RESEARCH

Evaluation of Two Transgenes for Aluminum Tolerance in Alfalfa

Rafael Reyno, Dong-Man Khu, Maria J. Monteros, Joseph H. Bouton, Wayne Parrott, and E. Charles Brummer*

ABSTRACT

Alfalfa (*Medicago sativa* L.) production is dramatically reduced in acidic, Al-rich soil. Transgenic plants of several species overexpressing 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 *Daucus carota* L. plasma membrane H^+-transporting adenosine triphosphatase ($H^+ATPase$) (DcPA1) transgenes on Al tolerance in alfalfa. Stem cuttings from a full-sib T_2 population including four isogenic groups (plants with neither transgene, CS only, DcPA1 only, and both transgenes) together with nontransformed check genotypes were evaluated for Al and acid soil tolerance in a greenhouse assay in limed and unlimed soil. Aluminum and acid soil tolerance was assessed by measuring the ratios of root and shoot dry weight in unlimed soil compared to limed soil. The three transgenic populations, CS, DcPA1, and CS plus DcPA1, all showed higher Al and acid soil tolerance and lower levels of Al in shoot tissue than the nontransgenic isogenic population or the nontransgenic parental genotypes. This suggests that an Al-exclusion mechanism could be driving Al and acid soil tolerance in this study. We observed no advantage of combining both transgenes in the same genetic background. These transgenes offer an efficient method to achieve enhanced Al and acid soil tolerant alfalfa cultivars, but more information is needed on their stability across generations and genetic backgrounds and their performance under field conditions.

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Abbreviations: CS, citrate synthase; DcPA1, *Daucus carota* plasma membrane H^+-transporting adenosine triphosphatase; DW, dry weight; G×E, genotype × environment; $H^+ATPase$, H^+-transporting adenosine triphosphatase; ICP, inductively coupled plasma; PCR, polymerase chain reaction; QTL, quantitative trait loci; RDW-L, root dry weight in limed soil; RDWR, root dry weight ratio; SDWR, shoot dry weight ratio; SDW-UL, shoot dry weight in unlimed soil.

Alfalfa is widely cultivated throughout the world and is the fourth most important crop in U.S. agriculture (USDA-NASS, 2010). Acid soils are a severe problem for alfalfa production in many parts of the world, including the southeastern United States (Bouton and Sumner, 1983). Acid-soil syndrome, which mainly compromises Al^{+3} and H^+ toxicity combined with deficiency of essential nutrients such as P, causes severe inhibition of root growth and development (Kochian et al., 2004). Two main mechanisms of Al tolerance have been described in higher plants—those that prevent Al uptake by the roots through the exudation of organic acids 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., 2004). Both mechanisms are complex and hard to dissect experimentally

Published in Crop Sci. 53:1–8 (2013).
doi: 10.2135/cropsci2012.12.0676
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(Kochian et al., 2005). Based on research in many species, Al 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., 2007; 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, 1983). Genetic tolerance to acidic conditions would be a more durable and less expensive method to increase alfalfa productivity and reduce production costs (Bouton, 1996; Bouton et al., 1986), but alfalfa germplasm has limited variability for Al tolerance (Bouton, 1996; Campbell et al., 1988). An additional method to modify Al tolerance is the use of transgenes or using transgenic methods to modulate expression of existing genes to a level not available in existing germplasm.

The Al-chelating ability of some organic acids, including $C_6H_8O_7$ (citric), $HO_2CCH_2CHOHCO_2H$ (malic), and $H_2C_2O_4$ (oxalic), appears to confer Al tolerance through the formation of stable complexes with Al^{+3} in the soil, making Al insoluble and preventing its uptake by the roots (Barone et al., 2008; Delhaize et al., 1993). Overexpressed transgenes for organic acid synthesis, such as citrate synthase (CS), have provided improved Al tolerance in several crops, including tobacco (*Nicotiana tabacum* L.), papaya (*Carica papaya* L.) (de la Fuente et al., 1997), canola (*Brassica napus* L.) (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., 2004). This approach has been effective in barley (*Hordeum vulgare* L.), an Al-sensitive cereal crop (Delhaize et al., 2004, 2009). The *Daucus carota* plasma membrane H^+-transporting adenosine triphosphatase (H^+ATPase) (DcPA1) gene may play a role in proton exudation (Ohno et al., 2004) and can increase Al tolerance in certain plants (Delhaize et al., 2009; Hoekenga et al., 2006; Ryan et al., 2001).

