate the mean values for SL501 and Koshihikari. *Horizontal lines* under the *arrows* indicate the standard deviations

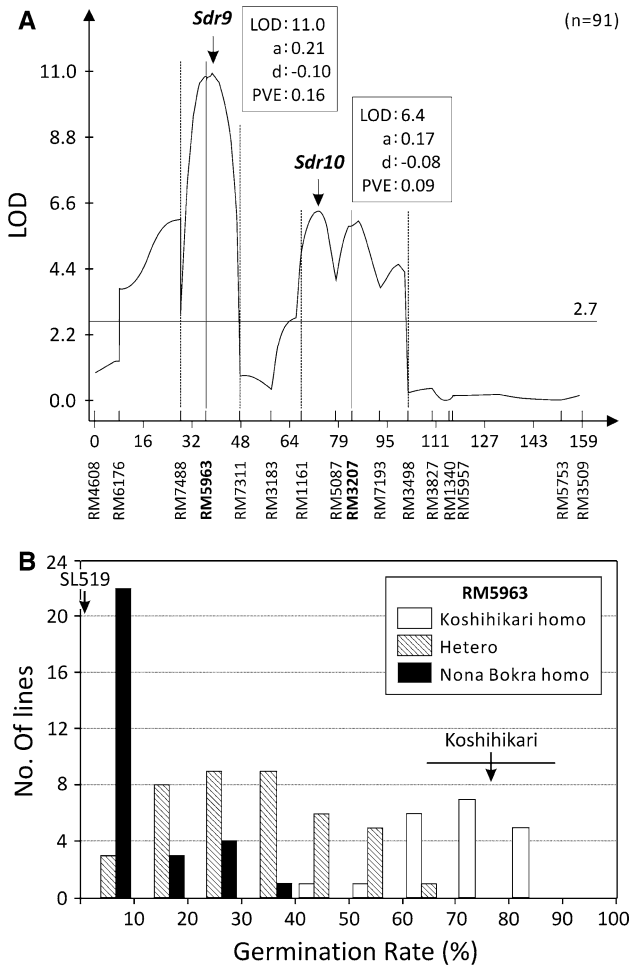

Fig. 4 Chromosomal locations of QTLs on the short arm of chromosome 6, and allelic differences in the case of MR5963. **a** The *log-likelihood curve* indicates putative QTL positions on chromosome 6 in the F_2 population of Koshihikari × SL-519. *LOD* logarithm of odds, *a* additive effect, *d* dominance effect, *PVE* percentage of variance explained. **b** Frequency distribution of seed germination rate in F_2 plants showing three genotype classes of the SSR marker RM5963, which was found to be linked to the QTL. Genotypes of RM6902 are represented as *white bars* (homozygous for Koshihikari allele), *hatched bars* (heterozygous allele), and *black bars* (homozygous for Nona Bokra allele). Germination rates of F_2 populations were determined 8 weeks after heading. *Arrows* indicate the mean values for SL519 and Koshihikari. *Horizontal lines* under the *arrows* indicate the standard deviations

Characterization of the selected CSSLs

To further characterize two CSSLs, germination tests were performed. To synchronize the heading dates, the seeds were harvested from plants grown in the greenhouse under controlled day length, as described in "Materials and methods".

To exclude noise from other chromosomal regions, we used SL502 to predict the function of *Sdr6* on chromosome 1, because the substituted region was smaller than that of SL501 (Fig. 2b). Koshihikari germinated faster than the two dormant lines SL502 (including the QTL on chromosome 1) and SL519 (including the two QTLs on chromosome 6) (Fig. 5a). In Koshihikari, germination was evident in 9% of the seeds 4 days after imbibition (DAI) and had reached nearly 40% by 8 DAI, whereas both CSSLs showed no germination until 6 DAI; the germination rate at 8 DAI was 2% in SL502 and 8% in SL519.

To survey the responses of the two CSSLs (SL502 and SL519) to ABA, we exposed AR seeds to various concentrations of ABA (0–100 μM) and plotted response curves (Fig. 5b). The germination rate of Koshihikari was consistently high (45–60%), even with 100 μM ABA

