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ORIGINAL PAPER

qLTG-9, a stable quantitative trait locus for low-temperature germination in rice (*Oryza sativa* L.)

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Abstract Low-temperature germination (LTG) is an important agronomic trait for direct seeding of rice in temperate regions of East Asia. To dissect the genetic control of LTG, we constructed a recombinant inbred line (RIL) population derived from a cross of *japonica* variety USSR5 and *indica* variety N22. Three putative QTL involved in LTG were detected and named *qLTG-7*, *qLTG-9* and *qLTG-12*. They explained 9.5, 12.12 and 7.08 % of the phenotypic variation, respectively, and the alleles from USSR5 enhanced LTG. A set of advanced backcross lines selected for the presence of *qLTG-9* (with the biggest contribution of the three QTL), by both linked markers and phenotype, was used to validate *qLTG-9* in different generations, years and locations. A near-isogenic line in USSR5 background with a *qLTG-9* insertion from N22 had retarded germination under low-temperature conditions. Finally, *qLTG-9* was fine mapped between markers L9-25D and ID-1, to a 72.3-kb region in chromosome 9, which in the Nipponbare genome contains five predicted genes. This result provides a springboard for map-based cloning of *qLTG-9* and is helpful in understanding the mechanism of seed germination under low-temperature conditions.

Abbreviations

AB	Advanced backcross
NIL	Near-isogenic line
MAS	Marker-assisted selection
MAB	Marker-assisted backcrossing
PS	Phenotypic selection
RIL	Recombinant inbred line
LTG	Low-temperature germination
QTL	Quantitative trait loci/locus
qLTG-*	Quantitative trait allele for low-temperature germination
Chr	Chromosome
SSR	Simple sequence repeat
PCR	Polymerase chain reaction

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Introduction

The ability of seeds to germinate at low temperatures is an important agricultural trait for direct seeding in rice, a practice that is becoming increasingly common in many Asian countries due to lower costs and operational simplicity (Teng et al. 2001; Fujino et al. 2004; Wan et al. 2006; Ji et al. 2009). Uneven seedling emergence and retardation of seedling growth at high altitudes in tropical and subtropical areas, and in areas with cold irrigation water, reduces yield significantly (Chen et al. 2006; Fujino et al. 2004, 2008; Hou et al. 2004; Sasaki and Honma 1974; Teng et al. 2001; Jiang et al. 2006; Sugimoto et al. 2010).

Therefore, research on the genetic mechanisms of low-temperature germination (LTG) is quite important.

LTG is affected by many factors, including genetic background and environment, during the seed development period (Sun et al. 2007). With the development of molecular markers and genome mapping, QTL analyses of LTG have been undertaken in a number of species, including *Medicago truncatula* (Dias et al. 2011), soybean (Hu et al. 2008; Zhang et al. 2012), lettuce (Argyris et al. 2005; Hayashi et al. 2008), and rice (Fujino et al. 2004, 2008; Jiang et al. 2009). QTL for LTG in rice were detected using a number of segregating populations; they were widely distributed on all 12 chromosomes revealing that LTG was polygenically controlled (Chen et al. 2006; Fujino et al. 2004; Hou et al. 2004; Ji et al. 2008, 2009; Jiang et al. 2006; Teng et al. 2001; Iwata and Fujino 2010). However, few of the QTL were confirmed to be stable across environments (Fujino et al. 2008; Ji et al. 2008; Jiang et al. 2009). The only cloned QTL for LTG in rice, namely *qLTG-3-1*, encodes a protein with an unknown function (Fujino et al. 2008), and microarray analysis indicated that a complex metabolic and signal pathway was involved (Fujino and Matsuda 2010).

The basis of a marker-assisted backcrossing (MAB) strategy is to transfer a specific allele at a target locus from a donor line to a recipient line while selecting against donor introgressions across the rest of the genome. The use of molecular markers permits genetic dissection of progeny in each generation and increases the speed of selection, thus increasing genetic gain per unit time (Tanksley et al. 1989; Thomson et al. 2010). These strategies are also suitable for QTL identification. Once a QTL is identified in a primary population, near-isogenic (NIL) and advanced backcross (AB) lines can be developed by MAB (Tanksley et al. 1996) for fine mapping. Cloning a gene from an NIL can be quite efficient as exemplified by genes *Sdr4* involved in dormancy (Sugimoto et al. 2010), *DOG1* for delay of germination in Arabidopsis (Bentsink et al. 2006), *RAS1* associated with salt tolerance and ABA sensitivity in Arabidopsis (Ren et al. 2010), *qSW5* for grain size in rice (Shomura et al. 2008), *PROG1* for plant architecture in rice (Jin et al. 2008) and DTH2, a minor QTL, for heading date in rice (Wu et al. 2013).

A study of varietal differences in LTG in rice revealed that USSR5 (*Oryza sativa* L. ssp. *japonica*) from the former Soviet Union had a high level of LTG, whereas N22 (ssp. *indica*) from India exhibited a low level of LTG, but a high level of seed dormancy (Jiang et al. 2006). The objectives of the study were (1) to detect the QTL associated with LTG in an RIL population from USSR5/N22; (2) to validate and evaluate the QTL using an AB population and an NIL developed from the same parents in different years and locations; and (3) to fine map the authentic and stable QTL. The results should be useful for further understanding of the genetic basis of LTG.

