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# Biodegradation of atrazine in transgenic plants expressing a modified bacterial atrazine chlorohydrolase (*atzA*) gene

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## Summary

Atrazine is one of the most widely used herbicides in the USA. Atrazine chlorohydrolase (AtzA), the first enzyme in a six-step pathway leading to the mineralization of atrazine in Gram-negative soil bacteria, catalyses the hydrolytic dechlorination and detoxification of atrazine to hydroxyatrazine. In this study, we investigated the potential use of transgenic plants expressing *atzA* to take up, dechlorinate and detoxify atrazine. Alfalfa, *Arabidopsis thaliana* and tobacco were transformed with a modified bacterial *atzA* gene, p-*atzA,* under the control of the cassava vein mosaic virus promoter. All transgenic plant species actively expressed p-*atzA* and grew over a wide range of atrazine concentrations. Thin layer chromatography analyses indicated that *in planta* expression of p-*atzA* resulted in the production of hydroxyatrazine. Hydroponically grown transgenic tobacco and alfalfa dechlorinated atrazine to hydroxyatrazine in leaves, stems and roots. Moreover, p-*atzA* was found to be useful as a conditional-positive selection system to isolate alfalfa and *Arabidopsis* transformants following *Agrobacterium*-mediated transformation. Our work suggests that the *in planta* expression of p-*atzA* may be useful for the development of plants for the phytoremediation of atrazine-contaminated soils and soil water, and as a marker gene to select for the integration of exogenous DNA into the plant genome.

## Introduction

Atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine] is a widely used herbicide for the control of broadleaf weeds in corn and sugarcane. It is the predominant member of a broad class of *s*-triazine herbicides, and approximately 76–100 million pounds of atrazine are applied annually in the USA. Atrazine is transformed relatively slowly in the environment (Erickson and Lee, 1989), with an average half-life ranging from 4 to 57 weeks (Anderson and Georgeson, 1989), and has been detected in soils as well as in ground and surface water in several countries (Belluck *et al*., 1991).

In *Pseudomonas* sp. strain ADP (Mandelbaum *et al*., 1995), as in many other Gram-negative atrazine-degrading soil bacteria (de Souza *et al*., 1998a; Sadowsky and Wackett, 2000; Wackett *et al*., 2002), atrazine catabolism is initiated by a plasmid-borne *atzA* gene (Martinez *et al*., 2001), encoding atrazine chlorohydrolase (AtzA). AtzA catalyses the hydrolytic dechlorination of atrazine, resulting in the production of hydroxyatrazine as the first intermediate in the degradation pathway (Figure 1). Hydroxyatrazine is non-herbicidal (Gysin and Knuesli, 1960), more biodegradable (Goswami and Green, 1971), non-toxic and more strongly sorbed to soils than atrazine (Clay and Koskinen, 1990). AtzA, which has been

 

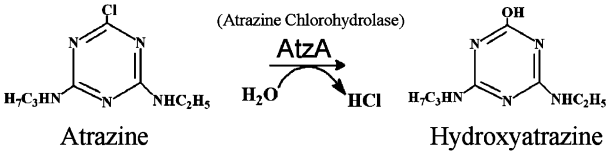


**Figure 1** Initiation reaction for the degradation of atrazine. Atrazine chlorohydrolase, encoded by the *atzA* gene in *Pseudomonas* sp. strain ADP, hydrolytically dechlorinates atrazine to hydroxyatrazine, a non-herbicidal product.

over-expressed in *Escherichia coli* and purified to homogeneity, is an Fe(II) metalloenzyme belonging to the amidohydrolase superfamily, which includes urease and cytosine deaminase (Seffernick *et al*., 2002; Wackett *et al*., 2002). AtzA catalyses the dechlorination of the herbicides atrazine, simazine and terbutylazine and the metabolites desethylatrazine and deisopropylatrazine (de Souza *et al*., 1996; Sadowsky and Wackett, 2001; Seffernick *et al*., 2000).

Several bacterial genes have been transformed into plants with the goal of enhancing tolerance to pesticides. Herbicide-tolerant plants have been produced by introducing bacterial genes encoding target proteins that are insensitive to herbicides, and by directly incorporating herbicide detoxification genes into plants (Mullineaux, 1992). For example, transgenic plant lines that have been transformed with the *bxn* (Stalker *et al*., 1988), *tfdA* (Streber and Willmitzer, 1989), *aroA* (Comai *et al*., 1985), *bar* and *pat* (Lutz *et al*., 2001), glutamylcysteine synthetase (a glutathione *S*-transferase precursor enzyme) (Gullner *et al*., 2001) and human and soybean cytochrome P450 monooxygenase (Siminszky *et al*., 1999; Bode *et al*., 2003) genes are resistant to herbicides. Likewise, tobacco plants transformed with a chimeric *psbA* gene are resistant to atrazine (Cheung *et al*., 1988), and the *Arabidopsis* protoporphyrinogen oxidase (*ppox*) gene renders tobacco tolerant to diphenylether herbicides (Lermontova and Grimm, 2000).

To date, however, only a few plants have been genetically engineered to degrade herbicides for the purpose of cleaning up the environment. Such phytoremediation approaches have received recent attention as lower cost and environmentally friendly alternatives to remediate pollutant-contaminated soils and water (Burken and Schnoor, 1997; Dietz and Schnoor, 2001). Although there have been several reports concerning the engineering of plants for the phytoremediation of metal-impacted environments (Bizily *et al*., 1999; Ruiz *et al*., 2003; Singh *et al*., 2003), fewer studies have been carried out on the production of transgenic plants designed to degrade and detoxify chlorinated and other organic compounds. Recently, Hannink *et al*. (2001) reported that tobacco plants transformed with a bacterial nitroreductase degraded and detoxified trinitrotoluene (TNT), and French *et al*. (1999) demonstrated that tobacco plants expressing a bacterial pentaerythritol tetranitrate reductase gene detoxified TNT and glycerol trinitrate. Although Shimizu *et al*. (2002) reported that transgenic rice plants containing a bacterial *cbnA* gene, encoding chlorocatechol dioxygenase, degraded 3-chlorocatechol, the transformed plants failed to dechlorinate, and produced 2-chloromuconate as a product. Similarly, transgenic tobacco plants containing the human cytochrome P450 2E1 gene transformed trichloroethylene, but only to the chlorinated products trichloroacetaldehyde and trichloroethanol (Doty *et al*., 2000). Recently, Bode *et al*. (2003) reported that tobacco cell cultures transformed with the human cytochrome P450 enzymes, CYP1A and CYP1A2, transformed atrazine to the chlorinated products deisopropylatrazine, deethylatrazine and deethyldeisopropylatrazine.