The practical value of the transgenes may not be manifested in plants that only contain a single gene. The optimum Al tolerance may arise from a strategy that combines organic acid production 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 previously developed (Shen, 2009). Under the control of the constitutive MtHP promoter from *Medicago truncatula* Gaertn. (Xiao et al., 2005), both genes showed high expression levels in alfalfa plants, but the plants containing the genes were not evaluated for Al tolerance (Shen, 2009).

We hypothesized that the CS and DcPA1 genes individually would confer improved Al tolerance to alfalfa compared with untransformed control genotypes but 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 CS and the DcPA1 genes were previously transformed into the Forage Genetics International proprietary alfalfa genotype R2336 under the control of three promoters (Shen, 2009). R2336, like most alfalfa genotypes, is noninbred. The transgenic (T_0) genotypes, CS-14 and DcPA1-85, with genes driven by the constitutive promoter MtHP, had the highest expression levels of their respective transgene (Shen, 2009) and therefore 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 genotype (R2336, which is self-incompatible), hybridization between them, even if possible, would result in progeny with significant inbreeding depression, potentially obscuring the phenotypes caused by the transgenes. Therefore, each T_0 plant was hybridized to an unrelated nontransgenic genotype as follows: CS-14 × 95-608 and DcPA1-85 × 60T180-14. Previous evaluations have identified the 95-608 (Khu et al., 2012a) and 60T180-14 (data not shown) genotypes as Al sensitive. The 95-608 genotype was derived from the nondormant synthetic cultivar CUF101 (Lehman et al., 1983) while 60T180-14 was selected under grazing and drought conditions in Tifton, GA (Dr. J.H. Bouton, unpublished data, 2007) from an Italian germplasm obtained from Forage Genetics International.

Twenty-five T_1 progeny from each cross were evaluated by polymerase chain reaction (PCR) (see below) for the presence or absence of the appropriate transgene. One highly expressing plant (see below for quantification methodology) from each cross was selected for hybridization. The genotype CS-16 was selected from the CS-14 × 95-608 cross and the genotype H^+-4 from the DcPA1-85 × 60T180-14 cross. These two genotypes, CS-16 and H^+-4, were reciprocally hybridized, emasculating the female parent, to generate a full-sib near-isogenic T_2 population consisting of four groups: (i) no transgene, (ii) CS only, (iii) DcPA1 only, or (iv) 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.

Polymerase Chain Reaction Screening of Putative Transgenic Plants

Genomic DNA extractions from T_0, T_1, and T_2 genotypes were done using a modified cetyltrimethyl ammonium bromide

Table 1. Properties of the soil collected from the University of Georgia Plant Science Farm in Watkinsville, GA, and same soil after modification for the limed and unlimed treatments used in this experiment.

Type of soil	pH $CaCl_2$†	Equiv. H_2O pH	Ca	K	Mg	Mn	P	Zn	Exchangeable Al
			Mehlich 1 (mg kg^{-1})						$cmol_c\ kg^{-1}$
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 (Kissel and Vendrell, 2012).

protocol (Murray and Thompson, 1980). About 50 ng of DNA from each alfalfa plant was used for PCR amplification reactions using primers CS-463F (5′-CCGAAGCATCGCGAAGTCTC-3′) and CS-1012R (5′-CCAGTTGCGGGTCGTTGATG-3′) or DcPA1-1342F 5′-ATTGATAAGTTTGCAGAGCGTGGGTT-GAG-3′) and DcPA1-1977R (5′-AAACACAATACGGATC-GTGATGGATACTGC-3′) as described by Shen (2009). The positive controls were plasmid DNA (about 1 ng) containing the corresponding expression cassettes and genomic DNA isolated from the wild-type R2336 alfalfa genotype as the negative control. Amplicons from the PCR were analyzed on a 1% agarose gel containing ethidium bromide and visualized under ultraviolet light. The segregation ratios for the T_1 and T_2 individuals were tested for deviation from expectations using the FREQ procedure of SAS version 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 Superfine Germinating Mix (Conrad Fafard Inc.) under greenhouse conditions. For citrate production, three replicate stem cuttings of 10 transgenic individuals originating from CS-14 × 95-608 containing the CS transgene together with two nontransgenic siblings, the nontransformed alfalfa genotype R2336, and a single genotype labeled as GA-AT, derived from a population with enhanced Al tolerance (Bouton and Radcliffe, 1989), were evaluated as described by Shen (2009). Briefly, 0.2 g of fresh active growing roots were harvested, ground in liquid N_2 with a mortar and pestle, and transferred to a 1.5 mL Eppendorf tube with 1 mL of ice-cold 0.6 M $HClO_4$ (perchloric acid) neutralized with 170 to 200 µL of 5 M K_2CO_3. The citrate acid quantification kit (RBiopharm Inc.) was used for the spectrographic assay of citrate, and the absorbance was measured using the Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc.).

