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ORIGINAL ARTICLE

The genetic basic and fine-mapping of a stable quantitative-trait loci for aluminium tolerance in rice

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Abstract Aluminium (Al) toxicity is a primary cause of low rice productivity in acid soils. We have mapped a number of quantitative-trait loci (QTL) controlling Al tolerance in a recombinant inbred line population derived from a cross between the tolerant *japonica* cultivar Asominori and the sensitive *indica* cultivar IR24. Tolerance was assessed on the basis of relative root elongation. QTL were detected on chromosomes 1, 9, and 11, with the percentages of phenotypic variance explained ranging from 13.5 to 17.7%. Alleles from Asominori at all three QTL were associated with increased Al tolerance. *qRRE-9* is expressed both in the genetic background of IR24 and in an Asominori/IR24-mixed background. *qRRE-9* was reduced to the single recessive Mendelian factor *Alt-9*. High-resolution genetic and physical maps were constructed for *Alt-9* in a BC_3F_2 population of 1,043 individuals. *Alt-9* maps between *RM24702* and *ID47-2* on chromosome 9, and co-segregates with *RM5765*.

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Keywords Aluminum tolerance · Fine-mapping · Indel marker · Rice (*Oryza sativa* L.) · Simple sequence repeats

Abbreviations

CSSL	Chromosome segment substitution line
LOD	Log of the odds ratio
QTL	Quantitative trait locus
RIL	Recombinant inbred line
RRE	Relative root elongation (%)
SSR	Simple sequence repeat
SRE	Stress root elongation at 100 μM Al (cm)
CRE	Control root elongation in control under non-stress condition (cm)

Introduction

Aluminum (Al) is one of the most abundant components of cultivated soil, occurring in various forms, depending on the local soil pH. Where this falls below 5.0, Al is ionized, and in this form is highly toxic to plants. Al toxicity is considered to be one of the primary causes of low rice (*Oryza sativa* L.) productivity in acid soils. The extent of tolerance among the small grain cereal crops follows the general pattern rice ≥ rye > wheat > barley, although some genetic variation for tolerance exists within each of these species (Foy 1988), and specifically in rice (Sivaguru et al. 1992). Molecular markers linked to genes or quantitative-trait loci (QTL) conferring Al tolerance have previously been identified in wheat (Riede and Anderson 1996), rye (Gallego et al. 1998; Miftahudin et al. 2002), maize (Sibov et al. 1999; Ninamango-Cardenas et al. 2003), and barley (Tang et al. 2000; Ma et al. 2004). In rice, a number of Al tolerance conferring QTL were identified in a random inbred mapping population derived from the cross

Azucena × IR1552 (Wu et al. 2000), while Nguyen et al. (2001) were able to map a major Al tolerance QTL on chromosome 1. Ten Al tolerance QTL, dispersed over nine chromosomes, were mapped in a doubled-haploid population derived from the cross CT9993 × IR62266 (Nguyen et al. 2002) and three were placed on chromosomes 1, 2, and 6 by Ma et al. (2002). The latter authors also used a cross between a susceptible mutant (als1) and a wild type to define a specific gene (*Als1*) mapping on the long arm of chromosome 6 (Ma et al. 2005). Finally, five QTL based on the measurement of relative root length (RRL) have been identified from various noncultivated *Oryza* species (Nguyen et al. 2003). Thus the Al tolerance trait is clearly controlled by multiple genes (Gallego and Benito 1997), and as a result, the mechanistic control of tolerance remains poorly understood.

Various approaches have been employed to attempt the isolation of Al tolerance genes, and these have led to the cloning of genes from wheat (Hamel et al. 1998; Sasaki et al. 2002, 2004), *Arabidopsis thaliana* (Richards et al. 1998), rye (Milla et al. 2002), and sugarcane (Watt 2003). However, Al-tolerance genes or QTL are yet to be fine-mapped and cloned in rice. Conventional biparental populations are not generally suitable for the fine mapping of QTL, as each segregant typically inherits multiple large chromosomal segments from one or other parent. On the other hand, chromosome segment substitution lines (CSSLs) or near-isogenic lines (NILs) are advantageous for QTL identification. Most importantly, in these materials, genetic interactions are limited to those involving genes present on a small number of regions, since each line carries only one, or at most a small number of introgressed segments in a homogeneous and common genetic background. Fine mapping then requires the construction of secondary populations through backcrossing a particular CSSL/NIL with the recurrent parent.

