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ORIGINAL PAPER

Biodegradation of atrazine by three transgenic grasses and alfalfa expressing a modified bacterial atrazine chlorohydrolase gene

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Abstract The widespread use of atrazine and other *s*-triazine herbicides to control weeds in agricultural production fields has impacted surface and groundwater in the United States and elsewhere. We previously reported the cloning, sequencing, and expression of six genes involved in the atrazine biodegradation pathway of *Pseudomonas* sp. strain ADP, which is initiated by *atzA*, encoding atrazine chlorohydrolase. Here we explored the use of enhanced expression of a modified bacterial atrazine chlorohydrolase, p-AtzA, in transgenic grasses (tall fescue, perennial ryegrass, and switchgrass) and the legume alfalfa for the biodegradation of atrazine. Enhanced expression of p-AtzA was obtained by using combinations of the badnavirus promoter, the maize alcohol dehydrogenase first intron, and the maize ubiquitin promoter. For alfalfa, we used the first intron of the 5′-untranslated region tobacco alcohol dehydrogenase gene and the cassava vein mosaic virus promoter. Resistance of plants to atrazine in agar-based and hydroponic growth assays was correlated with in vivo levels of gene expression and atrazine degradation. The *in planta* expression of p-*atzA* enabled transgenic tall fescue to transform atrazine into hydroxyatrazine and other metabolites. Results of our studies highlight the potential use of transgenic plants for bioremediating atrazine in the environment.

Keywords Atrazine · Transformation · Grasses · Transgenic plants · Biodegradation

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Introduction

Atrazine is one of the most widely used herbicides in the US, and is primarily applied for weed control in the production of corn, sorghum, and sugarcane. Atrazine is a predominant member of the *s*-triazine class of herbicides and functions by inhibiting photosynthetic electron transport. The US Environmental Protection Agency estimates that 33–35 million kg of atrazine were used in agriculture in 2007 (Grube et al. 2011). The extensive use of atrazine and its mobility in soil and water has contributed to non-source point pollution of surface and groundwater, especially during times of soil run off at spring planting (Solomon et al. 1996; Thurman et al. 1998). Several successful bioremediation studies done to treat atrazine-contaminated soils have been reported (Kontchou and Gschwind 1999; Strong et al. 2000). A number of these studies have used *Pseudomonas* sp. strain ADP (Mandelbaum et al. 1995) for the successful treatment of soil contaminated with atrazine (Shapir and Mandelbaum 1997; Kontchou and Gschwind 1999; Silva et al. 2004), while other studies have used *Arthrobacter* sp. strains AD26 and AD4 (Li et al. 2008; Liu et al. 2010). Killed *E. coli* overexpressing atrazine chlorohydrolase (AtzA) from *Pseudomonas* sp. strain ADP has also been used to bioremediate soil contaminated with atrazine (Strong et al. 2000).

Pseudomonas sp. strain ADP completely biodegrades atrazine to carbon dioxide and ammonia through the consecutive action of six catabolic enzymes, encoded by *atzABCDEF*, located on a self-transmissible plasmid, pADP-1 (Martinez et al. 2001). The first reaction is initiated by AtzA, resulting in the dechlorination of atrazine to yield hydroxyatrazine, which is non-herbicidal and non-phytotoxic (de Souza et al. 1996).

Atrazine contamination in fields may also be treated by using phytoremediation procedures, involving the use of wild-type and transgenic plants (Wang et al. 2005). Thus far, the majority of transgenic plants designed for remediation of atrazine in soil were model plant systems and expressed human cytochrome P450 monooxygenases (Kawahigashi 2009). Transgenic rice plants expressing a human cytochrome P450 gene (*CYP1A1*) were shown to metabolize atrazine and produce deethyl-, deisopropyl-, and deethyldeisopropyl-atrazine (Kawahigashi et al. 2003).Transgenic potato plants (*Solanum tuberosum*) expressing *CYP1A1* metabolized atrazine to deisopropylatrazine, deethyldeisopropylatrazine, and two unidentified metabolites (Inui et al. 1999). Tobacco cell cultures expressing CYP1A1 and CYP1A2 did not result in atrazine dechlorination (Bode et al. 2004), and the end products likely had residual herbicide activity. In contrast, a bacterial atzA gene modified for expression in plants, *p-atzA*, was expressed in tobacco (*Nicotiana tabacum*), alfalfa (*Medicago sativa*), and *Arabidopsis thaliana*, and these plants dechlorinated atrazine to non-herbicidal hydroxyatrazine (Wang et al. 2005, 2010). However, because these plants have low root area indices and do not explore a large soil area, they likely have limited use in remediating atrazine contaminated soil or preventing surface runoff. In contrast, due to their high root area indices, transgenic grasses may provide the ideal platform for bioremediating atrazine in soil systems.

Here we report the construction and evaluation of several transgenic grasses expressing a modified bacterial AtzA. Transgenic tall fescue (*Festuca arundinacea*), ryegrass (*Lolium perenne*), and switchgrass (*Panicum virgatum*) transformed atrazine into hydroxyatrazine. We also evaluated the use of a new promoter system to drive expression of p-*atzA* in alfalfa. As such, these transgenic plants may prove to be useful in removing atrazine from soils and reducing the potential for leaching of atrazine into water.

Materials and methods

Transformation vector construction

Biolistic transformation vectors were produced by directionally cloning a modified bacterial *atzA* gene (p-*atzA*) encoding atrazine chlorohydrolase (Wang et al. 2005) into the *Nco*I and *Eco*RI restriction sites of pScBV-3m (Topp et al. 1989) and the *Sma*I and *Sac*I restriction sites of pAHC25 (Christensen and Quail 1996) to yield plasmids pSAM1b and pUAM1b, respectively. The p-*atzA* gene was inserted behind the sugarcane bacilliform badnavirus promoter and maize alcohol dehydrogenase first intron (Tzafrir et al. 1998) in pSAM1b. In pUAM1b, the p-*atzA* gene was inserted behind the maize ubiquitin promoter (Ubi1) and its first intron (Christensen and Quail 1996).

