Characterization and plant expression of a glyphosate-tolerant enolpyruvylshikimate phosphate synthase†

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Abstract

BACKGROUND: Glyphosate tolerance is a dominant trait in modern biotech crops.

RESULTS: A gene encoding a glyphosate-tolerant EPSP synthase (*aro*A_{1398}) from bacterial strain ATX1398 was cloned and characterized. The protein is initiated at a GTG translational start codon to produce a protein that provides robust glyphosate resistance in *Escherichia coli* (Mig) Cast & Chalm. The *aro*A_{1398} protein was expressed and purified from *E. coli*, and key kinetic values were determined ($K_i = 161\,\mu M$; $K_m(PEP) = 11.3\,\mu M$; $k_{cat} = 28.3\,s^{-1}$). The full-length enzyme is 800-fold more resistant to glyphosate than the maize EPSP synthase while retaining high affinity for the substrate phosphoenol pyruvate. To evaluate further the potential of *aro*A_{1398}, transgenic maize events expressing the *aro*A_{1398} protein were generated. T_0 plants were screened for tolerance to glyphosate sprays at 1.3× commercial spray rates, and T_1 plants were selected that completely resisted glyphosate sprays at 1×, 2× and 4× recommended spray rates in field trials.

CONCLUSION: These data suggest that *aro*A_{1398} is a suitable candidate for conferring glyphosate tolerance in transgenic crop plants.

Keywords: glyphosate resistance; EPSP synthase; bacteria; *Escherichia coli*

1 INTRODUCTION

N-Phosphonomethylglycine, commonly referred to as glyphosate, is an important agricultural herbicide. Glyphosate inhibits the enzyme that converts phosphoenolpyruvic acid (PEP) and 3-phosphoshikimic acid to 5-enolpyruvyl-3-phosphoshikimic acid. Inhibition of this enzyme [5-enolpyruvylshikimate-3-phosphate (EPSP) synthase] kills plant cells by shutting down the shikimate pathway, thereby inhibiting aromatic amino acid biosynthesis.

Owing to its mode of action on aromatic amino acid biosynthesis, glyphosate is also toxic to bacterial cells when grown in appropriate media. However, certain bacterial EPSP synthases have high resistance to glyphosate. Several such bacterial EPSP synthase enzymes have been isolated previously and grouped into either a class I or a class II nomenclature.[1] Analysis of these EPSP synthase sequences identifies a number of conserved motifs that help define these classes. In particular, a series of four motifs found in class II enzymes has been described.[1]

Plant cells resistant to glyphosate toxicity can be produced by transforming plant cells to express glyphosate-resistant bacterial EPSP synthases. Notably, a class II bacterial gene from *Agrobacterium tumefaciens* strain CP4 confers herbicide tolerance in several plant species.

Here, the isolation, cloning and characterization of a glyphosate-tolerant bacterial EPSP synthase are described. This EPSP synthase, *aro*A_{1398}, has excellent kinetic qualities, as it possesses a stronger binding affinity for the natural substrate PEP while simultaneously binding glyphosate with 800-fold lower affinity than the maize EPSP synthase. The protein confers strong glyphosate tolerance on maize plants both in greenhouse and field conditions, allowing

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glyphosate to be sprayed at 4× recommended field spray rates. Furthermore, *aro*A_{1398} does not possess the protein sequence hallmarks of class II enzymes.

2 METHODS

2.1 Isolation of a glyphosate-resistant bacterial strain

Glyphosate-resistant microbes were isolated by plating environmental soil samples on an enriched minimal medium (EMM) (sucrose 10, NH_4Cl 1, $Mg_2SO_4.7H_2O$ 0.2, $FeSO_4.7H_2O$ 0.01, $MnSO_4.H_2O$ 0.007 g L^{-1} in water) containing glyphosate (10 mM) as the sole source of phosphorus. One particular strain, designated ATX1398, was selected for its ability to grow in the presence of high glyphosate concentrations.