The objectives of the current study were to (1) identify Al tolerance QTL/genes in a recombinant inbred (RIL) population, (2) confirm validate these QTL using CSSLs, and (3) attempt to reduce a specific QTL into a single Mendelian gene.

Materials and methods

Plant materials

A population of 71 RILs derived from the cross of *Oryza sativa* L. *japonica* variety Asominori × *Oryza sativa* L. *indica* variety IR24 (Tsunematsu et al. 1996a, b) were used for the initial QTL analysis. These QTL were then validated in a population of 66 CSSLs in an IR24 background (seeds obtained from A. Yoshimura, Kyushu University, Japan). The F_2 of the backcross (CSSL51 × IR24) × IR24 formed the fine-mapping population. In all, 192 BC_2F_2 progeny, together with 20 BC_2F_3 offspring per each BC_2F_2, were grown at the experimental station of the Jiangsu Academy of Agricultural Sciences. Later, 224 homozygous Al tolerant selections, selected from among 1,043 BC_2F_2 individuals, were used for fine mapping.

Phenotypic evaluation

Al tolerance was evaluated following the procedure of Ma et al. (2002), with minor modifications. A dose response experiment was first conducted to contrast the performance of Asominori and IR24. First, seeds were soaked overnight in de-ionized water and then allowed to germinate for 4 days at 27 ± 2°C on plastic nets floating over a solution of 0.5 mM $CaCl_2$ (pH 4.5). Ten uniformly germinated seedlings were exposed for 24 h to a 0.5 mM $CaCl_2$ (pH4.5) solution containing 0, 50, 100, 150 or 200 μM $AlCl_3$. Root length was measured both before and after the Al treatment. The largest effect on root elongation was observed under 100 μM $AlCl_3$ and so this concentration was chosen for the population screening.

The Al tolerance of the RILs, CSSLs, and parents was quantified by the difference in root elongation between the control (0 μM) and 100 μM $AlCl_3$. The experiments were arranged in a randomized block design with two replications (each of ten seedlings per line). The plants were maintained at 27 ± 2°C for 12 h under 300-μmol photons m^{-2} s^{-1} of light, and the culture solution (0.5 mM $CaCl_2$ pH4.5) was renewed daily. Root lengths were recorded before and 24 h after the onset of the treatment. A root tolerance index (RRE) was generated by SRE/CRE where SRE was root elongation under Al stress, and CRE the root elongation under non-stressed conditions. The same method was used to phenotype 20 seedlings of IR24, CSSL51 and each individual BC_2F_2 and BC_2F_3 progeny.

Genotypic analysis

DNA was extracted from fresh leaves following Dellaporta et al. (1983). The PCR protocol of Chen et al. (1997) was adopted, with a few minor modifications. PCR products were separated by 8% non-denaturing polyacrylamide gel electrophoresis, and detected by silver staining (Sanguinetti et al. 1994). To genetically saturate the target region in the centromeric region of rice chromosome 9, both published (Wu et al. 2000; McCouch et al. 2002) and newly developed molecular markers were employed. The latter were generated from the Nipponbare rice genome sequence (http://www.rgp.dna.affrc.go.jp), using Primer 5 and SSRIT software (http://www.gramene.org/db/searches/ssrtool). Loci likely to be polymorphic were identified by a comparison

between the genomic sequence of Nipponbare and the *indica* cultivar cv 93-11 (http://www.rice.genomics.org.cn/index.jsp).