For proton exudation, three replicate stem cuttings of eight transgenic and two nontransgenic individuals from the DcPA1-85 × 60T180-14 progeny, the nontransformed 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.) centrifuge tubes containing 20 mL of 0.5 mM $CaCl_2$ (pH 5.8) for 24 h in a growth chamber (14 h and 26°C day and 10 h and 22°C night regimen). Changes in the pH of the solution were measured using a Corning pH meter 240 (Corning Incorporated). The H^+ concentration in the solution was calculated using 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 version 9.2 (SAS, 2008) was used to evaluate the general statistics in each trait and to test for normal distribution using the QQPlot statement and the normal option. A generalized linear model was used to calculate the means for each genotype and to compute a mean comparison based on the 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 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 blot. Briefly, approximately 10 µg of purified genomic DNA were digested overnight at 37°C with 12 units of *Eco*RI (Promega) and the fractions separated in a 0.8% agarose gel with 1x Tris-acetate-ethylenediaminetetraacetic acid buffer at 30 V and 35 mA. After soaking the agarose gel in denaturation and neutralization buffers, DNA was transferred to a Hybond-N+ membrane (Amersham). The probes used in each case were an amplified 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 the primer pairs hph-117F (5′-CGATGTAGGAGGGCGTG-GATA-3′) and hph-938R (5′-CTTCTGCGGGCGATTT-GTG- 3′). Finally, after prehybridization and hybridization steps, the hybridized membrane was exposed to a Kodak BioMax Film at −80°C for 7 d.

Phenotypic Analysis of Aluminum Tolerance

Evaluation of soil-based Al tolerance of the T_2 isogenic populations was during November and December 2011, using two greenhouses as separate environments. We used the whole-plant assay in soil system (Khu et al., 2012a) modified to prevent waterlogging. A poorly aerated soil increases the toxic forms of Mn in the soil solution (Hue, 1988). Therefore, the modification consisted of placing racks with conetainers (Stuewe and Sons, Inc.) 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 prevents soil saturation after watering (Dr. M.E. Sumner, personal communication, 2009), thereby preventing Mn toxicity. The soil used, collected from the University of Georgia Plant Science Farm in Watkinsville, GA, had acidic pH and Al-toxic potential and was identical to that in Khu et al. (2012a), except that nutrients and lime were mixed immediately before these experiments (Table 1).

Rooted stem cuttings were used as previously described (Khu et al., 2012a). Briefly, conetainers were filled with either limed or unlimed soil leaving a space at the top for a 6-wk-old

cutting of a given genotype, which was transferred directly without washing off the Germinating 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 averaged 25°C day and 20°C night with a 16 h photoperiod supplemented by high-intensity lights. The plants were watered daily using distilled water, and the trays were rotated on the greenhouse benches weekly to diminish microenvironmental variation inside the greenhouse. Chlorfenapyr (4-bromo-2-(4-chlorophenyl)-1-ethoxymethyl-5-trifluoromethyl-1*H*-pyrrole-3-carbonitrile) (0.41 g L^{-1}) and avermectin (49.3 g L^{-1}) were applied to control thrips (*Caliothrips fasciatus* and *Frankliniella occidentalis*).

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 nontransformed genotypes were used as checks. Additional cuttings of some genotypes were included in each replication to fill the rack with a total of 60 plants evaluated in each conetainer rack. These 60 genotypes were randomized in a 98 (7 by 14) conetainer rack, with the outer cones planted as borders. The four isogenic populations and the check genotypes were evaluated in four replications per environment. Two racks, one with unlimed soil and the other with limed soil, represented one replication. Conetainers used in this experiment were the same as those used in Khu et al. (2012a).

