Identification and Confirmation of Aluminum Tolerance QTL in Diploid *Medicago sativa* subsp. *coerulea*

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ABSTRACT

The acid, aluminum (Al) toxic soils found throughout the USA are a major limitation to the productivity of cultivated alfalfa (*Medicago sativa* subsp. *sativa* L.). One strategy to overcome this limitation is to develop Al tolerant alfalfa cultivars. The objective of this study was to identify quantitative trait loci (QTL) controlling Al tolerance in diploid *M. sativa* subsp. *coerulea* genotypes, to be used for introgression of the QTL into cultivated, tetraploid alfalfa. Restriction fragment length polymorphism (RFLP) markers were used in conjunction with a callus growth bioassay to identify Al tolerance QTL in an F_2 population, and confirm them in a backcross population. Single marker analysis was used to find significant ($P < 0.05$) associations between RFLP markers and callus weight means. A soil-based study, conducted with selected diploid, backcross individuals, verified that QTL markers identified in tissue culture were also associated with Al tolerance in whole plants growing in soil. Two RFLP markers, UGAc471 and UGAc502, were associated with Al tolerance in the F_2 and backcross callus assays, and the study in soil. These RFLP markers can be used to introgress these QTL into cultivated, tetraploid alfalfa.

ALFALFA is the most important and widely grown forage legume in the world. In the USA, alfalfa is the fourth largest crop produced, surpassed in number of acres planted only by maize (*Zea mays* L.), soybean [*Glycine max* (L.) Merr.], and wheat (*Triticum aestivum* L.). Alfalfa produces more protein per hectare than grain or oilseed crops, making it highly desirable for hay production and pasture for livestock, especially dairy cows. In addition, alfalfa's ability to fix atmospheric nitrogen makes it valuable for use in crop rotations, increasing the productivity of crops grown after it (Barnes, 1993).

Alfalfa productivity can be limited by Al toxicity. Aluminum is the most abundant metal found in the earth's crust, comprising up to 7% of its mass. At low pH, Al becomes soluble and available to plants, resulting in inhibition of root elongation and reduced plant growth. Al toxicity is a major factor limiting the productivity of crops throughout the world (Kochian, 1995). This is also true in the USA, where for the past century, Al toxicity associated with acid soils has been a major obstacle in alfalfa production (Rechigl et al., 1988). Surface application of lime is typically used to raise soil pH and reduce Al toxicity. Liming, however, is expensive and does not affect the pH of the subsurface soil, which can substantially reduce yield in alfalfa (Sumner et al., 1986). An alternative to liming is the breeding of plants with higher tolerance to Al (Foy, 1988).

Many crop species exhibit variable levels of Al tolerance (Putterill et al., 1991). Al tolerant genotypes of alfalfa have been identified among noncultivated diploid *M. sativa* subspecies (Bouton, 1996). These noncultivated, Al-tolerant genotypes could be used as donor parents to transfer Al tolerance into cultivated alfalfa. Transfer of genes from the diploid level to the autotetraploid level is possible either by somatically doubling the chromosomes in the diploid, or by taking advantage of 2*n* gametes, which occur at a low but regular frequency in both cultivated and wild *M. sativa* germplasm (Veronesi et al., 1986).

In this study, Al tolerance, measured by means of a callus growth bioassay, was used in conjunction with a genetic map to identify QTLs associated with Al tolerance in diploid *M. sativa* subsp. *coerulea* germplasm. These QTLs were first identified in three small F_2 populations, and then confirmed in a larger backcross population. The effect of these QTLs on plant growth in acid, Al-toxic soil was also investigated.

MATERIALS AND METHODS

Plant Materials

Thirty-eight diploid alfalfa genotypes, representing 10 USDA plant introductions (PI) were screened for Al tolerance by means of a callus growth bioassay (Parrott and Bouton, 1990). An Al-sensitive, diploid genotype from *M. sativa* subsp. *coerulea*, PI 440501 (now identified as 440501-2), and an Al-tolerant, diploid genotype from PI 464724 (now identified as 464724-25), also *M. sativa* subsp. *coerulea*, were selected and reciprocal crosses made between them. The F_1 hybrids were screened with a random selection of RFLP probes to confirm that they were hybrids and not selfs. Three F_1 hybrids, designated Al-3, Al-4, and Al-5, were chosen at random and self-pollinated to produce three F_2 populations segregating for Al tolerance. Population one, consisting of 29 individuals, was derived from Al-3. Population two, consisting of 26 individuals, was derived from Al-4. Population three, consisting of 50 individuals, was derived from Al-5. Al-3 and Al-4 were the progeny of PI 440501-2 (Al sensitive) × PI 464724-25 (Al tolerant). Al-5 was from the reciprocal cross. Hybrid Al-4 was backcrossed to the Al-sensitive parent, 440501-2, to produce 121 backcross progeny.