The production of transgenic plants often requires the use of marker gene(s) for the selection of transformants following the introduction of foreign genes. These marker genes can be incorporated directly into the transformation vector containing the transgene, or indirectly used for cotransformation purposes. Although the majority of commercially produced transgenic plant varieties have used kanamycin, phosphinothricin or hygromycin as selectable markers, genes for herbicide resistance, and those encoding enzymes for the detoxification of metabolic analogues and other toxic compounds, can also be used for the conditional-positive and conditional-negative selection of transgenic plants (Erikson *et al*., 2004; Miki and McHugh, 2004). Issues of biosafety and toxicity in the use of human therapeutic agents often dictates which selectable markers are useful for plant transformation studies and for commercial applications. Consequently, phosphinothricin, glyphosate (Zhou *et al*., 1995), sulphonylurea (Olszewski *et al*., 1988), imidazolinone (Aragao *et al*., 2000), bromoxynil (Freyssinet *et al*., 1996), cyanamide (Weeks *et al*., 2000) and other herbicides and pesticides will most probably continue to be used as conditional selectable markers in plant transformation applications. As future plant biotechnological applications will probably continue to use cotransformation strategies, and require the pyramiding (Miki and McHugh, 2004) of multiple transgenes into plants, additional non-interacting selectable markers will be required.

In this study, transgenic alfalfa (*Medicago sativa*), *Arabidopsis thaliana* and tobacco (*Nicotiana tabacum*) plants maintained and expressed a modified bacterial *atzA* gene. Moreover, all three transgenic plant species grew in the presence of atrazine at levels killing wild-type parent plants. Tobacco and alfalfa plants transformed this herbicide to

hydroxyatrazine, a non-herbicidal and more easily degradable product. To our knowledge, this is the first report describing transgenic plants containing a single bacterial gene that has the ability to selectively and specifically dechlorinate atrazine and related chlorinated *s*-triazines using a hydrolytic mechanism. Moreover, the p-*atzA* gene was found to be useful as a conditional-positive selection system to isolate *Arabidopsis* transformants following *Agrobacterium*-mediated transformation. As such, this system holds promise as a means to phytoremediate atrazine-impacted environments and as a marker gene system to select for the integration of exogenous DNA into the plant genome.

## Results

Initial alfalfa transformation studies performed using wild-type *atzA* downstream of the cauliflower mosaic virus (CaMV) 35S promoter indicated that the bacterial gene failed to express *in planta* (data not shown). However, the *atzA* gene used in the subsequent studies reported here, p-*atzA,* was modified from the original wild-type bacterial *atzA* by changing 359 nucleotides (representing changes to 312 codons) to promote efficient translation, relative to codon usage in alfalfa (Figure 2). The p-*atzA* gene was also modified to contain a plant-like consensus sequence, ACC, before the translation start codon (Kozak, 1991), *Xba*I and *Bam*HI restriction enzyme sites and a TAA stop codon. The modified *atzA* gene was directionally cloned downstream of the cassava vein mosaic virus (CsVMV) promoter to produce pPW1 (Figure 3). This promoter has been shown to give high-level constitutive expression of several genes in alfalfa (Samac *et al*., 2004) and other plants (Verdaguer *et al*., 1996). Owing to substantial codon changes, p-*atzA* failed to express in *E. coli* or *Agrobacterium* strains, as evidenced by plate clearing (de Souza *et al*., 1995) and high-performance liquid chromatography (HPLC) assays.

### Incorporation and expression of p-*atzA* in $T_0$ transgenic plants

Following selection on kanamycin-containing medium and plant regeneration, transformed alfalfa and tobacco plants

```
                1                                                                                                  100
p-atzA  TCTAGACCCG GGACCATGCA AACTTTGTCC ATTCAACATG GCACTTTGGT AACAATGGAT CAGTATCGCA GAGTTTTGGG TGACTCATGG GTTCATGTGC
wt-atzA                .....  ...GC.CAG. ..C..G..C. .T..CC.C.. C..G...... .....C.... ....CC.T.. G..TAGC... .....C....
              101                                                                                                  200
AAGATGGACG TATTGTTGCA CTCGGTGTTC ATGCTGAAAG CGTGCCTCCT CCTGCAGACA GGGTCATAGA TGCCAGGGGT AAGGTTGTGT TACCCGGTTT
.G........ G..C..C..G .....A..G. .C..C..GTC G........G ..A..G..TC ....G..C.. ...AC.C..C .....C.... ..........
              201                                                                                                  300
CATCAACGCT CATACTCATG TCAACCAGAT TCTACTTAGA GGAGGTCCCT CTCACGGGAG GCAATTCTAC GATTGGCTGT TTAATGTCGT TTACCCAGGG
......T..C ..C..C.... .G........ C..C..GC.C .....G.... .G......C. T........T ..C....... .C..C..T.. G..T..G..A
              301                                                                                                  400
CAAAAGGCTA TGAGGCCTGA AGATGTTGCT GTTGCTGTGC GCTTGTACTG TGCAGAGGCA GTTCGCTCTG GAATAACTAC TATAAATGAG AATGCCGACT
........G. ....A..G.. G..C..A..G ..G..G...A .G.....T.. ...G..A..T ..G...AGC. .G..T..G.. G..C..C..A ..C.....T.
              401                                                                                                  500
CCGCTATTTA TCCAGGAAAT ATTGAAGCAG CTATGGCAGT ATACGGCGAA GTTGGTGTGA GAGTTGTGTA CGCTCGTATG TTCTTTGATA GGATGGATGG
.G..C..C.. C.....C..C ..C..G..C. .G.....G.. C..T..T..G ..G....... .G..C..C.. ...C..C... .........C .......C..
              501                                                                                                  600
TAGGATTCAG GGGTATGTTG ATGCTCTCAA GGCTAGATCT CCACAAGTTG AGTTATGCAG TATTATGGAA GAAACAGCCG TCGCCAAGGA TCGTATCACT
GC.C.....A ........G. .C..CT.G.. ....C.C... ..C.....C. .AC.G...TC G..C.....G .....G..T. .G.....A.. ...G.....A
              601                                                                                                  700
GCTCTGTCCG ATCAATATCA TGGAACAGCC GGAGGAAGAA TTTCAGTATG GCCAGCTCCA GCTACTACAA CTGCTGTGAC CGTTGAAGGA ATGCGCTGGG
..C.....A. ....G..... ...C..G..A .....TC.T. .A.....T.. ...C.....T ..C.....C. .G..G..... A......... .....A....
              701                                                                                                  800
CACAGGCCTT TGCTAGAGAT AGAGCTGTTA TGTGGACCCT GCACATGGCA GAGTCTGATC ATGATGAGAG AATACATGGA ATGTCCCCTG CAGAGTACAT
....A..... C..CC.T... C.G..G..A. .......G.. T........G ...AGC.... ........C. G..T.....G ...AGT..C. .C........
              801                                                                                                  900
GGAGTGTTAT GGCCTTCTTG ATGAAAGGTT ACAAGTAGCC CACTGTGTGT ATTTTGATAG AAAAGACGTG AGACTACTTC ACCGTCACAA CGTCAAGGTT
.........C ..A..CT.G. ....GC.TC. G..G..C..G ..T..C.... .C.....CC. G..G..T..T C.G..G..G. ....C..... T..G.....C
              901                                                                                                 1000
GCTTCACAGG TAGTTAGCAA TGCTTATCTT GGTAGTGGGG TTGCACCTGT ACCTGAAATG GTGGAACGTG GCATGGCTGT TGGAATCGGT ACTGACAATG
..G..G.... .T..G..... ...C..C..C ..CTCA.... .G..C..C.. G..A..G... .....G..C. .......C.. G..C..T..A ..A..T..C.
             1001                                                                                                 1100
GTAATAGCAA CGATTCAGTG AATATGATCG GTGACATGAA ATTCATGGCT CATATTCATA GGGCTGTTCA CCGTGATGCA GATGTCTTGA CACCAGAAAA
.G.....T.. T..C..C..A ..C....... .A........ G..T.....C ........CC .C..G..G.. T..G.....G ..C..GC... .C.....G..
             1101                                                                                                 1200
AATTCTTGAA ATGGCTACAA TTGATGGTGC TCGTTCTCTT GGAATGGATC ACGAAATCGG TAGTATCGAA ACTGGAAAAA GGGCAGATTT GATACTCCTA
G......... .....G..G. .C.....G.. G.....GT.G ........C. ....G..T.. .TCC...... ..C..C..GC .C..G..CC. T..C..G..T
             1201                                                                                                 1300
GACCTTCGTC ACCCTCAGAC CACCCCACAT CATCATTTAG CAGCAACAAT TGTTTTTCAA GCATATGGTA ACGAAGTCGA CACAGTTTTG ATCGACGGAA
.....G.... .......... G..T..T..C ........G. .G..C..G.. C..G.....G ..T..C..C. .T..G..G.. ...T..CC.. ..T.......
             1301                                                                                                 1400
ACGTTGTTAT GGAGAATCGC CGTCTCTCAT TCTTGCCTCC AGAGCGTGAG CTTGCATTTC TCGAGGAGGC CCAATCTAGG GCAACCGCTA TTTTGCAAAG
.......G.. ......C... ..CT.GAGC. .TC.T..C.. T..A...... T.G..G..C. .T.....A.. G..GAGCC.C ..C..A.... .......GC.
             1401                                          1452
AGCAAACATG GTGGCTAATC CAGCATGGAG GAGTCTTTAA GAGCTCGGAT CC
G..G...... ........C. ....T...C. C..C..C..G
```

