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Identification and fine mapping of quantitative trait loci for seed vigor in germination and seedling establishment in rice

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Abstract Seed vigor is an index of seed quality that is used to describe the rapid and uniform germination and the establishment of strong seedlings in any environmental conditions. Strong seed vigor in low-temperature germination conditions is particularly important in direct-sowing rice production systems. However, seed vigor has not been selected as an important breeding trait in traditional breeding programs due to its quantitative inherence. In this study, we identified and mapped eight quantitative trait loci (QTLs) for seed vigor by using a recombinant inbred population from a cross between rice (*Oryza sativa* L. ssp. *indica*) cultivars ZS97 and MH63. Conditional QTL analysis identified *qSV-1*, *qSV-5b*, *qSV-6a*, *qSV-6b*, and *qSV-11* influenced seedling establishment and that *qSV-5a, qSV-5c*, and *qSV-8* influenced only germination. Of these, *qSV-1*, *qSV-5b*, *qSV-6a*, *qSV-6b*, and *qSV-8* were low-temperature-specific QTLs. Two major-effective QTLs, *qSV-1*, and *qSV-5c* were narrowed down to 1.13-Mbp and 400-kbp genomic regions, respectively. The results provide tightly linked DNA markers for the marker-assistant pyramiding of multiple positive alleles for increased seed vigor in both normal and low-temperature germination environments.

Keywords: Conditional quantitative trait locus mapping; germination; seedling establishment; seed vigor; rice
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INTRODUCTION

Seed germination is a complex biological process, which involves three physiological phases (Figure 1): the rapid, passive imbibition of water by the dry seed until all of the seed organs are fully hydrated (Phase I); a plateau of hydration in which the water status of the seed remains unchanged (Phase II); and a much larger uptake of water due to the protrusion of radicle and coleoptile, which then elongate quickly (Phase III) (Bewley 1997; Nambara et al. 2010; Nonogaki et al. 2010). By strict definition, seed germination starts with the uptake of water by the mature dry seed (Phase I) and terminates with the protrusion of the radical (Phase II) (Rajjou et al. 2012). The growth of the radicle and coleoptile in Phase III is considered to occur post-germination, in the seedling establishment phase of development (Nonogaki et al. 2010).

Seed vigor is a complex and important agricultural trait that defines the seed properties, which determine the potential for rapid, uniform emergence, and for the development of normal seedlings under a wide range of field conditions (Association of Official Seed Analysis 1983). Seed vigor has both direct and indirect influences on yield (Dennis and Dennis 1991; Ellis 1992). Strong seed vigor is especially important in sub-optimal growth environments (Zhang et al. 2005a; Landjeva et al. 2010; Rajjou et al. 2012; Huang et al. 2013) and is considered to be the essential trait for seedling establishment in direct-sowing agronomic production systems. Low-temperature germination and seedling growth is an extremely important trait for rice production in temperate rice growing areas, at high altitudes in both tropical and sub-tropical areas, and in areas with a cold irrigation water supply (Fujino and Matsuda 2010).

Though seed vigor is crucial for successful rice production, there is no universally standardized system for seed vigor evaluation. This is likely due to the fact seed vigor is greatly influenced by factors such as dormancy, seed size, genotype, environment, the nutrition of the mother plant, and maturity at harvest (Milosevic et al. 2010). McDonald (1975) recommended three testing methods: physical tests, such as size and mass; physiological tests, using various germination and seedling growth parameters (optimal and cold temperatures, Hiltner testing in unfavorable conditions); and biochemical tests, such as conductometric measurements (study of integrity of cell membrane), tetrazolium tests (living or dead cell), and evaluation of enzyme activity. A rolled paper towel method is described to study seed vigor (http://seednet.gov.in/). With this method, seed lots producing taller seedlings are considered to be more vigorous than seed lots producing shorter seedlings. Seed germination (germination *sensu strict* or germination *per se*) in Phases I and II, and seedling growth (seedling establishment) in Phase III represent two different but related physiological phenomena that are likely controlled by different sets of genes (Bentsink and Koornneef 2008). In