Screening for Al Tolerance

The F_2 and backcross progenies were screened for Al tolerance by means of a callus growth bioassay (Parrott and Bouton, 1990) as described by Dall'Agnol et al. (1996). Leaf tissue was the source of callus. Briefly, calli were established by

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Abbreviations: Al, aluminum; QTL, quantitative trait loci; RFLP, restriction fragment length polymorphism.

growing on modified Blaydes medium for 28 d, as described by Parrott and Bouton (1990). Whole pieces of callus were then weighed and transferred to Blaydes medium, pH 4.0, both with and without the addition of 400 μM of Al. Callus from each individual genotype was grown on three plates with Blaydes plus Al, and three plates with Blaydes minus Al. Calli were transferred to fresh medium at 2-wk intervals, and final weights taken at the end of 8 wk. A ratio of growth on Al relative to growth without Al was scored as the Al tolerance present within each genotype. The experimental design was a complete randomization.

DNA Extraction, DNA Probes, and Southern Analysis

Methods for extraction of DNA, Southern blotting, and hybridization were as described by Brummer et al. (1991). The DNA probes were RFLPs from the UGA alfalfa genetic map (Brummer et al., 1993). Leaf material was collected and frozen in liquid nitrogen, then lyophilized for 24 to 48 h. Dried leaves were ground in a mortar and pestle with liquid nitrogen and a small amount of glass beads. DNA was extracted by a CTAB (hexadecyltrimethylammonium bromide) extraction method (Saghai-Maroof et al., 1984). Five to 10 μg of genomic DNA was digested with *Eco*RI, *Eco*RV, and *Hin*dIII. Digested DNA was separated by electrophoresis on 1.2% (w/v) agarose gels, and blotted onto GeneScreen Plus nylon membranes (PerkinElmer Life Sciences). For the F_2 population, cloned cDNA inserts from an alfalfa seedling library were PCR amplified, and hexamer-labeled with [^{32}P]dCTP, or with both [^{32}P]dCTP and [^{32}P]dATP. Hybridizations were carried out overnight at 65°C, followed by one wash with 2× SSC + 0.1% (w/v) SDS (1× SSC is 0.15 *M* NaCl plus 0.015 *M* sodium citrate) and two washes with 1× SSC + 0.1% SDS, 20 min each at 65°C. The membranes were then wrapped in plastic wrap and exposed to Kodak X-O Mat film; one intensifying screen was used for 7 d at −80°C. For the backcross population, probes were labeled with the DIG Luminescent Detection Kit [Roche Biomedical Supplies (cat. #1363514)]. Hybridizations were carried out in roller bottles overnight at 42°C, followed by two washes with 0.1× SSC + 0.1% SDS, antibody labeling, and luminescent detection, with reagents and instructions provided by the manufacturer. Membranes were then sealed in sheet protectors, and placed on autoradiographic film for 30 min to 1 h.

Marker Alleles

Alleles of markers UGAc044, UGAc141, UGAc471, and UGAc502 were named A through D, respectively. The subscripts "$_S$" and "$_T$" refer to the parent from which the allele was inherited, either the sensitive or tolerant parent. Markers with two alleles in one parent have an added subscript of "$_1$" or "$_2$" to distinguish the two alleles. Markers UGAc044 and UGAc141 each have an allele that is carried by both parents. That allele is named for the parent that is homozygous for that allele.

Soil Study

A study using soil was conducted as previously described (Smith, 1991; Dall'Agnol et al. 1996) with cuttings rather than seedlings. Thirty-two diploid, backcross genotypes were included in the soil assay. The soil, a Cecil sandy clay loam (clayey, kaolinitic, thermic, Typic, Kanhapludults), was collected from the Univ. of Georgia Plant Sciences Farm, near Athens, GA, and had the following characteristics: pH_{water} = 4.7; Al_{KCl} = 0.29 meq/100g; Ca = 0.283 meq/100g; Mg = 0.073 meq/100g; P = 7 kg ha^{-1}; and K = 104 kg ha^{-1}. For limed treatments, lime and nutrients were added to the soil and the following test values were recorded: pH_{water} = 6.5; Al_{KCl} = 0.0 meq/100g; Ca = 1.80 meq/100g; Mg = 0.56 meq/100g; P = 72 kg ha^{-1}; and K = 240 kg ha^{-1}. The experimental design was a randomized complete block with eight replications.

Cuttings were taken and rooted under mist for approximately 3 wk. The cuttings were inoculated with 10 mL of the Al-tolerant *Rhizobium meliloti* strain 59, at 10^7 CFU mL^{-1} (Dall'Agnol et al., 1996; Hartel and Bouton, 1991). At 12 wk, multiple cuttings of each genotype were washed free of soil, and the roots trimmed to 2.5 cm below the crown, and the stems trimmed 2.5 cm above the crown. The cuttings from each genotype were separated into eight classes on the basis of a visual assessment of the vigor of the cuttings, considering such factors as overall size, quantity of roots, stem number, and appearance of the leaves. The classes were then placed in blocks numbered from 1 to 8, ranging from the most vigorous class in block 1, down to the least vigorous class in block 8. Cuttings were placed in either limed, fertilized soil or unlimed, unfertilized soil. The soil and cuttings were placed in 0.72-L polystyrene cups, one cutting per cup, and watered by weight to 75% of field capacity every 2 to 3 d. After 6 wk, the plants were washed free of soil. The roots and shoots were separated, dried, and weighed.

