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Comparative mapping of QTLs for Al tolerance in rice and identification of positional Al-induced genes*

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Abstract: Aluminum (Al) toxicity is the major factor limiting crop productivity in acid soils. In this study, a recombinant inbreed line (RIL) population derived from a cross between an Al sensitive lowland indica rice variety IR1552 and an Al tolerant upland japonica rice variety Azucena, was used for mapping quantitative trait loci (QTLs) for Al tolerance. Three QTLs for relative root length (RRL) were detected on chromosome 1, 9, 12, respectively, and 1 QTL for root length under Al stress is identical on chromosome 1 after one week and two weeks stress. Comparison of QTLs on chromosome 1 from different studies indicated an identical interval between C86 and RZ801 with gene(s) for Al tolerance. This interval provides an important start point for isolating genes responsible for Al tolerance and understanding the genetic nature of Al tolerance in rice. Four Al induced ESTs located in this interval were screened by reverse Northern analysis and confirmed by Northern analysis. They would be candidate genes for the QTL.

Key words: Aluminum tolerance, Quantitative trait loci (QTL), Expressed sequence tag (EST), Gene, Rice (*Oryza sativa* L.)
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INTRODUCTION

Aluminum (Al) toxicity is one of the most important yield-limiting factors for crop grown on acid upland and lowland acid sulphate soils (IRRI, 1978). Al toxicity results in a reduced and damaged root system, which in turn causes the affected plants to be susceptible to drought stress and mineral nutrient deficiencies (Foy, 1988). The physiological and biochemical mechanisms of the toxic effect of Al on root elongation had been extensively investigated (Matsumoto, 2000). The genetic or molecular mechanisms controlling Al tolerance in plants, however, are poorly understood.

Al tolerance has been speculated to be the result of either exclusion of Al from the root apex and/or the tolerance for symplasmic Al. Detoxification of Al by releasing organic acids to chelate Al has been reported in wheat and several other plants (Delhaize *et al*., 1993; Ma *et al*., 2002). Furthermore, Al tolerance could be acquired in transgenic tobacco plants by alteration of citrate synthesis (de la Fuente *et al*., 1997). However, information on Al tolerance mechanisms in rice is limited. Ma *et al*. (2002) reported that no organic acid except small amount of citrate was induced by Al exposure in rice, and no significant effect on the Al detoxification in both Al-tolerant and Al-sensitive varieties. It means that rice may have different Al tolerance mechanism other than release of organic acids.

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Al tolerance is a complex trait controlled by multiple genes in rice (Ma *et al.*, 2002; Nguyen *et al.*, 2001; 2002; 2003; Wu *et al.*, 2000). Five genetic populations have been used for identifying QTLs for Al tolerance on rice. QTLs identified from different background were located on several chromosomes. But one QTL on chromosome 1 was identified in similar position across more than 3 populations.

Various techniques had been used to clone genes for Al tolerance and a number of Al induced genes had been isolated from Wheat (Hamel *et al.*, 1998; Sasaki *et al.*, 2002), *Arabidopsis* (Richards *et al.*, 1998), Rye (Milla *et al.*, 2002) and Sugarcane (Watt, 2003). It was reported that Al-induced genes could ameliorate Al stress (Ezaki *et al.*, 2000; 2001), which implicated that Al-induced genes could be tolerance genes.

The main objectives of this study were to compare mapping QTLs for Al tolerance across different genetic backgrounds or experimental conditions and to screen Al-induced genes in QTL interval on chromosome 1 for further studying. In this study, QTLs for Al tolerance in rice were mapped, and comparison between different genetic backgrounds was done. Four Al induced EST clones located within the QTL interval on chromosome 1 were identified by reverse Northern analysis.

MATERIALS AND METHODS

Plant material and growth conditions

A recombinant inbreed line (RIL) population composed of 96 lines derived from a cross between an Al-sensitive indica rice variety IR1552 and an Al-tolerant japonica rice variety Azucena developed by single seed decedent were used. Solution culture experiments were performed in a culture chamber at Zhejiang University. The day/night temperature was 30 °C to 24 °C and the relative humidity was 65%−70% and 12 h photoperiod of approximately 300−320 μmol/m^2·s provided by 20400 W sodium and metal haloid lamps.