Materials and methods

Plant materials

The *japonica* cultivar USSR5 has a high germination rate at low temperatures (15 °C), whereas *indica* cultivar N22 has poor germination under the same conditions. An RIL (F_7) population of 181 individuals was developed by single seed descent from a cross between USSR5 and N22. In order to fine map QTL related to LTG in USSR5/N22, we developed an AB population by backcrossing the USSR5/N22 F_1 to USSR5. Lines with low germination rates were selected for further backcrossing. The first four generations were selected according to the germination rates under low-temperature conditions. We used 113 SSR markers (part of 176 SSR markers used for linkage mapping of the RIL population) distributed over all 12 chromosomes for reconstitution of the genetic background, combined with phenotypic selection from the BC_4F_1 population. A BC_4F_1 line with a low germination rate under low temperatures and a purer genetic background was selected for later research. Several AB populations were developed from USSR5/N22 to validate *qLTG-9* in different genetic backgrounds. W907 (BC_4F_2 80 individuals), Y2469 (BC_4F_3 62 individuals) and Y2288 (BC_5F_2 219 individuals) were selected from different generations of the AB line development. These AB populations had USSR5 alleles at the *qLTG-7* (RM3394, RM5344 and RM427) and *qLTG-12* (RM247 and RM2935) loci. We then tried to isolate *qLTG-9* by selecting NILs (Y1751; there was a 20 cM introgression in the NIL). Finally, more AB populations in BC_6F_2 were generated for fine mapping of *qLTG-9*. The backgrounds of these BC_6F_2 lines were the same as the NIL (Y1751). A total of 7,218 BC_6F_2 plants segregating only with respect to the introgression in chromosome 9 were used for fine mapping. Recombinants were selected and their phenotypes validated by progeny testing (20–40 plants for each recombinant) in 2010 in Nanjing (240 recombinants) and Hainan (129 recombinants), and homozygotes were selected in Nanjing in 2011 (100 recombinants). Progeny tests involved 20–40 offspring of each recombinant planted in different environments. The average LTG rates of each recombinant population were used as phenotypes. Recombinants (W1050–F93) were selected from the 7,218 BC_6F_2 population.

All grains were harvested on the 35th day after heading. To break dormancy, seeds were incubated at 50° for 7 days.

Evaluation of low-temperature germination

Thirty seeds of each AB line (three replications) were placed on double sheets of moistened filter paper in 9 cm Petri dishes maintained at 15 °C and 100 % relative

humidity for several days. Germination was determined by the emergence of the radicle or/and plumule and the mean percentage of germinated seeds from three replications was used for QTL analysis (Fujino et al. 2004). After 5 days, the seeds were moved into a 30 °C incubator for another 4 days to determine total germination ability and secondary dormancy (Ji et al. 2008, 2009).

DNA template preparation and PCR

Protocols adapted from Dellaporta et al. (1983) were followed for DNA extraction. Following solubilization of the DNA in TE buffer, concentrations were determined spectrophotometrically and used to prepare working solutions of 20 ng/µl. PCRs were based on SSR primers documented in the Gramene database (http://www.gramene.org) and by McCouch et al. (2002). Amplification protocols were performed according to Chen et al. (1997) with minor modifications. Briefly, each 10 µl reaction contained 10 ng template DNA, 0.2 µM of each primer, 2.5 mM dNTP, 1 µl 10× Mg^{2+} free buffer, 2 mM $MgCl_2$ and 0.5 U rTaq DNA polymerase (Takara, USA). The cycling regime was a denaturation step (94 °C/5 min), followed by 35 cycles of 94 °C/30 s, 50–58 °C/30 s, 72 °C/50 s, and a final extension step of 72 °C for 7 min. The PCR products were separated by electrophoresis through 8 % non-denaturing polyacrylamide gels and visualized by silver staining (Sanguinetti et al. 1994).

Linkage mapping, QTL analysis and primer design for fine mapping

A linkage map was constructed using Mapmaker/EXP 3.0 with 176 SSR genotypes of the RIL population. The composite interval mapping procedure within Windows QTL Cartographer v2.5 (Wang et al. 2007) was used for QTL mapping. The average germination rate of each line was arc-sin transformed for the purpose of QTL analysis. Threshold LOD scores were calculated based on 1,000 permutations (Churchill and Doerge 1994). QTL nomenclature followed McCouch et al. (1997). Primers were designed by the software Primer 5.0. The genomic sequence was obtained from the National Center for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov/. Gene annotation within specific genomic regions according to Nipponbare sequence was carried out using RAP-DB (http://rapdb.dna.affrc.go.jp/).

Results

Phenotypic variation of the parents

After dormancy was broken, the germination ability of USSR5 was not different from N22 at the first and second days after imbibition (DAI) at 30 °C (Fig. 1); the germination rates of both reached nearly 100 % at the third DAI. A clear difference in the germination rate was observed at 15 °C (Fig. 1). USSR5 germinated from 3 DAI and almost all of the seeds had germinated at 8 DAI. In contrast, N22 germinated from 6 DAI, and only 15 % of seeds had germinated by 11 DAI (Fig. 1). When the seeds were moved to 30 °C for another 4 days, almost all seeds germinated (Fig. 1). These results showed a significant germination difference between USSR5 and N22 at low temperature with no interference from secondary dormancy.