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 at the soil line into root and stem fractions. The roots growing in the Germinating 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 unlimed or limed soil. After drying at 65°C for 72 h, the dry weight (DW) of roots and shoots was determined. Relative root weight was computed as the ratio of root DW in unlimed soil to the root DW in limed soil for each genotype in each replication. Relative shoot DW was computed analogously. Therefore, the traits analyzed were root DW in limed and unlimed soil, hereafter referred to RDW-L and RDW-UL, shoot DW in limed and unlimed soil, hereafter named as SDW-L and SDW-UL, and root and shoot DW ratios, hereafter named as RDWR and SDWR, respectively, representing the relative growth in unlimed vs. limed soil.

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 tissue analysis of 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 replications 3 and 4 from were consolidated to produce two samples per environment and type of soil for analysis. The analyses were performed at the Soil, Plant, and Water Laboratory of The University of Georgia, Athens, GA, following the inductively coupled plasma (ICP) method (Isaac and Johnson, 1985) by using the ICP Emission Spectrograph (Thermo Jarrel Ash Corp.).

Table 2. Observed and expected frequencies for the absence–presence and chi-square tests of both T_1 groups (citrate synthase [CS] and *Daucus carota* plasma membrane H^+-transporting adenosine triphosphatase [DcPA1]) and for the isogenic alfalfa populations.

Class	Number of individuals	Observed frequency (%)	Expected frequency (%)	$P > \chi^2$
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 plus DcPA1	13	17.1	25	0.085
CS	14	18.4	25	
DcPA1	26	34.2	25	
None	23	30.3	25	

Data Analysis

Data for each trait were 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. The environment effect was considered as a fixed effect because it represented 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 version 9.2 (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 2). In both cases the observed frequencies of the presence or absence of the transgenes fit the expected segregation ratio of 1:1 (Table 2). Citrate concentration or proton exudation varied among the T_1 individuals evaluated (Fig. 1 and 2). The transgenic genotype CS-16 showed a three-fold increase over the nontransgenic genotypes (Fig. 1). Two genotypes, H^+-2 and H^+-4, showed 4.5-fold higher proton exudation levels than the R2336 nontransgenic genotype (Fig. 2). Based on the citrate concentration in roots and proton exudation levels of 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 2). Four classes of progeny from

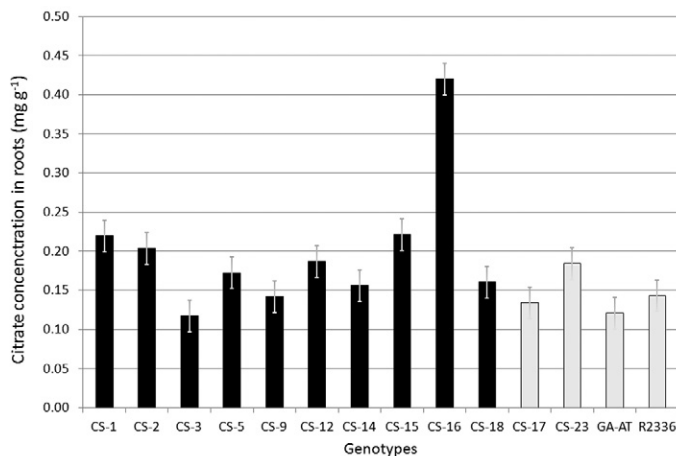

Figure 1. Citrate concentration in roots of 10 T_1 plants containing the citrate synthase (CS) transgene (black bars), two T_1 plants with no CS gene (white bars), and the nontransgenic genotypes GA-AT and R2336, Al tolerant, and Al sensitive, respectively, as checks. The genotype CS-16 was used as a parent for the T_2 population.

