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GENETIC TRANSFORMATION AND HYBRIDIZATION

Bacterial citrate synthase expression and soil aluminum tolerance in transgenic alfalfa

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Abstract Alfalfa is very sensitive to soil acidity and its yield and stand duration are compromised due to inhibited root growth and reduced nitrogen fixation caused by Al toxicity. Soil improvement by liming is expensive and only partially effective, and conventional plant breeding for Al tolerance has had limited success. Because tobacco and papaya plants overexpressing *Pseudomonas aeruginosa* citrate synthase (CS) have been reported to exhibit enhanced tolerance to Al, alfalfa was engineered by introducing the *CS* gene controlled by the *Arabidopsis Act2* constitutive promoter or the tobacco *RB7* root-specific promoter. Fifteen transgenic plants were assayed for exclusion of Al from the root tip, for internal citrate content, for growth in in vitro assays, or for shoot and root growth in either hydroponics or in soil assays. Overall, only the soil assays yielded consistent results. Based on the soil assays, two transgenic events were identified that were more aluminum-tolerant than the non-transgenic control, confirming that citrate synthase overexpression can be a useful tool to help achieve aluminum tolerance.

Keywords Acid soils · Aluminum toxicity · Citrate synthase · Genetic engineering · *Medicago sativa* L. · Transformation

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Introduction

Soil acidity is a global problem that limits crop productivity (von Uexkull and Mutert 1995). At low pH, aluminum (Al) becomes soluble and available to plants in the Al^{3+} and $Al(OH)^{2+}$ forms (Kinraide 1991). Micromolar concentrations of Al^{3+} can inhibit root growth and, as a consequence, yield is severely reduced as a result of insufficient uptake of water and other nutrients (Kochian 1995). It has been estimated that 56% of the soils in the humid tropics and 40% of the soils worldwide are affected by Al toxicity. Acid soils also cover vast areas in Northern Europe (Buol and Eswaran 1993).

The physiological mechanisms of Al toxicity and resistance have been studied by several groups. Organic

acid secretion is one of the principle mechanisms involved (Delhaize et al. 1993; Ryan et al. 1995; de la Fuente and Herrera-Estrella 1999; Lopez-Bucio et al. 2000; Matsumoto 2000; Yang et al. 2000; Ma et al. 2001; Pineros et al. 2002; Zhao et al. 2003). It is hypothesized that the Al-chelating ability of some organic acids, namely citrate, malate and oxalate, confers tolerance through the formation of stable complexes with Al^{3+} that are not toxic to plants or that cannot enter the roots. In some Al-tolerant plants, one or more of these organic acids are secreted by the root tip as a response to toxic Al, although organic acid exudation does not appear to be the only tolerance mechanism (Pineros et al. 2005; Hoekenga et al. 2006).

Improvement of Al tolerance in plants is a major plant-breeding objective worldwide (reviewed by Samac and Tesfaye 2003). Alfalfa (*Medicago sativa* L.) is very sensitive to Al^{3+}, and its yield and stand duration in acid soils are compromised because of inhibited root growth and reduced nitrogen fixation due to the acid sensitivity of the symbiotic bacteria (Hartel and Bouton 1989). Several approaches have been suggested to increase the yield of alfalfa in acid soils, including both soil and crop improvement. Soil improvement by liming is expensive and only partially effective (Devine et al. 1990). In addition, surface-liming is not very effective at raising the soil pH below the plow layer (Dall'Agnol et al. 1996).