Statistical Analysis

To identify probes associated with Al tolerance, one-way analysis of variance (ANOVA) was used. Data were analyzed one RFLP marker at a time, by means of the GLM Procedure of SAS (SAS Institute Inc., 1994). The marker genotype was used as the predictor variable and the Al tolerance score as the response variable. Means were obtained from averaging over the genotypic classes. A marker was considered to be associated with Al tolerance if there was a significant difference ($P < 0.05$) between marker genotype means for Al tolerance, as measured with the callus assay, or for differences in mean dry weights of roots or shoots for the soil assay, by an *F*-test from the type III mean squares obtained from the GLM Procedure of SAS. This probability level was chosen to enhance our ability to detect QTL across multiple experiments. The coefficient of determination (R^2) was used as a measure of the magnitude of the marker association. Fisher's Protected LSD was used to differentiate among the genotypic classes using the statistical software package StatView (SAS Institute Inc., 1998). Normality of the F_2 and backcross populations was tested with the Shapiro-Wilk test by the UNIVARIATE Procedure of SAS. Broad sense heritability was calculated as $H = V_G/[(V_E/r) + V_G]$, where V_G = component of variance due to genotypes, V_E = component of variance due to error, and r = replication (Wricke and Weber, 1986).

RESULTS AND DISCUSSION

F_2 Study

Some genotypes being tested for Al tolerance, for selection of parents, grew poorly in tissue culture. Therefore, an effort was made to choose parents that grew vigorously in tissue culture so that a lack of tissue culture vigor would not bias the Al tolerance score. The subsp. *coerulea* genotype 464724-25, chosen as the Al-tolerant parent, had an Al tolerance score of 0.72 and grew well in tissue culture. The subsp. *coerulea* genotype 440501-2, chosen as the Al-sensitive parent, grew well in tissue culture without Al, but had an Al tolerance score of only 0.48. Additionally, 440501-2 had the advantage of being one of the parents used in constructing the UGA diploid alfalfa map (Brummer et al. 1993) from which mapped cDNA probes were utilized in this study.

Because of the high levels of heterozygosity present in subsp. *coerulea*, some marker loci had up to four different alleles. If only a single F_1 had been chosen to derive an F_2 population, marker alleles with a positive influence on Al tolerance could have been missed. Therefore, we chose a combination of three F_1s to form multiple, small populations, from which we were able to detect effects from all alleles present in the two parents. Nevertheless, this complicated the molecular marker analysis and influenced the choice of QTL detection method. With three small populations, map construction and the use of interval analysis for QTL detection were not feasible. Single-marker ANOVA was therefore used to identify potential QTLs.

The Al tolerance distribution was not significantly different from normal in the three F_2 populations, suggesting that Al tolerance is a quantitative trait (Fig. 1). The Al tolerant parent had an Al tolerance score of 0.72, and the Al sensitive parent had an Al tolerance score of 0.48. The Al-3, Al-4, and Al-5 F_1s had Al tolerance scores of 0.55, 0.61, and 0.65, respectively, near the expected mid-parent value of 0.60. The populations derived from the F_1s had mean Al tolerance scores of 0.66, 0.57, and 0.65. The Al-3 and Al-5 populations had significantly higher Al tolerance means than the Al-4 population. Since Al-3 and Al-5 were derived from reciprocal crosses, these effects cannot be attributed to maternal or paternal effects. Rather, these differences probably reflect the different QTL alleles these three F_1s inherited from the parents.

Of the 146 alfalfa cDNA probes used to screen the parents and three F_1s, 58 were polymorphic, and were used to genotype the F_2 populations. Five RFLP markers, UGAc044, UGAc141, UGAc191, UGAc471, and UGAc502, were associated with Al tolerance (Table 1). Markers UGAc191 and UGAc471 map within 9.2 centimorgans of each other (Fig. 2) in another diploid *M. sativa* subsp. *sativa* × *M. sativa* subsp. *coerulea* population (Brummer et al. 1993) and probably represent a single QTL. Therefore, only marker UGAc471 will be discussed.

Marker UGAc044 had two alleles, A_S and A_T (Table 1). The Al-sensitive parent had the A_SA_T genotype, the Al-tolerant parent had the A_TA_T genotype, and the F_1s Al-4 and Al-5 had the A_SA_T genotype. The F_1 plant Al-3 had the A_TA_T genotype. The F_2s arising from Al-3, therefore, did not have segregating alleles, and could not be used in the analysis of this marker. This left a total of 52 F_2 individuals, from the other two F_1s, which could be scored for this marker. The Al tolerance means for the A_TA_T F_2 genotypes and for the A_SA_T F_2 genotypes were not significantly different. The A_TA_T and A_SA_S F_2 genotypes were also not statistically different with respect to Al tolerance. This is not consistent with either dominance or additive gene action, suggesting that this marker is a false positive and not actually associated with Al tolerance.