**Figure 2** Sequence of *p-atzA*. The bacterial wild-type *atzA* gene was modified according to plant codon usage and named p-*atzA*. A total of 359 nucleotides were changed, representing changes to 312 codons. Only changed nucleotides are indicated, and those represented by a dot are the same in both wild-type *atzA* and modified p-*atzA* sequences. Sequences upstream and downstream of the translation start and stop codons (shown in bold) are *Xba*I and *Bam*HI restriction enzyme sites, respectively, added to facilitate cloning.

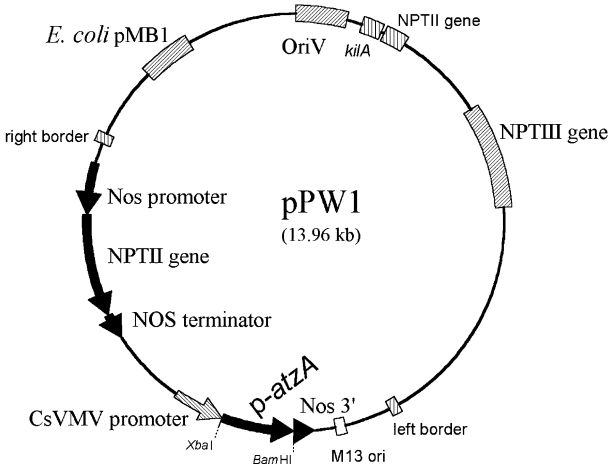


**Figure 3** Plant transformation vector pPW1. The modified *p-atzA* gene was directionally cloned into the *Xba*I and *Bam*HI restriction sites of the binary vector pILTAB357 to yield plasmid pPW1. Gene expression *in planta* is driven by the cassava vein mosaic virus (CsVMV) promoter.

were evaluated for the presence of p-*atzA*. Polymerase chain reaction (PCR) analyses performed using primers specific for p-*atzA* indicated that 13 of 18 (72%) and 27 of 28 (96%) of $T_0$ tobacco and alfalfa plants, respectively, contained p-*atzA*. Only two of 10 *Arabidopsis* seed lines (R4 and R10) that were generated from the *Agrobacterium*-mediated floral-dip transformation method germinated and survived on agar medium containing kanamycin; both tested positive for p-*atzA* by PCR. Expression analyses, performed using reverse transcriptase (RT)-PCR, indicated that 69% (nine of 13), 96% (26 of 27) and 100% (two of two) of the transgenic $T_0$ tobacco, alfalfa and *Arabidopsis* lines, respectively, expressed p-*atzA in planta*.

Western blot analysis of soluble proteins from tobacco $T_0$ plant lines indicated that the AtzA protein was present in crude protein extracts, but in low abundance (data not shown). Although no antibody-reactive proteins were detected in the transgenic alfalfa lines, subsequent thin layer chromatography (TLC) analyses indicated AtzA activity in crude extracts from alfalfa and tobacco lines (see below).

## Transformed alfalfa, tobacco and *Arabidopsis* plants expressing p-*atzA* are resistant to atrazine

Seeds of transgenic tobacco and *Arabidopsis* plant lines, and regenerated alfalfa plantlets from $T_0$ plants expressing p-*atzA*, were evaluated for their ability to grow in the presence of high concentrations of atrazine in agar medium. The results in Figure 4 show that the transgenic lines were able to toler-