Uniform seeds were rinsed with distilled water, and incubated at 30 °C for 2 days for germination. Germinated seeds were grown in distilled water for another 2 days at 27 °C±2 °C. Seedlings were then transferred to a plastic tray covered by a PVC sheet with nylon screen attached holes. Half strength nutrient solution was used (Yoshida *et al.*, 1976). The pH of the solution was adjusted daily to 4.0 with 1 mol/L NaOH or 1 mol/L HCl. For reverse Northern or Northern analysis, 4-day-old seedlings were used for Al-stress treatment as following: seedlings were exposed to 0.5 mmol/L $CaCl_2$ solution (pH 4.0) for 2 h, then exposed to 0.5 mmol/L $CaCl_2$ solution (pH 4.0) containing 0 or 183 μmol/L $AlCl_3$. Roots and shoots of seedlings sampled at 0 h, 0.5 h, 2 h, 12 h, 24 h, 48 h were cut and quickly frozen in liquid nitrogen, and stored at –70 °C for RNA extraction.

For QTL analysis, the experiment was arranged in a randomized complete block design with 3 replications. Seedlings were transferred to PVC sheet with one seedling per hole and three seedlings in one row per line in each replication. The PVC sheets were laid above a plastic tray with a 1/2 nutrient solution (Yoshida *et al*., 1976) containing either 0 (control) or 0.556 mmol/L $AlCl_3$. The pH of the solutions was adjusted daily to 4.0 with 1 mol/L NaOH or 1 mol/L HCl. The longest root of each seedling was measured after 1 and 2 weeks of growth in control (control root length) or stress (stress root length) solution. Relative root length of average root length under stressed versus control conditions for each line in each replication was used as a measure for Al tolerance.

Molecular map construction

A genetic linkage map was constructed based on a previous map (Zhang *et al.*, 2001), consisting of 260 marker loci including 114 restriction fragment length polymorphism (RFLP) markers, 104 amplified fragment length polymorphism (AFLP) markers, 41 microsatellite (SSR) markers and 1 CAPS marker using MAPMAKER/EXP version 3.0. The total map length was 2860 cM with an average distance of 11 cM between adjacent markers. Map units (cM) were derived using the Kosambi function. Forty-one SSR markers and 1 CAPS marker were mapped in this case according to the

methods described in the web sites (www.gramene.org) and (http://rgp.dna.affrc.go.jp/).

Statistical analysis

The concentration of free active Al^{3+} was determined by a computer software Geochem-PC program (Parker *et al.*, 1995). And free active Al^{3+} of 100 μmol/L was used for all the Al stress treatments in this paper.

One-way ANOVA (SAS/6.11, GLM) was performed to test the significance of variation among parents and RIL lines. QTLs for Al tolerance and the molecular markers linked to the putative QTLs, were analyzed by Qgene3.06 (Nelson, 1997). A LOD score ≥2.4 was considered significant for QTL detection.

Comparison across mapping population

QTLs for relative root length (RRL) were compared across different genetic populations with the same markers. Markers flanking similar QTL interval were mapped to a genetic map using a BC_1F_6 population (http://rgp.dna.affrc.go.jp/) derived from Nipponbare/Kasalash//Nipponbare to construct the integrated map. In *silico* mapping were performed by sequence alignment of objective clone to a mapped BAC or PAC clone in two web sites (http://rgp.dna.affrc.go.jp/) or (http://www.genome.arizona.edu/fpc/rice/).

