Theor Appl Genet (2011) 122:687–694
DOI 10.1007/s00122-010-1478-3

ORIGINAL PAPER

The genetic control of tolerance to aluminum toxicity in the 'Essex' by 'Forrest' recombinant inbred line population

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Received: 13 October 2009 / Accepted: 11 October 2010 / Published online: 9 November 2010

Abstract Aluminum (Al) toxicity to plant roots is a major problem of acidic soils. The main chemical reaction involved is Al hydrolysis. Application of lime or nitrate fertilizers to raise soil pH reduces Al toxicity but not as economically as a plant genotypes with natural tolerance against this stress. Ammonium fertilization of crops and assimilation of ammonium (even that derived from dinitrogen) are particularly acidifying of the root zone. The aims of the present study were to find genotypes of soybean tolerant to aluminum stress and identify QTL underlying that trait. Used were recombinant inbred lines (RILs) derived from the cross of 'Essex' by 'Forrest'. RILs were grown in a greenhouse for 3 weeks and then transferred to hydroponics in a growth chamber. Root lengths (RL) were measured before and 72 h after Al treatment. RL before and after Al treatment were measured and used to calculate root tolerance index (RTI) and relative mean growth (RMG). RILs 1, 85, 40 and 83 had significant ($P < 0.005$) tolerance to Al stress judged by RL after Al, RTI and RMG. Eleven minor but significant marker–trait associations ($P < 0.05$) were detected using one-way ANOVA but only two major loci were significant in composite interval maps (LOD >3.0). The QTL on linkage group F (chromosome 13) was in the interval Satt160–Satt252 with a peak at 24 cM (peak LOD was 3.3). The QTL underlay 31% of trait variation and the Essex allele provided an additional 1.61 cm of root growth over 72 h in the presence of Al. The QTL on linkage group C2 (probably chromosome 4) was in the interval from Satt202 to Satt371 with a peak at 3.2 cM (peak LOD was 14.7). The QTL underlay 34% of trait variation or 1.81 cm of growth over 72 h in the presence of Al. Both loci encompassed genes implicated in citrate metabolism, a method of aluminum detoxification known to vary among soybean cultivars. Two major loci and at least nine minor loci were inferred to underlie tolerance to Al. RILs and markers may be used to select alleles that increase tolerance to soybean against Al stress.

Introduction

In the solid phase aluminum (Al) is a normal constituent of every soil but in solution phase it can become toxic to plants even at low concentrations (Foy 1974; Sanchez and Salinas 1981; Delhaize and Ryan 1995). Al is the most abundant metal in the earth's crust. Even at low concentrations, it is toxic to the roots of many plant species and interferes with phosphate uptake. Plants can sequester up to 1% of their dry weight as Al in vacuoles to avoid toxicity. Fortunately, most of the Al in soils occurs in forms like

Communicated by M. Bohn.

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aluminosilicates and precipitates which are non-phytotoxic. However, low pH results in more availability of Al, that can result in low plant production on acidic soils due to Al toxicity (Foy 1974; Delhaize and Ryan 1995).

Acidic soils, which are soils with a pH of 5.5 or lower, present an important class of problem soils that cause a serious limitation to agricultural production worldwide (reviewed by Kochian 2004). Acidic soils constitute 30% of the world's total land area. Farming practices like extensive use of ammonia fertilizer caused acidification of agricultural soils. In developed countries like the USA, where high input farming practices were widespread by 2010, acidification of soils was a common phenomenon. Liming of acidic soils can alleviate this problem, but this is not a viable option for poor farmers due to economic concerns and has been found to be a less effective strategy to counter subsoil acidity.

Part of the difficulty of studying Al-related processes in plants can be attributed to the complex chemistry of Al (Kinraide 1991). Aluminum hydrolysis is the dominant chemical reaction. Al hydrolyzes in solution such that the trivalent Al species, Al^{3+}, dominates in acid conditions ($pH < 5$), whereas the $Al(OH)^{2+}$ and $Al(OH)_2^+$ species form with the increase in pH. At near-neutral pH, the solid phase $Al(OH)_3$, or gibbsite occurs, whereas $Al(OH)_4^-$, or aluminate, dominates in alkaline conditions. Equilibrium constants can be used to predict the relative concentrations of the monomeric Al species and other Al compounds in solution (Parker and Bertsch 1992). Al toxicity has a primary effect on root growth and hence inhibition of root growth has become a widely accepted measure of Al stress in plants. In simple nutrient solutions micromolar concentrations of Al can begin to inhibit root growth within 60 min. The root apex accumulates Al and hence was more susceptible to physical damage than the mature root tissues. Indeed, only the apical 2–3 mm of the roots (in maize) need be exposed to Al for growth to be inhibited (Ryan et al. 1993). Under field conditions, it is generally difficult to observe Al toxicity symptoms on crop roots because the plants generally die quickly due to water stress (Foy 1974; Delhaize and Ryan 1995; Kochian 2004).

