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ORIGINAL PAPER

QTL mapping and confirmation for tolerance of anaerobic conditions during germination derived from the rice landrace Ma-Zhan Red

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Abstract Wide adoption of direct-seeded rice practices has been hindered by poorly leveled fields, heavy rainfall and poor drainage, which cause accumulation of water in the fields shortly after sowing, leading to poor crop establishment. This is due to the inability of most rice varieties to germinate and reach the water surface under complete submergence. Hence, tolerance of anaerobic conditions during germination is an essential trait for direct-seeded rice cultivation in both rainfed and irrigated ecosystems. A QTL study was conducted to unravel the genetic basis of tolerance of anaerobic conditions during germination using a population derived from a cross between IR42, a susceptible variety, and Ma-Zhan Red, a tolerant landrace from China. Phenotypic data was collected based on the survival rates of the seedlings at 21 days after sowing of dry seeds under 10 cm of water. QTL analysis of the mapping population consisting of 175 $F_{2:3}$ families genotyped with 118 SSR markers identified six significant QTLs on chromosomes 2, 5, 6, and 7, and in all cases the tolerant alleles were contributed by Ma-Zhan Red. The largest QTL on chromosome 7, having a LOD score of 14.5 and an R^2 of 31.7 %, was confirmed using a BC_2F_3 population. The QTLs detected in this study provide promising targets for further genetic characterization and for use in marker-assisted selection to rapidly develop varieties with improved tolerance to anaerobic condition during germination. Ultimately, this trait can be combined with other abiotic stress tolerance QTLs to provide resilient varieties for direct-seeded systems.

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Introduction

Direct-seeded rice (DSR) cultivation is increasingly being practiced among farmers in both rainfed and irrigated ecosystems (Pandey and Velasco 2002). Labor scarcity, water shortage, and high production cost have become the major driving forces of this shift. However, large scale adoption of DSR practices has been held back by poor crop establishment or a total loss of the crop stand due to improperly leveled fields, heavy rainfall and poor drainage, which lead to the accumulation of water of varying depths immediately after sowing or during the early stages of seedling growth. Damage by excessive water can even take place in drought-prone areas with localized heavy rainfall or due to runoff from surrounding high lands. The hazards of flooding right after sowing or during germination can discourage farmers from adopting DSR technology or force them to abandon this practice (Konchan and Kono 1996). On the other hand, flooding the fields shortly after sowing has the benefit of helping in weed control, another major constraint in DSR ecosystems (Tuong et al. 2000; Ismail et al. 2012). Consequently, developing high yielding varieties that can withstand flooding during germination and

early growth is essential for sustainability of practicing direct seeding for rice crop establishment.

Tolerance to anaerobic conditions during germination (here referred to as anaerobic germination, AG) is a complex trait controlled by several families of genes that are involved in essential processes such as breakdown of starch, glycolysis, fermentation, and other biochemical and metabolic processes (Bailey-Serres and Chang 2005; Ismail et al. 2009, 2012). The breakdown of starch is a complex biochemical process modulated by both hormonal and metabolic regulations (Perata et al. 1997). Amylases are the key enzymes for starch degradation in germinating seeds, and rice varieties that have greater ability to degrade starch even under oxygen deprivation through successful production of α-amylase are more likely to vigorously germinate and survive the stress (Loreti et al. 2003). Oxygen deprivation triggers the switching from mitochondrial respiration through the Krebs cycle to fermentative metabolism, and under this condition, glucose supply from starch degradation, mainly through the action of the α-amylase genes in the *Amy3* subfamily, especially *Amy3D*, would allow ATP production through fermentative metabolism. Fermentation processes with ethanol as the main product are absolutely dependent on the availability of carbohydrates (Drew 1997). Key enzymes in the alcoholic fermentation pathway, alcohol dehydrogenase (ADH) and pyruvate decarboxylase (PDC), are induced by anoxia stress (Ricard et al. 1986). This pathway recycles nicotinamide adenine dinucleotide (NAD) to maintain glycolysis and substrate level phosphorylation, which could provide energy for successful germination through coleoptile elongation in the absence of oxygen (Matsumura et al. 1998; Saika et al. 2006; Shingaki-Wells et al. 2011; Ram et al. 2002). This in turn provides a higher probability for the germinating seed to make contact with air or the better-aerated surface water (Kordan 1974), thus increasing the chance for the young seedling to survive and to continue to grow.