QTL analysis

The RIL population linkage map consists of 375 loci (Tsunematsu et al. 1996, b; Kubo et al. 2002). QTL mapping was performed using QTL Cartographer software v1.13 (Liu 1997; Basten et al. 1999). Permutation tests defined a LOD threshold of 2.94 to ensure a type I error of <0.05. This threshold rejects the QTL *qRRE-9*, but as this QTL has been identified in a number of other independent studies, a less-stringent LOD threshold of 2.6 was adopted. The additive effects and relative contributions of each QTL were analyzed using composite interval mapping. Although epistatic QTL × QTL interactions may be of some importance, these were ignored, as the major objective was to identify major QTL contributing stably to Al tolerance. In any case, statistical methods capable of mapping epistasis are not yet fully developed. The standard *t* test was used to test for significance of differences in Al tolerance. QTL nomenclature followed the suggestions of McCouch et al. (1997).

Mapping of *alt-9*

The response to Al stress of the BC_2F_2 progeny was characterized as being either tolerant (similar to Asominori or CSSL51) or sensitive (like IR24). Based on the pattern of segregation in the BC_2F_3 generation, the BC_2F_2 individuals were further classified as fixed tolerant plants (*alt-9alt-9*), segregating (*Alt-9alt-9*) or fixed Al sensitive (*Alt-9Alt-9*). Genetic mapping of *Alt-9* was performed with Mapmaker/Exp 3.0 (Lander et al. 1987). Fine mapping used the bulked-extreme and recessive-class approaches, as described by Zhang et al. (1994), and this allowed for the estimation of recombination frequencies (*c*) between *Alt-9* and molecular markers for the 224 (out of 1,043) *alt-9alt-9* individuals. *c* is given by $(N_1+N_2/2)/N$, where *N* is the overall number of tolerant plants, N_1 the number of tolerant individuals having the sensitive parent's molecular marker profile, and N_2 the number of tolerant plants having a heterozygous molecular marker profile.

Results

Phenotypic performance

The CRE of the RILs varied from 0.58 to 3.43 cm, a range, which includes that of both IR24 (2.01 ± 0.16 cm) and Asominori (1.42 ± 0.12 cm). The mean RIL CRE lay closer to that of Asominori than to IR24 (Fig. 1a). The SREs of IR24 and Asominori were, respectively, 0.82 ± 0.08 and 1.24 ± 0.11 cm (Fig. 1b). Among the RILs, the distribution was continuous, ranging from 0.25 to 1.65 cm, with a mean of 0.83 cm. The mean and variance of RRE in the RIL population are 0.54 and 0.0487, respectively. The average RRE values are 0.41 and 0.87, for the