Leguminous plants like soybeans (*Glycine max* (L.) Merr.) are particularly acidifying because ammonium assimilation causes ion exchange from soil to be more imbalanced toward cations (Delhaize and Ryan 1995). Hence there are more hydrogen ions in the root vicinity in legumes compared to non-leguminous plants. In contrast, during nitrate nutrition of plants, three hydrogen ions are removed from the root per N assimilated increasing soil pH. Multiple Al tolerance mechanisms are used by different plant species. However, the most studied was root exclusion depending on an Al-activated organic acid (OA) exudation from root apex (Delhaize and Ryan 1995). In soybeans, citrate is the OA exuded (Matsumoto 2000) and the degree of exudation was cultivar dependent (Shen et al. 2005).

In soybean, selection for Al tolerance has been hampered by inadequate screening methodologies (Villagarcia et al. 2001). Hydroponics was an attractive alternative to soil- based screening for Al tolerance (Bianchi-Hall et al. 2000). It was particularly important in evaluation of a large number of genotypes quickly and has been used to identify parental stock for soybean breeding.

Soybean seedlings grown in hydroponics have been used to identify QTL underlying tolerance to aluminum from the cross of resistant 'PI 416937' and susceptible cultivar 'Young' (Bianchi-Hall et al. 2000). Four major loci and two minor loci were reported. The major loci were located on linkage groups (LG) A2; LG B1; LG F; and LG J. The minor loci were on LG B1 and LG L. Plants grown in sand have been used to identify QTL underlying tolerance to aluminum from the cross of resistant 'Kefeng No. 1' × 'Nannong 1138-2' (Qi et al. 2008). Two to three major loci and several minor loci were reported. The major loci were located on LG B1; LG L; and LG D1b. Both studies agreed that about a third of the trait was explained by a small number of major loci and about two thirds by several polygenes.

The hypothesis formulated here was that the tolerance to Al toxicity in soybean is a genetically programmed quantitative trait underlain by variation among alleles of loci that alter root growth and agronomic potential. Root growth was measured after Al treatment in a recombinant inbred line population (RIL). QTL underlying root growth and aluminum tolerance were identified and correlations with other agronomic traits made.

Materials and methods

Materials

In this study, seed of the $F_{5:13}$ RIL population derived from a cross of 'Essex' by 'Forrest' (EF96; Lightfoot et al. 2005) were sown in sand media in the month of February, 2007. The 42 selected genotypes used were the same as for Hnetkovsky et al. (1996) and Kassem et al. (2004). No external nutrient solution was applied and plants were germinated purely based on the reserves in their seeds. Plants were watered daily, to avoid moisture stress. After 3 weeks of germination, the progeny was evaluated in hydroponics to assess Al tolerance in comparison with the parents. Initial root length (RL) (root tip to hypocotyl) was recorded, after washing the roots through a sieve. After measuring initial RL, plants were shifted to growth chamber (Percival Scientific; AR-75L; Perry, IA).

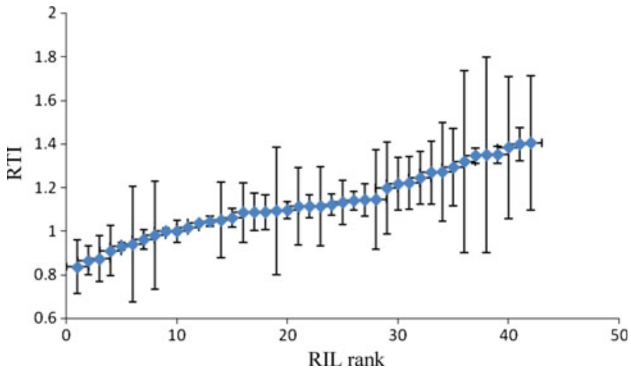


Fig. 1 Root tolerance index (RTI) ± SEM of 42 ranked RILs. RTI was final root length with Al/final root length − Al. RTI for Essex was 1.30 ± 0.01, for Forrest; it was 1.03 ± 0.05. Lines that segregated for the two major loci had large SEMs