Breeding DSR varieties with AG tolerance has been difficult in the past due to the limited number of tolerant donors and the complexity of the trait (Biswas and Yamauchi 1997; Yamauchi et al. 1993; Yamauchi and Winn 1996). Most rice varieties fail to germinate and thrive under anaerobic conditions in waterlogged and puddled fields, leading to poor or complete failure of seedling establishment. More recently, after screening thousands of rice accessions from the IRRI Genetic Resources Center (GRC), several landraces that are tolerant of AG stress have been identified (Angaji et al. 2010). Since then, genetic studies to unravel the molecular mechanisms of AG stress tolerance and breeding of AG tolerant varieties have been intensified (Septiningsih et al. 2012a). Exploration of novel QTLs for tolerance of anaerobic conditions during germination from diverse germplasm is essential to capture the complexity of the trait, to unravel the molecular basis of tolerance, and to simultaneously provide additional QTL targets for marker-assisted breeding. Khao Hlan On, a tolerant donor originating from Myanmar, was investigated for AG tolerance, leading to the identification of five QTLs, the largest of which was mapped on the long arm of chromosome 9 (*qAG-9-2*), having a LOD score of 20.3 and explaining 33.5 % of the variation for this trait (Angaji et al. 2010). The effect of this QTL was successfully confirmed, fine mapped and validation of the gene(s) underlying the QTL is underway (unpublished data). Other studies have reported a total of five QTLs identified on chromosomes 1, 2, 5, and 7 from recombinant inbred lines (RILs) of Kinmaze (*japonica*) and DV85 (*indica*) (Jiang et al. 2004), and two QTLs on chromosomes 5 and 11 from an F_2 population of USSR5 (*japonica*) and N22 (*indica*) (Jiang et al. 2006). Thus far, very few accessions have been explored through QTL studies for tolerance of AG stress conditions, hence, genetic studies using additional sources of tolerance are needed to identify novel QTLs.

The objectives of the present study were to identify QTLs in an $F_{2:3}$ population from Ma-Zhan Red, an AG tolerant landrace from China, and to confirm the presence of selected target QTL(s) in a backcross population. These QTLs will then provide the foundation for further investigation into the molecular mechanisms underlying the tolerance and to provide additional targets for varietal improvement through molecular breeding.

Materials and methods

Plant material and population development

F_1 seeds were developed from a cross between IR42, an AG susceptible variety, and Ma-Zhan Red, a highly AG tolerant landrace from China (IRGC 60184, Angaji et al. 2010). F_2 seeds were collected from an F_1 SSR marker-validated plant, and a total of 180 F_2 individuals were used to develop $F_{2:3}$ families for gathering phenotype data by screening under AG stress conditions. Plantings of all materials were conducted in the IRRI field using standard practices.

Screening for tolerance to anaerobic conditions during germination

Dry seeds were sown on trays filled with 1.5 cm of fine soil and the seeds were then covered by another layer of 1 cm of soil. Each tray has 34 × 17 holes and was sown with 11 entries, including 2 of the parental controls, IR42 and

Ma-Zhan Red, with 30 seeds used for each entry, and with one empty row between entries and on the outer sides to minimize errors during phenotyping. Twenty trays were used per replication to accommodate the 180 families and the controls. An Alpha Plus design was used to enable the randomization of all entries, including the two controls used in each tray. The trays were then laid on two concrete benches in a greenhouse, and submerged with 10 cm of tap water. Two replications were used (40 trays) and survival was counted 21 days after sowing. A seed germination test under normal conditions was conducted to examine the seed vigor and quality in petri dishes lined with a sheet of moist filter paper and incubated at 30 °C. A total of 30 seeds per entry were used, and the germination rate was scored after 7 days.

Linkage map construction

Leaf samples were collected from a bulk of $\sim$20 plants per family from the 180 $F_{2:3}$ families. Total genomic DNA was extracted according to Zheng et al. (1995). Genotyping was performed using SSR markers, and the PCR reaction was as described in Septiningsih et al. (2012b). Following this protocol, the PCR reaction was performed in a G-Storm GS1 thermal cycler (G-Storm Ltd., UK) in a total volume of 10 µl with 25 ng genomic DNA, 0.25–5 µM each of SSR primer, 200 µM dNTP mix, 1× PCR buffer (containing 50 mM KCl, 10 mM TRIS–Cl, pH 8.3, 3 mM MgCl), and 1 U of taq polymerase. The PCR profile was performed by initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 10 s, annealing at 55 or 60 °C for 10 s, and extension at 72 °C for 10 s, and a final extension at 72 °C for 2 min. However, for some primers with higher molecular weight or are difficult to amplify, we used the PCR reaction setting and the PCR profile described in Thomson et al. (2006) with slight modification, with a total volume of 10 µl instead of 15 µl was used. PCR reactions were performed using 96-well plates, the DNA fragments were separated on 6 % acrylamide gels (C.B.S. Scientific, USA), and stained with SYBR-Safe (Invitrogen) for manual allele scoring. A parental survey was conducted to identify the polymorphic SSR markers between the two parents, and a set of 1,066 SSR markers was used for the survey. Polymorphic markers that are relatively easy to score were selected for genotyping to assemble the linkage map. The linkage map was constructed using Map Manager QTX, vQTXb20 with Kosambi mapping function (Manly et al. 2001).

QTL analyses

The phenotypic and genotypic data were first analyzed for QTL mapping by interval mapping (IM) and composite interval mapping (CIM) using QGene v4.3.6 (Nelson 1997). Permutations of 10,000 iterations were used to determine the significance threshold to call the QTLs in QGene. For CIM, the method used was the stepwise cofactor selection, in which markers were used as cofactors, and maximum number of cofactor was selected automatically (F to add = 0.01 and F to drop = 0.01). The permutation LOD value at $p \leq 0.05$ was used as the threshold to declare the significance of the QTLs. For comparison, data were also analyzed with QTL Cartographer v2.5 (Wang et al. 2010) using both IM and CIM and 1,000 permutations. The cofactors in CIM were selected automatically using forward–backward stepwise regression with F-in = 0.01 and F-out = 0.01. The phenotypic and genotypic data were also analyzed using QTLNetwork-v2.1 (Yang and Zhu 2005; Yang et al. 2007; Yang et al. 2008). Permutation of 1,000 iterations was used to determine the threshold of the F-value of the QTLs. Digenic interactions were also analyzed using this program. QTL names were designated following the standard rice QTL nomenclature (McCouch 2008).