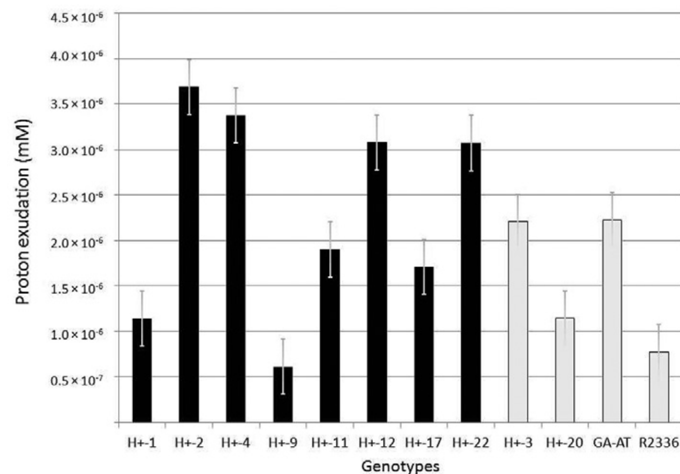

Figure 2. Proton exudation by roots of eight T_1 plants containing the *Daucus carota* plasma membrane H^+-transporting adenosine triphosphatase (DcPA1) gene (black bars), two T_1 plants with no DcPA1 gene (white bars), and the nontransgenic genotypes GA-AT and R2336, Al tolerant and Al sensitive, respectively, as checks. The genotype H^+-4 was used as a parent for the T_2 population.

Table 3. Least square means for root and shoot dry weight (DW) of four isogenic T_2 populations, the T_1 parents of the T_2 populations (CS-16 and H^+-4), and the parental genotypes of the T_1 parents (the nontransgenic 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 genotype NECS-141.

	Root DW			Shoot DW		
	mg per plant		Unlimed:limed	mg per plant		Unlimed:limed
Entry†	Limed	Unlimed	ratio‡	Limed	Unlimed	ratio‡
			Isogenic T_2 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 plus 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

†None, nontransgenic; CS, citrate synthase (only CS transgene present); DcPA1, *Daucus carota* plasma membrane H^+-transporting adenosine triphosphatase (only DcPA1 transgene present); CS plus DcPA1, both transgenes present.

‡The unlimed:limed ratios were computed for each replication to enable a statistical analysis. Therefore, they will not equal to the ratio of unlimed:limed least-square means.

§Means followed by the same letter (within a column) are not significantly different ($P < 0.05$).

the CS-16 × H^+-4 cross were expected in equal frequencies, assuming that each transgene was present as a single insertion and as a simplex (i.e., Tttt) and the observed progeny frequencies fit the expected 1:1:1:1 ratio (Table 2).

The isogenic population evaluation in soil showed significant genotype effects for most of the traits, except for RDW-L, SDW-UL, Al and Mn content in limed soil, and Al content for the unlimed:limed ratio (data not shown). No significant G×E effect was observed for RDW and SDW. However, significant G×E effect was observed for Al and Mn content in unlimed soil (data not shown). The isogenic T_2 population without any transgenes had similar root weight in limed soil but lower root weight in unlimed soil when compared to the populations with transgenes although the isogenic populations with neither or both genes did not differ under unlimed conditions (Table 3). However, Al tolerance of the unlimed:limed 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

Table 4. Aluminum and Mn content in the shoot fraction of the four isogenic populations (none, citrate synthase [CS], *Daucus carota* plasma membrane H^+-transporting adenosine triphosphatase [DcPA1], and CS plus DcPA1), the parents of the populations (H^+-4 and CS-16), and the six nontransgenic lines used as checks.

Entry	Al			Mn		
	Limed	Unlimed	Unlimed:limed ratio†	Limed	Unlimed	Unlimed:limed ratio†
	Isogenic T_2 populations					
	—— mg kg^{-1} ——			—— mg kg^{-1} ——		
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^{bc}	2.49^{b}	51.1^{a}	59.6^{de}	1.27^{bc}
CS plus 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}

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

either soil but those containing a transgene (CS-16 and/or H^+-4) had higher ratios than those without transgenes. Shoot DW 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 of an Al-tolerance mapping population (Khu et al., 2012b), showed the same Al tolerance (unlimed:limed ratio) as the transgenic lines and populations (Table 3). The genotype NECS-141, the Al-sensitive parent of a mapping population (Khu et al., 2012b), was Al sensitive in this experiment, as expected. The GA-AT genotype showed an intermediate performance between Altet-4 and NECS-141 (Table 3).

Aluminum and Mn content in the shoots did not differ among the isogenic populations when grown in limed soil (Table 4). However, in unlimed soil, the transgenic populations had generally lower Al in the tissue than the nontransgenic population. The transgenic parental genotypes were similar to the transgenic populations and generally lower in Al content than the nontransgenic genotypes. The Al-tolerant, nontransgenic genotype Altet-4 had high levels of Al in its shoot tissue. Trends for Mn were less clear than those for Al (Table 4). The nontransgenic 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 shoot tissue.