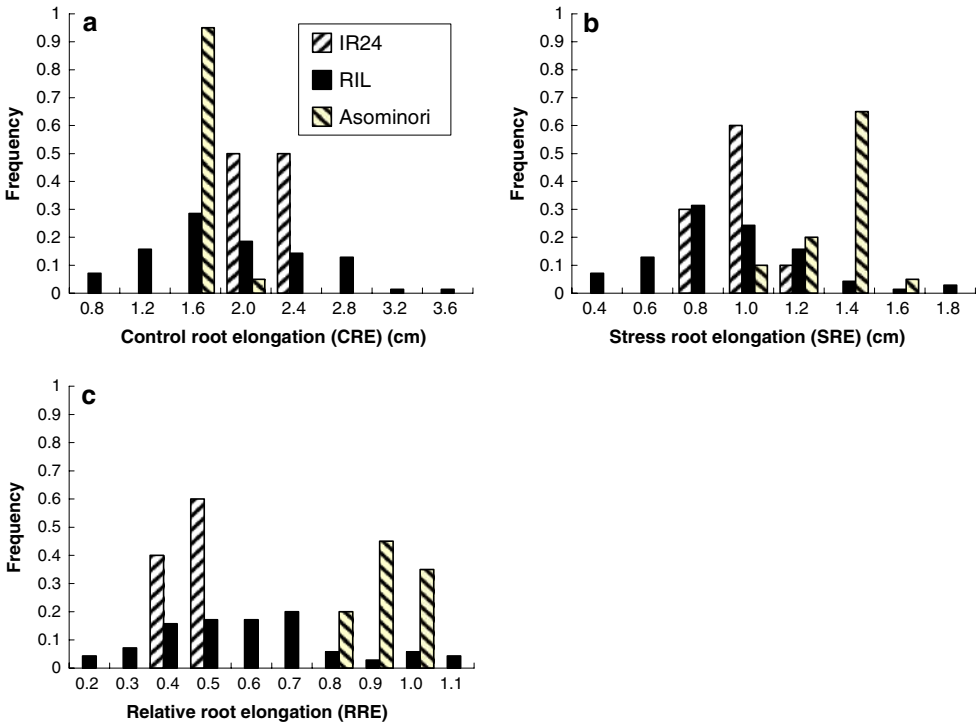

Fig. 1 Frequency distribution of 71 RILs for CRE, SRE and RRE

two parents IR24 and Asominori, respectively. Under the assumption of the normal distribution of RRE in the RIL population, the probability that an RIL has an RRE value less than IR24 is 0.27, and the probability that an RIL has an RRE value greater than Asominori is 0.07. Both probabilities are higher than 0.05, indicating the transgressive segregation in both directions, i.e. less than the lower parent IR24 and greater than the higher parent Asominori. RILs showed a certain transgressive segregation and normally distributed for the three traits, indicating they were controlled by multiple genes (Fig. 1). Although the IR24 CRE is higher than that of Asominori, the latter showed both a higher SRE and RRE. This demonstrates that Asominori is more tolerant of Al toxicity than IR24.

QTL mapping in the RIL population

Among the three root growth metrics, RRE is considered to be the best assessor of Al tolerance. In our experiments, this trait had a broad-sense heritability of 79.6%. Three QTL (denoted *qRRE-1*, *qRRE-9*, and *qRRE-11*) were identified, mapping to, respectively, chromosomes 1, 9, and 11. The LOD score of these loci ranged from 2.64 to 3.60, and the proportion of the phenotypic variance explained from 13.5 to 17.7% (Table 1; Fig. 2). As Asominori contributed the positive alleles (less affected by stress) at all three loci, these QTL cannot explain the transgression observed in the population. However, a number of minor (non-significant) QTL peaks, where Asominori contributed a negative effect, were also observed, and segregation for these is therefore the most likely basis for the transgression observed in the RIL population.

To validate the three QTL, 11 CSSLs containing the critical QTL segments were identified. In CSSL2, -41 and -45, the IR24 segment on chromosome 1 defined by *R2635* and *C1370* is replaced by one derived from Asominori. The RREs of CSSL2 and -41 were both significantly higher than that of CSSL45. The overlapping of introgressed segments allows the inference that *qRRE-1* maps close to marker locus *R2635* (Fig. 3). *qRRE-9*, is located between *C609* and *C1263* on chromosome 9. Six CSSLs (CSSL46, -47, -49, -50, -51, and -58) all carry a segment flanked by *C1263* and *XNpb293* (Fig. 2), indicating that this QTL lies close to