Binary vectors were constructed for *Agrobacterium*-mediated transformation of grasses using the

expression cassette from pSAM1b and pUAM1b, in which the expression cassette included the promoter, intron, and p-*atzA* gene. The expression cassettes of pSAM1b and pUAM1b were directionally cloned into the *Sma*I and *Hin*dIII restriction sites, respectively, of the multicloning site in the T-DNA region of pCAMBIA1305.1 (CAMBIA, Canberra, Australia) to yield plasmids pSAM1a and pUAM1a, respectively. For alfalfa transformation, the vector pPW1 (Wang et al. 2005) was modified by the addition of the 5′-untranslated region of the tobacco alcohol dehydrogenase gene (*NtADH*) from the ADH NF construct (Satoh et al. 2004) by directionally cloning into the *Xba*I and *Sma*I sites between the cassava vein mosaic virus promoter and the p-*atzA* gene to yield pPW1Plus.

Plant transformation

Tall fescue (*Festuca arundinacea* Schreb. cv. KY-31), ryegrass (*Lolium perenne* L.), switchgrass (*Panicum virgatum* L. cv. Alamo), and alfalfa (*Medicago sativa* L. cv. Regen-SY) were transformed with vectors containing p-*atzA*. Cells from tall fescue calli (ca. 5 mm in diameter) were transformed with pSAM1b together with pAHC25 (Suppl. Fig. 1, A) or pUAM1b (Suppl. Fig. 1, B) by the biolistic method as described previously (Bai and Qu 2001). Calli from tall fescue, perennial ryegrass, and switchgrass were transformed using *Agrobacterium tumefaciens* strain AGL1, containing pSAM1a (Suppl. Fig. 2, A) or pUAM1a (Suppl. Fig. 2, B) (An et al. 1988) as previously described (Dong and Qu 2005). Alfalfa was transformed with *A. tumefaciens* strain LBA4404 (Hoekema et al. 1983) containing pPW1Plus (Suppl. Fig. 3) (An et al. 1988) as previously described (Austin et al. 1995).

Atrazine tolerance and growth assays

Regenerated plants were evaluated for atrazine resistance using an agar-based assay (Wang et al. 2005). Plants or vegetative cuttings were transferred from rooting media to a modified Murashige and Skoog medium (Austin et al. 1995) supplemented with 2 or 10 μg/ml atrazine. Tall fescue and perennial ryegrass were incubated at 24 °C for 4 weeks and alfalfa was incubated at 24 °C for 3 weeks. Switchgrass was incubated at 27 °C for 3 weeks. Only T_0 lines were evaluated in these initial studies. Alfalfa lines were derived from cuttings. Resistance to atrazine and overall plant appearance and growth were scored by visual inspection after the incubation period. Only plants growing in the greatest concentration of atrazine were used for subsequent analyses.

A growth assay using hydroponic medium supplemented with atrazine was performed using wild type and selected transgenic lines of tall fescue, switchgrass, and alfalfa. Hydroponic chambers consisted of a Kerr® quart mason jar fitted with a rubber stopper containing two holes for an aerator and air-vent, and one slit to accommodate the plant. The bottom of the rubber stopper was lined with fiberglass tape coated with non-stick Teflon® PTFE (M-Pak Systems, Inc., Greensboro, NC). Plants were acclimated in hydroponic chambers with 650 ml modified Hoagland's nutrient solution (Epstein 1972) and incubated at 25 °C at 70 % relative humidity with a 16 h photoperiod. After acclimating, the plants were transferred to hydroponic chambers with fresh nutrient solution supplemented with atrazine. Hydroponic chambers with tall fescue, switchgrass, and alfalfa were incubated for 23, 25, and 16 days, respectively, under the same conditions as described above. Controls consisted of hydroponic chambers with nutrient solution and atrazine, but without plants.

PCR and reverse transcriptase-PCR assays

DNA from 100 mg of leaf tissue from wild type (WT) and transgenic plants was isolated using the Qiagen DNeasy® Plant Mini Kit (Qiagen Sciences, Valencia, CA). Polymerase chain reaction (PCR) amplification of the p-*atzA* gene in plant DNA was done using primers patzA-F126 (5′-TGTTCATGCTGAAAGCG TGCCT-3′) and patzA-R584 (5′-GCGACGGCTGTT TCT TCCATAA-3′) and the following PCR conditions: 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 2 min as described previously (Wang et al. 2005).

Total RNA from 100 mg of young leaf tissue from WT and transgenic plants was isolated using Qiagen RNeasy® Plant Mini Kit (Qiagen Sciences). Reverse transcriptase-PCR was performed using the TaKaRa RNA PCR Kit (AMV) Ver.3.0 (Takara Bio Inc., Shiga, Japan) with DNase-treated RNA samples and primers patzA-F126 and patzA-R584. The following conditions were used: reverse transcription at 55 °C for 30 min, 99 °C for 5 min, and 5 °C for 5 min,

followed by PCR using the conditions described above, except that a 1.5 min extension was used.

Atrazine degradation in vitro

The in vitro hydrolytic dechlorination of atrazine in wild-type and selected transgenic lines of tall fescue, switchgrass, and alfalfa was assessed using crude protein extracts from plants, [UL-ring-^{14}C]-atrazine, and thin layer chromatography (TLC) analyses, as previously described (Wang et al. 2005) with slight modifications. Protein extraction buffer contained 100 mM Tris–HCl, pH 8.5, 3 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride, 3 % (w/v) bovine serum albumin, 1 % (w/v) polyvinylpolypyrrolidone, 2 μM antipain, 1 μM pepstatin, and 100 μM $FeSO_4$. A 400 μl aliquot of the crude protein extract was incubated overnight at 25 °C with 10 μl of ^{14}C-UL-ring-atrazine to give a final concentration of 10 μg/ml atrazine. The atrazine standard was made by mixing 10 μl of 10 μg/ml [UL-ring-^{14}C]-atrazine and 400 μl extraction buffer and incubating overnight at 25 °C. The mixture was evaporated to dryness under nitrogen gas and resuspended in 50 μl methanol. The hydroxyatrazine standard was prepared by growing recombinant *E. coli* DH5α (pMD4) containing the *atzA* gene (de Souza et al. 1995) overnight at 37 °C in LB medium containing 34 μg/ml chloramphenicol. A 1 ml culture was centrifuged at 14,000×*g* for 1 min, washed, and resuspended in 900 μl phosphate buffered saline (pH 8.0). The resuspended culture was incubated with 100 μl of 10 μg/ml ^{14}C-UL-ring-atrazine overnight at 30 °C. The culture was centrifuged at 14,000×*g* for 2 min and 100 μl of the supernatant was evaporated to dryness under nitrogen gas then resuspended in 50 μl methanol. The TLC analyses were done by spotting 50 μl aliquots of standards and reactions onto precoated Uniplate silica gel GF channeled plates with a preadsorbent zone (Analtech, Newark, DE). The solvent system used was a mixture of chloroform:methanol:formic acid:water (75:20:4:2). The TLC plates were scanned using a Molecular Dynamics Storm Trooper—Storm 860 Densitometer (Sunnyvale, CA) to identify radioactive spots as previously described (Wang et al. 2005).