QTL for LTG in the USSR5/N22 RIL population

Germination rates were determined after 4 and 5 days at 15 °C, identified as the most significant period of difference among USSR5, N22 and RILs. The germination rates of RILs varied between 0 and 100 % on the fourth and fifth days at 15 °C (Fig. 2). No secondary dormancy was detected when seeds were incubated for another 4 days at 30 °C. Germination rates of all RILs were above 98 % (data not shown).

Of the 789 SSR loci covering the rice genome, 176 showed polymorphisms between the parents. A linkage map of the 176 SSR markers was 1,702.8 cM with an average interval between markers of 9.68 cM. The order of

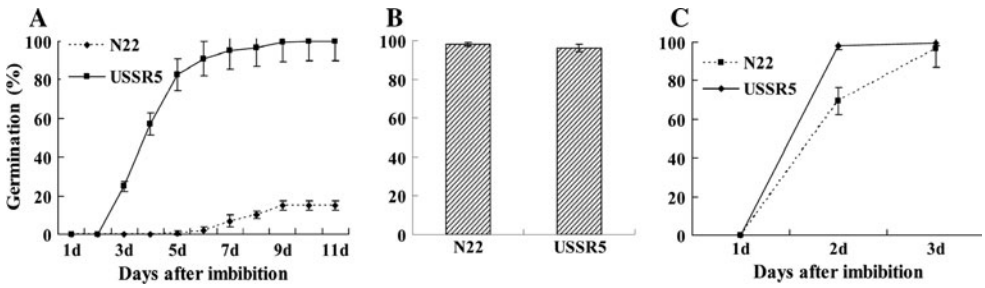

Fig. 1 Germination rates of USSR5 and N22 at low temperatures (15 °C) for 11 days (**a**), low temperatures for 11 days, then transfer to high temperatures (30 °C) for another 4 days (**b**), and high temperatures (30 °C) for 3 days (**c**). Values are mean ± SD of three different experiments

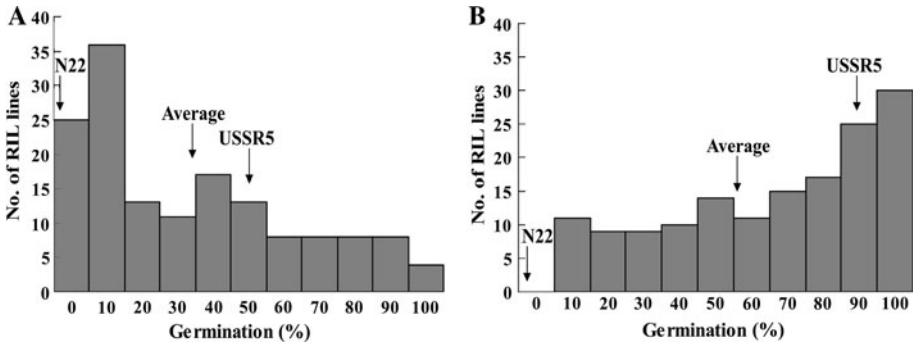

Fig. 2 Frequencies of germination at 15 °C of individuals in the USSR5/N22 RIL population scored at 4 DAI (**a**) and 5 DAI (**b**)

markers was consistent with a previously published rice map using an F_2 population (Jiang et al. 2006).

Three putative QTLs, *qLTG-7*, *qLTG-9* and *qLTG-12*, associated with LTG were detected in the RIL population. *qLTG-9*, with the largest effect, was located on chromosome 9 between RM7038 and RM434, and was detected at both 4 and 5 DAI (Fig. S1; Table 1). The phenotypic variations explained (PVE) by *qLTG-9* were 12.12 and 6.81 % and the LOD values were 4.6 and 2.73, respectively. Additional QTL were detected on chromosomes 7 and 12, and both alleles enhancing LTG were from USSR5. *qLTG-7* was detected on both days and accounted for 9.5 and 6.81 % of total phenotypic variations, respectively. *qLTG-12* was detected only on the fourth day and explained 7.08 % of the phenotypic variation.

Genotypic analysis, validation of *qLTG-9* using different AB populations and fine mapping of *qLTG-9*

The LTG ability of AB populations with and without the USSR5 *qLTG*-9 allele was tested to validate the stability of *qLTG*-9 in seed produced over three seasons in Nanjing and Hainan (Table 2). In 2007, the effects of *qLTG-9* were detected in W907 grown in Hainan; 22.2 % of the phenotypic variation was explained and the LOD value was 6.65. In seed produced in Nanjing in 2008, 19.44 % of the variation was explained and the LOD value was 4.27 in Y2469. From the winter 2008 season in Hainan, the variation explained was 45.7 % and the LOD value was 15.69 in Y2288. *qLTG-9* was located between markers RM105 and RM434 (Fig. 3; Table 2). Thus, the *qLTG-9* allele in the AB populations was confirmed in different backgrounds, years and locations (Fig. 4). The phenotypic variance explained in the AB populations increased with increasing background purity (Table 2; Fig. S2). Recombinants in the AB populations (W907 BC_4F_2, Y2469 BC_4F_3 and Y2288 BC_5F_2) were selected for validation by progeny testing to narrow the *qLTG-9* interval to between L9-25 and L9-6 (Fig. 3).