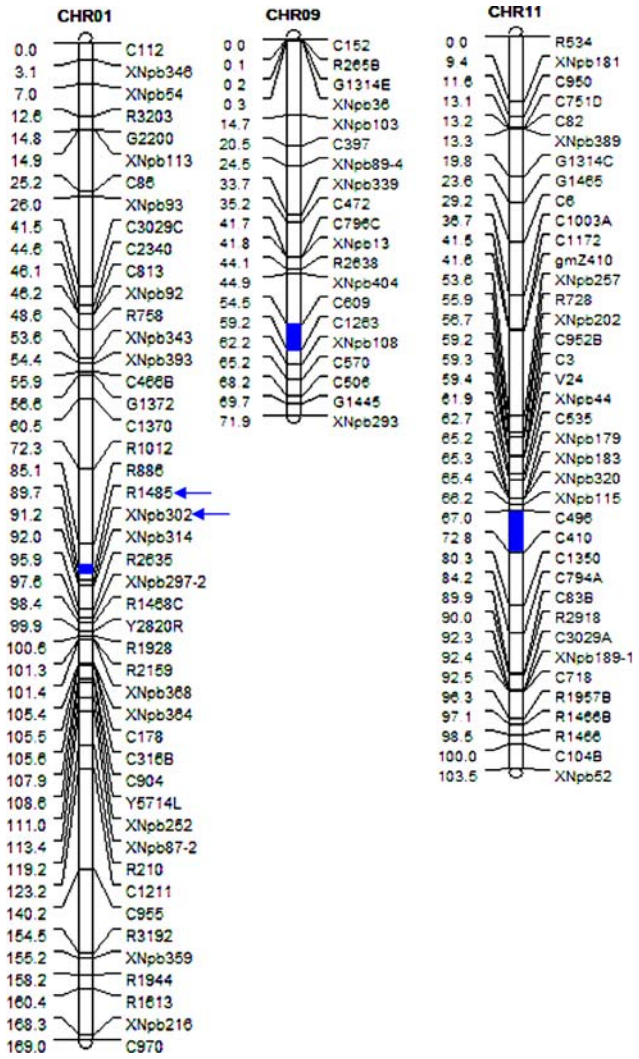

Fig. 2 QTL location of Al tolerance on the Asominori × IR24 linkage map. *Vertical bars* indicate the position of RRE QTL. *Arrows* indicate the flanking markers of the QTL on chromosome 1

C1263. On chromosome 11, the segment *C1350-XNpb320* includes *qRRE-11*. The RREs of CSSL58, -59, and -60 suggest the presence of *qRRE-11*. Note that CSSL58, which carries two QTL segments derived from Asominori, had a higher RRE than any line carrying only a single QTL, indicating that *qRRE-9*, and *qRRE-11* act additively to increase RRE.

Table 1 Putative QTLs for relative root elongation (RRE) detected in the RILs of Asominori and IR24

QTL	Chromosome	Marker interval	LOD score	Percentage of variance explained (%)	Additive effect
qRRE-1	1	R1485-XNpb302	3.60	17.68	0.26
qRRE-9	9	C609-C1260	2.71	13.90	0.23
qRRE-11	11	C496-C410	2.64	13.53	0.22

Additive effect was associated with Asominori alleles

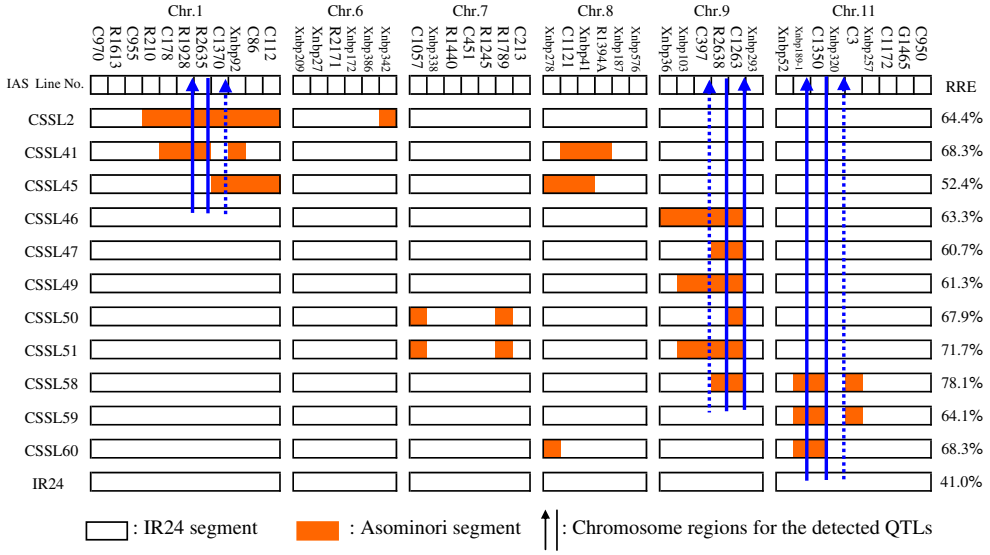

Fig. 3 Graphical genotypes of the Asominori/IR24 CSSLs. The CSSLs harboring RRE QTL are indicated on the *left* and the RRE for each CSSL on the *right*

qRRE-9 is a single Mendelian factor

The RRE frequency distribution among the BC_2F_2 individuals was bimodal, with a discontinuity around 0.65 (Fig. 4; Wang and Gai 2001). The ratio of sensitive to tolerant individuals is consistent with the presence of a single locus ($\chi^2(3:1) = 0.840 < 3.84$, $P = 0.05$). Similarly, the BC_2F_3 families fell into 54 fixed tolerant, 98 segregating and 40 fixed sensitive (Table 2), fitting the monogenic 1:2:1 segregation pattern ($\chi^2(1:2:1) = 1.825 < 5.99$, $P = 0.05$). As *qRRE-9* could therefore be considered as a single Mendelian factor, it was re-designated *Alt-9*. Linkage analysis allowed it to be mapped to a 6.7-cM region flanked by *RM533* and *RM215* (Fig. 5).