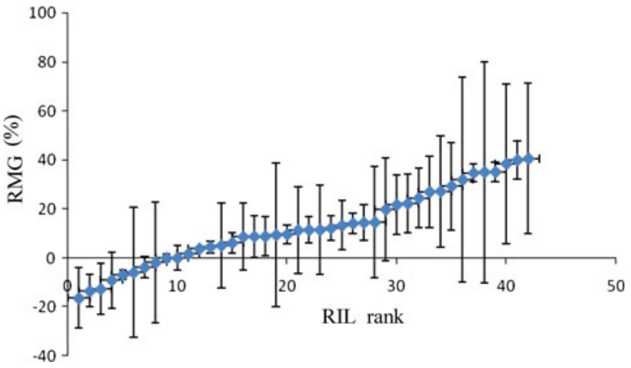


Fig. 2 Relative mean growth ± SEM of ranked RILs. RMG (%) was final root length − initial root length/initial root length × 100. RMG for the Essex parent was highest at 31 ± 8% while that for Forrest parent was 3 ± 4%. Lines that segregated for the two major loci had large SEMs

Hydroponics and trait measurement

In the growth chamber, light intensity ranged from 135 to 188 μmols m^{-2} s^{-1} and temperature ranged from 24 C (night) to 26 C (day). Sixteen round tanks (7.5 l) aerated at 1 l per min with air by fish tank pumps were used. Five plants per tank were supported with perforated Styrofoam rings floating on the media. For providing an acidic medium 80 ml of 0.025 M hydrochloric acid was used to set up a pH of ∼4.3 in all the tanks. The solution contained 7 l of water having 0.95 g calcium sulfate ($CaSO4 \cdot 2H_2O$). The pH was measured daily and kept at pH 4.3 by titration with dilute hydrochloric acid. Plants were grown in growth chamber for 24 h without Al to promote acclimatization with the environment. The aluminum treatments consisted of 0 and 2 μM/l aluminum sulfate (Al_2SO_4 at 1.4 g/tank) and the final RLs were recorded after 72 h of Al exposure. The experiment was repeated twice or three times with from 2–8 plants per line. The third run with four plants was used if there was heterogeneity of root growth in the first two experiments.

The root tolerance index (RTI), calculated as the maximum RL in Al stressed hydroponics divided by maximum RL in non-stressed controls (Moore et al. 1990).

$$\mathrm{RTI} = \text{Final root length with Al}/\text{Final root length minus Al.}$$

The relative mean growth (RMG) was calculated by using this formula:

$$\begin{aligned}\mathrm{RMG}\,(\%) = {} & \text{Final root length} \\ & - \text{Initial root length}/\text{Initial root length} \\ & \times 100.\end{aligned}$$

Table 2 Skewness and kurtosis of nine traits with Mapmaker/QTL (version 1.1b)

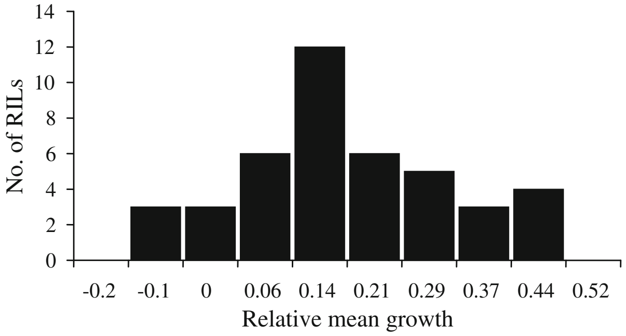
Trait	Distribution mean	Skewness	Kurtosis
Relative mean growth	0.14	0.20	−0.67
Root length mean without Al	7.45	−0.62	1.56
Root length mean with Al	8.29	−0.87	4.36