The LTG based on the genotypes of the AB populations are shown in Fig. 5. In Hainan 2007, the LTG of the homozygous individuals with the N22 intervals detected by RM434 and RM7038 were 42 % lower than those of homozygous individuals with the corresponding USSR5 intervals in the W907 population (Fig. 5). In Nanjing 2008, the LTG of the individuals with the N22 interval detected by RM434 and RM105 were 30 % lower than those of individuals with the corresponding USSR5 interval in the Y2469 population (Fig. 5). In Hainan 2008, the comparative difference for the Y2288 population was 38 % (Fig. 5). The mean LTG rates of the homozygous USSR5 and N22 classes were significantly different according to *t* tests (data not shown). Heterozygotes had intermediate phenotypes, indicating that the USSR5 allele was incompletely dominant. However, differences between individuals homozygous and heterozygous for the USSR5 interval were not significantly different in Nanjing 2008 (Fig. 5). These results revealed that *qLTG-9*

Table 1 QTL for LTG identified at 15 °C using RILs derived from USSR5/N22 at 4 DAI and 5 DAI

Days	Locus	Chromosome	Marker interval	LOD score	PVE[b] (%)	AE[c]
4 d	*qLTG-7*	7	RM3394[a]–RM427	4.15	9.5	6.80
	qLTG-9	9	RM7038–RM105[a]–RM434	4.6	12.12	7.67
	qLTG-12	12	RM19–RM247[a]–RM2395	3.25	7.08	5.84
5 d	*qLTG-7*	7	RM3394[a]–RM427	2.74	6.81	5.73
	qLTG-9	9	RM105[a]–RM434	2.73	6.81	5.28

[a] Nearest marker

[b] Phenotypic variation explained

[c] Additive effects of USSR5 allele after arc-sine transformation of germination rates

Table 2 Chromosomal locations and biometrical characteristics of *qLTG-9* in AB populations over three seasons

Years	Place	Population	Maker interval	Interval (cM)	LOD value	PVE[a] (%)	AE[b]
2007–2008	Hainan	USSR5/N22//USSR5 NIL (BC4F2)	RM7038–RM434	27.3–56.3	6.65	22.2	11
2008	Nanjing	USSR5/N22//USSR5 NIL (BC4F3 Y2469)	RM105–RM434	34.8–56.3	4.27	19.44	6
2008–2009	Hainan	USSR5/N22//USSR5 NIL (BC5F2)	L9-25–L9-6	40.3–49.1	15.69	45.73	18

[a] Phenotypic variation explained

[b] Additive effects of USSR5 allele after arc-sine transformation of germination rates

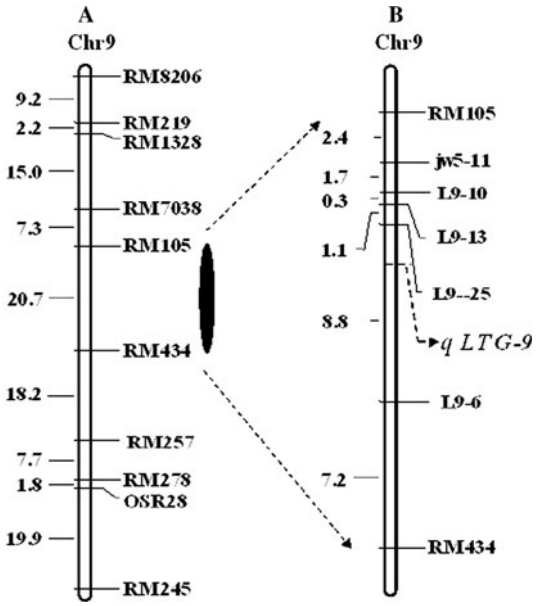

Fig. 3 Genetic location of *qLTG-9* on chromosome 9. **a** *qLTG-9* location in the map based on the RIL population. **b** *qLTG-9* mapped to an 8.8 cM interval between markers of L9-25 and L9-6 based on 361 AB individuals (80 BC_4F_2 (W907), 62 BC_4F_3 (Y2469) and 219 BC_5F_2 (Y2288). Genetic distances are at the *left* and markers are on the *right* of each chromosome

was a functional and stable QTL for LTG, and the broad sense heritability for LTG was 72.7 %.

To further localize the position of *qLTG-9*, several AB populations and a larger BC_6F_2 population were constructed in 2009. Fifteen BC_6F_2 populations comprising a total of 7,218 plants were used for fine mapping, and 240 recombinants between markers RM105 and RM434 were selected. The germination percentage of each recombinant under low temperature was tested, and markers were used to identify the genotypes. Recombinant lines with the most extreme phenotypes and appropriate recombination lines were progeny tested in Nanjing and Hainan, and homozygotes were compared in Nanjing. The extreme high and low LTG lines are shown in Fig. 4. Primers used in fine mapping are listed in Table S1. Finally, *qLTG-9* was located between markers 9-L25D and ID-1 (Figs. 4, 6).

Candidate genes

qLTG-9 was located between markers 9-L25D and ID-1. This region corresponded to a 72.3-kb region in the Nipponbare genome (Fig. 6). Os09g0395600, Os09g0396300, Os09g0396900, Os09g0395700 and Os09g0395800 were annotated in this region by RAP-DB (http://rapdb.dna.affrc.go.jp/) (Fig. 6). There are two transcriptions of Os09g0395600, one is Concanavalin A-like lectin/glucanase domain containing protein, and the other is Xyloglucan endotransglucosylase/hydrolase 13. Os09g0396300 is a peptidase C15, pyroglutamyl peptidase I family protein. Os09g0396900 is a protein of unknown function (DUF125), a transmembrane family protein. There is no gene function information for Os09g0395700 and Os09g0395800.