Conventional plant breeding has had limited success in addressing Al susceptibility in alfalfa. A breeding line improved for yields in acid soils ("GA–AT") yields significantly more than unimproved ones in the presence of toxic Al, but the yields remain too low, only 20% of normal (Bouton and Sumner 1983; Bouton et al. 1986; Hartel and Bouton 1989; Bouton and Parrott 1997). An Al-tolerant diploid *M. coerulea* genotype from a Turkish germplasm (PI 464724) was selected using callus production on tissue culture medium containing toxic Al as a screening test (Parrott and Bouton 1990; Dall'Agnol et al. 1996). Tolerance was mapped in a cross with an Al-sensitive *M. coerulea* (PI 440501), and two RFLP markers were found to be associated with the trait in F_2 and backcross populations, using both tissue culture and soil tests. This Al tolerance was then introgressed into tetraploid cultivated alfalfa using 2*n* gametes and marker-assisted selection (Sledge et al. 2002). Nevertheless, this breeding strategy will still require additional sources of Al tolerance to achieve agronomically useful levels of tolerance. Genetic engineering provides another opportunity to enhance Al tolerance through the expression of endogenous or foreign genes associated with the biosynthesis of organic acids involved in the Al-chelating and detoxifying process.

In alfalfa, overexpression of a nodule-enhanced form of malate dehydrogenase induced a significant increase of the concentration of malate and other organic acids in tissues of some transgenic lines, resulting in Al tolerance (Tesfaye et al. 2001).

Tobacco and papaya plants overexpressing a bacterial citrate synthase (*CS*) gene exhibited citrate overproduction and enhanced tolerance to Al (de la Fuente et al. 1997). A subsequent study by Delhaize et al. (2001) on the same and other tobacco lines expressing CS at higher levels was unable to confirm the findings. These authors also reported that two CS-expressing alfalfa events did not show improved Al tolerance.

Given the contradictory nature of the information, additional information was necessary to further assess the usefulness of citrate synthase overexpression. Accordingly, a similar genetic engineering approach to Al tolerance was carried out in alfalfa by introducing the *CS* gene from *Pseudomonas aeruginosa* controlled by the *Arabidopsis actin 2 (Act2)* constitutive promoter or the tobacco *RB7* root-specific promoter. The results presented show that some alfalfa plants expressing CS can have significantly better root and plant growth in Al toxic soil in a greenhouse test, possibly due to exclusion of Al^{3+} from the root tip.

Materials and methods

Construction of transformation vectors

The coding region of the *P. aeruginosa* CS was amplified from the plasmid pPKB (provided by H. W. Duckworth, University of Manitoba, Canada) with the primers forward 5′-ATA*GGATCC*CATCATGGCTGAC-3′ and reverse 5′-GA*GAGCTC*AGCCGCGATCCTTG-3′. Inclusion of *Bam*HI and *Sac*I restriction sites (in italic) in the primers allowed the PCR products to be ligated (Fast-Link DNA ligation kit, http://www.epicentre.com) into the *Bam*HI and *Sac*I site of the vector pAPCK between the *Act2* constitutive promoter (An et al. 1996) and the *NOS* terminator. Vector pAPCK contains the *NptII* gene as selectable marker for kanamycin resistance under the control of the potato *ubiquitin3* promoter and terminator (Garbarino and Belknap 1994).

A second CS expression cassette was obtained by substituting the *Act2* promoter with the tobacco *RB7* root-specific promoter (Yamamoto et al. 1991). This was excised from the pBluescript-TobRB7 plasmid (provided by M. A. Conkling, North Carolina State University) with *Bam*HI and *Xba*I and ligated into *pAPCK–Act2–CS* from which the *Act*2 promoter had been removed with *Bam*HI and *Spe*I. Each expression cassette was separately subcloned into the *Asc*I and *Pac*I sites of the binary vector pPZP201BK (Covert et al. 2001), thus obtaining the transformation vectors *pPZP201BK–Act2–CS* and *pPZP201BK–RB7–CS* (Fig. 1). The integrity of the

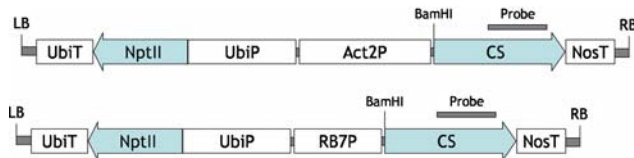

Fig. 1 T-DNAs used for alfalfa transformation. *UbiT*, potato ubiquitin3 terminator; *NptII*, neomycin phosphotransferase coding sequence; *UbiP*, potato ubiquitin3 promoter; *Act2P*, *Arabidopsis Actin 2* promoter; *CS*, citrate synthase coding sequence; *NosT*, Nopaline synthase terminator; *RB7P*, tobacco *RB7* gene promoter

cassettes was assessed by sequencing, after which they were separately introduced into *Agrobacterium tumefaciens* strain LBA4404 via electroporation (Gene pulser, http://www.biorad.com) according to the manufacturer's instructions.