Significant genotype effect was observed for the concentrations of Ca, Mg, K, and Fe in limed and unlimed soils, except for Ca in unlimed, Fe in limed soil, and P in both soils (data not shown). The concentration of Ca showed no differences among isogenic populations in limed and unlimed soil (Supplemental Table S1). The CS plus DcPA1 population had higher concentrations of Mg than the CS, DcPA1, and nontransgenic populations in limed soil but only higher than CS population in unlimed soil. Concentrations of K and P in shoot tissue did not differ between limed and unlimed soil or among the isogenic populations within type of soils. Iron had a similar pattern to that of Al, showing two-fold higher concentration in unlimed than limed conditions, and a similar ranking of the isogenic populations as for the Al concentration in unlimed soil. In unlimed soil, the concentration of Fe in shoots was generally high in all the populations and genotypes (Supplemental Table S1).

DISCUSSION

Organic acids play a key role in Al tolerance (Kochian et al., 2004), and the role of organic acids in alfalfa was shown previously by expressing a gene for CS (Barone et al., 2008). However, Barone et al. (2008) also suggested that tolerance could be improved further by also expressing a citrate transporter gene that would enable an increased secretion of citric acid from the roots, thereby imparting a greater level of Al tolerance than either gene alone could provide.

This experiment confirmed that the CS gene improves Al tolerance as had previously been shown in a different, less agronomically desirable 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). This difference in the magnitude of Al tolerance 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 compared to the previous experiment. We also used soil with slightly different characteristics and a modified methodology to evaluate Al tolerance in this experiment. Regardless, both experiments showed that expression of the CS gene imparts a measure of Al tolerance in alfalfa.

The DcPA1 transgene had been previously introduced into alfalfa (Shen, 2009), but the effect of the gene on Al tolerance was not evaluated. 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 nontransgenic population (Table 3). 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, with no additional tolerance achieved by expressing both genes in the same plants. Apparently the existing transporter is sufficient if citrate is overexpressed, but if not overexpressed, the transporter transgene helps export whatever organic acids the plant produces normally.

The Al tolerance, measured as the ratio of growth in unlimed vs. limed soil, of the transgenic populations evaluated here was similar to that of the nontransgenic, Al-tolerant genotype Altet-4, which was used as a parent of a genetic mapping population (Khu et al., 2012b). 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 that in the nontransgenic isogenic population. However, the concentration of shoot Al in Altet-4 was similar to that of 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 in the shoot 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, 2001, 1997). These results suggest that combining quantitative trait loci (QTL) for Al tolerance derived from Altet-4 (Khu et al., 2012b) with the transgenes may result in superior Al tolerance to that observed in this experiment although we have not yet empirically tested this hypothesis.

The Mn concentrations observed in unlimed soil were similar to those in limed soil, in which no toxicity was observed. Therefore, even though some differences among populations and genotypes were observed in unlimed soil, the magnitude of Mn concentrations suggests that Mn is not a major factor affecting the results.

CONCLUSIONS

We have confirmed that two transgenes, *Pseudomonas aeruginosa* CS and DcPA1, improved alfalfa Al and acid soil tolerance either singly or in combination. The Al tolerance conferred by the transgenes was similar to that observed in a nontransgenic Al tolerant genotype, but based on different levels of Al accumulation in shoot tissue, the tolerances may result from different mechanisms. Efforts are underway to pyramid the transgenes with QTL identified in a segregating population derived from Altet-4 (Khu et al., 2012b) to determine if additional Al tolerance can be obtained. Additional research is also needed to determine the performance of the transgenic plants under field and commercial conditions.

Supplemental Information Available

Supplemental material is included with this manuscript.

Acknowledgments

The authors would like to thank Donald Wood, Jonathan Markham, and Wesley Dean for their support with the greenhouse screening, Dr. Nathan Hancock for helping with the Southern blot analysis, Yanling Wei for laboratory assistance with gene expressions, and Maria Ortega for helping with the RT-PCR. We would also like to thank Forage Genetics Intl. for providing plant materials R2336 and 60T180-14. This study was funded by the Georgia Agricultural Experiment Stations and The Samuel Roberts Noble Foundation.

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