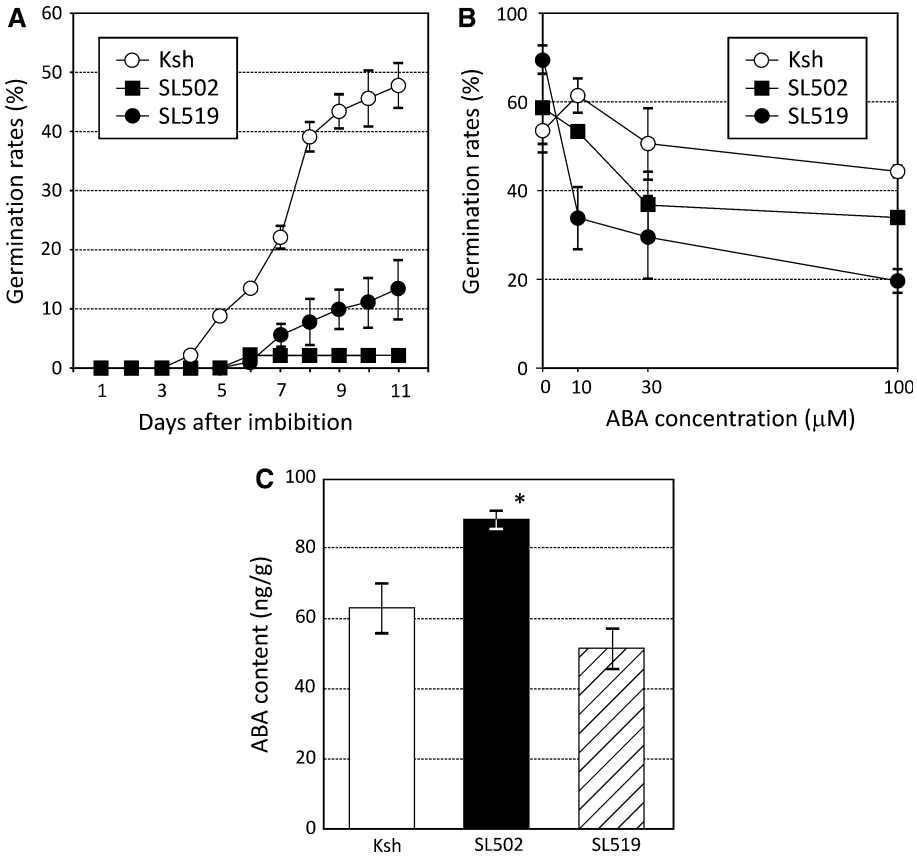

Fig. 5 Physiological characterization of two selected CSSLs and Koshihikari. **a** Temporal changes in germination rates of three lines (SL502, SL519, and Koshihikari) after imbibition. Germination rates were determined 5 weeks after heading. **b** Effect of ABA treatment on germination rates of the three lines. Ripened seeds were used. **c** ABA contents of seeds of the three lines 4 weeks after heading. Values are mean ± standard deviation. *$P < 0.05$ by the t test

treatment. SL502 was slightly more sensitive than Koshihikari to ABA. SL519 was even more sensitive than Koshihikari and SL502 and had the lowest germination rates.

We measured the endogenous ABA contents in the seeds of the two CSSLs and the recurrent parent, Koshihikari, harvested at 4 WAH. Significantly greater ABA accumulation was detected in SL502 (90 ng/g) than in Koshihikari (62 ng/g) (Fig. 5c; $P < 0.05$).

In higher plants, many genes are involved in the ABA biosynthesis pathway. Of these, the genes encoding NCED are considered to be of key importance. To investigate the expression of *OsNCEDs*, we used line #6095, which was derived from the SL502 F_2 population and carried the reduced-size chromosome segment from SL502 (Fig. 6a). This line had a lower germination rate than that of the isogenic control, Koshihikari except 6 WHA (Fig. 6b). Among them, higher expression of *OsNCED3* and *OsNCED5* were detected. Furthermore, the *OsNCED3* was significantly higher in the endosperm of plant #6095 than in the endosperm of Koshihikari. The level of expression of *OsNCED3* was significantly higher in #6095 than in Koshihikari ($P = 0.01$), but *OsNCED1*, *2*, and *4* were expressed at uniformly very low levels in both #6095 and Koshihikari (Fig. 6c).