QTL comparisons

The QTLs detected in this study were compared with previously published QTLs to help determine whether QTLs in similar regions have been detected in other genetic backgrounds or for closely related traits, and whether the newly identified QTLs were novel. To enable the QTL comparison, the markers were placed on the physical map, in most cases using the Gramene website (http://www.gramene.org/markers/) or BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) performed against the reference rice sequence. If no sequence information was available for the peak marker, the nearest flanking markers with sequence information were used as a guide.

Development of advanced backcrossed populations for confirmation of the largest QTL, *qAG7.1*

Near isogenic line (NIL) development was initiated for the major QTL, *qAG7.1*, by backcrossing selected progenies to IR42, while maintaining a small introgression of Ma-Zhan Red in the QTL region and selecting against the rest of the genome using background markers. The pre-NILs of BC_2F_3 families derived from two individuals with the largest proportion of IR42 background (Suppl. Figs. 1, 2) were used to confirm the presence and effect of the QTL *qAG7.1*. For this QTL confirmation experiment, 76 lines with 2 replications of 30 individuals each were screened for AG tolerance (as described above), to assess the QTL effect. The 76 BC_2F_3 lines were derived from two individuals, MR1-5-5-9 and MR154-13-26-7. Based on the

background genotyping, MR1-5-5-9 had Mazhan Red introgressions on the top of chromosome 4 (RM335–RM5749), the top of chromosome 5 (RM5361–RM13), and in the middle of chromosome 10 (Suppl. Fig. 1). MR154-13-26-7 has background introgressions on the top part of chromosome 4 (RM518–RM5479) and in the middle of chromosome 8 (RM547–RM210) (Suppl. Fig. 2). The Tukey–Kramer test was used to compare the mean among the genotype categories using the flanking markers RM3583–RM21427 to determine the presence of the QTL.

Results

Phenotyping of the QTL mapping population

The germination tests under normal conditions showed that both parents, IR42 and Ma-Zhan Red, had a germination rate close to 100 %, on the other hand 5 out of 180 families had less than 80 % germination. The rest of the 175 families had a range of germination rates between 80 and 100 % with an average of 96.6 %. To improve the quality of the QTL analysis, families with low germination under normal conditions were removed, with only the 175 families with germination rates above 80 % used in the QTL study. The average survival rates of the parents, IR42 and Ma-Zhan Red, under submerged conditions were 3.3 and 63 %, respectively. Among the selected 175 families that were screened under submergence during germination, the range of survival rates was between 0 and 95 % (Fig. 1). The mapping population showed transgressive segregation in both directions, where 26 out of 175 families (14.9 %) had a survival rate higher than that of Ma-Zhan Red, while only 7 out of the 175 families (4 %) had a survival rate less than that of IR42.

Construction of the linkage map and QTL identification

A parental survey of IR42 and Ma-Zhan Red was performed using 1066 SSR markers, and out of these, 118 (11.1 %) were polymorphic and easy to score, and were subsequently used for the linkage map. The software MapManager QTX vQTXb20 (Manly et al. 2001) with the Kosambi function was used for constructing the map, resulting in a total length of 1,869.6 cM and an average of 17.6 cM distance between markers (Fig. 2).

At the significant levels of $p \leq 0.05$ and $p \leq 0.01$, the threshold LOD scores of 3.69 and 4.41 for IM and 3.72 and 4.55 for CIM, respectively, were established using 10,000 permutations in QGene (Nelson 1997). Similarly, the permutation LOD scores were 3.63 and 4.20 for IM, and 3.63 and 4.44 for CIM, respectively, using QTL Cartographer with 1,000 permutations (Wang et al. 2010). Based on the analysis, six QTLs above the permutation threshold were identified, and in all cases, the tolerant alleles came from Ma-Zhan Red. The largest QTL, located on the short arm of chromosome 7, *qAG7.1*, was consistently detected by both methods (IM and CIM) using both software packages (QGene and QTL Cartographer) with high LOD scores (permutation $p \leq 0.01$). The detection by QGene CIM showed a LOD score of 14.5 and a phenotypic variance (R^2) of 31.7 % (Table 1; Fig. 2, Suppl. Fig. 3). The presence of this QTL was also confirmed by QTL Network, with an additive effect (A) of 16.4 ($p \leq 0.001$) and a heritability for the additive effect ($h^2(a)$) of 0.27.