Atrazine degradation in vivo

Hydroponically-grown WT and selected transgenic tall fescue and alfalfa lines were evaluated for in vivo hydrolytic dechlorination of atrazine using ^{14}C-UL-ring-atrazine. Tillers or cuttings were transferred to 650 ml modified Johnson's nutrient solution (1.2 mM KNO_3, 0.8 mM $Ca(NO_3)_2$, 0.1 mM $NH_4H_2PO_4$, 0.2 mM $MgSO_4$, 50 μM KCl, 12.5 μM H_3BO_3, 1 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.4 μM $CuSO_4$, 0.1 μM Na_2MoO_4, 0.1 μM $NiSO_4$) in hydroponic chambers (Pence et al. 2000). Wild type and transgenic lines of alfalfa (AP-44) and tall fescue (TF-2008) were allowed to adapt for 7 and 14 days, respectively, at 25 °C with a 16 h photoperiod. After acclimation, 650 ml fresh nutrient solution containing 0.5 μg/ml ^{14}C-UL-ring-atrazine was added and plants were grown for 14 days under the same temperature and photoperiod as described above. Controls consisted of nutrient solution only, with and without added radiolabeled atrazine. Three 1 ml aliquots of plant nutrient solution containing ^{14}C-UL-ring-atrazine were taken before and after incubation. After the 14 days of incubation, leaves, stems, and roots were harvested and frozen at −20 °C until analyzed.

Samples of leaves, stems, and roots (100 mg) from replicate WT and transgenic lines were ground to a fine powder under liquid nitrogen, resuspended in 1 ml methanol, vortexed, and centrifuged at 10,000×*g*. Radioactivity in triplicate 20 μl aliquots of extracted plant material and hydroponic solution was determined using a Packard 1900 TR Liquid Scintillation Counter (Perkin Elmer, Boston, MA). Sub-samples from plant extracts and hydroponic solutions (10,000 counts of radioactivity) were evaporated under nitrogen gas and resuspended in 50 μl methanol. TLC analyses were done as described above. Spot density analysis of atrazine and atrazine by-products on the TLC plates were determined using a Molecular Dynamics Storm Trooper—Storm 860 Densitometer and ImageJ processing and analysis software (Abramoff et al. 2004).

TLC and mass spectroscopic analysis of unknown metabolite

To determine if an unknown metabolite produced in transgenic tall fescue was a compound produced from the same first three enzymatic reactions in the *Pseudomonas* sp. strain ADP atrazine degradation pathway, sub-samples of blade and crown extracts from transgenic tall fescue plants were grown with [UL-ring-^{14}C]-atrazine. Blade and crown tissue samples (100 mg) from transgenic line TF-2008 were ground under liquid nitrogen, suspended in 1 ml

methanol, and spotted on TLC plates. Plant extracts and [^{14}C] radiolabeled standards of atrazine (CIET), hydroxyatrazine (OIET), *N*-isopropylammelide (OOIT) and cyanuric acid (OOOT) standards (Syngenta Crop Protection) were evaluated by TLC analyses as described above using 10,000 counts of radioactivity of each compound. After measuring R_f values, the areas corresponding to the unknown metabolite band(s) were scraped from TLC plates into 1.5 ml centrifuge tubes. The material was suspended in 1 ml methanol-formic acid (98:2) and vortexed vigorously. Supernatants were combined and evaporated under nitrogen gas and the dried sample was resuspended in 200 µl methanol-formic acid (98:2). Analytical standards of 2-hydroxy-4-(ethylamino)-6-amino-*s*-triazine (OEAT), 2-hydroxy-4-isopropylamino-6-amino-*s*-triazine (OIAT), 2-hydroxy-4,6-diamino-*s*-triazine (OAAT) (Pestenal®, Ridel-de Haën, Sigma-Aldrich Laborchemikalien GMBH, Germany), and atrazine were obtained to compare with mass spectra of the compound(s) in the unknown band(s). The analytical standards and the extract from the TLC scrapings containing the unknown compound were evaporated under liquid nitrogen and acidified with 0.1 % formic acid. HPLC coupled to a Waters MicroMass ZMD mass spectrometer (Waters Corporation, Milford, MA) with an electrospray interface and photodiode array was used to identify the unknown compound. Acidified standards and the unknown metabolite were directly infused via syringe pump injection into the mass spectrometer at 0.3 ml/min. Injections (25 µl) of atrazine, OEAT, and the unknown were separated using a Zorbax—RX-C8 column (2.1 mm ID × 150 mm long × 5 µm film thickness). The mobile-phase flow rate was 0.2 ml/min with the following mobile-phase gradient: starting with 95 % water (0.1 % formic acid) (A): 5 % methanol (B), 95 % A at 0 min, 95 % A at 5 min, 50 % A at 10 min, 3 % A at 15 min, 3 % A at 20 min, 95 % A at 25 min, and 95 % A at 30 min. Samples were maintained at 8 °C in the autosampler to minimize decomposition. Atrazine, the known standards, and the unknown compound were analyzed with positive ion electrospray ionization at the same retention time to determine ion fragmentation. A full scan spectrum (100–300 amu) for each compound was acquired at 2 scans/s. The capillary exit and entrance voltages were selected to optimize formation of fragment ions while keeping $[MH]^+$ at 100 % relative abundance.

Results

Verification of incorporation and expression of the p-*atzA* gene in plants

Following initial selection on tissue culture media and plant regeneration, DNA from potentially transformed tall fescue, perennial ryegrass, switchgrass, and alfalfa plants was evaluated for the presence of the p-*atzA* gene by using the PCR technique and primers specific for p-*atzA*. The total numbers of transgenic plant lines constructed and tested for PCR are shown in Table 1. Results of PCR analyses indicated that 67 % of pUAM1a in tall fescue (4 of 6), 100 % of pUAM1a in perennial ryegrass (2 of 2), 71 % of pSAM1a (5 of 7) or 100 % of pUAM1a in switchgrass (14 of 14), and 55 % of pPW1Plus (11 of 20) in alfalfa lines contained p-*atzA* via *A. tumefaciens*-mediated transformations. Expression analyses, performed using reverse transcriptase (RT)-PCR, indicated that 67 (4 of 6), 100 (2 of 2), 71 (5 of 7), 100 (14 of 14), and 79 % (11 of 14) of T_o tall fescue, perennial ryegrass, switchgrass, and alfalfa lines, respectively, expressed of p-*atzA* mRNA *in planta*. The single tall fescue line transformed with p-*atzA* using a biolistc approach, with either pSAM1b or pUAM1b, expressed AtzA activity *in planta*.