LTG of AB lines carrying the N22 allele of *qLTG-9*

To further confirm the role of *qLTG-9* in low-temperature germination, USSR5, N22 and Y1751 carrying the *qLTG-9* allele from N22 were compared in germination tests at 15° and 30°. Separate tests indicated that there was no seed dormancy in the materials under investigation (data not shown). USSR5 and Y1751 began to germinate from 3 DAI at 15°, whereas N22 began to germinate from 5 DAI (Figs. 1, 7). After 11 days, seeds were moved to 30° for another 4 days to determine whether the seeds had secondary dormancy. The final germination rates at 30° were all above 98.5 %. These results showed that seeds used in the low-temperature test were non-dormant and were not influenced by secondary dormancy.

At 4 DAI, the germination rates of USSR5 and Y1751 were 56.92 and 27.12 %; at 5 DAI, the germination rates were 82.65 and 56.17 %, respectively. N22 began to germinate from 5 DAI and the germination rate was 0.67 % (Fig. 7). Figure 7 shows the typical growth of both parents and Y1751 at 12 DAI when incubated at 15°. The AB line carrying the *qLTG-9* allele from N22 showed germination delayed for 1 day and slow growth compared to USSR5

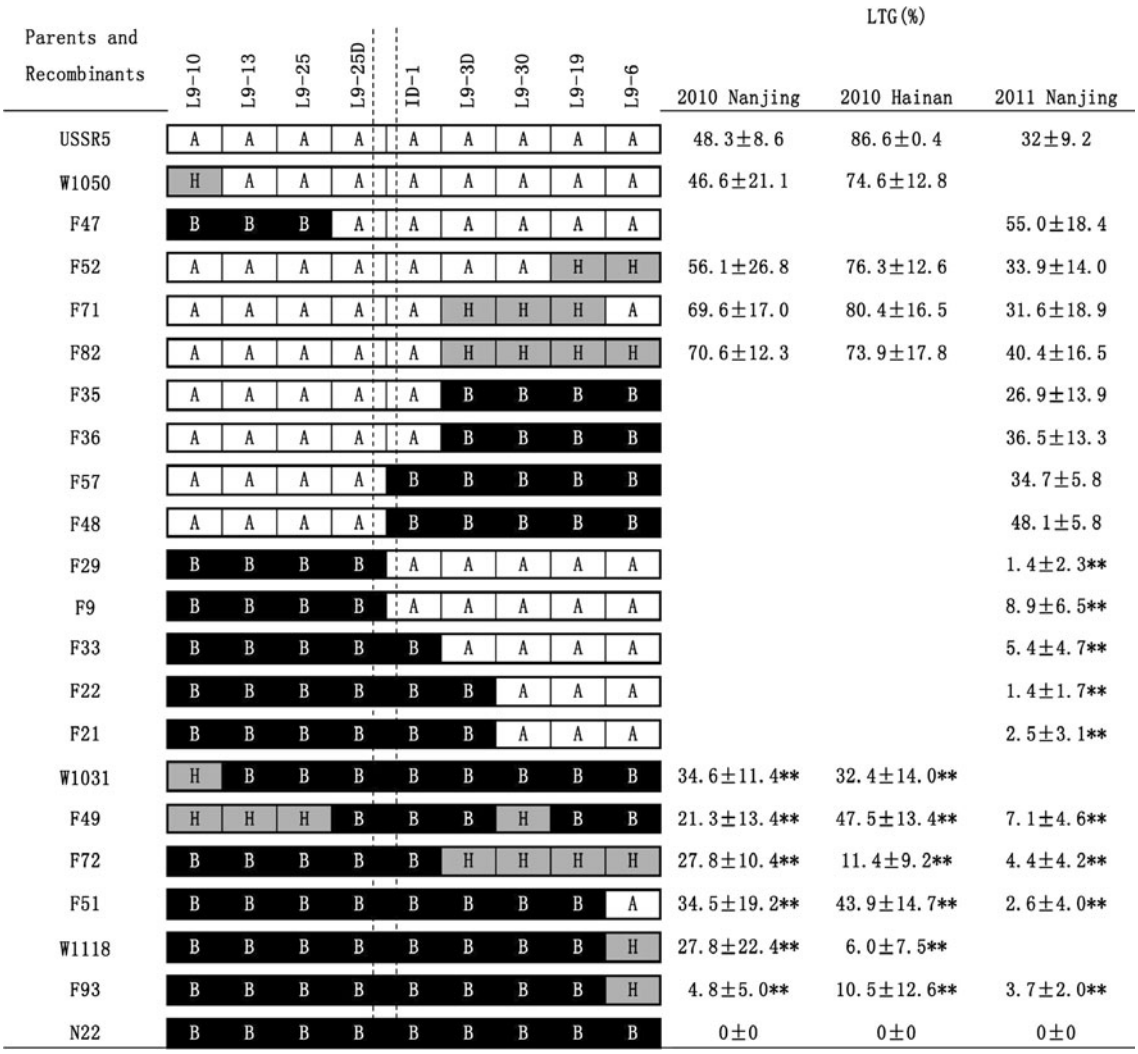

Fig. 4 Molecular marker genotypes and phenotypes of the recombinants identified in fine mapping. *White* (*A*), *gray* (*H*) and *black* (*B*) *rectangles* correspond to homozygous 'USSR5' allele, heterozygous 'USSR5'/'N22' alleles and homozygous 'N22' allele. Analysis of both the donor region and the phenotype of each recombinant permitted fine mapping of *qLTG-9*. These recombinants were selected from the BC_6F_2. The average LTG (±SD) at 5 DAI for each recombinant is listed on the *right*. **Significantly different from USSR5 at $P < 0.05$

(Fig. 7). These results confirmed that the LTG phenotype of USSR5 was reduced by insertion of the *qLTG-9* allele from N22.