Data analysis and QTL programs

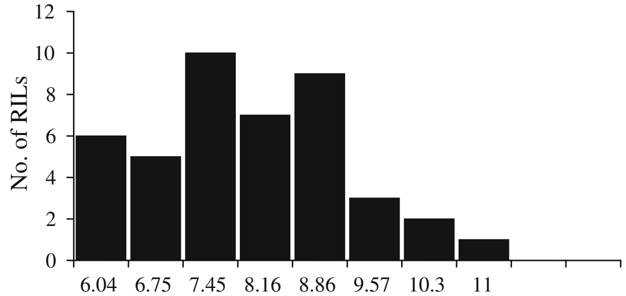
The mapping programs used were Mapmaker EXP (3.0), Mapmaker QTL (1.1) (Lander et al. 1987) for formation of linkage groups and marker–trait associations; SAS and WinQTL Cartographer (2.5; Wang et al. 2005) for single point analysis (SPA). The map data used were described previously (Kassem et al. 2006). A significant difference ($P < 0.05$) was considered to be a preliminary indication of an association between a marker and a QTL for the trait in question because trait data were from highly controlled reproducible growth chamber conditions. For major QTL a value of $P \leq 0.0005$ was suggested by an approximate Bonferroni correction ($P < 0.05/100$) for the set of about 100 independent (unlinked or >10 cM apart) DNA markers (from the 144 mapped). However, when using a smaller population ($n = 40$) and at genomic regions where markers were sparse and gaps between adjacent markers were >10 cM associations in the range $0.05 > P > 0.005$ were considered significant associations (Kassem et al. 2004). In this study, under controlled conditions and with a small population, if the pair interval was large and one of the

Table 1 Selected RILs with elite performance for both RTI and RMG parameters

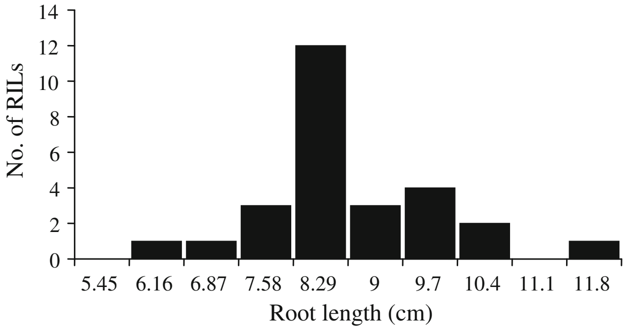
RIL	1	85	40	83
RTI	1.35 ± 0.03	1.38 ± 0.4	1.40 ± 0.07	1.40 ± 0.5
RMG (%)	35 ± 4	43 ± 32	42 ± 7	38 ± 30



(A) Relative mean growth for two sets



(B) Mean root length without Al (cm)



(C) Mean root lengthwith Al

Fig. 3 Trait distributions for tolerance to Al. **a** The relative mean growth for two runs, **b** the mean root length without Al, **c** the mean root length with Al in the media

flanking markers was significant at $P < 0.05$ the uncorrected P value of <0.60 was accepted for the second marker. Precedents with first-pass mapping of other quantitative traits (Hnetkovsky et al. 1996; Chang et al. 1997; Njiti et al. 2002; Kassem et al. 2004) have shown these criteria to be valid during subsequent saturation mapping of intervals inferred at marginal P values (Njiti et al. 1998; Meksem et al. 2001; Yuan et al. 2002; Triwitayakorn et al. 2005; Kassem et al. 2006; Ruben et al. 2006).

Interval maps of QTL

Maps of all linked markers and trait data were simultaneously analyzed with Mapmaker/QTL 1.1 using the F_2-backcross genetic model for trait segregation (Kassem et al. 2006). Putative QTL were inferred when LOD scores exceeded 2.0 at some point in an interval. LOD 2.0 was empirically determined to be equivalent (but not equal) to a single marker $P < 0.005$. The position of each QTL was inferred from LOD peaks at individual loci detected by maximum likelihood tests at positions every 2 cM between adjacent linked markers.

Composite interval maps of QTL

For more accurate location of QTL among sets of linked markers, the composite interval map (CIM) function of WinQTL Cartographer (version 2.5) was used (Wang et al. 2005). Following Kassem et al. (2006) a walk speed of 2 cM and the forward regression method were selected. QTL were inferred when LOD score peaks exceeded 2.0 for the traits studied, considering a $P < 0.05$ corrected for the use of about 100 independent markers. To confirm linkage, an experiment-wise threshold was calculated from 1,000 permutations of each genotype marker against the phenotype in the population.