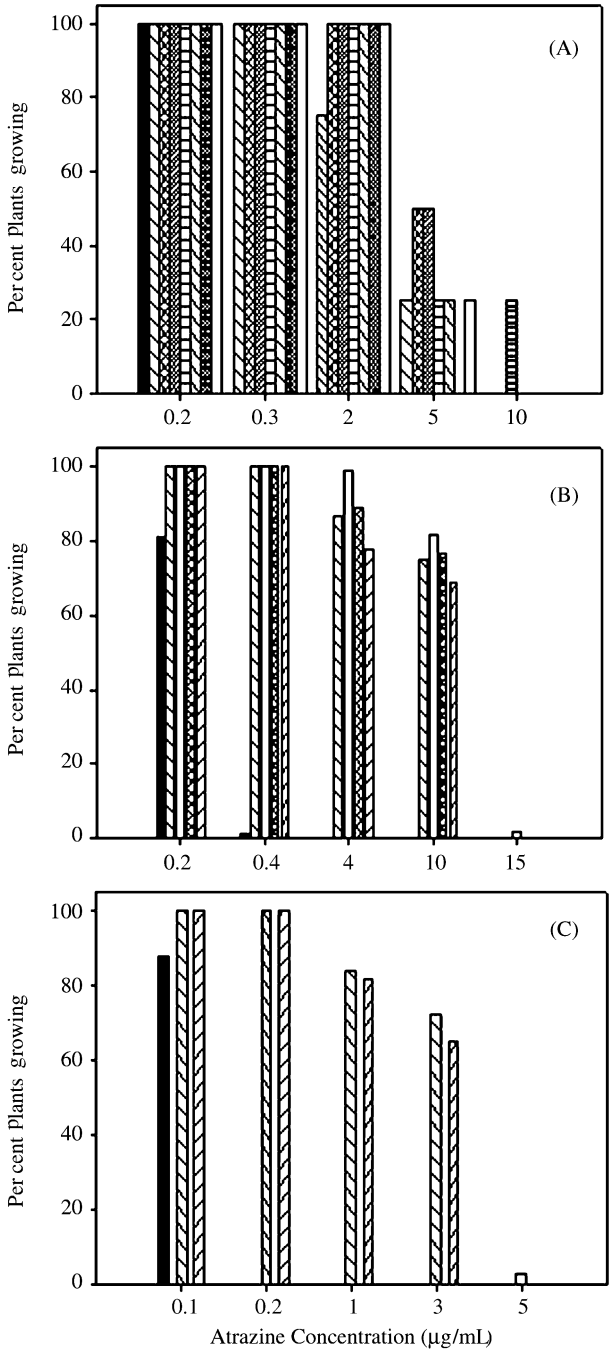


**Figure 4** Atrazine toxicity studies. Growth responses of (A) wild-type (WT) and transgenic alfalfa, (B) WT and transgenic tobacco and (C) WT and transgenic *Arabidopsis* to different concentrations of atrazine in agar medium. The percentage of each plant type surviving is indicated. (A) Alfalfa: wild-type parent ■ and transgenic lines A1 ▨, A2 ▩, A3 ▨, A4 ▤, A5 ▨, A6 ▩ and A13 □. (B) Tobacco: wild-type parent ■ and transgenic lines P9 ▨, P10 □, P12 ▩ and P16 ▨. (C) Arabidopsis: wild-type parent ■ and transgenic lines AR10 ▨ and AR4 ▨.

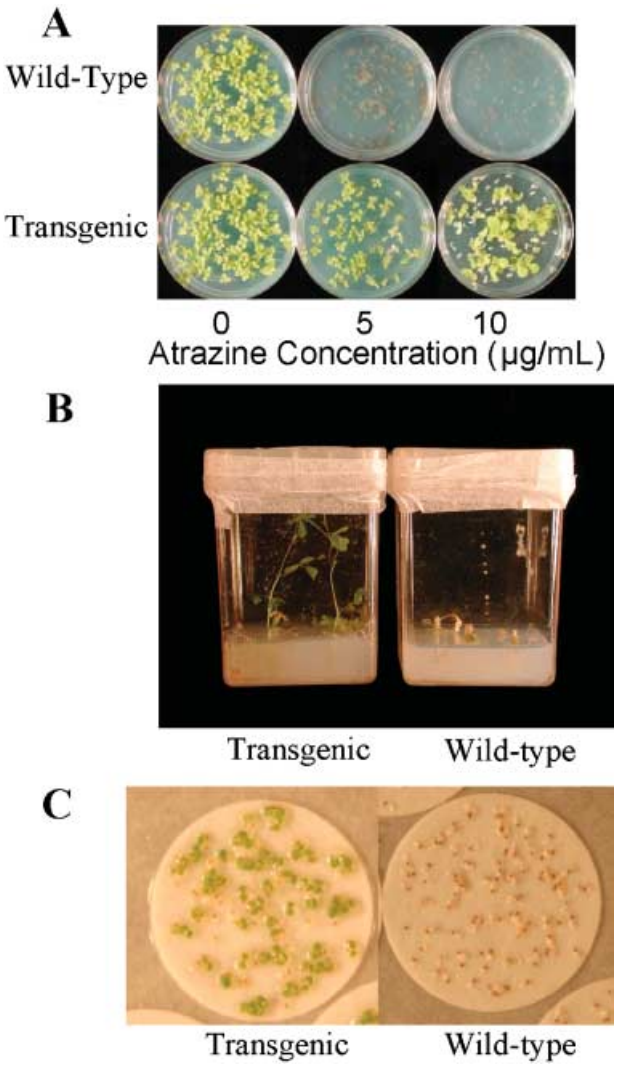


**Figure 5** Resistance of transgenic tobacco, alfalfa and *Arabidopsis* plants to growth in the presence of atrazine. (A) Transgenic tobacco line P10 and wild-type (WT) plants growing in medium containing 0, 5 or 10 μg/mL atrazine. (B) Transgenic alfalfa line A3 (left) and WT (right) plants growing in medium containing 5 μg/mL atrazine. (C) Transgenic *Arabidopsis* line R4 (left) and WT (right) plants grown in the presence of 3 μg/mL atrazine.

ate a significantly higher concentration of atrazine relative to the wild-type parent plants. The best transgenic $T_0$ lines of tobacco, alfalfa and *Arabidopsis* were able to survive and grow in the presence of 15, 10 and 5 μg/mL atrazine, respectively, whereas wild-type tobacco, alfalfa and *Arabidopsis* plants survived only in medium containing 0.4, 0.2 and 0.1 μg/mL atrazine, respectively. This represents a 38-, 50- and 50-fold increase in atrazine tolerance for tobacco, alfalfa and *Arabidopsis*, respectively, relative to wild-type plants. The typical results in Figure 5 show the response of wild-type and transgenic tobacco plants to 0, 5 or 10 μg/mL atrazine, alfalfa plants to 5 μg/mL atrazine and *Arabidopsis* plants to 3 μg/mL atrazine.