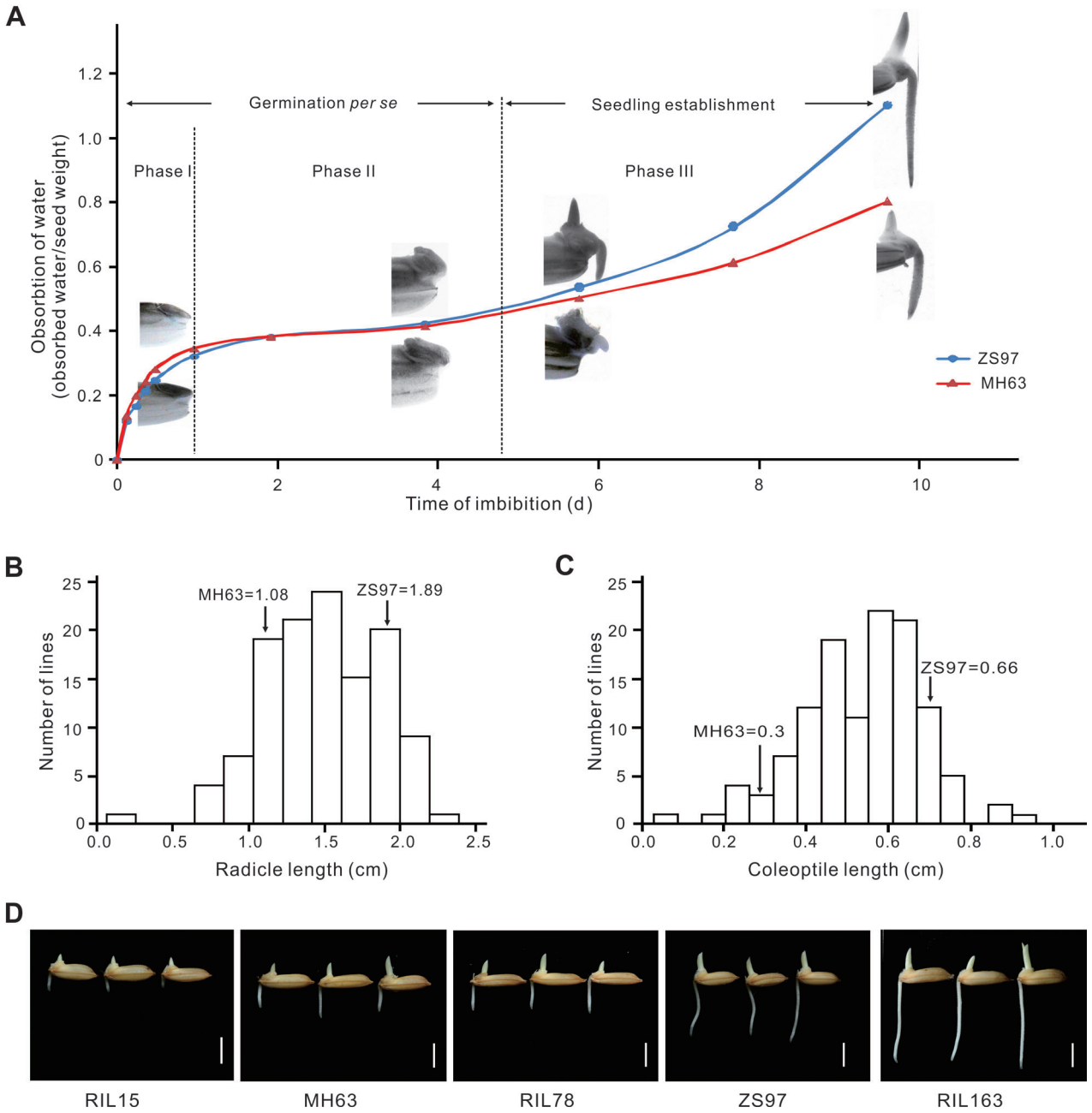

Figure 1. The phenotype of recombinant inbred (RI) lines during seed germination in low temperature (15 °C)
(A) The uptake of water of parental lines during germination. **(B)** The frequency distribution of radicle length of the RI population grown at 15 °C for 10 d. **(C)** The frequency distribution of coleoptile length of the RI population grown at 15 °C for 10 d. **(D)** Seedlings of parental and typical progeny lines.

practice, germination rates and seedling growth are indistinguishably used in the evaluation of seed vigor.

Quantitative trait loci (QTL) analysis has been widely used to study complex traits, for example, quantitative disease resistance (Wang et al. 2010; Cao et al. 2012) and yield-related traits (Bai et al. 2012; Hu et al. 2012; Liu et al. 2013). For rice seed vigor, at least 38 QTLs for germination rate, germination index, germination percentage, and mean germination time have been identified (Miura et al. 2002; Fujino et al. 2004; Zhang et al. 2005a; Ji et al. 2009; Wang et al. 2011) (see Table S1). Further, 75 QTLs for seedling morphological traits, such as shoot, root, coleoptile, and mesocotyl length, and physiological traits, such as reducing sugar, root activity, amylase activity, etc., have been mapped on rice chromosomes (Redone and Mackill 1996; Cui et al. 2002; Zhang et al. 2005b, 2005c; Zhou et al. 2007) (see Table S1). Of them, more than 30 QTLs were

identified in a recombinant inbred (RI) population derived from a cross between two rice (*Oryza sativa* L. ssp. *indica*) cultivars ZS97 and MH63 (Cui et al. 2002). This study identified QTLs for traits such as root length, root dry weight, and total seedling dry weight after 5 d growth of seedlings at 30 °C, α-amylase activity, total amylase activity, and reducing sugar content after continuous incubation for 6 d in the dark at 30 °C.

A map-based cloning study (Fujino et al. 2008) revealed that a major-effective QTL for seed germination, *qLTG-3-1* (Fujino et al. 2004), encodes an unknown protein that is specifically expressed in the embryo, panicle, and shoot. Histological analysis suggested that the function of *qLTG3-1* may be to accelerate vacuolation and that it may be involved in the weakening of the tissues covering the embryo during seed germination. A later study (Fujino and Matsuda 2010) showed that *qLTG3-1* has a role in the upregulation of genes involved in phytoalexin biosynthesis. Further, *qLTG3-1* was shown to increase the expression of probenazole-induced protein (PBZ1), which might induce programmed cell death. In spite of the importance of seed germination and seedling growth, the understanding of the genetic and molecular basis of these traits is limited.

Seed germination in Phases I and II and seedling growth in Phase III are traits that are controlled by many different genes. Developmental phenotypes such as radicle and coleoptile length often represent the cumulated effects of gene networks expressed gene during the entire germination/seedling establishment process phase. However, previous studies did not clearly distinguish QTLs for germination *per se* and seedling growth. A conditional QTL mapping method was proposed to reveal dynamic gene expression for the development of quantitative traits (Zhu 1995). Using this method, Yan et al. (1998) identified several plant height QTLs that had effects in discrete developmental stages in rice. Zhang et al. (2013) reported that maize grain filling rate was regulated by a series of QTLs that were selectively expressed at specific grain filling stages, each of which likely controlled unique physiological processes.

In this study, we conducted dynamic seed vigor evaluations by measurement of radicle and coleoptile lengths of an RI population from a cross between ZS97 and MH63 in low-temperature (15 °C) and optimal-temperature (25 °C) conditions. QTL mapping based on phenotypic data led to the identification of low-temperature-specific QTLs for seed germination and seedling growth. By using a conditional QTL mapping strategy, QTLs for germination and seedling establishment were successfully identified and distinguished. Moreover, fine mapping narrowed down a QTL for germination to a 0.4-Mbp region on chromosome 5 and narrowed a QTL for seedling establishment at low temperature to a 1.13-Mbp region on chromosome 1. These results provide tightly linked DNA markers for marker-assistant selection in rice breeding programs and will serve as the basis for further understanding the genetic and molecular basis of seed germination and seedling growth.

RESULTS

Assessment of seed vigor in low- and optimal-temperature conditions

Our experiment monitoring seed germination and seedling growth of the RI mapping population and the two parental lines at 15 °C showed a typical three-phase physiological process (based on water uptake), and there were clear differences in radicle and coleoptile lengths between ZS97 and MH63 (Figure 1A). In Phase I, a rapid initial water uptake was observed within about 24 h. A plateau of water uptake, Phase II, was observed between Days 1 and 5. A further increase in water uptake that occurred after Day 5 was considered as Phase III (radicle and coleoptile elongation). Differences in radicle and coleoptile lengths between ZS97 and MH63 were initially observed at the early stage of Phase III (Day 6), and obvious differences were apparent at Day 10. The observed longer radical and coleoptile lengths of ZS97 as compared to MH 63 in the Phase III likely result from the accumulative effects of both the seed germination (Phases I and II) and seedling growth (Phase III) phases.