Results

Root tolerance index

A range of 0.87–1.35 ± 0.05 (RTI) was observed among the RILs (Fig. 1). RTI for Essex was 1.30 ± 0.01 while that for Forrest was 1.03 ± 0.05. On the basis of the RTI criterion the Essex parent performed better than Forrest. This cultivar difference may be due to allelic variation intolerance to Al stress. Alternately the cultivar performance may be the product of a interactions with a wider range of mineral nutritions, especially phosphorus, calcium and magnesium, during stress. RILs 1, 85, 40 and 83 performed better than either parent and showed continued growth even under Al stress (Table 1). RILs 85 and 83 showed wide variations among plants that suggested the RILs might each segregate for a major QTL underlying tolerance of Al stress.

Relative mean growth

Another parameter that focused on growth during Al treatment was RMG. The RMG curve (Fig. 2) showed the same trends observed for RTI. RMG for Essex parent was highest at 31% while that for Forrest parent was 3%. Some RIL roots shrank (negative response) due to Al stress. RILs 1, 85, 40 and 83 performed better than either parent and showed continued growth even under Al stress (Table 1). These observations correlated well

Table 3 Single point analysis by one-way ANOVA for the association of markers with seedling mean root length after aluminum addition for 72 h

Marker	*P*(*F*)	R^2	LG	Essex ± SEM (cm)	Forrest ± SEM (cm)	cM position
Satt122	0.011	0.18	B2	8.75 ± 0.80	7.81 ± 0.64	72.457
Satt202	0.013	0.17	C2	6.67 ± 1.15	7.94 ± 0.89	126.236
Satt310	0.045	0.10	D2	7.89 ± 1.10	8.84 ± 0.69	107.491
Satt252	0.011	0.16	F	7.77 ± 1.04	8.99 ± 0.87	16.084
Satt334	0.024	0.15	F	8.98 ± 0.80	8.14 ± 0.61	78.055
Satt610	0.008	0.24	G	8.90 ± 1.00	7.71 ± 0.53	10.923
Satt138	0.040	0.18	G	7.01 ± 1.38	9.05 ± 1.01	55.985
Satt232	0.028	0.11	L	7.59 ± 1.22	8.62 ± 0.82	10.345
Satt152	0.039	0.10	N	8.98 ± 0.89	8.14 ± 1.08	22.673
Satt159	0.039	0.15	N	8.74 ± 0.89	7.79 ± 1.08	27.132
Satt123	0.050	0.13	O	7.72 ± 1.11	8.90 ± 1.08	86.859

Table 4 Composite interval map of QTL that underlie root tolerance to Al

Trait	LG	Marker/interval	Peak cM position	LOD	R^2	Forrest ± SEM (cm or %)	Essex ± SEM (cm or %)
RLMN	F	Satt160–Satt252	24	3.3	0.31	7.57 ± 1.01	9.20 ± 0.75
RLMN	C2	Satt202–Satt371	3.2	14.7	0.34	6.35 ± 1.03	8.16 ± 0.77
RMG	C2	Satt202–Satt371	3.4	14.1	0.26	9.01 ± 4.59	20.5 ± 3.17

the previous parameter RTI but allowed the genotypic difference in root growth in hydroponics among RILs to be reduced. RILs 85 and 83 showed wide variations among plants that suggested the RIL might segregate for a major QTL underlying tolerance of Al stress. RMG ranking differed only slightly from RTI rankings showing the traits were dependent and highly correlated. The close correlation of traits showed that growth before Al addition was not a major determinant of growth after Al was added. Therefore, only RMG and RL 72 h after Al addition were used further. Among the 83 traits previously reported for this population (Lightfoot et al. 2005; Kassem et al. 2006, unpublished) none were significantly correlated with RMG, RTI or growth after Al treatment (not shown).

Analysis of distributions

As a preamble to QTL mapping it is important to analyze trait distributions for skewness and kurtosis (Table 2). For root length mean (RLM) both with and without Al (Fig. 3); majority of RILs were found to be negatively skewed but positively kurtotic. Therefore, the RL distribution was not altered by Al. RMG (and RTI not shown) was nearly normal showing only a slight positive skew and negative kurtosis. The distributions suggested some major loci and several minor loci might underlie tolerance to Al.

QTL analysis

Since RL following Aluminum addition was the primary measure of tolerance RLM and RMG were used for QTL analysis. The 218 BARC-Satt marker data set and linkage groups developed by Kassem et al. (2006) were used to scan for QTL. Table 3 shows all markers associated with the trait by ANOVA. By using PROC GLM option of SAS, significant marker–trait associations were found for mean RL in the presence of aluminum.