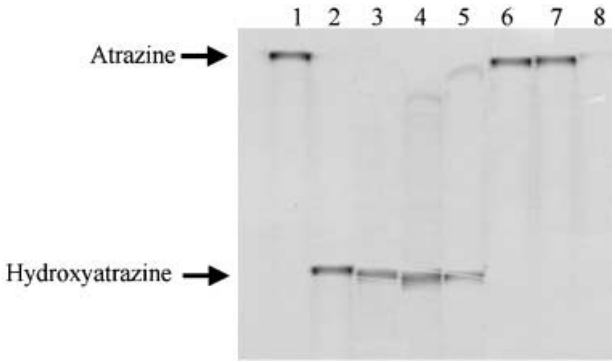


**Figure 6** *In vitro* activity of p-AtzA. Cell-free extracts from leaf, root and stem samples of transgenic tobacco line P10 and wild-type (WT) tobacco were analysed for atrazine dechlorination using $^{14}$C-UL-ring-atrazine and thin layer chromatography (TLC) analyses. All samples contained $^{14}$C-UL-ring-atrazine. Lanes: (1) control without cell extract; (2) cell-free extract from *E. coli*(*atzA*); (3–5) cell-free extracts from P10 leaves, stems and roots, respectively; (6) cell-free extract from WT tobacco leaves; (7) cell-free extract from WT tobacco stems; (8) cell-free extract from WT tobacco roots.

### *In vitro* activity of AtzA

To link the growth responses of transgenic plants to the detoxification of atrazine, we investigated whether cell-free extracts obtained from transgenic $T_1$ seedlings from tobacco line P10 plants growing on an agar medium would dechlorinate $^{14}$C-UL-ring-atrazine to hydroxyatrazine. The results of TLC analyses showed that cell-free extracts from leaves, stems and roots of transgenic tobacco plants were capable of dechlorinating atrazine to hydroxyatrazine (Figure 6). The identity of the hydroxyatrazine product was verified in TLC by comigration with an authentic radiolabelled standard, and by mass spectrometry [*m/z*(M + 1) = 198]. No transformation activity was seen when cell-free extracts from wild-type tobacco leaves, stems or roots were incubated with $^{14}$C-labelled atrazine. The extracts from the leaves of tobacco line P10 dechlorinated all of the added atrazine, whereas a small amount of residual labelled parent compound was seen when using extracts from P10 stems and roots (Figure 6). We obtained similar results using other tobacco lines. These results indicate that the transgenic lines produce active p-AtzA, and also suggest that AtzA activity may be greater in leaves than in stems or roots.

### Hydroponic growth of transgenic tobacco and alfalfa plants and *in vivo* p-AtzA activity

Transgenic tobacco (P9 and P10) and alfalfa (A1 and A3) were grown hydroponically in the presence of 0.5 μg/mL

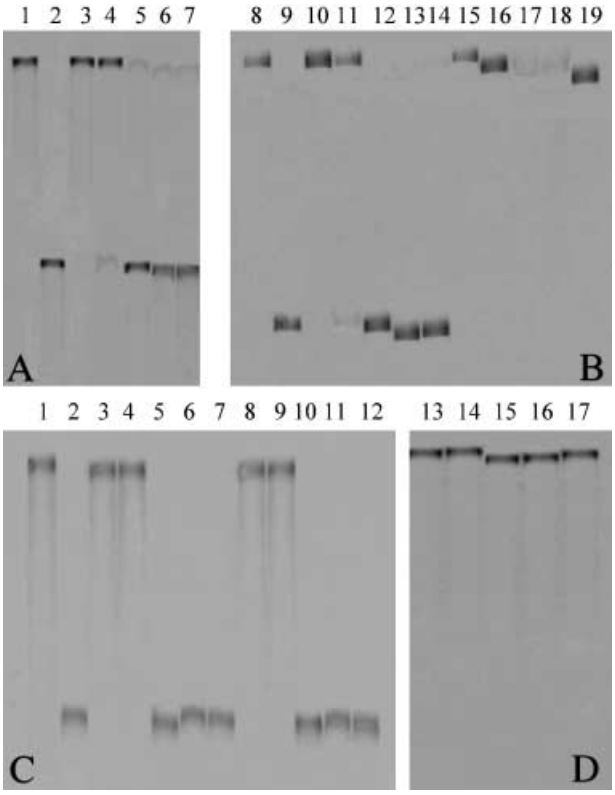


**Figure 7** *In planta* activity of p-AtzA. Thin layer chromatography (TLC) analyses of tissue extracts from transgenic and wild-type (WT) alfalfa and tobacco plants hydroponically grown in the presence of $^{14}$C-UL-ring-atrazine. Panels A and B contain extracts from transgenic alfalfa lines A1 and A3, respectively. Lanes: (1) labelled atrazine control; (2) atrazine plus AtzA from *E. coli*(pMD4); (3) and (4) nutrient solution before and after plant A1 growth, respectively; (5–7) A1 leaves, stems and roots, respectively; (8) labelled atrazine control; (9) atrazine plus AtzA from *E. coli*(pMD4); (10) and (11) nutrient solution before and after A3 growth, respectively; (12–14) A3 leaves, stems and roots, respectively; (15) and (16) nutrient solution before and after WT alfalfa growth, respectively; (17–19) WT alfalfa leaves, stems and roots, respectively. Panels C and D contain tissue extracts from WT and transgenic tobacco lines P9 and P10. Lanes: (1) labelled atrazine control; (2) atrazine plus AtzA from *E. coli*(pMD4); (3) and (4) nutrient solution before and after plant P9 growth, respectively; (5–7) P9 leaves, stems and roots, respectively; (8) and (9) nutrient solution before and after P10 growth, respectively; (10–12) P10 leaves, stems and roots, respectively; (13) and (14) nutrient solution before and after WT tobacco growth, respectively; (15–17) WT tobacco leaves, stems and roots, respectively.

$^{14}$C-UL-ring-atrazine, and evaluated for their ability to take up and dechlorinate atrazine to hydroxyatrazine. Both transgenic alfalfa and tobacco plants had the ability to grow in the presence of 0.5 μg/mL atrazine, whereas the wild-type parent plants died (data not shown). Moreover, TLC and liquid scintillation studies performed using leaf, stem and root extracts from hydroponically grown transgenic alfalfa (A1 and A3) and tobacco (P9 and P10) lines indicated that p-AtzA activity was present in all plant samples examined (Figure 7).

**Table 1** Primary selection of p-*atzA* in *Arabidopsis* transformants using agar and soil selection systems

| *Arabidopsis* line | Seeds analysed | Plantlets surviving | Percentage survival | Percentage p-*atzA* in survivors |
|---|---|---|---|---|
| Agar plate selection | | | | |
| R4* | 500 | 382 | 76.4 | 95 |
| R10 | 500 | 371 | 74.2 | 100 |
| Wild-type | 500 | 0 | 0 | –† |
| Soil-spray selection | | | | |
| R4 | 50 | 38 | 76 | 93 |
| R10 | 50 | 34 | 68 | 100 |
| Wild-type | 50 | 0 | 0 | – |

*R4 and R10 refer to transformed *Arabidopsis* lines.
†Not determined.

