IMPROVING ACID AND ALUMINUM TOLERANCE IN ALFALFA USING BREEDING AND GENOMICS

by

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(Under the Direction of H. Roger Boerma)

ABSTRACT

Alfalfa, *Medicago sativa* L., is an important forage crop in the U.S. and worldwide. However, in acid soil, its productivity and persistence dramatically decrease. Acid-soil syndrome causes a severe toxicity that inhibits root growth and development exacerbated by Al^{+3}. Breeding and genomic approaches to improve acid/Al tolerance provide new opportunities to grow alfalfa in lands considered marginal for alfalfa growth. In this study, three different approaches were used to identify and to evaluate acid/Al tolerance in alfalfa: (1) identification and mapping of quantitative trait loci (QTL) associated with acid/Al tolerance, (2) evaluation of transgenic plants over-expressing *Pseudomonas aeruginosa* citrate synthase (CS) and/or a gene coding for a plasma membrane H^{+}-ATPase from *Daucus carota* (DcPA1), and (3) comparison of genetic gain for acid/Al tolerance as assessed by seedling biomass production in acidic soil under greenhouse conditions. Single-factor analysis and interval mapping identified QTL for Al tolerance on Altet-4 and on NECS-141 linkage groups. The phenotypic variation explained by individual QTL ranged from 9.5 to 35.3%. Some of these QTL were detected in multiple environments and for multiple traits while others were environment-specific. Four isogenic T_2 populations, containing neither, one, or both transgenes (CS+DcPA1) were evaluated for acid/Al

tolerance. The transgenic populations containing either CS or DcPA1 or both genes showed higher acid/Al tolerance than the non-transgenic population. No advantage of combining both transgenes in the same genetic background was observed. Lower levels of Al in shoot tissue were observed for the transgenic populations over the non-transgenic population. The comparison of selection methods showed that the phenotypic recurrent selection with gridding in unlimed soil was most effective at improving the Bulldog 805 population for shoot dry weight. This method of selection was the most effective in terms of the resources used and the responses achieved. Direct selection in unlimed soil resulted in increased alfalfa growth in acid and Al-rich soils compared to selection in limed soil. The information generated in these three experiments can be useful in improving our understanding of acid/Al tolerance in alfalfa and in developing cost-effective and efficient methods to obtain enhanced alfalfa germplasm in the future.

INDEX WORDS: alfalfa, greenhouse soil-based assay, unlimed and limed soils, quantitative trait loci, transgenic, citrate synthase, plasma membrane H^+-ATPase, recurrent selection, grid selection, among-within family selection

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DEDICATION

This dissertation is dedicated to my wife Barbara, my son Emiliano, and my daughter Julieta. Without their support, nothing would have been possible. I also want to dedicate this work to my family members and friends in Uruguay.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Importance of alfalfa

Alfalfa, *Medicago sativa* L., is one of the most important forage legumes worldwide and is the fourth most important crop in U.S. agriculture in terms of both farm gate value and hectares under cultivation (USDA, 2009). Alfalfa is successfully cultivated throughout most of the country, under very different management and environmental conditions, ranging from intensively irrigated alfalfa in the Southwest, to dryland cultivation in the northern Great Plains. Commercial cultivars have been developed to accommodate specific growing conditions throughout the country.

Acid soils, aluminum toxicity, and tolerance mechanisms

Acid soils are a limitation for alfalfa cultivation in many parts of the world, including the southeastern USA (Bouton and Sumner, 1983b). Under low pH soil conditions, aluminum (Al) and manganese (Mn) become soluble causing a severe toxicity that inhibits root growth and development (Kochian et al., 2004a). Two different types of Al tolerance mechanisms exist; those that exclude Al from the root apex through organic acid exudation and those that allow the plant to tolerate Al accumulation in roots and shoots (Kochian et al., 2004a). The predominant mechanism varies among and within species (Kochian et al., 2005). Studies in barley, wheat, sorghum, rye, soybean, rice, and Arabidopsis indicate that aluminum tolerance is controlled by

few genes of large effect, but other studies, sometimes in the same species, have reported that Al tolerance is a more complex trait (Kochian et al., 2005).

Soil acidity can be ameliorated by liming, effectively eliminating Al toxicity in the plow layer (Foy, 1992) but not in the subsoil (Bouton and Sumner, 1983b). The increased costs of lime and fertilizer have increased interest in identifying genetic tolerance to acidic conditions (Bouton and Sumner, 1983b). Genetic selection for acid soil tolerance can increase alfalfa productivity and reduce production costs, with previous experiments showing moderate success (Bouton and Radcliffe, 1989; Bouton, 1996).

Methods to evaluate acid soil and aluminum tolerance

In *Medicago,* various methods have been used to measure Al tolerance some of which are discussed individually below.

Callus culture. Parrott and Bouton (1990) developed a tissue culture bioassay in which callus growth on an aluminum toxic cell culture medium relative to growth on media without Al was used as a measure of Al tolerance. The method consists of inducing callus from seedling hypocotyls on modified Blaydes medium, and splitting the resulting callus 30 d after induction into equal pieces that are transferred to separate plates with and without aluminum. The calli are transferred onto fresh media every 15 d for 8 wk and weighed at each transfer. The technique proved useful to distinguish between an acid-tolerant (AT) and an acid-sensitive (AS) *M. sativa* germplasm (Parrott and Bouton, 1990). The limitation of the method is that the number of genotypes that can be evaluated is limited because of the time and resources needed. Al tolerance was also observed at the callus level in tomato (Meredith, 1978) and sorghum (Smith et al., 1983).

Soil “cup test”. This assay was initially developed to assess acid tolerance of the alfalfa core collection (Bouton, 1996). The method evaluates seedlings grown in 720-ml Styrofoam cups filled with 930 g of Cecil sandy clay loam soil. The method consists of the following treatments: (1) an unlimed treatment with 730 g of unlimed and unfertilized soil overlaid with an upper layer of 200 g of the same soil limed to a near neutral pH, and (2) a limed treatment in which the entire cup is filled with soil amended with lime to increase the pH to near 7.0. The cups are watered by weight until 90% of their field capacity with deionized water to prevent leaching and soil saturation, which causes increased acidic conditions and Mn toxicity (Foy and Brown, 1964a; Sumner et al., 1986). The seedlings are allowed to grow for 8 wk at which time the plants are destructively harvested. Shoots are separated from roots at the soil surface. The intact cup is then cut at the point between soil types in the unlimed treatment and the same level is used for the limed treatment. Roots from both portions (i.e., the top is limed in both treatments; the bottom is either limed or unlimed) are then washed free of soil, dried, and weighed. This procedure was also used as a screening method to develop alfalfa germplasm tolerant of acid and aluminum toxic soils (Dall'Agnol et al., 1996). Aluminum tolerance is assessed as the total root growth, total shoot growth, and the ratio between growths in unlimed vs. limed soil (Bouton, 1996; Dall'Agnol et al., 1996; Narasimhamoorthy et al., 2007b; Sledge et al., 2002). In the soil assay, relative growth was found to be a poor indicator of Al tolerance, as genotypes with poor vigor and limited growth in limed, fertilized soil, could have high relative root and shoot growth (Dall'Agnol et al., 1996). In addition, the ratios show very low narrow sense heritability. Therefore, root growth in unlimed soil is considered a more effective estimate of Al tolerance. The soil assay was also used with rooted stems and could successfully discriminate among genotypes differing in Al tolerance (Sledge et al., 2002). The soil based assay showed greater

discriminating power among genotypes than either hydroponic or root staining methods (Narasimhamoorthy et al., 2007b). The soil assay requires close monitoring of water content by weight or by electronic sensors, which is highly time consuming, and the cups themselves are rather large. As a consequence, the soil assay is low throughput and thus limited in the number of entries that are able to be evaluated simultaneously. For example, evaluating mapping populations of 200 individuals is almost impossible with this method.

Hydroponic systems. Hydroponic screening techniques have been extensively used to evaluate Al tolerance in many species, including soybean, rice, wheat, and maize (Bianchi-Hall et al., 2000; Nguyen et al., 2002; Ninamango-Cárdenas et al., 2003; Xue et al., 2008; Zhou et al., 2007). These evaluation systems have also been used to assess Al responses in alfalfa and *Medicago truncatula* (Barone et al., 2008; Narasimhamoorthy et al., 2007b; Scott et al., 2008; Sledge et al., 2005; Zhang et al., 2007). The method evaluates seedlings grown on floating devices for short periods of time, from 5 to 7 d. The general method consists of growing the seedlings initially in an Al-free solution and then either transferring them to new solutions containing Al or placing them in a shock solution with high Al content for 1 d before growing in Al solutions with a more moderate concentration. The pH and Al concentration in the solution are monitored and adjusted every day to maintain constant levels. The length of roots at the end time point serves as the measure of Al tolerance. In some cases, the Al concentration in the root may be estimated by root staining (Narasimhamoorthy et al., 2007b; Scott et al., 2008).

This methodology is rapid, in terms of time, and hence able to screen large numbers of accessions or genotypes (Narasimhamoorthy et al., 2007b; Scott et al., 2008), and it has some advantages compared to soil assays for measuring the effect of added Al (Sledge et al., 2005). However, this methodology has not shown high correlations with soil assays (Narasimhamoorthy

et al., 2007b). General combining ability was observed for root length growth, suggesting that recurrent selection would be successful in improving root length in Al solution (Zhang et al., 2007), but whether this is related to enhanced Al tolerance in the field remains unclear. Root length in Al solution has been increased in populations selected using the hydroponic system (Scott et al., 2008).

Root staining. This method estimates the amount of Al present in root tips based on stains which react with the Al absorbed and accumulated by the plants. Many stains can be used, but the most commonly used have been lumogallion and hematoxylin (Barone et al., 2008). Plants are exposed to Al for short a period, root tips are sampled and sectioned longitudinally, and root sections are washed with buffer solutions, and stained. Al forms a complex with the stain solution reflecting a fluorescence color. Digital images are taken and the quantification of the stain in the roots is achieved by measuring the intensity of the fluorescence. The more intense the fluorescence, the more Al is accumulated in the root tips, and hence, the more Al sensitive the plant. The absence of color indicates that organic acids (citrate or malate) has chelated the Al and prevented its accumulation in the root apices. This technique has been used in wheat, barley, *M. truncatula,* and alfalfa to identify superior genotypes related to their Al tolerance (Barone et al., 2008; Delhaize et al., 2004; Narasimhamoorthy et al., 2007b; Ryan et al., 1995).

Genetic engineered barley genotypes were successfully discriminated based on this technique (Delhaize et al., 2004). A modified technique, staining root tips with 0.1% solution of Eriochome cyanine R, was used to detect Al accumulation in transformed wheat genotypes (Ryan et al., 1995). Root staining was capable of identifying genotypes with higher tolerance to Al, when analyzing 15 transgenic alfalfa plants transformed for citrate synthase (Barone et al., 2008). A wide range in fluorescence between the most sensitive and the most tolerant accessions

was found using seedlings of *M. truncatula* (Narasimhamoorthy et al., 2007b). Since this technique correlated well with hydroponics, it could be an alternative to screen for Al tolerance, but it is time-consuming and labor intensive (Narasimhamoorthy et al., 2007b). No consistency was found between root staining methodologies and the soil based assay (Barone et al., 2008). The value of root staining to assess Al tolerance is thus not clear due to its highly variable results and inconsistency with soil evaluations.

Whole plant culture. Screening methods which can discriminate between the effects of acidity and Al on root growth and development would provide a better understanding of the process involved in Al/acid tolerance. A new methodology has been described to differentiate between these two factors using rooted stems grown in a $CaCl_2$ culture media containing 1% Gelrite (Khu et al., 2012). Plants can be grown under neutral pH, acidic pH without Al, or acidic pH with Al. The genotypes are grown for 18 d, at which time the plants are harvested and the root length, lateral root number, and root branching are quantified using the winRHIZO software (Regent Instruments, Québec, Canada). In addition, absolute root growth and ratio of roots growing in Al-containing media and Al-free media are determined to quantify the Al tolerance.

Summary of methods. All these methods are capable of identifying variation between and within populations. However, their utility for use in selection programs needs further analysis, since they have shown contradictory results when evaluated on the same genotypes. In general, the correlations between the culture (tissue or hydroponics) or root staining assays and soil assays are low. Although we assume that the soil assay may be most relevant for field conditions, we do not know how selection using any of these methods relates to progress for Al tolerance in the field.

Genetic mapping for acid soil/aluminum tolerance

The identification and evaluation of quantitative trait loci (QTL) associated with traits of interest enables the effective introgression of favorable alleles from any source into elite breeding germplasm (Bernardo, 2002). Aluminum tolerance QTL have been identified in rice (Nguyen et al., 2002; Xue et al., 2008), wheat (Zhou et al., 2007), maize (Ninamango-Cárdenas et al., 2003), soybean (Bianchi-Hall et al., 2000), and *Arabidopsis thaliana* (Ikka et al., 2008a) by phenotyping in nutrient solutions with different concentrations of Al^{+3}. These studies have identified three (Bianchi-Hall et al., 2000) to nine (Nguyen et al., 2002) QTL associated with Al tolerance. One QTL in a wheat doubled haploid mapping population explained 49% of the phenotypic variation in the population (Navakode et al., 2009).

Aluminum tolerance QTLs based on the callus assay were indentified in diploid alfalfa F_2 populations and confirmed in a backcross population using both callus and soil assays (Sledge et al., 2002). The effects of marker alleles were consistent in both F_2 and backcross callus studies. In addition, two alleles that had not been confirmed in the callus assay of the backcross population were identified in the soil study. More information is needed about the regions surrounding the QTL to precisely locate them on the linkage group and estimate their effects. A further mapping study using the backcross population first evaluated by Sledge et al. (2002) added 162 EST-SSR marker loci to the population and identified QTL for Al tolerance based on the callus assay on linkage groups LG 1, LG 2, LG 3, LG 4, and LG 5 (Narasimhamoorthy et al., 2007a). A major QTL explained 37.9% of the variation for Al tolerance in this population.

Transgenesis to improve acid soil/aluminum tolerance

The exudation of organic acids by root tips plays an important role in conferring Al tolerance to plants, and Al tolerant plants can secrete these acids in response to Al in the rhizosphere (Barone et al., 2008; Delhaize et al., 1993a). The Al^{+3} present in acid soil forms a chelate in the presence of organic acids such as citrate, malate, and oxalate (Barone et al., 2008; Kochian et al., 2004a). Transgenic plants over-expressing organic acids have shown improved Al tolerance in several crops, including tobacco and papaya (de la Fuente et al., 1997) and canola (Anoop et al., 2003). The over-expression of citrate synthase was also introduced into alfalfa (Barone et al., 2008).

A second transgenic approach to improving Al tolerance is to introgress organic acid transporters (Kochian et al., 2004a). This approach has been effective in barley (*Hordeum vulgare*), a very Al-sensitive cereal crop (Delhaize et al., 2004). The DcPA1 gene coding for a plasma membrane H^{+}ATPase from *Daucus carota* was shown to play a role in the proton exudation (Ohno et al., 2004). The optimum Al tolerance may result from a strategy that integrates the organic acid over-expression with a gene encoding the corresponding organic acid transporter (Barone et al., 2008).

Breeding to improve acid soil/aluminum tolerance

Field based selection. In the field, aluminum or acid soil tolerance is manifested as higher biomass yield in tolerant relative to sensitive germplasm when grown in acidic and Al-rich soils. Recurrent selection based on field performance was used to develop a tolerant germplasm, Georgia - Acid Tolerant (GA-AT), derived from U.S. cultivars (Bouton and Radcliffe, 1989). GA-AT was the result of 3 cycles of selection in acid soil and showed enhanced top-root growth

and nodulation compared with unimproved control and aluminum susceptible (AS) germplasm selected for 3 cycles in limed soil, when tested in acid soil with pH 4.6 and Al at 32 µg g^{-1}. The levels of tolerance realized in GA-AT were not high enough to be economically useful in low pH environments because yields were far below those observed under limed conditions. No other selection in the field for Al tolerance has been reported, and no Al tolerant cultivar is currently available (Bouton and Radcliffe, 1989; Dall'Agnol et al., 1996; Narasimhamoorthy et al., 2007b).

Greenhouse soil and cell culture selection. A selection program was performed using the greenhouse based soil assay and the callus assay (Dall'Agnol et al., 1996). From the same base population (GA-TE, Georgia Tifton elite germplasm), selections were performed by identifying seedlings grown in completely unlimed soil or in limed and fertilized top-soil with an unlimed sub-soil layer, or by selecting callus based on a high ratio (+Al/-Al) in cell culture, a low ratio (+Al/-Al) in cell culture, high net growth in cell culture, or low net growth in cell culture. Based on one cycle of selection for all the methodologies, selection in completely unlimed soil was the most effective in terms of success, time, and resources (Dall'Agnol et al., 1996).

Hydroponic selection. Selection for acid and Al tolerance in a hydroponic system improved root regrowth after an Al shock treatment, after 2 cycles of selection. However, relative value to field performance is not known (Scott et al., 2008). Higher selection intensity may need to be used because the Al/acid tolerance alleles appear to be rare in alfalfa populations (Scott et al., 2008).

Summary of selection for acid/Al tolerance. Direct selection in the target environment has often been found to be the most effective way to improve populations for stressful environments (Ceccarelli, 1989). In the greenhouse soil-based experiment, selection for better Al tolerance in low pH conditions did not negatively affect the yield performance in limed soil environments in the greenhouse (Dall'Agnol et al., 1996). Selection for shoot growth in acid soil (pH levels below

5) may be more effective than selection for root growth, which is highly variable in acid soils (Simpson et al., 1977). The improvement in Al tolerance could be limited by the lack of genetic variability in the alfalfa core collection (Bouton, 1996), the lack of understanding of the mechanism(s) controlling the trait, and the lack of long term breeding efforts (Dall'Agnol et al., 1996). In general, the most common breeding method in alfalfa has been phenotypic recurrent selection (PRS) using broad-based populations (Brummer, 1999). Most of the cultivars registered for Plant Variety Protection in 2008 were selected using some sort of PRS (USDA, 2008). Some variant of half-sib progeny testing has also been used in cultivar development, although not as commonly as PRS. Yield improvement in alfalfa has been extremely low compared with the improvements in annual crops during the last century (Brummer, 1999). The increase in resistance to biotic factors has preoccupied alfalfa breeders, with less effort focused specifically on increasing forage yield (Lamb et al., 2006). Undoubtedly, tetrasomic inheritance has played some role in limiting genetic gain, due to the presence of a large genetic load (Hill et al., 1988).

Although PRS can be effective in alfalfa, better genetic gain for yield could be achieved using progeny test methods (Fehr, 1993). Among and within family selection (AWFS) and PRS achieved twice the gain of mass, half-sib, and half-sib progeny test (HSPT) selection when improving ease of floret tripping in the CUF101 alfalfa population (Knapp and Teuber, 1993). This result is unexpected based on theory (Fehr, 1993), which suggest that on a per cycle basis, a higher gain would be observed using half-sib family selection. The efficiency of AWFS versus HSPT is a function of the intensities of selection among and within families (Casler and Brummer, 2008).

The key to family-based methods of selection is the estimation of the breeding value of an individual. Methods such as best linear unbiased prediction (BLUP) (Henderson, 1975), could

be useful in improving the estimation of breeding value. BLUP, which considers genotypes as random effects, maximizes the correlation between the true genotypic value and the predicted genotypic value (Piepho et al., 2008; Searle et al., 1992). An optimum selection strategy would be AWFS based on genotypic values that have been predicted by BLUP since it would simultaneously consider information on the family and the individual (Resende, 2002). However, when evaluating many families, recording individual information within families could be highly unpractical and time consuming.

Overall objectives of this research

This project pursues three avenues to enhance the aluminum and acid tolerance in alfalfa. First, we will identify QTL for aluminum tolerance, which could be integrated into elite breeding lines. Second, we will evaluate two transgenes that have been previously inserted into alfalfa, to determine if they improve aluminum/acid soil tolerance. Finally, we will evaluate classical breeding methods to assess genetic gain from greenhouse based selection in Al rich, acid soils. Exploring the potential of the three different approaches proposed here will give us a set of tools to obtain enhanced alfalfa cultivars.