The second largest QTL was also detected on chromosome 7, *qAG7.2*, with a LOD score of 8.0 and an R^2 of 19 %; however, this QTL was only detected by IM in QGene (Fig. 2, Suppl. Fig. 3). The third largest QTL, located on the short arm of chromosome 6, *qAG6*, was identified by IM in both QGene and QTL Cartographer and

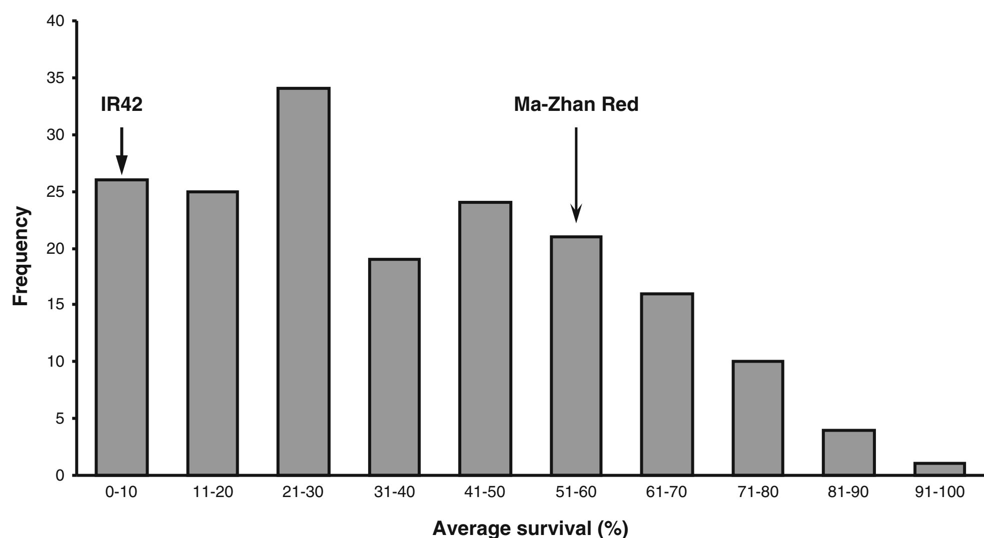

Fig. 1 Survival rate of the $F_{2:3}$ mapping population under flooded conditions during germination. The phenotypic distribution of the 175 families is shown, along with the average survival of the parents, IR42 and Ma-Zhan Red