Transgenic grass and alfalfa plants are resistant to atrazine growing in agar and in hydroponic assays

The T_o transgenic grass and alfalfa lines were evaluated for their ability to grow in the presence of 2 and 10 µg/ml atrazine in agar growth medium. Results from this experiment showed that 75 (3 of 4), 100 (1 of 1), 96 (22 of 23) and 59 % (10 of 17) of the transgenic tall fescue, perennial ryegrass, switchgrass and alfalfa, respectively, were resistant to 10 µg/ml atrazine. Wild-type (WT) tall fescue and perennial ryegrass were tolerant to 2 µg/ml atrazine, whereas WT switchgrass was resistant to 10 µg/ml atrazine in agar medium.

Selected transgenic plant lines, resistant to atrazine in agar, were evaluated for their ability to grow in hydroponic nutrient solution supplemented with atrazine. As shown in Table 2, a comparison of average fresh weight changes and average water usage of triplicate plants were used to determine the differences between WT and transgenic plants under the same

Table 1 Total number of plant lines obtained from transformation and regeneration

Plant	Transformation method	Plasmid	No. lines tested by PCR	Plants testing positive by PCR and RT-PCR
Tall fescue	Biolistic	pSAM1b[a]	1	1/1
	Biolistic	pUAM1b	1	1/1
	Agrobacterium	pUAM1a	6	4/6
Switchgrass	*Agrobacterium*	pCAMBIA[b]	7	0/7
	Agrobacterium	pSAM1a	7	5/7
	Agrobacterium	pUAM1a	14	14/14
Perennial rye grass	*Agrobacterium*	pUAM1a	2	2/2
Alfalfa	*Agrobacterium*	pPW1Plus	20	11/20[c]

[a] Plasmid pSAM1b was co-bombarded with pAHC25

[b] Control vector pCAMBIA 1305.1

[c] About 79 % of plants (11 of 14) tested positive by RT-PCR for AtzA expression

conditions. Tall fescue line TF-2008 was exposed to various atrazine concentrations and plants grew at a concentration of 6.5 μg/ml of atrazine (Fig. 1A). Conversely, WT tall fescue was severely damaged or killed by the minimum concentration of atrazine tested 0.5 μg/ml. However, the formation of yellow and dead blades in transgenic tall fescue was visible at atrazine concentrations ≥2.5 μg/ml. Although the WT switchgrass parent line was tolerant of atrazine, the results show transgenic lines (SG-U7, SG-U10, and SG-U21) growing in 25 μg/ml atrazine were able to increase 2.2–3.4 times in fresh weight and 1.8–2.7 times in water usage compared to WT switchgrass. Brown spots were present on the blades and tillers of both WT and transgenic switchgrass lines, but were more prevalent on the WT switchgrass than the transgenic lines. The WT alfalfa plants were either damaged or killed in the presence of 0.5 μg/ml atrazine, while the transgenic lines grew in 1 μg/ml atrazine. Transgenic alfalfa lines (AP-14, AP-22, AP-44) developed in this study (Fig. 1B) had a better growth response in 1 μg/ml atrazine than the previously described line A1 (Wang et al. 2005) showing a 7–13 % increase in water usage and 22–55 % increase in fresh weight.

In vitro activity of p-AtzA

In vitro activity of p-AtzA was examined by incubating crude plant extracts of blades (or leaves), crown (or stem), or roots with ^{14}C-labeled atrazine. TLC analyses of extracts from transgenic alfalfa, tall fescue, and switchgrass revealed that the transgenic lines produced active p-AtzA, which catalyzed the dechlorination of atrazine to produce hydroxyatrazine. This was not observed using extracts from the wild-type parents. The identity of the hydroxyatrazine product was verified by its co-migration with authentic standard. When [UL-ring-^{14}C]-atrazine was incubated with cell-free extracts from the leaves, stems, and roots of alfalfa line AP-44, hydroxyatrazine was observed (Suppl. Figure 4). Similarly, the dechlorination of atrazine to yield hydroxyatrazine occurred in blades, crown, and roots of tall fescue line TF-2008 and switchgrass blades (Suppl. Table 1).

In vivo activity of AtzA

Transgenic and WT tall fescue and alfalfa plants were grown hydroponically in the presence of [UL-ring-^{14}C]-atrazine (0.5 μg/ml) and evaluated for the presence of p-AtzA activity *in planta.* Liquid scintillation counting of plant growth medium and plant tissue extracts for triplicate plants was used to determine a mass balance based on radioactivity. The results of the mass balance study indicated that 10.3 ± 0.4 and 19.6 ± 3.3 % of accumulated counts were taken up into WT tall fescue and transgenic tall fescue line TF-2008, respectively. Similarly, the accumulated counts in WT alfalfa and transgenic alfalfa line AP-44 were 3.8 ± 1.8 and 34.1 ± 4.0 %, respectively.

TLC analysis indicated that *in planta* activity of p-AtzA transforming atrazine to hydroxyatrazine was present in the leaves, stems, and roots of transgenic alfalfa line AP-44 (Fig. 2A) and in the blades, crowns, and roots of tall fescue line TF-2008 hydroxyatrazine was found in the plant nutrient reservoir of transgenic alfalfa (Fig. 2A) and tall fescue (data not shown) but

Table 2 Change in plant fresh weight and water usage of transgenic and wild type tall fescue, switchgrass, and alfalfa in hydroponic growth assays