Fourteen markers indicated significant association ($P < 0.05$) with roots length after aluminum addition on eight LGs. There appeared to be 3 linked QTL on LG G and 2 on F judged by position and beneficial allele so 11 QTL in total. Each QTL explained from 9 to 24% of the variation in RLM after Al addition. Five loci had beneficial alleles from Essex and six from Forrest. The many weak associations suggested a large number of loci underlay tolerance of Al.

At the more stringent $P < 0.015$ four QTL were detected on LGs B2, C2, F and G (Table 3). At the very stringent LOD >3.0 (about $P < 0.0001$) QTL Cartographer was used to detect which QTL could be found in significant intervals (Table 4). Only the QTL on LG C2 and F were significant (LOD >3.0). Both beneficial alleles were from Essex. These two loci may represent major genes segregating in RILs 85 and 83 (Table 1). When RMG was measured only the interval on C2 was significant (Table 4).

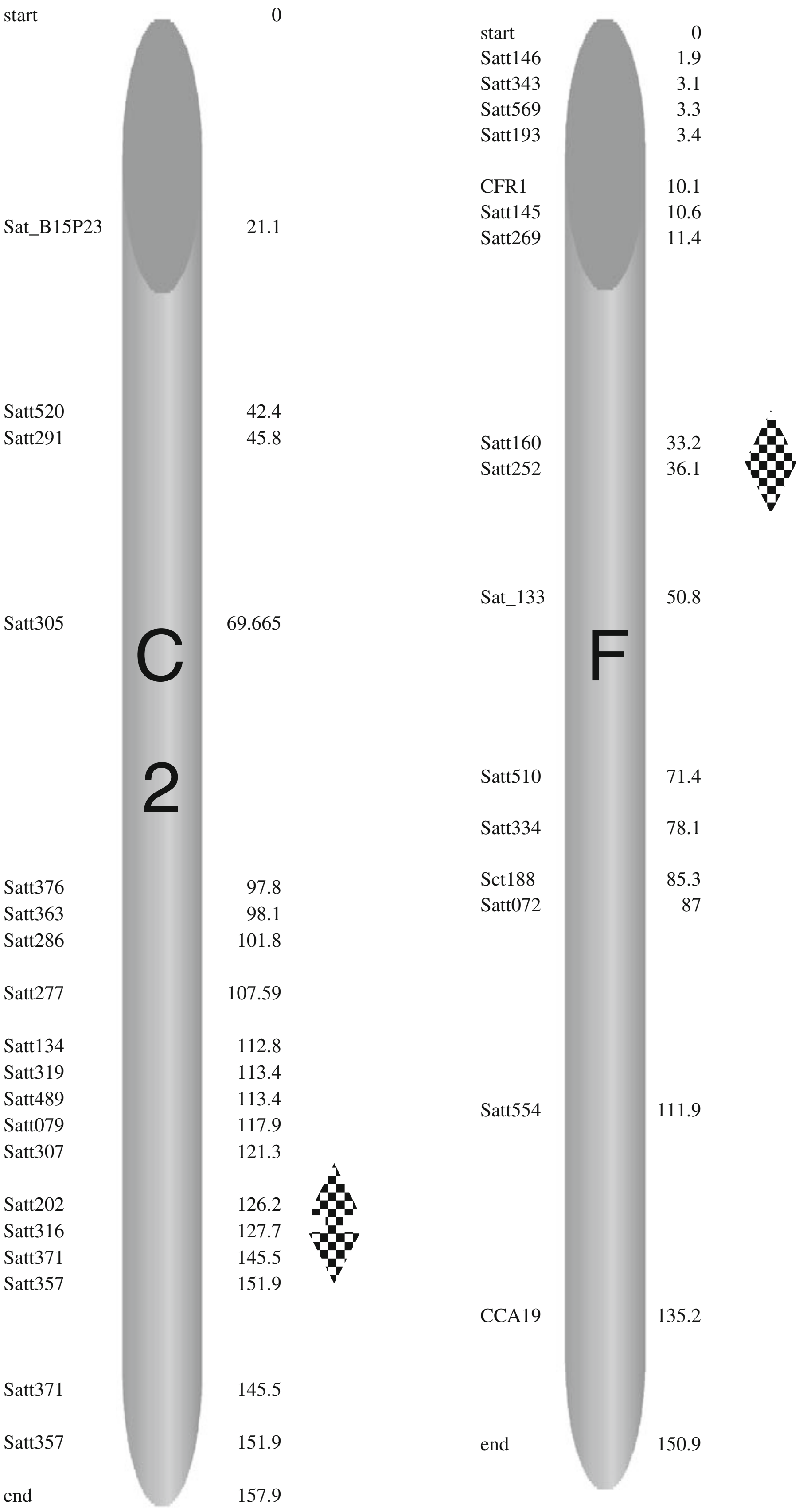


Fig. 4 The positions of the major QTL underlying tolerance to aluminum on linkage groups C2 and F. *Hatched arrows* indicate the intervals within which the loci were identified by CIM

Discussion

Soybean was considered a non-tolerant species with respect to aluminum (Sanchez and Salinas 1981). However, significant cultivar variation in tolerance to Al has been reported (Villagarcia et al. 2001). Cultivar variation in root growth may be underlain by citrate excretion at the root tip (Shen et al. 2005). Linkage group F was assigned to chromosome 13, C2 putatively to chromosome 6. In the interval on F encompassed by Satt160–Satt252 was a mitochondrial citrate synthase [EC 2.3.3.1 (previously 4.1.3.7)]. In the interval on C2 encompassed by Satt202–Satt371 was a gene encoding a cytoplasmic citrate hydro-lyase (aconitase; EC 4.2.1.3). Therefore, genes conditioning citrate excretion may underlie the two major QTL detected on LGs C2 and F (Table 4). The QTL on LG F was reported in PI 416937 (Bianchi-Hall et al. 2000) and so this report confirms that QTL (Fig. 4).

Previous reports identified major Al tolerance QTL on linkage groups LG B1; LG L; and LG D1b2 from Kefeng No. 1 (Qi et al. 2008); LGA2; LG B1; LG F; and LG J from in PI 416937; along with minor loci on LG B1 and LG L (Bianchi-Hall et al. 2000). All three studies agreed that about a third of the trait was explained by a small number of major loci and several polygenes.