There were two UGAc141 marker alleles (Table 1). The Al-sensitive parent had the B_SB_S genotype and the Al-tolerant parent had the B_SB_T genotype. All three F_1s had the B_SB_T genotype. The Al tolerance means for the B_TB_T F_2 genotypes and for the B_SB_T F_2 genotypes were

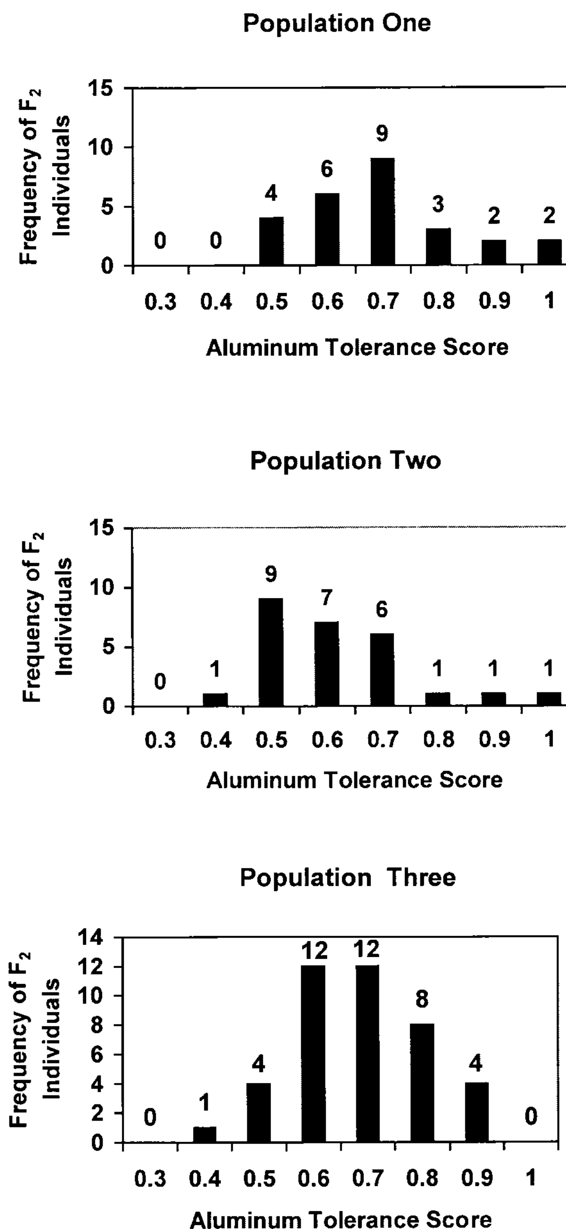

Fig. 1. Frequency distribution of Al tolerance, as measured with a callus bioassay, in three F_2 populations of *M. sativa* subsp. *coerulea*.

not significantly different. The B_TB_T and B_SB_S F_2 genotypes were also not different statistically with respect to Al tolerance. As for UGAc044, this is not consistent with either dominance or additive gene action, suggesting that this marker is also a false positive.

There were three alleles for marker UGAc471, two inherited from the Al-sensitive parent (alleles C_{S1} and C_{S2}) and one inherited from the Al-tolerant parent (C_T) (Table 1). The Al-sensitive parent had the $C_{S1}C_{S2}$ genotype, the Al-tolerant parent had the C_TC_T genotype, and the two F_1s Al-3 and Al-5 had the $C_{S1}C_T$ genotype, while the F_1 Al-4 had the $C_{S2}C_T$ genotype. The Al tolerance means for F_2 genotypes $C_{S1}C_{S1}$, C_TC_T, $C_{S1}C_T$, and $C_{S2}C_T$ were not significantly different, and were higher than the means for genotypes $C_{S2}C_{S2}$. The C_{S1} and C_T alleles appear to have positive effects on Al tolerance.

Marker UGAc502 had four different alleles, the D_{S1}

Table 1. Summary of results from a callus bioassay showing RFLP markers associated with Al tolerance by single marker ANOVA, marker genotypes, and Al tolerance means in a diploid, F_2 population of *M. sativa* subsp. *coerulea*.

Marker locus	Parental genotypes (sensitive × tolerant)	F_1 genotypes	F_2 genotypes	Number of individuals	Aluminum tolerance means	$P > F$	$R^2 \times 100$
UGAc044	$A_SA_T \times A_TA_T$	A_SA_T	A_SA_T	29	0.65 A	0.043	11.56
			A_TA_T	9	0.59 A B		
			A_SA_S	14	0.56 B		
UGAc141	$B_SB_S \times B_SB_T$	B_SB_T	B_SB_T	47	0.65 A	0.034	8.70
			B_TB_T	14	0.63 A B		
			B_SB_S	16	0.55 B		
UGAc471	$C_{S1}C_{S2} \times C_TC_T$	$C_{S1}C_T$	$C_{S1}C_{S1}$	7	0.66 A	0.027	13.74
		$C_{S2}C_T$	$C_{S1}C_T$	33	0.66 A		
			C_TC_T	18	0.66 A		
			$C_{S2}C_T$	16	0.58 A B		
			$C_{S2}C_{S2}$	5	0.50 B		
UGAc502	$D_{S1}D_{S2} \times D_{T1}D_{T2}$	$D_{S1}D_{T1}$	$D_{S1}D_{S1}$	8	0.73 A	0.031	17.79
		$D_{S2}D_{T1}$	$D_{T2}D_{T2}$	8	0.70 A B		
		$D_{S2}D_{T2}$	$D_{S2}D_{T2}$	14	0.63 B C		
			$D_{S1}D_{T1}$	12	0.62 B C		
			$D_{S2}D_{S2}$	13	0.60 B C		
			$D_{T1}D_{T1}$	12	0.59 C		
			$D_{S2}D_{T1}$	8	0.55 C		