Discussion

Phenotypic expression of dormancy is often affected by environmental conditions—in particular, temperature—as well as by genotype. Differences in heading dates among diverse cultivars have prevented the reliable measurement of levels of seed dormancy, owing to differences in temperature during the ripening period (Allen et al. 2007). This is also true in primary mapping populations, such as F_2 plants and recombinant inbred lines showing a wide range of variation in heading date (Uga et al. 2007). To overcome this problem, we used CSSLs for the genetic analysis of seed dormancy. Nona Bokra has strong photoperiod sensitivity and shows extremely late heading (more than 200 days to heading required) under natural field conditions in summer in Japan. Under SD conditions, the difference in heading date between Koshihikari and Nona Bokra was only 6 days. We were, therefore, able to check for potential differences in seed dormancy levels between Nona Bokra and Koshihikari under SD conditions by using SD equipment in the field (Fig. 1). We used the CSSLs to identify the chromosomal regions influencing this difference in dormancy. Because each of the CSSLs has a single, or a few, segment substitutions, it is possible to detect QTLs with minor effects generated by the substituted

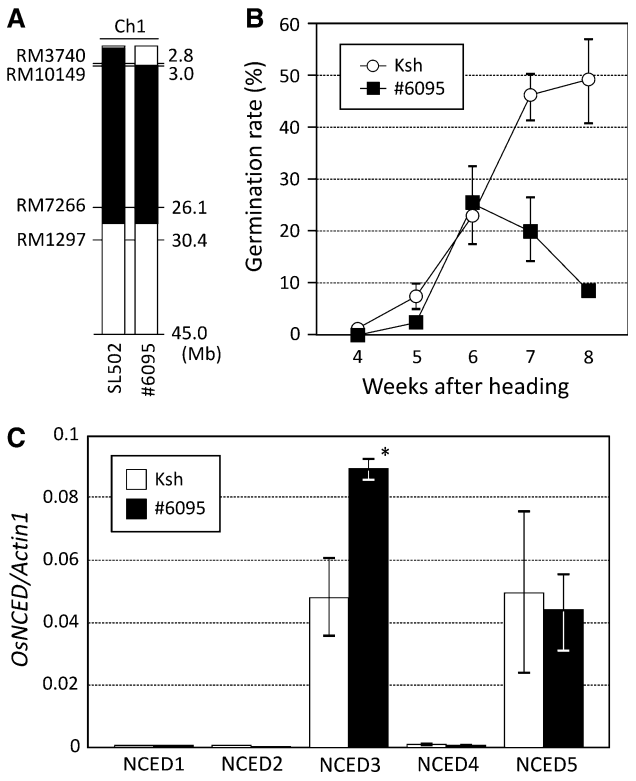

Fig. 6 Expression analysis using a substitution line. **a** Genotype of SL502 and one of the progeny lines, #6095, derived from the population used in the F_2 analysis. **b** Temporal changes in germination rates during maturation. **c** *OsNCED* expression levels in the endosperm at 4 weeks after heading. $^*P < 0.05$ by the *t* test

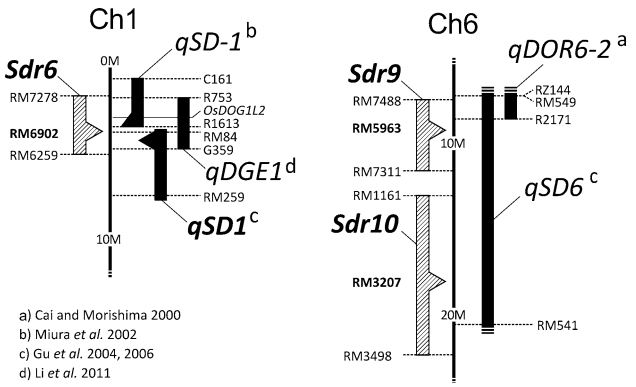

Fig. 7 Positions of QTLs and rice orthologs of Arabidopsis genes involved in seed dormancy and germination regulation. Previously reported QTLs on chromosomes 1 and 6 associated with seed dormancy and germination are indicated to the *right* of each chromosome. The location of each marker is indicated by the physical position determined on the basis of the RAP-DB Build 5 rice genome sequence (RAP 2008). *Bars* show the positions of the flanking markers and *triangles* next to the *bars* show the nearest markers. *Hatched boxes* show QTLs reported in this study. QTLs indicated in *bold type* have been reported as Mendelian factors. Position of *DOG1L-2* is indicated

segments (Ebitani et al. 2005; Fukuoka et al. 2010; Kubo et al. 2002; Takai et al. 2007). Although the F_2 population showed a wide range of variation in heading date, 26 lines among the 44 CSSLs did not differ significantly from each other in heading date (Uga et al. 2007). Even in the case of CSSLs with different heading dates, heading occurred within 30 days after that in Koshihikari (Uga et al. 2007). We were, therefore, able to perform more reliable seed germination testing and successfully identified three QTLs for seed dormancy. In addition, as the CSSLs carried one or a few particular chromosome segment substitutions, these CSSLs could be used to predict the putative allele effects at the putative QTLs.