Seeds of the mapping population were multiplied in two locations in China: Sanya and Beijing. The radicle and coleoptile lengths of dormancy-free seeds harvested from Hainan were evaluated daily after germination at 15 °C from Days 6 to 10. The frequency distributions of the radicle and coleoptile lengths at Day 10 were approximately normal (Figure 1B, C). The mean values of the radicle and coleoptile lengths of parental line ZS97 were 1.89 and 0.66 cm, respectively, which were significantly longer than MH63's radical (1.08 cm) and coleoptile (0.3 cm). The parental lines had significant differences in these traits based on the *t*-test. Interestingly, lines with radicle and coleoptile that were both longer and shorter than those of either of the parents were observed in the RI population, for example, RIL15 and RIL163 (Figure 1D). A normal frequency distribution of radicle and coleoptile lengths was observed for seeds following 3 d incubation in the optimal-germination condition (25 °C) (Figure S1A). The radicle and coleoptile lengths in the population grown at 25 °C ranged from 1.43 to 4.28 and 0.19 to 0.86 cm, respectively. ZS97 had significantly longer radicle (4.2 cm) and coleoptile (0.78 cm) than did MH63 (radicle length of 3.18 cm and coleoptile length of 0.67 cm). The frequencies of RI lines classified according to radicle and coleoptile lengths of the seeds harvested in Beijing in optimal-germination condition (25 °C) were also normally distributed (Figure S1B), but slight skew in low germination temperature condition (15 °C) (Figure S1C).

Identification of QTLs for seed germination and seedling establishment

Our comprehensive QTL analysis based on the radicle and coleoptile lengths assessed daily during the 6th to 12th d of seed germination and seedling development in low (15 °C) and the 2nd and 3rd d at optimal- (25 °C) temperature conditions identified eight map positions that were significantly associated with seed vigor (Figure 2; Table 1). Three major-effective QTLs were identified and designated as *qSV-5c*, *qSV-11*, and *qSV-1*. These QTLs explained maximum phenotypic variances of 30.5%, 28.8%, and 22.6%, respectively. The additional five QTLs with Logarithm of the Odds (LOD) values greater than 5.0 had moderate effects that explained more than 12% of phenotypic variance. Alleles of MH63 at the *qSV-1*, *qSV-5b*, and *qSV-11* positions increased radicle and coleoptile lengths, whereas alleles of ZS97 at the *qSV-5a*, *qSV-5c*, *qSV-6a*, *qSV-6b*, and *qSV-8* positions had positive additive effects (Table 1). QTL analysis of both low- and optimal-temperature germination conditions showed that three QTLs, *qSV-5a*, *qSV-5c*, and *qSV-11*, were

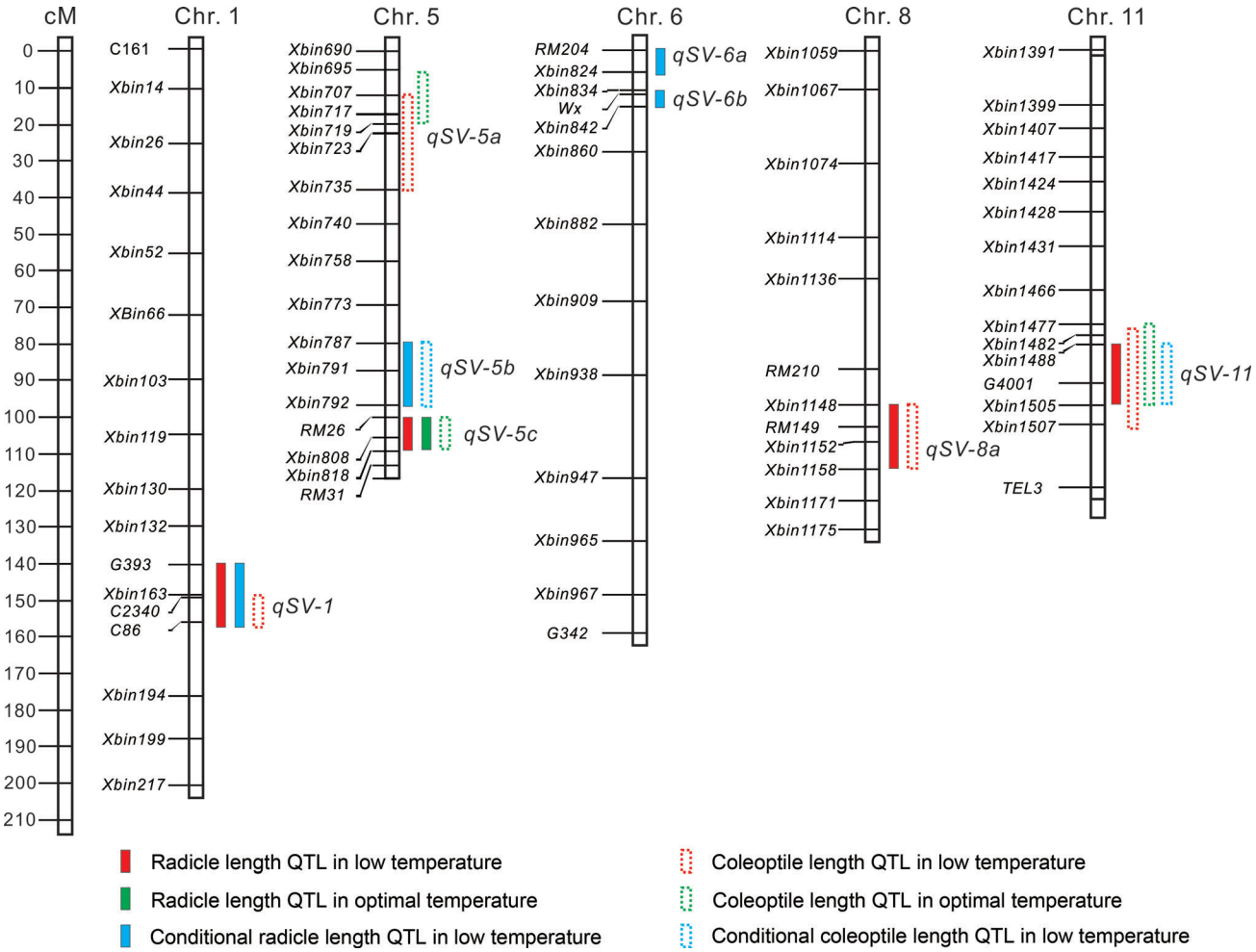

Figure 2. Map positions of quantitative trait loci for seed vigor
Length of bar represents a two Logarithm of the Odds (LOD) support interval of QTLs.

consistently effective in both low and optimal conditions, whereas *qSV-1*, *qSV-5b*, *qSV-6a*, *qSV-6b*, and *qSV-8*, were effective only in low temperature (Table 1).

Seed vigor is greatly affected by seed quality and physiology state during seed maturation. In this study, seeds of the RI mapping population were multiplied and harvested in Sanya in 2009 and in Beijing in 2011. Three QTLs, *qSV-1*, *qSV-5c*, and *qSV-11*, were consistently detected from the seeds harvested in both locations (Tables S2, S3). However, *qSV-5a* was only detected in the seeds harvested in Sanya; *qSV-8*, which regulates both radicle and coleoptile elongation, was specific to the seeds multiplied in Beijing (Tables Table S2, S3).