Plant	Line	Atrazine concentration (mg/l)	Change in plant fresh weight[a] (%)	Water usage[c] (ml/g)
Tall fescue	TF-WT	0.5	−38.6 (9.7)[b]	38.9 (15.7)
	TF-2008		132.5 (12.9)	76.7 (11.1)
	TF-WT	2.5	−42.8 (14.3)	21.7 (3.1)
	TF-2008		72.5 (6.9)	99.4 (1.7)
	TF-WT	4.5	−48.2 (9.3)	13.1 (1.9)
	TF-2008		37.8 (2.3)	57.3 (6.0)
	TF-WT	6.5	−55.3 (7.4)	13.8 (4.9)
	TF-2008		14.6 (15.6)	68.4 (4.1)
Switchgrass	SG-WT	25	52.6 (15.8)	17.2 (0.7)
	SG-U7		126.3 (35.6)	39.9 (2.2)
	SG-U10		177.1 (24.0)	47.1 (5.1)
	SG-U21		116.9 (112.7)	317 (16.7)
Alfalfa	AF-WT	0.5	−0.6 (32.0)	27.3 (2.7)
	AP-14		169.5 (71.1)	68.3 (13.5)
	AP-44		82.9 (33.2)	52.5 (24.2)
	AF-WT	1.0	−9.8 (20.6)	17.6 (1.5)
	AP-14		95.1 (59.5)	49.0 (23.6)
	AP-22		72.4 (30.1)	53.0 (9.7)
	AP-44		65.8 (10.2)	46.6 (6.4)
	A1		43.5 (14.7)	40.1 (6.4)

[a] Values are means of triplicate assays

[b] SD are given in parentheses

[c] Water usage values were corrected for initial fresh weight

was not seen in WT controls. Furthermore, densitometric studies of triplicate transgenic lines from TLC plates indicated that hydroxyatrazine accounted for 25.5 ± 4.5, 41.0 ± 1.0, and 25.7 ± 2.9 % of accumulated counts in transgenic tall fescue blades, crown and roots, respectively. Additionally, hydroxyatrazine accounted for 75.7 ± 0.6, 58.5 ± 3.3, and 61.7 ± 1.6 % of accumulated counts in transgenic alfalfa leaves, stems, and roots, respectively. In contrast, atrazine accounted for 16.4 ± 3.0, 6.9 ± 4.2, and 44.1 ± 2.1 % in transgenic tall fescue blades, crown, and roots, respectively and 1.9 ± 0.2, 2.1 ± 0.6, and 2.1 ± 0.2 % in transgenic alfalfa leaves, stems and roots, respectively. Moreover, transgenic plant lines TF-2008 and AP-44 were able to further transform hydroxyatrazine into one to two other unidentified compounds, respectively. The WT parent plants did not produce these novel metabolites (Fig. 2B). No data was collected for switchgrass in these analyses.

A major metabolite produced by transgenic tall fescue line TF-2008 (Fig. 3) was not identified as either *N*-isopropylammelide or cyanuric acid (Fig. 2C), but was tentatively identified by high-performance liquid chromatography coupled with mass spectroscopy (LC–MS) as 2-hydroxy-4-(ethylamino)-6-amino-*s*-triazine (OEAT). Identification of OEAT was based on retention times being ±2 % of those of the corresponding authentic standard compound (Fig. 3A, B), and by comparison of the molecular ion m/z at 156. In addition, two fragment ions (m/z at 118 and 125) were seen at the same retention time by using positive ion electrospray ionization LC–MS (Fig. 4A, B).

Discussion

Atrazine is one of the most prevalent herbicides used in the past several decades for preventing the

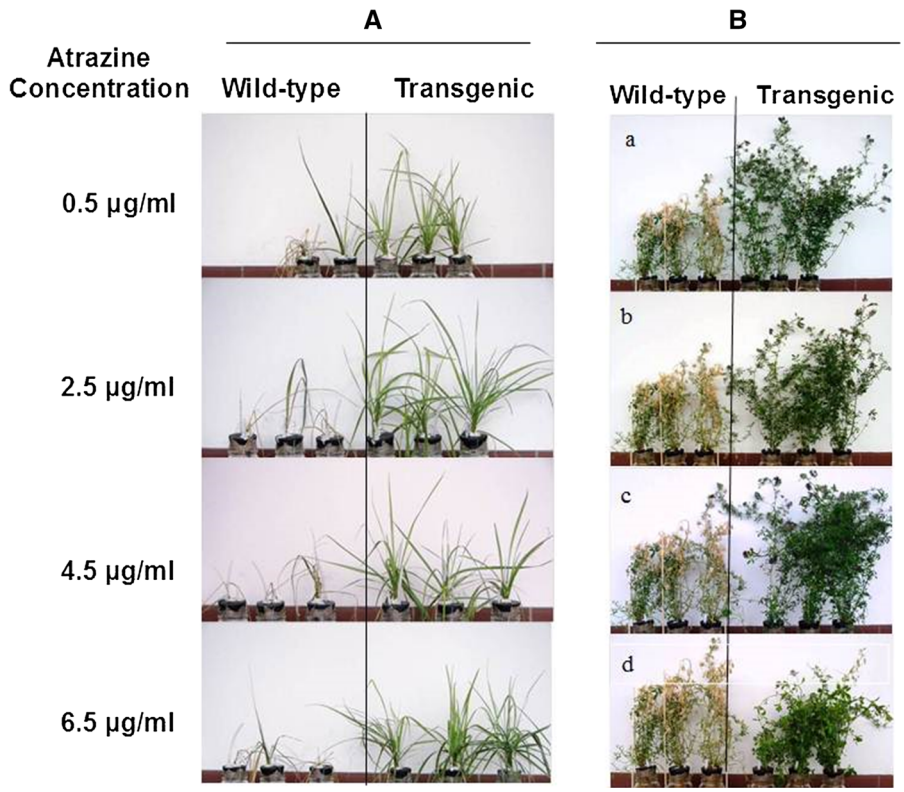

Fig. 1 Growth response of transgenic plants in atrazine-amended media. **A** Growth response of wild-type (WT) and transgenic tall fescue (TF-2008) to various concentrations of atrazine in a hydroponic plant growth system. Triplicate plants of wild-type (*left-side* of **A**) and transgenic tall fescue lines (*right-side* of **A**) after incubation. **B** Growth response of WT and transgenic alfalfa in hydroponic plant growth medium supplemented with 1 µg/ml atrazine. Triplicate plants of wild-type (*left-side* of **B**) and transgenic alfalfa lines (*right-side* of **B**) after incubation. Sections a-d show transgenic alfalfa lines AP-14, AP-22, AP-44, and A1 (Wang et al. 2005), respectively

emergence of broad-leaf weeds, and the detection of atrazine in drinking water and soil has prompted interest in development of methods to clean up or prevent environmental contamination. A growing interest in phytoremediation approaches for large scale clean-up efforts have involved the development of transgenic plants that are designed to specifically degrade or detoxify environmental contaminants such as atrazine (Schwitzguébel et al. 2006; Henderson et al. 2007; Lin et al. 2008; Kawahigashi 2009; Merini et al. 2009; Murphy et al. 2009; Reinhold et al. 2010; Guimaraes et al. 2011; Murphy and Coats 2011; Bicalho and Langenbach 2012; Marecik et al. 2012; Wang et al. 2012; Albright III et al. 2013). In previous work, transgenic plants expressing a modified bacterial AtzA were designed to dechlorinate atrazine to produce hydroxyatrazine in model plant systems (Wang et al. 2005). However, the type of application and chemical properties of the contaminant directly affects the choice of plants used to help remediate soils or prevent run-off into waterways.