Discussion

The ability to germinate under low-temperature conditions is a major determinant of stable plant establishment for direct seeding in temperate regions and at high altitudes in tropical regions. Many QTL associated with LTG were identified in recent years (Chen et al. 2006; Iwata and Fujino 2010; Fujino et al. 2004; Ji et al. 2008, 2009; Teng et al. 2001; Jiang et al. 2006) confirming that it is a complex agronomic trait (Ji et al. 2008; Sasaki and Honma 1974). In our work, three QTL for LTG (*qLTG-7*, *qLTG-9* and *qLTG-12*) were detected in a USSR5/N22 RIL population. Among them, *qLTG-7* and *qLTG-9* were detected at 4 and 5 DAI. *qLTG-7* might be an allele of a QTL reported by Jiang et al. (2006), but is not the same as two other QTL associated with LTG on chromosome 7 reported by Ji et al. (2008, 2009). The location of *qLTG-12* borders upon *qGR-12* (Ji et al. 2009) and may be the same gene. *qLTG-9* corresponds with the interval RM219–RM434 reported by Jiang et al. (2006), and differs from a QTL located in the interval C397B–RZ617B in a ZYQ8/JX17 DH population (Teng et al. 2001). In the Jiang et al. (2006) study using F_2 individuals from the same cross (USSR5/N22) as reported here, 11 putative QTL for LTG were detected, among which one was on each of chromosomes 3, 4, 7, 9 and 10, two were on

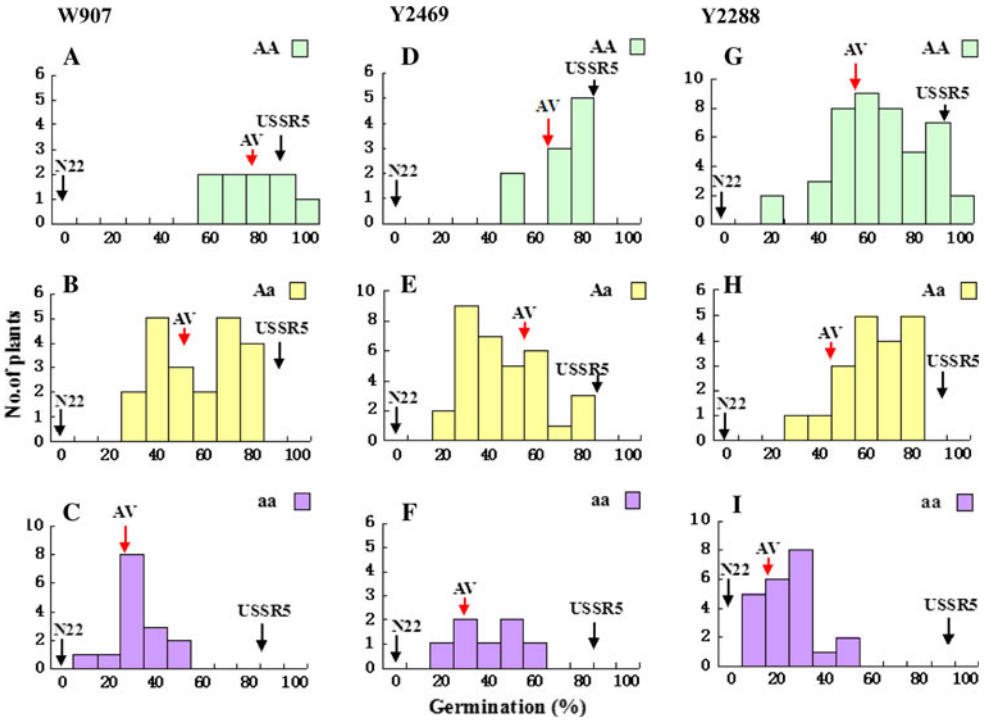

Fig. 5 Frequency distributions of LTG for three genotypic groups at 5 DAI. Genotypic detection was by RM434–RM 7038 in W907 (**a**–**c**), RM434–RM105 in Y2469 (**d**–**f**) and 9-L6/9–L25 in Y2288 (**g**–**i**). **a**, **d** and **g** were homozygous for the USSR5 segments, **b**, **e** and **h** were heterozygous, and **c**, **f** and **i** were homozygous for the inserted N22 segment. Average germination (AV) rates are indicated by *red arrows* (color figure online)