2.2 Cloning of a glyphosate-tolerant EPSP synthase

Strain ATX1398 was grown in EMM, and cells were pelleted by centrifugation. Genomic DNA was extracted from ATX1398, partially digested with the enzyme Sau3A I, ligated into a cosmid vector (Supercos 1; Stratagene, La Jolla, CA) and packaged into phage particles. An aliquot of the phage was transfected into *Escherichia coli* strain JM101 and plated on MOPS agar medium[2] containing 50 μg mL^{-1} kanamycin to select for colonies containing cosmids. Approximately 700 kanamycin-resistant colonies were replica plated onto MOPS agar containing 50 μg mL^{-1} kanamycin and 2 mM glyphosate. Four clones grew in the presence of 2 mM glyphosate, and one of these cosmids was observed to grow in the presence of 5 mM glyphosate. DNA from this cosmid was subjected to *in vitro* transposon mutagenesis using the Primer Island Kit (PE Biosystems, Foster City, CA) and transformed into *E. coli* strain XL1 Blue MRF' (Stratagene) by electroporation. Clones containing a transposon insertion were selected by plating on MOPS containing 50 μg mL^{-1} carbenicillin plus 50 μg mL^{-1} trimethoprim, then replica plated onto MOPS agar medium containing carbenicillin, trimethoprim and 2 mM glyphosate. Three colonies were identified that contained a transposon insertion and that did not grow in the presence of 2 mM glyphosate but did grow in its absence, indicating that the insertions were probably in or near the gene responsible for resistance to glyphosate. The DNA flanking the transposon insertions was sequenced, and the transposon insertions were all found to reside in a single open reading frame encoding an EPSP synthase, *aro*A_{1398}.

2.3 *aro*A_{1398} glyphosate tolerance in *E. coli*

The open reading frame encoding *aro*A_{1398} was subcloned into a plasmid (pAX306) that placed protein expression under the control of the *tac* promoter. The native GTG initiation codon was changed to ATG in this expression construct. Plasmids were then introduced into *E. coli* strains DH5α and XL1 Blue MRF', and individual colonies were plated onto M63 agar medium[3] containing 0, 1, 5, 10, 20, 50, 100 or 200 mM glyphosate and grown at 37 °C. Colony growth was scored after 48 h.

2.4 Kinetic characterization of *aro*A_{1398}

The *aro*A_{1398} gene was subcloned into a plasmid (pRSF1b; Novagen, Darmstadt, Germany) that allows strong protein expression via T7 RNA polymerase following IPTG induction in *E. coli*. The enzyme was purified in a single step using a cobalt chromatography column (Clontech, Palo Alto, CA), and was dialyzed into 50 mM Hepes/100 mM KCl (pH 7.0) for enzyme activity assays. The protein was greater than 95% pure as assessed by PAGE, and was quantified by Bradford's method[4] using bovine serum albumin as standard.

All kinetics measurements (K_m(PEP), K_i and V_{max}) were carried out using a coupled fluorescence enzymatic assay under the following conditions: Hepes 50 mM (pH 7.0), KCl 50 mM, shikimate-3-phosphate 500 μM, xanthine oxidase 1 U mL^{-1}, purine nucleoside phosphorylase 2 U mL^{-1}, inosine 2.25 mM, horseradish peroxidase 1 U mL^{-1} and Amplex Red (Invitrogen, Carlsbad, CA) 1100 μM. Purified enzyme was added to initiate the reactions. The enzymatic rate was measured at phosphoenol pyruvate (PEP) concentrations ranging from 2.5 to 600 μM, and the data points were fitted to the Michaelis–Menten equation to calculate V_{max}. The titration was then repeated in the presence of glyphosate (100, 200, 500, 800 μM) and the K_m(app) value was calculated at each glyphosate concentration. A plot of K_m(app) vs glyphosate was used to calculate the K_m and K_i values (y-intercept and $-1/x$-intercept respectively).