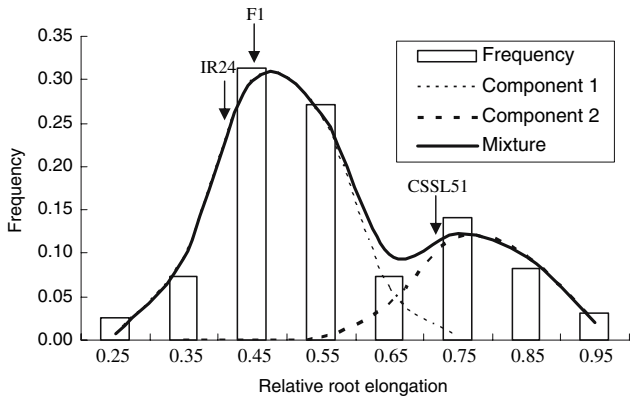

Fig. 4 Frequency distribution of 192 BC_2F_2 (CSSL51 × IR24) × IR24 individuals for RRE. Component 1, Al-sensitive individuals; component 2, Al-tolerant individuals. Mix indicates a bimodal model with a discontinuity around an RRE of 0.65

High-resolution mapping of *Alt-9*

Recombination between *Alt-9* and various molecular markers in the *RM533-RM215* segment is depicted in Fig. 5. The novel markers consisted of one indel and four SSRs. Among these, *RM24702* and *ID47-2*, each showed three recombinants out of the 224 homozygous tolerant plants, allowing *Alt-9 to* be localized within the 0.9 cM *RM24702-ID47-2* region. *RM5765* was completely linked to *Alt-9*.

Discussion

Measuring Al tolerance

A reliable assay is essential to accurately phenotype the response to Al stress. Both absolute root length and relative root length (RRL), and the derived root tolerance index (RTI), have been widely used for determinations (Wu et al. 2000; Nguyen et al. 2001, 2002, 2003). The parameter RRL is the ratio between maximum root length under Al treatment and the maximum root length of control plants after 2 to 4 weeks. This value is affected not only by Al stress, but also by factors such as differential nutrient uptake (Ma et al. 2002). Previous studies have shown that Al toxicity rapidly (within 1 h) inhibits root elongation (Ryan et al. 1992). Therefore we evaluated tolerance by following root elongation over a 24-h period. RRE was used as the primary parameter in the QTL analysis to overcome any potentially confounding effect of differences in inherent root growth rate among the various genotypes.

Table 2 Performance of aluminum tolerance in BC_3F_2 and BC_3F_3 populations derived from CSSL51 and the recurrent parent IR24

a. BC_3F_2 population (in 2004 and Nanjing) ($\chi_{3:1}$ test)

Types	No. of plants observed	No. of plants expected	|O-E|-1/2	$[\|O-E\|-1/2]^2/E$
Al-resistant (0.65–0.90)	54	48	5.5	0.630
Al-sensitive (0.20–0.65)	138	144	5.5	0.210
Total number	192	192		0.840

b. BC_3F_3 population (in 2005 and Nanjing) ($\chi_{1:2:1}$ test)

Types	No. of lines observed	No. of lines expected	|O-E|-1/2	$[\|O-E\|-1/2]^2/E$
Non-segregating lines with Al-resistant	54	48	5.5	0.630
Segregating lines with Al-sensitive	98	96	1.5	0.023
Non-segregating lines with Al-sensitive	40	48	7.5	1.172
Total number	192	192		1.825