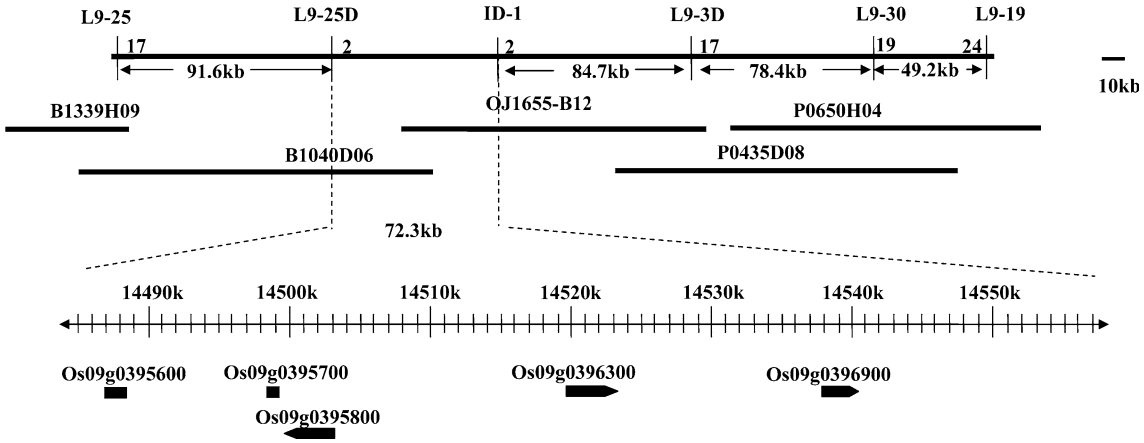

Fig. 6 High-resolution genetic and physical maps of *qLTG-9*, and gene prediction in the critical region of chromosome 9. The numbers of recombinant individuals are shown below the *markers*. Os09g0395600, Os09g0396300, Os09g0396900, Os09g0395700 and Os09g0395800 are genes annotated by RAP-DB in the critical 72.3-kb DNA fragment in Nipponbare

chromosome 11, and four were on chromosome 5. However, *qLTG-5-2* (with the maximum effect) and *qLTG-3* identified by Jiang et al. (2006) were not detected in the present work using an RIL population from the same cross. In the present research, *qLTG-9* was the most stable allele in the RIL population. A set of advanced backcross lines was selected by both marker-assisted and phenotypic selection, and the presence of *qLTG-9* was validated in different generations, years and locations. In all instances of a common main-effect QTL across populations, the direction of the parental contribution was the same. Although some QTL were detected only in F_2 or RILs, various factors, such as population size, population type, number of molecular markers, environmental conditions, methods, and critical standards of the germination test, may have affected the mapping results. Similar results were also reported by Yano et al. (1997) with regard to heading date in rice. They found different QTL for heading date using an F_2 population or BILs; some QTL for heading date were detected in the BILs, but not in the F_2 population, while other QTL detected in the F_2 population were not identified in the BILs. Some major QTL were detected in both populations (Yano et al. 1997; Lin et al. 1998). Later fine mapping using advanced backcross progeny or NILs confirmed most of

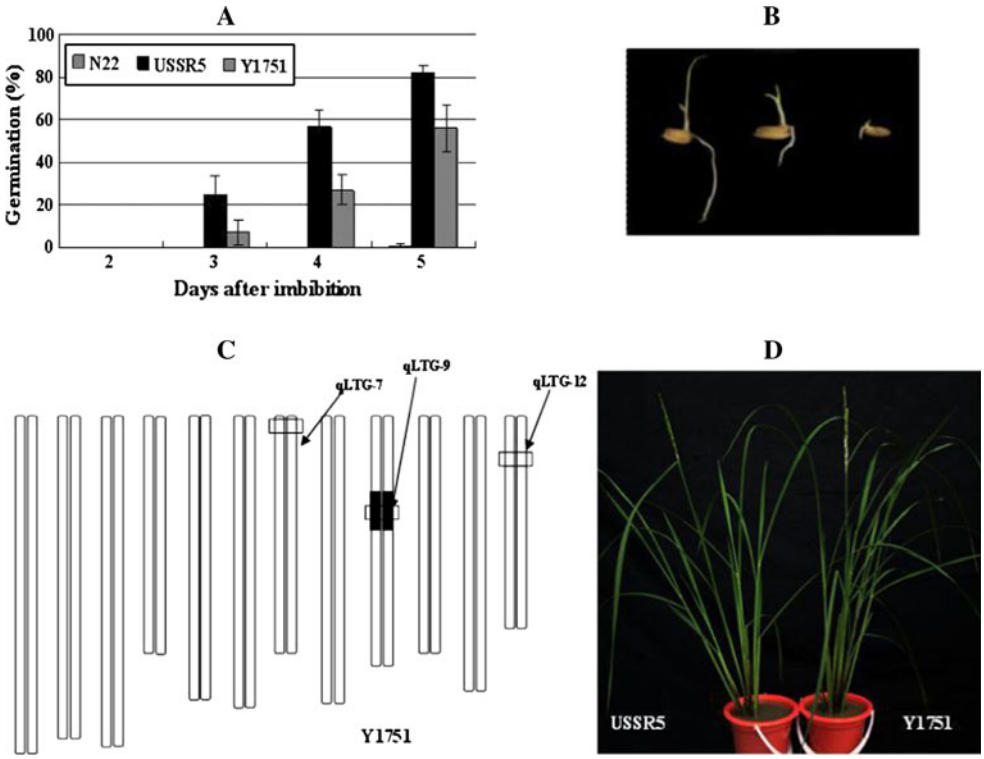

Fig. 7 Germination under low temperatures. **a** Low-temperature germination of USSR5, N22 and 1751 at 2 DAI and 5 DAI. *Error bars* indicate the SD. **b** L to R, USSR5, Y1751 and N22 at 12 DAI. **c** Genetic background of Y1751 and QTL identified in the USSR5/N22 RIL population. *Black square* represents the N22 insertion. *Hollow squares* are QTL detected in RIL. **d** Field phenotypes of USSR5 and Y1751 were very similar

the QTL as single Mendelian factors (Lin et al. 2002; Yamamoto et al. 2000).