2.5 Expression of *aro*A_{1398} in maize plants

An expression cassette containing a novel constitutive promoter from *Tripsacum dactyloides* (L.) L.[5] was placed upstream of a chloroplast transit peptide-*aro*A_{1398} gene fusion to direct expression and translocation of *aro*A_{1398} to the maize chloroplast, as described.[6] The cassette was then subcloned into pSB11 shuttle vector and the plasmid was introduced into *Agrobacterium tumefaciens* strain LBA4404 which also harbors the plasmid pSB1 (Japan Tobacco, Inc., Tokyo, Japan), using triparental mating. Positive cointegrates were identified as colonies on agar media containing spectinomycin, tetracycline, streptomycin and rifampicin. The *Agrobacterium* strain harboring the cointegrate was used to transform maize embryos.[7,8] Transgenic plants were confirmed by Southern analysis and immunoblotting (data not shown).

Transgenic plants generated by this protocol were tested for protein expression by Western blot using rabbit polyclonal antibodies generated against *aro*A_{1398}, and the glyphosate tolerance of the T_0 plants was assessed after spraying with glyphosate. A generic glyphosate solution was mixed with surfactant (TWEEN 20, 1 mL L^{-1}) and applied to plants via

a spray table at a rate corresponding to 1.3× field application rate, where a 1× application correlates with a spray rate of $1.78\,L\,ha^{-1}$ of a $480\,g\,AE\,L^{-1}$ commercial glyphosate formulation. Several plants sprayed in this manner did not display any visible damage or growth setback when examined at 1, 2 and 3 weeks post-spray. These plants produced a robust seed set following pollination, and the resulting T_1 seeds were collected for field trials.

Field trial glyphosate sprays were carried out as follows. Multiple stands were sprayed at the V4 leaf stage with glyphosate formulations in water at rates of 0, 1.78, 3.55, or $7.10\,L\,ha^{-1}$ (= 0, 1, 2 and 4× label rate). A non-ionic surfactant was added to the spray mixture at a concentration of $0.04\,mL\,L^{-1}$. Spray mixtures were prepared individually for each rate, and a backpack sprayer was used to apply the mixtures. To determine the tolerance to the glyphosate applications, plots were inspected visually 14 days post application. The number of dead plants in each row was recorded to determine the segregation ratios in the glyphosate sprayed rows. The surviving plants were rated using a visual scale.[9]

3 RESULTS

3.1 *aro*A_{1398} confers glyphosate resistance in *Escherichia coli*

Bacterial strain ATX1398 was isolated from an environmental sample owing to its ability to grow in the presence of glyphosate. Cosmid libraries were generated, and a gene encoding a glyphosate-tolerant EPSP synthase, *aro*A_{1398}, was isolated.[6] The glyphosate tolerance of the protein was further assessed by characterizing the growth of an expression clone transformed into glyphosate-sensitive *E. coli* strains. In this format, *aro*A_{1398} was found to allow robust colony growth through a glyphosate concentration of 200 mM (Table 1).

Alignments of the *aro*A_{1398} amino acid sequence and examination of the DNA sequence context around potential start codons suggest that translation initiates at a GTG, rather than the first in-frame ATG codon at amino acid 23 in the native strain (Fig. 1). Structure-function studies with the *E. coli* EPSP synthase enzyme (AROA) have identified Lys-22 as being critical for enzyme function.[10] Furthermore, crystal structure data for *E. coli* AROA identify Lys-22 as being positioned in the active site of the enzyme.[11]

Table 1. Growth of *Escherichia coli* strains containing empty vector, *aro*A_{1398} or *aro*A_{1398}(Δ1–22) on a defined medium containing glyphosate

	Plasmid construct[a]					
	Empty vector		*aro*A_{1398}		*aro*A_{1398}(Δ1–22)	
Glyphosate (mM)	DH5α	XL1 Blue	DH5α	XL1 Blue	DH5α	XL1 Blue
0	++	++	++	++	++	++
1	+	+	++	++	+	+
5	−	−	++	++	−	−
10	−	−	++	++	−	−
20	−	−	++	++	−	−
50	−	−	++	++	−	−
100	−	−	++	++	−	−
200	−	−	++	++	−	−

[a] ++ = strong growth; + = weak growth; − = no growth

The residue Lys-20 in *aro*A_{1398} aligns with Lys-22 in *E. coli* AROA, suggesting that the 22 amino acids preceding Met-23 are required for function in *aro*A_{1398}. These observations suggested that Met-23 is not the natural translational start site in *aro*A_{1398}, and that proteins generated from Met-23 would be incapable of providing tolerance since the resulting enzyme lacks a critical part of the active site.