Reverse Northern analysis

Eighty-three ESTs were selected from Comprehensive Rice Transcript Map (Wu *et al.*, 2002), offered by MAFF DNA bank. All EST clones were amplified by PCR with M_{13} primer, then blotted to a Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech) using a Bio-Dot® Microfiltration Apparatus (BIO-RAD) according to the instruction manual after being checked by 0.8% Agrose gel. Total RNA was extracted from root tissues by using the Trizol reagent (GIBCO, Germany). Treated RNA and control RNA of Azucena and IR1552 were prepared by pooling equal amount of RNA at the 4 time points (0.5 h, 2 h, 12 h, 24 h). Treated RNA and control RNA were used to purify Poly $(A)^+$ RNA with the Oligotex mRNA Mini Kit (QIAGEN, Germany). Poly $(A)^+$ RNA of treatment and control were used as probes for reverse Northern analysis. Probe labeling was performed according to molecular cloning (Sambrook and Russeel, 2001). Prehybridization (8 h) and hybridization (24 h) were carried out at 65 °C in 7% (w/v) SDS, 0.191 mol/L Na_2HPO_4, 0.058 mol/L NaH_2PO_4, 1% (w/v) bovine serum albumin, and 100 μg/ml denatured salmon sperm DNA. Washes were done at room temperature for 20 min in 2×SSC, 0.1% (w/v) SDS and 60 min in 0.1×SSC, 0.1% (w/v) SDS.

Northern analysis

Total RNAs (20 μg) were separated on 1.2 % formaldehyde-agarose gels and transferred onto Hybond-N^+ nylon membranes (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Probe labeling and hybridization was the same as described in Reverse Northern analysis. Washes were done at room temperature for 15 min in 2×SSC, 0.1% (w/v) SDS and 10 min in 1×SSC, 0.1% (w/v) SDS.

Sequence analysis

The EST clones with different expression pattern were sequenced using MegaBASE™ 1000 (Amersham Pharmacia, USA). Nucleotide sequences and translated sequences were compared with sequences of the GenBank by using the BLAST sequence alignment program. Function of genes was determined according to the BLAST results (BLASTX, E values less than 1e-5) (Ditt *et al.*, 2001)

RESULTS

Experimental conditions

To assess root growth after different Al stress time, Al tolerant variety Azucena and Al sensitive variety IR1552 were used for stress treatment (100 μmol/L active Al^{3+}). The results showed that IR1552 was more sensitive than Azucena (Fig.1). Root growth of IR1552 was severely inhibited (75.8%) after 1 week stress treatment. While in Azucena, root length was almost not inhibited in the first two weeks (5.7% and 0%) of Al stress treatment, and

slightly inhibited in the next 2 weeks (10.9% and 12.2%). Therefore, the first two weeks of stress treatment was used in the phenotyping experiment to assess the Al tolerance of this two varieties and the population derived from these two varieties.

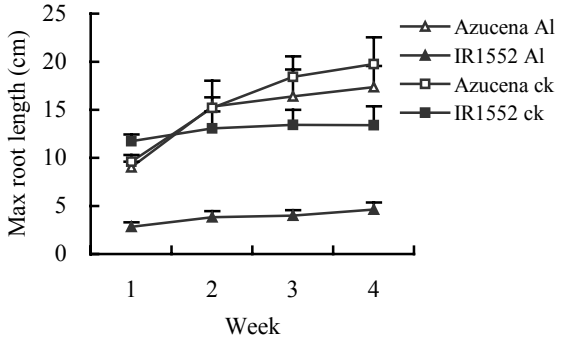

Fig.1 Root length of Azucena and IR1552 of different time after growth on Al and CK (without Al) condition, Data are means (+SE) of results from 15 seedlings

Phenotypic performance

To evaluate the effects of Al stress on root growth, relative root length (RRL) defined as a ratio of root length under Al stress to root length under normal condition was used to evaluate genotypic variation in Al tolerance. The mean, range, and distributions of RRL among RIL population and of their parents are shown in Fig.2. The mean RRL of IR1552 and Azucena were about 0.2 and 0.9 after 1 week stress treatment, 0.3 and 1.0 after 2 weeks stress treatment, respectively; while the average RRL of 96 RIL lines after 1 and 2 weeks stress treatment were 0.68 and 0.71, respectively. The RRL of the population lines was normally distributed according to Shapiro-Wilk test. Control root length (CRL) and stress root length (SRL) were also evaluated. The distributions of CRL and SRL were also normal (data not shown). The range of progeny means appreciably exceeded that of their parents for the three traits, suggesting transgressive variation among the population.