The development of advanced backcross lines is an efficient procedure for map-based fine mapping of quantitative trait genes (Fujino et al. 2008; Sugimoto et al. 2010). To fine map and clone *qLTG-9*, it is necessary to construct NILs. Considering that N22 was strongly dormant and USSR5 strongly non-dormant (Lu et al. 2011; Jiang et al. 2006; Xie et al. 2011), we chose USSR5 as the backcross parent to exclude any influence on low-temperature germination from seed dormancy during construction of the AB populations. Phenotypic selection was conducted in the first four generations; then, marker-assisted selection combined with phenotypic selection was used in later generations to select the desired phenotypes and to purify the genetic backgrounds. The average LTG rate of lines carrying the N22 allele at the *qLTG-9* locus was decreased by 30–42 % at 5 DAI compared with the line carrying the USSR5 allele (Fig. 6). The allele from N22 delayed germination by 1 day, similar to the effect of *qLTG-3-1* (Fujino et al. 2008) from Livorno which accelerated LTG of Hayamasari by almost 2 days. So far, it is the QTL with the largest contribution ($R^2 = 35$ %) to phenotypic variation in LTG (Fujino et al. 2004; Fujino et al. 2008). Although the *qLTG-9* allele from N22 had an effect of a 1-day delay in germination, the NIL with the N22 allele showed a significant difference in germination from its counterpart in the 4–5th day after germination (Fig. 7). Because *qLTG-9* was the main QTL associated with faster germination and growth under low-temperature conditions, it could be useful for improving germination and early growth rate under low-temperature conditions in breeding programs. Moreover, it should be feasible to pyramid other QTL with *qLTG-9* to improve overall LTG. Fifteen BC_6F_2 populations comprising a total of 7,218 individuals were used for fine mapping and the extreme low and high LTG lines were used for fine mapping. Each recombinant was validated by progeny testing in multiple years to exclude environmental effects. We tested for significant differences using *t* tests and tried to distinguish different genotypes using not only average LTG rates, but also the distribution of LTG values within offspring populations to exclude environmental interference. Although the LTG values (34.6 %) of some recombinants (F51 and W1031) with the N22 (*qLTG9*) insertion were high (Fig. 4), the phenotypes of these recombinants were validated by progeny testing in multiple years. We also considered genetic background interference and used markers for selection. The BC_6F_2 population had a pure genetic background, and all lines used in fine mapping had a uniform genetic background of USSR5 except for the RM105–RM434 region (Fig. 7). Finally, *qLTG-9* was located in a 72.3-kb region with five potential candidate genes, among which we hope to clone and identify the presumed single gene responsible for low-temperature germination and then to elucidate its biological functions.

The mechanism of LTG is complex. Germination encompasses various events starting with the uptake of water by the mature dry seed and terminating with the

protrusion of the radicle through the seed coat. This process can be divided into three phases, a rapid initial uptake of water (phase I), a plateau phase (phase II) and then a further increase in water uptake after germination is complete and as the embryonic axes elongate (phase III). During each phase, many new mRNAs are transcribed. Although the *qLTG-3-1* gene (Fujino et al. 2004, 2008) was cloned, it had an unknown function. Other attempts at cloning have failed (Chen et al. 2006; Hou et al. 2004; Iwata and Fujino 2010; Jiang et al. 2006; Ji et al. 2008, 2009; Teng et al. 2001). Correlation between the tissue-specific expression of *qLTG3-1* and vacuolation of tissues covering the embryo strongly suggested that *qLTG3-1* was involved in tissue weakening, resulting in reduced mechanical resistance to growth of the coleoptile (Fujino et al. 2004, 2008), and microarray analysis indicated gene expression changes indicative of complex metabolic and signal pathways (Fujino and Matsuda 2010). Elucidation of the molecular mechanisms of low-temperature germination may occur if more genes can be cloned and characterized.

There are five candidate genes in the interval between markers 9-L25D and ID-1 in our study. One gene is Os09g0395600, the 13th member of xyloglucan endotransglucosylase (XTH/XET), which hydrolyzes O-glycosyl compounds and participates in carbohydrate metabolism. There is little information about the function of XTH/XET 13, but it is recognized as a cell wall-modifying enzyme that participates in diverse physiological roles (Cho et al. 2006; Yokoyama et al. 2010), including a role in endosperm cap weakening, a key process in regulation of seed germination in tomato (Chen et al. 2002). *CaXTH1*, *CaXTH2* and *CaXTH3* in capsicum have roles in early events of abiotic-related defense responses (Cho et al. 2006). Os09g0396300 is a member of the pyroglutamyl peptidase I family and participates in proteolysis. It participates in the degradation of thyrotropin-releasing hormone, which is thought to play an important role in the development of organs and tissues in rats (De Gandarias et al. 1994, 2000; Fuse et al. 1990; Agirregoitia et al. 2007). Os09g0396900 is a member of transmembrane family proteins with unknown function (DUF125). Expression of DUF125 was down-regulated in silicon-amended plants in rice (Brunings et al. 2009). The functions of the other two genes are unknown. Therefore, a transgenic complementation assay is proposed to gain an understanding of the mechanisms underlying regulation of low-temperature germination.

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