The conditional QTL analysis that employs the final phenotype value conditioned on the phenotype values observed at each day during seed germination and seeding growth (from the 6th d to 10th d) identified five seedling establishment QTLs (Table 1). *qSV-1* was identified from populations harvested in both Sanya and Beijing; *qSV-5b* was specific to the Sanya population; *qSV-6a*, *qSV-6b*, and *qSV-11* were specific to the Beijing population (Tables 1, S4, S5). Interestingly, three QTLs (*qSV-5b*, *qSV-6a*, and *qSV-6b*) were detected only by the conditional QTL analysis. Further, these three QTLs were effective specific to the seedling establishment in developmental Phase III. The LOD value for *qSV-1* using the normal composite interval mapping (CIM) method was 11.26 (Table S2). This value was estimated by QTL analysis of the final radicle length at the 10^{th} d. Conditional QTL analysis using phenotypic data obtained from 6^{th} to 10^{th} d (Day 10|Day 6) gave an LOD value of 10.38 for *qSV-1* (Table S4). This closed LOD values suggested that *qSV-1* mainly contributed to seedling establishment (the 6^{th} d to 10^{th} d). *qSV-5b* was detected by conditional QTL analysis using the coleoptile lengths observed from 6^{th} to 10^{th} d (Day 10|Day 6). However, it was not detected at 9^{th} to 10^{th} d (Day 10|Day 9) time interval, indicating that *qSV-5b* influences the elongation of coleoptiles only during the 6^{th} to 9^{th} d (Table S4). *qSV-5a*, *qSV-5c*, and *qSV-8* were not detected by the conditional QTL analysis. The fact that these QTLs were only identified by the normal CIM method, suggests that these three QTLs likely only influence the germination (Tables 1, S2, S4, S5).

Table 1. Summary of quantitative trait loci (QTL) for seed vigor in low- and optimal-temperature conditions

QTL	Chr.	Marker interval	Max. LOD value[a]	R^{2b}	Additive effects[c]	Traits and conditions[d]	Response to temperature	Effective stages
qSV-1	1	*G393–C86*	11.86	22.6	0.145	RL/LT/d 9/SY	Specific to low temperature	Seedling establishment
qSV-5a	5	*Xbin695–Xbin735*	6.78	12.7	−0.016	CL/LT/d 6/SY	Optimal and low temperature	Germination *per se*
qSV-5b	5	*Xbin787–Xbin792*	6.53	14.9	0.030	CL/LT/d 10|d 6/SY	Specific to low temperature	Seedling establishment
qSV-5c	5	*RM26–Xbin818*	14.8	30.5	−0.159	RL/OT/d 2/SY	Optimal and low temperature	Germination *per se*
qSV-6a	6	*RM204–Xbin824*	5.33	12.6	−0.039	RL/LT/d 12|d 9/BJ	Specific to low temperature	Seedling establishment
qSV-6b	6	*Xbin834–Xbin842*	5.63	13.3	−0.040	RL/LT/d 12|d 9/BJ	Specific to low temperature	Seedling establishment
qSV-8	8	*Xbin1148–Xbin1158*	6.03	13.4	−0.025	CL/LT/d 9/BJ	Specific to low temperature	Germination *per se*
qSV-11	11	*Xbin1477–Xbin1505*	13.02	28.8	0.025	CL/LT/d 6/SY	Optimal and low temperature	Germination *per se*
			5.27	13.4	0.020	CL/LT/d 12|d 9/BJ		Seedling establishment

Note: [a]The maximum Logarithm of the Odds (LOD) value of a QTL estimated from data of the time-course assessment of seed vigor at low and normal temperatures by composite interval mapping and conditional QTL analysis. [b]The proportion of phenotypic variance explained by the locus. [c]Effects of the alleles from MH63. [d]The seed vigor traits observed on a given day (i.e., "d9" means the 9th d) at different germination temperatures. BJ, Beijing; CL, coleoptile length; LT, low temperature; OT, optimal temperature; RL, radicle length; SY, Sanya; d10|d6 indicates the data observed at the 6th and 10th d were used in conditional the QTL analysis.

Fine-mapping *qSV-5c*

In order to finely map the major-effective QTL *qSV-5c* (Figure 3A), four MH63 BC_4F_1 introgression lines in the ZS97 genetic background having heterozygous segments in the *RM26–Xbin818* region were selected and used to generate a BC_4F_2 segregation population containing 440 F_2 plants. Two markers, *CAPS1* and *dCAPS2*, flanking *qSV-5c*, and two more markers, *dCAPS1* and *CAPS2*, in the region were developed. In total, 68 F_2 plants that had recombination between *CAPS1* and *dCAPS2* were identified from the segregation population. Among the 68 recombinants, six recombinant lines were identified between *CAPS1* and *dCAPS1*, 16 were between *dCAPS1* and *CAPS2*, and 46 were between *CAPS2* and *dCAPS2* (Figure 3B, C). Radicle lengths of the 68 F_2 families were referred by that of the corresponding F_3 seedlings (48 seedlings) (Figure 3C). ANOVA of the 11 genotypes of the 68 recombinant F_2 families and non-recombinant lines representing the two parental genotypes revealed two distinguishable and significantly different phenotypic groups. The radicle length of the long-radicle groups showed no difference to that of the ZS97 genotype, and the short phenotype groups were not statistically different to the MH63 genotype (Figure 3C). The genotype data and corresponding phenotypic results allowed us to map *qSV-5c* between *dCAPS1* and *dCAPS2* (Figure 3B). Notably, lines heterozygous between *dCAPS1* and *dCAPS2* had similar short-radicle length with those of the MH63 genotype, indicating that *qSV-5c* may have a dominant allele from MH63 for short-radicle length. Further, it may contain a recessive allele from ZS97 for long-radicle length.

For further fine mapping of *qSV-5c*, we developed seven single nucleotide polymorphism (SNP) markers between *dCAPS1* and *dCAPS2* (Figure 3B). Four representative lines (F_2 families) having recombination in this region were evaluated for radicle length (Figure 3C). The obvious long and short phenotypes of the radicle in these lines allowed us to locate *qSV-5c* between *SNP3* and *CAPS2*, a genomic region of approximately 400 kbp (Figure 3B, C).