QTL analysis

Interval mappings were used to detect putative QTLs for Al tolerance after 1 week or 2 weeks stress treatment. Three parameters of stress root length, control root length and relative root length were

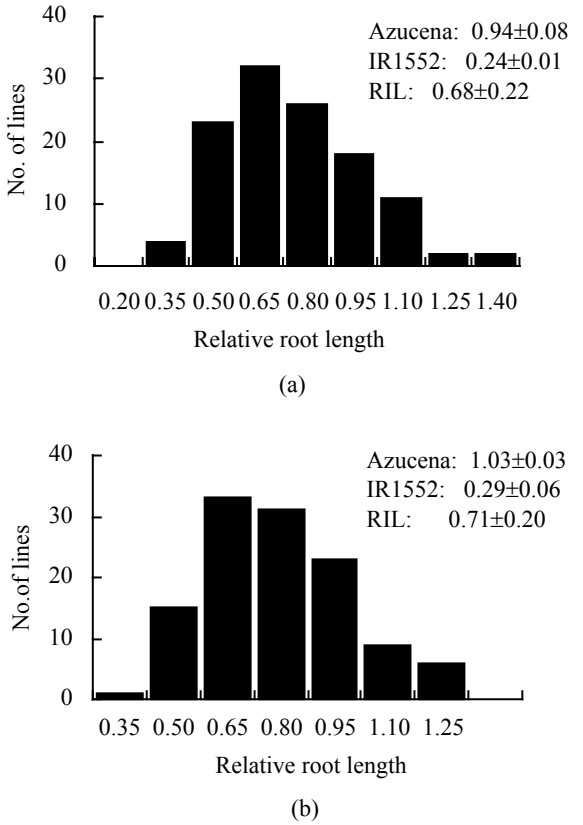

Fig.2 Means (±SE) of the relative root length of the parents and the frequency distribution for relative root length after 1 week (a) and 2 weeks (b) of stress treatment

used for QTL analysis. The results are shown in Table 1 and Fig.3. QTLs detected after 1 and 2 weeks stress treatment were almost in the same map positions. No QTL was detected for control root length. And only one QTL was detected for stress root length (Table 1, Fig.3). This QTL contributed 15% and 11% of the phenotypic variation after 1 and 2 weeks stress treatment, respectively. Three QTLs for RRL were detected which could explain about 39% and 35% of the total variation in RRL after 1 and 2 weeks stress treatment, respectively (Table 1, Fig.3). The three QTLs were mapped at the interval RG381−RZ801 on chromosome 1, RZ698−ACA-CTA1 on chromosome 9 and ACA-CTT1−RM117 on chromosome 12. The positive allele(s) at the QTL on chromosome 1 was from Azucena, and this QTL explained 16% and 9% of phenotypic variation in RRL after 1 and 2 weeks stress treatment, respectively. The positive allele(s)

Table 1 Putative QTLs detected for stress root length (SRL) and relative root length (RRL) after one and two weeks Al stress. Numbers at the end of each trait indicate different stress time (1 for one week and 2 for two weeks)

Trait	Chrom.	LOD value[a]	QTL interval	Var (%)	Additive effect[b]
SRL1	1	2.6	RZ801/RG323	15%	–0.70
RRL1	1	2.5	RZ801/RG381	16%	–0.08
	9	2.9	RZ698/ACA-CTA1	13%	0.08
	12	2.4	ACA-CTT1/RM117	10%	–0.08
SRL2	1	2.4	RZ801-RG323	11%	–0.88
RRL2	1	2.4	RZ801/RG381	9%	–0.07
	9	3.1	RZ698-ACA-CTA1	15%	0.07
	12	2.5	ACA-CTT1/RM117	11%	–0.08

[a]: Maximum-likelihood LOD (likelihood odds ratio) score for the individual QTL
[b]: Negative value means the positive allele(s) is from Azucena