Alfalfa transformation

One hundred pieces approximately 0.25 cm^2 in size, from young, fully expanded leaves of the highly regenerable RSY1 genotype of Regen-SY germplasm (Bingham 1991) were transformed for each construct, using the media, procedures and conditions as described by Austin et al. (1995). About 3 g l^{-1} of GelRite (http://www.caissonlabs.com) were used as the gelling agent in all tissue culture media; 250 mg l^{-1} cefotaxime were used for *Agrobacterium* control. Mature embryos were obtained on selective (25 mg l^{-1} kanamycin) growth medium, isolated, and subjected to partial desiccation by placing them in groups of 5–15 into a Petri dish containing a piece of solidified B5HO medium in the middle (approx. 0.5 cm^3, not touching the embryos) and sealed with Nescofilm (http://www.karlan.com). After 2 days, the embryos were transferred to germination medium (1/2 MS basal medium with B5 vitamins) in Petri dishes for 2 weeks; single plants were then transferred to Magenta GA-7 boxes (http://www.magentacorp.com).

One leaflet was aseptically excised from each in vitro plantlet, and divided into two parts that were used for in vitro regeneration as described, but omitting *Agrobacterium* treatment; one part was placed on selective medium (50 mg l^{-1} kanamycin), the other on kanamycin-free medium. Multiple plants from each event were obtained through this second cycle of regeneration under selective conditions (T_0-II). One or two plants per transformation event were transferred to soil and then to a greenhouse.

Preliminary screening of transformants by PCR

DNA was isolated with a CTAB protocol (Murray and Thompson 1980) from leaf tissue of the putative transformants. Equal amounts of DNA (200 ng) of the putative transformed and control plants and 1 ng of the binary vector *pPZP201BK–Actin–CS* were used for PCR with the primers *Npt*IIA (5′-AGAGGCTATTTCGGCTATGAC-3′) and *Npt*IIB (5′-CGAATATCATGGTGGAAAATGG-3′), giving an expected 553-bp amplicon, and with the primers CS-1F (5′-TGA TCA TCG AGG GCT CAG CCC CCG-3′) and CS-1R (5′-CTT GAG GGC GGT GAA GTC GCG CTG-3′), giving an expected 1,252-bp amplicon. The reactions were performed as follows: 5 min at 94°C, followed by thirty-five 45 s cycles at 94°C, 45 s at 67°C (*CS*) or 55°C (*NptII*), 90 s at 72°C, and a final extension of 5 min at 72°C.

Southern analysis

Total plant genomic DNA (10 μg per sample) and plasmid pPZP201BK–Actin–CS (10 pg) digested with *Bam*HI (that cuts the T-DNAs between the Act2 or the Rb7 promoter and the CS coding sequence, Fig. 1), were separated by electrophoresis on a 0.8% TAE agarose gel and transferred to a positively charged nylon membrane (Hybond+, http://www.amersham.com) according to Sambrook et al. (1989). A 576-bp PCR fragment of the *CS* gene was amplified with the primers CS-2F (5′-GGACATCAATAA CCCGAAG-3′) and CS-2R (5′-TTCATCGCCAGTTC-CAGT-3′) and used as a probe. The probe was labeled with α-[^{32}P] dCTP (http://las.perkinelmer.com) using the Rediprime labeling system (http://www.gelifescience.com). Prehybridization and hybridization were performed at 65°C The membranes were washed twice in 2× SSC 0.1% SDS at 65°C for 30 min each, once in 0.5× SSC, 0.1% SDS at 65°C for 15 min, and once in 0.1× SSC, 0.1% SDS at room temperature for 30 min. The membrane was subjected to autoradiography using Kodak BioMax Film at −80°C for 3 days.