Densitometric analyses of TLC plates and liquid scintillation counting of extracts and plant growth solutions revealed that hydroxyatrazine accounted for 98.1 ± 0.3%, 98.6 ± 0.1% and 82.9 ± 0.3% of the accumulated counts in transgenic tobacco leaves, stems and roots, respectively, and 94 ± 1.5%, 91.4 ± 0.8% and 85.7 ± 1.4% of the accumulated counts in transgenic alfalfa leaves, stems and roots, respectively. Hydroxyatrazine accounted for only 1.3 ± 0.5% and 4.9 ± 0.9% of the counts in the plant growth solution, following growth of tobacco and alfalfa plants, respectively, indicating that the majority of dechlorination activity takes place *in planta*, and that little hydroxyatrazine leaches into the environment.

### Selection of transformants using p-*atzA*

The growth of seeds on agar medium containing atrazine and the spraying of plantlets with atrazine following growth in soil were effective methods for the selection of *Arabidopsis* transformants following *Agrobacterium*-mediated transformation with p-*atzA* (Table 1). Results from the agar plate and plantlet-spraying methods showed that about 68%–76% of the seeds from transformed lines were capable of germinating and growing in the presence of atrazine, whereas no resistant plants were detected when wild-type seeds were examined. PCR analyses indicated that 95%–100% of the tested survivors contained p-*atzA*, indicating that the selection methods were fairly efficient in ensuring only a limited number of escapes in these lines (Table 1). Agar plates containing 1.5 μg/mL atrazine were also effective in differentiating transgenic from wild-type alfalfa lines. After 3 weeks of growth on modified B5h selection medium, calli from transgenic alfalfa line A3 began to produce embryos, whereas calli from wild-type alfalfa appeared dried out and brownish in colour (data not shown).

## Discussion

Atrazine and simazine are widely used herbicides for the control of broad-leaf weeds and, owing to their widespread use, these *s*-triazine herbicides are sometimes detected in drinking water supplies and soil at levels exceeding the maximal concentrations set by the US Environmental Protection Agency (EPA). Although bioremediation strategies have been applied to clean up atrazine from soil (Strong *et al*., 2000), such approaches are not feasible when large areas are impacted, or if these herbicides have leached into the soil profile. In these cases, phytoremediation approaches may hold promise as a means to reduce the environmental impact of herbicide use, and the production of transgenic plants that actively degrade and detoxify *S*-triazine herbicides may be one way to achieve this goal.

The main purpose of this study was to determine whether transgenic plants expressing a bacterial AtzA were able to dechlorinate atrazine to the non-herbicidal compound hydroxyatrazine. To test this, we used *Agrobacterium*-mediated transformation to introduce wild-type bacterial *atzA,* under control of the CaMV 35S promoter, into alfalfa plants. Unfortunately, these plants failed to degrade atrazine, most probably due to a lack of adequate gene expression. To overcome this problem, we modified *atzA* for plant codon usage, which resulted in changes to 312 of the 474 codons, modified the context before the translation initiation codon and changed the translation stop codon. This more 'plant-friendly' construct, p-*atzA* (Figure 2), was introduced into three genetically divergent dicotyledonous plants: alfalfa, tobacco and *Arabidopsis*.

The p-*atzA* gene, under the control of the constitutive CsVMV promoter, was expressed in all three plant species, resulting in the production of transgenic tobacco, alfalfa and *Arabidopsis* plants that were capable of growing in the presence of 38, 50 and 50 times more atrazine, respectively, than the wild-type parent plants. Atrazine resistance was observed at a concentration 5–30 times greater than the normal field application rates, making these plants potentially useful for remediating contaminated soils. Interestingly, although p-*atzA* was expressed *in planta*, it was not expressed in *E. coli* and *Agrobacterium tumefaciens*, most probably due to incompatible codon usage issues. This is similar to that observed for a modified *bar* gene (Lutz *et al*., 2001), and has the advantage of reducing the potential of gene expression in non-target soil microorganisms if rare horizontal gene transfer events from plant to bacteria were to occur (Schluter *et al*., 1995).

Transgenic tobacco and alfalfa lines dechlorinated atrazine to hydroxyatrazine in leaves, stems and roots. Thus, in addition to growing in the presence of atrazine, both transgenic plants transformed the herbicide to a non-herbicidal compound. Although herbicide tolerance remains an effective strategy for crop production purposes, and atrazine-tolerant plants have been produced previously (Cheung *et al*., 1988), herbicide degradation and detoxification have obvious advantages for future phytoremediation technologies. Although results from our studies showed that hydroxyatrazine accumulates in these tissues, the ultimate fate of this compound *in planta* must await future studies. Nevertheless, as hydroxyatrazine is non-phytotoxic, its accumulation in plant tissues did not apparently harm the growth of any of the tested plant species over the time course of these experiments.

Although other transgenic plants that transform atrazine have been constructed previously (Inui *et al*., 1999, 2001; Yamada *et al*., 2002; Bode *et al*., 2003), to our knowledge, this is the first example of the incorporation and expression of a substrate-specific bacterial dechlorinating gene in plants that results in the complete detoxification of atrazine and its conversion to a single non-chlorinated product. Although trichloroethylene-transforming transgenic tobacco plants expressing mammalian cytochrome P450 2E1 have been reported, the primary degradation products were halogenated: chloral (2,2,2-trichloroacetaldehyde) and trichloroethanol (Doty *et al*., 2000). Similarly, Naested *et al*. (1999) reported that transgenic *Arabidopsis* plants expressing bacterial *dhlA* (haloalkane dehalogenase) hydrolysed 1,2-dichloroethane to a chlorinated product, and Shimizu *et al*. (2002) reported that transgenic rice plants expressing chlorocatechol dioxygenase (*cbnN*) transformed 3-chlorocatechol to the chlorinated metabolite 2-chloromuconate.

In the studies reported here, we have shown that atrazine is nearly completely detoxified *in planta*, resulting from its dechlorination to hydroxyatrazine, a non-herbicidal metabolite. This accounts for our observation of robust plant growth even in the presence of very high concentrations of atrazine. Previous efforts to produce transgenic atrazine-degrading plants using human P450 cytochrome oxidases have resulted in plants producing some herbicidal and non-herbicidal, but chlorinated, metabolites (Inui *et al*., 1999, 2001; Bode *et al*., 2003). Yamada *et al*. (2002) reported that transgenic potato plants expressing a rat P450 cytochrome monooxygenase (CYP1A1), under the control of the sucrose-inducible patatin promoter, transformed about 50% more atrazine than untransformed plants; however, the metabolites produced were not described.