Confirmation of *qSV-5c* for germination *per se*

Based on conditional QTL mapping, *qSV-5c* was effective in germination. In order to confirm this result, we conducted germination assay at 25 °C. In our fine mapping of *qSV-5c*, three near isogenic lines (BC_4F_3), no. 118 and no. 233 having the homozygous alleles of ZS97 and no. 234 having the homozygous alleles of MH63, were generated and used for germination analysis. The germination rates of no. 118 and no. 233 were approximately 26% at the 2nd d after imbibition and 88% at the 3rd d, while only 5% and 66% germination rates were observed in line no. 234. At the 4th d, germination rate of all three lines reached to 100% (Figure 4). The significant differences in germination rates between lines having ZS97 alleles and lines having MH63 allele confirmed that *qSV-5c* did indeed influence germination.

Fine-mapping *qSV-1*

qSV-1 is a low-temperature-specific seed vigor QTL that mainly contributes to seedling establishment (Figure 5A). It was mapped to genetic region between marker *G393* and *C86*. Similarly, an MH63 BC_4F_1 introgression line in the ZS97 genetic background having a heterozygous segment in this QTL region was self-pollinated, giving a segregating F_2 population containing 720 plants. *CAPS3* and *CAPS10*, two markers flanking

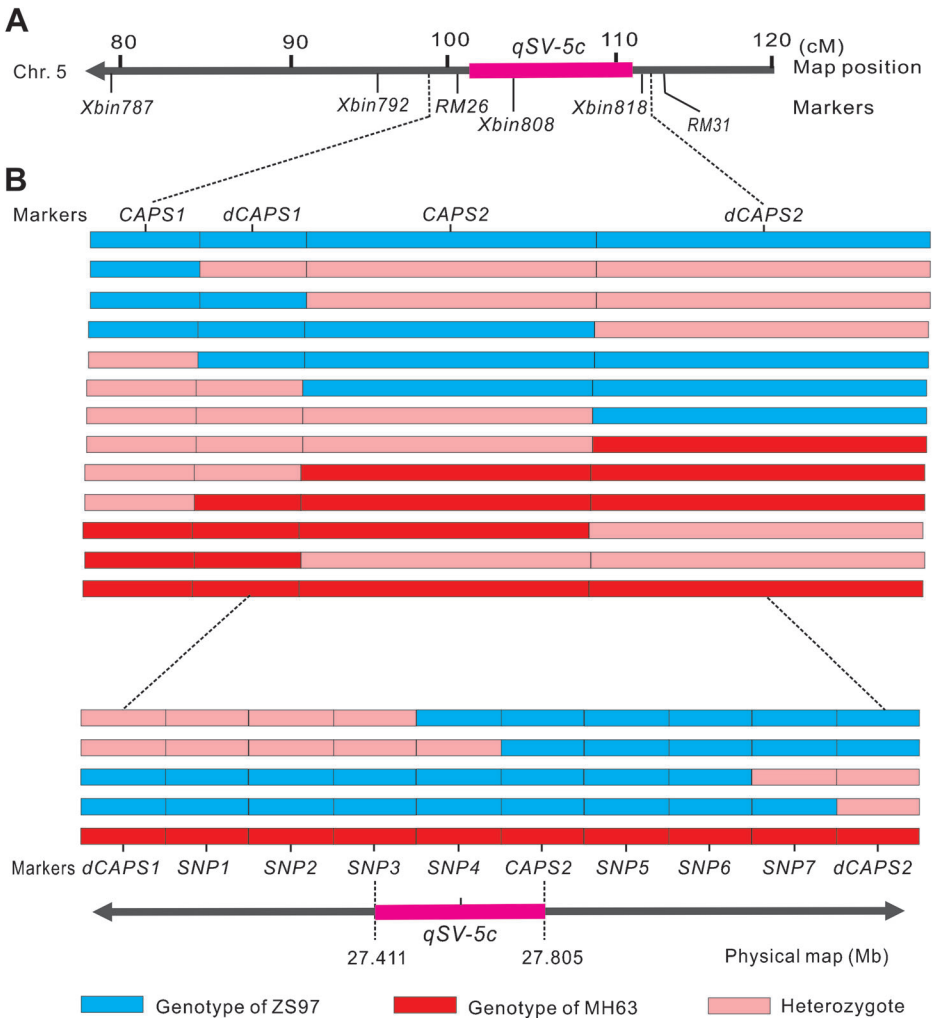

Figure 3. Fine mapping of *qSV-5c*
(A) The position of *qSV-5c* on a linkage map of chromosome 5. **(B)** Genotypes of recombinant lines. **(C)** Phenotypes of groups of recombinant lines.

the *qSV-1* containing region (*G393-C86*) that is approximately 9 Mbp physical distance, were developed and used to identify recombinant lines (Figure 5A). In total, 136 lines were identified and genotyped using seven additional DNA markers (Figure 5B). Our single-marker linkage analysis between genotypes and radicle length showed that markers *CAPS9* and *CAPS10* had no linkage with radicle length, while the other seven makers had significant linkages with the phenotype ($P < 0.01$) (Figure 4B). To further map *qSV-1*, the radicle lengths of the 11 F_2 lines that had recombination between *CAPS3* and *CAPS8* were assessed. Three distinguishable phenotypes, short, medium, and long radicle were identified (Figure 4C). This genotype and phenotype data showed a co-segregation between radicle length phenotype and markers *CAPS5*, *RM302*, and *CAPS6*, locating *qSV-1* in a genome region of approximately 1.13 Mbp (Figure 5B). Interestingly, *qSV-1* showed additive quantitative effect; the long-radicle allele is from MH63 and the short-radicle allele from ZS97.

DISCUSSION

In this study, we identified eight QTLs regulating seed vigor in low and optimal temperature: *qSV-1*, *qSV-5a*, *qSV-5b*, *qSV-5c*, *qSV-6a*, *qSV-6b*, *qSV-8*, and *qSV-11*. The positions of *qSV-5a*, *qSV-5c*, and *qSV-6b* have been identified as QTLs for seed vigor in previous reports (Figure 6; Table S1). The locus of *qSV-5a* is a hot spot for seed vigor QTLs. Many QTLs, including those for seed dormancy (Miura et al. 2002), germination rate, shoot/root dry weight, and some physiological traits such as reducing sugar and total amylase activity (Cui et al. 2002; Zhang et al. 2005a, 2005b) were previously mapped to a region similar to *qSV-5a*. The *qSV-5c* region contains QTLs for germination rate, reducing sugar content, root dry weight, total dry weight (Cui et al. 2002), and shoot length (Redone and Mackill 1996). These findings suggest that this region may contain genes that are interactively effective to seed germination and seedling growth (Cui et al. 2002). *qSV-6b*, near the waxy gene (*wx*)