Reverse transcriptase-PCR

Total RNA was extracted from tissue of young leaves of transgenic and control plants with TRIZOL Reagent (http://www.gibcobrl.com) according to the manufacturer's instructions and treated with RNase-free DNase to remove contaminating DNA. Then, cDNAs were synthesized from 1 μg of total RNA by reverse transcription in a 50-μl reaction containing 12 units of AMV reverse transcriptase (http://www.usbweb.com), 0.3 μg oligo dT primer, 10 mM of each dNTP and 5 μl of 10× reverse transcriptase buffer. Residual RNA was removed by incubating the cDNA in 2 units of *E. coli* RNase H (http://www.invitrogen.com) for 20 min at 37°C. One twentieth of the reaction was amplified with the citrate synthase-specific primers CS-1F and CS-1R (see above), with 3 min at 94°C, 35 cycles at 94°C, 45 s; 67°C 45 s; 72°C 90 s. The reaction outcome was visualized by ethidium bromide staining after 1% agarose

gel electrophoresis. A PCR reaction on total RNA treated with RNase-Free DNase was performed with the same primers without the reverse transcriptase step to confirm the absence of any genomic DNA contamination in the samples. The 18*S* RNA gene was used as an internal control to normalize for sample to sample variations in total RNA amounts and for reaction efficiency. A 210-bp fragment of the *18S* gene was amplified using primers 18S-F 5′-AAGGAATTGACGGAAGGGCACCA-3′ and 18S-R 5′-TAAGAACGGCCATGCACCACC-3′.

Aluminum tolerance assessment

In vitro root growth

To obtain a preliminary estimate of Al tolerance, ten somatic embryos from each transgenic plant and from control plants obtained in a second regeneration cycle were converted into plants on Al-toxic and Al-free modified Blaydes medium (Parrott and Bouton 1990) in 10-cm Petri plates. When the first true leaf was completely expanded, the length of the longest root was measured on each plantlet.

Exclusion of Al from the root tip

Roots from three in vitro T_0-II plants per each of seven transgenic events (all the events for which we had three vigorous in vitro plants) and three control plants were excised and treated as described by Polle et al. (1978). The roots were rinsed in distilled water and immersed into 5 ml of one of the three liquid growth media: 1/2 MSO, 1/2 MSO + Al (400 μM $AlCl_3$) or modified Blaydes + Al (40 μM $AlCl_3$) in 50-ml plastic tubes. After 2 h, the roots were rinsed five times in distilled water and treated in the same tubes with 5 ml staining solution (2 g l^{-1} hematoxylin and 0.2 g l^{-1} $NaIO_3$) for 15 min. After five rinses in distilled water, the roots were left in water overnight with gentle agitation. The root tips were then excised, placed on blotting paper and photographed immediately.

Evaluation in soil

Rooted cuttings of the 15 transgenic plants confirmed by molecular analysis were used in a greenhouse trial to test for Al tolerance; five of the plants were lost due to sensitivity to bacterial wilt. The plants were grown in a soil mixture containing toxic levels of Al due to its acidity, with and without liming, as described by Dall'Agnol et al. (1996) in 0.72-l polystyrene cups using a randomized complete block design with six replicates. After 6 weeks, the plants were washed free of soil, the root biomass was scored in a 1 (minimum) to 5 (maximum) scale, and the plants were dried and weighed. Data were subjected to the analysis of variance using the SAS GLM procedure (SAS Institute, Cary, NC, USA, 1995).

Citrate concentrations in roots of transgenic plants

Eight transgenic lines and one control plant (RSY1) were grown in aerated hydroponic culture using Hoagland's solution, with three clonal replications and the roots excised. One gram of roots was ground to powder in liquid nitrogen, extracted in two volumes of 0.6 M perchloric acid, and neutralized with 2 M KOH before assaying for citrate using the enzymatic procedure described in Bergmeyer et al. (1974).