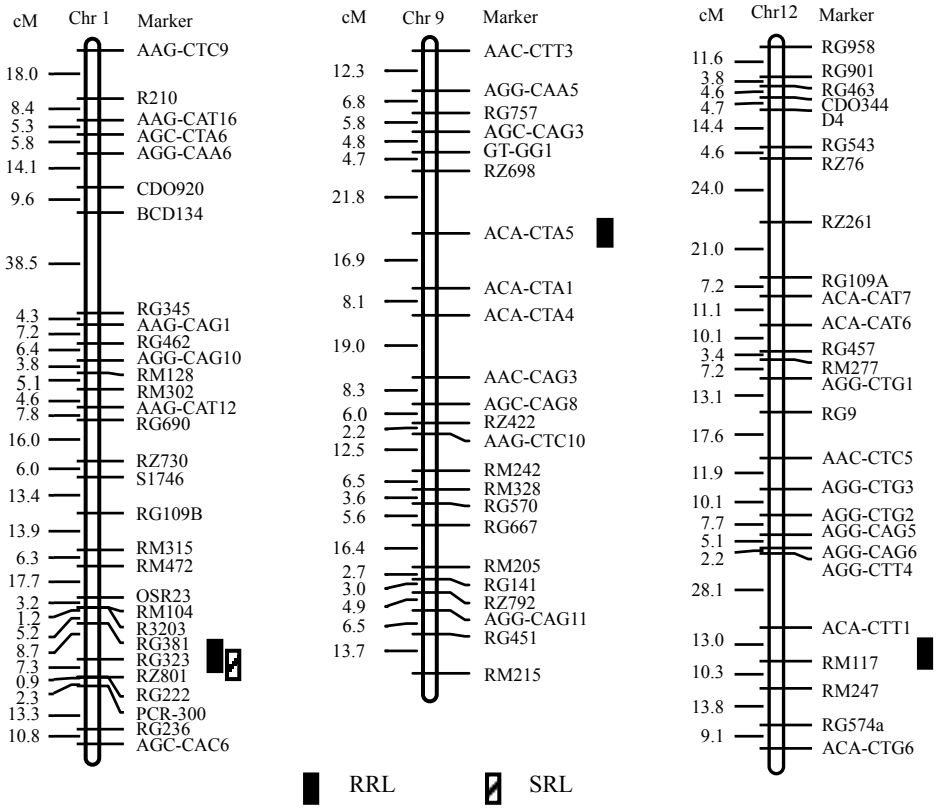

Fig.3 Chromosomal locations of putative QTLs for Al tolerance. Because QTLs detected after 1 and 2 weeks stress treatment were located almost in the same positions; it is not shown separately

at the QTL on chromosome 9 was from IR1552, and this QTL explained about 13% and 15% of total variation in RRL after 1 and 2 weeks stress treatment, respectively. The positive allele(s) at the QTL with 10% and 11% variance explained on chromosome 12 was from Azucena after 1 and 2 weeks stress treatment, respectively.

Comparative mapping of QTL for Al tolerance across different populations

The QTLs for Al tolerance in rice have been reported from different genetic populations. RRL

had been used as a common parameter for assessing Al tolerance in different studies. QTLs for RRL in this study were compared with other reported QTLs for Al tolerance. One identical QTL was found at the interval on chromosome 1 flanked by C86 and RZ801 across 5 populations (Fig.4). About 9% to 25% of the total variation in RRL was explained by the QTL from different reports. The result suggested that this QTL interval was a conserved genome region associated with Al tolerance across different genetic backgrounds.

Identification of positional Al-induced genes

In the comprehensive rice transcript map (Wu *et al.*, 2002), 83 ESTs were found between C86 and C742. Reverse Northern analysis was used for identifying Al induced genes among these ESTs (Fig.5a). Four ESTs that showed differential expression pattern in two replications were selected as Al induced genes. All 4 ESTs were sequenced and Blast search showed their homology to putative amino acid transport protein (C50531), putative Rho GDP-dissociation inhibitor (E1391), nonspecific lipid transfer protein (E30131), and ubiquitin-like protein SUMO-1 (E61853). The chromosomal locations of the ESTs are shown in Fig.5b.

Northern blot analysis was performed to identify the expression patterns of the 4 ESTs (Fig.6). C50531 mainly expressed in root and was induced by Al in both Azucena and IR1552, but weakly and constitutively expressed in shoot. E1391 was constitutively expressed in shoot in Azucena and a little up-regulated in IR1552 (0.5 h, 2 h), but was increasingly induced in root of Azucena, while up-regulated in early time (0.5 h) then down-regulated later in root in IR1552. E30131 was up-regulated increasingly in root of Azucena, and down-regulated in root of IR1552, but weakly expressed in shoot in both varieties. E61853 were up-regulated in both roots and shoots in Azucena and IR1552. The Al-induced expressions of the gene in roots of Azucena were stronger than that of IR1552, while IR1552 accumulated its transcripts to the peak earlier than Azucena.