To characterize the functional effect of the downstream ATG start site of *aro*A_{1398}, *E. coli* expression clones for the full-length enzyme (pAX306) and the enzyme starting at Met-23 [*aro*A_{1398}(Δ1–22)] (pAX1640) were created. Heterologous protein expression was driven by the Ptac promoter for both clones. These clones were then introduced into glyphosate-sensitive *E. coli* strains and grown on M63 agar medium containing various concentrations of glyphosate. As shown in Table 1, *aro*A_{1398} conferred tolerance to high concentrations of glyphosate, while *aro*A_{1398}(Δ1–22) did not provide tolerance beyond the negative control.

3.2 Glyphosate resistance of *aro*A_{1398} vs maize EPSP synthase

A fluorescence-based assay method was modified to quantify phosphate generation by EPSP synthase enzymes, and used to generate a kinetic profile for purified *aro*A_{1398} (Fig. 2) in the presence and absence of the inhibitor glyphosate. The data align well with published models in which glyphosate binding competes with phosphoenolpyruvate in the

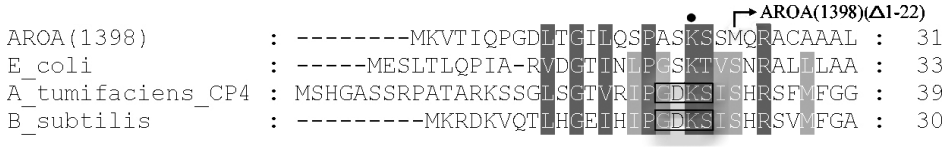

Figure 1. Protein sequence alignment of *aro*A_{1398} and EPSP synthase enzymes from *Escherichia coli, Agrobacterium tumifaciens* CP4 and *Bacillus subtilis*. A downstream methionine in the *aro*A_{1398} sequence (Met-23) is indicated as the translational start site for *aro*A_{1398}(Δ1–22), and a conserved active site lysine residue (●) that influences enzymatic activity in the *E. coli* enzyme is indicated. A conserved 'GDKS' sequence that is present in class II enzymes contains the conserved lysine residue.

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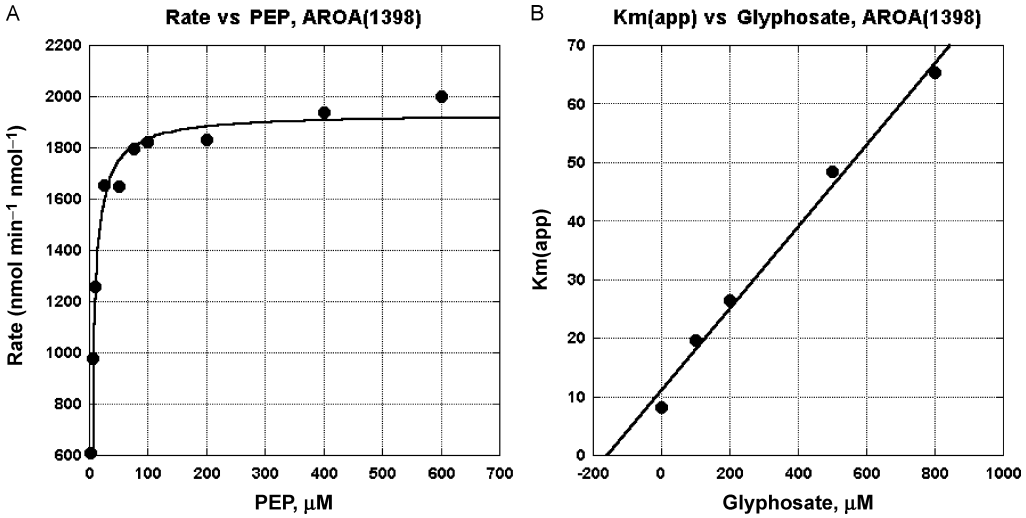