Results

Alfalfa transformation and molecular analyses of transgenic plants

The *CS* gene sequence was identical to that from the complete genome of *P. aeruginosa* (Stover et al. 2000, GenBank AE004091.2), revealing a few errors in the originally published sequence (GenBank M29728.1, Donald et al. 1989).

Forty-one putative transgenic alfalfa plants were regenerated, of which 20 were retained following PCR and a functional test of the *NptII* gene expression through regeneration of leaf explants on kanamycin-containing medium as described. Of these plants, 15 were confirmed to be transgenic for the gene of interest by Southern analysis. A high percentage of escapes was reported previously in RSY1 alfalfa transformation with this protocol (Rosellini et al. 2007). Five of these contained the Actin-CS (labeled with an "A" prefix) construct, and the other ten contained the RB7-CS T-DNA (labeled with a "T" prefix). The CS copy number per event varied between 1 and 6 (Fig. 2). No correlation was observed between the *CS* gene copy number and the results of Al tolerance tests (not shown).

When the transgenic plants were tested for *CS* gene expression by RT-PCR, a 1,252-bp fragment was amplified from all transgenic plants, while no fragment was detected in the non-transgenic control (Fig. 3). Because Northern blot assays failed to detect the transcript (not shown), it is probable that the transcription level or the transcript stability was low.

Al tolerance assays

The internal citrate content of roots as estimated in hydroponic culture was generally lower in transgenic than

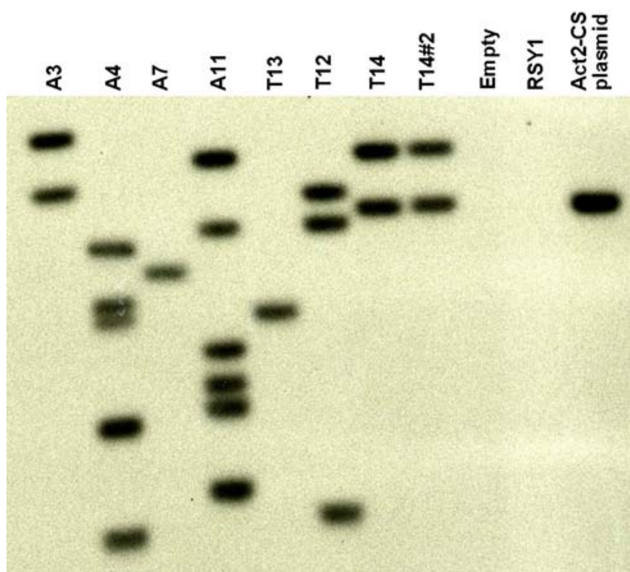

Fig. 2 Southern blot analysis of some of the transgenic plants. The probe was part of the CS coding sequence, as shown in Fig. 1. *RSY1*: non-transgenic control. The rightmost lane was loaded with a one-copy equivalent amount of the *pPZP201BK–Act2–CS* vector

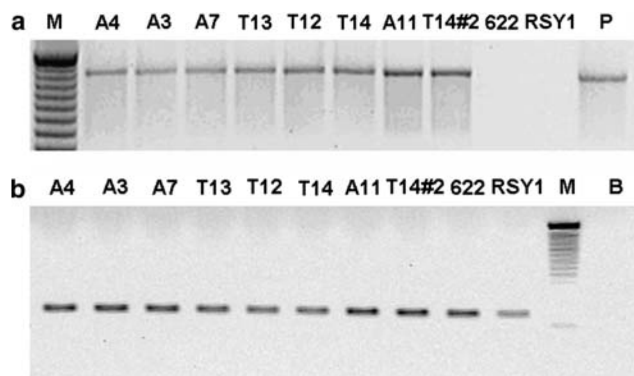

Fig. 3 RT-PCR analyses. **a** The expected 1.252-kb amplicon from the *CS* gene was obtained from eight of the transgenic plants, whereas no amplification was detected in the controls. **b** A 210-bp fragment from the *18S* gene was amplified in the same conditions. M (**a**): 100-bp DNA ladder; A4-T14#2: transgenic plants; 622 and *RSY1*: untransformed controls; *P* plasmid *pPZP201BK–Act2-CS*. M (**b**): 25-bp DNA ladder; *B* water