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CHAPTER 2

GENETIC MAPPING OF TOLERANCE TO ACID-SOIL SYNDROME IN TETRAPLOID ALFALFA USING A WHOLE-PLANT ASSAY IN SOIL[1]

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Abstract

Alfalfa, *Medicago sativa* L., is one of the most important forage legumes worldwide. However, acid soils and aluminum toxicity dramatically decrease its productivity and persistence. Limited variability for Al tolerance exists within tetraploid germplasm, and no aluminum tolerant cultivar is commercially available. The tetraploid aluminum tolerant genotype Altet-4, derived from a source of tolerance identified in diploid *M. sativa* subsp. *caerulea*, was crossed to NECS-141, a semi-dormant breeding line, to produce a mapping population segregating for Al tolerance. The population was evaluated using a modified soil-based greenhouse assay to assess individual genotype performance in unlimed, aluminum rich, acid soil compared with performance in limed soil without Al toxicity. Rooted stem cuttings were grown for 6 wk in both types of soil and harvested. Root and shoot dry weight in both soil treatments were measured and the ratio of unlimed:limed of these traits was determined. Linkage maps were constructed using molecular markers, generating a composite map length of 840 cM and 749 cM for Altet4 and NECS-141, respectively. Single-factor analysis and interval mapping identified quantitative trait loci (QTL) on Altet-4 linkage groups 1, 2, 5, 6, and 7, and on NECS-141 linkage group 5. The phenotypic variation explained by individual QTL ranged from 9.5 to 35.3%. Some of these QTL were detected in multiple environments and for multiple traits while others were environment-specific, suggesting that multiple mechanisms of Al tolerance exist. Introgression of these Al-tolerance QTL into elite breeding lines can result in cultivars with enhanced Al tolerance.

Introduction

Alfalfa, *Medicago sativa* L., is one of the most important forage legumes worldwide and is the fourth most important crop in U.S. agriculture in terms of hectares under cultivation (USDA-NASS, 2009). Acid soils limit alfalfa cultivation in many parts of the world, including the southeastern USA (Bouton and Sumner, 1983a). At low pH, aluminum (Al) and manganese (Mn) become soluble, causing severe toxicity that inhibits root growth and development (Kochian et al., 2004a). The most common form of aluminum released from soil minerals is $Al(H_2O)_3^+$, which is commonly referred to as Al^{3+} (Kinraide, 1991). Two different types of Al tolerance mechanisms exist; those that exclude Al from the root apex through organic acid exudation, and those that allow the plant to tolerate Al accumulation in roots and shoots (Kochian et al., 2004a). Studies in barley, wheat, sorghum, rye, soybean, rice, and arabidopsis indicate that aluminum tolerance is controlled by a few genes with large effects (Delhaize et al., 2004; Hoekenga et al., 2003; Magalhaes et al., 2004; Pineros et al., 2002), while others, sometimes in the same species, suggests the complexity of the Al trait (Bianchi-Hall et al., 2000; Ikka et al., 2008b; Liu et al., 2009; Narasimhamoorthy et al., 2007a; Xue et al., 2008).

Acid soil syndrome (Al^{+3} and H^+ toxicity) can be ameliorated by applying lime and P fertilizer, effectively eliminating Al toxicity in the plow layer, but not changing the subsoil (Bouton and Sumner, 1983a). The rising costs of lime application and fertilizer have made these practices less affordable (Bennett et al., 2008), thus increasing the interest in genetic tolerance to acidic conditions. Genetic selection for acid soil tolerance can increase alfalfa productivity and reduce production costs, but only limited variation has been detected in alfalfa germplasm (Bouton, 1996; Campbell et al., 1988).

The success for developing Al tolerance alfalfa cultivars depends on reliable phenotypic assays. Phenotypic assays traditionally used to evaluated Al tolerance in alfalfa have included a callus bioassay (Parrott and Bouton, 1990), hydroponic systems (Barone et al., 2008; Narasimhamoorthy et al., 2007b; Scott et al., 2008; Sledge et al., 2005), root-staining (Barone et al., 2008; Narasimhamoorthy et al., 2007b), and soil-based assays (Bouton, 1996; Dall' Agnol et al., 1996; Sledge et al., 2002). Results among the various assays are not highly correlated, suggesting that they may be capturing different mechanisms of Al tolerance (Barone et al., 2008; Narasimhamoorthy et al., 2007b). We recently described two new screening assays to assess Al tolerance in alfalfa more efficiently, one using a whole-plant assay in media, and one that is a modification of the Bouton (1996) soil assay that enables high-throughput analysis (Khu et al., 2012). Although we anticipate the soil assay to be most relevant for field conditions, we do not know how selection using any of these methods relates to progress for Al tolerance in the field.

Understanding the genetic basis of Al tolerance can further improve selection strategies. Al tolerance QTL were identified in several genomic regions in diploid alfalfa based on callus assays (Narasimhamoorthy et al., 2007a; Sledge et al., 2002). However, since cultivated alfalfa is tetraploid, those QTL need to be integrated and evaluated at the tetraploid level. Genetic maps in tetraploid alfalfa (Julier et al., 2003; Robins et al., 2007b; Sledge et al., 2005) were constructed and used for mapping traits of interest, including yield, persistence, and others (Brower et al., 2000; Robins and Brummer, 2010; Robins et al., 2007a; Robins et al., 2008; Robins et al., 2007b).

Our hypothesis is that QTL associated with Al/acid soil tolerance can be identified in a tetraploid alfalfa breeding population using a modified soil-based assay, and the QTL identified previously at the diploid level will also be identified at the tetraploid level. The objective of this

study was to evaluate Al/acid soil tolerance in a tetraploid alfalfa mapping population using a soil-based assay.

Materials and Methods

Plant Materials

The autotetraploid genotypes NECS-141 and Altet-4 were reciprocally crossed, emasculating the female parent in each case, generating a full-sib F_1 population of 185 individuals. Altet-4 is a tetraploid genotype derived from an interploidy cross between Al-4, a diploid plant of *M. sativa* subsp. *caerulea* used to identify QTL alleles for Al tolerance (Narasimhamoorthy et al., 2007a; Sledge et al., 2002), and an Al-sensitive tetraploid genotype derived from the non-dormant synthetic cultivar CUF101 (Lehman et al., 1983). The Al-sensitive parental genotype NECS-141 (Khu et al., 2012) is a semi-dormant (3-4 dormancy group) genotype with high yield, derived from a breeding population previously evaluated for yield at three locations (Li et al., 2011).

Genotyping and Map Construction

Genomic DNA extractions from all individuals and the parental genotypes were performed using the DNeasy Plant Kit® (QIAGEN, Valencia, CA, USA). The NECS-141×Altet-4 population was initially screened using 20 SSR primer pairs to confirm the hybrid origin of the individuals in the population. Amplicons from 266 loci, including SSR markers (Sledge et al., 2005) and candidate genes involved in organic acid secretion (Narasimhamoorthy et al., 2007a), were detected using ABI3730 DNA analyzer, and then scored using GeneMapper 3.7^{TM} and GeneMarker V1.5 software. The software TetraploidMap (Hackett and Luo, 2003), previously used for map construction and QTL analysis in alfalfa (Julier et al., 2003; Robins and Brummer,

2010; Robins et al., 2007a; Robins et al., 2008), was used to infer allele dosage in each parent and the most likely parental genotype, and to estimate recombination frequencies and to cluster markers into linkage groups. Marker order and distances were estimated using the simulated annealing algorithm from the TetraploidMap suite (Hackett and Luo, 2003). MapChart (Voorrips, 2002) was used to draw the resulting linkage groups, and to draw the QTL identified using the TetraploidMap software.

Eight linkage groups, corresponding to the basic chromosome number in alfalfa, were obtained from each parent. Each linkage group contains four co-segregating groups corresponding to the four homologous chromosomes, and they were randomly numbered from 1 to 4 within each linkage group (Supplemental Fig. 2.1). Different allelic dosages and multi-allelic markers are expected in autotetraploid alfalfa. The markers were placed into co-segregating groups based on their linkage with simplex markers (only one copy of a given allele present in only one parent) (Hackett and Luo, 2003). Most of the alleles were allocated into co-segregating groups based on their coupling or repulsion phases using a LOD score ≥ 3.0, previously utilized in alfalfa (Robins et al., 2007b).

Phenotyping

Experimental design and data collection

Phenotyping was performed in the Crop and Soil Sciences Department greenhouses at the University of Georgia, Athens, GA. The entire experiment was performed in the following three environments: Nov-Dec 2009, Mar-Apr 2010, and Apr-May 2011. The use of cups as described by (Bouton, 1996) for soil-based Al tolerance evaluations is time-consuming and space-intensive, and thus cups were replaced with conetainers (Stuewe and Sons, Inc., Tangent, OR) using the whole-plant assay in soil as previously described (Khu et al., 2012). Briefly, each

conetainer (3.8 cm diameter × 21 cm long, with a volume of 164 ml) was filled with 140 g of soil leaving approximately 5 cm space at the top of each cone. Individual conetainers were placed in 30 cm wide × 61 cm long × 18 cm high racks, each of which held 98 (7 × 14) conetainers. Half of each rack (49 cones in a 7 × 7 square) was filled with unlimed (UL) soil and the other half with limed (L) soil. The outer cones in each half were utilized for border rows thus leaving 25 cones (5 × 5) per rack evaluated for each soil type. Each soil type per rack included two clones of each parental genotype along with 21 genotypes from the mapping population. The same 21 genotypes were evaluated in both soil treatments within the same rack. To prevent waterlogging, the racks with the conetainers were placed into plastic trays that were filled with 15 cm of sterile sand, and with drain holes at the bottom. The conetainers used had drain holes at the bottom and were placed 5 cm into the sand to ensure contact between cone soil and sand. The sand was used to prevent soil saturation after watering (Dr. M.E. Sumner, pers. comm.), thereby preventing Mn toxicity. One replication of the entire mapping population consisted of nine incomplete blocks (racks) of 21 genotypes from the population and two clones of each parent (NECS-141 and Altet-4) per rack for a total of 25 individuals evaluated in each soil treatment per rack. Four replications of the entire population were evaluated in each environment.

The soil used for these experiments and the macro and micro nutrients used for amendments were previously described (Khu et al., 2012). Although the same soil, obtained from the UGA Plant Sciences Farm near Watkinsville, GA, was used for each environment, nutrients and lime were mixed into the soil prior to each experiment, resulting in minor variations in the soil analysis (Table 2.1). After the addition of soil amendments, the soil was air-dried and sieved using a 3-mm screen (Dall' Agnol et al., 1996) prior to filling the conetainers.

Rooted stem cuttings were used for all evaluations. Stem cuttings were vegetatively propagated in 96-cell plastic flats growing in Fafard Super-fine Germinating Mix® (Conrad Fafard Inc., Agawam, MA). Each cutting was grown in a cell with top dimensions of 2.5 cm × 2.5 cm and 5 cm deep, tapering to a point and grown for 6 wk. Uniform 5-wk old stem cuttings from each genotype in the population were identified, and the clonal plugs were transferred directly to the conetainers without washing off the germination mix in which the cuttings were grown. The treated soil, L or UL, depending on the treatment was used to fill the area around the plug at the top of the conetainer. The experiment was watered every 1 to 2 d using distilled water, and the trays were rotated inside the greenhouse benches at weekly intervals to diminish micro-environmental variation inside the greenhouse. Day and night temperatures were 25°C and 20°C, respectively, with a day length of 16 hr. During the course of the experiment one or two applications of chlorpenapyr (0.41 g/l) and avermectin (49.3 g/l) for thrips (*Caliothrips fasciatus* and *Frankliniella occidentalis*) control were required.

After growing for 6 to 8 wk, plants and soil were removed from the conetainers and the roots of each plant were gently washed to remove the excess soil. Plants were separated into root and stem fractions at the soil line. The top 5 cm of the root fraction, corresponding to the roots growing in the germination mix, was discarded and not considered for further analysis. The remaining roots below 5 cm represented the roots growing in the UL or L soil, were retained as the root fraction for analysis. The dry weight (DW) of roots and shoots was determined after drying at 65ºC for 72 hr. Relative root weight was determined as the ratio of root dry weight in UL soil compared to the root dry weight in L soil for each genotype at the block level. Relative shoot dry weight was computed analogously. Therefore, the traits analyzed were root dry weight in L and UL soil, hereafter referred to as RDW-L and RDW-UL, shoot dry weight in L and UL

soil, hereafter referred to as SDW-L and SDW-UL, and root and shoot dry weight ratios representing the relative growth in UL vs L soil, referred to as RDWR and SDWR, respectively.

Data analysis

The UNIVARIATE procedure of SAS v. 9.1 (SAS Institute, 2007) was used to determine general statistics for each trait and to test for normality using the QQPlot statement and the normal option. The MIXED procedure also in SAS was used to calculate the least-square means for each genotype for each trait. The full model considered genotypes, environments, and genotypes × environments (G×E) interaction as fixed effects, while incomplete blocks (nested in replications) and replications (nested in environments) were designated as random effects. The environment effect represents the greenhouse conditions under which this experiment was conducted and thus, was considered as a fixed effect. Based on the overall analysis, a significant G×E effect for all traits suggested we analyze environments separately, using an analogous model. Progeny data were analyzed using the VARCOMP procedure of SAS in an all-random model to estimate the genetic, G×E interaction, and the residual error variance components. The broad sense heritability (H) for traits evaluated was calculated using the following equation:

$$H = \frac{\sigma^2_{genotypes}}{\left[\sigma^2_{genotypes} + \frac{\sigma^2_{gxe}}{e} + \frac{\sigma^2_{residual}}{re}\right]}$$

where $\sigma^2_{genotypes}$ is the variance due to genotypes, $\sigma^2_{g\times e}$ is the variance due to the two-way G×E interaction, $\sigma^2_{residual}$ is variance due to the error, *e* refers to the number of environments, and *r* is the number of replications within environments. Heritabilities were also estimated for each environment modifying the previous equation by removing the $\sigma^2_{g\times e}$ and *e* variables.

Phenotypic and genetic correlations among traits were estimated for each environment. The CORR procedure of SAS was used to estimate phenotypic correlations based on mean values within environments. The MANOVA option from the GLM procedure was used to obtain the variance-covariance matrix and the genetic correlations were calculated using the equation:

$$r_{G12} = \frac{Cov_{1-2}}{\sqrt{Var_1 * Var_2}}$$

where $r_{g\ x\text{-}y}$ represents the genetic correlation between traits x and y, $Cov_{x\text{-}y}$ is the covariance of traits x and y, Var_x is the variance associated with trait x, and Var_y is the variance associated with trait y. For all statistical analyses, we assessed significance at the 5% probability level unless otherwise indicated.

QTL analyses

Single-factor analysis of variance and the nonparametric Kruskal-Wallis test (Siegel, 1956) were performed using the ANOVA module of the TetraploidMap software (Hackett and Luo, 2003). This analysis was performed for each trait in each of the three environments. Single-factor analysis compares the least-square mean of the genotypes with the presence of a particular allele against the least-square mean of the genotypes without that allele. Marker alleles were declared significantly associated with a trait at $P \leq 0.01$. Single-factor analysis was used for a preliminary identification of markers associated with traits, highlighting relevant linkage groups for further analysis. Interval QTL mapping was performed using TetraploidMap (Hackett et al., 2001; Hackett et al., 2007). Linkage groups from both parents were analyzed to identify QTL for each trait. Experiment-wise thresholds were set by running 1000 permutations, where putative QTL showing a LOD score over the 5% experiment-wise threshold were declared as significant QTL.

Results

Trait variation and correlations among traits

The overall analysis of variance across all three environments showed that most traits exhibited significant G×E interaction (data not shown). Therefore, we evaluated each environment independently. Although the experiments were conducted in the greenhouse, the external environment, particularly day length and ambient temperature, were quite variable throughout the different environments, which likely affected plant growth. In addition, soil properties for the L and UL treatments were slightly different across environments (Table 2.1).

All the traits in each environment were normally distributed (data not shown). Genotypes differed for all traits in all environments, except for SDWR in Environment 1 and RDWR 2 in Environment 2 (Table 2.2). For the primary Al trait phenotype – the ratio of root dry weight in UL vs. L soil (UL:L) – the two parental genotypes differed as expected in Environments 2 and 3, with Altet-4 showing a higher ratio, suggesting more Al tolerance, than NECS-141; the parents performed similarly in Environment 1. The mapping population means for all traits were close to the mid-parent value, but the range largely exceeded the parent values (Table 2.2).

Broad-sense heritablities were moderate to low for all the traits (Table 2.3). In the overall analysis, the G×E and error variances were large. In L soil, the genotype variance component was larger than G×E variance component, but these were reversed in UL soil (Table 2.3). Heritability for root dry weight was higher in L than UL conditions, but the heritability of shoot dry weight was similar for the two soils. Heritabilities for all traits were very low for Environment 1. Environment 2 had higher heritabilities for root and shoot dry weights in UL soils than in L soil. The heritability of the ratios was lower in all cases than the corresponding dry weight heritability.

Phenotypic correlations among the traits were positive and moderate in each environment (Table 2.4). Root and shoot dry weights had similar correlations between L and UL conditions. Correlations of RDW with SDW in L or UL soil were higher than the correlations of individual traits across soil conditions. RDWR and SDWR were moderately correlated in all environments. Genetic correlations were generally similar than phenotypic correlations. In all the cases, genetic correlations were positive and moderate to high depending on the trait.

Linkage Map and QTL Analyses

The genetic map of each parent consists of eight linkage groups corresponding to the eight alfalfa chromosomes, each containing four homologous chromosomes. The composite map lengths were 840 cM and 749 cM for the Altet-4 and NECS-141, respectively (Supplementary Fig. 2.1).

Single-factor ANOVA identified 36 marker alleles, 24 from Altet-4 and 12 from NECS-141 that showed an association with RDW or SDW in one or both soil treatments (Table 2.5). Marker alleles from all linkage groups (LG) except LG 2 and LG 3 were associated with RDW or SDW. Most of the associations are environment and soil specific, but a few marker alleles are associated with multiple traits and/or with the same trait in multiple environments or soils (e.g., marker allele BF119-159 on LG 1 is associated with SDW and RDW in UL soil in Environments 1 and 2, and with SDW in L soil in Environment 1). In general, few marker associations with RDW in L soil were identified.

Both parents contributed marker alleles associated with RDW and SDW (Table 2.5). While most of the associations identified for RDW and SDW in L and UL conditions showed negative allele effects, each parent contributed alleles with both positive and negative effects,

and alleles from one parent with the same effect within a linkage group appeared to be clustered (Table 2.5).

The ratio of dry weight in UL vs. L soil was used to estimate the Al tolerance. Thirty-three marker alleles associated with RDWR and SDWR were identified from both parents and on all linkage groups and with both positive and negative effects (Table 2.6). The marker allele effects range from -0.15 to +0.13, and in general, alleles with same effect tend to be clustered within a LG. All of the associations were trait and environment specific, but in the case of NECS-141 LG 4, a cluster of marker alleles with a positive effect were located in the interval between positions 4.1 to 21.9 cM across all environments evaluated (Table 2.6).

Interval mapping identified 16 QTL associated with dry weight or dry weight ratios in Altet-4 and nine QTL for those traits in NECS-141 (Table 2.7; Fig. 2.1; Fig. 2.2). QTL for dry weight and Al tolerance were located on Altet-4 LGs 1, 2, 5, 6, 7, and 8, and on NECS-141 LGs 1, 4, 5, and 7. Individual QTL explained between 7 and 41% of the phenotypic variation (Table 2.7). As suggested by the single factor analysis, limited correspondence across environments was seen (Fig. 2.1; Fig. 2.2). However, the genomic region located in the middle of LG 1 of Altet-4 is associated with traits in several soil types, traits, and environments (Table 2.7; Fig. 2.1). Most of the QTL detected by interval mapping correspond to alleles identified by single factor analysis (Fig. 2.1a and 2.1b).

The gene action of these QTL is quite complex. For instance, the RDWR QTL on Altet-4 LG 2 explained 30% of the phenotypic variation (Table 2.7) and had a positive effect when homologues 2 and 3 from Altet-4 were inherited by the F_1 individuals (data not shown). This QTL is flanked by the duplex markers RCS4209-157A and AC155884-162A which showed positive effects in the single-factor analysis (Fig. 2.1 and Table 2.6). Even though this QTL was

detected only in Environment 3 using interval mapping, AC155884-162A was associated with RDWR in Environment 1 as well (Table 2.6). A QTL for RDWR in Environment 1 was located on LG 5 of Altet-4 at 48 cM, and explains 35% of the phenotypic variation. For this QTL, the F_1 individuals that inherited homologues 1 and 3 showed higher Al tolerance than those with other homologous chromosome combinations (data not shown). The simplex marker-allele BF106-214B, located at 54.9 cM position on homologue 4 (Supp. Fig. 2.1), was negatively associated with Al tolerance for RDWR, while no marker from homologues 1 and 3 was associated with this trait.

Putative QTL for Al tolerance were also identified in the Al-sensitive parent NECS-141. On LG 5, QTL for Al tolerance (RDWR and SDWR) were identified at 75 and 77 cM in Environment 3. For both QTL, F_1 individuals that inherited NECS-141 homologue 2 had lower Al tolerance (data not shown). This result is in agreement with the presence of simplex marker Mstri10686-126N on that homologue (Supp. Fig. 2.1), which was highly negatively associated with these traits in Environment 3 (Table 2.6).