and D_{S2} alleles from the Al-sensitive parent, and D_{T1} and D_{T2} alleles from the Al-tolerant parent (Fig 3). The sensitive parent had the $D_{S1}D_{S2}$ genotype, and the tolerant parent had the $D_{T1}D_{T2}$ genotype. The Al-3 F_1 had the $D_{S1}D_{T1}$ genotype, the Al-4 F_1 had the $D_{S2}D_{T1}$ genotype, and the Al-5 F_1 had the $D_{S2}D_{T2}$ genotype. The $D_{S1}D_{S1}$ F_2 genotypes had higher Al tolerance means than the $D_{S1}D_{T1}$ F_2 genotypes, suggesting additive rather than dominance gene action. There was no significant difference in Al tolerance means between the $D_{T2}D_{T2}$ and $D_{S2}D_{T2}$ F_2 genotypes, suggesting that these alleles have similar, positive effects on Al tolerance. The D_{S1}, D_{S2}, and D_{T2} alleles all appear to be positively associated with Al tolerance.

Backcross Study

To confirm the QTL identified in the F_2 populations, a backcross population was created. The backcross population was screened with the four RFLP markers associated with Al tolerance in the F_2 populations. Because of the heterozygosity of marker alleles, no single F_1 was ideal for confirming all of the potential QTLs identified. Nevertheless, all alleles except UGAc502 D_{T2} were represented in the backcross population. A disadvantage of this backcross population was that it was not possible to compare all combinations of alleles. The backcross population did have the advantage of being a single large population, rather than three small populations, as in the F_2 generation.

Al tolerance was normally distributed in the backcross population (Fig. 4). Heritability for Al tolerance as measured with the callus bioassay was 93%, a relatively high value. This high heritability could be a result of using a tissue culture assay, with well defined growth conditions, rather than a field experiment, where environmental conditions could have resulted in large G × E interactions and lower heritability.

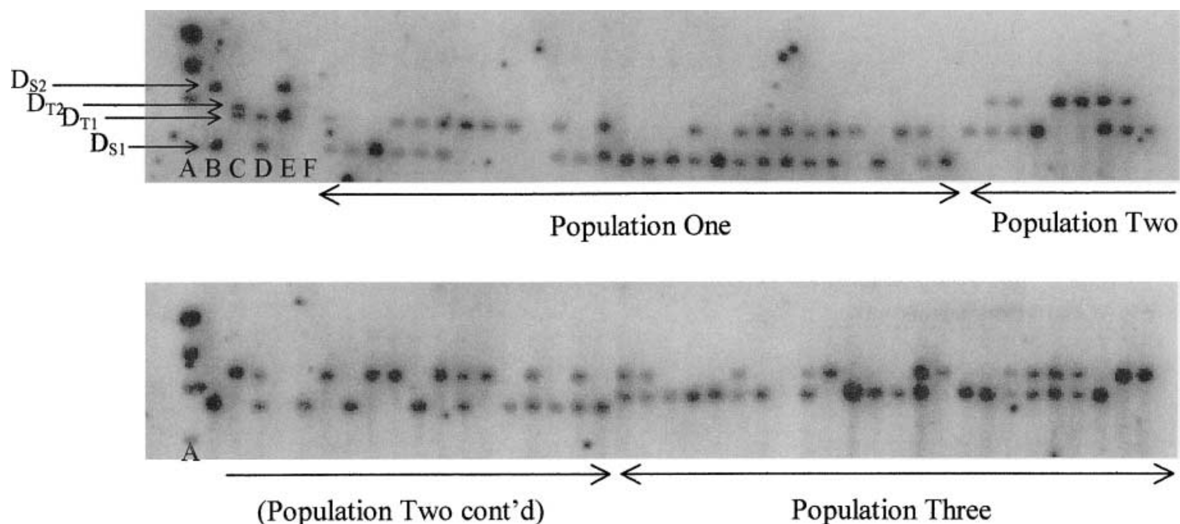

Fig. 2. Segregation of Al tolerance associated RFLP marker UGAc502 in three F_2 populations. A is *Hin*dIII-cut λ DNA; B is the Al sensitive parent *M. sativa* subsp. *coerulea* 440501-2, showing the D_{S1} and D_{S2} alleles; C is the Al tolerant parent *M. sativa* subsp. *coerulea* 464724-25, showing the D_{T1} and D_{T2} alleles; D is F_1 Al-3; E is F_1 Al-4; F is F_1 Al-5. Population One is an F_2 population derived from selfing Al-3, Population Two is from Al-4, and Population Three is from Al-5.