Both RT-PCR and metabolic analyses indicated that the p-*atzA* transgene was expressed in leaves, stems and roots. This was expected as the CsVMV promoter used is known to

be constitutively expressed in many plant tissues (Verdaguer *et al*., 1996; Samac *et al*., 2004). This is in contrast with other dechlorinating enzymes that failed to adequately express in roots (Shimizu *et al*., 2002). Nevertheless, any atrazine escaping dechlorination in the root will most likely be transformed in leaves, the site of action of this herbicide (Arntzen *et al*., 1982). As greater than 90% of the labelled atrazine in leaves and stems was in the dechlorinated, non-herbicidal form, and triazine herbicides are taken up by plants with water via transpiration activity (Burken and Schnoor, 1997; Dietz and Schnoor, 2001), we propose that the majority of atrazine transported in water and transpired by all three transgenic plant species will be converted to hydroxyatrazine *in planta*. This, coupled with the fact that relatively little hydroxyatrazine was found in the plant nutrient solution reservoir, suggests that these and other transgenic plants containing p-*atzA* may prove to be useful in the phytoremediation of atrazine-impacted soil and water environments.

As only a very small proportion of plant cells are actually transformed following interaction with *Agrobacterium* or particle bombardment, both conditional-positive and conditional-negative selection strategies have been developed to select for transformants (Erikson *et al*., 2004; see Miki and McHugh, 2004). Although the majority of transformed plant lines have thus far been selected using kanamycin, phosphinothricin or hygromycin as marker genes (Miki and McHugh, 2004), future applications of plant biotechnology will require the use of several non-interacting and harmless selectable markers to introduce multiple genes into the plant genome. Thus, there is a need to increase the number and types of available marker genes for future plant transformation protocols (Birch, 1997). In the studies presented here, p-*atzA* was successfully used as a marker gene to allow conditional-positive selection of *Arabidopsis* transformants. Selection of *Arabidopsis* transformants was achieved by growing seedlings directly on agar medium containing atrazine, or in soil followed by spray application of atrazine to plantlets. The former method also appeared to be useful to select for alfalfa transformants from callus tissue. As atrazine disrupts the photosynthetic electron flow in diverse plant species, the p-*atzA* marker gene may find widespread use in the selection of transformants following the transformation of a variety of plants. Both agar and soil methods proved to be equally effective as a means to select for *Arabidopsis* transformants, allowing only a limited number of escapes. We postulate that this number may be further reduced by increasing the concentration of the selective agent, atrazine. As the costs associated with screening plant populations for undamaged transformants often exceeds the cost of transformation (Birch, 1997), it is desirable to have a selection strategy that is cost effective, easy to perform on large numbers of plants and does not result in unintended collateral genetic damage to selected populations. The use of the atrazine soil-spray selection strategy, in conjunction with the p-*atzA* marker gene, appears to satisfy all of these criteria.

## Experimental procedures

### Strains, plasmids and media used in these studies

*E. coli* strains were grown on Luria–Bertani (LB) medium (Sambrook *et al*., 1989) at 37 °C, with antibiotic selection as needed. *Agrobacterium tumefaciens* strain LBA4404 (Hoekema *et al*., 1983) was grown at 30 °C on LB medium containing 25 μg/mL rifampicin. *Agrobacterium tumefaciens* strain C58C1 (pMP90) (Koncz and Schell, 1986) was used for vacuum transformation of *Arabidopsis* and grown at 30 °C on yeast extract peptone (YEP) medium (Lichtenstein and Draper, 1986) containing 25 μg/mL gentamycin.

### Construction of pPW1

The atrazine chlorohydrolase gene, *atzA* (GenBank accession U55933), was obtained from *Pseudomonas* sp. strain ADP as described previously (de Souza *et al*., 1995). The *atzA* gene was modified for plant codon usage, now referred to as p-*atzA*, by Integrated DNA Technologies (Coralville, IA, USA). The modified *atzA* gene (Figure 2) was directionally cloned into the *Xba*I and *Bam*HI restriction sites of the binary vector pILTAB 357 (Verdaguer *et al*., 1996) behind the CsVMV promoter to yield plasmid pPW1 (13.96 kbp), which also contained the *nptII* gene controlled by the *nos* promoter for the selection of transgenic plants. Plasmid pPW1 was transformed (Sambrook *et al*., 1989) into *E. coli* DH5α and selected on LB medium containing 50 μg/mL kanamycin. For plant transformation, pPW1 was conjugated into *Agrobacterium tumefaciens* strains LBA4404 and C58C1 (pMP90) by triparental mating.

### Plant transformation

*N. tabacum* cv. Samsun, *M. sativa* cv. Regen SY (Bingham, 1991) and *A. thaliana* ecotype Columbia were used in all studies. Plants were grown in a growth chamber at 22–25 °C at 80% relative humidity in Sunshine® mix SB300 universal plant growth mixture (Sun Gro Horticulture, Bellevue, WA, USA) with 16 h of light per day. Alfalfa leaf pieces were

cocultivated with *Agrobacterium tumefaciens* LBA404(pPW1) and transgenic plants were regenerated essentially as described previously (Austin *et al*., 1995). Transgenic tobacco plants were generated after cocultivation of leaf pieces with *Agrobacteium tumefaciens* LBA404(pPW1) as described previously (Horsch *et al*., 1986). *Arabidopsis* was transformed with *Agrobacterium tumefaciens* strain C58C1 (pMP90)(pPW1) using the floral-dip vacuum infiltration method as described previously (Ye *et al*., 1999).

### Atrazine tolerance assays

Transgenic and wild-type tobacco, alfalfa and *Arabidopsis* plants were evaluated for their ability to grow in the presence of different concentrations of atrazine. Approximately 50 and 100 seeds from transgenic and wild-type tobacco and *Arabidopsis* lines, respectively, were placed on to the surface of agar germination medium [2.5 g/L Peter's 20-10-20 water-soluble fertilizer (Scotts Co., Marysville, OH, USA)] amended with 0–30 μg/mL atrazine. Plants were incubated at 25 °C for 2 weeks and visually evaluated for growth. Leaves were excised from five confirmed transgenic alfalfa lines and wild-type control plants, surface sterilized, cut into 1 $cm^2$ explants and incubated for 3 weeks on B5h medium containing 50 μg/mL kanamycin (Austin *et al*., 1995). Somatic embryos were induced from calli, converted to plantlets on MMS (modified Murishige & Skoog) medium (Austin *et al*., 1995) and 20 plants from each line were transferred to Magenta boxes containing MMS medium supplemented with various concentrations of atrazine.

### PCR and RT-PCR assays

DNA from wild-type and transgenic plants was isolated from 1 g of leaves using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). p-*atzA* was amplified from tissues by PCR using primers patzAF 5′-GATAGGATGGATGGTAGG-3′ and patzAR 5′-GTCAGTACCGATTCCAAC-3′, as described previously (Miller *et al*., 1998). Longer PCR products from transgenic plants were obtained using primer pairs Atzam 0006f 5′-AGATCTGATTCTAGACC-3′ and Atzam0105f 5′-GGTTCATGTGCAAGATGG-3′, and Atzam1467r 5′-TCCGAGCTCCTATTAAAG-3′ and Atzam1373r 5′-AATGCAAGCTCAGGCTCT-3′, and the following PCR conditions: 98 °C for 5 min, 35 cycles of 95 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min, and a final 7 min extension at 72 °C.