Figure 2. Kinetic characterization of *aro*A$_{1398}$. Panel A – the enzymatic activity of *aro*A$_{1398}$ was measured at varying PEP concentrations and used to plot rate vs PEP. The apparent Michaelis–Menten binding constant for PEP [K_m(app)] and maximal rate (V_{max}) were calculated from the half-maximal and maximal rates respectively. Panel B – the K_m(app) was measured at glyphosate concentrations ranging from 0 to 800 µM, and was plotted against the glyphosate concentration to derive the K_m and K_i for the enzyme.

active site of the enzyme, but not with shikimate-3-phosphate.[12,13] The enzyme exhibits excellent glyphosate tolerance ($K_i = 161\,\mu M$) (Table 2) while retaining a strong binding affinity for substrate PEP ($K_m = 11.3\,\mu M$) (Table 2). Since glyphosate competes with PEP for binding in the active site of EPSP synthase enzymes, this binding discrimination between glyphosate and PEP ($K_i/K_m = 14$) is critical to maintaining enzymatic activity in the presence of inhibitor. In contrast, the binding discrimination for the maize EPSP synthase ($K_i/K_m = 0.01$) is 1400-fold lower. In addition to these binding properties, the purified *aro*A$_{1398}$ has a robust catalytic rate ($k_{cat} = 28.3\,s^{-1}$). This kinetic profile suggests that *aro*A$_{1398}$ can substitute well for the maize EPSP synthase *in planta*.

3.3 Transgenic maize expressing *aro*A$_{1398}$

Transgenic maize plants expressing the *aro*A$_{1398}$ protein were produced and evaluated for tolerance to glyphosate in greenhouse and field tests. PCR analysis confirmed the presence of *aro*A$_{1398}$ in these plants, and Western blot analysis confirmed that the plants generated by these constructs expressed *aro*A$_{1398}$ (data not shown).

T_0 plants derived from tissue culture were sprayed at 1.3× glyphosate concentration, and several dozen *aro*A$_{1398}$ events were identified that were scored as a '0' (no apparent leaf damage) on a visual rating scale.[9] A representative sprayed plant is shown in Fig. 3 (panel A) alongside an unsprayed plant and a sprayed untransformed control plant.

Glyphosate-resistant T_1 lines were then generated by crossing to the parental line. Non-transgenic controls were generated alongside. All T_1 plants were sprayed at 0×, 1×, 2× and 4× glyphosate concentrations in field trials, and segregation was assessed by spray tolerance at 1× glyphosate. Of the T_1 plants generated from the glyphosate-tolerant lines, 54% survived the spray, which suggests that segregation took place at a 1:1 ratio and that the majority of the T_0 plants contained a single copy of *aro*A$_{1398}$. None of the non-transgenic control plants survived the 1× glyphosate spray. The segregation data from representative lines are shown in Table 3. At the highest glyphosate concentration tested (4×), it was observed that 38% of the positive segregant events had less than 5% leaf chlorosis in the field, and, among those, 22% had no leaf damage. A representative series of plants that were sprayed at 4× glyphosate are shown in Fig. 3 (panel B), alongside control plants that were killed at 1× glyphosate spray (panel C).

Table 2. Kinetic constants for *aro*A$_{1398}$ and maize EPSP synthase

	K_m(PEP) (µM)	K_i (µM)	V_{max} (nmol min^{-1} µg^{-1})	k_{cat} (s^{-1})
*aro*A$_{1398}$	11.3	161	35.9	28.3
Maize EPSPS	18.1	0.2	47.5	38.4

4 DISCUSSION

Glyphosate-tolerance has become a predominant train in modern transgenic crops. The majority of

Table 3. Glyphosate resistance of segregating T_1 plants

T_0 line	Total plants sprayed	Surviving plants	Survival (%)
5106	34	17	50.0
5131	36	20	55.6
5177	27	14	51.9
5196	35	16	45.7
5203	32	15	46.9
Hi-II (neg. ctrl)	16	0	0.0