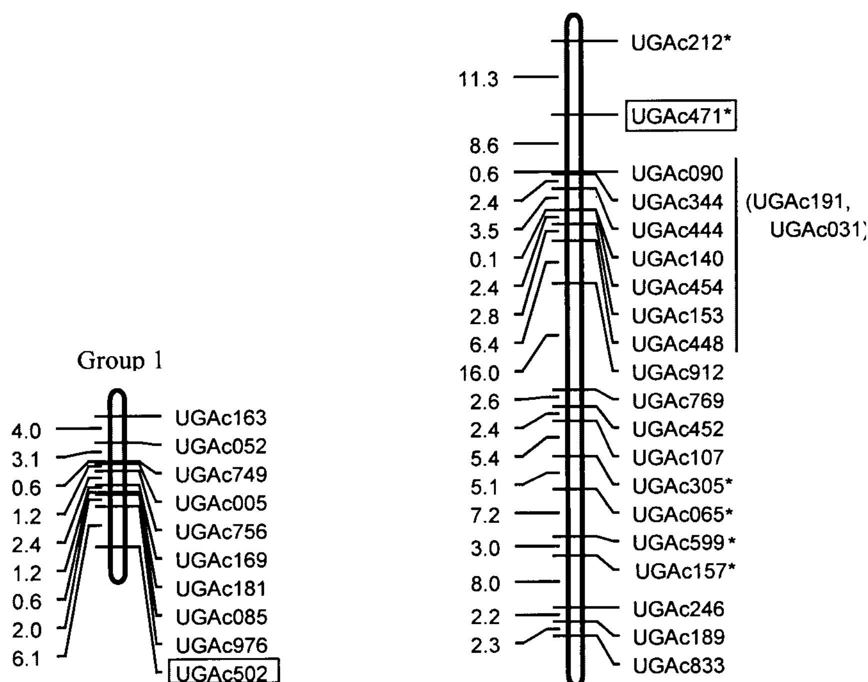

Fig. 3. Linkage groups 1 and 8 of the UGA Alfalfa RFLP map, showing the locations of markers UGAc471 and UGAc502, which are associated with Al tolerance.

RFLP banding patterns were more complex in the backcross population than in the F_2 population. In the F_2 population, two segregating alleles were scored per locus even though there were other nonsegregating restriction fragments present. Brummer et al. (1993) found that 41% of cDNA clones tested in a diploid population had one to two restriction fragments, 27% had three to four restriction fragments, 24% had five to eight restriction fragments, and 8% had greater than eight restriction fragments per locus. Nevertheless, it was relatively simple to pick out the two segregating alleles from a background of nonsegregating restriction fragments. In the backcross population, however, three of the five potential QTL markers had three segregating alleles and four genotypes, as well as multiple, nonsegregating bands. Distinguishing which alleles should be scored from this background of nonsegregating fragments was difficult. For this reason, each of the markers was given a presence–absence score for each restriction fragment, and individual backcross genotypes were inferred from the presence–absence scores. Al tolerance means for both genotypes and alleles are presented (Table 2).

There were no marker genotypes or individual restriction fragments significantly associated with Al tolerance in the backcross population for markers UGAc044 or UGAc141 (data not shown). This supports evidence from the F_2 study that these markers are false positives.

The marker UGAc471 genotypes were not associated significantly with Al tolerance in the backcross population (Table 2). The $C_{S1}C_T$ genotypes, however, had significantly higher Al tolerance means than the $C_{S2}C_{S2}$ genotypes. When scored as single alleles, absence of the C_{S2} allele was associated with higher Al tolerance means, whereas presence of the C_T allele was associated with

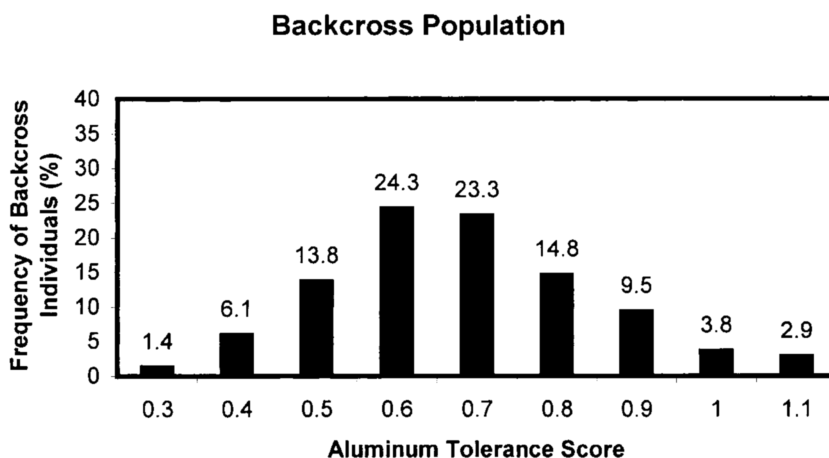

Fig. 4. Frequency distribution of aluminum tolerance, as measured with a callus bioassay, in a backcross population of *M. sativa* subsp. *coerulea*.