RT-PCR was performed using total plant RNA extracted from 2 g of young leaves from transgenic and wild-type plants using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Samples were treated with DNAse prior to RT-PCR. RT-PCR was performed using the patzAF and patzAR primers described above and a OneStep RT-PCR Kit (Qiagen, Valencia, CA, USA) with the following conditions: 50 °C for 30 min, 95 °C for 15 min, 35 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min, and a final 10 min extension at 72 °C.

### Western blot analyses

Total plant proteins were extracted from 200–600 mg of leaves, stems and roots from transgenic and wild-type plants, Western blotted as described previously (Miller *et al*., 1998) and hybridized to anti-AtzA polyclonal antiserum produced in rabbits as described previously (de Souza *et al*., 1998b). Goat anti-rabbit horseradish peroxidase (Bio-Rad, Hercules, CA, USA) was the secondary antibody.

### Atrazine degradation assays

p-*atzA*-dependent hydrolytic dechlorination of atrazine by agar- and hydroponically grown transgenic and wild-type alfalfa and tobacco plants (see below) was assessed using TLC and $^{14}$C-UL-ring-atrazine as described previously (de Souza *et al*., 1995). Frozen and ground roots, leaves or stems (1 g) from single plants or multiple plants from the same line were added to 10 mL of extraction buffer (100 mM Tris-HCl, pH 8.5, 3 mM dithiothreitol, 1 mM phenylmethylsulphonyfluoride, 3% (w/v) bovine serum albumin, 1% (w/v) polyvinylpolypyrrolidone, 2 μM antipain and 1 μg/mL pepstatin) and centrifuged to remove plant debris. The crude protein extract (100 μL) was added to 500 μL of 100 mM Tris buffer, pH 8.5, containing 0.01 μCi of $^{14}$C-UL-ring-atrazine (specific activity, 17.1 mCi/mmol) and incubated overnight at 25 °C. The mixture was evaporated to dryness under nitrogen gas, and suspended in 50 μL of methanol. Radiolabelled hydroxyatrazine standard was prepared by mixing 500 μL of uniformly ring-labelled $^{14}$C-UL-ring-atrazine (40 000 dpm) with 50 μL of purified AtzA (de Souza *et al*., 1996) in 100 mM Tris buffer, pH 8.5. The reaction mixture was incubated overnight at 25 °C, 50 μL samples were loaded on to precoated silica gel 60 F254 TLC plates (Alltech Associates, Chicago, IL, USA) and developed using a chloroform–methanol–formic acid–water (75 : 20 : 4 : 2) solvent system. Radioactive spots on TLC plates were scanned using a Molecular Dynamics/Amersham Storm Trooper –840 Imager (Sunnyvale, CA, USA).

The *in vivo* dechlorination of atrazine by wild-type and transgenic alfalfa (lines A1 and A3) and tobacco (lines P9 and P10) plants was evaluated in hydroponic experiments. Seedlings

or vegetative cuttings were transferred to hydroponic chambers consisting of Mason® jars containing 400 mL of plant growth medium (Wych and Rain, 1978). Jars were fitted with lids containing a two-hole rubber stopper: one hole was slit vertically to accommodate the plant, and the other held a glass tubing and an air stone connected to a pump for aeration. Hydroponic chambers were incubated at 27 °C, with an 18 h photoperiod, allowed to adapt for 4 days, and fresh hydroponic solution containing 0.5 μg/mL $^{14}$C-UL-ring-atrazine (specific activity, 17.1 mCi/mmol) was added. Controls were hydroponic chambers with plant growth medium containing no plants or no atrazine. The solution in each jar was sampled immediately and 3 days after the addition of atrazine. Leaves, stems and roots were harvested after 3 days of incubation of the plants with atrazine. Samples were ground to a fine powder in liquid nitrogen, suspended in 10 mL of methanol, mixed for 5 min and centrifuged for 2 min at 10 000 *g*. Supernatants were evaporated to dryness, residues were resuspended in 200 μL of methanol and the radioactivity in 20 μL aliquots of plant samples and hydroponic solutions was determined using a Packard 1900 TR Liquid Scintillation Counter (Perkin Elmer, Boston, MA). Combined tissues from three plants of each line and hydroponic solutions were analysed for $^{14}$C-labelled atrazine and hydroxyatrazine by TLC analyses as described above. The ratio of hydroxyatrazine to atrazine in plant samples was determined by calculating the density of corresponding spots on TLC plates using a Storm Trooper Imager (Sunnyvale, CA, USA).

### Selection of primary transformants using the p-*atzA* marker gene

*A. thaliana* ecotype Columbia was transformed, using the floral-dip vacuum infiltration method, with *Agrobacterium tumefaciens* strain C58C1 (pMP90)(pPW1) as described by Ye *et al*. (1999). Five hundred seeds from wild-type and transformed *Arabidopsis* $T_0$ lines R4 and R10 were surface sterilized by immersion in sodium hypochlorite (50% Chlorox bleach) for 10 min, followed by extensive washing with sterile water. Seeds were positioned on to the surface of filter paper discs (50 seeds per filter) which were placed on the surface of Murashige and Skoog basal medium, without sucrose, containing 0.4 μg/mL atrazine. Plates were incubated at 4 °C for 3 days and then at 25 °C, with a 16 h photoperiod. Seeds were scored for germination and plant growth after 2 weeks of incubation. The selection of transformants using p-*atzA* was also evaluated using a soil germination assay. Fifty surface-sterilized seeds from wild-type and transformed *Arabidopsis* lines R4 and R10 were sown in pots containing Metromix® 200 (Scotts Company, Marysville, OH, USA) and incubated for 2 weeks at 25 °C, with a 16 h photoperiod. Two-week-old plantlets were sprayed with atrazine solution (10 μg/mL) and plant survival was visually scored after further incubation for 7 days under the same conditions. The presence of p-*atzA* in DNA from *Arabidopsis* transformants that were selected following growth on agar medium (20 plants) and in soil (15 plants) was evaluated using PCR and the primers described above.

Ten leaves from wild-type and transgenic alfalfa line A3 (containing p-*atzA*) were surface sterilized and transgenic callus was generated as described previously (Austin *et al*., 1995). Callus pieces were transferred to agar plates containing B5h medium with 5% sucrose, kinetin, 2,4-dichlorophenoxyacetic acid and 1.5 μg/mL atrazine. Each agar plate containing 10–14 calli was incubated at 25 °C, with a 16 h photoperiod, and visually scored for the production of somatic embryos after 3 weeks of growth.

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