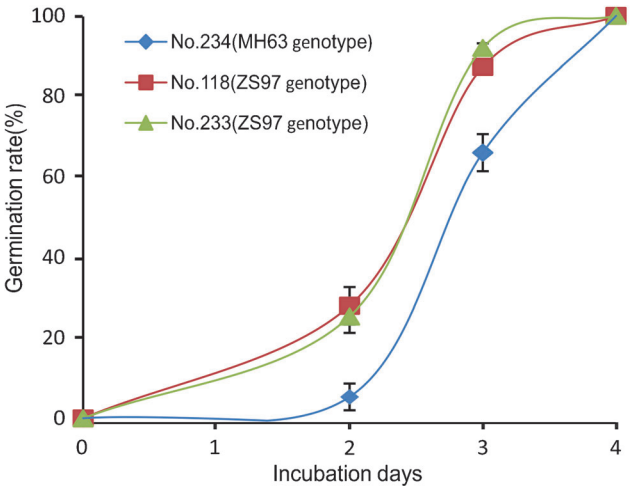

Figure 4. Comparison of germination rate of two homozygous alleles coming from two parents

(Figure 2), co-localizes with QTLs for total amylase activity, reducing sugar content, total dry weight, and shoot dry weight (Cui et al. 2002).This indicates that these physiological traits might have a close relationship with seed vigor and may possibly supply the energy that is required for germination. *qSV-6a* was a newly identified QTL in this study (Figure 6). *qSV-1*, *qSV-8*, and *qSV-11* overlapped with the edges of interval regions of previously identified QTLs for seed vigor. These three QTLs may represent different QTLs, but allelic tests or fine mapping is required to confirm this.

The assessment of radicle and coleoptile lengths at Phase III reflects the accumulative effects of germination and seedling development (Figure 1). In this study, we used a conditional QTL mapping method to successfully identify QTLs for seedling establishment, and compared the effects of QTLs for the accumulated radicle and coleoptile lengths in Phase III to distinguish QTLs for germination *per se* (Table 1). Three QTLs for seedling establishment, *qSV-5b*, *qSV-6a*, and *qSV-6b*, which were not detected in traditional QTL analysis were, however, unambiguously identified by the conditional QTL analysis. This may be due to the fact that certain epistatic genetic effects expressed at different developmental phases from intra-QTL elements counteract each other and result in failure to detect a QTL for cumulative effects (Yan et al. 1998). In contrast, *qSV-5a*

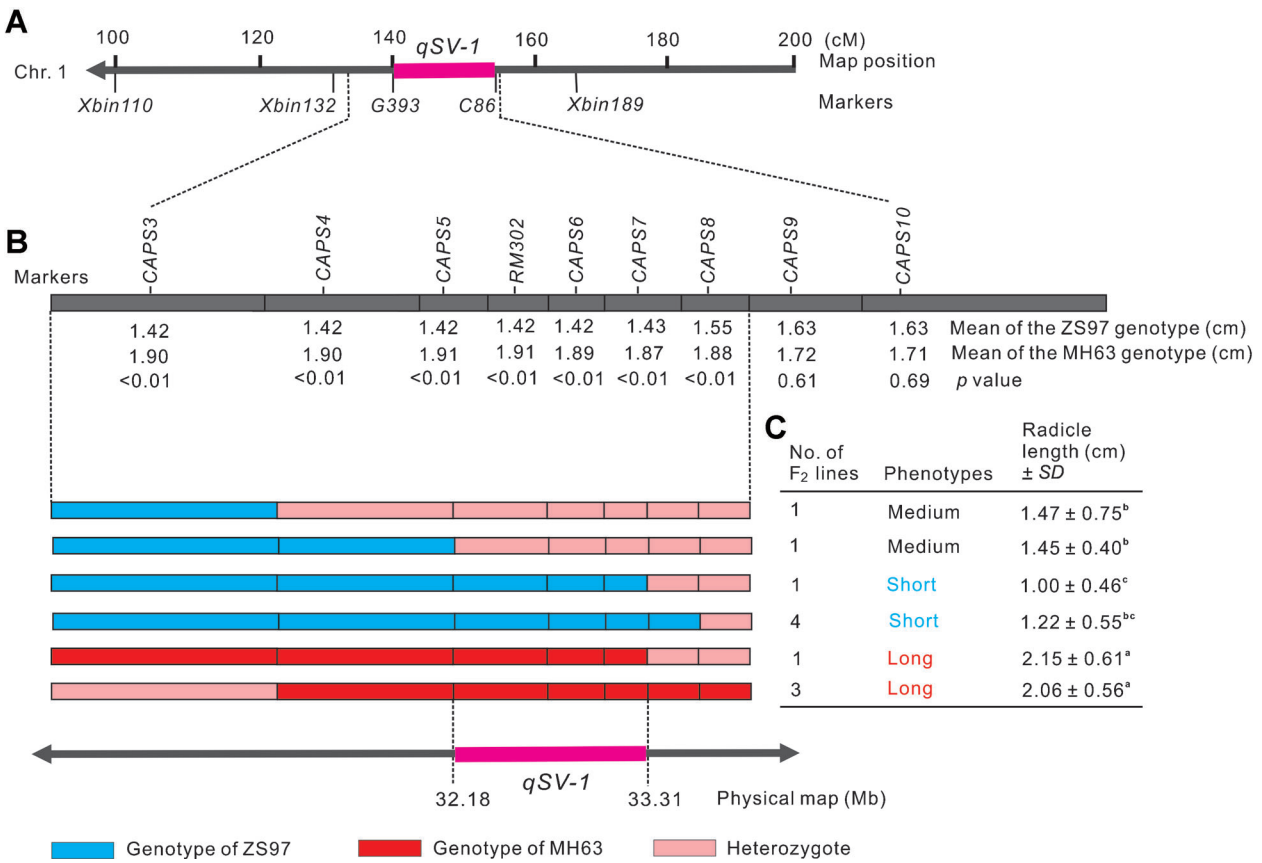

Figure 5. Fine mapping of *qSV-1*

(A) The position of *qSV-1* on a linkage map of chromosome 1. **(B)** Single-marker analysis shown *qSV-1* tightly linked to *CAPS3*, *CAPS4*, *CAPS5*, *RM302*, *CAPS6*, *CAPS7*, and *CAPS8*. **(C)** The phenotype of recombinant lines between *CAPS3* and *CAPS8*.