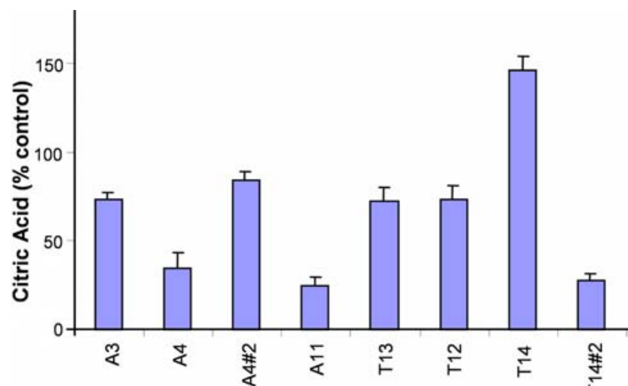

Fig. 4 Internal root citrate concentration in transgenic plants compared with the non-transgenic control (=100). Data represent the mean ± SD of three independent measurements

in the non-transgenic control plants (Fig. 4), though the differences were not statistically significant. Different cuttings of the same transgenic events (A4 and A4#2; T14 and T14#2) differed markedly, suggesting that either gene expression is highly variable, or the citrate levels within the cells are highly variable.

All plant cells produce citric acid as part of the citric acid or tricarboxylic acid (TCA) cycle, the regulation of which is still poorly understood (Siedow and Day 2000). Many of the TCA enzymes are subject to negative feedback by high levels of ATP or a high ratio of NADH/NAD+. Such feedback would be triggered by increased levels of citric acid in transgenic cells, and lead to dynamic levels within the cell. Such cell-level regulation of internal citrate concentration could easily explain the variability observed in the levels of citric acid, also reported in the literature.

Likewise, in vitro root length in the presence of toxic Al levels was not significantly higher than that of the non transgenic control for any of the transgenic plants, even though the difference between transgenic plants and controls in the Al-toxic growth medium was much higher than in the control medium (Table 1).

Exclusion of Al from the root tip was suggested by the consistent lack of hematoxylin staining of the root tips shown by some of the transgenic plants (T5 and T8 in Fig. 5). However, root-tip staining was variable (A3, T25) in some transgenic events, and was comparable to that of the non-transgenic control in some others (A4, T7).

The soil test showed that root and shoot biomass did not significantly differ between any of the transgenic plants and the control at a neutral pH (limed soil), which is as expected. However, in the absence of lime (i.e., in acid, aluminum toxic soil), four transgenic plants had significantly higher root mass (Table 1; Fig. 6), and three had significantly higher shoot biomass with respect to the non-transgenic control (Table 1), suggesting Al tolerance. Two plants, A3 and A4, were significantly better than the control for both traits: showing 62 and 37% greater root biomass, and 56 and 121% greater shoot biomass, respectively. Lines A5 and A7 appeared to be Al-tolerant at the root level, but this did not result in increased shoot biomass. In contrast, the increased shoot biomass of T8 was not accompanied by higher root biomass.

Because aluminum toxicity affects all aspects of plant growth, and because the different transgenic events can behave differently, absolute shoot or root mass comparisons to a non-transgenic control are not always appropriate.