DISCUSSION

Different experiment condition may affect QTL detection

Five studies reported QTL analysis using RRL as measurement of Al tolerance, but different resu-

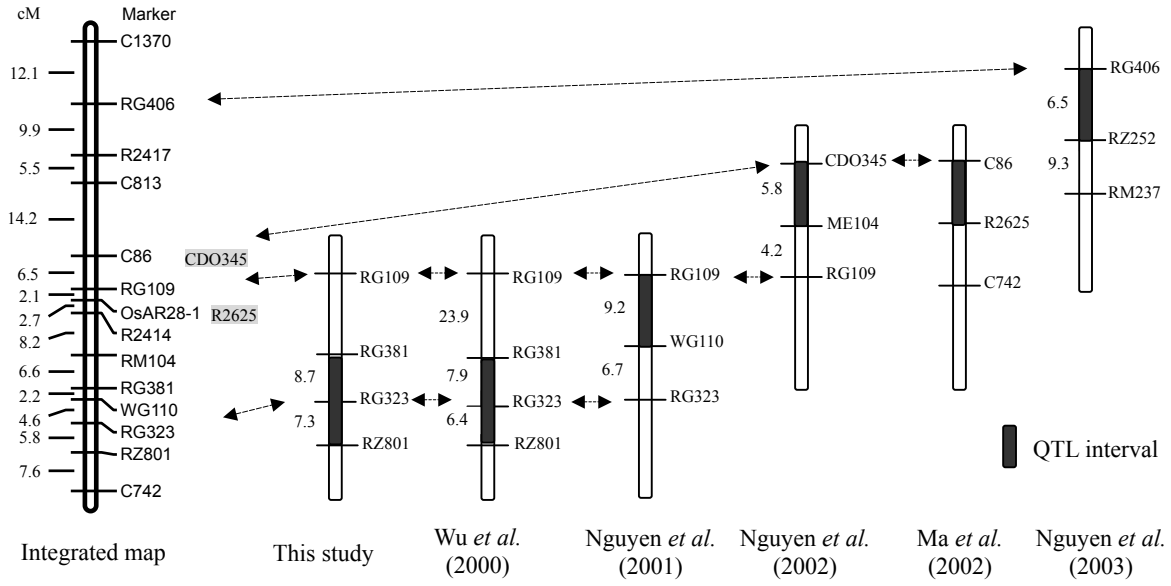

Fig.4 Comparison of QTL for Al tolerance on chromosome 1 across different genetic populations [Integrated map was drawn using a BC_1F_6 population derived from Nipponbare/Kasalash//Nipponbare. Mapmaker 3.0 was used for linkage analysis. Shaded markers beside the markers on the integrated map were by *in silico* mapping (in the same BAC clone with the marker nearby). Other partial maps were redrawn according to different study shown at the bottom of each map]

Table 2 Function analysis of Al-induced EST at the QTL interval on chromosome 1

Clone name	Score	E-value	Functional homology
C50531	159	8E-67	Putative amino acid transport protein
E1391	138	1E-31	Putative Rho GDP-dissociation inhibitor
E30131	55.1	1E-06	Non-specific lipid transfer protein
E61853	150	1E-044	Ubiquitin-like protein SUMO-1