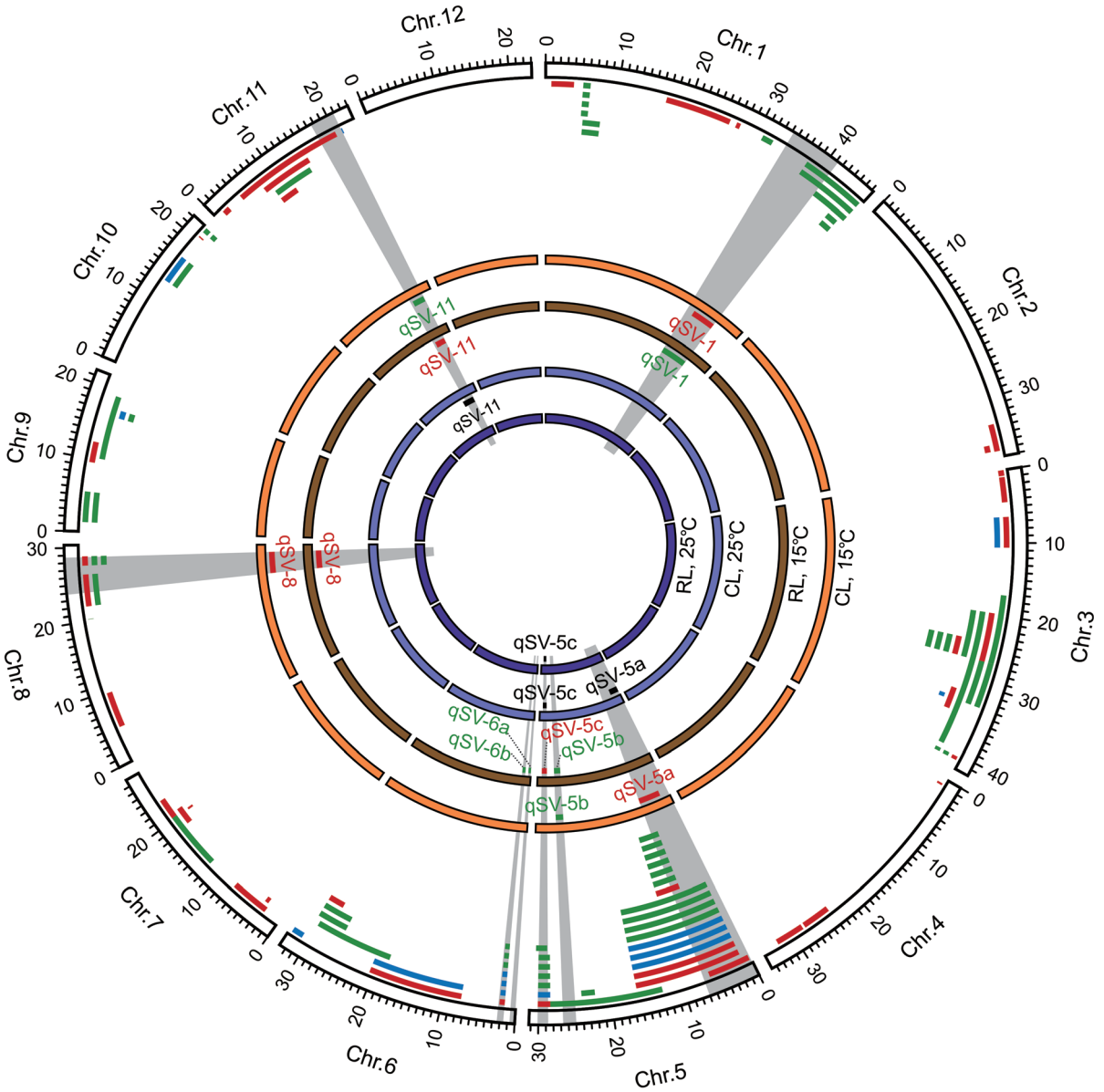

Figure 6. Comparison of quantitative trait loci (QTLs) for rice seed vigor identified in this study with previously reported QTLs
Detailed information for all of the QTLs is listed in Table S1. QTLs identified in previous studies are shown on the outside circle (red represents QTLs related to germination, green represents QTLs related to seedling growth, black represents undefined QTLs). QTLs for coleoptile length at low temperature (15 °C), radicle length at 15 °C, coleoptiles length at normal temperature (25 °C), radicle length at 25 °C are shown on the second, third, fourth, and fifth interior circles, respectively. RL, radicle length; CL, coleoptile length.

and *qSV-5c* were only identified by traditional QTL analysis, suggesting that these QTLs were only effective on germination. These QTLs were identified in previous QTL analyses of seed germination (Cui et al. 2002) and confirmed by our germination assay using near isogenic lines having homozygous genotypes of two parents (Figure 4). The use of conditional QTL methods to distinguish the discrete roles of particular QTLs in separate developmental processes enables finer resolution (temporal, spatial) in association between genotypes and phenotypes. For example, QTLs effective on germination *per se* may be related to seed maturation and after-ripening, while QTLs for seedling development may be associated with cell division, shoot (root) elongation, and metabolite mobilization.

Low-temperature-induced retardation of seedling growth is a common problem in temperate rice growing areas, at high altitudes of both tropical and sub-tropical regions, and in areas with a cold irrigation water supply (Fujino et al. 2008). Vigorous seed germination and growth in low temperatures is essential for the stable establishment of seedlings in the direct-sowing

cultivation of rice in sub-tropical areas (Fujino et al. 2004). In this study, we identified five low-temperature-specific QTLs for seed vigor, *qSV-1*, *qSV-5b*, *qSV-6a*, *qSV-6b*, and *qSV-8* (Table 1). *qSV-6b* for seedling establishment is co-localized with a QTL for total dry weight, shoot dry weight, total amylase activity, α-amylase activity, and reducing sugar content that was mapped from the same population in a previous study (Cui et al. 2002). It is not clear whether this locus has a pleiotropic effect or whether it contains a gene cluster of many loci, which control several traits.

The *qLTG-3-1* QTL on rice chromosome 3 controlling seed germination was cloned previously (Fujino et al. 2008), but the molecular mechanism regulating seed vigor is still not clearly characterized (Fujino and Matsuda 2010). However, this QTL was not identified in the RI mapping population of ZS97 × MH63. In this study, the major-effective QTL *qSV-1* for seedling establishment in low temperature on chromosome 1 and the major-effective *qSV-5c* for germination on chromosome 5 were finely mapped to 1.13-Mbp and 400-kbp genome regions, respectively. Map-based cloning these two QTLs using the results from this study will facilitate our understanding the molecular mechanism of seed germination and seedling growth in vascular plants (Lucas et al. 2013). Among the eight identified QTLs, alleles of MH63 at *qSV-1*, *qSV-5b*, and *qSV-11* and ZS97 at *qSV-5a*, *qSV-5c*, *qSV-6a*, *qSV-6b*, and *qSV-8* have positive effects to seed vigor. Pyramiding all eight alleles with positive effects by selection with the identified tightly linked markers would allow the generation of breeding lines with more vigorous seedlings than either ZS97 or MH63 in both optimal- and low-temperature growth conditions.