Table 2. Single factor ANOVA of molecular markers scored as single genotypes and as alleles, and Al tolerance scores in a backcross population of *M. sativa* supsp. *coerulea*.

Molecular marker loci					
UGAc471 *P*-Value = 0.1191			UGAc502 *P*-Value = 0.0144		
Backcross genotypes	Al tolerance means		Backcross genotypes	Al tolerance means	
$C_{S1}C_T$	0.79 A		$D_{S2}D_{S2}$	0.80 A	
$C_{S2}C_T$	0.73 AB		$D_{S2}D_{T1}$	0.75 A	
$C_{S1}C_{S2}$	0.71 AB		$D_{S1}D_{S2}$	0.71 AB	
$C_{S2}C_{S2}$	0.68 B		$D_{S1}D_{T1}$	0.64 B	
Marker Allele‡	+§	−	Marker Allele	+	−
C_{S1}	0.75	0.70	D_{S1}	0.68	0.77**
C_T	0.76†	0.70	D_{T1}	0.69	0.76*
C_{S2}	0.71	0.79*	D_{S2}	0.74*	0.67

* Indicates significance at $P = 0.05$.
** Indicates significance at $P = 0.01$.
† Significant at the 0.10 probability level.
‡ Each marker allele was treated as a single marker for ANOVA.
§ A1 tolerance means are for individuals with presence (+) or absence (−) of a marker allele.

higher Al tolerance means. It is possible that genotypes with the C_TC_T genotypes, which did not occur in this backcross, would have a stronger association with Al tolerance.

UGAc502 has four marker alleles. The D_{T2} allele, identified as a positive allele in the F_2 callus study, did not occur in the backcross population (Table 2). Confirmation of this allele would require the construction of a different population. The $D_{S1}D_{T1}$ genotypes had significantly lower Al tolerance means than the other genotypes. When scored as alleles, presence of the D_{T1} allele is associated with Al tolerance. This allele however, also occurs in the $D_{S2}D_{T1}$ genotype, which is not significantly different than the $D_{S2}D_{S2}$ and $D_{S1}D_{S2}$ genotypes. The D_{S2} allele is the only allele clearly associated with Al tolerance, as measured with the callus bioassay, in the backcross population.

Soil Study

Liming of soil is the method used in the field to raise soil pH and eliminate Al toxicity. We used the backcross genotypes to determine if RFLP markers identified as being associated with Al tolerance by the callus bioassay would also be associated with traits typically used to measure Al tolerance in whole plants, such as root growth in unlimed soil.

Genotypes for the soil study were chosen on the basis of the marker alleles of markers UGAc471 and UGAc502. There were no genotypes that had only favorable or only unfavorable alleles. An attempt was made, however, to select plants that had mostly favorable or mostly unfavorable markers alleles for both markers. Al tolerance scores from the callus assay were not used to select genotypes. Therefore, marker-assisted selection, rather than selection based on phenotype, was used to select plants for this study. The purpose was not only to confirm positive alleles, but also to test the effectiveness of marker-assisted selection.

There were no alleles significantly associated with Al tolerance in the soil-based study for markers UGAc044 or UGAc141 (data not shown). This supports evidence from the F_2 and backcross studies that suggest that these markers are false positives.

Marker allele C_T of UGAc471 was associated positively with the dry weight of roots and tops grown both in unlimed and limed soil (Table 3). This result is consistent with both the F_2 and backcross callus studies. The C_{S1} marker allele, which was positively associated with Al tolerance in the F_2 callus study, but not in the backcross callus study, was not associated with growth in either limed or unlimed soil. The C_T allele appears to be the only positive allele for UGAc471 and is the allele that should be used for marker-assisted introgression of Al tolerance into cultivars of alfalfa.

Marker allele D_{S2} of UGAc502 is positively associated with dry weights of roots and tops in both limed and unlimed soils (Table 3). Marker allele D_{S1} was associated with dry weight of roots grown in unlimed soil and dry weights of roots and tops in limed soil. Results from the two callus bioassays were not conclusive for these markers. In the F_2 callus assay D_{S1}, and to a lesser degree D_{S2}, were associated with Al tolerance. In the backcross callus assay, D_{S2} but not D_{S1} was associated with Al tolerance. On the basis of the soil assay, it appears that both alleles have a positive effect on Al tolerance. The D_{S2} allele has a greater probability of association with Al tolerance than the D_{S1} allele, but it is possible that both alleles could be used for marker-assisted selection.

A ratio of growth in unlimed soil to growth in limed soil is not typically an effective measure of Al tolerance (Dall'Agnol et al., 1996; Smith, 1991). It is often the case that genotypes with poor vigor (i.e., dry matter yield) and poor growth in both unlimed and limed soil

Table 3. Average dry weight growth of diploid, backcross *M. sativa* subsp. *coerulea* genotypes grouped according to presence (+) or absence (−) of a marker allele.