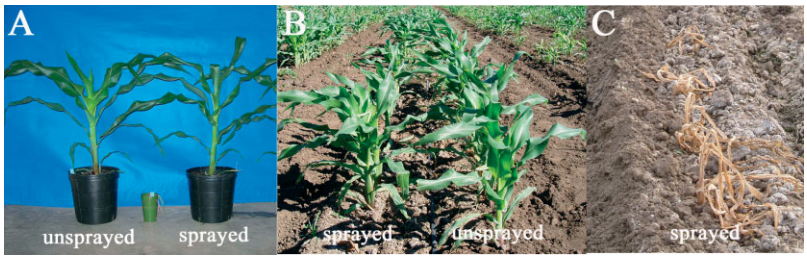

Figure 3. Glyphosate resistance, *aro*A_{1398} expressed in maize. Panel A – glyphosate spray tolerance of greenhouse maize plants. A representative plant expressing *aro*A_{1398} (right) is compared with an unsprayed *aro*A_{1398} plant (left) and a sprayed control plant without *aro*A_{1398} (center). Both plants expressing *aro*A_{1398} were transplanted into larger pots following spraying, and no growth setback or leaf damage was observed in the sprayed *aro*A_{1398} plant when compared with the unsprayed *aro*A_{1398} plant. Panel B – glyphosate spray tolerance of field trial plants. T_1 plants from the same event were sprayed with 4× glyphosate spray (left row) or were not sprayed (right row). No growth setback or leaf damage is observed at 2 weeks post-spray in the surviving positive segregants. Panel C – control plants in a field trial without *aro*A_{1398}, sprayed with 1× glyphosate.

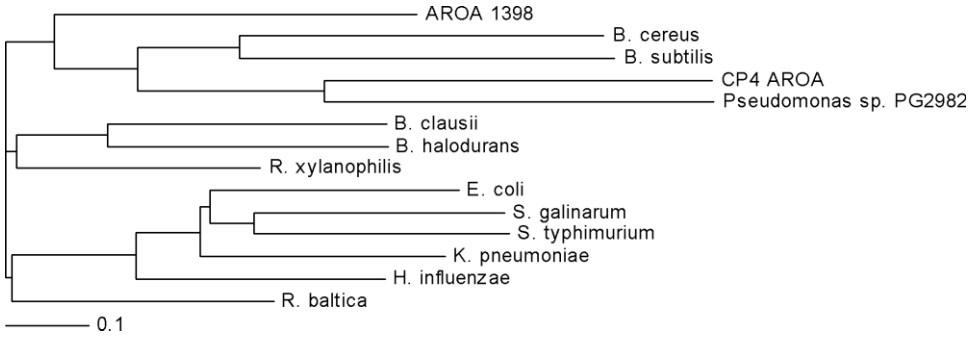

Figure 4. Phylogenic comparison of bacterial EPSP synthases. The phylogenic differences between *aro*A_{1398} and representative class I EPSP synthase enzymes (e.g. *Escherichia coli, Rubtobacter xylanophilis*) and class II enzymes (e.g. AROA-CP4, *Bacillus subtilis*) were visualized by dendrogram analysis (AlignX software; InforMax Inc. Frederick, MD).

soybeans grown in the USA and cotton grown today contain the glyphosate-resistant EPSP synthase from *Agrobacterium tumifaciens* CP4.[14] Transgenic corn resistant to glyphosate is also rapidly being adopted. While this transgene provides a weed control solution for these crops, it also creates certain limitations to introducing additional input traits or output traits into these crops. For example, hybrid seed lines that stack traits such as insect resistance, drought tolerance or yield improvements would also be very desirable for growers. However, these lines must be combined with glyphosate tolerance for broad market adoption. Consequently, access to a glyphosate resistance trait acts as a 'gatekeeper' that limits the adoption of other traits in the market. For this reason, additional solutions for glyphosate resistance are needed.

aroA_{1398} encodes a novel bacterial EPSP synthase that is highly tolerant to the widely used herbicide glyphosate. The primary sequence of *aro*A_{1398} is quite divergent from other known bacterial EPSP synthases, sharing only 25% protein sequence identity with the *E. coli* AROA protein, and 22% identity with the *Agrobacterium tumafaciens* CP4 protein. A dendrogram (Fig. 4) illustrates the differences between *aro*A_{1398} and several class I and class II enzymes. In particular *aro*A_{1398} has none of the four protein sequence domains identified in class II EPSP synthases, a previously described class of glyphosate-resistant EPSP synthases, including the *Agrobacterium tumafaciens* sp. CP4 enzyme.