Discussion

Aluminum tolerance was evaluated in a tetraploid alfalfa mapping population grown in soil using conetainers. This new methodology (Khu et al., 2012) allowed us to screen a mapping population using a soil assay, something precluded using previous methods. Substantial G×E was observed in this experiment for root and shoot dry weights and for the ratios of dry weights from UL vs. L soils. Significant G×E interactions for dry weight were also found in tetraploid alfalfa in a QTL mapping analysis of yield (Robins et al., 2007b), where location and year combinations were analyzed independently. Broad-sense heritabilities within environments were

moderate to low (Table 2.3). In this experiment, the causes of G×E include slight variations in the soil analysis (Table 2.1) and also differences in photoperiod responses. Although we supplemented plants with lights, the ambient day-length varied considerably between environments, and probably affected the rate of plant growth. The parents differ in autumn dormancy, and therefore, may be expected to respond to photoperiod differently during the duration of the experiment.

The traits measured in this population in each of the three environments showed normal and continuous distribution in agreement with polygenic inheritance cited in alfalfa and other species for Al tolerance and for biomass (Narasimhamoorthy et al., 2007a; Robins et al., 2007b; Sledge et al., 2002). The density of the genetic maps used in this study were 4.0 cM/marker in Altet-4 and 3.2 cM/marker in the NECS-141 parental map. These genetic maps were sufficiently dense to detect marker-trait associations though single-factor analysis and interval mapping.

Transgressive segregation was observed in all evaluated traits in this population (Table 2.2), thus supporting our findings of marker-alleles and QTL with positive and negative effects being contributed from both parents. Even though NECS-141 has lower Al tolerance compared with Altet-4, it contributed positive alleles for Al tolerance. Similar findings of transgressive segregation and beneficial alleles from the agronomically inferior parent were reported studying Al tolerance in alfalfa at the diploid level (Narasimhamoorthy et al., 2007a), persistence and other agronomic traits at the tetraploid level (Robins et al., 2007a; Robins et al., 2008; Robins et al., 2007b), and in other species (Bianchi-Hall et al., 2000; Hoekenga et al., 2003).

Phenotypic and genetic correlations among shoot and root dry weights and their ratios were moderate to high in all the cases in each of the three environments (Table 2.4). Linkage or pleiotropic effects drive genetic correlations. Thus, these positive and moderate to high

correlations suggest that in this study these traits are being controlled to some extent by the same genomic regions. We have observed marker-alleles and QTL associated with different traits located in the same LG and in similar genomic regions likely representing the same QTL.

In this study, we identified QTL associated with RDW and SDW in limed conditions. Under limed conditions the soil pH was almost neutral and the exchangeable Al was absent (Table 2.1). Therefore, these QTL likely represent growth traits (biomass yield) that are not necessarily related to Al tolerance. Thus, QTL identified in the same locations in UL soil are also probably related to yield *per se*, rather than to Al tolerance. For instance, the cluster of QTL on LG 1 of the Altet-4 parent (Fig. 2.1) includes dry weight QTL in both L and UL soils, and these may represent growth QTL, not Al tolerance QTL.

In the Environment 1, parents did not perform as anticipated in terms of the Al tolerance type (Table 2.2). As stated above, parents differ in autumn dormancy, and thus because the experiment was performed during the fall-winter season we likely observed confounding effects due to dormancy responses. In alfalfa, QTL associated to winter hardiness, fall growth, and freezing injury were identified on LGs 1, 3, 5, and 8 (Brouwer et al., 2000). QTL identified only in Environment 1 may be affected by dormancy effects. However, the lack of common markers between this study and the Brouwer map makes confirmation difficult.

A previous study to evaluate Al tolerance based on a callus bioassay, identified QTL on LGs 1, 2, and 3 in diploid alfalfa (Narasimhamoorthy et al., 2007a). The parental genotype Al-4 had tolerance alleles at these QTL, and as it is a parent of Altet-4 thus, we anticipate that QTL in these regions would also be identified in the tetraploid population provided that markers segregate in this region. Al tolerance QTL *per se* are those assuming associated with RDWR and SDWR. However, QTL for root or shoot growth characteristics could also indirectly affect Al

tolerance through improved growth *per se*. A major QTL for Al tolerance was identified at the diploid level in the region near marker AW11 (Narasimhamoorthy et al., 2007a); we also identified a QTL for SDWR in the region of AW11 (Table 2.7 and Fig. 2.1). The second QTL found on LG 1 in the diploid population may correspond to the large cluster of QTL we identified on LG 1 in this study. However, different markers were used in both studies limiting our ability to determine whether these QTL co-locate to the same position on the same LG. An Al-tolerance QTL at the diploid level on LG 2 of Al-4 was localized at the marker locus AL99 (Narasimhamoorthy et al., 2007a); we identified a QTL on Altet-4 LG 2 close to this marker (Fig. 2.1).

The position of a QTL on a LG identified in different environments may be affected by the G×E interaction, thus slightly shifting its specific map position. This phenomenon was reported for persistence QTL in tetraploid alfalfa (Robins et al., 2008). Similar results were found on Altet-4 LG 1, were two QTL detected in two different environments range in map position between 77 to 91 cM (Fig. 2.1), suggesting that these may be under the same genetic control.

In addition to the presence of two of the three Al tolerance QTL previously reported at the diploid level, we identified three additional LGs (5, 6, and 7) associated with Al tolerance in Altet-4 not previously reported at the diploid level. Different Al tolerance mechanisms have been described acting at different levels of the plant structure, and these can be cell-based and whole-plant based. Those mechanisms include Al-induced exudation of organic acids from the root apex and/or internal detoxification of Al via complexation with organic ligands, which allows the plant to accumulate Al in roots and shoots (Kochian et al., 2004a). Evidence for the later mechanism includes some plant species which are able to accumulate high levels of Al in roots

and shoots (Ma et al., 1998; Ma et al., 2001; Ma et al., 1997). Therefore, the QTL identified can be a result of different mechanism of Al tolerance captured by the different assays and genetic maps used. Alternatively, these represent Al tolerance mechanism relevant at the tetraploid level based on the higher order allelic interaction present in tetraploid alfalfa (Bingham et al., 1994).

Single factor ANOVA was used to identify several alleles with positive and negative effects being closely linked (Table 2.5 and 2.6). In most of the cases, the alleles with the positive and negative effects are localized in different co-segregating homologous on a linkage group (Supp. Fig. 2.1). The same condition was observed in the homologous combination inherited by the F_1 individuals, where some homologues contain alleles with a positive effect and others with negative effects in the same genomic region.

This study has identified several genomic regions associated with Al/acid soil tolerance using a recently described whole-plant assay in soil that allowed screening at a mapping population (Khu et al., 2012). Sources of Al/acid soil tolerance were identified, but their value in a range of alfalfa germplasm with different background remains unknown.

Conclusion

Multiple QTL for Al/acid soil tolerance were identified in a tetraploid alfalfa mapping population. Our results suggest that two QTL identified for Al tolerance at the diploid levels were also present in tetraploid alfalfa, with the addition of QTL identified from the Al-tolerant parent, not previously reported, and from the Al-sensitive parent. Individual QTL explained between 8 and 35% of the phenotypic variation, but their modes of action together with the QTL×E interaction suggest the complexity behind Al tolerance in alfalfa. Further research is needed to refine the genomic regions associated with Al tolerance and to understand Al tolerance

mechanisms in alfalfa, including identification of and identify candidate genes from which to develop new molecular markers. The introgression of some of these QTL in elite breeding alfalfa germplasm and the development of a marker-assisted selection program can be used to increase their frequency in elite alfalfa populations thus enhancing Al tolerance.

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Table 2.1. Soil properties of soil collected from the Plant Sciences Farm near Watkinsville, GA and the same soil after adding soil amendments to limed and unlimed soil.

Type of soil	Env.	pH $CaCl_2$*	Equiv. H_2O pH	Ca	K	Mg	Mn	P	Zn	Exchangeable Al $cmol_c/kg$
				Mehlich 1 mg/kg (ppm)						
Farm soil	-	4.43	5.03	103.41	32.50	37.31	5.26	2.21	1.44	0.85
Limed	1	5.94	6.54	468.80	173.45	118.40	2.31	27.84	2.62	0.06
Unlimed	1	4.59	5.19	172.70	167.15	34.02	2.09	22.79	2.70	0.72
Limed	2	6.28	6.88	589.90	187.50	140.95	3.26	33.70	4.03	0.05
Unlimed	2	4.53	5.13	171.45	173.80	38.09	2.17	23.55	2.83	0.74
Limed	3	6.10	6.70	629.97	207.19	138.05	8.05	32.80	5.47	0.02
Unlimed	3	4.63	5.23	191.73	176.47	43.97	8.92	30.43	2.40	0.63

* Soil Testing: Soil pH and salt concentration (http://pubs.caes.uga.edu/caespubs/pubcd/C875/C875.htm)

Table 2.2. Effect of each trait in each environment due to genotype, means for each parent and the F_1 progeny.

		Root Dry Weight			Shoot Dry Weight		
		Limed	Unlimed	UL:L Ratio§	Limed	Unlimed	UL:L Ratio§
	Genotype effect	<.0001†	<.0001	0.05	<.0001†	<.0001	0.10
		mg plant^{-1}			mg plant^{-1}		
Env. 1	Altet-4	187^{b}‡	155^{b}	0.80^{a}	373^{a}‡	260^{a}	0.77^{a}
	NECS-141	252^{a}	195^{a}	0.71^{a}	363^{a}	250^{a}	0.66^{a}
	F_1 Mean	217	170	0.77	348	258	0.79
	Genotype effect	<.0001†	<.0001	0.09	<.0001†	<.0001	0.02
		mg plant^{-1}			mg plant^{-1}		
Env. 2	Altet-4	294^{a}‡	211^{a}	0.74^{a}	420^{a}‡	260^{a}	0.72^{a}
	NECS-141	221^{b}	108^{b}	0.54^{b}	219^{b}	95^{b}	0.47^{b}
	F_1 Mean	349	228	0.68	393	210	0.57
	Genotype effect	<.0001†	<.0001	.0001	<.0001†	<.0001	0.05
		mg plant^{-1}			mg plant^{-1}		
Env. 3	Altet-4	325^{a}‡	290^{a}	0.81^{a}	445^{a}‡	397^{a}	0.84^{a}
	NECS-141	285^{a}	156^{b}	0.53^{b}	352^{b}	214^{b}	0.64^{b}
	F_1 Mean	312	220	0.75	433	312	0.74

† p-values for genotype effect.
‡ Means followed by the same letter (within a column) are not significantly different ($P < 0.05$).
§The UL:L ratios were computed for each replication to enable a statistical analysis. Therefore, they will not equal the ratio of UL:L LS means.

Table 2.3. Variance components and broad-sense heritabilities (H) for root and shoot dry weights traits in each environment and for the overall data across environments.

		Root Dry Weight			Shoot Dry Weight		
		Limed	Unlimed	UL:L Ratio	Limed	Unlimed	UL:L Ratio
Env.1	$\sigma^2_{genotypes}$	1330	722	0.001	906	828	0.007
	$\sigma^2_{residual}$	13324	11430	0.181	29592	17350	0.153
	H	0.29	0.20	0.02	0.11	0.16	0.15
Env.2	$\sigma^2_{genotypes}$	7108	6810.2	0.003	12721	7902	0.010
	$\sigma^2_{residual}$	16647	5876	0.115	14183	4610	0.088
	H	0.63	0.82	0.11	0.78	0.87	0.31
Env.3	$\sigma^2_{genotypes}$	2604	5069	0.020	4582	7562	0.005
	$\sigma^2_{residual}$	33949	15330	0.150	45659	25163	0.136
	H	0.23	0.57	0.35	0.29	0.55	0.14
Overall	$\sigma^2_{genotypes}$	1775	999	0.007	3173	2737	0.007
	$\sigma^2_{G\times E}$	470	2332	0.002	1723	3081	0.005
	$\sigma^2_{residual}$	24451	12443	0.151	30994	15464	0.131
	H	0.45	0.36	0.34	0.50	0.54	0.36

Table 2.4. Phenotypic and genetic correlations among traits in each environment.

	Environment 1		Environment 2		Environment 3	
	r_P†	r_G‡	r_P	r_G	r_P	r_G
RDW-L vs. RDW-UL	0.49***	0.50	0.56***	0.65	0.66***	0.62
SDW-L vs. SDW-UL	0.52***	0.52	0.56***	0.67	0.66***	0.61
RDW-L vs. SDW-L	0.76***	0.74	0.80***	0.80	0.73***	0.73
RDW-UL vs. SDW-UL	0.74***	0.73	0.74***	0.77	0.72***	0.72
RDWR vs. SDWR	0.54***	0.50	0.52***	0.58	0.38***	0.51

† Phenotypic correlations; ‡ Genetic correlations; *** Significant at $P \leq 0.001$.

Table 2.5. Allele effects of markers associated with root and shoot dry weight (in mg plant^{-1}) in L and UL soil in each environment identified using the nonparametric Kruskal-Wallis test and SF-ANOVA (P value ≤ 0.01).

				Environment 1				Environment 2				Environment 3			
				Limed		Unlimed		Limed		Unlimed		Limed		Unlimed	
Marker allele	Parent	LG	Position (cM)	RDW	SDW	RDW	SDW	RDW	SDW	RDW	SDW	RDW	SDW	RDW	SDW
3e10.cag.6-1-196	Altet-4	1	18.4		-55.4[§]		-47.5							-39.9	
BG234-251A	Altet-4	1	23.1							-42.8	-56.0				-46.2
BF119-159	Altet-4	1	38.5		-56.3	-29.8	-49.0			-25.7	-38.3				
BG142-251A	Altet-4	1	51.5								+37.0				
1h03.ata.9-1-303	Altet-4	1	66.4								-29.4				
AW11-233	Altet-4	1	78.4								-34.6				
BG180-159	Altet-4	1	81.8											-44.9	
BG285-309A	Altet-4	1	88.3											+47.3	
BG249-284A	Altet-4	1	90.2								+33.6			+57.7	
BG248-284A	Altet-4	1	91.0											+50.5	
BE105-236	NECS-141	1	66.3		-42.7					-36.6	-33.7				
BE105-235	NECS-141	1	74.4								-30.9				
Mstri10456-283N	NECS-141	1	83.3		+48.5										
AW11-337N	NECS-141	1	84.9		+48.3										
1a09ggt5-1-252B	NECS-141	4	0.0							-40.1	-32.1				
1g05tct12-1-277	NECS-141	4	83.3		+43.3										
Mstir11314-131	Altet-4	5	16.1			-30.4		-41.5							
AW196-212-M	Altet-4	5	18.8								-29.6				
BF106-214B	Altet-4	5	54.9			-33.1	-60.4								
TC105099-111	Altet-4	5	72.0										-60.1		
TC105099-117	NECS-141	5	23.3										-63.5		
1c12tgt5-1-100	NECS-141	5	29.9				+34.1								
AW369-169	NECS-141	5	44.1				-39.2								

BE92-199	Altet-4	6	6.8						-36.5	-40.2		
1f11aatt4-1-192A	Altet-4	6	14.3						-28.0			
Mstri8733-18805A	Altet-4	6	51.2						-38.5	-52.9		
BE123-211B	Altet-4	7	100.7									+49.3
BF65-391	NECS-141	7	37.6	-40.2	-79.9	-49.7						
AW325-172A	Altet-4	8	10.1	-29.6								
BF218-243A	Altet-4	8	60.8		-52.0		-33.5					
BI116-225	Altet-4	8	83.0				-36.6					
Mstir11470-306A	Altet-4	8	86.1							-46.2		
AW201-296A	Altet-4	8	87.6							-42.2		
BI86-223	Altet-4	8	88.8				-43.4	-43.9				
Mstri9820-120N	NECS-141	8	30.4				-48.8	-47.4				
Mstri7807-242	NECS-141	8	58.5				-45.2					

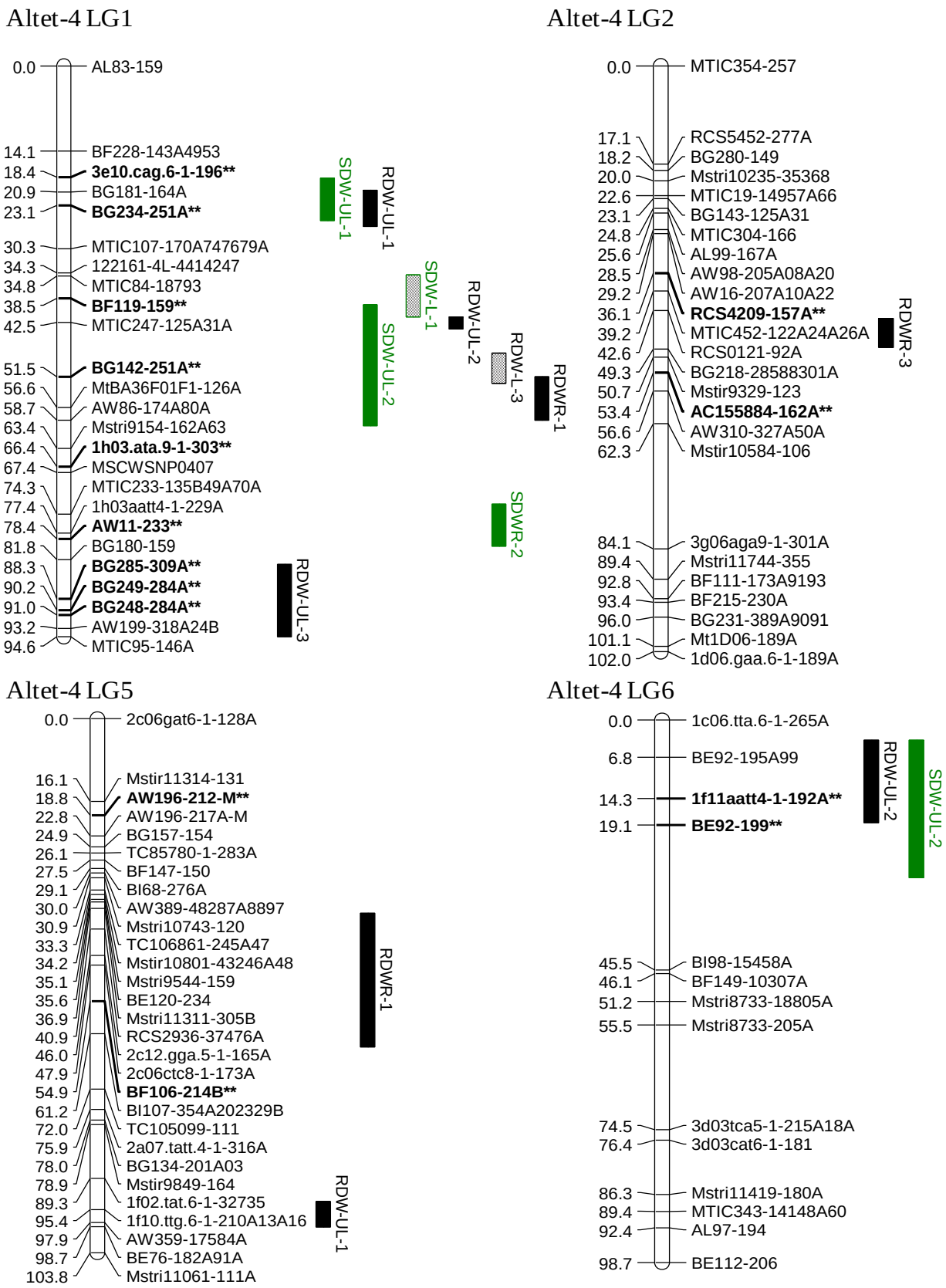
[§] (-) or (+) indicates the negative or positive effects of the alleles.

Table 2.6. Allele effects of markers associated with Al tolerance identified based on root and shoot dry weight ratios (RDWR and SDWR), in each environment based on the nonparametric Kruskal-Wallis test and SF-ANOVA (*P* value ≤ 0.01).