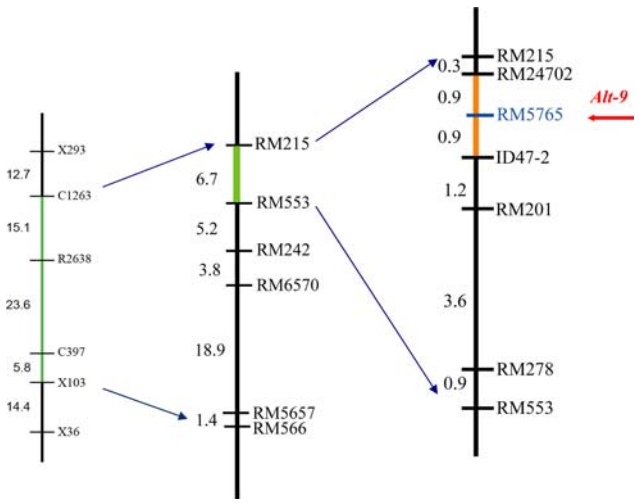

Fig. 5 Fine mapping of *Alt-9*. The *bar* on the left indicates CSSL51 chromosome 9, *the shaded* area on the left the Asominori chromosome substitution segment, *the other shaded areas* the mapped QTL region, and the *arrow* the position of *Alt-9*

QTL analysis for rice Al tolerance

A major QTL for Al tolerance has been located on chromosome 1 (Wu et al. 2000; Nguyen et al. 2001, 2002, 2003; Ma et al. 2002, 2003; Mao et al. 2004), and this location includes *qRRE-1* in the present experiments. Integrated genetic maps (http://www.shigen.lab.nig.ac.jp/rice/oryzabase/) show that the flanking marker *R1485* lies some 5.4-cM away from *RZ154*, which itself maps 4.5 cM away from *RZ252*, a flanking marker of the chromosome 1 QTL in IR1552 × Azucena (Wu et al. 2000), CT9993 × IR62266 (Nguyen et al. 2002), OM269 × Chiembau (Nguyen et al. 2001) and IR64 × *O. rufipogon* (Nguyen et al. 2003; Fig. 6a). Similarly, the location of *qRRE-9*/*Alt9*, which was responsible for 13.9% of phenotypic variation, corresponds to QTL identified in IR1552 × Azucena (Wu et al. 2000), CT9993 × IR62266 (Nguyen et al. 2002) and IR64 × *O. rufipogon* (Nguyen et al. 2003). *qRRE-9*/*Alt9* lies in the region defined by *C609* and *C1263* (Fig. 6b). According to the SSR marker map of McCouch et al. (2002), *C609* maps 3.3 cM from *RM201*, which is one of the flanking markers of an Al tolerance QTL mapped to chromosome 9 in IR64/*O.rufipogon* population (Fig. 6b). This genetic interval is also closely linked (2 cM) to an ortholog of WALI7, an Al-induced wheat cDNA.

Two major genes for Al tolerance, *Alm1* and *Alm2*, have been located on, respectively, maize chromosomes 10 and 6 (Sibov et al. 1999). *Alm1* lies 20.1 cM from *UMC130*, which in rice co-segregates with *RZ141* (Wilson et al. 1999) on chromosome 11 (Causse et al. 1994). *Alm2* maps 18.5 cM from *CSU70*, which is closely linked to *CDO580* (Wilson et al. 1999); the rice ortholog maps to chromosome 5 (Causse et al. 1994). Rice chromosome 5 was not identified as a source of Al tolerance QTL in our populations, but an Al tolerance QTL was located on chromosome 11, flanked by *C496* and *C410*, a region, which appears to be syntenic with the one carrying *Alm1*. Nguyen et al. (2001) also detected an Al tolerance QTL on rice chromosome, but an analysis of flanking markers indicates that these two QTL are unlikely to be identical to one another. Thus *qRRE-11* appears to be a new Al tolerance locus in rice.