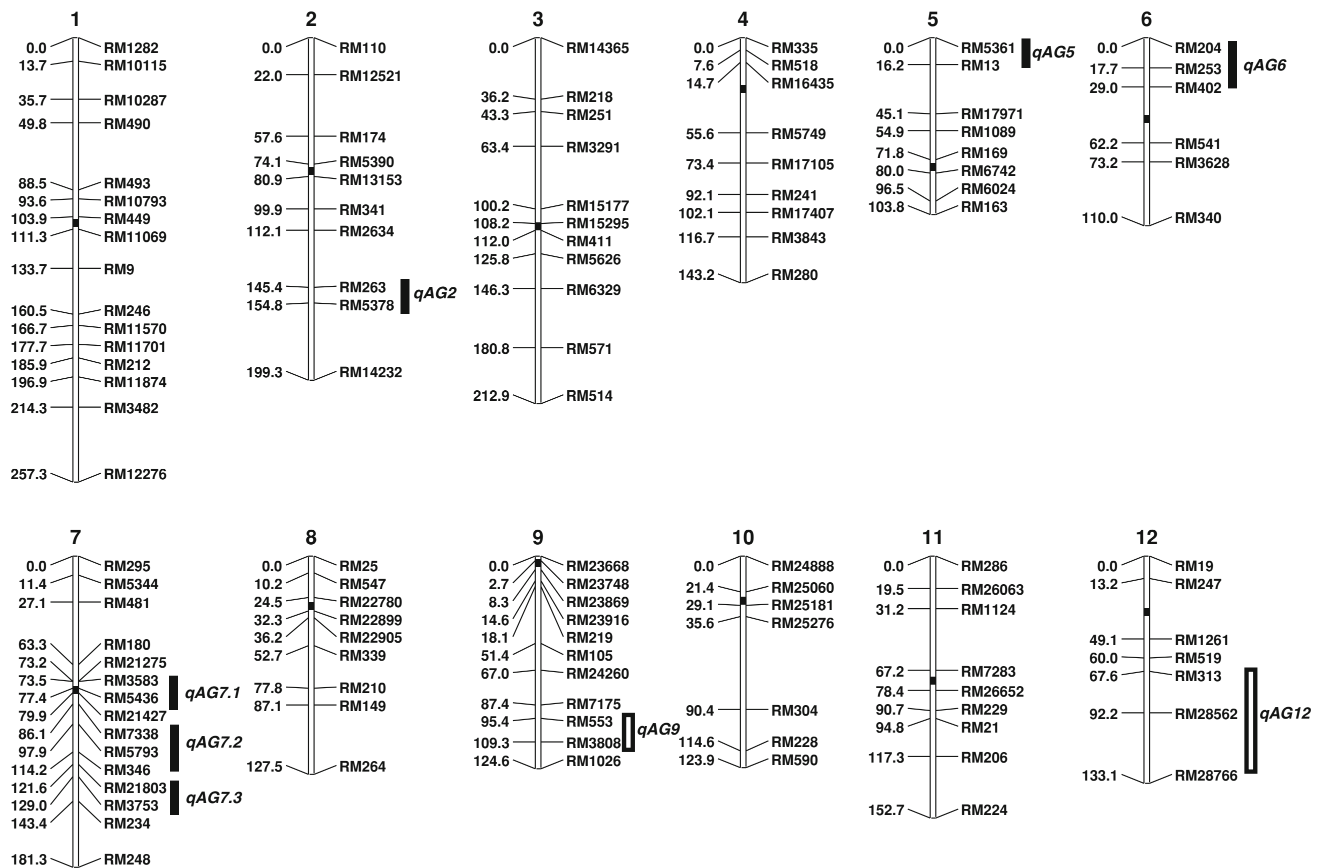

Fig. 2 Mapping of QTLs for tolerance of flooding during germination using a population derived from IR42 and Ma-Zhan Red. The molecular linkage map of an $F_{2:3}$ mapping population was constructed with 118 SSR markers. The position of six significant QTLs on chromosomes 2, 5, 6, and 7 are illustrated by *solid black bars* next to the chromosomes, while the position of two QTLs detected under permutation threshold but above LOD score of 3 on chromosomes 9 and 12 are depicted by *hollow black bars*. Centromeres are shown as *black boxes*

Table 1 QTLs for submergence tolerance during germination identified using the IR42/Ma-Zhan Red population

QTL	Chr.	Flanking markers	Peak marker	QGene IM			QGene CIM			QTL Cart. IM			QTL Cart. CIM		
				LOD	R^2	Add	LOD	R^2	Add	LOD	R^2	Add	LOD	R^2	Add
qAG2	2	RM263–RM5378	RM263/RM5378	*2.9*[a]	*7.4*	*7.9*	*2.9*	*7.5*	*7.1*	***3.3***[b]	***13.4***	**12.4**	**3.7**[c]	**9.3**	**10.3**
qAG5	5	RM5361	RM5361	*2.2*	*5.6*	*7.7*	**3.9**	**9.8**	**7.7**	*2.3*	*8.8*	*9.2*	**5.1**[d]	**11.8**	**10.7**
qAG6	6	RM204–RM402	RM253	**4.6**	**11.3**	**6.5**	***3.5***	***8.8***	***5.4***	**5.2**	**1.5**	**3.7**	**4.5**	**1.5**	**3.7**
qAG7.1	7	RM3583–RM21427	RM5436	**13.7**	**30.3**	**18.9**	**14.5**	**31.7**	**17.7**	**12.2**	**23.5**	**15.8**	**16.5**	**26.7**	**16.9**
qAG7.2	7	RM7338–RM346	RM5793	**8**	**19**	**18.1**	–	–	–	–	–	–	–	–	–
qAG7.3	7	RM21803–RM234	RM3753	**3.8**	**9.6**	**10.4**	–	–	–	**3.9**	**8.6**	**9.4**	–	–	–
qAG9	9	RM553–RM3808	RM553	–	–	–	–	–	–	–	–	–	***3.1***	***1.7***	***4.3***
qAG12	12	RM313–RM28766	RM28562	–	–	–	***3.2***	***8.2***	***3.7***	–	–	–	***3.5***	***5.3***	***7.3***

[a] Italicized LOD scores were below the experiment-wise significant threshold using permutation analysis but LOD >2

[b] Bold and italicized LOD scores were below the threshold but LOD >3

[c] Bold LOD scores were above the $p = 0.05$ threshold

[d] Underlined and bold LOD scores were above the $p = 0.01$ threshold

by CIM only in QTL Cartographer with LOD above $p \leq 0.01$. However, when this QTL was detected by CIM in QGene, the LOD was slightly below the permutation threshold but still above a score of 3 (LOD of 3.5). This QTL was detected by QGene IM with a LOD of 4.6 and an R^2 of 11.3 % (Table 1; Fig. 2). Detection of this QTL was

also confirmed by QTL Network with the nature of the QTL effect being a mixture of additive and dominant, with the additive effect of 8.4 ($p \leq 0.001$) and the dominant effect of 8.1 ($p \leq 0.05$). The fourth largest QTL on the short arm of chromosome 5, *qAG5*, was detected only by CIM method in both QGene ($p \leq 0.05$) and QTL Cartographer ($p \leq 0.01$). This QTL had a LOD value of 5.1 and R^2 of 11.8 % by CIM QTL Cartographer. The fifth largest QTL on the long arm of chromosome 7, *qAG7.3*, was detected only by IM in both QGene and QTL Cartographer (Fig. 2, Suppl. Fig. 3). In QTL Cartographer, this QTL had a LOD value of 3.9 and an R^2 of 8.6 %. The last QTL was on the long arm of chromosome 2, but was only detected by CIM QTL Cartographer with a LOD score of 3.7 and an R^2 of 9.3 %. This QTL was detected below the permutation threshold but above a LOD score of 3 by IM QTL Cartographer (LOD = 3.3). In addition to the total six QTLs detected above the permutation threshold, there were two additional QTLs detected with LOD >3, but below the threshold of $p \leq 0.5$. They were *qAG9* on chromosome 9 (LOD = 3.1 by Qgene CIM) and *qAG12* on chromosome 12 (LOD = 3.5 by QTL Cartographer CIM). These QTLs were also included in Table 1 and presented in Fig. 2 for the sake of comparisons with QTLs identified in other studies. In all cases, the tolerant alleles came from Ma-Zhan Red. There were no digenic interactions detected by QTLNetwork.