				Environment 1		Environment 2		Environment 3	
Marker allele	Parent	LG	Position (cM)	RDWR	SDWR	RDWR	SDWR	RDWR	SDWR
AL81-228	NECS-141	1	0.0					+0.11	
MTIC247-128	NECS-141	1	8.3					-0.15	
RCS4209-157A	Altet-4	2	36.1	+0.12					
AC155884-162A	Altet-4	2	53.4		+0.12				
3g06aga9-1-301A	Altet-4	2	84.1					-0.10	
1g05cata17-1-139	NECS-141	2	66.2				-0.07		
Mstri11090-187	NECS-141	2	78.8				-0.07		
MTIC124-179B	Altet-4	3	38.1		-0.11				
Mstri11067-147N	NECS-141	3	65.9					+0.11	
MSCWSNP0413	NECS-141	3	89.4			-0.08			
Mstir12038-216	Altet-4	4	12.1						-0.10
AW317-158	Altet-4	4	36.5			+0.09			
prs186-229A	Altet-4	4	96.7		-0.10				
prs353-212A	Altet-4	4	98.9		-0.10				
BG171-192	NECS-141	4	4.1					+0.13	
AL99-176	NECS-141	4	10.1					+0.11	
BF184-299	NECS-141	4	13.1					+0.12	
AW317-158	NECS-141	4	17.3			+0.09			
AA04-315	NECS-141	4	21.9		+0.10				
2c06gat6-1-128A	Altet-4	5	0.0	-0.09					
Mstri10686-126N	NECS-141	5	76.4					-0.11	-0.14
Mstri8733-18805A	Altet-4	6	51.2				-0.08		
BG281-195	Altet-4	7	34.8					+0.10	
BE123-211B	Altet-4	7	100.7		-0.14				
2a09.aac.6-1-282	Altet-4	8	0.0						-0.09
BI113-241	Altet-4	8	41.5						-0.09
BF218-243A	Altet-4	8	60.8		+0.11				
AL79-259A	Altet-4	8	80.2		-0.11				
BI116-225	Altet-4	8	83.0		+0.10				
BG186-189	NECS-141	8	61.7			-0.09			
1e08.gat.5-1-241	NECS-141	8	62.3				+0.11		
1e08.tttc.4-1-342	NECS-141	8	66.3			-0.09			

Table 2.7. QTL associated with root and shoot dry weights, and Al tolerance in tetraploid alfalfa identified by interval mapping. The parent column indicates the parent in which each QTL was detected, QTL position in each LG, maximum LOD value of the QTL and LOD threshold computed based on 1000 permutations (value in parentheses). R^2 indicates the percentage of the variation explained by the QTL.

Trait	Linkage Group	Environment	Parent	QTL position (cM)	LOD value	R^2 (%)
Root DW ratio	1	1	Altet-4	57	5.0 (3.9)	11
Root DW ratio	2	3	Altet-4	48	4.7 (4.1)	30
Root DW ratio	4	2	NECS-141	18	4.1 (3.9)	28
Root DW ratio	5	1	Altet-4	48	5.0 (3.8)	35
Root DW ratio	5	3	NECS-141	77	3.6 (3.6)	19
Root DW-L	1	3	Altet-4	49	3.6 (3.5)	14
Root DW-L	1	1	NECS-141	101	4.3 (4.1)	9
Root DW-L	4	2	NECS-141	22	3.8 (3.5)	15
Root DW-UL	1	1	Altet-4	23	4.5 (4.0)	10
Root DW-UL	1	2	Altet-4	43	3.4 (3.4)	7
Root DW-UL	1	3	Altet-4	91	4.0 (3.6)	12
Root DW-UL	5	1	Altet-4	96	4.2 (3.5)	18
Root DW-UL	6	2	Altet-4	15	4.0 (3.5)	8
Root DW-UL	7	3	Altet-4	71	3.9 (3.3)	12
Shoot DW ratio	1	2	Altet-4	77	3.9 (3.4)	9
Shoot DW ratio	4	2	NECS-141	18	3.3 (3.2)	28
Shoot DW ratio	5	3	NECS-141	75	5.6 (3.7)	15
Shoot DW ratio	8	1	Altet-4	72	3.3 (2.8)	7
Shoot DW-L	1	1	Altet-4	39	3.8 (3.4)	7
Shoot DW-L	4	2	NECS-141	22	4.0 (3.6)	21
Shoot DW-L	7	1	NECS-141	52	4.0 (3.6)	41
Shoot DW-UL	1	1	Altet-4	23	4.5 (3.8)	11
Shoot DW-UL	1	2	Altet-4	27	4.7 (3.9)	12
Shoot DW-UL	1	2	NECS-141	83	3.7 (3.5)	8
Shoot DW-UL	6	2	Altet-4	7	4.7 (3.5)	11


Altet-4 LG1
0.0 AL83-159
14.1 BF228-143A4953
18.4 3e10.cag.6-1-196**
20.9 BG181-164A
23.1 BG234-251A**
30.3 MTIC107-170A747679A
34.3 122161-4L-4414247
34.8 MTIC84-18793
38.5 BF119-159**
42.5 MTIC247-125A31A
51.5 BG142-251A**
56.6 MtBA36F01F1-126A
58.7 AW86-174A80A
63.4 Mstri9154-162A63
66.4 1h03.ata.9-1-303**
67.4 MSCWSNP0407
74.3 MTIC233-135B49A70A
77.4 1h03aatt4-1-229A
78.4 AW11-233**
81.8 BG180-159
88.3 BG285-309A**
90.2 BG249-284A**
91.0 BG248-284A**
93.2 AW199-318A24B
94.6 MTIC95-146A
SDW-UL-1
RDW-UL-1
SDW-L-1
RDW-UL-2
SDW-UL-2
RDW-L-3
RDWR-1
SDWR-2
RDW-UL-3
Altet-4 LG2
0.0 MTIC354-257
17.1 RCS5452-277A
18.2 BG280-149
20.0 Mstri10235-35368
22.6 MTIC19-14957A66
23.1 BG143-125A31
24.8 MTIC304-166
25.6 AL99-167A
28.5 AW98-205A08A20
29.2 AW16-207A10A22
36.1 RCS4209-157A**
39.2 MTIC452-122A24A26A
42.6 RCS0121-92A
49.3 BG218-28588301A
50.7 Mstir9329-123
53.4 AC155884-162A**
56.6 AW310-327A50A
62.3 Mstir10584-106
84.1 3g06aga9-1-301A
89.4 Mstri11744-355
92.8 BF111-173A9193
93.4 BF215-230A
96.0 BG231-389A9091
101.1 Mt1D06-189A
102.0 1d06.gaa.6-1-189A
RDWR-3
Altet-4 LG5
0.0 2c06gat6-1-128A
16.1 Mstir11314-131
18.8 AW196-212-M**
22.8 AW196-217A-M
24.9 BG157-154
26.1 TC85780-1-283A
27.5 BF147-150
29.1 BI68-276A
30.0 AW389-48287A8897
30.9 Mstri10743-120
33.3 TC106861-245A47
34.2 Mstir10801-43246A48
35.1 Mstri9544-159
35.6 BE120-234
36.9 Mstri11311-305B
40.9 RCS2936-37476A
46.0 2c12.gga.5-1-165A
47.9 2c06ctc8-1-173A
54.9 BF106-214B**
61.2 BI107-354A202329B
72.0 TC105099-111
75.9 2a07.tatt.4-1-316A
78.0 BG134-201A03
78.9 Mstir9849-164
89.3 1f02.tat.6-1-32735
95.4 1f10.ttg.6-1-210A13A16
97.9 AW359-17584A
98.7 BE76-182A91A
103.8 Mstri11061-111A
RDWR-1
RDW-UL-1
Altet-4 LG6
0.0 1c06.tta.6-1-265A
6.8 BE92-195A99
14.3 1f11aatt4-1-192A**
19.1 BE92-199**
45.5 BI98-15458A
46.1 BF149-10307A
51.2 Mstri8733-18805A
55.5 Mstri8733-205A
74.5 3d03tca5-1-215A18A
76.4 3d03cat6-1-181
86.3 Mstri11419-180A
89.4 MTIC343-14148A60
92.4 AL97-194
98.7 BE112-206
RDW-UL-2
SDW-UL-2

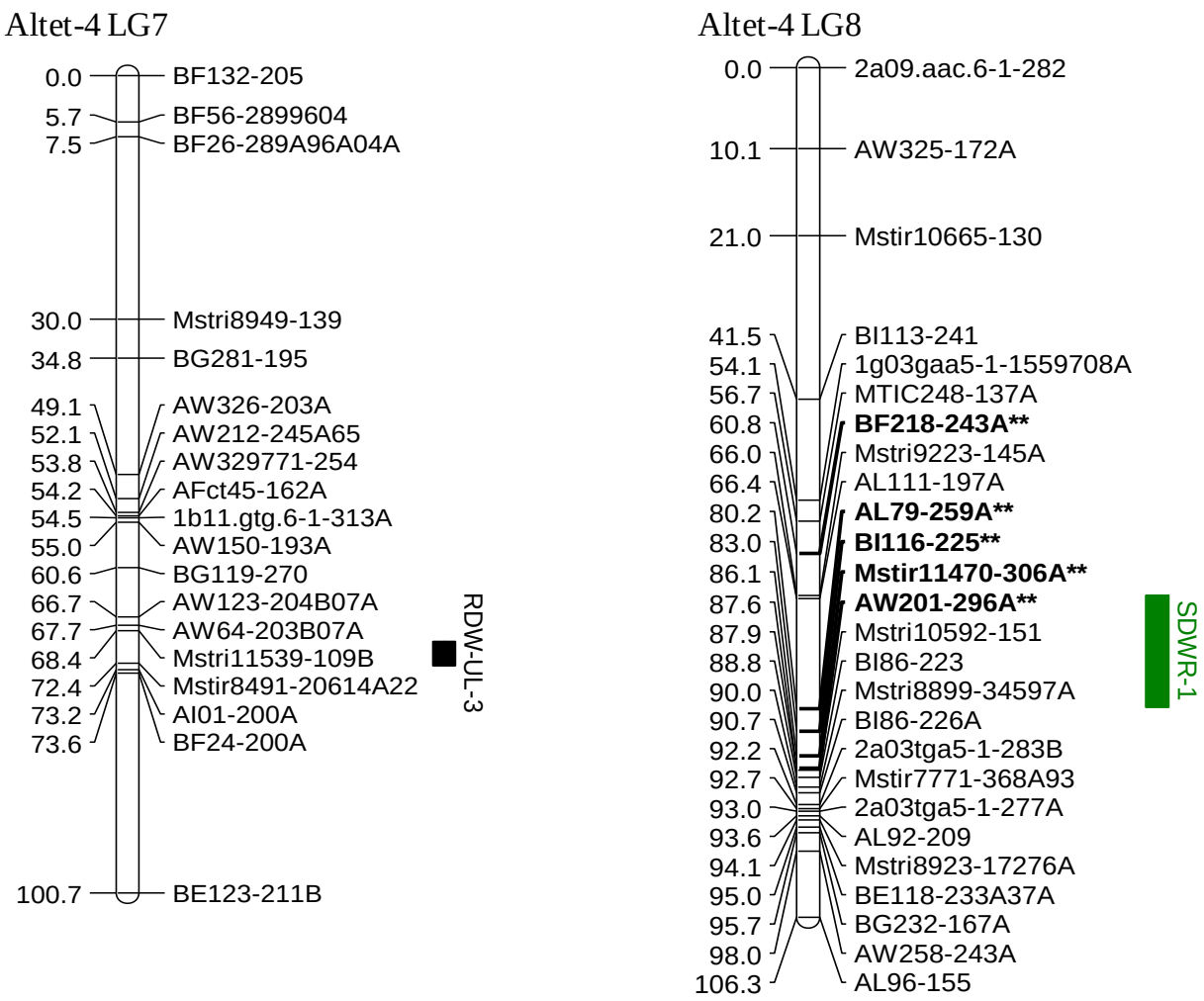


Fig. 2.1. QTL identified using interval mapping in Altet-4 linkage map. Bars with solid bars represent dry weight in UL soil and their ratios. Stripped bars represent dry weight in L soil. Green and black bars represent shoot and root traits respectively. Marker-alleles in bold and with asterisks represent association at *P* value ≤ 0.01, based on the nonparametric Kruskal-Wallis test and on SF-ANOVA.

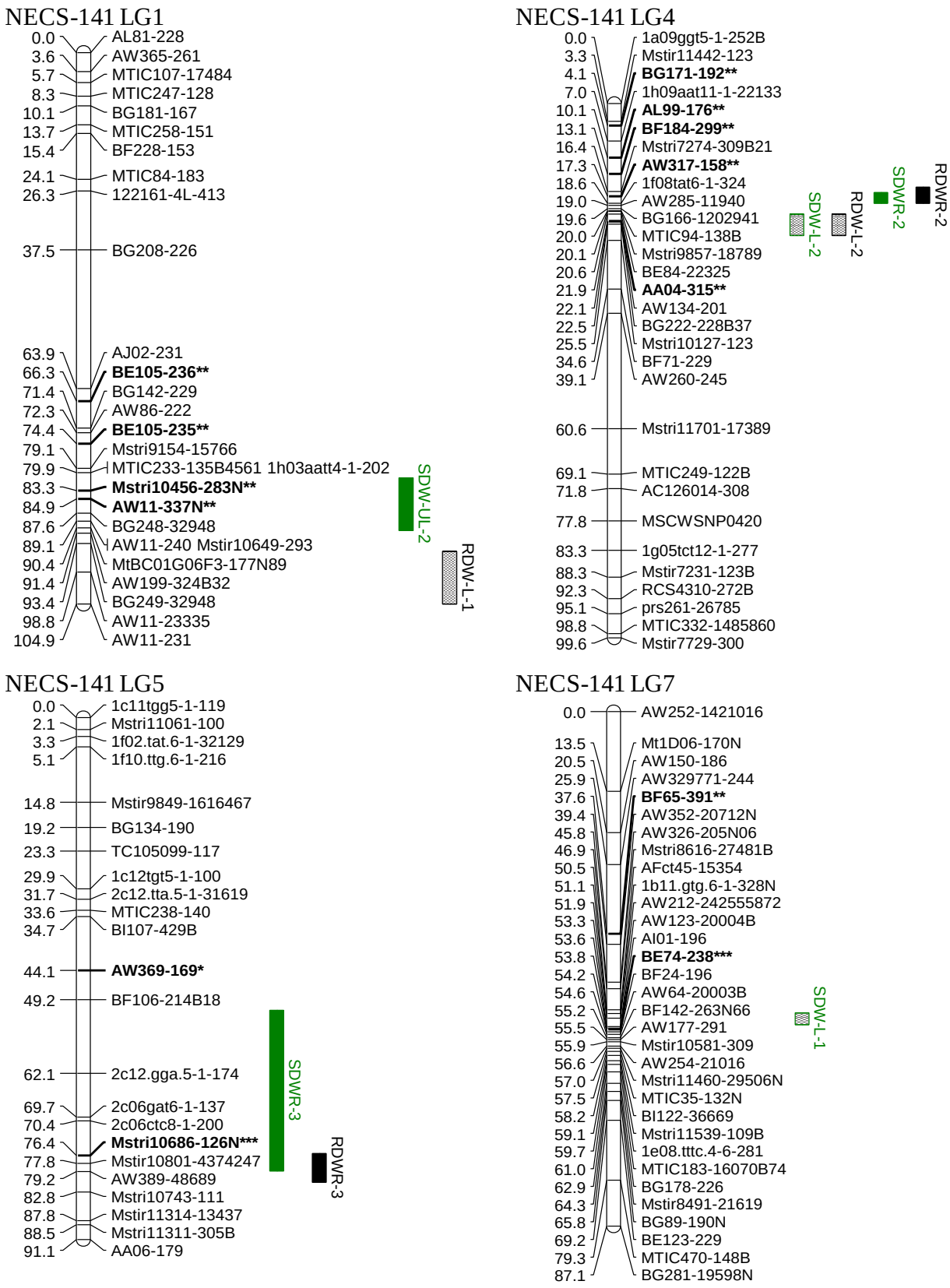
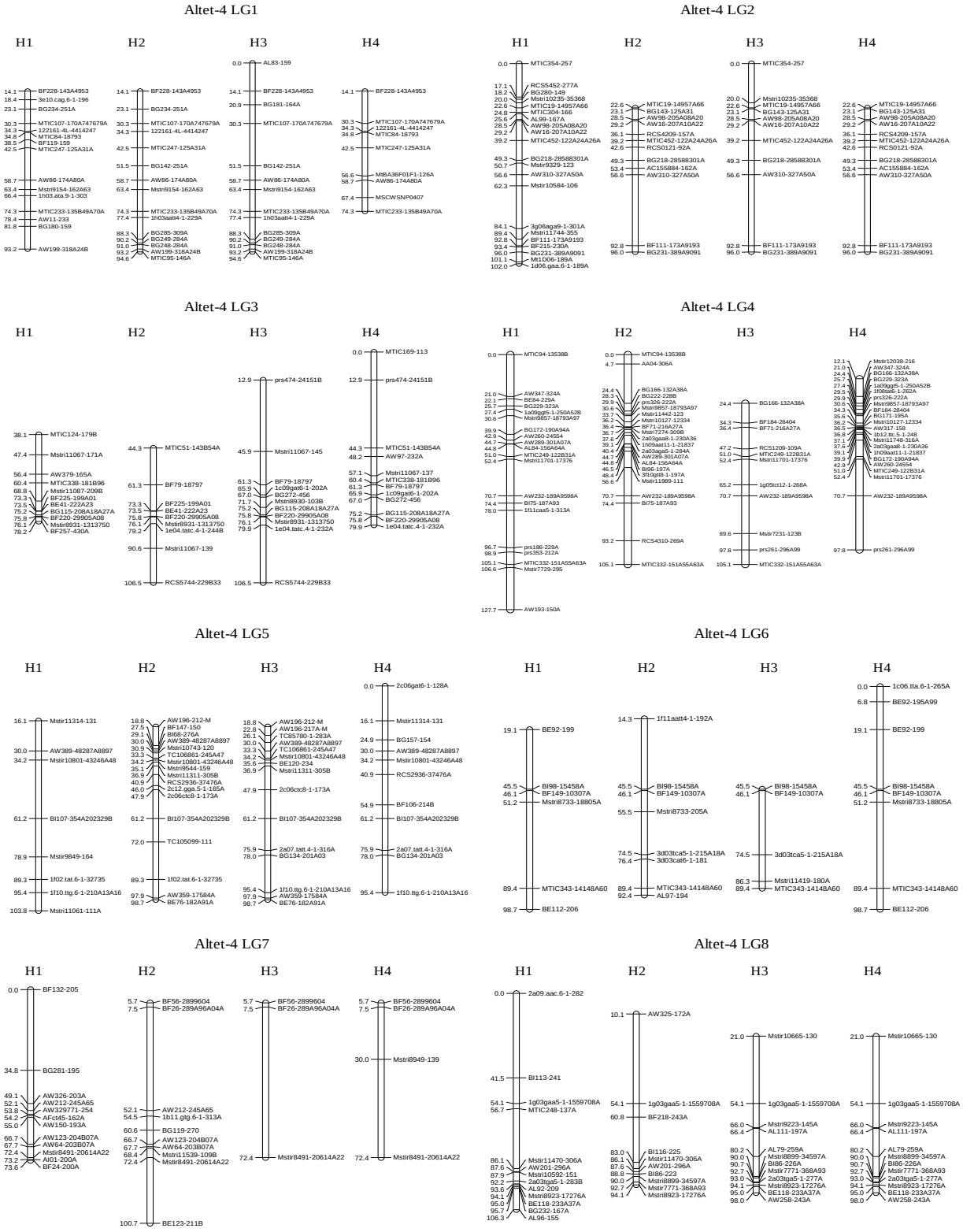
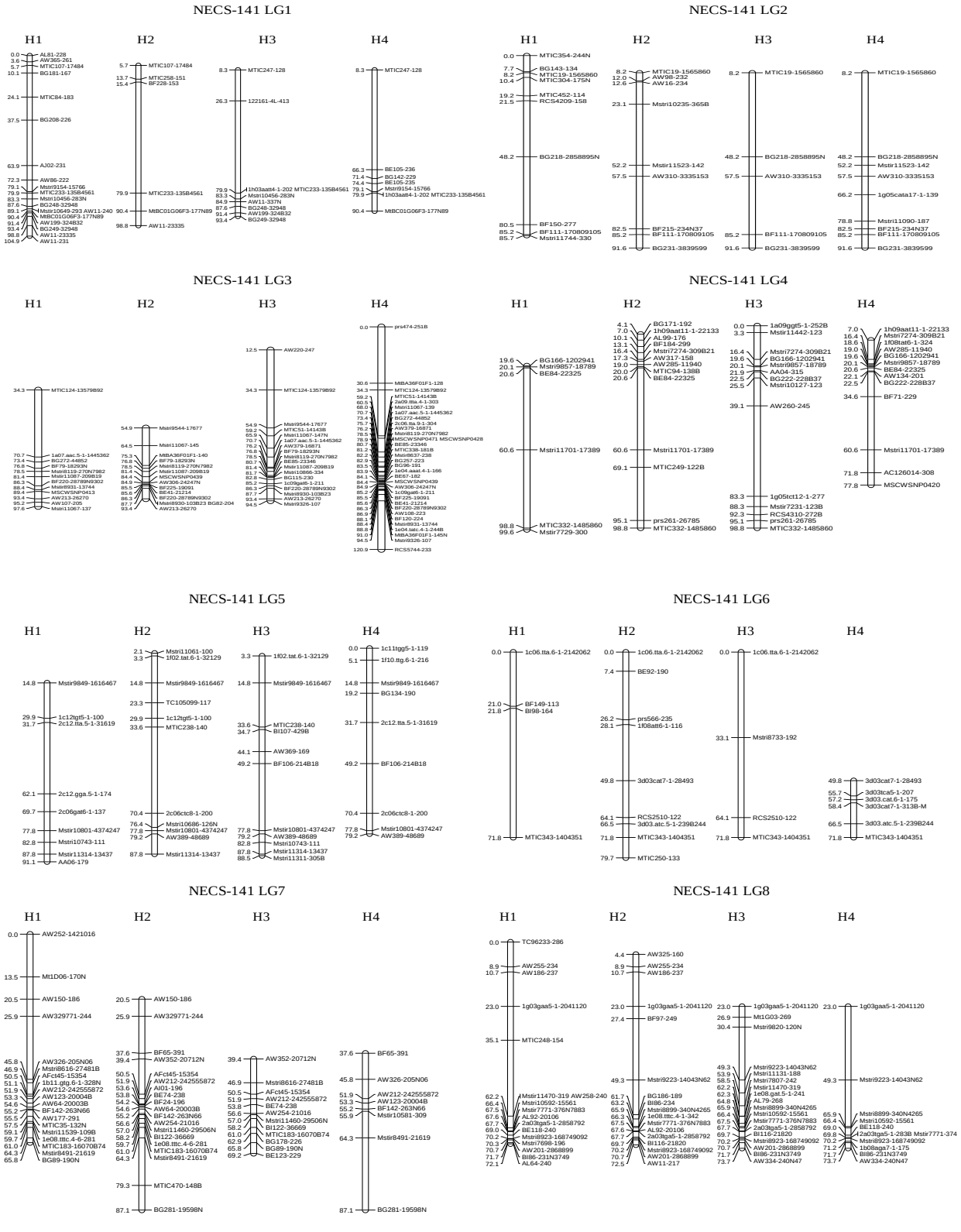