In this study, we showed that a modified bacterial atrazine chlorohydrolase gene was expressed in transgenic tall fescue, switchgrass, and perennial ryegrass. Hydroponic assays showed that several of these transgenic grasses grew better than their WT parents in the presence of atrazine. Moreover, the transgenic plants were shown to degrade atrazine into hydroxyatrazine and tall fescue, perennial ryegrass, and alfalfa further degraded hydroxyatrazine into one or two other metabolites *in planta*. Switchgrass was not tested.

The in vivo degradation studies demonstrated uptake, dechlorination, and detoxification of atrazine in transgenic tall fescue. Wang et al. (2005) reported similar findings in transgenic alfalfa, *Arabidopsis,* and tobacco expressing the p-*atzA* gene, but degradation was not as extensive as reported here. Transgenic potato and rice plants expressing a human cytochrome P450 (*CYP1A1*) gene were shown to transform atrazine to deethylatrazine, deisopropylatrazine, and/or deethyldeisopropyl atrazine (Inui et al. 1999, 2001), but not non herbicidal hydroxyatrazine. Similarly, transgenic rice plants expressing human cytochrome P450 genes *CYP1A1*, *CYP2B6*, and *CYP2C19* in rice plants were found to produce *N*-dealkylated metabolites when grown in the presence of atrazine (Kawahigashi

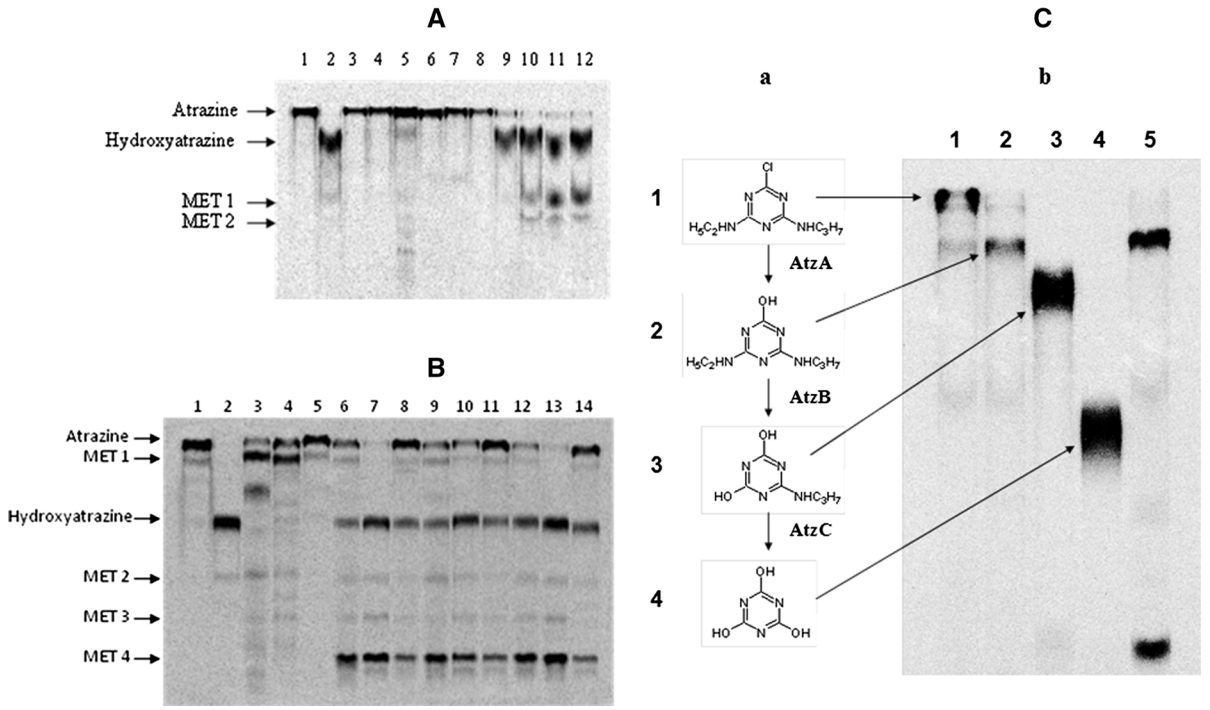

Fig. 2 *In planta* activity of p-AtzA. **A** TLC analyses of tissue extracts from wild-type (WT) transgenic alfalfa line AP-44 plants hydroponically grown in the presence of ^{14}C-UL-ring-atrazine. *Lanes 1* atrazine control; *2* atrazine incubated with *E. coli* (pMD4); *3* and *4* nutrient solution before and after WT alfalfa growth, respectively; *5–7* WT alfalfa leaves, stems and roots, respectively; *8* and *9* nutrient solution before and after AP-44 growth, respectively; *10–12* AP-44 leaves, stems and roots, respectively. **B** TLC analyses of tissue extracts from wild-type (WT) and transgenic (TF-2008) tall fescue plants grown hydroponically in the presence of ^{14}C-UL-ring-atrazine. *Lanes 1* atrazine control; *2* atrazine incubated with *E. coli* (pMD4); *3–5* WT tall fescue leaves, stems and roots, respectively; *6–14* triplicate plants of line TF-2008 leaves, stems and roots, respectively. **C** Identification of unknown metabolite. Subset (*a*) represents the first three enzymatic reactions in the atrazine degradation pathway of *Pseudomonas* sp. strain ADP. Subset (*b*) is the TLC of ^{14}C-radiolabelled standards and blade tissue extract from transgenic tall fescue. *Lanes 1* atrazine; *2* hydroxyatrazine; *3 N*-isopropylammelide; *4* cyanuric acid; and *5* transgenic tall fescue blade tissue extract

2009). However, these transgenic plants expressing human cytochrome P450 failed to dechlorinate atrazine.