MATERIALS AND METHODS

Mapping population and linkage map

The RI population used in this study was derived from a cross between ZS97 (*Oryza sativa* L. ssp. *indica*) and MH63 (*O. sativa* L. ssp. *indica*). This population was initially developed by Xing et al. (2002). The seeds of 120 RI lines were multiplied in Sanya, Hainan province of China in 2009, and in Beijing in 2011. After the seeds were harvested, they were held at room temperature for 2 months to break dormancy.

An ultra-high density map was constructed based on the previously developed 220 SSR/RFLP markers (Yu et al. 1997; Xing et al. 2002) and the genotype data of the 1619 "bins" (Xie et al. 2010; Yu et al. 2011) by using JoinMap 4 (Van Ooijen 2006). This bin map was developed by whole-genome, low-coverage sequencing analysis of this RI population. In this study, each bin represented a marker locus, and was defined as "*Xbin*."

Germination assay and trait evaluation

Seed dormancy has significant influence on the evaluation of seed germination and seed vigor. Seeds used in the seed vigor assessment in this study were tested for seed dormancy. The germination test for dormancy was conducted 25 °C for 4 d. Seeds with a visible radicle and coleoptile (length ≥1 mm) were considered to be germinated. The results of this germination assay indicated that the germination rate of all of the RI lines (120 lines) and both of the parental lines was greater than 95% (Figure S2), indicating that dormancy had been broken prior to the seed vigor assessment.

For the seed vigor assessment, 12 seeds from each line were soaked in water for 24 h. The soaked seeds were surface-sterilized with 70% ethanol for 10 min and rinsed three times with sterile, deionized water. The seeds were placed on moistened filter paper on top of 0.8% agar medium in sterile plastic Petri dishes at low (15 °C) or optimal (25 °C) temperature, in incubators in the dark. The radicle and coleoptile lengths of each seed were recorded daily from the 6^{th} to 12^{th} d after incubation for the low-temperature germination condition, and recorded at the 2^{nd} d and the 3^{rd} d after incubation for the optimal-germination temperature condition. Three replications were performed for the seed vigor assessment of each of the 120 RI lines and the two parent lines. In the fine-mapping experiments, four replications were used. The phenotypic data were assessed at 15 °C for fine-mapping *qSV-1* that was only effective at low-temperature condition. For *qSV-5c*, which was effective at both low and optimal temperature, the phenotype was evaluated at 25 °C.

QTL mapping and conditional QTL analysis

Quantitative trait loci mapping analysis for each trait was performed with the CIM method using WinQTLCart2.5 software (Wang et al. 2007). The LOD threshold at the significant level of 0.05 was determined based on 1,000 permutations. For conditional QTL analysis, the conditional phenotype values were obtained using a mixed-model approach with the QGA station 1.0 (Zhu 1995). Briefly, the conditional genotypic values at time t reflect the net genetic effects during $(t-1) \sim t$ stages and the variance of the conditional genotypic values at time $(t-1)$ is independent of the phenotypic variation at time t. Thus, the genotypic values at time t conditional on the phenotypic mean measured at time $(t-1)$ can be expressed by the following formula (Zhu 1995):

$$y_{(t-t-1)} = \mu_{(t-t-1)} + G_{(t-t-1)} + E_{(t-t-1)} + GE_{(t-t-1)} + \varepsilon_{(t-t-1)}$$

where, $y_{(t|t-1)}$ represents the genotypic value at time t conditional on phenotypic value of $(t-1)$; $\grave{i}$ represents the conditional population mean; $G_{(t|t-1)}$ represents conditional genetic effects; $E_{(t|t-1)}$ represents conditional environmental effects; $GE_{(t|t-1)}$ represents conditional interaction effects of $G \times E$; and $\mathring{a}_{(t|t-1)}$ represents conditional random effects.

Construction of fine-mapping populations and marker development

Fine-mapping populations were constructed for each of the two targeted QTLs. Introgression lines that were heterozygous for the targeted QTL region were selected from a set of introgression lines (BC_4F_1) covering the rice genome. These lines were generated by a cross between ZS97 and MH63, and then backcrossed using ZS97 as the recurrent parent for four generations. For *qSV-5c*, a fine-mapping "F_2" population containing 440 plants was generated by self-pollination of the BC_4F_1 plants having heterozygous segments in the QTL region. Similarly, a population containing 720 "F_2" individuals was constructed for *qSV-1*. DNA markers were genotyped using individual "F_2" plants, and the phenotypes, including radicle and coleoptile length, were assessed using the seeds from the corresponding F_3 families.

Single nucleotide polymorphisms, CAPS, and dCAPS markers were used for fine mapping of *qSV-5c* and *qSV-1*.

These markers in the QTL regions were developed based on the marker data and sequence information available from http://www.OryzaSNP.org. The polymorphic markers between ZS97 and MH63 in target regions were confirmed by sequencing, and CAPS or dCAPS markers were subsequently developed (Neff et al. 2002). The information of markers developed in this study was shown in Table S6.

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SUPPORTING INFORMATION

Additional supporting information can be found in the online version of this article.

Figure S1. Frequency distribution of radicle and coleoptile length of the RI population

(A) Frequency distribution of radicle length of the recombinant inbred (RI) population harvested in Sanya at 25 °C. **(B)** Frequency distribution of coleoptile length of the RI population harvested in Sanya at 25 °C. **(C)** Frequency distribution of radicle length of the RI population harvested in Beijing at 25 °C. **(D)** Frequency distribution of coleoptile length of the RI population harvested in Beijing at 25 °C. **(E)** Frequency distribution of radicle length of the RI population harvested in Beijing at 15 °C. **(F)** Frequency distribution of coleoptile length of the RI population harvested in Beijing at 15 °C.

Figure S2. Frequency distribution of the germination rate of the recombinant inbred (RI) population at optimal temperature (25 °C)

Table S1. Summary of quantitative trait loci (QTL) regulating to seed vigor in previous studies

Table S2. Quantitative trait loci (QTLs) for low-temperature seed vigor in time series of rice germination by composite interval mapping using an RI population harvested in Sanya in 2009 and Beijing in 2011

Table S3. Quantitative trait loci (QTLs) for optimal temperature seed vigor by composite interval mapping using an recombinant inbred (RI) population harvested in Sanya in 2009 and in Beijing in 2011

Table S4. Conditional quantitative trait loci (QTLs) for low-temperature seed vigor in time series of rice germination by composite interval mapping in an recombinant inbred (RI) population harvested in Sanya in 2009

Table S5. Conditional quantitative trait loci (QTLs) for low-temperature seed vigor in time series of germination by composite interval mapping using an recombinant inbred (RI) population harvested in Beijing environment in 2011

Table S6. Information of markers used in this study