NECS-141 LG1
0.0 AL81-228
3.6 AW365-261
5.7 MTIC107-17484
8.3 MTIC247-128
10.1 BG181-167
13.7 MTIC258-151
15.4 BF228-153
24.1 MTIC84-183
26.3 122161-4L-413
37.5 BG208-226
63.9 AJ02-231
66.3 BE105-236**
71.4 BG142-229
72.3 AW86-222
74.4 BE105-235**
79.1 Mstri9154-15766
79.9 MTIC233-135B4561 1h03aatt4-1-202
83.3 Mstri10456-283N**
84.9 AW11-337N**
87.6 BG248-32948
89.1 AW11-240 Mstir10649-293
90.4 MtBC01G06F3-177N89
91.4 AW199-324B32
93.4 BG249-32948
98.8 AW11-23335
104.9 AW11-231
SDW-UL-2
RDW-L-1
NECS-141 LG4
0.0 1a09ggt5-1-252B
3.3 Mstir11442-123
4.1 BG171-192**
7.0 1h09aat11-1-22133
10.1 AL99-176**
13.1 BF184-299**
16.4 Mstri7274-309B21
17.3 AW317-158**
18.6 1f08tat6-1-324
19.0 AW285-11940
19.6 BG166-1202941
20.0 MTIC94-138B
20.1 Mstri9857-18789
20.6 BE84-22325
21.9 AA04-315**
22.1 AW134-201
22.5 BG222-228B37
25.5 Mstri10127-123
34.6 BF71-229
39.1 AW260-245
60.6 Mstri11701-17389
69.1 MTIC249-122B
71.8 AC126014-308
77.8 MSCWSNP0420
83.3 1g05tct12-1-277
88.3 Mstir7231-123B
92.3 RCS4310-272B
95.1 prs261-26785
98.8 MTIC332-1485860
99.6 Mstir7729-300
SDW-L-2
RDW-L-2
SDWR-2
RDWR-2
NECS-141 LG5
0.0 1c11tgg5-1-119
2.1 Mstri11061-100
3.3 1f02.tat.6-1-32129
5.1 1f10.ttg.6-1-216
14.8 Mstir9849-1616467
19.2 BG134-190
23.3 TC105099-117
29.9 1c12tgt5-1-100
31.7 2c12.tta.5-1-31619
33.6 MTIC238-140
34.7 BI107-429B
44.1 AW369-169*
49.2 BF106-214B18
62.1 2c12.gga.5-1-174
69.7 2c06gat6-1-137
70.4 2c06ctc8-1-200
76.4 Mstri10686-126N***
77.8 Mstir10801-4374247
79.2 AW389-48689
82.8 Mstri10743-111
87.8 Mstir11314-13437
88.5 Mstri11311-305B
91.1 AA06-179
SDWR-3
RDWR-3
NECS-141 LG7
0.0 AW252-1421016
13.5 Mt1D06-170N
20.5 AW150-186
25.9 AW329771-244
37.6 BF65-391**
39.4 AW352-20712N
45.8 AW326-205N06
46.9 Mstri8616-27481B
50.5 AFct45-15354
51.1 1b11.gtg.6-1-328N
51.9 AW212-242555872
53.3 AW123-20004B
53.6 AI01-196
53.8 BE74-238***
54.2 BF24-196
54.6 AW64-20003B
55.2 BF142-263N66
55.5 AW177-291
55.9 Mstir10581-309
56.6 AW254-21016
57.0 Mstri11460-29506N
57.5 MTIC35-132N
58.2 BI122-36669
59.1 Mstri11539-109B
59.7 1e08.tttc.4-6-281
61.0 MTIC183-16070B74
62.9 BG178-226
64.3 Mstir8491-21619
65.8 BG89-190N
69.2 BE123-229
79.3 MTIC470-148B
87.1 BG281-19598N
SDW-L-1

Fig. 2.2. QTL identified with interval mapping in NECS-141 linkage map. Solid bars represent dry weight in UL soil and their ratios. Stripped bars represent dry weight in L soil. Green and black bars represent shoot and root traits respectively. Marker-alleles in bold and with an asterisk represent association at *P* value ≤ 0.01, based on the nonparametric Kruskal-Wallis test and SF-ANOVA.

A. Altet-4 linkage map.



B. NECS-141 linkage map.



Supplementary Fig. 2.1. Linkage maps of (A) Altet-4 and (B) NECS-141 showing the four homologous chromosomes (H1-H4) for each linkage group.

CHAPTER 3

EVALUATION OF TWO TRANSGENIC EVENTS FOR ALUMINUM TOLERANCE IN ALFALFA[2]

[2] Rafael Reyno, Dong-Man Khu, Maria J. Monteros, Joseph H. Bouton, Wayne Parrott, and E. Charles Brummer. To be submitted to *Crop Science*

Abstract

Acid-soil syndrome inhibits root growth and development of many plant species due to Al^{+3} and H^{+} toxicity and essential nutrients deficiencies. Alfalfa, *Medicago sativa* L., production is dramatically reduced in acidic, Al-rich soil. Transgenic plants of several species over-expressing organic acid synthesis and/or organic acid transporter genes have shown enhanced tolerance to Al. The objective of this research was to evaluate the effect of the citrate synthase (CS) and the plasma membrane H^{+}-ATPase (DcPA1) transgenes on Al tolerance in alfalfa when evaluated in a greenhouse soil assay. Transgenic alfalfa plants containing a single insertion of either CS or DcPA1 were crossed to the Al-sensitive genotypes 95-608 and 60T180-14, respectively, and a highly expressing transgenic progeny genotype from each cross was used to generate a full-sib T_2 population including four isogenic groups. Stem cuttings from 48 T_2 genotypes, consisting of 13 with neither transgene, 11 with CS only, 14 with DcPA1 only, and 10 with both transgenes, together with 8 non-transformed check genotypes were evaluated for Al/acid soil tolerance in a greenhouse assay in limed (L) and unlimed (UL) soil. Al/acid soil tolerance was assessed by measuring the ratios of root and shoot dry weight in UL soil compared to L soil. The three transgenic populations, CS, DcPA1, and CS+DcPA1, all showed higher Al/acid soil tolerance than the nontransgenic isogenic population or the nontransgenic parental genotypes. We observed no advantage of combining both transgenes in the same genetic background. Levels of tolerance achieved by the transgenic populations were high, with ratios of 0.91 to 0.98. Lower levels of Al in shoot tissue were observed for the transgenic populations compared to the non-transgenic population, suggesting that the Al-exclusion mechanism could be driving Al/acid soil tolerance in this study. These transgenes offer an efficient method to achieve enhanced Al/acid

soil tolerant alfalfa cultivars, with more information needed on the stability of these transgenes and their performance under field conditions.

Introduction

Alfalfa, *Medicago sativa* L., is widely cultivated worldwide and is the fourth most important crop in U.S. agriculture (USDA-NASS, 2009). Acid soils are a severe problem for alfalfa production in many parts of the world, including the southeastern U.S. (Bouton and Sumner, 1983b). Acid-soil syndrome, which mainly compromises Al^{+3} and H^{+} toxicity and a deficiency of essential nutrients such as phosphorus (P), causes severe inhibition of root growth and development (Kochian et al., 2004b). Two mechanisms of Al tolerance have been described in higher plants – those that prevent Al uptake by the roots, through the exudation of organic acids from the roots or by raising the pH of the rhizosphere, and those that internally detoxify Al, allowing the plant to tolerate Al accumulation in roots and shoots (Kochian et al., 2004b). Both mechanisms are complex and hard to dissect experimentally (Kochian et al., 2005). Based on research in many species, aluminum tolerance appears to be controlled by few genes with large effects, and organic acid exudation plays an important role (Delhaize et al., 2004; Hoekenga et al., 2003; Magalhaes et al., 2004; Pineros et al., 2002). However, other studies, sometimes in the same species, have reported that Al tolerance is more complex, with potentially multiple mechanisms interacting (Bianchi-Hall et al., 2000; Liu et al., 2009; Narasimhamoorthy et al., 2007a; Xue et al., 2008).

Traditionally, soil acidity can be ameliorated by liming, effectively eliminating Al toxicity in the plow layer, but not in the subsoil (Bouton and Sumner, 1983b). Genetic tolerance to acidic conditions would be a more durable and less expensive method to increase alfalfa productivity and reduce production costs in acid soils (Bouton, 1996; Bouton et al., 1986), but

limited variability has been observed in alfalfa germplasm for Al tolerance (Bouton, 1996; Campbell et al., 1988). An alternative, or additional, method of modifying Al tolerance of alfalfa is through the insertion of transgenes, introduces genes or modulates expression of existing genes to a level not available in existing germplasm.

The Al-chelating ability of some organic acids, including citrate, malate, and oxalate appears to confer Al tolerance through the formation of stable complexes with Al^{+3} in the soil, making it insoluble and preventing its uptake by the roots (Barone et al., 2008; Delhaize et al., 1993b). Transgenic plants over-expressing genes coding for organic acid synthesis, such as citrate synthase (CS), have shown improved Al tolerance in several crops, including tobacco, papaya (de la Fuente et al., 1997), canola (Anoop et al., 2003), and alfalfa (Barone et al., 2008). A complementary transgenic approach to improve Al tolerance is to use organic acid transporters (Kochian et al., 2004b). This approach has been effective in barley (*Hordeum vulgare*), a very Al-sensitive cereal crop (Delhaize et al., 2004; Delhaize et al., 2009). The DcPA1 gene coding for a plasma membrane H^{+}ATPase from *Daucus carota* may play a role in the proton exudation (Ohno et al., 2004).

The practical value of the transgenes may not be manifested in plants only containing a single gene. The optimum Al tolerance may arise from a strategy that combines the organic acid over-expression with a gene encoding a compatible transporter (Barone et al., 2008). The transporter gene is hypothesized to be necessary to export citrate out of the plant cells, and consequently, both transgenes could perform better than either one alone.

Engineered alfalfa plants with CS and DcPA1 under the control of three different promoters were developed (Shen, 2009). The MtHP promoter, a constitutive promoter from *M. truncatula* (Xiao et al., 2005), showed higher expression levels for both genes, CS and DcPA1,

compared to the untransformed control, R2336 alfalfa line (Federal Register /Vol. 70, No. 122 /Monday, June 27, 2005 /Notices 36917-9) and the GA-AT breeding line (Shen, 2009). However, transgene-containing plants were not evaluated for aluminum tolerance.

We hypothesized that both the CS and DcPA1 gene individually would confer Al tolerance on alfalfa, and that the combination of both genes would further improve Al tolerance. Therefore, the objective of this experiment was to evaluate isogenic alfalfa populations containing neither transgene, each transgene individually, or both transgenes for their Al tolerance using a soil-based assay in the greenhouse.

Materials and Methods

Plant material and population development

The citrate synthase (CS) and the plasma membrane H^+ATPase (DcPA1) genes were previously transformed into the Forage Genetics Intl. proprietary alfalfa genotype R2336 under the control of three promoters (Shen, 2009). R2336, like many alfalfa genotypes, is non-inbred. The transgenic (T_0) genotypes, CS-14 and DcPA1-85, with genes driven by the constitutive promoter MtHP, showed higher expression levels of their respective transgene than other genotypes when compared to the untransformed R2336 control (Shen, 2009), and thus were selected for further evaluation.

Our goal was to evaluate the performance of these genes singly and in combination in isogenic backgrounds. Because both genes were transformed into the same alfalfa line (R2336), they cannot be crossed due to self-incompatibility, and if the hybridization would have been successful, the progeny would have resulted with significant inbreeding depression, potentially obscuring the phenotypes caused by the transgenes. Therefore, avoid low vigor and other

problems due to inbreeding depression; we hybridized each transgenic genotype to an unrelated genotype in the following combination: CS-14×95-608 and DcPA1-85×60T180-14. Previous evaluations have identified the 95-608 (Khu et al., 2012) and the 60T180-14 (data not shown) genotypes as Al-sensitive. The 95-608 genotype was derived from the non-dormant synthetic cultivar CUF101 (Lehman et al., 1983), while 60T180-14 was selected under grazing and drought conditions in Tifton GA (Dr. J.H. Bouton, pers. comm.) from an Italian germplasm (Forage Genetics Intl.).

Twenty-five T_1 progeny from each cross were evaluated by PCR (see below) for the presence or absence of the appropriate transgene. The genotype CS-16 derived from CS-14×95-608 was hybridized to the genotype H^+-4 from DcPA1-85×60T180-14 to generate progeny that fell into one of four isogenic T_2 populations: (1) no transgene, (2) CS only, (3) DcPA1 only, or (4) both transgenes. Because every plant in the population is genetically unique, the comparison among the transgene groups has to be done at the population level to minimize background variation. The resulting populations are expected to be isogenic except for the transgene. The genotypes CS-16 and H^+-4 were reciprocally hybridized, emasculating the female parent, to generate a full-sib F_1 population of 76 individuals.

PCR screening of putative transgenic plants

Genomic DNA extractions from T_0, T_1, and T_2 genotypes were done using a modified CTAB protocol (Murray and Thompson, 1980). About 50 ng of DNA from each alfalfa plant was used for PCR. Amplification reactions used primers CS-463F (5'-CCGAAGCATCGCGAAGTCTC-3') and CS-1012R (5'-CCAGTTGCGGGTCGTTGATG-3') or DcPA1-1342F (5'-ATTGATAAGTTTGCAGAGCGTGGGTTGAG-3') and DcPA1-1977R (5'-AAACACAATACGGATCGTGATGGATACTGC-3') as described by Shen (2009). The

positive controls were plasmid DNA (about 1 ng) containing the corresponding expression cassettes, and the negative control was genomic DNA isolated from the wild type R2336 alfalfa genotype. Amplicons from the PCR were analyzed on a 1% agarose gel containing ethidium bromide and visualized under UV light. The segregation ratios for the T_1 and T_2 individuals were tested for deviation from expectations using the FREQ procedure of SAS v. 9.2 (SAS, 2008) with the chi-square test option.

Expression levels

To quantify citrate production and proton exudation, we evaluated 5-wk-old rooted stem cuttings grown in a Fafard Super-fine Germinating Mix® (Conrad Fafard Inc., Agawam, MA) in the UGA Crop and Soil Sciences greenhouses, Athens GA. For citrate production, three replicate stem cuttings of 10 transgenic individuals originating from CS-14×95-608 containing the CS transgene together with two non-transgenic siblings, the non-transformed alfalfa genotype R2336, and a single genotype from the GA-AT alfalfa population (Bouton and Radcliffe, 1989) (labeled as GA-AT) were evaluated as described by Shen (2009). Briefly, 0.2 g of fresh active growing roots were harvested, ground in liquid nitrogen with a mortar and pestle, and transferred to a 1.5 ml Eppendorf tube with 1 ml of ice-cold 0.6 N perchloric acid neutralized with 170-200 µl of 5 N K_2CO_3. The citrate acid quantification kit (RBiopharm Inc., Marshall, Michigan) was used for the spectrographic assay of citrate, and the absorbance was measured using the Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT).

For proton exudation, three replicate stem cuttings of eight transgenic and two non-transgenic individuals from the DcPA1-85×60T180-14 progeny, the non-transformed R2336 alfalfa genotype, and the GA-AT genotype were evaluated as described by Shen (2009). Briefly, a uniform set of stem cuttings with 5 cm long roots were placed in 30 ml COREX® (Krackeler

Scientific Inc., Albany, NY) centrifuge tubes containing 20 ml of 0.5 mM $CaCI_2$ (pH 5.8) for 24 hours in a growth chamber (14h/26°C day and a 10 h/22°C night regimen). Subsequently, changes in the pH of the solution were measured using a Corning pH meter 240 (Corning Incorporated, Corning, NY). The H^+ concentration in the solution was calculated based on the following equation, $[H^+]$ $(mol^{-1}) = 10^{-pH}$.

For both assays, a completely randomized design with three biological and three technical replications was used. The UNIVARIATE procedure of SAS was used to evaluate the general statistics in each trait and for testing for normal distribution using the QQPlot statement and the normal option. The GLM procedure of SAS v. 9.1 (SAS Institute, 2007) was used calculate the means for each genotype and to compute a mean comparison based on the least significant difference (LSD) at the 5% probability level.

Southern blot analysis

The CS-16 and the H^+-4 genotypes were evaluated to confirm the number of insertion sites. The Southern blot analysis protocol used was previously described by Shen (2009). Negative controls for each transgene and positive controls of plasmid DNA containing the corresponding expression cassettes were included in the analysis. Briefly, about 10 µg of purified genomic DNA were digested overnight at 37°C with 12 units of *Eco*RI (Promega, Madison, WI), and fractions separated in a 0.8% agarose gel with 1×TAE buffer at 30V, 35 mAmps. After soaking the agarose gel in denaturation and neutralization buffers, DNA was transferred to a Hybond™-N+ membrane (Amersham, Piscataway, NJ). The probes used in each case amplified a fragment of the CS and DcPA1 genes, and were amplified using the primers pairs CS-463F (5'-CCGAAGCATCGCGAAGTCTC-3') and CS-1012R (5'-CCAGTTGCGGGTCGTTGATG-3'), and hph-117F (5'-CGATGTAGGAGGGCGTGGATA-3') and hph-938R (5'-

CTTCTGCGGGCGATTTGTG- 3'). Finally, after prehybridization and hybridization steps, the hybridized membrane was exposed to a Kodak BioMax Film at -80^{o}C for 7 d.

Phenotypic analysis of Al tolerance

Evaluation of soil-based Al tolerance of the T_2 isogenic populations was performed in the UGA Crop and Soil Sciences greenhouses, Athens, GA during Nov. and Dec. 2011, using two greenhouses as separate environments. We used the whole-plant assay in soil system (Khu et al., 2012) modified to prevent waterlogging. The modification consisted of placing racks with conetainers into a plastic tray with drain holes at the bottom and filled with 15 cm of sterile sand. The conetainers were placed 5 cm into the sand to ensure contact between cone soil and sand. The sand was used to prevent soil saturation after watering (Dr. M.E. Sumner, pers. comm.), thereby preventing Mn toxicity. The soil we used was identical to that in Khu et al. (2012), except that nutrients and lime were mixed prior to these experiments (Table 3.1).

Rooted stem cuttings were used as previously described (Khu et al., 2012). Briefly, conetainers were filled with either limed (L) or unlimed (UL) soil leaving a space at the top for a 6-wk-old clonal plug of a given genotype, which was transferred directly without washing off the germination mix in which the cuttings were grown. The appropriate soil was added around the plug to fill the space at the top of the conetainer. Greenhouse temperatures were 25°C day and 20°C night with a 16 hr photoperiod supplemented by high-intensity lights. The experiments were watered daily using distilled water, and the trays were rotated on the greenhouse benches weekly to diminish micro-environmental variation inside the greenhouse. Chlorpenapyr (0.41 g/l) and avermectin (49.3 g/l) were applied to control thrips (*Caliothrips fasciatus* and *Frankliniella occidentalis*). After growing for 6 wk, plants and soil were removed from cones, and the soil was gently washed off from the roots. Plants were then separated into root and stem

fractions at the soil line. The roots growing in the germination mix, corresponding to the top 5 cm of the root fraction, were discarded and not considered for further analysis. Therefore, the root fraction that we analyzed and discussed here corresponds to the roots below 5 cm, growing in the UL or L soil. After drying at 65ºC for 72 hr, the dry weight (DW) of roots and shoots was determined. Relative root weight was computed as the ratio of root dry weight in UL soil to the root dry weight in L soil for each genotype in each replication. Relative shoot dry weight was computed analogously.