Here the locus on LG L was found for the third time, but as a minor locus, by IM but not CIM, suggesting a lesser difference between Essex and Forrest or PI 416937 and Young alleles than the Kefeng No. 1 and Nanong 1138-2 alleles. The loci not found in EF94 (LG A2, B1 and J) may be fixed since the parents are closely related (0.25 coefficient of parentage; reviewed by Lightfoot et al. 2005)

Under Al stress, plant behavior will be strongly affected by root morphology and mineral nutrition. In large amounts, Al may inhibit Ca^{2+} uptake by blocking Ca^{2+} channels in the plasma membrane (Foy 1974, Huang et al. 1992) and magnesium uptake by blocking binding sites of transport proteins (Rengel and Robinson 1989). A large number of genes are expected to be involved in these traits. These may underlie the many QTL of small effect detected (Table 3).

Considering a genetic model of two major genes and many minor genes RILs showing better RTI might be useful for breeding. For root growth RILs 40, 83 and 85 (ranked 13th, 15th and 3rd best) but not RIL 1 (ranked 5th worst) appear to have inherited alleles underlying both better root growth and better root growth in the presence of Al than their counterparts. Such lines are better than Essex and have acquired additional beneficial alleles from Forrest. Therefore, the RILs may be used to breed and select for improved mineral nutrition in soybean.

Acknowledgments This research was funded over the past 11 years in part by grants from the NSF 9872635, ISA 95-122-04; 98-122-02 and 02-127-03 and USB 2228-6228. The integrated genetic and physical map was based upon work supported by the National Science Foundation under Grant No. 9872635. Any opinions, findings, and conclusions or recommendations expressed in this material were those of the author(s) and do not necessarily reflect the views of the National Science Foundation or United States Department of Agriculture. The support of SIUC, College of Agriculture and Office of the Vice Chancellor for Research to DAL was appreciated. The authors thank James H. Klein III, Drs. P. Gibson, O. Myers Jr. and M. Schmidt for assistance with germplasm development and maintenance from 1991 to 2000. We thank the "Soybean Genome Project", at "DoE Joint Genome Institute" for release of the WGS reads, scaffolds and genome sequence.

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