Table 1 Characteristics of the transgenic plants

Transgenic event[a]	CS copy number	In vitro root length (mm)[b]			Root mass[c]			Shoot DMY (g)[d]		
		Al	No Al	Al/No Al (%)	UL (Al)	L (no Al)	UL/L (%)	UL	L	UL/L(%)
A3	2	6.6	21.4	31	2.54*	4.25	60	0.64*	3.15	20
A4	4	4.8	20.1	24	3.00*	2.42	124	0.91*	1.35	67
A5	4–5	nt	nt	nt	2.67*	4.17	64	0.56	2.98	19
A7	1	4.7	14.6	32	2.75*	3.75	73	0.61	2.37	26
A11	6	6.4	23.6	27	2.19	4.17	53	0.55	2.59	21
T2	1	nt	nt	nt	2.08	4.17	50	0.47	2.39	20
T4	1–2	4.2	19.1	22	1.50	1.58	95	0.45	0.67	67
T5	1	7.8	16.8	46	2.17	4.42	49	0.57	3.18	18
T7	3–4	4.7	26.7	18	2.08	4.08	51	0.56	3.19	18
T8	1	5.3	19.9	27	2.00	3.67	54	0.68*	2.41	28
T12	3	9.6	28.9	33	1.75	3.33	53	0.49	2.01	24
T13	1	nt	nt	nt	1.92	3.92	49	0.55	3.15	17
T14	2	6.0	28.2	21	1.94	4.08	48	0.54	2.56	21
T15	1	7.0	17.6	40	1.75	4.17	42	0.49	2.89	17
T25	4–5	3.0	17.4	17	2.25	4.33	52	0.60	2.75	22
WT	–	3.4	20.6	17	1.85	3.29	56	0.41	2.01	20

UL unlimed, *L* limed, *nt* not tested

[a] A, plants with Actin2–CS; T, plants with RB7–CS

[b] Mean length of the longest root, in the presence of toxic Al (means of 9–10 roots)

[c] 1, lowest, to 5, highest; means of 6–18 replications; 24 replications were used for the non transgenic control

[d] Means of 6–18 replications; 24 replications were used for the non transgenic control

*Significantly higher than the non transgenic control at $P < 0.05$

Instead, each transgenic event can serve as its own best control if its performance is measured as a ratio of the amount of growth a given event has in aluminum-toxic soil as compared to the growth of that same event in soil with a neutral pH in replicated trials.

These ratios are provided in Table 1. A ratio of 100 would indicate the transgenic event performed as well under limed as under unlimed conditions. Most events were comparable to the non-transgenic control, and only produced about half the root mass in unlimed soil as they did in limed soil. Notable exceptions were events A4 and T4, which essentially did as well in limed soil as in unlimed soil.

Shoot mass ratios for most events were also similar to those of the non-transgenic control, and under unlimed conditions only produced about 20% of the shoot mass they produced under limed conditions. Again, events A4 and T4 were the notable exceptions, as their shoot masses under unlimed conditions were about 67% of what they were under the limed conditions.

Given all these criteria, event A4 showed resistance when compared to the non-transgenic control under soil conditions and when compared to itself under limed and unlimed conditions. Event T4 only showed resistance when compared to itself under limed and unlimed conditions.

Discussion

Two conclusions are evident from the results presented here. First, it is clear that enhanced plant growth under acidic, aluminum-toxic conditions can be obtained in transgenic alfalfa plants expressing citrate synthase. Secondly, the type of assay used is critical to properly evaluate the transgenic phenotype.

The widely differing results between de la Fuente et al. (1997) and a subsequent study by Delhaize et al. (2001) have called the strategy of citrate overproduction into question. Overexpression of mitochondrial CS has also been carried out, with encouraging results. Increased CS activity and/or exudation with a concomitant increase in Al tolerance was observed in *Arabidopis thaliana* (Koyama et al. 2000), cultured carrot cells (Koyama et al. 1999) and in canola plants (Anoop et al. 2003), but not in *Aspergillus niger* (Ruijter et al. 2000). It is not clear to what extent, if any, the *Aspergillus* results apply to plants.