Therefore, the traits analyzed were root dry weight in L and UL soil, hereafter referred to RDW-L and RDW-UL, shoot dry weight in L and UL soil, hereafter named as SDW-L and SDW-UL, and root and shoot dry weight ratios, the relative growth in UL vs L soil, hereafter named as RDWR and SDWR, respectively.

Forty-eight genotypes from the CS-16×H^+-4 progeny were used in this experiment, consisting of 13 with neither transgene, 11 with the CS transgene, 14 with the DcPA1 transgene, and 10 with both transgenes. Eight non-transformed genotypes were used as checks. Some of the genotypes were present in more than one copy per replication, for completing a total of 60 plugs evaluated in each conetainer rack. These 60 genotypes were placed in a 98 (7 × 14) conetainer rack, where the outer cones were planted as borders. The four isogenic populations and the check genotypes were evaluated in four replications per environment. Two racks, one with UL soil and the other with L soil represented one replication. Conetainers used in this experiment were the same as those used in Khu et al. (2012).

Besides the traits previously described, we also measured the concentration of macronutrients (Ca, K, Mg, N, P, and S) and Al, Fe, and Mn accumulated by the plants using a tissue analysis on the entire shoot fraction of plants from the four isogenic populations and the

eight checks in both types of soil. In each environment, shoot fractions from replications 1 and 2, and 3 and 4 from were consolidated, therefore two samples per environment and type of soil were analyzed. The analyses were performed at the Soil, Plant, and Water Laboratory of The University of Georgia, Athens, GA, following the inductively coupled plasma method by using the ICP Emission Spectrograph (Thermo Jarrel Ash Corp., Franklin, MA).

Reverse transcriptase PCR

Total RNA was extracted from young leaves from the four isogenic populations (CS-DcPA1, CS, DcPA1, and non-transgenic), and the eight genotypes used as checks growing in L and UL soil in the fifth week of the soil assay using the TRI Reagent® protocol (Ambion, Austin, TX). Tissue samples from the isogenic populations were collected from two replications per environment in each type of soil, while for the check genotypes samples from both environments and replications were pooled. The concentration of extracted total RNA was measured using the Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT). About 1 μg of total RNA was used to synthesize cDNAs with GoScript™ Reverse Transcription System (Promega Corporation, Madison, WI). The cDNA product was used as the template in a PCR reaction using the same primers and PCR conditions described for the DNA screening section. PCR amplicons were analyzed on a 1% agarose gel containing ethidium bromide and visualized under UV light.

Data analysis

Data for each trait was tested for normality, and all traits were normally distributed. We used a mixed model containing effects for replications (nested in environments), genotypes, environments, and genotype × environment (G×E) interaction; all effects were considered fixed except replications. Environment effect was considered as fixed effect since they represent

highly controlled greenhouse conditions. For each trait, least square means were obtained for genotypes and compared using the least significant difference. Mixed models were evaluated using the MIXED procedure of SAS v. 9.1 (SAS, 2008). For all statistical analyses, significance was determined at the 5% probability level unless otherwise indicated.

Results

Twenty-three hybrid T_1 plants were obtained from each cross, CS-14×95-608 and DcPA1-85×60T180-14, and were screened for the presence or absence of the appropriate transgene (Table 3.2). In both cases the observed frequencies of the presence/absence of the transgenes fit the expected segregation ratio of 1:1 (Table 3.2). Citrate concentration or proton exudation varied among the T_1 individuals (Figures 3.1 and 3.2). The transgenic genotype CS-16 showed a three-fold increase over the non-transgenic genotypes (Figure 3.1). Two genotypes, H^+-2 and H^+-4, showed 4.5-fold higher proton exudation levels than the R2336 non-transgenic genotype (Figure 3.2). Based on the expression levels showed by CS-16 and H^+-4, they were used as parents to develop the isogenic populations.

Southern-blot analysis indicated that CS-16 contained a single insertion of the CS transgene and that H^+-4 likely contained a single DcPA1 insertion (data not shown). The segregation ratios observed for the T_1 plants were congruent with this result (Table 3.2). We expected four classes of progeny from the CS-16 × H^+-4 cross in equal frequencies, assuming that each transgene was present as a single insertion and in a single dose (i.e., Tttt) and the observed progeny frequencies fit the expected 1:1:1:1 ratio (Table 3.2).

The isogenic population evaluation in soil showed significant genotype effects for most of the traits, except for RDW-L and SDW-UL (Table 3.3). No significant genotype×environment

(G×E) effect was observed for RDW and SDW. The isogenic T_2 population without transgenes had similar root weight in L soil, but lower root weight in UL soil when compared to the populations with transgenes (Table 3.4). The Al-tolerance phenotype of the UL:L ratio was lower for the isogenic T_2 population without transgenes than for the other three populations, which had similar ratios. Parental genotypes did not differ for root weight in either soil but those containing a transgene (CS-16 and/or H^+-4) had higher ratios than those without transgenes. Shoot dry weight results similarly showed that the populations or parental genotypes containing at least one transgene had superior Al-tolerance than those without a transgene. The Al-tolerant genotype Altet-4, a parent in our Al-tolerance mapping population (see Chapter 2), showed the same Al tolerance (UL:L ratio) as the transgenic lines and populations (Table 3.4). The genotype NECS-141, the Al-sensitive parent of our mapping population, was Al-sensitive in this experiment, as expected.

Aluminum (Al) and manganese (Mn) content in the shoots did not differ among the isogenic population when grown in L soil (Table 3.5). However, in UL soil, the transgenic populations had lower Al in the tissue than the non-transgenic population. The transgenic parental genotypes were similar to the transgenic populations and generally lower than the non-transgenic genotypes. The Al-tolerant, non-transgenic genotype Altet-4 had high levels of Al in its shoot tissue. Trends for Mn were less clear than those for Al (Table 3.5). The non-transgenic population had higher Mn than the CS population, but did not differ from the other two transgene containing populations. The parents showed no consistent relationship between Mn content and transgene content. The Al-tolerant genotype Altet-4, showed the highest levels of Mn in root/shoot tissue from all entries evaluated.

The concentration of Ca and Mg were generally higher in L versus UL soil. The CS+DcPA1 population had higher concentrations of Mg than the CS, DcPA1, and non-transgenic populations in L and UL soils. Concentrations of K and P in shoot tissue did not differ between L and UL soil or among the genotypes within type of soils. Iron (Fe) had a similar pattern to that of the Al, showing two-fold higher concentration in UL than L conditions, and also a similar ranking of the isogenic populations for the Al concentration in UL soil. In UL soil, the non-transgenic population showed higher concentration of Fe in shoots than the other three transgenic populations. No differences were observed in L soil (Supplementary Tables 3.1 and 3.2).

Discussion

Organic acids play a key role in aluminum tolerance (Kochian et al., 2004), and the role of organic acids in alfalfa was shown by expressing a gene for citrate synthase (Barone et al., 2008). In this experiment, we wanted to confirm the value of citrate synthase for improving Al tolerance in a different, more agronomically desirable genetic background and to determine if tolerance could be improved further by also expressing a citrate transporter gene, as suggested by Barone et al. (2008). Therefore, we developed isogenic T_2 populations in order to compare Al tolerance of the genes singly or in combination with a non-transgenic control. Our hypothesis was that combining the CS gene with a transporter would enable more organic acid to be secreted from the roots, thereby imparting a greater level of Al tolerance than either gene alone could provide.

We confirmed that the CS gene improves Al tolerance as had previously been demonstrated in a different genetic background (Barone et al., 2008). The range of Al tolerance,

measured as RDWR and SDWR, observed in the population having only the CS transgene was higher in this study than that previously reported (Barone et al., 2008). The difference in the magnitude of Al tolerance reported may be because we used a different transgenic construct with a different promoter driving the CS gene and/or because we evaluated completely unrelated germplasm in the two experiments. We also used soil with slightly different characteristics and a modified methodology to evaluate Al tolerance, both of which could also have caused variations between the experiments. Regardless, in both cases, expression of the CS gene imparts Al tolerance in alfalfa.

The DcPA1 transgene had been previously introduced into alfalfa (Shen, 2009), but the effect of the gene on Al tolerance had not been evaluated in alfalfa. In this study, the DcPA1 isogenic T_2 population showed similar Al tolerance as the CS isogenic population and higher RDWR and SDWR than the non-transgenic population (Table 3.4). When we compared the isogenic populations with individual genes to that containing both genes, we found that all three isogenic populations gave the same level of Al tolerance, but no additional tolerance was achieved by expressing both genes in the same plants.

The Al tolerance, measured as the ratio of growth in unlimed vs limed soil, of our transgenic populations was similar to that of the nontransgenic, Al tolerant genotype Altet-4, which we are using as a parent for a genetic mapping population. We evaluated the concentrations of Al in the shoot tissue to get an indication of the mechanism of Al tolerance – either exclusion of Al from the plant or detoxification inside the plant (Kochian et al., 2004). The Al concentration in the transgenic isogenic populations was lower than the nontransgenic isogenic population. Interestingly, the concentration of shoot Al in Altet-4 was similar to the nontransgenic population. This suggests that the transgenic populations are achieving Al

tolerance through the exclusion of Al from the plant, or at least from the shoot. In contrast, Altet-4 appears to be able to accumulate Al and still maintain Al tolerance. This result suggests that detoxification of Al within Altet-4 is a likely mechanism of Al tolerance, as previously described in other plant species (Ma et al., 1998; Ma et al., 2001; Ma et al., 1997).

The Mn concentrations observed in UL soil were similar to those in L soil, where no toxicity effects were observed. Thus, even though some differences among populations and genotypes were observed in UL soil, the magnitude of Mn concentrations suggests that Mn was not a major factor affecting the results.

Conclusion

We have confirmed that two transgenes, *Pseudomonas aeruginosa* citrate synthase (CS) and *Daucus carota* plasma membrane H^+ATPase (DcPA1) improved alfalfa Al/acid soil tolerance either singly or in combination. The Al tolerance conferred by the transgenes was similar to that observed in a non-transgenic Al tolerant genotype, but based on different levels of Al accumulation in shoot tissue, the tolerances may result from different mechanisms. We are now attempting to pyramid the transgenes with quantitative trait loci (QTL) identified in a segregating population derived from Altet-4 to determine if additional Al tolerance can be obtained. Additional research is also needed to determine the stability of these transgenes and their performance under field and commercial conditions.

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Table 3.1. Properties of the soil collected from the UGA Plant Science Farm in Watkinsville, GA and same soil after limed and unlimed treatments.

Type of soil	pH $CaCl_2$ *	Equiv. H_2O pH	Ca	K	Mg	Mn	P	Zn	Exchangeable Al $cmol_c/kg$
			Mehlich 1 mg/kg (ppm)						
Farm soil	4.44	5.04	167.8	59.1	76.4	9.8	4.5	3.3	0.68
Unlimed	4.53	5.13	164.2	134.8	42.0	8.1	15.4	3.6	0.60
Limed	6.01	6.61	448.9	129.8	102.9	10.9	13.6	2.1	0.03

* Soil Testing: Soil pH and salt concentration (http://pubs.caes.uga.edu/caespubs/pubcd/C875/C875.htm)

Table 3.2. Observed and expected frequencies for the absence-presence, and chi-square tests of both T_1 groups (CS and DcPA1), and for the isogenic alfalfa populations.

Class	Number of individuals	Observed Frequency (%)	Expected Frequency (%)	$Pr>X^2$
CS-14×95-608 T1 population				
No-CS	12	52.2	50	0.835
CS	11	47.8	50	
DcPA1-85×60T180-14 T1 population				
No-DcPA1	14	60.9	50	0.297
DcPA1	9	39.1	50	
CS-16×H+-4 T2 population				
CS+DcPA1	13	17.1	25	0.085
CS	14	18.4	25	
DcPA1	26	34.2	25	
None	23	30.3	25	

Table 3.3. *P*-values of the genotype, environment, and G×E effects for root and shoot dry weight (DW), Al and Mn content in limed and unlimed soil, and UL:L ratios.

Trait		Genotype	Environment	G×E
Root dry weight	Limed	0.054	0.045	0.720
	Unlimed	0.036	0.150	0.150
	UL:L Ratio	<.0001	0.490	0.780
Shoot dry weight	Limed	<.0001	0.031	0.079
	Unlimed	0.095	0.064	0.300
	UL:L Ratio	<.0001	0.300	0.450
Aluminum	Limed	0.806	0.722	0.884
	Unlimed	0.021	0.584	0.019
	UL:L Ratio	0.235	0.685	0.647
Manganese	Limed	0.487	0.410	0.316
	Unlimed	0.003	0.865	0.005
	UL:L Ratio	0.050	0.247	0.036

Table 3.4. Least square means of the four isogenic populations (None: non-transgenic; CS: only CS transgene present; DcPA1: only DcPA1 transgene present; CS+DcPA1: both transgenes present), the T_1 parents of the populations (CS-16 and H^+-4), and the genotypes which originated the T_1 parents, the non-transgenic genotype R2336, and the Al-sensitive genotypes 95-608 and 60T180-14, the Al-tolerant genotypes GA-AT and Altet-4, and the Al-sensitive breeding line NECS-141.

	Root DW			Shoot DW		
	mg plant^{-1}		UL:L Ratio§	mg plant^{-1}		UL:L Ratio§
Entry	Limed	Unlimed		Limed	Unlimed	
Isogenic T2 populations						
None	179a‡	115b‡	0.63b‡	877a‡	444bcd‡	0.66b‡
CS	175a	138a	0.83a	601b	504ab	0.91a
DcPA1	163a	139a	0.90a	578b	572a	0.98a
CS+DcPA1	167a	130ab	0.92a	569b	548b	0.95a
Parental and grandparental genotypes						
H+-4	156a	128ab	0.98a	456bc	462abc	1.09a
CS-16	148a	106ab	0.92a	429bc	463abc	1.02a
R2336	182a	122ab	0.60bc	765ab	401bcd	0.56bc
95-608	174a	116ab	0.63b	659b	314d	0.40bc
60T180-14	189a	103ab	0.43c	838ab	555abc	0.69b
Unrelated check genotypes						
GA-AT	148a	102ab	0.62b	550bc	308d	0.54bc
Altet-4	167a	140a	0.85a	794ab	620a	0.74ab
NECS-141	184a	131ab	0.61b	869a	431bcd	0.43c

‡ Means followed by the same letter (within a column) are not significantly different ($P < 0.05$).
§The UL:L ratios were computed for each replication to enable a statistical analysis. Therefore, they will not equal the ratio of UL:L LS means.

Table 3.5. Aluminum and manganese content in the shoot fraction of the four isogenic populations (None, CS, DcPA1, and CS+DcPA1), the parents of the populations (H^+-4 and CS-16), and the eight non-transgenic lines used as checks.

Entry	Aluminum			Manganese		
	Limed	Unlimed	UL:L Ratio§	Limed	Unlimed	UL:L Ratio§
	Isogenic T2 populations					
	ppm			ppm		
None	424.0[a‡]	1549.3[b‡]	3.64[a‡]	54.5[a‡]	64.3[cd‡]	1.36[b‡]
CS	425.3[a]	741.0[c]	1.80[bc]	60.2[a]	46.2[e]	0.87[bc]
DcPA1	452.3[a]	1103.8[c]	2.49[b]	51.1[a]	59.6[de]	1.27[bc]
CS+DcPA1	732.5[a]	865.5[c]	1.72b[c]	75.5[a]	52.3[de]	0.74[c]
	Parental and grandparental genotypes					
H+-4	818.5[a]	1075.1[c]	1.39[c]	89.4[a]	70.3[c]	0.73[c]
CS-16	552.0[a]	997.1[c]	1.71[bc]	83.8[a]	83.7[b]	1.14[bc]
R2336	508.0[a]	1660.6[ab]	3.11[a]	87.1[a]	65.5[c]	0.74[bc]
60T180-14	529.0[a]	1091.6[bc]	2.09[bc]	78.3[a]	87.9[b]	1.11[bc]
95-608	657.0[a]	2146.6[a]	2.93[ab]	77.6[a]	52.0[cde]	0.70[c]
	Unrelated check genotypes					
GA-AT	734.5[a]	1029.6[bc]	1.42[c]	76.7[a]	53.7[cde]	0.65[c]
Altet-4	364.0[a]	1547.6[ab]	3.86[a]	78.1[a]	118.5[a]	1.52[a]
NECS-141	542.0[a]	1164.6[bc]	2.12[bc]	80.0[a]	74.0[b]	0.91[bc]

‡ Means followed by the same letter (within a column) are not significantly different ($P < 0.05$).
§The UL:L ratios were computed for each replication to enable a statistical analysis. Therefore, they will not equal the ratio of UL:L LS means.

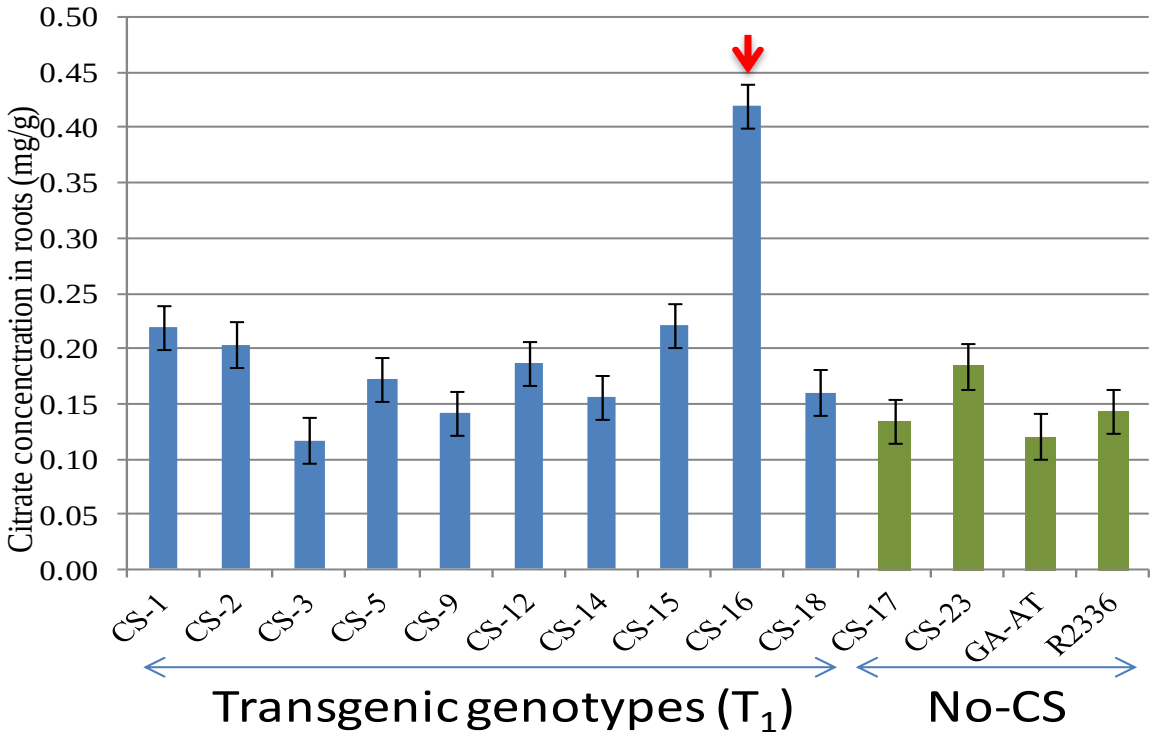

Figure 3.1. Citrate concentration in roots of 10 T_1 plants containing the CS transgene (blue bars), two T_1 plants with no CS gene (green bars), and the GA-AT and R2336 non-transgenic genotypes as checks. The red arrow indicates the genotype used as a parent for the T_2 population.