Marker allele	Unlimed root means		Unlimed top means		Limed root means		Limed top means	
	g							
	+	−	+	−	+	−	+	−
UGAc471 CS1	0.17	0.15	0.32	0.26	0.27	0.22	0.71	0.75
UGAc471 CS2	0.16	0.17	0.30	0.31	0.25	0.27	0.71	0.75
UGAc471 CT	0.18***	0.15	0.33***	0.28	0.29***	0.23	0.85**	0.64
UGAc502 DS1	0.16*	0.12	0.28	0.23	0.28*	0.21	0.72*	0.50
UGAc502 DS2	0.17***	0.11	0.33***	0.18	0.30***	0.19	0.81***	0.41
UGAc502 DT1	0.15	0.13	0.28	0.23	0.26	0.23	0.68	0.54

* Indicates significance at $P = 0.05$.
** Indicates significance at $P = 0.01$.
*** Indicates significance at $P = 0.001$.

have high ratios. These ratios, therefore, may reflect low vigor rather than high Al tolerance (Scott and Fisher, 1989). Use of ratios was also not an effective measure of aluminum tolerance in our study. None of the markers showed any association with Al tolerance based on a ratio of dry weight in unlimed soil to dry weight in limed soil (data not shown).

Broad sense heritability in the soil-based assay was previously reported as 0.66 and 0.77 for root growth in unlimed and limed soil, respectively (Smith, 1991). Although Smith (1991) used *M. sativa* subsp. *sativa* and we used *M. sativa* subsp. *coerulea*, this suggests that the soil-based assay is as effective a selection strategy for Al/acid soil tolerance as the callus bioassay. Dall'Agnol et al. (1996) compared the soil and cell culture methods of selecting genotypes for Al tolerance and found a greenhouse soil-based assay to be the most efficient way to screen and select alfalfa for tolerance to acid soil and Al toxicity. The advantage of the soil-based assay is that it is less expensive, as well as less labor intensive than the callus bioassay. On the basis of the molecular marker associations observed in this soil-based study, the soil-based assay yields molecular marker associations that are consistent with those associations identified in the callus-based assays.

Efficiency of QTL experiments is difficult to measure. One method used is to sum cumulative phenotypic variance attributable to a combination of all significant QTLs (Tanksley, 1993). Where complete maps have been used for quantitative studies, these values have ranged from 10 to 95%, but typically average from 30 to 40% (Tanksley, 1993; Kearsey and Farquhar, 1998). The variation explained by markers UGAc471 and UGAc502 cumulatively accounted for 31.53% of the variation (Table 1). Single marker detection of QTLs using ANOVA, however, does not give an accurate estimation of the amount of variation explained by each QTL, since the exact location of the QTL is not known; therefore, it is not possible to distinguish between tight linkage to a QTL of small effect, and loose linkage to a QTL of large effect (Lander and Botstein, 1989). High resolution mapping of the regions surrounding the identified QTL, and interval analysis, could give a more accurate location of the QTLs and estimation of the magnitude of their effects.

Uncertainty as to the exact location of a QTL has consequences for marker-assisted breeding. The further a marker is from a QTL, the more likely it is that a recombination event could separate the QTL from the marker allele. Flanking markers tightly linked to the QTL would allow recombination between the QTL and the markers to be detected, and could also be used for marker-assisted backcrossing. It is possible that UGAc191 and UGAc471 are flanking a single QTL. The other QTL identified, however, is linked to only a single marker, UGAc502. High resolution mapping of the regions surrounding the identified QTL could be used to identify flanking markers for this QTL.

One of the two Al tolerance QTLs identified in these experiments, linked to UGAc502, was derived from the Al sensitive parent. It has been observed that unadapted germplasm, with poor trait phenotypes, often contain alleles that are actually favorable for the trait (Moncada et al., 2001). This is the basis for advanced backcross QTL breeding (Tanksley and Nelson, 1996) in which these alleles are introgressed into elite cultivars that allow for favorable expression of these alleles. While advanced backcross QTL breeding is not amenable to heterozygous, outcrossing crops such as alfalfa, this study demonstrates that these unexpressed, favorable alleles do exist in unadapted *M. sativa* germplasm.

SUMMARY AND CONCLUSIONS

Four unlinked RFLP markers were identified as being associated with Al tolerance in three diploid F_2 populations by means of a callus bioassay. Two of these markers were confirmed in a diploid backcross population. Marker UGAc471, allele C_T, and marker UGAc502, alleles D_{S1} and D_{S2} were positively associated with Al tolerance. A study in soil, utilizing diploid, backcross genotypes, was conducted to demonstrate that these QTL also had value to whole plants growing in soil. Future work in this area will consist of high resolution mapping of the regions surrounding the three confirmed QTL to locate more accurately the QTL and estimate the magnitude of their effects. This will facilitate the introgression of these marker alleles into cultivated, autotetraploid alfalfa via $2n$ gametes and marker-assisted selection.

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