In the present study, an additional atrazine degradation metabolite was found in the tissue of transgenic tall fescue. Some studies have shown that hydroxylated atrazine intermediates in maize were further catabolized into other metabolites by endogenous plant enzymes (Shimabukuro 1968). In vivo degradation studies of atrazine, reported here, demonstrated that the majority of atrazine taken up in the plant was degraded in these transgenic plants. While transgenic tall fescue blades showed some damage at higher concentrations of atrazine, a greater concentration of atrazine was found in the blades than in the crown of transgenic tall fescue. This suggests that atrazine accumulated in the transgenic tall fescue blades and transgenic tall fescue blade tissue damage was caused by atrazine-induced chlorosis at increasing atrazine concentrations. Thus, additional metabolites from hydroxylated atrazine degradation produced from endogenous plant metabolism may not affect the growth of these transgenic plants. Additional studies, however, are needed to determine the identification of these unknown compounds and examine their potential to cause plant injury.

One metabolite produced in transgenic tall fescue plants at levels similar to or higher than hydroxyatrazine was identified as 2-hydroxy-4-ethylamino-6-amino-*s*-triazine (deisopropylhydroxyatrazine, OEAT). Several C_3 species have been shown to detoxify triazine herbicides by *N*-dealkylation, including annual ryegrass and hybrid poplar (Burnet et al. 1993; Burken and Schnoor 1997). Burnet et al. (1993) studied simazine resistance in annual ryegrass and tentatively identified de-ethyl simazine derivatives as

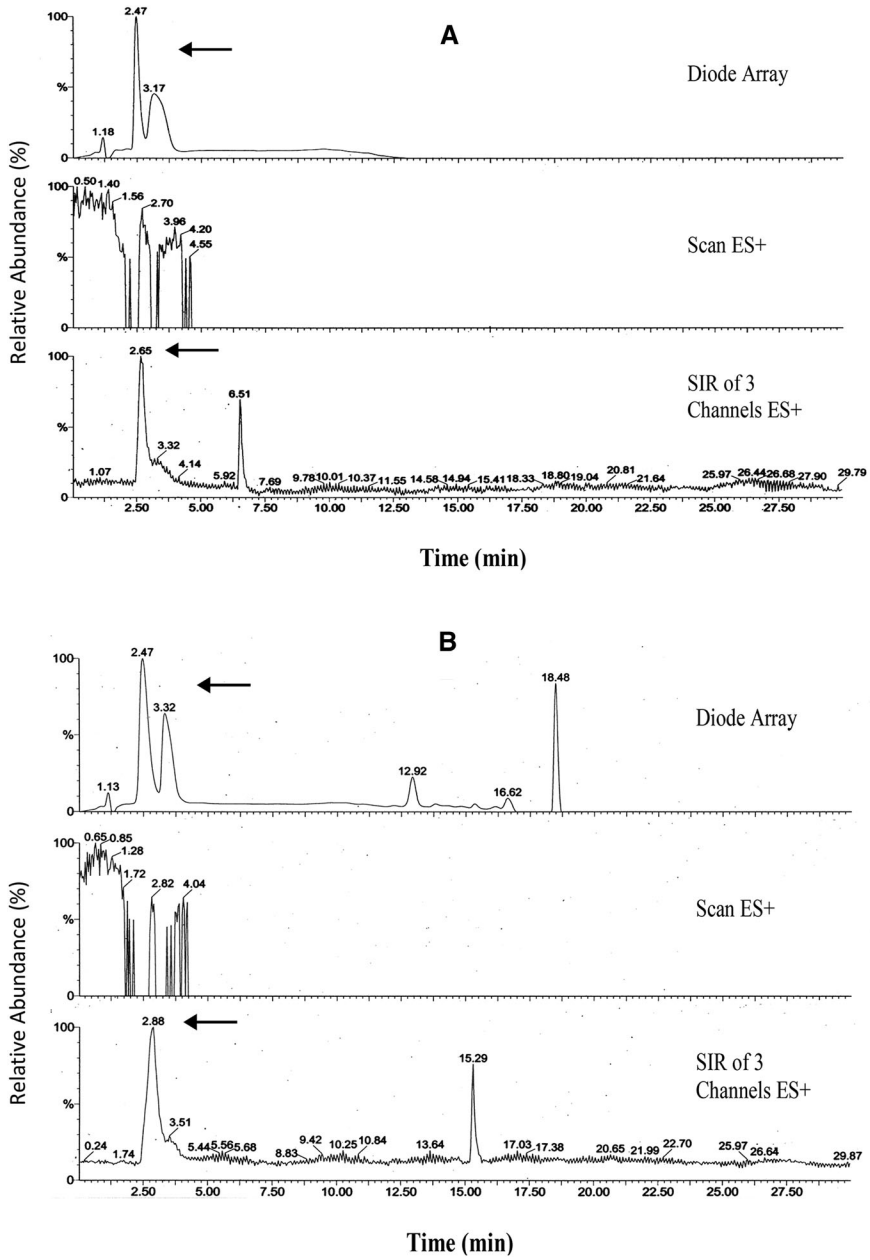

Fig. 3 Chromatograms of high-performance liquid chromatography coupled with mass spectroscopy (LC-MS). Panels **A** and **B** are the LC-MS chromatograms of 2-hydroxy-4-ethylamino-6-amino-*s*-triazine and the unknown sample (Met 4 from TLC analysis of transgenic tall fescue), respectively. Abbreviations: ES+, positive electrospray ionization; SIR, single ion recording

the primary products of *N*-dealkylation that may be associated with cytochrome P450 activity. Burken and Schnoor (1997) studied atrazine uptake and degradation in hybrid poplar, but did not detect the presence of OEAT in poplar tissue. Shimabukuro (1968) indicated that *N*-dealkylation of hydroxyatrazine occurs in corn and sorghum with higher levels of deethylhydroxyatrazine than deisopropylhydroxyatrazine. However, Lin et al. (2008) performed a field lysimeter study with five forage grasses and indicated that the predominant atrazine metabolites in tall fescue were hydroxylated atrazine degradation products, and the major degradation product of atrazine degradation in tall fescue was OEAT.