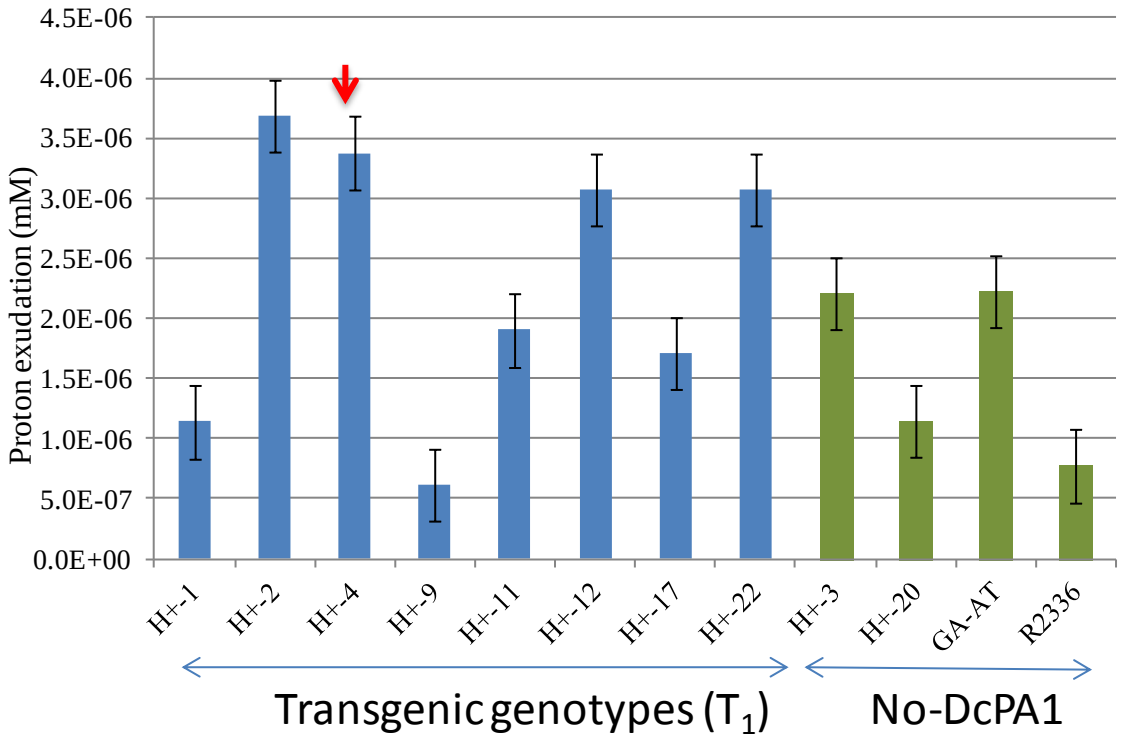

Figure 3.2. Proton exudation by roots of 8 T_1 plants containing the DcPA1 gene (blue bars), two T_1 plants with no DcPA1 gene (green bars), and the GA-AT and R2336 non-transgenic genotypes as checks. The red arrow indicates the genotype used as a parent for the T_2 population.

Supplementary table 3.1. *P*-values of the genotype, environment, and G×E effects for calcium (Ca), magnesium (Mg), potassium (K), phosphorus (P), and iron (Fe) content in limed and unlimed soil.

Nutrient	Type of soil	Genotype	Environment	G×E
Ca	Limed	0.016	0.285	0.157
	Unlimed	0.125	0.287	0.387
Mg	Limed	0.021	0.784	0.287
	Unlimed	0.004	0.514	0.024
K	Limed	0.003	0.523	0.013
	Unlimed	0.039	0.720	0.105
P	Limed	0.074	0.653	0.213
	Unlimed	0.089	0.158	0.435
Fe	Limed	0.291	0.998	0.347
	Unlimed	0.001	0.393	0.001

Supplementary table 3.2. Calcium (Ca), magnesium (Mg), potassium (K), phosphorus (P), and iron (Fe) content (in ppm) in the shoot fraction for the four isogenic populations (None, CS, DcPA1, and CS+DcPA1), the parents of the populations (H^+-4 and CS-16), and the eight non-transgenic lines used as checks measured in limed (L) and unlimed (UL) soil.

	Ca		Mg		K		P		Fe	
	L	UL	L	UL	L	UL	L	UL	L	UL
	Isogenic T2 populations									
	ppm									
None	1.15	1.17	0.39	0.43	2.15	2.57	0.37	0.18	403	1095
CS	1.13	1.04	0.36	0.38	1.92	2.70	0.31	0.19	317	571
DcPA1	1.15	1.15	0.41	0.43	1.95	2.42	0.32	0.17	357	941
CS+DcPA1	1.53	1.15	0.50	0.48	2.10	2.58	0.36	0.18	548	640
	Parental and grandparental genotypes									
H+-4	1.46	1.18	0.49	0.53	2.20	2.51	0.30	0.21	822	870
CS-16	1.06	1.06	0.42	0.44	2.11	2.48	0.34	0.26	444	856
R2336	1.25	1.13	0.47	0.46	2.16	2.57	0.23	0.15	647	1689
60T180-14	1.02	1.12	0.37	0.45	2.17	3.10	0.42	0.23	872	1022
95-608	1.34	0.72	0.37	0.36	1.73	2.65	0.33	0.13	619	2305
	Unrelated check genotypes									
GA-AT	1.20	0.93	0.44	0.42	2.29	2.40	0.24	0.19	660	827
Altet-4	0.69	0.95	0.30	0.44	1.39	2.75	0.26	0.14	313	2043
NECS-141	1.04	1.11	0.39	0.45	2.16	2.40	0.33	0.20	458	1111

CHAPTER 4

SELECTION FOR TOLERANCE TO ACIDIC, ALUMINUM-RICH SOIL IN ALFALFA[3]

[3] Rafael Reyno, Daniel Real, and E. Charles Brummer. To be submitted to *Crop Science*

Abstract

Alfalfa (*Medicago sativa* L.) is an important forage crop in U.S. and worldwide but its productivity is greatly affected in acid soils. In acid soils (pH below 5), aluminum (Al) and manganese (Mn) become toxic, which affects plant growth and development. In the southeastern U.S., Al and Mn toxicity, together with calcium (Ca) and phosphorus (P) deficiencies are the most significant soil factors limiting alfalfa production. Selection in the field is difficult due to heterogeneity of pH, Al concentration, and other microenvironmental variables. The aim of this study was to compare genetic gain for acid/Al tolerance as assessed by seedling biomass production in acidic soil under greenhouse conditions using phenotypic recurrent selection (PRS) and among and within family selection (AWFS) in the cultivar Bulldog 805, and PRS in the germplasm Cultivated Alfalfa at the Diploid Level (CADL) for two cycles. Selection was performed based on aerial biomass production or visual score 60 d after germination in an Al-rich acidic (unlimed or UL) and a limed (L) soil. Cycles 0, 1, and 2 (C0, C1, and C2) from each method and population were evaluated for their root and shoot dry weight (RDW and SDW) after 60 days of growth in UL and L soils using a greenhouse soil-based assay. Relative root and shoot ratios, DW in UL relative to L soil were computed. The diploid CADL cultivar did not respond to selection likely due to the lack of initial variability for Al tolerance. Bulldog 805 selected in L soil did not result in improvement in L or UL conditions, but selection conducted in UL soil successfully improved performance in acid Al-rich soil after two cycles of selection, and did not negatively affect its growth when evaluated in L soil. The PRS method in Bulldog 805 resulted in the largest response per cycle, over 20% per cycle over C0. Direct selection in UL soil was the best way to improve alfalfa growth in acid Al-rich soils.

Introduction

Alfalfa (*Medicago sativa* L.) is the most important forage crop in U.S. and worldwide. Under acidic conditions at a soil pH below 5, aluminum (Al) and manganese (Mn) become toxic affecting plants growth and development; alfalfa is particularly sensitive to low pH and Al toxicity. Aluminum toxicity decreases root elongation and branching, thus reducing water and nutrient uptake (Kochian et al., 2004a). In the southeastern U.S., Al and Mn toxicity, together with calcium (Ca) and phosphorus (P) deficiencies are significant factors limiting alfalfa production (Foy and Brown, 1964b). Usually, soil acidity is addressed by using lime and fertilizers to correct acidity and nutrient deficiencies at the plow layer, but this does not solve the problem at the subsoil layer which remains unamended and thus acid and highly Al-toxic (Bouton and Sumner, 1983b; Foy and Brown, 1964b). Therefore, genetic improvement of alfalfa cultivars with tolerance to acid Al-rich soils offers an alternative to overcome this problem (Bouton, 1996; Bouton et al., 1986).

In the field, aluminum or acid soil tolerance is manifested as higher biomass yield in tolerant relative to sensitive germplasm when grown in acidic and Al-rich soils. Recurrent selection based on field performance has been used to develop a tolerant germplasm, Georgia - Acid Tolerant (GA-AT), derived from U.S. cultivars (Bouton and Radcliffe, 1989). GA-AT was the result of three cycles of selection in acid soil and showed enhanced tap-root growth and nodulation over an unimproved control and aluminum sensitive (AS) germplasm selected for 3 cycles in limed soil, when tested in acid soil with pH 4.6 and 32 μg g^{-1} Al (Bouton and Radcliffe, 1989). The levels of tolerance realized in GA-AT were not high enough to be economically useful at low pH environments, because yields are far below those observed under limed

conditions. No other selection in the field for Al tolerance has been reported, and no Al tolerant cultivar is currently commercialized (Bouton and Radcliffe, 1989; Dall' Agnol et al., 1996).

A number of screening methodologies has been developed for detecting Al tolerance in alfalfa. Selection of seedlings using two greenhouse soil-based assays and on four callus bioassay characteristics for one cycle indicated that selection in completely UL soil was the most effective in terms of success, time, and resources (Dall' Agnol et al., 1996). Selection for acid and Al tolerance in a hydroponic system improved root regrowth after 2 cycles of selection, but the relation to field performance was not evaluated (Scott et al., 2008). Because the Al/acid tolerance alleles appear to be rare in alfalfa populations, a higher selection intensity may be necessary to concentrate useful alleles (Scott et al., 2008). The hydroponic-based selection was validated in alfalfa using populations derived from selection in high Al-solution culture which showed improved root and shoot growth in acid soil (Hayes et al., 2011). However, a lack of correlation among hydroponic systems, root staining evaluations, and acid-soil screenings, suggest the use of soil methods, since they may be the most relevant for field performance, although even that has to be rigorously evaluated (Barone et al., 2008; Narasimhamoorthy et al., 2007b).

Direct selection in the target environment has often been found to be the most effective way to improve populations for stressful environments (Atlin and Frey, 1989; Ceccarelli, 1989). In the greenhouse soil-based experiment, selection for better Al tolerance at low pH conditions did not negatively affect the yield performance in L soil environments in the greenhouse (Bouton and Sumner, 1983b; Dall' Agnol et al., 1996). Selection for shoot growth in acid soil (pH levels below 5) may be more effective than selection for root growth, which is highly variable in acid soils (Simpson et al., 1977). The improvement in Al tolerance is probably limited by the lack of

genetic variability in the alfalfa germplasm (Bouton, 1996), the lack of understanding of the mechanism(s) controlling the trait, and the lack of long term breeding efforts (Dall' Agnol et al., 1996).

Theoretical responses to selection for quantitatively inherited traits like yield show that phenotypic recurrent selection (PRS) can be effective. However, in general, better genetic gain could be achieved using progeny test methods (Fehr, 1993; Hill and Haag, 1974). Phenotypic recurrent selection can be improved by gridding to control environmental variability when doing selection based on single plant performance (Burton, 1982; Missaoui et al., 2005). The amount of improvement realized from each method of evaluation depends on the population, trait of interest, and intensity of selection applied (Celami, 1996; Haag and Hill, 1974; Knapp and Teuber, 1993). Among and within family selection (AWFS) and PRS achieved twice the gain of mass, half-sib, and half-sib progeny test (HSPT) selection when improving ease of floret tripping in the CUF101 alfalfa population (Knapp and Teuber, 1993). The similar performance of AWFS and PRS is not expected (Fehr, 1993). The efficiency of AWFS versus HSPT is a function of the intensities of selection among and within families (Casler, 2008; Casler and Brummer, 2008). An optimum selection strategy would be AWFS based on genotypic values predicted by best linear unbiased predictor (BLUP), since it would simultaneously use information on family and individuals (Resende, 2002). However, when evaluating many families, recording individual information within families could be highly unpractical and time consuming.

In this experiment we attempted to use a simple soil-based assay in the greenhouse to control soil variation to a much greater extent than is possible in the field, but still evaluate large populations while selecting for Al tolerance. Our hypotheses were that using a family based selection method like AWFS would improve our genetic gain for shoot biomass in acid, Al-rich

soil compared to an individual-based method like PRS, and that selecting directly in unlimed soil would be lead to greater improvement in shoot biomass than selecting in limed soil. Therefore, the objective of this experiment was to test these two hypotheses by selecting for shoot dry weight with PRS and AWFS in the tetraploid alfalfa cultivar, Bulldog 805, which is adapted to the acidic subsoils of the southeastern USA. In addition, we also evaluated PRS in unlimed soil in the diploid population, CADL.

Materials and Methods

Base populations

The tetraploid cultivar Bulldog 805 (Bouton et al., 1997), a non-dormant cultivar developed in southern Georgia, and the diploid germplasm CADL, Cultivated Alfalfa at the Diploid Level (Bingham and McCoy, 1979), were used for the selection experiments. About 120 plants per population (Bulldog 805 and CADL) were grown in 750-ml plastic pots filled with Fafard Super-fine Germinating Mix® (Conrad Fafard Inc., Agawam, MA) and were intercrossed to develop half-sib families. For each population, the 80 families producing the most seed were retained.

Soil for selection and evaluation

Selection was performed in the Crop and Soil Sciences Department greenhouses at the University of Georgia, Athens, GA using a Cecil sandy clay loam soil (clayey, kaolinitic, thermic, Typic Kanhapludul) collected from the UGA Plant Sciences Farm near Watkinsville, GA, which is naturally acidic and high in exchangeable Al. Prior to the experiment, this soil was amended with macro and micro nutrients as previously described (Khu et al., 2012) to generate unlimed (UL) and limed (L) soils. After the addition of soil amendments, the soil was air dried

and sieved using a 3-mm screen according to Dall'Agnol et al. (1996). Amendments were mixed into the soil prior to each cycle of selection and prior to the evaluation of selection response, resulting in minor variations in the soil analysis (Table 4.1).

Selection Methodologies and Experimental Design

Two cycles of selection for shoot biomass were performed in Bulldog 805 and CADL using among-and-within family (AWFS) recurrent selection (Casler and Brummer, 2008; Vogel and Pedersen, 1993) and phenotypic recurrent selection with gridding (Bos and Caligari, 1995; Gardner, 1961) (Table 4.2). Cycle 1 was performed during summer 2010 and Cycle 2 during spring 2011.

Phenotypic recurrent selection (PRS): An equal amount of seed from each of the 80 families of Bulldog 805 and of CADL was pooled to form the Cycle 0 populations used for selection. In each cycle of selection, seeds were planted in 128-cell (8 × 16) plastic flats, in which each seedling was grown in a 2.5 cm × 2.5 cm × 5 cm cell tapering to a point. Seedlings were watered every 1 to 2 d using distilled water, and the flats were rotated inside the greenhouse benches at weekly intervals to diminish micro-environmental greenhouse variation. Day and night temperatures were on average 25°C and 20°C, respectively, but during Cycle 1 selection, maximum temperatures during the day went up to 33 to 35°C. Supplemental lights were added to provide 16 hr of light. During the course of the selections one or two applications of chlorpenapyr (0.41 g/l) and avermectin (49.3 g/l) for thrips (*Caliothrips fasciatus* and *Frankliniella occidentalis*) control were required.

In each flat, the outer rows were considered as borders, and thus not included in the evaluation, keeping a core of 84 plants per flat for the evaluation. Six flats containing a total of 1008 plants from Bulldog 805 and from CADL Cycle 0 were placed in each of two greenhouses

(environments) and evaluated after growing for 60 d in UL soil. Shoots for each plant were visually scored from 1 (low vigor) to 5 (highly vigorous). Each flat was then divided into seven grids consisted of 12 plants (6 × 2), and the visual scores of plants within each grid were standardized as follows:

$$\frac{p_{ij} - \overline{p_i}}{s_{Pi}}$$

where p_{ij} is the phenotypic value of plant j in grid i, $\overline{p}_{i.}$ is the mean phenotypic value of all plants in grid i, and s_{pi} is the standard deviation of the plants in grid i. Based on standardized values, truncation selection was imposed to select the most vigorous 80 plants across all flats in both environments. Each environment and flat within environment contributed roughly equal numbers of plants for the next cycle. The selected 80 plants in each cycle were intercrossed using bumble bees (*Bombus impatiens*) to form the seed for the next cycle. Each plant contributed the same amount of seed for the new population, but no control of the paternal contribution to the next generation was made.

Among and within family selection: The AWFS method was performed in Bulldog 805 in both L and UL soil (Table 4.2). General seedling growth methods were the same as described for grid selection above. Border plants were again grown on all sides of flats. Two greenhouse environments were used. Fourteen families were grown per flat, considered as a block in the statistical model. Each half-sib family consisted of six plants growing in a row per replication with four replications per environment. At 60 d after planting, for the families grown in UL soil, individual plants within families were visually scored, and the two most vigorous individuals across replications in each environment (8.3%) within the 20 (25%) superior families were selected for the next cycle. Each environment contributed equal number of plants. For the

families grown in L soil, plants were larger than in UL soil, so shoot biomass was harvested by row at 2.5cm above the soil level, dried at 65ºC for 72 hr, and weighed. The 20 (25%) highest yielding families were selected based on biomass and then the two (8.3%) individuals within these families across replications in each environment with the most visually vigorous regrowth were selected for intercrossing. Thus, eighty plants were selected each cycle and intercrossed in bee cages to produce seed for the next cycle. A linear statistical model was developed including effects of families, environments, genotypes × environments (G×E) interaction, incomplete blocks (nested in replications), and replications (nested in environments). All effects were considered random except families, which were fixed. The visual score or the SDW least square mean for each family was estimated using the MIXED procedure of SAS v. 9.1 (SAS, 2008).

Evaluation and genetic gain estimation

In order to evaluate gain from selection, we first manually recombined about 60 plants from each of the eight populations generated by selection (Table 4.2) and the two base populations (Cycle 0) of Bulldog 805 and CADL to increase seed quantity, to produce seeds under similar environmental conditions and of the same age, and to avoid any heterotic effects present in the Syn-1 generation. From the 60 plants planted per population, between 40 and 50 contributed most of the seeds in each population, and those seed were used for evaluating the cycles and for genetic gain estimations.

Response to selection in each method was evaluated by growing Cycles 0, 1, and 2 from both germplasms in L and UL soil for 65 d during the winter of 2011-2012 in the Crop and Soil Sciences Department greenhouses at the University of Georgia, Athens, GA. Evaluations were performed using conetainers (Stuewe and Sons, Inc., Tangent, OR) as previously described for Al tolerance evaluation in soil (Khu et al., 2012). Each conetainer (3.8 cm diameter × 21 cm

long, with a volume of 164 ml) was filled with 140 g of soil. Individual racks (30 cm wide × 61 cm long × 18 cm high) held 98 (7 × 14) conetainers. The outer cones in each rack were used for border rows, leaving 60 cones (12 × 5) per rack. Half of each rack was filled with L and the other half with UL soil (Table 4.1). In the case of the UL treatment, a top-layer of 2.5 cm of limed soil was added to allow proper germination and initial growth of the seedlings. To prevent waterlogging and manganese toxicity, racks with the conetainers were placed into a plastic tray with drain holes at the bottom that were filled with 15 cm of sterile sand (Khu et al., 2012). In a single rack in each type of soil, two rows (named as replications) of five plants each for Cycle 0, 1, and 2 for a given selection method and a given germplasm were randomized. Seeds were scarified by lightly rubbing with sandpaper prior to planting. Each method of selection was evaluated in three blocks in each of two greenhouses, denoted as environments.

After growing for 65 d, plants were removed from the conetainers and roots gently washed to remove excess soil. Plants were separated into root and stem fractions at the soil line, and both fractions placed in a forced-air dryer at 65ºC for 72 hr to determine the root and shoot dry weight (RDW and SDW) for each method in each type of soil for each individual plant. Plant dry weights were summed for each row, and the root and shoot weight ratio (RDWR and SDWR) on an entry basis was determined as the ratio of root or shoot dry weight in UL soil compared to the dry weight in L soil. Therefore, the traits analyzed include SDW, which was basically the trait used for selection, and RDW in both UL and L soil, and RDWR and SDWR, representing the relative growth in UL vs. L soil.

Data analysis

The data for each trait in each method and cycle were tested for normality using the UNIVARIATE procedure of SAS v. 9.2 (SAS, 2008) with the QQPlot statement and the Normal

option. Because RDW and SDW were not normally distributed, they were transformed using a square-root transformation. The data were fit to a mixed linear model consisting of the fixed effects of cycle, environments, and cycle × environments (G×E) interaction, and the random effects of replications (nested in blocks) and blocks (nested in environments). The environment effect represents the greenhouse conditions under which this experiment was conducted and thus, was considered as a fixed effect. The degrees of freedom for cycle were partitioned into two orthogonal contrasts to test for linear and quadratic effects. A second all-random effects model was subsequently used to estimate the cycle, cycle by environment (G×E) interaction, and the residual error variance components. The broad sense heritability (H) for traits in each method and cycle were calculated using the following equation:

$$H = \frac{\sigma^2_{cycles}}{\left[\sigma^2_{cycles} + \frac{\sigma^2_{gxe}}{e} + \frac{\sigma^2_{residual}}{re}\right]}$$

where σ^2_{cycles} is the variation due to cycles of selection, $\sigma^2_{g\times e}$ is the variation due to the two-way G×E interaction, $\sigma^2_{residual}$ is error variation, *e* refers to the number of environments, and *r* is the number of replications within environments.