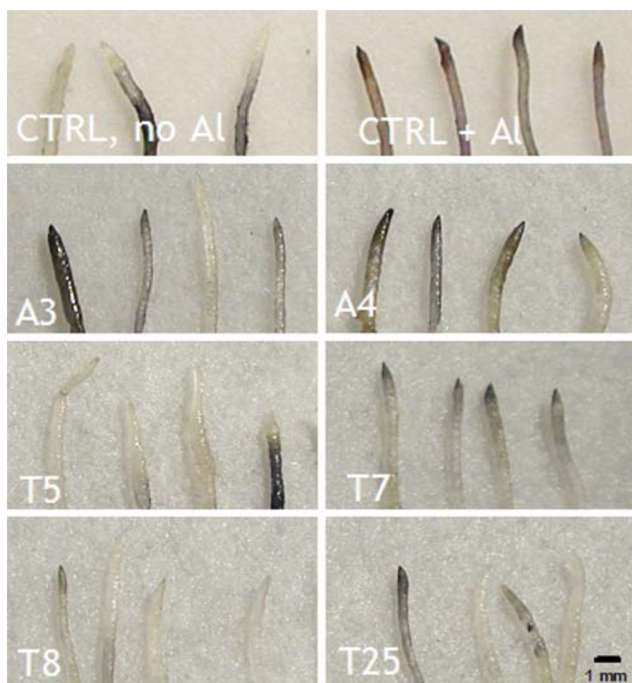

Fig. 5 Root tips of transgenic and control plants stained by hematoxylin for Al uptake following exposure to Al. Plants *T5* and *T8* show reduced coloration of the root tips; *A4* and *T7* stained similarly to the Al-treated, non transgenic control; *A3* and *T25* have stained and non stained root tips

The results presented here agree with those of Delhaize et al. (2001), but differ markedly from those of de la Fuente et al. (1997), in that there was no consistent detection of increased citrate content within the tissues, leading to inconsistent results between the in vitro assays and the performance of plants in soil assays. For example, the events that performed best in acid soil, A3 and A4, did not clearly differ from the non-transgenic control for root-tip staining or for in vitro root growth in the presence of toxic Al (Fig. 5; Table 1). Plant T8 showed both decreased root-tip staining and improved shoot biomass, but its roots were not larger than those of the control. The in vitro staining test samples roots during a short, 2-h period, while the soil assay tests the root performance over several weeks. Likewise, the use of root growth in a medium designed for plant cell culture may not accurately represent root growing conditions in soil, illustrating how the type of assay used can have a great influence on the results obtained.

In addition, the negative results obtained by Delhaize et al. (2001) may have been due to the fact that they only presented the analysis for two transgenic alfalfa events. The work presented here found great variability between the 15 different events tested. The two events tested by Delhaize et al. (2001) may simply be too few to capture the full extent of phenotypes possible. Furthermore, their characterization was based on Western blot ands root elongation in hydroponic culture in the presence and absence of Al. In contrast, a soil assay was more reliable than a hydroponic one to measure aluminum tolerance in the work reported here.

Root citrate secretion was not tested in this study, so we cannot exclude that Al tolerance is due to increased citrate exudation by the root tip. Increased citrate secretion in plants overexpressing *P. aeruginosa* CS was demonstrated in tobacco (de la Fuente et al. 1997).

Fig. 6 Examples of root mass formed in limed or unlimed soil by one transgenic plant (*A5*) and the non-transgenic control (*RSY 1*). *L*, plants grown in soil limed to obtain a neutral pH; *UL*, plants grown in unlimed soil

Overexpression of mitochondrial CS genes also resulted in increased citrate efflux in cultured carrot cells (Koyama et al. 1999), *Arabidopsis* (Koyama et al. 2000), and canola (Anoop et al. 2003) plants. The enhanced aluminum tolerance found in a few of the transgenic events here suggests that this CS overexpression technique is promising, and germplasm with agronomically useful levels of tolerance might be obtained with further refinements. Additional results from the published literature suggest that citric acid may be stabilized if there were a mechanism whereby the cells could excrete the elevated levels of citric acid. Recently, it was demonstrated that overexpression of the ALMT1 gene of wheat, encoding a malate transporter, induced Al tolerance in barley through Al-activated efflux of malate (Delhaize et al. 2004). A strategy that combines citrate overproduction and a way to facilitate its excretion might provide a strategy that can stabilize citrate levels within the cell and lead to increased aluminum tolerance levels.

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