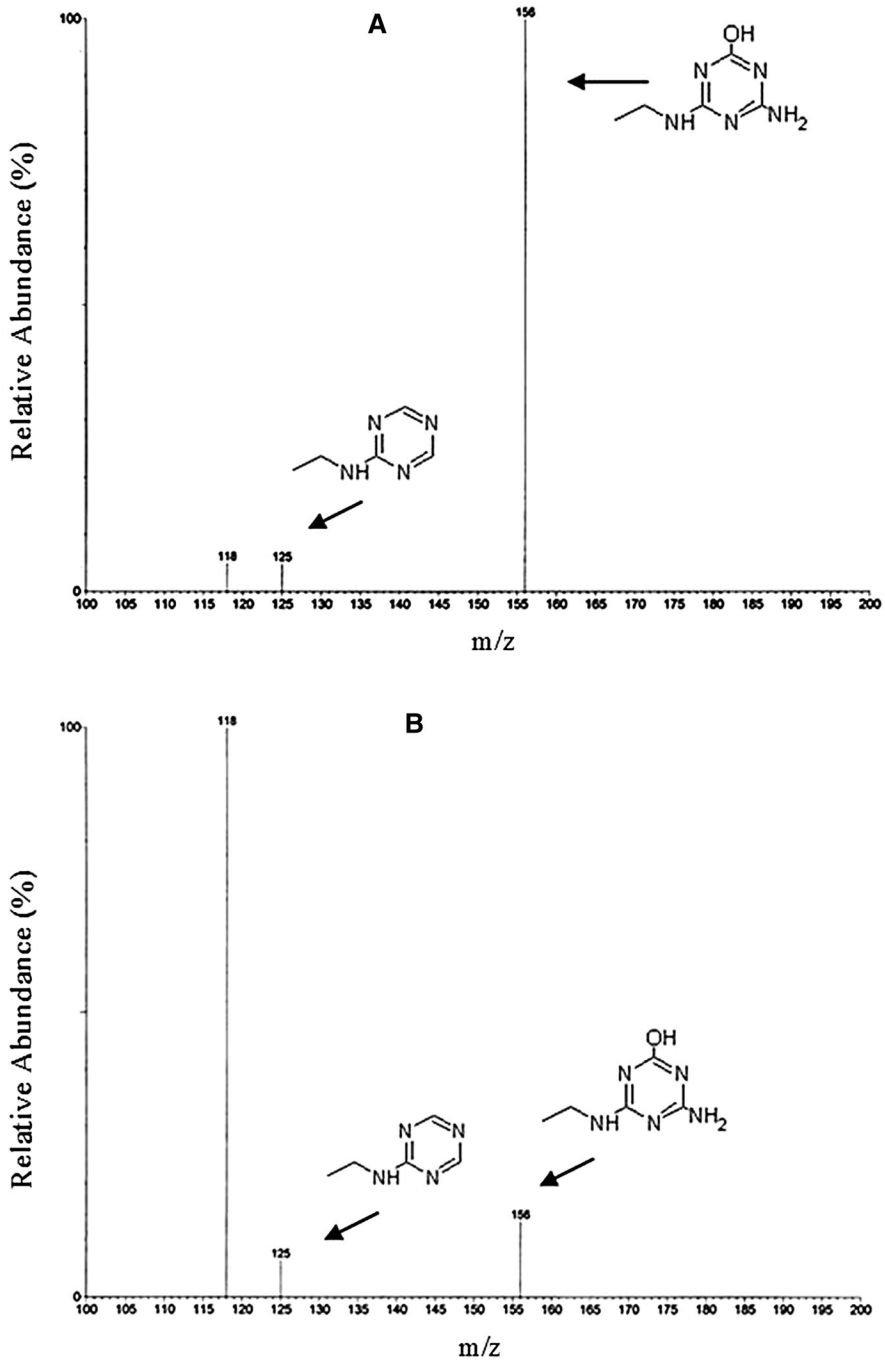

Fig. 4 The LC/MS single ion recording (SIR) chromatograms. Panels **A** and **B** are the LC/MS SIR of three channel positive electrospray ionization of 2-hydroxy-4-ethylamino-6-amino-*s*-triazine and the unknown sample (Met 4 from TLC analysis of transgenic tall fescue), respectively, at the retention time of 2.725 min

Hydroxyatrazine was found in the plant nutrient reservoir of transgenic tall fescue and alfalfa, but was not seen in WT controls. Data shown in Fig. 2, and our previous results shown in Wang et al. (2005), showed that WT plants do not metabolize atrazine to hydroxyatrazine, and thus cannot excrete this metabolite to

the nutrient reservoir. Similarly, Kawahigashi et al. (2003) reported transgenic rice plants expressing human *CYP1A1* excreted atrazine metabolic intermediates and these were found to be at a greater concentration in the culture medium than in the plants themselves. Likewise, Uchida et al. (2005) observed a greater concentration of 1-butanol, the by-product of DhaA catalyzed degradation of 1-chlorobutane, in hydroponic medium, but DhaA activity was not detected. Conversely, Wang et al. (2005) reported relatively little hydroxyatrazine present in the plant nutrient reservoir, which may have been due to the short incubation time (3 days) that was used in their studies. However, hydroxyatrazine is non-herbicidal. Also, hydroxyatrazine shows a greater propensity to sorb to soil than does atrazine (Moreau and Mouvet 1997) and less substituted *s*-triazines are more readily metabolized by soil microorganisms. Coupled with the idea that plants can stimulate microbial metabolism of organic pollutants, these results suggest that the transgenic plants not only take up and dechlorinate atrazine, but may also help increase the degradation of atrazine by soil microorganisms. However, further studies are needed to determine if this occurs at an environmentally significant rate.

Although transgenic alfalfa lines containing a substrate-specific bacterial dehalogenase gene have been previously constructed (Wang et al. 2005), the new transgenic alfalfa lines reported here containing p-*atzA* under the control of the cassava mosaic virus promoter with the 5′-UTR of *NtADH*, had greater growth responses in the presence of atrazine, indicating greater degradation activity than the previously developed lines (Wang et al. 2005). Satoh et al. (2004) reported that the *NtADH* 5′-UTR enhances foreign gene expression in dicotyledonous plants. Thus, the newly created transgenic lines containing the *NtADH* 5′-UTR may have a greater tolerance to atrazine and degradation ability due to enhanced p-*atzA* gene expression.

The capability of each transgenic grass line constructed in this study for biodegradation of atrazine in soil and the fate of the degraded atrazine will be tested in the near future. The synergy of these transgenic plants and the associated rhizobacteria during bioremediation of atrazine in soil also needs to be investigated (Segura et al. 2009; Segura and Ramos 2013). Moreover, in the future, plant lines with highest efficiency of atrazine biodegradation need to be selected in order to develop vegetative buffer strips (VBS) for atrazine filtering, degradation, and removal from the environment.

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