Multivariate analysis of variance was conducted in an all-random model (MANOVA option from the GLM procedure of SAS) to estimate phenotypic correlations based on mean values within environments and to obtain the variance-covariance matrix to enable computation of genetic correlations as follows:

$$r_{G12} = \frac{Cov_{1-2}}{\sqrt{Var_1 * Var_2}}$$

where $r_{g\ x\text{-}y}$ represents the genetic correlation between traits x and y, $Cov_{x\text{-}y}$ is the covariance of traits x and y, Var_x is the variance associated with trait x, and Var_y is the variance associated with trait y.

Although our goal was to improve biomass production in unlimed soil, we also conducted selection for biomass production in limed soil. This enabled us to test the efficiency of selecting for SDW in L soil vs. UL soil when the ultimate goal is SDW in UL soil. We computed efficiency (the ratio of correlated response from indirect selection to direct response) using the following equation:

$$\frac{R_L^C}{R_{UL}} = \frac{r_G h_L}{h_{UL}}$$

where r_G represents the genetic correlation of SDW measured in L and UL soils, h_L is the square-root of the heritability of SDW in L soil, and h_{UL} is the square-root of the heritability of SDW in UL soil. The heritabilities were computed as described above, and are therefore broad sense heritabilities rather than the typically used narrow sense values. For all statistical analyses, we assessed significance at the 5% probability level unless otherwise indicated.

Results

We evaluated progress from selection by measuring shoot dry weight on original and selected populations. For practical purposes, plants were visually evaluated during selection in UL soil, simply because plants were very small and not large enough to enable both harvest and regrowth for subsequent selection. Therefore, in UL soil, we actually indirectly selected for shoot dry weight using visual vigor scores.

The Bulldog 805 population selected based on shoot dry weight by AWFS in L soil did not show any progress in L soil or in UL soil (Table 4.3). Of the populations selected based on

visual vigor in UL soil and evaluated in UL soil, both AWFS and PRS in Bulldog 805 resulted in a linear increase in shoot dry weight across cycles (Table 4.3). In L soil, the AWFS population did not show any response, but the PRS population showed a positive quadratic response over cycles. Phenotypic recurrent selection in CADL was not effective at improving performance in UL soil but did result in a small linear increase in L soil (Table 4.3). Broad sense heritabilities computed based on the evaluation experiment were generally small, as would be expected based on the selection response results.

The Bulldog 805 populations AWFS-UL and PRS-UL, which showed a positive selection response for SDW, also showed a correlated response for increased RDW in both soils (Table 4.4). Both root and shoot dry weight ratios (UL/L soil) improved linearly in both UL and L soil for these populations (Table 4.5).

Phenotypic and genetic correlations were in general lower between SDW assessed in L vs UL soil than for RDW and SDW assessed within soil type (Table 4.6). In general, the correlated response to selection for SDW was lower than directly selecting for SDW within a particular soil (Table 4.6).

Discussion

Selection has successfully improved alfalfa grown in acidic Al-rich soils or solutions (Bouton and Sumner, 1983b; Dall' Agnol et al., 1996; Hayes et al., 2011; Scott et al., 2008; Zhang et al., 2007). Because selecting alfalfa for improved Al and/or low pH tolerance is generally difficult in the field due to microenvironmental heterogeneity of Al concentration and pH, controlled environment methodology should improve our ability to develop Al tolerant cultivars. In this experiment, we selected for increased shoot growth directly in unlimed soil in

the greenhouse, using AWFS and PRS. Selection in Bulldog 805, selected in south Georgia in soils which naturally have acidic subsoils with high Al, was effective at improving shoot biomass by 8 to 20% per cycle (Table 4.3). A 22% gain in shoot dry weight was reported in a previous study, in which one cycle of selection was done in unlimed, unfertilized soil using a different germplasm than we used in this experiment (Dall' Agnol et al., 1996). In our experiment, phenotypic recurrent selection with gridding was more effective than AWFS. The diploid germplasm, CADL, did not respond to PRS, perhaps because of limited variability for Al tolerance, a problem noted previously in most alfalfa germplasm (Bouton, 1996).

In Bulldog 805, we also attempted to select indirectly for shoot growth in UL soil by conducting selection in L soil. Our hypothesis was that selection for general plant vigor and growth may translate into more vigorous plants in either soil. This was not the case. Our results conclusively show that in order to improve growth in UL soil, selection needs to be made in UL soil, at least in Bulldog 805.

In a previous study, it was observed that selection for root and shoot growth in UL, unfertilized soil was the most effective way to achieve acid soil stress tolerance, and that selection for acid soil did not negatively affect root and shoot yields in L and fertilized soil (Dall' Agnol et al., 1996). In our experiment, selection for shoot growth actually improved root growth as well, and also improved the ratio of root and shoot growth in UL soil relative to L soil. Thus, our results suggest that selecting for shoot growth in UL soil will improve overall plant performance in UL soil, and in some cases in L soil as well. Selection for shoot growth in acid soil may be more effective than selection for root growth, which is highly variable in acid soils (Simpson et al., 1977), and our experiment supports that contention.

The lack of improvement observed in AWFS-L when evaluated in L soil, may be due to the fact that under L conditions, most plants performed well, making identification of superior families or individuals within family at the seedling stage difficult. When the plants were grown in UL soil, most plants were greatly affected by the stressful conditions, making identification of superior individuals easier.

Based on theoretical expectations, a higher genetic gain for yield, typically a low heritability quantitative trait, would be expected using family test methods (Fehr, 1993; Hill and Haag, 1974). The use of gridding in a phenotypic recurrent selection program (Bos and Caligari, 1995; Gardner, 1961) can effectively control environmental variability when selection is done on single plant performance, thus resulting in improved populations (Burton, 1982; Missaoui et al., 2005). Selection performed in the CUF101 alfalfa population to improve ease of floret tripping, showed that AWFS and phenotypic recurrent selection achieved twice the gain of mass, half-sib, and half-sib progeny test selection (Knapp and Teuber, 1993). Our results showed that PRS in UL soil showed higher responses on cycle basis than the AWFS-UL. Apparently, under the conditions of this experiment, gridding was efficiently able to reduce environmental variance to a point where PRS was more effective than AWFS for acid Al-rich soil.

Some studies have concluded that direct selection in the target environment was the most effective way to improve populations for stressful environments (Atlin and Frey, 1989; Ceccarelli, 1989). In our study, selection directly in the unlimed soil resulted in better performance for UL conditions than selection in limed soil (Table 4.6). This result agrees with a previous study using a greenhouse soil-based experiment, which concluded that selection in UL soil was the most effective way for achieving acid/Al tolerance in alfalfa (Dall' Agnol et al., 1996). Therefore, selection to improve alfalfa yields in acid Al-rich soils should be conducted

directly in the target soil and using refined selection techniques in order to reduce the environmental variance as much as possible.

Conclusions and Future Implications

The results we found in this experiment need to be validated in the field. If selection in UL soil in the greenhouse can improve field tolerance to Al, then additional cycles of selection should be undertaken. The progress we noted was quite good, but whether greenhouse selection of seedling biomass translates into adult plant biomass and Al tolerance also needs to be tested. We did not study physiological aspects of acid/Al tolerance in this investigation. However, other experiments we have conducted have identified molecular markers associated with acid/Al tolerance and have documented Al tolerance from different transgenes (Chapters 2 and 3). Thus, combining the breeding methods with marker-assisted recurrent selection could accelerate genetic gain for this trait. Breeding programs could be developed to select families based on their yield and individuals within families based on molecular markers (Casler, 2012). Further research on all these avenues is needed.

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Table 4.1. Soil properties of soil collected from the UGA Plant Sciences Farm near Watkinsville, GA after adding soil amendments to limed and unlimed treatments.

Cycle / Type of soil	pH $CaCl_2$ *	Equiv. H_2O pH	Ca	K	Mg	Mn	P	Zn	Exchangeable Al $cmol_c/kg$
			Mehlich 1 mg/kg (ppm)						
C1†-Limed	6.8	7.4	576.3	169.0	111.7	1.1	27.6	2.2	0.02
C1-Unlimed	4.7	5.3	151.5	161.4	22.5	2.3	23.9	2.9	0.48
C2-Limed	6.3	6.8	711.4	339.9	206.7	4.6	43.7	2.7	0.05
C2-Unlimed	4.5	5.1	186.2	225.4	36.5	4.6	38.3	2.3	0.63
Comparsion-Limed	6.0	6.6	448.9	129.8	102.9	10.9	13.6	2.1	0.03
Comparsion-Unlimed	4.5	5.1	164.2	134.7	42.0	8.1	15.4	3.6	0.60

* Soil Testing: Soil pH and salt concentration http://pubs.caes.uga.edu/caespubs/pubcd/C875/C875.htm)
† C1: Cycle 1; C2: Cycle 2.

Table 4.2. Selection scheme for each method and cycle.

Cycle	Cultivar†	Selection method‡	Soil used for selection	Traits§	Families tested	Plants tested	Selection intensity#	Plants selected and recombined
					No.	No.	%	No.
1	BG805	AWFS	Limed	DW	80	3840	25.0 - 8.3	80
1	BG805	AWFS	Unlimed	VS	80	3840	25.0 - 8.3	80
1	BG805	Grid	Unlimed	VS	NA¶	1008	8.0	80
1	CADL	Grid	Unlimed	VS	NA	1008	3.7	37
2	BG805	AWFS	Limed	DW	80	3840	25.0 - 8.3	80
2	BG805	AWFS	Unlimed	VS	80	3840	25.0 - 8.3	80
2	BG805	Grid	Unlimed	VS	NA	1008	8.0	80
2	CADL	Grid	Unlimed	VS	NA	1008	8.0	80

† BG805=Bulldog 805; CADL=Cultivated alfalfa at the diploid level.
‡ AWFS=among-and-within family selection; Grid=phenotypic recurrent selection with gridding.
§ DW=dry weight; V=visual score of 1=low vigor to 5=highly vigorous.
¶ Not applicable.
# For AWFS method, the first number shows among-family and the second corresponds to within-family intensity of selection.

Table 4.3. Least square means for shoot dry weight for each cycle of selection, the linear response to selection, and the broad sense heritabilities for several methods of selection in two populations evaluated in two different soil conditions.

	Unlimed soil				Limed soil			
	Bulldog 805			CADL	Bulldog 805			CADL
	AWFS-L†	AWFS-UL	Grid-UL	Grid-UL	AWFS-L	AWFS-UL	Grid-UL	Grid-UL
	mg plant^{-1}							
Cycle 0	41.6	41.6	41.6	30.5	64.5	64.5	64.5	40.3
Cycle 1	38.4	45.1	46.0	34.3	55.5	57.8	57.3	41.7
Cycle 2	42.0	48.3	58.5	31.3	59.6	70.9	69.5	42.0
Linear Response‡	0.2^{ns}	3.3*	8.5*	0.4^{ns}	-2.1^{ns}	1.2^{ns}	1.6^{ns}	0.5*
Quadratic Response	ns	ns	ns	Ns	ns	ns	*	ns
Percentage of C0§	0.5	8.0	20.3	0.6	-3.0	1.7	2.3	1.1
H	0.11	0.28	0.36	0.03	0.26	0.12	0.23	0.12

†AWFS=among and within family selection; Grid=phenotypic recurrent selection with gridding; L=selection in limed soil; UL=selection in unlimed soil.
‡Response per cycle as mg plant^{-1} cycle^{-1}.
§Linear response per cycle as the percentage of C0.
*Linear response across cycles is significant at the 5% probability level.
ns=not significant.

Table 4.4. Least square means for root dry weight for each cycle of selection and the linear response to selection for several methods of selection in two populations evaluated in two soils.

	Unlimed soil				Limed soil			
	Bulldog 805			CADL	Bulldog 805			CADL
	AWFS-L†	AWFS-UL	Grid-UL	Grid-UL	AWFS-L	AWFS-UL	Grid-UL	Grid-UL
	mg plant^{-1}							
Cycle 0	42.9	42.9	42.9	21.6	74.5	74.5	74.5	32.1
Cycle 1	42.8	43.3	47.0	21.4	66.9	69.7	66.5	35.8
Cycle 2	51.0	60.0	65.9	24.8	71.2	103.2	102.1	36.6
Linear Response‡	6.0^{ns}	10.1*	11.5*	2.2^{ns}	-3.6^{ns}	11.4^{ns}	10.9*	1.8^{ns}
Quadratic Response	ns	ns	ns	ns	ns	*	*	ns

†AWFS=among and within family selection; Grid=phenotypic recurrent selection with gridding; L=selection in limed soil; UL=selection in unlimed soil.
‡Response per cycle as mg plant^{-1} cycle^{-1}.
*Linear response across cycles is significant at the 5% probability level.
ns=not significant.

Table 4.5. Least square means for root and shoot DW ratios (UL/L soil) for each cycle of selection and the linear response to selection for several methods of selection in two populations evaluated in two soils.

	Root DW Ratio (DW in UL/L soil) §				Shoot DW Ratio (DW in UL/L soil) §			
	Bulldog 805			CADL	Bulldog 805			CADL
	AWFS-L	AWFS-UL	Grid-UL	Grid-UL	AWFS-L	AWFS-UL	Grid-UL	Grid-UL
Cycle 0	$0.55^{b‡}$	$0.55^{b‡}$	$0.55^{b‡}$	$0.64^{a‡}$	$0.65^{b‡}$	$0.65^{b‡}$	$0.65^{b‡}$	$0.78^{a‡}$
Cycle 1	0.72^{a}	0.64^{b}	0.69^{b}	0.65^{a}	0.85^{a}	0.73^{b}	0.86^{a}	0.86^{a}
Cycle 2	0.81^{a}	0.77^{a}	0.78^{a}	0.79^{a}	0.88^{a}	0.99^{a}	0.92^{a}	0.80^{a}
Linear Response‡	0.13^{ns}	0.11*	0.12*	0.07^{ns}	0.11^{ns}	0.14**	0.13*	0.01^{ns}
Quadratic Response	ns	ns	ns	ns	ns	ns	ns	ns

†AWFS=among and within family selection; Grid=phenotypic recurrent selection with gridding; L=selection in limed soil; UL=selection in unlimed soil.
‡Linear response per cycle as mg plant^{-1} cycle^{-1}.
*, ** Linear response across cycles is significant at the 5% and 1% probability level respectively. ns=not significant.
§The UL:L ratios were computed for each replication to enable a statistical analysis.

Table 4.6. Phenotypic (r_P) and genetic (r_G) correlations, and ratio of correlated response of selection in L soil to direct response in UL soil in AWFS-UL and Grid-UL for Bulldog 805 population.

	AWFS-UL			Grid-UL		
	r_P†	r_G‡	R^{C}§	r_P†	r_G‡	R^{C}§
SDW-L vs. SDW-UL	0.09^{ns}	0.13	0.08	0.01^{ns}	0.41	0.33
RDW-L vs. SDW-L	0.66***	0.97		0.75***	0.98	
RDW-UL vs. SDW-UL	0.72***	0.75		0.75***	0.98	
RDWR vs. SDWR	0.51**	0.75		0.73***	0.99	

† Phenotypic correlations.

‡ Genetic correlations.

* Significant at $P \leq 0.05$, ** Significant at $P \leq 0.01$, *** Significant at $P \leq 0.001$.

§ Ratio of correlated response of selection in L soil to direct response in UL soil.

CHAPTER 5

CONCLUSIONS

Alfalfa, *Medicago sativa* L., is one of the most important forage legumes worldwide. However, in acidic soils the productivity and persistence of alfalfa decreases markedly due to Al^{+3} and H^{+} toxicity and essential nutrients deficiencies that inhibit root growth and development. Low pH and Al toxicity can be ameliorated by liming the soil, but acidic subsoil layers will persist. Therefore, plant breeding to develop cultivars tolerant of low pH and Al would be valuable. Genomics technologies can help dissect the genetic basis of acid/Al tolerance, and can augment plant breeding programs to achieve tolerance in an efficient and durable manner.

The first objective of this research was to identify sources of acid/Al tolerance in tetraploid alfalfa through genetic mapping of quantitative trait loci (QTL). The tetraploid aluminum tolerant genotype Altet-4, derived from a source of tolerance identified in diploid *M. sativa* subsp. *caerulea*, was crossed to NECS-141, a semi-dormant breeding genotype, to produce a mapping population segregating for Al tolerance. Linkage maps were constructed using single-sequence repeat (SSR) molecular markers, generating a composite map length of 840 cM for Altet4 and 749 cM for NECS-141. Single-factor analysis and interval mapping identified QTL for root and shoot weight or root and shoot weight ratio between unlimed and limed soil on Altet-4 LGs 1, 2, 5, 6, and 7, and on NECS-141 LG 5. Our results suggest that two of the three QTL previously detected for Al tolerance at the diploid level were also present in this mapping population, together with new QTL identified from the Al-tolerant and from the Al-

sensitive parent. Individual QTL explained between 8.0 and 35.3% of the phenotypic variation. Some of these QTL were detected in multiple greenhouse environments and for multiple traits while others were environment-specific, suggesting that multiple mechanisms of Al tolerance exist in alfalfa.

The second goal was to assess the acid/Al tolerance of transgenic alfalfa plants over-expressing *Pseudomonas aeruginosa* citrate synthase (CS) and a plasma membrane H^+-ATPase from *Daucus carota* (DcPA1). Because membrane transporters are thought to enhance organic acid transportation and exudation, we hypothesized that combining both genes into a common background would increase Al tolerance over either gene individually. We generated a full-sib population with individual genotypes being allocated to one of four isogenic T_2 populations: (1) no transgene, (2) CS only, (3) DcPA1 only, or (4) both transgenes. Based on the segregation ratios of T_1 and T_2 generations and Southern blot analysis, both transgenes are likely present as a single copy.

The evaluation of these populations was done using rooted stem cuttings growing in unlimed and limed soil in the greenhouse. All three transgenic populations (CS, DcPA1, and CS+DcPA1) showed higher Al/acid soil tolerance measured as the ratios of root and shoot dry weight in UL vs. L soil than the isogenic non-transgenic population. However, we did not observe an advantage of combining both transgenes compared to the single transgenes. Levels of tolerance achieved by the transgenic populations were high, with UL:L ratios near unity (0.91 to 0.98), showing the potential of a transgenic approach. Lower levels of Al in shoot tissue were observed for the transgenic populations over the non-transgenic one, suggesting that the Al-exclusion mechanism could be driving Al/acid soil tolerance in this study. Interestingly, the level of tolerance we observed by transgenesis was equal to that observed in the non-transgenic

genotype Altet-4, but Altet-4 had high levels of Al in its shoot tissue. This suggests that combining both transgenes and QTL into a common background may lead to superior Al tolerance.

Finally, the third objective of this study was to compare genetic gain for acid/Al tolerance as measured by seedling biomass production in acidic soil under greenhouse conditions from phenotypic recurrent selection with gridding (PRS) and among and within family selection (AWFS) in the tetraploid cultivar Bulldog 805 and the diploid germplasm CADL (Cultivated Alfalfa at the Diploid Level). Two cycles of selection were performed either based on shoot dry weight or a visual score of shoot vigor 60 d after germination. Cycles 0, 1, and 2 (C0, C1, and C2) from each method and population were evaluated for their root and shoot dry weight (RDW and SDW) after 60 d of growth in UL and L soils using a greenhouse soil-based assay. The diploid CADL cultivar did not respond to selection likely due to a lack of variability. Bulldog 805 selected for aerial biomass by either method in UL soil had higher shoot biomass when grown UL soil after two cycles of selection. Interestingly, its performance in L soil was not negatively affected. The results also suggest that direct selection in UL soil was the most efficient method to improve alfalfa's performance in acid Al-rich soils. Under the conditions of this experiment, the PRS method in UL soil was the most effective in terms of the resources used and the responses achieved.

The transgenic approach offers a fast, efficient way to achieve enhanced acid/Al tolerant alfalfa cultivars, but more information is needed especially related to their performance under field and commercial conditions. However, the difficulty and costly regulatory process make this approach less affordable for small seed companies or public breeding programs, and may preclude commercialization altogether. The identification of molecular markers associated with

acid/Al tolerance in this study will provide the opportunity to introduce marker-assisted recurrent selection to the breeding programs. This genomic information together with the development of quick and easy selection methodology to assess the current existing variability in tetraploid germplasm will allow us to speed up the progress to integrate acid/Al tolerance in alfalfa.