Confirmation of the largest QTL, *qAG7.1*

The presence of this QTL was confirmed in BC_2F_3 families segregating for *qAG7.1* (Fig. 3). Introgression lines harboring homozygous alleles of Ma-Zhan Red in the region of *qAG7.1* have significantly higher percentage survival (47.58 ± 4.72) compared to their counterparts with homozygous IR42 alleles across the QTL region (16.67 ± 2.99) with $p < 0.001$. Likewise, the difference between families carrying heterozygous alleles of the QTL (37.88 ± 3.80) with the families carrying the homozygous IR42 alleles were also highly significant ($p < 0.001$). However, the difference in survival rate between the heterozygous genotypes and those with the homozygous allele of Ma-Zhan Red in the QTL region was not statistically significant at $p < 0.05$. These results confirmed the QTL effect, with 30 % higher survival for the lines containing the homozygous Ma-Zhan Red introgression over the lines with IR42 alleles at *qAG7.1*. Moreover, the donor parent, Ma-Zhan Red (73.7 ± 14.19) had significantly higher survival than the introgression lines having the homozygous Ma-Zhan Red allele in the *qAG7.1* QTL region ($p < 0.05$), confirming that the other QTLs also contributed to the increased tolerance in addition to *qAG7.1*.

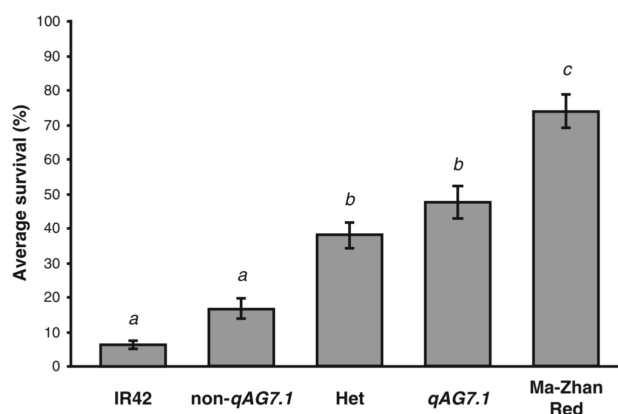

Fig. 3 Selected BC_2F_3 introgression lines in the background of IR42 were used for confirmation of the *qAG7.1*. The percent survival of the three genotype categories confirmed a 30 % higher survival for the Ma-Zhan Red homozygous allele (labeled as *qAG7.1*) over the lines with IR42 alleles at the target QTL (labeled as non-*qAG7.1*). The distinct letters have significantly different values based on the Tukey–Kramer test, and the *error bars* indicate the standard errors of each category

Discussion

With the increasing interest in DSR in both irrigated and rainfed systems, more attention is needed to improve AG tolerance for better crop establishment. Most of the tolerant donors we identified, however, are landraces that are not adapted to intensive systems (Angaji et al. 2010; unpublished data). These traditional varieties have several undesirable characters, including poor yield, susceptibility to lodging, long awns, and red or dark colored grains, which requires considerable time and effort to breed improved varieties using these donors. Therefore, identifying the major QTLs from these tolerant landraces and developing NILs in breeding-ready genetic backgrounds is essential to enable a more effective and efficient breeding strategy for DSR.

The survival rate of most of the families fell within the range of the parental values; however, a small number of the families (7 %) had lower survival rate than the susceptible parent IR42, while some of them (15 %) had higher survival rate than the donor parent Ma-Zhan Red. This phenomenon demonstrated some degree of transgressive segregation in the mapping population. In this case, even though IR42 is very susceptible and Ma-Zhan Red is highly tolerant, there were still some genetic factors from IR42 or a more favorable combination of alleles from the two parents contributing to higher tolerance in their progenies. Several studies have demonstrated similar transgressive variation, such as the QTL studies on yield and yield components, and grain quality from wild rice relatives (McCouch et al. 2007; Septiningsih et al. 2003; Thomson et al. 2003), and the recent QTL study on

submergence tolerance (Septiningsih et al. 2012b). However, as expected, all of the tolerant QTLs detected above the LOD score of 3 were derived from the tolerant donor Ma-Zhan Red.

The results of the QTL analysis revealed six QTLs above the permutation threshold located on chromosomes 2 (*qAG2*), 5 (*qAG5*), 6 (*qAG6*), and 7 (*qAG7.1*, *qAG7.2*, and *qAG7.3*) (Fig. 2). The largest QTL was *qAG7.1* with a LOD score of 14.5 and an R^2 of 31.7 % (Table 1). In addition, we have also identified two QTLs that were detected under the permutation threshold, but were above a LOD score of 3, located on chromosomes 9 and 12. In total, there were four QTLs above the permutation threshold detected by IM in QGene; however, only two QTLs were detected by CIM in QGene. In this case, only one QTL was commonly detected by both methods (*qAG7.1*). QTL Cartographer IM detected three QTLs, and all of them were also detected by QGene IM. However, QTL Cartographer CIM detected four QTLs, and one of them was novel (*qAG2*) and was only detected by QTL Cartographer IM with a LOD score of 3.3, which is under the permutation threshold. Therefore, the use of both QTL programs is useful for conformation and comparison of the detected QTLs. In most cases, by using both methods, IM and CIM, both QTL programs detected similar QTLs (Table 1). QTL Network, however, only detected two QTLs above the permutation threshold. It seems that this program uses more stringent criteria for declaring a significant QTL. However, in our study, we preferred to use all the three QTLs software, since they can complement each other.