Fine mapping of *Alt-9* and its breeding application

A QTL has been consistently identified in the region of rice chromosome 9. But a basic question about this QTL still remains, that is, whether the QTL corresponds to a single locus, or whether it represents a cluster of genes, each contributing a small additive effect. This problem has to be resolved before map-based cloning can be initiated. For this purpose, it is necessary to reduce the QTL to a single Mendelian factor and to localize it precisely on the genetic linkage map. But populations such as F2:3, RIL, BC and DH

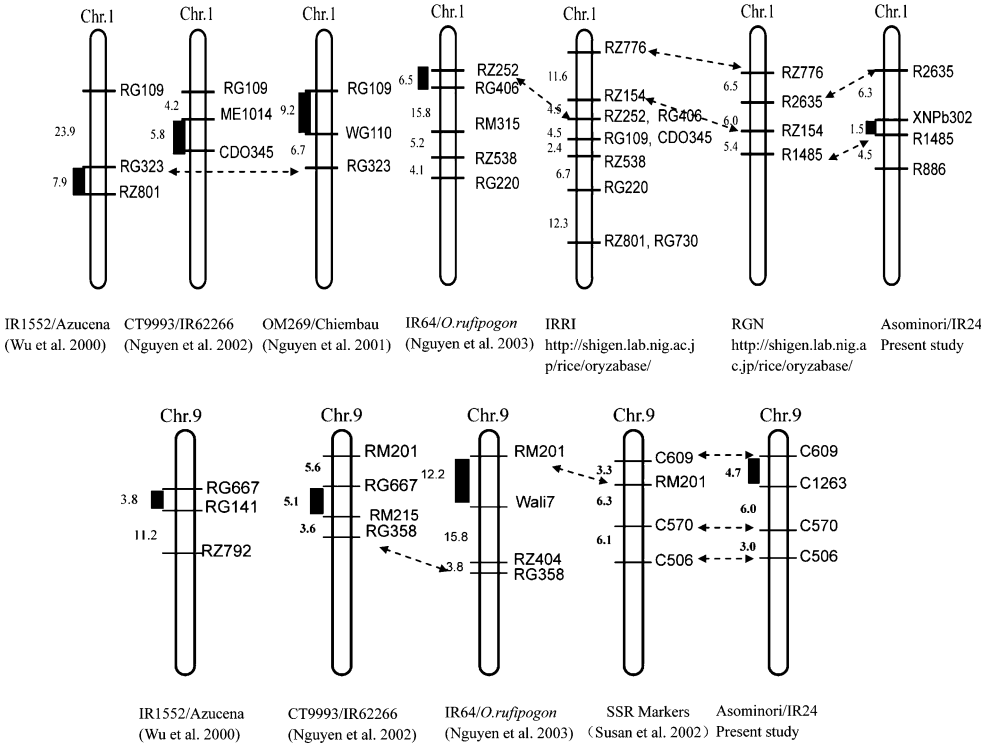

Fig. 6 A comparison of Al tolerance QTL across various rice mapping populations. **a** Chromosome 1. **b** Chromosome 9. *Vertical bars* indicate the position of Al tolerance QTL

could not be used for fine mapping of one of multiple QTL, because they segregate multiple genetic factors on the whole genome simultaneously.

Unlike populations mentioned above, chromosome segment substitution lines (CSSLs) have distinct advantages for QTL identification. Most importantly, genetic interactions between donor alleles are limited to those between genes on homozygous substituted tracts since each CSSL carries one or a few donor segments in the near-isogenic background of a recurrent genotype, thus reducing the effects of interferences from genetic background (Howell et al. 1996). Fine mapping of QTL has been achieved in rice using secondary populations derived by backcrossing specific materials (Yano et al. 2000; Li et al. 2004). We have followed this same strategy to define the location of *qRRE-9/Alt9* to 1.8 cM region. Genes for tolerance typically act in a dominant fashion, but the *qRRE-9/Alt9,* tolerance is recessive. Recessive tolerance could come about through the loss of function Al transporter activity, or stress signaling; alternatively, dominance may be dependent on the level of stress, being dominant at low levels and recessive at high ones.

Comparative mapping has shown that *qRRE-9/Alt9* can be identified in various mapping populations. A number of PCR-based assays (such as RM215, RM24702, RM5765, ID47-2, RM201, RM278 and RM553), which all amplify loci tightly linked to *qRRE-9/Alt9*, are available. Although the phenotypic effect of this locus has yet to be validated in the field, it is likely to represent a gene involved in the determination of Al tolerance in rice. The map-based cloning of *Alt9* is presently under way.

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