The kinetics for *aro*A_{1398} in Table 2 describe an enzyme that is comparable with plant enzymes in K_m and k_{cat} while maintaining an 800-fold higher tolerance to glyphosate than that of maize EPSP synthase. This makes *aro*A_{1398} a suitable candidate for expression in transgenic crop plants for glyphosate resistance. Transgenic maize plants expressing *aro*A_{1398} were produced and characterized in greenhouse and field environments. The T_0 and T_1 plants expressing *aro*A_{1398} were highly tolerant to glyphosate herbicide formulations. In field tests, several lines were scored with no visible damage 2 weeks after spraying at 4× normal field rates. These lines were selected for further field testing and development. This indicates that *aro*A_{1398} is an outstanding alternative gene for transgenic maize crops.

REFERENCES

1 Barry GF, Kishore GM and Padgette SR, DNA encoding Class II 5′-enolpyruvyl shikimate-3-phosphate synthase for producing plants and bacteria tolerant to glyphosate herbicides. World Patent WO 9 204 449 (1992).
2 Neidhardt FC, Block PL and Smith DF, Culture medium for enterobacteria. *J Bacteriol* **119**:736–747 (1974).
3 Ebling K and Brent R, Media preparation and bacteriological tools, in *Current Protocols in Molecular Biology*, Vol. 1. John Wiley & Sons, New York, 1.1.2 (2001).
4 Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248–254 (1976).
5 Hammer PE, Cis-acting regulatory elements from *Tripsacum dactyloides*. US Patent Application 11/377318 (2006).

Pest Manag Sci **64**:340–345 (2008)
DOI: 10.1002/ps

6 Hammer PE and Duck NB, Genes conferring herbicide resistance. US Patent Application 10/739610 (2004).
7 Ishida Y, Saito H, Ohta S, Hiei Y, Komari T and Kumashiro T, High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. *Nature Biotech* **14**:745–750 (1996).
8 Hiei Y, Ohta S, Komari T and Kumashiro T, Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J* **6**:271–282 (1994).
9 Meilan R, Han K-H, Ma C, DiFazio SP, Eaton JA, Horen EA, *et al*, The CP4 transgene provides high levels of tolerance to Roundup herbicide in field-grown hybrid poplars. *Can J For Res* **32**:967–976 (2002).
10 Shuttleworth WA, Pohl ME, Helms GL, Jakeman DL and Evans JNS, Site-directed mutagenesis of putative active site residues of 5-enolpyruvylshikimate-3-phosphate synthase. *Biochemistry* **38**:296–302 (1999).
11 Schonbrunn E, Eschenburg S, Shuttleworth WA, Schloss JV, Amrhein N, Evans JNS, *et al*, Interaction of the herbicide glyphosate with its target enzyme 5-enolpyruvylshikimate-3-phosphate synthase in atomic detail. *Proc Natl Acad Sci USA* **98**:1376–1380 (2001).
12 Steinrucken HC and Amrhein N, The herbicide glyphosate is a potent inhibitor of 5-enolpyruvyl-shikimic acid-3-phosphate synthase. *Biochem Biophys Res Comm* **94**:1207–1212 (1980).
13 Boocock MR and Coggins JR, Kinetics of 5-enolpyruvylshikimate-3-phosphate synthase inhibition by glyphosate. *FEBS Lett* **154**:127–133 (1983).
14 Fernandez-Cornejo J, *Adoption of Genetically Engineered Crops in the U.S.* [Online]. Available: http://www.ers.usda.gov/Data/BiotechCrops/ [12 March 2007].