Up to now, there have been limited QTL studies performed for tolerance to AG stress (Jiang et al. 2004, 2006; Angaji et al. 2010). The QTL detected on the long arm of chromosome 2 in this study, *qAG2*, is located in the similar region of *qAG2* (LOD = 4.1 and R^2 = 15.6 %) derived from Kinmaze identified between R418–C560 by Jiang et al. (2004). Our largest QTL, *qAG7.1* on chromosome 7, appeared to coincide with *qAG7.1* (LOD = 5.05, R^2 = 9.9 %) detected in our previous study (Angaji et al. 2010) between RID12–RM5606 with the peak marker RM7571, where the QTL originated from the tolerant donor Khao Hlan On. However, in the Angaji et al. (2010) study, this QTL was detected slightly below the permutation threshold of a LOD score 5.53. Likewise, the QTL *qAG7.3* identified here overlaps with *qAG7.2* (LOD = 9.68 and R^2 of 19.43 %; Angaji et al. 2010) between RM21868–RM172, where the tolerant allele was also derived from the tolerant parent Khao Hlan On. It will be interesting to investigate whether these QTLs located in the similar regions are controlled by the same gene(s). If that is the case, it will be intriguing to further examine the allelic differences between them and the relative contribution of each allele to tolerance.

Ella et al. (2010) reported that under AG stress, survival rates decreased significantly with older seeds and seeds stored at warmer temperature. This rapid loss of viability was associated with a decrease in superoxide dismutase and catalase activities and an increase of lipid peroxidation. This phenomenon suggested that varieties tolerant of aging would be useful in breeding for DSR. It is interesting to note that the largest QTL for AG stress in our previous study, *qAG-9-2* (Angaji et al. 2010), coincided with two large QTLs for seed longevity in different studies, *qLG-9* (Miura et al. 2002) and *RC9-2* (Sasaki et al. 2005). In this study, one QTL, *qAG7.2*, is located in a similar region as the QTL *RC7* for longevity with a LOD of 3.9 and an R^2 of 9.6 %; and another QTL between XNpb33–XNpb91 for germination and normal seedling growth of 2-year-old seeds, with a LOD of 4.7 and an R^2 of 9.6 % (Sasaki et al. 2005). It will be ideal to breed improved varieties that are tolerant to AG stress and also tolerant to seed aging, since this means seeds that have been stored for some time still could be used for DSR and the AG stress tolerance trait will be an important complementary trait to significantly improve seedling establishment.

Seed longevity and dormancy are especially important in the regions that have high temperatures and high humidity, such as in the tropics, because most seeds rapidly deteriorate under such conditions (McDonald 1999). Several studies showed that *Indica*-type seeds have better tolerance to seed aging than *Japonica*-types (Chang 1991; Ellis et al. 1992). Even though there are some exceptions, cultivars having high dormancy tend to have high longevity, while those with low dormancy quickly lose their viability (Ikehashi 1973; Siddique et al. 1988). The position of *qAG6.1* on the short arm of chromosome 6 was similar to that of the dormancy QTL reported by Cai and Morishima (2002) located near RZ144. In addition, the QTL *qAG7.2* overlapped with the dormancy QTL on chromosome 7 near R1440 identified by Lin et al. (1998). Likewise, *qAG7.3* was detected in similar regions as *qSD-7-2* near RM346 (Gu et al. 2004, 2005) and the QTL detected by Lin et al. (1998) close to R1245. Our QTL *qAG9* overlaps with *DORd* near RZ792 for seed dormancy (Cai and Morishima 2002). Lastly, the QTL *qAG12* was detected in a similar position as *DORc* close to G148 (Cai and Morishima 2002) and *qSD12* near RM270 (Gu et al. 2004, 2005). Interestingly, the gene underlying *qSD12* was cloned and found to promote production of ABA in early seed development to induce primary seed dormancy (Gu et al. 2010). Since the enhancement of coleoptile growth might be linked to a lower ABA synthesis under anoxia (Hoffmannbenning and Kende 1992), the effect of *qSD12* is opposite of what is needed for AG tolerance, indicating that an alternate allele is probably involved in our population, or that the gene underlying *qAG12* is

different from *qSD12*. It is also interesting to note that *qAG12* is located in a similar region as a QTL for GA3 response, *qGAR-12*, and a QTL for seedling height, *qSPH-12*, detected between C751–XNpb402 (Dong et al. 2006). According to Horton (1991), GA is one of the important plant hormones that promote coleoptile growth under anaerobic conditions. Therefore, it is possible that the gene(s) related to GA3 could be responsible for controlling *qAG12*, which warrants further investigation.