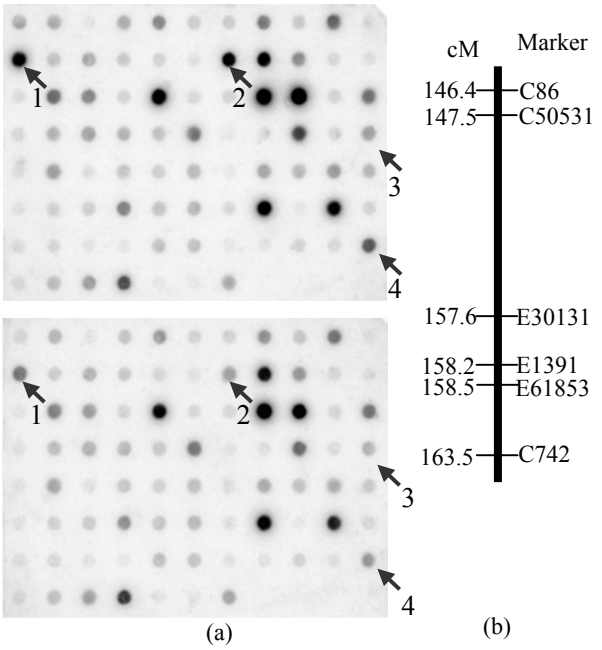

Fig.5 (a) Results of reverse Northern analysis. Labeled probe of treated mRNA (upper panel) and control mRNA (lower panel) sampled from the root region of Al tolerant variety were hybridized with two nylon membranes that have equal amount of PCR products of each EST blotted onto the same position. Differential expressed clones are shown in black arrows. The last dot in each membrane is actin. Numbers below each arrow indicate different ESTs, 1, E61853; 2, C50531; 3, E30131; 4, E1391; (b) *in silico* mapping of Al induced EST clones of E61853; C50531; E30131; E1391

lts were observed; which indicated that Al tolerance in plant could differ with nutrient concentration, solution pH, genotype and seedling age (Wu *et al.*, 2000). Using the same genetic population, we detected different QTLs associated with Al tolerance from these reported by Wu *et al.*(2000). The experiment in their case was conducted in greenhouse, the roots of 7-day old seedlings were cut off, leaving 1 cm before stress treatment, Al stress treatment was carried out with 1 mmol/L Al^{3+} in full strength nutrient solution (100 μmol/L active Al^{3+}). The experiment in this study was conducted in a growth chamber. Geminated seeds were used for Al treatment, and 1/2 nutrient solution with 0.556 mmol/L Al^{3+} (100 μmol/L active Al^{3+}) was used in Al stress treatment. Three QTLs were detected after 2 weeks stress treatment in both experiments with phenotypic variation explained for 38% and 35%. Three QTLs were located on chromosome 1, 3, 12 in Wu's study while on chromosome 1, 9, 12 in this case. The QTL on chromosome 1 was identical in the two experiments, while QTL on chromosome 12 mapped on different interval in different experiments. The results suggested that different growth conditions should affect QTL detection. RRL of initial root and re-growth root may have different potential for evaluating Al tolerance. It may

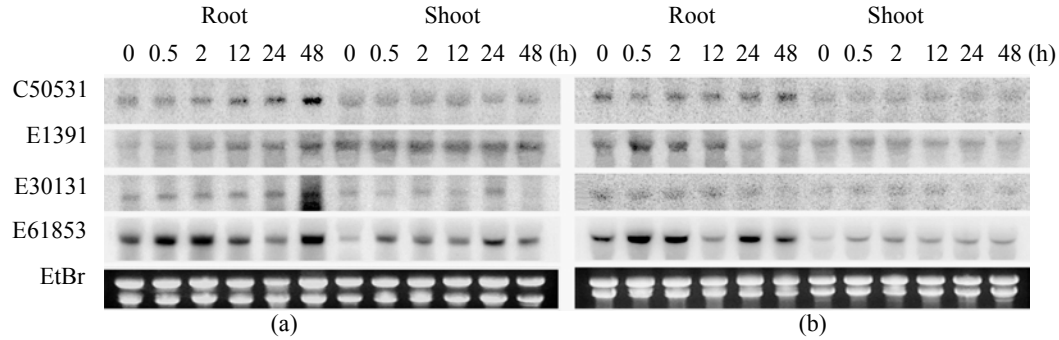

Fig.6 Northern blot analysis of Al induced EST clones. (a) Azucena; (b) IR1552

also reflect that different seedling stage or Al treatment may induce different Al tolerance genes. It was reported that a QTL for root growth under upland condition was closely linked with RG109B and RZ730 (within C86−RZ801 on chromosome 1) (Zhang *et al.*, 2001) and a QTL for tolerance to Fe^{2+} toxicity also located in this interval (Wu *et al.*, 1998). The results suggested that the gene (s) at the QTL might be for multiple tolerances to Al, Fe^{2+} and drought stresses.