One QTL was mapped on the short arm of chromosome 1 (*Sdr6*) from SL501, and two were mapped on chromosome 6 of SL519 (*Sdr9* and *Sdr10*). The Nona Bokra alleles of these three QTLs were associated with decreased germination rate.

During the last decade, many QTLs for seed dormancy in rice have been mapped using genetic markers. Genetic dissection of seed dormancy using isozyme markers has revealed five QTLs (Wan et al. 1997). By using RFLP markers, five QTLs have been detected in backcrossed inbred lines (BILs) (Lin et al. 1998). Subsequent studies have identified many QTLs controlling seed dormancy (e.g., Sugimoto et al. 2009). It should be noted that, in our study, *Sdr6* was co-localized with the QTLs *qSD-1*, *qSD1*, and *qDGE1* (Miura et al. 2002; Gu et al. 2006; Li et al. 2011) (Fig. 7). Although Miura et al. (2002) and Lin et al. (1998) used the same materials derived from Nipponbare/Kasalath//Nipponbare backcrossed inbred lines, Miura et al. detected *qSD-1* but Lin et al. did not. This inconsistent detection of QTLs in this region was likely a result of differences in environmental conditions. In fact, we found that the temporal changes in the germination rate of line #6095 were complex: during maturation, the germination rate until 6 WAH resembled that of Koshihikari, whereas after 6 WAH, with increasing maturation, the germination rate dramatically decreased (Fig. 6b). This suggests that the expression of *Sdr6* is strongly affected by environmental conditions such as temperature and moisture.

We also surveyed homologs of Arabidopsis genes associated with seed dormancy and germination. A homolog of Arabidopsis *DOG1* (a regulator of seed dormancy; Bentsink et al. 2006), namely *OsDOG1L-2* (Os01g0159000) (Sugimoto et al. 2010), is co-localized with *Sdr6* and *qDGE1* (Li et al. 2011). *OsDOG1L-2* is a target of the recently reported PHS resistance gene, *Sdr4* (Sugimoto et al. 2010). Interestingly, *OsDOG1L-3* (Os05g0560200) and *DOG1-like* (Os05g0492000) have been found in the candidate region for *qDGR5b* (Li et al. 2011), where they are co-localized with a QTL, *qSdn-5* (Lu et al. 2010).

There is considerable circumstantial evidence that ABA is involved in regulating the onset of dormancy and in maintaining the dormant state (Bewley 1997), and ABA levels are finely controlled by the balance between the rates of ABA biosynthesis and catabolism (Nambara and Marion-Poll 2005). Previous studies have shown that NCED is a key enzyme in ABA biosynthesis (Seo and Koshiba 2002). We observed greater ABA accumulation in SL502 and greater expression of *OsNCED3*, a key gene of ABA biosynthesis, in line #6095, which also harbored *Sdr6* at 4 WAH. We speculated that the reduced germination rate of #6095 at 4 and also 5 WAH may have resulted from the accumulation of *OsNCED3* and ABA predicted from hyperaccumulation detected on SL502 at 4 WAH. However, because we had no data showing ABA content and *OsNCED* expression between 5 and 8 WAH of #6095, the relationship between ABA and germination rate was difficult to predict. Although we have no direct evidence of the relationship between the allele effects of *Sdr6* and ABA accumulation.

The candidate region for *Sdr9* on chromosome 6 overlapped with the region in which *qDOR6-2* and *qSD6* are located (Cai and Morishima 2000; Gu et al. 2004). On the basis of the data reported in the other studies, it is very difficult to clarify the allelic relationships, and to do this the QTLs will need to be cloned. The other QTL, *Sdr10*, was also detected on chromosome 6, and this QTL was co-localized with *qSD6* (Gu et al. 2004). Physiological analysis of SL519 revealed that it had a hypersensitive phenotype to ABA. This observation may help to predict the functions of *Sdr9* and/or *Sdr10*. As mentioned before, differences in heading dates might affect the levels of seed dormancy. In fact, two heading date QTLs, corresponding to *Sdr9* and *Sdr10*, have been mapped on chromosome 6 (Uga et al. 2007), and we speculated that both QTLs were apparent because of variations in heading date. However, our germination tests using seeds harvested from greenhouse-grown plants, the heading dates of which were synchronized, clearly showed the dormant phenotype of SL519; we therefore ruled out the effect of heading date in this analysis.

Despite the comparisons with the above-mentioned Arabidopsis experiments, the relevance of ABA to seed dormancy in rice remains unclear. Further analysis and cloning of *Sdr6*, *Sdr9*, and *Sdr10* will help to reveal the seed dormancy mechanism in rice.

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