In addition to tolerance to AG stress, it is essential to breed improved varieties that can tolerate low temperature during germination (LTG) and early seedling establishment, especially for temperate or high altitude tropical regions, because rice seeds are more sensitive to LTG under flooded conditions (Ella et al. 2010). Several of our QTLs are mapped in similar regions as QTLs identified by Iwata et al. (2010) for stable seedling establishment (SES) under direct seeding in flooded fields and low temperature. In this case, *qAG2* is in a similar region as *qSES2* between RM3515–RM5706, and *qAG7.2* is in a similar position as *qSES7-2* between RM5543–RM6394. The tolerant allele for these SES QTLs was derived from Arroz Da Terra, the tolerant parent. Likewise, *qAG5* is in a similar region as *qSES-5-1* between RM4777–RM3906, and the tolerant allele in this QTL was from Italica Livorno, another tolerant parent. The QTL *qAG7.2* also overlapped with the QTL for shoot length of seedling under low temperature, mapped between C285–RM11 (Zhang et al. 2005a), while the QTL *qAG12* was mapped in the similar position as a QTL for seedling height under low temperature, *qCSH12* mapped between RM270–RM17 (Han et al. 2007). Lastly, the QTL *qAG5* overlapped with a QTL for tolerance of LTG, *qLTG-5-1* near RM405 (Jiang et al. 2006).

Seedling vigor is an essential trait for DSR. Together, this trait and tolerance to AG stress requires energy generated from the breakdown of starch in the endosperm, before the seedlings become autotrophic. It is expected that amylase genes and other related downstream genes are associated with germination rate and early seedling growth (Williams and Peterson 1973; Sasahara et al. 1986; Hwang et al. 1999; Ismail et al. 2009). Since weed invasion is one of the main problems in DSR (Chauhan 2012; Ismail et al. 2012), varieties with high early seedling vigor and tolerance to AG stress are desired to ensure enhanced crop establishment and better weed competitiveness. Our QTL *qAG2* co-localized with a QTL for seedling/early vigor (SEV), *qSEV-2-2* between RM221–RG139 (Lu et al. 2007), while the QTL *qAG7.2* mapped in a similar position as a QTL for shoot length between RG678–C285 (Zhang et al. 2005b). Whether the gene(s) underlying these overlapping QTLs are the same gene(s) having pleiotropic effects or different genes that are tightly linked, remains to be seen. However, these QTLs that localize with others identified in the current study seem to control traits that are relevant to seed germination and seedling growth under early flooding and variable weather and management conditions (Ismail et al. 2012; Ella et al. 2010, 2011).

QTL confirmation is an important step before selecting a target QTL for further study or for use in breeding, to ensure that the QTL is not a statistical artifact and to more accurately measure the size of its effect. Using BC_2F_3 families segregating for the Ma-Zhan Red introgression at RM3583–RM21427, the major QTL *qAG7.1* was confirmed to function in a largely IR42 background (Fig. 3). On average, *qAG7.1* provided a 30 % increase in the rate of survival compared to IR42 alleles at *qAG7.1*, which was similar to the phenotypic effect detected in the original QTL analysis based on the additive effect of 16.4 % higher survival for each allele (or 32 % for the homozygous Ma-Zhan Red introgression). However, the difference in survival rate between the heterozygous genotypes and those with the homozygous allele of Ma-Zhan Red in the QTL region was not statistically significant, indicating a partial dominance effect, in contrast to the original QTL study which showed a largely additive effect for *qAG7.1*. Currently we are developing NILs to further measure the precise allele effect at *qAG7.1* and to characterize the molecular and physiological mechanisms underlying this QTL.

Thus far, there have been no major QTLs for tolerance of AG stress that possess sufficient phenotypic value to be introgressed individually for marker-assisted breeding as the case with the *SUB1* QTL (Xu et al. 2006; Septiningsih et al. 2009). All the AG QTLs reported in the literature or the ones we have identified so far (Angaji et al. 2010, and herewith) have smaller effects than *SUB1*. Therefore, an appropriate breeding strategy could involve combining multiple AG QTLs in a suitable background. However, the best and most effective combination of these QTLs still needs to be determined. Fine mapping and ultimately cloning and gene characterization will be conducted for the most promising QTL targets. The current set of flanking markers, however, can immediately be used for marker-assisted breeding activities. Once the QTLs are fine mapped, more closely flanking markers can be used and ultimately, once the gene(s) underlying the QTL(s) have been isolated and their functions have been determined, functional markers can be used for more precise marker-assisted breeding. Gene-based markers and closely linked flanking markers are important in the context of QTL pyramiding, to reduce negative linkage drag from multiple QTLs being introduced. Furthermore, once the genes underlying the QTLs can be cloned and characterized, we will be able to deepen our understanding of the molecular mechanisms of tolerance to AG stress.

The QTLs identified in this study and in our previous study using Khao Hla On (Angaji et al. 2010) are

promising targets for pyramiding of multiple QTLs through molecular breeding. If the selected major QTLs from multiple donors are complementary to each other, resilient varieties suitable for DSR even under less favorable ecosystems can be developed. Currently, the two QTLs with the largest effects on AG are *qAG-9-2* derived from Khao Hla On and *qAG7.1* derived from Ma-Zhan Red. These two loci are now being pyramided into improved breeding lines to determine if the resulting lines will combine the additive effects of both QTLs.

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