Al tolerance in rice is a complex trait

It had been suggested that natural variability of Al tolerance was controlled by 3 to 10 QTLs across different genetic backgrounds (Ma *et al.*, 2002; Nguyen *et al.*, 2001; 2002; 2003; Wu *et al.*, 2000). Three QTLs detected in this case, which totally explained less than 40% phenotypic variation in RRL. Epistasis analysis (QTLMapper V 1.0) showed that epistasis effects at 1 and 2 weeks stress could explain about 39% and 40% of the total phenotypic variation. Wu *et al.*(2000) also found that 20% and 32% of the phenotypic variation were contributed by epistasis effect. It suggested that epistasis is important to Al tolerance in rice and could imply that Al tolerance in rice was contributed by the interaction of many genes involved in different metabolic pathways. The effect of the QTL on chromosome 1 were decreased in 2 weeks stress treatment compared to 1 week stress treatment, whereas, the effects of QTLs on chromosome 9 and 12 were increased (Table 1). It suggested that the effect of QTL on Al tolerance should be changed at different stress time, or that these QTLs should be developmentally dependent (Wu *et al.*, 2000).

Positional Al induced genes would be candidate gene for Al tolerance

No Al-tolerance gene or QTL have been cloned so far in rice. It was known that the accuracy and reliability of QTL with minor effects would be relatively weak, which needs to be confirmed by different experiments (Mao and Cheng, 1999). By comparison, an identical QTL for Al tolerance in rice was revealed between C86 and RZ801 on chromosome 1 across different genetic backgrounds and experimental conditions. Because of the possibly not fine scale mapping in different experiments, the accurate position of this QTL is still unclear. We screened 15 SSR and RFLP markers and 1 CAPS marker near this interval, but could not shorten this QTL interval (data not show). Near-isogenic lines (NILs) development is necessary for fine mapping of the QTL.

Candidate gene is an alternative approach for isolation of Al tolerance gene. In this study, four ESTs with differential expression pattern were selected. Researchers will be greatly interested in the discovery of the extent of correlation between phenotypic variation and each of the Al induced ESTs that align in this region. E30131 showed significant homology with non-specific lipid transfer protein (nsLTPs). nsLTPs are involved in cutin biosynthesis, surface wax formation, pathogen-defence reactions, or the adaptation of plants to environmental changes. It can be induced by extreme temperature and salt or drought stresses (Kader, 1997). nsLTPs play a defensive role in plant against pathogens (Kristensen *et al.*, 2000). It was up-regulated stronger in Azucena than in IR1552 (Fig.6). E1391 showed significant homology with Rho-GDI (GDP dissociation inhibitor). It was found that Rho GDI system plays an important role in spatial determination in the actin cytoskeletal control (Sasaki and Takai, 1998). Expression of the tobacco GDI1 gene in yeast confers Al tolerance by promoting release of Al ions after uptake (Ezaki *et al.*, 1999). It was induced increasingly in root of Azucena, and up-regulated in early time (0.5 h) but down-regulated later in root of IR1552. From its expression pattern between Al tolerance and Al sensitive varieties, it implied that Rho-GDI might contribute to Al tolerance in rice. E61853 encodes an ubiquitin-like protein SUMO-1 (Small ubiquitin-like modifier-1), Small ubiquitin-like modifier (SUMO) is a member of the superfamily of ubiquitin-like polypeptides that become covalently attached to various intracellular target proteins as a way to alter their function, location, and/or half-life. It can be induced by stress such as heat shock, H_2O_2 and ethanol etc. It may have important function in stress protec-

tion and/or repair (Kurepa *et al.*, 2003). C50531 showed significant homology with a putative amino acid transport protein. Amino acid transporters are essential participants in the resource allocation processes that support plant growth and development. It also plays a key role in leaf senescence and seed germination (Ortiz-Lopez *et al.*, 2000). Gene expression of amino acid transporter can be regulated by environmental conditions, such as water and salt stress (Ortiz-Lopez *et al.*, 2000). According to the expression pattern of C50531 between Azucena and IR1552, it can be speculated that the induction of amino acid transporter might be a response of Al affected resource allocation in rice root.

To further investigate the genes for possible Al tolerance in rice, genetic transformation and complementation experiments should be performed, which can demonstrate conclusively if the candidate gene approach is an alternative for gene discovery for Al tolerance.

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